Integrin affinity modulation and survival signalling.

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Declaration

This thesis is my own work. Where work has been performed by other members of the research group it is clearly indicated. This work has not been submitted for any other degree or professional qualification.

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Abstract

Integrins are heterodimeric transmembrane proteins that provide a bi-directional link between the cell's internal biological mechanisms and the extracellular environment. During *inside-out signalling*, intracellular messages converge on the integrin cytoplasmic domain to induce a conformational change. This is transmitted to the extracellular domain where it results in an alteration in affinity for integrin ligands such as fibronectin and laminin. In this way the cell has developed the ability to modulate the critical functions of adhesion and cell movement. In *outside-in signalling*, the integrin performs a more complex function than simple adhesion; upon binding to ligand, the integrin extracellular domain undergoes a conformational change which is transmitted to the cytoplasmic domain. This alters the integrin's cytoplasmic domain affinity for intracellular signalling proteins and results in the activation of intracellular second messenger pathways. In this way, the extracellular milieu is able to influence intracellular signalling including those involved in apoptosis.

This thesis demonstrates data which provide original insights into bi-directional integrin signalling:

Inside-out signalling: Constitutively active Notch1 increases β3-integrin affinity and abrogates Hras-mediated integrin suppression without increasing expression of β3-integrin. Dominant-Negative Rras blocks Notch-mediated integrin activation and Notch1-mediated reversal of Hras and Raf-mediated integrin suppression and this is independent of erk phosphorylation. Notch1 induces Rras activation. Functional adhesion assays confirm that $Notch1^{IC}$ increases K562 adhesion in a β1-integrin dependent manner and this is abrogated by Dominant-Negative Rras. This data supports a mechanism in which Notch1 increases integrin affinity via activation of Rras.

Outside-in signalling: Evidence is presented demonstrating that extracellular matrix proteins, laminin and fibronectin, activate β1-integrins to protect SCLC cells against the apoptotic effects of etoposide and ionizing radiation via PI3Kinase activation. This occurs in two ways: 1) PI3Kinase-dependent β1-integrin signalling resulting in phosphorylation of Bad and reduced caspase-9 cleavage and 2) a β1-integrin-mediated over-riding of etoposide and radiotherapy-induced cell cycle S phase delay and G2/M arrest. β1-integrin-mediated outside-in survival signalling was investigated further in the *in vivo* setting; Matrigel™, a basement membrane product rich in extracellular matrix proteins, promoted SCLC xenograft survival and growth in a β1-integrin and tyrosine kinase-dependent manner.

This data provides novel insights into the critical functions that integrins play in adhesion and survival signalling.

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a.a.	amino acid
ABC	ATP binding cassette
Ab	Antibody
abl	Abelson tyrosine kinase
ADAM	a disintegrin and metalloprotease
ADMIDAS	adjacent to MIDAS
AF-6	ALL1 fused gene from chromosome 6
ANK	ankyrin repeats
AP-1	c-jun/c-fos dimer
Apaf-1	apoptotic protease activating factor
Aph1	Artemia POU-Homeoprotein
APX-1	ascorbate peroxidase-1
ARG-2F16	C.elegans Notch ligand
ATM	ataxia telangiectasia mutated
ATR	ATM-related protein
Aup1	Ancient Ubiquitous Protein1
B12.2	C.elegans Notch ligand
BCA	bicinchoninic acid
bHLH	basic Helix-Loop-Helix
BRCA1	breast cancer gene-1
BRCP	Breast cancer resistance protein
BSA	Bovine serum albumin
C3G	guanine nucleotide exchange factor
CAAX	cysteine, aliphatic, aliphatic, any
CalDAG-GEF	calcium and DAG-regulated GEF
CBF	Core Binding Factor
CBP/p300	CREB-binding protein
cdc25	GEF for Saccharomyces.cerevisiae
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
Ce	Caenorhabditis elegans
Chk1	Checkpoint kinase
CIB	calcium and integrin-binding protein
CIR	CBF1-interacting corepressor
CLL	chronic lymphocytic leukaemia
CSL	Core Binding Factor, Su(H), Lag-1
DAG	diacylglycerol
Del-1	developmental endothelial locus-1
DHR	discs-large homology region
DLL	Delta-like ligand
DMEM	Dulbecco's modified Eagle's Medium
DNA-PKcs	DNA Protein kinase catalytic subunit
DSL	Delta, Serrate, Lag-2

EBD	effector binding domain
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
Epac	exchange protein directly activated by cAMP
erbB2	erythroblastic leukaemia viral oncogene homolog 2
Erk1/2	extracellular signal-related kinase 1/2
F3	FERM 3
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
FAK	focal adhesion kinase
FBS	foetal bovine serum
FCS	foetal calf serum
FERM	four point one, ezrin, radixin, moesin
FGF	Fibroblast growth factor
FLIP	FLICE-like inhibitory protein
FRET	fluorescence resonance energy transfer
GABP-β	growth-associated binding protein beta
GAP	GTPase Activating Protein
GAP1m	mammalian homolog of Ras-GTPase activating protein
GCN5	histone acetyl-transferase
GDP	guanosine diphosphate
GEFs	guanidine Exchange Factors
GFFKR	Gly-Phe-Phe-Lys-Arg
GFP	Green Fluorescent Protein
GLGF	gly-leu-gly-phe
GLP-1	Germline proliferation 1
Grb2	growth factor receptor-bound protein 2
GSK	glycogen synthase kinase
GST	glutathione-s-transferase
GTP	guanosine triphosphate
Gy	Gray (joule/kg)
γH2Ax	histone H2Ax phosphorylated on Serine[139]
H+E	haematoxylin and eosin
hASH1	human Achaete-scute homolog
HDAC	histone deacetylase
Hes	Hairy enhancer of Split
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HERPs	Hes-related proteins
HR	homologous recombination
HRP	horseradish peroxidase
HSP60	heat shock protein 60
Hus1	DNA damage checkpoint protein

IAP	integrin-associated protein
IC1n	integrin IIb 3-associated protein
IGF-1	insulin-like growth factor 1
ICAM	intercellular adhesion molecule
ICAP-1α	integrin cytoplasmic domain-associated protein-α
IgG	immunoglobulin G
Il-6	interleukin 6
ILKBP	ILK-binding protein
ILK	Integrin linked kinase
JNK	Jun N-terminal kinase
p27Kip1	cyclin-dependent kinase inhibitor, 27kDa
Ku70	70kDa subunit of DNA-protein kinase complex
Lag	Lin and GLP
LFA1	lymphocyte function-associated antigen
LIMBS	ligand-induced metal-binding site
LIN-3	C.elegans EGF homolog
LIP-1	C.elegans MAPkinase phosphatase
LNR	Lin12/Notch repeat
LRP	lung cancer resistance protein
Mac-1/CR3	integrin αMβ2/complement receptor 3
MAML	mastermind-like; transcriptional coactivator for Notch
MAPK	mitogen activated protein kinase
Mash1	mouse Achaete-scute homolog
MDC1	mediator of DNA-damage checkpoint
MDR	multidrug resistance
MEK	mitogen activated protein kinase
MEKK	MEK kinase
MHC-I	major histocompatibility complex 1
MIDAS	metal ion-dependent adhesion sites
MKP3	mapk phosphatase
Mre11	meiotic recombination 11
MR-GEF	Mras-regulated GEF
mSin3A	mammalian self-inactivating 3A
mTOR	mammalian target of rapamycin
MRP	multi-drug resistance-associated proteins
MVP	major vault protein
MyoD	myogenic differentiation protein
Nbs1	Nojmegen breakage syndrome 1
NcoR	nuclear corepressor
NFκB	nuclear factor kappa B
NGF	nerve growth factor
NHEJ	non-homologous end-joining

Nore1	novel ras effector 1
NPxY	Asn-Pro-x-Tyr
Nrarp	Notch-regulated ankyrin repeat protein
NSCLC	non -small cell lung cancer
OPA	poly-glutamine sequence
p21Cip1	cyclin-dependent kinase inhibitory protein 1, 21kDa
p56lck	leukocyte-specific protein tyrosine kinase, 56kDa
Pac1	Platelet activation complex; αIIβ3 monoclonal antibody
PARP	poly-ADP ribose polymerase
PBS	phosphate buffered saline
pCAF	p300/CBP-associated factor
PDGF	platelet-derived growth factor
PEA-15	phosphoprotein enriched in astrocytes
PECAM	platelet endothelial cell adhesion molecule-1
pEGFP1	EGF-encoding expression vector
Pen-2	presenilin enhancer-2
PEST	Proline, Glutamate, Serine, Threonine
PFA	phosphonoformic acid
PgP	P-glycoprotein
PH	Pleckstrin homology
PI	phosphoinositol
PI3K	phosphoinositol-3-kinase
PI4K	phosphoinositol-4-kinase
PI45P2	phosphoinositol-4,5-bisphosphate
PINCH	binding protein for ILK
PIP5-kinase	phosphoinositol-4-phosphate 5-kinase
PIPKIγ90	typeI gamma phosphatidylinositol phosphate kinase isoform γ90
PIPKIγ-661	typeI gamma phosphatidylinositol phosphate kinase isoform γ661
PKB	Protein kinase B
PKCε	protein kinase C epsilon
PLCε	phospholipase C epsilon
PLL	poly-l-lysine
PP2A	protein phosphatase 2A
PTB	phosphotyrosine-binding
Rad50	protein involved in DNA double strand break repair
RA-GEFII	Ras-associated guanine exchange factor
RalGDS	Ral guanine nucleotide dissociation stimulator
RalGEF2	Ral guanine exchange factor 2
RalGPS	Ral-GEFs with PH domain and SH3-binding motif
RAM	regulation of amino-acid metabolism
RapI-GAPII	Rap1 GTPase-activating protein 2
RasGRF1	Ras-specific guanine nucleotide-releasing factor 1
Rap	Ras-related protein
RapL	regulating of cell adhesion and polarisation enriched in lymphoid tissues

RasGRF1	Ras-specific guanine nucleotide-releasing factor 1
RasGRP2/3	Ras guanyl releasing protein 2/3
Rb	retinoblastoma protein
RBD	ras-binding domains
RBPJκ	recombination signal binding protein for immunoglobulin kappa J region
RGD	Arg-Gly-Asp
Rgl	Ral GDP dissociation stimulator-like
Rgr	RalGDS-related
Rlf	RalGEF-like
RP-A	replication protein A
RPE	P-phycoerythrin
RPMI	Roswell Park Memorial Institute
RQRR	arg-gln-arg-arg
SAP30	Sin3A binding protein, 30kDa
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sel-10	F-Box protein
SH2/3	Src-homology 2/3
SITA	selenium, insulin, transferrin, albumin
SMC	Sensory Organ Mother Cell
smgGDS	small G-protein dissociation stimulator
SMRT	silence mediator for retinoic acid and thyroid receptors
Sos	son of sevenless
SPA	Rap1-GAP in lymphocytes
Src	family of tyrosine kinases
ST6Gal-1	beta galactosidase α-2,6-sialyltransferase 1
Su(H)	Suppressor of Hairless
SUP-17	suppressor 17, an ADAM protein
TACE	Tumor necrosis factor-alpha-converting enzyme
TAD	Transcriptional activation domain
TC21	teratocarcinoma oncogene 21
TCR	T-cell receptor
TEMED	N,N,N',N'-tetramethylethylenediaine
TGF	Transforming Growth Factor
Tiam-1	T lymphoma invasion and metastasis-inducing gene 1
TLE	Transducin-like enhancer of split
TopoII	Topoisomerase 2
TPA	tissue plasminogen activator
Vav	Rho-family GEF
V-CAM1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VLA-1	integrin α1β1
VPCs	vulval precursor cells
WAF1	wild-type p53-associated fragment 1

WEE1	cdc2 inhibitor
XRCC	Xray cross complementing
z-VAD	z-valine-Ala-Asp(Ome)-fluoromethyl ketone inhibitor

Chapter 1

Introduction

Inside-Out Modulation of Integrin Affinity

1.1 Integrins

Integrins are a family of adhesion receptors which mediate cell-extracellular matrix and cell-cell interactions. They consist of heterodimers containing non-covalently linked α and β subunits which are single-pass transmembrane proteins. The invertebrates Caenorhabditis.elegans (worm) and Drosophila.melanogaster (fly) possess 2α/1β and 5α/2β subunits respectively and the vital roles that these proteins play in cell development and signalling is highlighted by the frequent early non-viable phenotype of knockout mutants [(Brown et al., 2000)]. To date 18α/8β subunits and 24 distinct heterodimers have been identified in vertebrates, these interacting with extracellular matrix (ECM) proteins and cell surface receptors/proteins with a varying degree of promiscuity (**see Appendix: Table 1**). Integrin subunits typically contain a large extracellular domain, a single-pass transmembrane domain and a short cytoplasmic tail of 20-60 amino acids [(Hynes, 2002)]. A significant advance in the understanding of integrin-ligand interactions was provided by the demonstration of the X-ray structure of the extracellular domains of integrin αVβ3 [(Xiong et al., 2001)] (**see Appendix: Fig 1**). The integrin structure resembles a "head" connected to two legs. The head piece contains a seven-bladed β-propeller in the α-subunit closely juxtaposed with a von Willebrand Factor (vWF) type A domain in the β-subunit (βA

also referred to as the "I" domain or βI; several α subunits also contain vWF type A domains). A groove formed in the upper surface by the apposition of these two domains is able to bind peptides containing the RGD (Arg-Gly-Asp) sequence common to many extracellular matrix proteins. The βA domain is connected to an immunoglobulin-like "hybrid" domain and thence via EGF (epidermal growth factor) repeats and a β-tail domain to its transmembrane domain. The α-subunit is connected to its transmembrane domain by 3 β-sandwich modules (thigh, calf1, calf2). The top surface of the βA domain also contains three metal ion-dependent adhesion sites; MIDAS, adjacent to MIDAS (ADMIDAS) and ligand-induced metal-binding site (LIMBS) [(Chen et al., 2006),(Xiong et al., 2002)]. Cation binding to all three of these sites is able to influence movements of the α-helices of the βA domain and thus to influence affinity states. Cations can increase (Mn^{2+}/Mg^{2+}) or decrease (Ca^{2+}) affinity for ligands and can also modulate ligand specificity [(Plow et al., 2000)]. In the inactive state (low affinity for ligands) the αVβ3 integrin adopts a folded structure bent at the "genu" which occurs at the thigh/calf1 level of the α-subunit and between the Hybrid domain and EGF repeats of the β-subunit [(Xiong et al, 2001)]. In this inactive state the ligand-binding groove faces towards the plasma membrane and is inaccessible to ligands. The "legs" of this inactive integrin are stabilised by the conformation of the α and β-subunit cytoplasmic tails. Alterations in the conformational state of the cytoplasmic tail by "inside-out" signalling are propagated to the head resulting in shifts of α-helices in the βA domain, a swing of the hybrid domain away from the α-subunit and extension of the "genu" to an obtuse angle. As a result of this unfolding the ligand binding groove is "activated" to a higher affinity, exposed state [(Takagi et al., 2002)]. Recently FRET (fluorescence resonance energy transfer) has provided an

addition to this model which includes intermediate "primed" conformations [(Chigaev et al., 2003)]. In general, higher affinity states correlate with a greater distance of head domain movement away from the membrane.

A key feature of integrins is their ability to participate in bidirectional transmembrane signalling [(Shattil et al., 2004),(Calvete, 2004)]. Far from being inert anchorage proteins, integrins are able to transmit "Inside-Out" and "Outside-In" signals by virtue of a transmission of conformational changes from the cytoplasmic to extracellular domains (inside-out) and vice-versa (outside-in). This fundamental feature of integrins is most well characterised in the platelet integrin, αIIbβ3. When platelets are activated by agonists (e.g. thrombin), intracellular signals converge on the αIIbβ3 integrin cytoplasmic tail and via integrin conformational change modify the extracellular domain from a low to a high affinity state - "Inside-Out" signalling [(Byzova et al., 2000),(Bertoni et al., 2002)]. Conversely, ligand binding to the extracellular domains results in transmission of altered conformation to the intracellular cytoplasmic tails. This modifies the binding characteristics and activity of intracellular signalling proteins and transduces "Outside-In" signalling [(Ginsberg et al., 2005)].

The membrane-proximal sequences of integrin cytoplasmic tails are highly conserved [(Calderwood, 2004)] (**see Appendix: Fig 2**). The α-subunits have a highly conserved GFFKR (Gly-Phe-Phe-Lys-Arg) amino-acid sequence that auto-inhibits integrin activation and deletion of this sequence results in an increase in integrin affinity [(O'Toole et al., 1994)]. Deletion of membrane proximal portions of the β-integrin similarly increase affinity whilst deletion of membrane distal portions inhibit integrin activation [(Lu et al., 2001)]. In integrin-αIIbβ3, a salt bridge between R995 of αIIb and D723 of β3 stabilises the integrin in a low affinity state [(Hughes et al., 1996)]. Forced

association of the cytoplasmic regions of αLβ2 and αMβ2 blocks activation and forced dissociation promotes activation [Lu et al., 2001),(Kim et al., 2003)]. FRET has shown that higher affinity states are associated with a reduction in FRET indicative of cytoplasmic domain spatial separation. The high affinity state associated with deletions of the membrane-proximal regions is independent of cell type or metabolic energy whereas the more carboxy-terminal modulation of integrin affinity is both energy and cell-type dependent [O'Toole et al., 1994),(Hughes et al., 1995)]. Specific sequences in the β tail outside the membrane-proximal region do modulate integrin affinity; mutations in the conserved NPxY (Asn-Pro-x-Tyr) motif reduce binding of many signalling proteins [Liu et al., 2000)], induce structural changes in the cytoplasmic domain and inhibit integrin activation [Ulmer et al., 2001)]. Deletion of α-chain amino acids distal to the GFFKR sequence inhibits inside-out affinity modulation [Tohyama et al., 2003),(Kassner et al., 1993)]. i.e. the membrane-proximal amino acids stabilize the integrins in a low affinity state whilst the sequence of amino acids distal to these regions regulate activation via interactions with inside-out signalling proteins.

[In addition to integrin affinity changes, integrin clustering (or "avidity") is a complementary mechanism for increasing integrin adhesion and signalling. Clustering may be promoted by several mechanisms including ligand binding [Loftus et al., 1984)], ligand self-association [Simmons et al., 1996)], lateral interactions of integrins with other membrane proteins [Hemler, 1998)], integrin linkages to the cytoskeleton [Bennett et al., 1999)] and homomeric interactions of integrin transmembrane domains [Li et al., 2003)]. As this thesis is concerned with integrin affinity change, clustering changes will not be discussed further].

1.2 Inside-out signalling

In recent years many intracellular signalling proteins have been found to be implicated in inside-out integrin affinity modulation. These are summarised in **Appendix: Table 2**. The most widely studied, Talin and Ras moieties (Hras, Rras, Rap1), are discussed in more detail below.

1.2.1 Talin

Talin is a 235kDa protein which exists as a homodimer that links integrin cytoplasmic domains to the actin cytoskeleton [(Kobayashi et al., 2001)]. Talin binds strongly to β1A, β1D, β2, β3, β5 and weakly to β7 integrin cytoplasmic tails via the talin head [(Calderwood, 2004)]. The talin head domain contains a FERM (four point one, ezrin, radixin, moesin) domain composed of three subdomains (F1,2,3) [(Calderwood et al., 2002)]. F3 has the highest affinity of the three fragments for the cytoplasmic tail of integrin β3, recognising the NPxY motif. Studies using laser tweezer microscopy showed that talin is essential for integrin-cytoskeletal structural linkage [(Jiang et al., 2003)] but as well as a structural role in tethering integrins to the cytoskeleton, talin also plays a key role in regulating integrin activation [(Tadokoro et al., 2003),(Calderwood et al., 2002),(Vinogradova et al., 2002),(Knezevic et al., 1996)] by increasing integrin affinity. Talin knockdown using a RNAi approach directly correlated with a reduction in integrin activation that was not rescued by other integrin activators such as Rras confirming its role as the final common step in inside-out activation of integrins [(Tadokoro et al., 2003)]. Upon binding to talin the α and β-cytoplasmic tails undergo a spatial separation which is transmitted as a conformational change in the extracellular domain resulting in increased integrin affinity [(Kim et al., 2003)].

Talin-mediated modulation of integrin affinity can be further influenced by several intracellular mechanisms; The talin head domain is basic (Rees et al., 1990) enabling it to interact with negatively charged phosphoinositides such as PI45P2 (phosphoinositol-4,5-phosphate). Integrin-ligand binding results in increased PI45P2 synthesis (McNamee et al., 1993) by sequential phosphorylation of PI by PI4K and PI4P5Ks (Anderson et al., 1999). The integrin binding activity of talin is enhanced by its interaction with this membrane phospholipid PI45P2 (Martel et al., 2001). PI45P2 binding to talin induces a conformational change in talin which unmasks the integrin binding site within the FERM domain and increases its affinity for the β1 integrin cytoplasmic tail promoting integrin activation. Talin also binds to and activates a splice variant of the PI45P2-producing enzyme PIPKIγ-90 (Ling et al., 2002),(Di Paolo et al., 2002) raising the possibility of a positive feedback loop. However, PIPKIγ-90 and β3-integrin tails also compete for identical F3 binding sites on talin and may therefore act to inhibit integrin activation in some settings. Another phosphoinositide pathway may act in opposition: Integrin-ECM engagement induces focal adhesion formation. FAK-induced (Focal Adhesion Kinase) association of Src and PIPKIγ-661 (typeI gamma phosphatidylinositol phosphate kinase) and subsequent Src-mediated phosphorylation of PIPKIγ-661 on tyrosine^{644} increases PIPKIγ-661's affinity for talin 15-fold and consequently blocks talin binding to the cytoplasmic tail of β1 integrin (Ling et al., 2003). i.e. Src may inhibit talin-integrin interaction to act as a switch in focal adhesion turnover.

Other phosphotyrosine-binding (PTB) domains can bind NPxY motifs raising the possibility of competition with talin e.g. integrin cytoplasmic domain-associated protein-α (ICAP-1α) binds to β1A via a PTB-domain/NPxY interaction and inhibits

talin association [Bouvard et al., 2003]. Calcium-calmodulin dependent protein kinase II phosphorylates ICAP-1α and regulates β1-integrin-mediated adhesion [Bouvard et al., 1998]. Talin binds to the first NPxY motif of β1-integrin whilst tyrosine phosphorylation of the second NPxY motif inhibits ICAP-1α binding [Chang et al., 2002].

1.2.2 Hras, Rras and Rap

Since its discovery in 1964, the *Ras* superfamily has expanded to a group of over 150 small GTPases (distinct from the large heterotrimeric αβγ G-proteins) that are implicated in many signalling processes in cells. They can be assigned to 6 families; Ras, Rho, Ran, Rab, Arf and Rad/Rem/Kir which are closely interrelated phylogenetically [Ehrhardt et al., 2002] (**see Appendix: Fig 3**).

Within the Ras family are 5 subfamilies: 1) p21ras proteins – Hras, Nras, Kras4A, Kras4B, 2) Rras/TC21 (Rras2), 3) Mras (Rras3), 4) Rap, 5) Ral. All contain a carboxy CAAX (cysteine, aliphatic, aliphatic, any) domain and exhibit a high degree of amino acid sequence homology across a wide variety of species from yeast Saccharomyces.cerevisiae [Haney et al., 1994], to Drosophila.melanogaster [Brock, 1987] and humans [Lowe et al., 1987a].

Although the *Ras* superfamily members have independent and differing functions, the mechanism by which they signal to effectors follows a similar pattern: In the inactive state *Ras* members are bound to GDP. To become activated, Guanidine Exchange Factors (GEFs) catalyse the release of GDP which is then replaced by the more abundant GTP [Lenzen et al., 1998]. GTP-bound *Ras* moieties undergo activating allosteric changes in two regions (termed SwitchI and SwitchII) which facilitate binding and signalling to multiple effectors. The *Ras* family members possess

intrinsic GTPase activity. The dephosphorylation of GTP to GDP results in the insertion of an arginine side chain into the active site of *Ras* resulting in reduced activity. GTPase Activating Proteins (GAPs) increase the rate of dephosphorylation of GTP-GDP and thus play an important role in modifying the rate of conversion of *Ras* moieties to their inactive state. Several important Ras mutants have been developed: A (G12V)Hras mutation inhibits intrinsic GTPase activity resulting in failure to dephosphorylate active GTP-Hras to inactive GDP-Hras. (G12V)Hras is therefore constitutively active. (G38V)Rras is constitutively active in an identical manner. Conversely, (T43N)Rras binds GDP with higher affinity than GTP, rendering it Dominant-Negative [(Huff et al., 1997)].

Cellular Factors Influencing Ras Function

Guanidine Exchange Factors (GEFs)

Nearly all GEFs activate *Ras* by binding via a highly conserved region termed the minimal *Ras* domain of cdc25 (the GEF for Saccharomyces.cerevisiae).

In addition to this cdc25 domain, GEFs contain multiple other domains (**see Appendix: Fig 4**) which enable binding to protein/lipid, diacylglycerol (DAG) and calcium, indicating a much greater capacity for signalling complexity and crosstalk with other pathways. For example, receptor tyrosine kinases acting through effector proteins such as Grb2 can activate Sos (GEF) via interaction of SH2 and SH3 domains [(Gale et al., 1993),(Li et al., 1993)].

Structure-function studies have identified four regions of *Ras* that interact with GEFs; the switchI region (a.a. 25-40), switchII region (a.a. 57-65) and alpha helix3

(a.a. 92-104) plus loop7 (a.a. 105-9). However, the specificity of *Ras* for individual GEFs is poorly understood; for example, the helix3 region of Rras (a.a. 91-103) is responsible for binding of RasGRF1 but not Sos (two GEFs for Rras) [(Tian et al., 2001)]. Each *Ras* moiety is able to bind to several GEFs (**see Appendix: Table 3**) and vice versa and interestingly, the only GEF that does not possess a cdc25 domain, smgGDS, has the broadest *Ras* specificity [(Vikis et al,. 2002)].

Difficulty exists in the interpretation of *Ras* research: Nearly all studies utilise models which involve overexpression of *Ras* moieties. This overexpression may cause mislocalisation resulting in inappropriate recruitment of GEFs and other effectors. This may explain, in part, GEFs apparent marked promiscuity for multiple *Ras* members. Furthermore, overexpression of constitutively active *Ras* for chronic periods at supraphysiological levels may not represent the physiological state. Similarly, Dominant-Negative *Rases* produce their effect by inhibiting release of GDP which causes sequestration of GEFs. Since GEFs interact with several *Ras* members it is possible that the effect of Dominant-Negative overexpression in these experiments is to inhibit other *Ras* pathways. Many published experiments also utilise monoclonal antibodies considered specific to an epitope of p21Ras. However, the epitope consists of residues in SwitchII which are very similar to sequences in Mras and TC21, resulting in cross-reactivity [(Ehrhardt et al., 1999),(Feig, 1999)].

GTPase Activating Proteins (GAPs)

GAPs catalyse the hydrolysis of *Ras*-bound GTP to GDP resulting in a return of *Ras* to the inactive state. This mechanism involves the insertion of an arginine side-chain into the active site of *Ras* [(Sermon et al., 1998)]. Known GAPs involved in Hras, Rras and

Rap signalling are summarised in **Appendix: Table 3**. Similar to GEFs, GAPs exhibit broad *Ras*-binding specificity and are able to interact with other signalling pathways e.g. GAP1m and GAP1^{IP4BP}/Rras-GAP) can be regulated via binding to inositol phosphates and GAP1m may interact with the large heterotrimeric GTPases by binding directly to Gα12 [(Wittinghofer, 1998),(Jiang et al., 1998b)].

Membrane targeting via CAAX and posttranslational modification

The carboxy-terminal CAAX motif of *Ras* family members is a target for enzymic processing which results in the addition of a farnesyl or geranylgeranyl group [(Casey, 1995)] essential for *Ras* localisation to the plasma membrane. Inhibitors of this process block *Ras* function. Further modifications influence the activity of *Ras* moieties [(Hancock et al., 1990),(Hancock et al., 1989)]; for example Hras, Nras, Kras4A and TC21 become palmitoylated and this results in transport via the Golgi into cholesterol-rich membrane lipid rafts [(Hancock et al., 1990)]. The localisation of Hras to lipid rafts is essential for its activation of downstream effectors, possibly by regulating the proximity to other signalling proteins and GEFs [(Roy et al., 1999),(Jaumot et al., 2002)]. In contrast Kras4B and Mras lack palmitoylation sites but have multiple basic residues that stabilise non-raft membrane localisation; these moieties bypass the Golgi and transport to bulk plasma membrane in a tubulin-dependent manner [(Apolloni et al., 2000),(Thissen et al., 1997)].

Recently, further insights into subcellular movements of palmitoylated *Ras* members have been elucidated [(Rotblat et al., 2006)]; palmitoylated Hras and Nras are transported through the cytoplasm on 80-100nm cytoplasmic "rasosomes" that are not typical plasma membrane or Golgi-derived vesicles. This mechanism is dependent on the

hypervariable region of Ras and is not inhibited by actin cytoskeleton or microtubule disruption.

1.2.3 Ras Effectors

Many proteins have *Ras*-binding Domains (RBDs) capable of interacting with Ras proteins (**see Appendix: Table 4**) though not all of these have been proven to be involved in *Ras*-mediated signalling:

GEFs

A number of Ral-GEFs (e.g. RalGDS, Rgl, Rlf/Rgl2, RPM/Rgl3, RalGPS/Ral-GEF2, Rgr [Albright et al., 1993),(Kikuchi et al., 1994),(Spaargaren et al., 1994),(Shao et al., 2000),(D'Adamo et al., 1997] are also effectors for Ras, Rap and Rit and this has been shown to result in, for example, Ras-mediated activation of RalA [Rosario et al., 2001]. Similarly, two Rap-GEFs (MR-GEF and RA-GEFII) are known to function as Mras effectors; overexpression of Mras and RA-GEFII results in activation of Rap1A [Gao et al., 2001]. Similarly, activated Hras stimulates PLCε-mediated hydrolysis of PI45P2 [Kelley et al., 2001].

Raf-family serine-threonine kinases (Raf1, A-Raf, B-Raf)

p21Ras members activate mitogen activated protein kinases (MAPKs) to promote proliferation via a cascade involving initial activation of Raf [Vojtek et al., 1993),(Zhang et al., 1993]. The precise mechanism of activation of Raf is not known though this may involve direct activation by *Ras* binding and indirect via recruitment of Raf to the plasma membrane [Rizzo et al., 2000]. Kras and Hras are more efficient activators of Raf than Rras and Mras [Voice et al., 1999]. The amino-acid residues of Hras critical to binding

Raf include a.a. 31-45 (which neighbours the effector region) and a.a. 56-59 (n-terminal of switchII region) [Shirouzu et al., 1994].

MEKK-1

Another serine-threonine kinase, MEKK-1, an activator of MEK1/2 and Erk1/2, JNK and p38mapk [Lange-Carter et al., 1993],[Xu et al., 1996], binds directly to and is activated by the effector domain of active p21Hras [Russell et al., 1995].

Phosphoinositol-3-Kinase (PI3K)

This critical enzyme with lipid and protein kinase activity binds directly to the effector domain of GTP-bound Ras via its catalytic p110α [Rodriguez-Viciana et al., 1994] subunit, catalysing the activation of PI3K effectors such as PKB [Katso et al., 2001] which promote cell survival. Activated Rras and Mras are more efficient activators of PI3K than Hras [Kimmelman et al., 2000]. *Ras* members can activate Rho via PI3K; PI3K binds to PH domains of Sos1/2, Vav and Tiam-1 [Nimnual et al., 1998],[Han et al., 1998],[Michiels et al., 1997] which stimulate nucleotide exchange on Rac resulting in activation and increased mobility.

Nore1

In a yeast two-hybrid screen a presumed adaptor protein containing several SH3-domain binding sites and a DAG-binding site, Nore1, was identified as a protein that interacts with GTP-bound Hras, Mras and Kras4B [Vavvas et al., 1998] though its function remains unknown.

AF-6

AF-6 is the mammalian homologue of the Drosophila protein Canoe and is believed to participate in cell-cell adhesion regulation downstream of Ras [(Taya et al., 1998)]. Ras and Canoe are functionally linked in Drosophila ommatidial development [(Gaengel et al., 2003)], though in Drosophila dorsal closure AF-6 binds preferentially to activated Rap1 and acts as a Rap1 effector [(Boettner et al., 2003)].

1.2.4 Functions of Ras

Ras moieties have multiple well documented functions in development, transformation, growth stimulation and inhibition, differentiation and adhesion [(Ehrhardt et al., 2002)]. Hras and Rras share approximately 55% sequence homology with the main region of divergence being the c-terminal 26 amino acids [(Lowe et al., 1987a)]. Despite similar amino-acid sequences, Hras and Rras expression in mammalian cells produces significantly different responses. Though both are able to induce cellular transformation dependent upon conserved sequences in their effector binding domain (amino acids 32-40 of Hras [(Huff et al., 1997)]) and the integrity of their c-terminal CAAX domains [(Buss et al., 1989)], Hras is able to cause cellular transformation in a wide range of cells whilst Rras is much more cell type specific in this respect. Furthermore, Rras-transformed NIH3T3 cells do not display all of the morphological features associated with Hras transformation [(Cox et al., 1994)]. Amino acids 5-120 of Hras share 71% homology with Rras [(Lowe et al., 1987a)]. However, amino acids 121-189 show the greatest divergence with only 41% homology. Lowe et al. [(Lowe et al., 1988)] substituted the aligned sequences of Hras and Rras that showed the greatest sequence divergence (Rras1-30/Hras1-4, Rras52-57/Hras26-31, Rras67-78/Hras41-52, Rras112-

124/Hras86-98, Rras137-218/Hras111-end) to assess which amino acid regions were most important in Hras/Rras-mediated transformation and integrin affinity modulation. Exchanging amino-acids 1-30 of Rras for 1-4 of Hras did not alter the Hras transforming phenotype. It is not surprising that the first 26 amino acids of Rras are not involved in signalling function since Xray crystallography predicts that these 26 amino acids extend away from the GTP binding site [(McCormick et al., 1985)]. Only substitution of the c-terminal tail altered Hras transformation to an Rras phenotype, though it is unclear whether this relates to alterations in c-terminal-dependent membrane targeting. Also, replacing amino acids 137-218 of Rras with 111-189 of Hras does not induce a Hras-transforming phenotype. i.e. amino acids 111-189 are essential but not solely responsible for a Hras transformation phenotype [(Lowe et al., 1988)].

Hras and Rras also produce different responses to serum deprivation; whereas Hras induces survival in response to growth factor withdrawal [(Chou et al., 1997)], Rras increases apoptosis in response to serum deprivation [(Wang et al., 1995)]. Furthermore, Rras differs from Hras in its failure to induce neurite outgrowth in PC12 cells [(Rey et al., 1994)]. These differences may, in part, relate to differences in specificity for shared downstream effectors as described above.

In the following section Ras-mediated integrin affinity modulation and adhesion will be discussed in more detail:

Three Ras moieties have been shown to play a critical role in integrin affinity modulation; Hras, Rras and Rap. In most model systems Hras decreases whilst Rras and Rap increase integrin affinity.

Hras

Hughes et al showed that constitutively active (G12V)Hras reduced integrin affinity in Chinese Hamster Ovary cells and this was not related to integrin phosphorylation or dependent on de novo mRNA or protein synthesis [(Hughes et al., 1997)]. Hras residues 148-171 were critical for this suppression [(Hughes et al., 2002)].

Initially it was considered that the canonical ras-raf-mapk pathway was likely to be implicated in integrin affinity suppression. However, although Raf1, the downstream effector of Hras, is required for Hras-mediated integrin suppression [(Hughes et al., 1997)], erk phosphorylation and integrin suppression were able to be uncoupled using Hras mutants. Also α5β1 suppression by constitutively active Raf1 is not inhibited by MKP3 (a mapk phosphatase) or MEK inhibitors (e.g. PD98059) and overexpression of activated MEK does not suppress integrin affinity [(Hughes et al., 2002)]. Thus it appears that Hras and Raf1 suppress integrins independent of erk/mapk activation. An alternative hypothesis is that erk/mapk *are* involved, but in specific cellular compartments that are not accurately assessed in experiments assessing bulk cellular activation. It is important to note, however, that Hras-mediated suppression is highly contextual; Hras suppresses integrin affinity in fibroblasts but activates in other cell types [(Kinbara et al., 2003)]. For example, Dominant-Negative Hras reduces α4/5β1 affinity in haemopoietic Baf-3 cells [(Shibayama et al., 1999)] and decreases ICAM (intercellular adhesion molecule) binding in eosinophils [(Myou et al., 2002)]. LFA1 (lymphocyte function-associated antigen, also known as αL/β2) is activated by Hras in adult T cell leukaemia cells [(Tanaka et al., 1999)]. In model systems where Hras activates integrins it is usually via PI3K since it is blocked by inhibitors of PI3K, Ly294002 and

wortmannin, and reproduced by overexpression of p110α. Models demonstrating integrin suppression are usually mediated via Raf1.

Also note that in some models integrin affinity modulation may occur via non-Raf mediated mechanisms e.g. (G12V)Hras causes α2-6 sialylation of β1-integrins due to altered expression of ST6Gal-1 (a sialylator) and increased sialylation modulates collagen binding in a HD3 colonocyte overexpression model (Seales et al., 2003).

Rras

In contrast to Hras, Rras increases integrin affinity. Ruoslahti showed initially that constitutively active Rras converted mouse 32D and human myeloid cells from suspension to adherent phenotype (Zhang et al., 1996). Dominant-Negative (S43N)Rras produced the opposite effect. In these models Rras increased the affinity of integrins αIIbβ3 and α5β1 for fibronectin. Subsequently it was shown that stably expressed, constitutively active Rras stimulates α2β1-dependent migration of breast epithelial cells across collagen (Keely et al., 1999) and this is reversed by Dominant-Negative Rras. Rras also induces extensive spreading in PC12 cells (Self et al., 2001), neurite outgrowth in embryonic retinal cells (Ivins et al., 2000) and mast cell adhesion to fibronectin (Kinashi et al., 2000). Rras antagonises the Hras/Raf1-mediated integrin suppression pathway, though this effect is not due to simple competition for the common effector Raf1 since Rras can reverse suppression of a Raf mutant that does not possess a Ras-binding domain (Sethi et al., 1999a), and acts independently of erk/mapk since it does not correlate with erk activation. Unexpectedly, mutations of the effector binding domain (EBD), which mediates interaction with Rras's known effectors (Raf/PI3K/Nore1/RalGDS), did not correlate well with abrogation of integrin affinity

modulation [Sethi et al., 1999a),(Oertli et al., 2000),(Hansen et al., 2002] suggesting that known effectors are not involved. Using Hras/Rras chimaeras Hansen et al [Hansen et al., 2002] showed that the terminal 26 amino acids are sufficient to confer differential integrin affinity modulation, though again this may relate to alterations in subcellular localisation; Hras undergoes farnesylation and palmitoylation whilst Rras undergoes palmitoylation and geranylgeranylation [Lowe et al., 1987b] and Oertli et al showed that a c-terminal cysteine to serine mutation that blocked prenylation was also able to inhibit constitutively active (G38V)Rras-mediated affinity upregulation [Oertli et al., 2000].

Although little is known about the mechanisms mediating Rras's modulation of integrin affinity, several groups have identified downstream mediators: In macrophages, Rras activates integrin αMβ2 via Rap1 resulting in increased red blood cell phagocytosis [Self et al., 2001),(Caron et al., 2000]. In haemopoietic and epithelial cells a known Rras effector, PI3K, has been shown to be involved [Keely et al., 1999] in integrin affinity upregulation. Berrier et al [Berrier et al., 2000] also demonstrated that Rras propagates signals via PI3K to Rac and PKCε to regulate integrin-dependent cell spreading.

Rras possesses a proline rich sequence in amino acids 199-206 that binds the SH3 domain of Nck, a likely adaptor protein (acts via SH2 domain with p130Cas/PTKs/FAK). When this site was mutated in a mouse monocyte adhesion model, Rras was no longer able to increase integrin affinity for fibronectin [Wang et al., 2000].

Rap

Rap was originally identified as a reverser of Kras phenotype [(Kitayama et al., 1991)]; it does this by competing for Raf and reducing erk phosphorylation (though this may be an overexpression phenomenon). Rap GEFs include C3G, Epac, RasGRP2/3, RA-GEFI/II, MR-GEF and PLCε. Rap-GAPs are Rap1-GAPII and SPA-1 and GAP^{1P4BP} (**see Appendix: Table 3**). Rap works downstream of many cytokines and growth factors to increase integrin affinity e.g. Erythropoietin and Interleukin-3 cause Rap-mediated activation of α4/5β1-integrins and this is blocked by PLCγ inhibition [(Arai et al., 2001)]. Overexpression of constitutively active (G12V)Rap in macrophages increases αMβ2-mediated binding to C3bi-opsonised particles [(Caron et al., 2000)]. CD98, a multispan transmembrane protein can reverse Hras-induced integrin suppression [(Fenczik et al., 1997)] and CD98 crosslinking causes Rap activation to sustain αLβ2-dependent cell adhesion [(Suga et al., 2001)]. PECAM (platelet endothelial cell adhesion molecule-1) in neutrophils increases αLβ2 + α4β1-mediated adhesion in a Rap-dependent manner; Rap expression obviates the need for PECAM and Rap-GAP abrogates this [(Reedquist et al., 2000)]. TCR crosslinking also causes Rap activation [(Sebzda et al., 2002)]. Constitutively active Rap1 increased αIIbβ3 affinity in megakaryocytes [(Bertoni et al., 2002)] and αLβ2 affinity in Jurkat cells [(Katagiri et al., 2000),(Kinashi et al., 2000)].

Indirect confirmation of Rap's role in integrin control is provided by experiments manipulating Rap-GEFs and GAPs: C3G (a Rap-GEF) knockout causes reduced adhesion which is rescued by (G12V)Rap or other Rap-GEFs (e.g. Epac) [(Ohba et al., 2001)]. SPA1 (a Rap-GAP) overexpression reduces Rap activity and decreases β1-mediated cell adhesion to fibronectin [(Tsukamoto et al., 1999)] and αLβ2/α4β1-mediated adhesion [(de Bruyn et al., 2002)]. However, not all of Rap's effects on adhesion and motility

are affinity-mediated; Rap can also affect avidity in a cell-type specific manner [Sebzda et al., 2002].

Little is known about Rap's effectors: Peterson et al [Peterson et al., 1996] showed that the CAAX box was not essential and hypothesised that Rap's reversal of Hras-mediated integrin suppression involved EBD-dependent competition for other GEFs such as Rgl2. Nore1B (also known as RapL) interacts with active Rap in a yeast 2 hybrid screen and binds to GTP-Rap via a Ras association (RalGDS/AF-6) domain, resulting in hydrolysis to GDP-Rap. Nore1B coimmunoprecipitates with integrin αLβ2 and Nore1B overexpression causes αLβ2 clustering and increased affinity [Katagiri et al., 2003],[Tommasi et al., 2002]. An amino terminal-truncated Nore1B inhibits TCR-induced, Rap-dependent T cell adhesion though this may be through sequestration of Rap rather than direct Nore1 involvement. However, although Rras binds Nore1, an EBD mutation that inhibits Nore1 binding did not block Rras-mediated integrin activation [Oertli et al., 2000].

Hypothesis:

Extracellular matrix binding to β1-integrins prevents apoptosis in response to chemotherapy and radiotherapy. This is a result of β1-integrin-mediated modulation of classical apoptotic signalling proteins (Bax/Bcl and caspases), overriding of cell cycle checkpoints and alterations in DNA damage and repair mechanisms.

Chapter 2

Materials and Methods

2.1 Reagents

Dulbecco's Modified Eagles Medium (DMEM); penicillin; streptomycin; dialysed foetal bovine serum (FBS); BCA protein reagent; L-glutamine; 30% albumin (w/v); non-essential amino acids; 30% (w/v) acrylamide/bis solution and LipofectAMINE^{TM} Plus reagent were purchased from Life Technologies (Paisley, UK). Wortmannin and Ly294002 were purchased from Calbiochem-Novabiochem Corporation (Nottingham, UK). Hybond C nitrocellulose membrane and enhanced chemiluminescence (ECL) reagent were purchased from Amersham Life Science Ltd. (Cheshire, UK). JET-PEI^{TM} transfection reagent was purchased from Q-BIOgene. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (Dorset, UK).

2.2 Antibodies

Anti-HA (Y-11), anti-myc (9E10 and A14) and anti-ERK2 (C-14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-ERK1/2 (ERK-PT115), anti-FLAG M2 monoclonal antibody, anti-actin (AC-40), anti-mouse IgG, mouse anti-p21Cip1/WAF1 (clone CP74) and mouse anti-p27Kip1 (clone DCS-72) were purchased from Sigma (Dorset U.K). All anti-species specific horseradish peroxidase-conjugated antibodies and RPE-Conjugated monoclonal mouse anti-

Human CD25 Interleukin-2 receptor were purchased from DAKO (Bucks, UK). All anti-species specific Alexa-Fluor-conjugated antibodies were purchased from Molecular Probes (The Netherlands). Function-blocking β1-antibody, 4B4, was purchased from Coulter (Hialeah, Florida). Function-activating β1-antibody, TS2-16, was obtained from an in-house hybridoma. Anti-PAC1 (activation specific αIIbβ3 antibody) and mouse anti-DNA-PKcs (p350 clone 6) were purchased from BD Biosciences (Oxford, UK). Rabbit anti-cleavage site-specific caspase-9 antibody (CCSA 315/316), rabbit anti-caspase-3 (Asp179) (5A1), anti-GSK-3α/β and rabbit anti-phospho-GSK-3β (pS9) were purchased from BioSource International. Mouse anti-cyclin A was purchased from Oncogene Research Products, USA. Mouse anti-cyclin B was purchased from BD Transduction Labs, USA. Mouse anti-cyclin D1 (clone AM29) was purchased from Zymed. Mouse anti-cyclin E (clone HE12) and mouse anti-γH2Ax were purchased from Upstate. Rabbit anti-phospho-CDK2 (Thr160), rabbit anti-phospho-CDK1 (Tyr15), rabbit anti-phospho-Bad (Ser136) and rabbit anti-Bad (9292) were purchased from Cell Signaling Technology, USA.

2.3 DNA constructs

Mouse Notch1^{IC}V1744 construct was a generous gift from R.Kopan (Washington University School of Medicine, St Louis, USA) and was provided carboxy-terminal myc-tagged in a pEGFP1 vector (with a stop codon proximal to the GFP sequence). To standardise controls, Notch1^{IC}V1744 was cloned from pEGFP1 into the HindIII/Apa1 sites of pcDNA3.1^{+}. For coimmunoprecipitation and immunofluorescence experiments which employed cotransfection of Notch1^{IC}V1744 and myc-tagged Ras moieties, Notch1^{IC}V1744 was myc-untagged by digestion with

BamH1 and Sal1 prior to cloning into pCMV-Tag4A (which contains a Flag-tag at the 3-primed end). HA-tagged pDCR-(G12V)Hras and Myc-tagged pSG5-(G38V)Rras genes were kindly donated by Dr. M.Ginsberg (Scripps Research Institute, La Jolla, USA). These genes were previously subcloned into pCDNA3.1^{+} in this laboratory by Dr J.Love. Myc-tagged (wt)Rras and (T43N)Rras in pSG5 were kindly donated by Hall (Scripps, La Jolla, CA). Interleukin-2 receptor subunit reporter construct, pBR322-Tac-α5 was kindly donated by S. E. LaFlamme (Centre for Cell Biology and Cancer research, NY, USA). All plasmids were sequenced prior to use and the integrity of subsequent maxiprep products were reconfirmed by electrophoresis of endonuclease digested samples.

2.4 Cell Culture

Chinese Hamster Ovary cells stably expressing the chimaeric integrin αIIbβ3/α6β1 (CHOαβpy) in association with a gene resistant to G418 were a generous gift from Professor Mark Ginsberg. These were cultured in CHO complete media: DMEM supplemented with 10%(v/v) heat-inactivated foetal calf serum (Labtec), 5mg/ml L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin (Invitrogen Ltd, UK), 400μg/ml G418 (Invitrogen) and 1% NEAA (Life Technologies). Cells were passaged on reaching confluence every three to five days. Cells beyond passage 12 were discarded and new stocks brought up from frozen. In experiments where CHO cells were quiesced, CHO quiescent media contained DMEM supplemented with 0.25%(w/v) BSA, 5mg/ml L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 1% non-essential amino acids.

K562 cells were cultured in K562 complete media: HEPES buffered RPMI 1640 medium (Invitrogen Ltd, UK) supplemented with 10%(v/v) heat-inactivated foetal calf serum (Labtec), 5mg/ml L-glutamine (Life Technologies, Paisley, UK) , 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen Ltd, UK).

GD25 Null cells are murine embryonic fibroblasts which do not express β1-integrin and GD25 β1 cells stably express β1-integrin in association with a puromycin resistance gene. GD25 Null cells were cultured in GD25 complete media: DMEM (Life Technologies) supplemented with 10%(v/v) heat-inactivated foetal calf serum (Labtec), 5mg/ml L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin (Invitrogen Ltd, UK) and 1% non-essential amino acids (NEAA-Life Technologies). GD25 β1 cells were cultured in the same media plus 10μg/ml puromycin (Sigma). In experiments where GD25 cells were quiesced, GD25 quiescent media contained DMEM supplemented with 0.25%(w/v) BSA, 5mg/ml L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 1% non-essential amino acids.

NCI-H69, NCI-H345 and NCI-H510 small cell lung cancer lines were obtained from American Type Tissue Culture Collection (ATCC - Rockville, Maryland, USA). These were cultured in SCLC complete media: HEPES buffered Rosewell Park Memorial Institute Medium 1640 (RPMI) 1640 medium (Invitrogen Ltd, UK) supplemented with 10%(v/v) heat-inactivated foetal calf serum (Labtec), 5mg/ml L-glutamine (Life Technologies, Paisley, UK) , 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen Ltd, UK). For experiments, cells were cultured in either SITA (RPMI supplemented with 30nM selenium, 5μg/ml insulin, 10μg/ml transferrin, 0.25%(w/v) BSA, 100U/ml penicillin and 100μg/ml streptomycin) or

SCLC quiescent media; RPMI medium supplemented with 0.25%(w/v) BSA, 100U/ml penicillin and 100μg/ml streptomycin.

2.5 Transformation

Frozen Escherichia coli aliquots (TOP10, Groningen, Netherlands and DH5α, Life Technologies, Paisley, UK) were thawed on ice and transferred to chilled eppendorf tubes. Purified plasmid DNA (1-10ng) was gently mixed with the cells and incubated on ice for 30 minutes. Cells were then heat-shocked at 42°C in a thermomixer for 60 seconds and returned to ice for a further 2 minutes. Transformed cells were grown in antibiotic free SOC media for 60 minutes at 37°C at 500rpm in a thermomixer, prior to spreading onto appropriate antibiotic selection plates. Plates were incubated at 37°C overnight. Individual colonies were picked into Luria-Bertani (LB) broth containing selection antibiotics and grown at 37°C overnight. Appropriate plasmid expression was confirmed by commercial DNA sequencing analysis (Stratagene).

2.6 DNA purification, cloning and Agarose Gel Electrophoresis

Mini-prep (1-10ml) DNA purifications were performed using the Wizard SV Miniprep kit (Promega) as per manufacturer's instructions. Large scale (100-500ml) DNA purifications were performed using the Qiagen Endotoxin Free Maxi-prep kit (Qiagen, Crawley, UK) as per manufacturer's instructions.

Diagnostic restriction digests were performed on purified DNA with the appropriate restriction enzymes (Promega) and resolved on 1-2% agarose (Seakem, Rockland, Maine, USA) gels containing 0.3μg/ml ethidium bromide to enable UV visualisation.

A Pharmacia Biotech Ultraspec 2000 UV spectrophotometer was used to quantify purified DNA.

When cloning, restriction digest products were gel purified using the Quick Gel extraction kit (Qiagen), as per manufacturer's instruction. Ligation into second vector was performed using T4 DNA ligase (Promega) as per manufacturer's instructions.

2.7 Transfection

Transient transfections of αβ-py and CHO-K1 cells were performed with LipofectAMINE^{TM} Plus reagent as per manufacturer's instruction. Cells were seeded at a density to achieve ~50% confluency overnight in 100mm or 60mm dishes (Corning, High Wycombe, UK). Purified DNA was placed in 5ml BD Falcon tubes (Becton Dickinson, Oxford, UK) in a volume ranging from 1–40µl. The DNA was pre-complexed with 110µl of the Plus reagent mixture (comprising Plus reagent 0.25%(v/v) diluted in DMEM containing 1%(v/v) non-essential amino acids) and gently mixed. After a 15 minute incubation at room temperature, 110µl of LipofectAMINE^{TM} mixture (comprising LipofectAMINE^{TM} 0.25%(v/v) diluted in DMEM containing 1%(v/v) non-essential amino acids) was added and incubated for a further 15 minutes at room temperature. The mixture was then made up to a total volume of 4ml with pre-warmed DMEM containing 1%(v/v) non-essential amino acids and placed onto the washed cells. Cells were cultured for five hours at 37ºC, before addition of complete media (4ml), containing 10% FCS. After 24 hours, media containing DNA was removed and replaced with fresh complete media. For experiments where protein kinase activities were to be assessed, the transfection

media was replaced with quiescent media. Cells were assessed 48 hours after transfection, either for lysis and SDS-PAGE analysis, or for integrin affinity determination. For some transfections JET-PEI™ was used as per manufacturer's guidelines.

K562 cells were transfected by AMAXA electroporation (program P13) according to manufacturer's instructions. 5μg of test DNA was used per transfection of 5 million cells.

2.8 Cell lysis

Cells were washed with ice cold PBS and lysed in buffer containing 50mM HEPES pH 7.4, 0.3M NaCl, 1.5mM $MgCL_2$, 1.2mM EDTA, 0.5% Triton X-100, 20mM β-glycerophosphate, 100mM sodium fluoride, 10mM sodium pyrophoshphate, 1mM sodium vanadate, 0.5mM dithiothreitol. One Complete™ protease inhibitor tablet (Boehringer Mannheim, Lewes, UK) was added per 50ml of lysis buffer. Lysates were clarified by centrifugation at 13,000rpm for 10 minutes at 4ºC and analysed for protein concentration. Samples were heated in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1 x SDS-PAGE sample buffer: 50mM Tris-HCl, 10%(v/v) glycerol, 2%(v/v) SDS, 0.1%(v/v) bromophenol blue, 10%(v/v) β-mercaptoethanol, pH 6.8) at 99ºC for 10 minutes.

2.9 Protein Assay

Protein concentrations of lysates were quantified using a BCA protein assay (Pierce, IL, USA). Samples were diluted 1:5 or 1:10 in distilled H_2O and 10μl incubated with 200μl of test solution for 30 minutes at 37ºC in 96-well plates. Plates were read

using an automated plate reader (MRX microplate reader, Dynatech, Chantilly, USA). Samples were assayed in triplicate and graded against a standard curve (0.1–0.5mg/ml) using bovine serum albumin.

2.10 SDS polyacrylamide gel electrophoresis and Western blotting

Samples were resolved on SDS polyacrylamide gels (Separating Gel: 0.375M Tris base (pH 8.8), 0.1%(v/v) SDS, 8–12%(v/v) acrylamide, 0.1%(v/v) ammonium persulphate, 0.02%(v/v) TEMED. Stacking Gel: 0.13M Tris base (pH 6.8), 0.15%(v/v) SDS, 4.6%(v/v) acrylamide, 0.11%(v/v) ammonium persulphate, 0.02%(v/v) TEMED) using a vertical electrophoresis tank Biorad Mini Protean II system (Biorad, Hemel Hempstead, UK). Samples were electrophoresed at 80–150 volts using electrophoresis buffer (50mM Tris base, 250mM glycine, 0.1%(v/v) SDS) for 1–2 hours, adjacent to pre-stained molecular weight markers (Life Technologies, Paisley, Glasgow). For optimal protein separation, 8% gels were used for 60–120kDa proteins, 10% gels for 40–70kDa and 12% gels for 10–40kDa proteins.

Proteins were transferred onto Hybond C nitrocellulose membranes in transfer buffer (210mM glycine, 24.7mM Tris base, 20%(v/v) methanol) at 100volts for 60–90 minutes, in a Mini Protean II blotting tank (Biorad, UK). Protein loading was confirmed by staining with 1% Ponceau S for 5 minutes to visualize protein bands. Non-specific binding sites were blocked by incubation with PBS-Tween 20 (PBS containing 0.2%(v/v) Tween) containing 5% non-fat dried milk powder for 1 hour at room temperature. Membranes were probed with appropriate antibodies diluted in PBS-Tween 20 containing 5% non-fat dried milk powder overnight at 4ºC. They

were then washed vigorously in PBS-Tween 20 for 1 hour, before exposure to appropriate species-specific horseradish peroxidase-conjugated (HRP) secondary antibodies for 1 hour, diluted in PBS-Tween 20 containing 5% non-fat dried milk powder. Membranes were then washed further in PBS-Tween 20 and immunoreactive bands identified using enchanced chemiluminescence (ECL) according to the manufacturer's instructions. For assessment of phosphorylated proteins, blocking and subsequent incubation steps were carried out using 3% albumin in PBS.

2.11 Integrin affinity determination by flow cytometry

The integrin affinity status of transfected αβ-py cells was assessed by three-colour flow cytometry. Cells were transfected as described above with 0–1μg test DNAs (Notch, Ras, Raf) and 0.75μg Tac-α5 transfection reporter construct. After 48 hrs, transfected cells were trypsinised and resuspended in a total volume of 50μl of HEPES/NaCl buffer (20mM HEPES, 140mM NaCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$ and 2mg/ml Glucose, pH 7.4) containing PAC1 antibody (5μg/ml) for 30 minutes at room temperature. Internal controls were performed for each sample with 5mM EDTA (for maximal integrin suppression) or 100μM $MnCl_2$ (for maximal integrin activation). Cells were washed in cold PBS and all subsequent steps were carried out on ice. Samples were incubated in 50μl DMEM containing 4%(v/v) anti-mouse IgM-FITC (Biosource, Nivelles, Belgium) for 30 minutes in the dark. Cells were washed again with cold PBS and were incubated for 30 minutes in 50μl DMEM containing 2%(v/v) anti-Tac-R-phycoerythrin (R-PE) (DAKO, Ely, UK). Cells were finally washed and resuspended in cold PBS. 5 minutes prior to analysis on a FACS-Caliber

flow cytometer, ToPro3 (Molecular Probes, Leiden, The Netherlands) was added to a final concentration of 1μM per sample. Integrin affinity was determined by flow cytometric assessment of PAC1 binding in live and highly transfected cells (ToPro3 negative and high Tac binding respectively). The integrin Activation Index (AI) was calculated by: Activation Index (%) AI = $(F_N - F_I) / (F_A - F_I)$ x 100. This was then used to calculate the percentage integrin suppression: $((AI_0\text{-}AI)/(AI_0)*100)$. [$F_N$: Geometric mean fluorescence intensity (MFI) of PAC1 binding to native integrin. F_I: MFI of PAC1 binding in the presence of 5mM EDTA, i.e. maximally suppressed. F_A: MFI of PAC1 binding in the presence of 100μM $MnCl_2$, i.e. maximally activated. AI_0 is the Activation index with the control vector and AI is the Activation index with DNA under test].

2.12 Flow cytometric analysis of β1-integrin expression on the surface of GD25 Null and GD25 β1 cells

$5x10^5$ GD25 cells were trypsinised, washed twice with PBS, resuspended in 100μl PBS and incubated with 1μg of rat anti-mouse β1-integrin (9EG7 - BD PharMingen, San Diego, CA) or rat anti-mouse IgG1 at room temperature for 1 hour. After two washes with cold PBS, cells were incubated with species-specific fluorescein isothiocyanate-conjugated secondary antibody (1:50) for 30 min at 4°C and again washed twice with cold PBS. Samples were finally resuspended in PBS at 4°C and analyzed by flow cytometry using FACSCalibur™ (BD Biosciences).

2.13 Proliferation assays

GD25: GD25 β1 and GD25 Null growth curves were performed on plastic and fibronectin. 10,000 cells were plated per 10cm plate, suspended in 15mls of GD25 complete media and incubated at 37^{o}C. When counting, media was removed and the adherent cells were washed twice with filtered PBS. After resuspension in filtered PBS, cells were counted using a Coulter counter.

SCLC: 0.5x10^{4} H345 cells were plated into 24-well plates coated with poly-l-lysine (10μg/ml) or laminin (10μg/ml) and following disaggregation, cell numbers were counted at times indicated using a haemocytometer.

2.14 Apoptosis

GD25 cells: 96 well plates were pre-coated by incubation at 37^{o}C for 1 hour with or without 10μg/ml laminin or 20μg/ml fibronectin. 2x10^{4} GD25 cells were plated per well either a) in GD25 complete media and allowed to adhere for 6 hours followed by overnight quiescence in GD25 quiescent media or b) in SITA with cells remaining in SITA for the duration of the experiment. Cells were then treated with etoposide 0-20μg/ml. The percentage of apoptotic cells was assessed 0-48 hours later a) morphologically by immunofluorescence microscopy after addition of 1μl of ethidium bromide (1mg/ml) and 1μl of acridine orange (1mg/ml) [Small orange = late apoptosis, large orange = necrosis, small green = early apoptosis, large green = healthy cell] and b) by flow cytometric analysis of AnnexinV-PE and Topro3 staining after trypsinisation and resuspension in AnnexinV buffer according to the manufacturers instructions.

SCLC cells: were quiesced overnight at 37°C in RPMI supplemented with 0.25% bovine serum albumin, 5mg/ml L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cells were washed twice and seeded ($4x10^4$ cells in 200µl of RPMI SITA per well) into 96 well plates which had been pre-coated by incubation at 37°C for 1 hour with poly-l-lysine (10µg/ml), laminin (10µg/ml), fibronectin (20µg/ml), or TS2/16 (10µg/ml). Cells were allowed to settle for 1 hour at 37°C and then treated with sodium orthovanadate (Na_3VO_4, 200µM), Ly294002 (30µM), tyrphostin-25 (25µM) or etoposide (25µg/ml) as specified. Cells were treated with ionising radiation (1-16Gray) using a 6MV linear accelerator. In experiments using 4B4 to block β1 integrin function, cells were incubated with 4B4 (10µg/ml) at 37°C for 30 minutes prior to seeding onto ECM. The percentage of apoptotic cells was assessed morphologically 0-72 hours later by immunofluorescence microscopy after addition of 1µl of ethidium bromide (1 mg/ml) and 1µl acridine orange (1mg/ml). Apoptosis was also determined using a cell death detection ELISA™ kit (based on the quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes) according to the manufacturer's instructions and by flow cytometric analysis of Annexin V and Topro3 staining according to the manufacturer's instructions. In the Caspase-3 activity assay quiesced SCLC cells were adhered to poly-l-lysine (10µg/ml), laminin (10µg/ml), fibronectin (20µg/ml), or TS2/16 (10µg/ml) as specified and treated with sodium orthovanadate (Na_3VO4) (200µM), Ly294002 (30µM), tyrphostin-25 (25µM), etoposide (25µg/ml) or z-VAD (100µM) as indicated. Cells were lysed at 48 hours and caspase-3 activity was assessed using a caspase-3 cellular activity kit according to the manufacturer's

instructions. Specific caspase-3 activity (pmol/min/mg protein) in each experiment was normalised to untreated cells.

K562 cells: 24-72 hrs after AMAXA electroporation the percentage of apoptotic cells was assessed morphologically by immunofluorescence microscopy after addition of 1µl of ethidium bromide (1 mg/ml) and 1µl acridine orange (1mg/ml).

2.15 Immumnofluorescent Microscopy

Notch expression; wild-type (wt) Chinese Hamster Ovary (CHO) cells were plated onto alcohol-sterilised coverslips in 6 well plates and transfected with $Notch1^{IC}V1744$ construct using jet-PEI lipofection reagents according to manufacturer's instructions. After 48 hours cells were washed twice with phosphate buffered saline (PBS), fixed with 3% paraformaldehyde, washed with PBS, permeabilised with 0.1% Triton, washed with PBS and blocked with 3% fish skin gelatin. Cells were probed with 1:2000 anti-myc (9E10) antibody for 1 hour at room temperature and washed with PBS. Secondary species-specific antibody labelled with ALEXA-flour488 (1:500) was applied for 1 hour at room temperature. After washing the slides with PBS, coverslips were fixed with mowiol. Slides were viewed with a Zeiss Axioskop 2 microscope and image analysis was performed using Openlab software from Improvision.

Visualisation of DNA double-strand breaks; SCLC Cells were quiesced overnight and seeded into 8 well coverslides (Nalge NUNC) at a density of $2.5x10^5$ cells/cm^2 pre-coated with laminin or poly-l-lysine. Cells were allowed to adhere for 2 hours at 37^oC and then treated as specified. Cells were fixed at 0-24 hours after treatment with 3% paraformaldehyde, washed with phosphate buffered saline, permeabilised

with 0.1% Triton and blocked with 3% fish skin gelatin. Cells were probed with 1:500 dilution of anti-γH2Ax for 2 hours at room temperature and washed with PBS. Secondary antibody labelled with ALEXA-fluor 488 (1:500) was applied for 1 hour at room temperature, slides were washed with PBS and coverslips were fixed. The slides were viewed with a Zeiss Axioskop 2 microscope. Image analysis was performed using Openlab software from Improvision.

2.16 Cell Cycle Analysis

SCLC cells were quiesced overnight and seeded into 6 well plates pre-coated with laminin, fibronectin or TS2/16 and treated as specified. In some experiments cells were treated with Nocodazole 2μM for 24 hours prior to seeding onto ECM. Cells were fixed 0-24 hours later in 70% ice-cold ethanol (for at least two hours), stained with propidium iodide and subjected to flow cytometric analysis (pulse processing) with a FACSCalibur flow cytometer. Cell cycle parameters from at least 10,000 events were analysed using CELLQuest software.

2.17 Rras activation assay

The GST linked Ras binding domain (RBD) of Raf was expressed from pGEX-Raf RBD (donated by W. Kolch) and linked to glutathione-agarose beads. CHOαβpy cells (2 x10^5/well in growth media) were transfected using JetPEI^{TM} with (wt)Rras and test DNA as specified. As a positive control cells were transfected with constitutively active (G38V)Rras (0.25μg/2ml of media). After 24 hours, growth media was replaced with serum free DMEM (1% BSA, 1% L-Glutamine, 1% Penicillin/Streptomycin, 1% non-essential amino acids and G418 antibiotic at

400µg/ml (Invitrogen)). After a further 24 hours cells were lysed in buffer (140mM NaCl, 1.5mM MgCl2 15% glycerol, 1% nonidet P40 and 1x protease inhibitor) at 4^{o}C for 20 min, centrifuged at 13,000 rpm for 10 min and protein balanced by BCA protein assay (Pierce, USA). An aliquot of the whole cell lysate was retained and boiled in Laemmli sample buffer. The remainder of the cell lysate was incubated with RBD-agarose beads (30µl/lysate of a 1:1 slurry beads:lysis buffer) for 2 hours at 4^{o}C. The beads were washed twice in lysis buffer and twice in ice cold PBS and protein was eluted by boiling in Laemmli sample buffer. The RBD pulldown and whole cell lysate were analysed by Western blotting with anti-myc (9E10) antibody.

2.18 Cell Adhesion Assay

24-well cell culture plates were coated with poly-l-lysine (10µg/ml) or fibronectin (10µg/ml) in PBS for 60 min at 37°C. Wells were washed and blocked with 3% (w/v) bovine serum albumin in PBS for 60 min at room temperature. 2x10^{5} K562 cells, treated as specified, were resuspended in 500µl of HEPES/NaCl buffer and seeded into the wells. EDTA (5mM final concentration) or Mn2+ (100µM final concentration) was added as indicated. Where specified K562 cells were treated with 4B4 (10µg/ml 45 min 37^{o}C) or MW167 (50µM 4 hours 37^{o}C) prior to seeding. Cells were allowed to adhere for 30 min at 37°C. Non-attached cells were then removed by gentle washing with PBS x2. Adhered cells were fixed with 3% paraformaldehyde (5 min) and stained with 0.4% methylene blue (5 min). Intracellular methylene blue was eluted with 0.1M HCl and the optical density (OD) (λ 560nM) of each sample was determined using an automated plate reader. Cell adhesion for each condition was

expressed as a percentage of total cellular adhesion to poly-l-lysine (i.e. OD sample / OD poly-lysine x 100).

2.19 SCLC xenograft experiments

$5x10^6$ H345 or H69 SCLC cells suspended in PBS or Matrigel (1:1 vol of cells:Matrigel/PBS mixed at 4^0C, total volume 200µl) were implanted subcutaneously in contralateral flanks of Nude nu/nu:Balb(c) mice (10 mice per condition) suspended in PBS or Matrigel (1:1 vol of cells:Matrigel mixed at 4^0C), laminin (20µg/ml), fibronectin (40µg/ml) or collagen (20µg/ml) with or without pre-incubation with mouse anti-human function-blocking β1-integrin antibody (4B4), IgG1-isotype antibody, genistein (50µM with the Matrigel/cells at the time of implantation) or genistein vehicle (10% DMSO in PBS). Some mice subsequently received 50mg/kg/day i.p. genistein or Vehicle in 200µl injections day2-8. Tumour size was assessed manually with calipers every 3-4 days and xenograft volume calculated by: Volume = ($Width^2$ x Length)/2. Mean ± s.e. shown. Statistical analysis: 2-way Anova. At the time of sacrifice, xenografts were dissected, halved and parallel samples were stored in formalin (haematoxylin and eosin staining and immunohistochemical staining for Ki67, CD34 and CD56 was kindly performed by Dr W.Wallace in the pathology department, New Royal Infirmary of Edinburgh) and liquid nitrogen (lysates were protein equilibrated and submitted to Western blot analysis as described above).

Chapter 3

Notch1 increases integrin affinity via activation of Rras.

3.1 Introduction

Notch proteins are large (~2500 amino acids), highly conserved, single-pass transmembrane proteins that play a vital role in cell differentiation and development. The major components of the Notch signalling pathway were originally recognised genetically following the discovery of loss of function mutant Drosophila.melanogaster fruit flies which possessed notches within their wing outlines [(Morgan, 1917)] and exhibited excessive neuronal differentiation [(Poulson DF, 1945)]. i.e. Notch represses neuronal differentiation. The core members of the Notch signalling pathway are 1) a Delta-type Notch ligand, 2) a Notch-type receptor [(Kopan et al., 1993)] and 3) a transcription factor of the CBF1/Su(H)/Lag-1 family (**see Appendix: Table 5**). Notch is initially produced as a single protein strand but undergoes three cleavages during its metabolic lifetime (**see Appendix: Fig 5**): After production in the nucleus, S1 cleavage occurs in the Golgi by a furin convertase after recognition of a RQRR sequence [(Logeat et al., 1998)]. The two fragments are held together by a calcium-coordinated bond [(Rand et al., 2000)] and Notch is then presented at the cell surface as a heterodimeric molecule [(Blaumueller et al., 1997)]. Notch ligands of the Delta and Serrate/Jagged family are transmembrane proteins with a small intracellular domain and a large extracellular domain containing epidermal growth factor-like repeats and

an amino-terminal DSL (Delta, Serrate, Lag-2) region specific to this class of proteins [Lendahl, 1998]. Notch ligands interact with the extracellular domain of Notch receptors via the EGF repeats and DSL domain [Rebay et al., 1991],[Kopan, 2002], resulting in the S2 cleavage, by a protease of the ADAM (a disintegrin and metalloprotease) family, TACE (Tumor necrosis factor-alpha-converting enzyme), at a site immediately external to the transmembrane domain [Mumm et al., 2000],[Brou et al., 2000]. The extracellular cleaved portion of the Notch receptor is transendocytosed into the Notch ligand-bearing cell [Parks et al., 2000],[Kramer, 2001] and the removal of the inhibitory LNR (Lin12/Notch repeat) sequence enables the remaining membrane-tethered Notch receptor fragment to be cleaved constitutively [Kimble et al., 1998],[Kopan et al., 1996] within its transmembrane domain at valine 1744 [Schroeter et al., 1998] by a 5-600kDa γ-secretase protein complex containing presenilin [Kaether et al., 2002],[Bergman et al., 2004], nicastrin, Aph1 and Pen2 [Song et al., 1999],[Struhl et al., 1999),[De Strooper et al., 1999),[De Strooper, 2003),[Shirotani et al., 2003] in a process termed Regulated Intramembrane Proteolysis (RIP). This process can be inhibited by a substitution at amino acid 1744 or the use of biochemical inhibitors of γ-secretase [Struhl and Greenwald, 1999),[Mizutani et al., 2001]. This S3 cleavage results in release of the intracellular portion ($Notch^{IC}$) which translocates to the nucleus [Kopan et al., 1996),[Schroeter et al., 1998] where it acts at subdetectable levels. $Notch^{IC}$ lacks intrinsic enzymatic or DNA binding capacity but influences transcription of many genes by modulating the balance of corepressor complexes (e.g. SMRT(silence mediator for retinoic acid and thyroid receptors)/NCoR(nuclear receptor corepressor)/HDAC1 or CIR/HDAC2/SAP30 [Espinosa et al., 2003),[Kao et al., 1998),[Hsieh et al., 1999] and coactivator complexes (e.g. p300, pCAF and MAML1) via binding to CSL (Core Binding Factor, Su(H), Lag-1) proteins in the nucleus [Henkel et

al., 1994). Several nuclear localisation sites have been identified and using deletion mutants (Dumont et al., 2000) it has been shown that the RAM domain (Tamura et al., 1995),(Chu et al., 2004) is the major domain involved in binding to RBPJ but also that the ankyrin repeats are essential for Hes1+5 activation. In addition other c-terminal sequences termed the OPA and PEST (Proline, Glutamate, Serine, Threonine) domains further enhance nuclear signalling. Coactivator complexes contain histone acetylases such as pCAF and GCN5 (Kurooka et al., 2000) which promote nucleosome disruption and chromatin relaxation resulting in upregulated transcription of target genes. Only a limited number of Notch effectors have been identified; the best described of these being the basic Helix-Loop-Helix (bHLH) repressor proteins Hes (Artavanis-Tsakonas et al., 1999) (Hairy/Enhancer of Split) and Hes-related proteins (HERPs) initially identified in Drosophila from the Enhancer of split Complex (E(spl)-C) gene region (Bailey and Posakony, 1995) that encodes many proteins that cause neural repression. Hes and HERP (Parks et al., 1997),(Bailey and Posakony, 1995),(Lecourtois et al., 1995),(Iso et al., 2003) transcription is switched on by Notch by virtue of CSL binding sites in their promoters. They function as suppressors of neurogenic differentiation in Drosophila by silencing proneural genes such as Achaete-scute (Ball, 2004) (Mash1 and HASH1 are mouse and human homologs). Hes can repress *actively* by recruiting corepressor complexes (e.g. Groucho (Dras/TLE/HDAC) for Hes or NCoR/mSin3A/HDAC1 for HERP) (Iso et al., 2003) or *passively* by sequestration of proteins e.g. Hes1 can heterodimerise with E47 (which is a MyoD and Mash homodimer partner) and thus MyoD/Mash signalling is reduced (Kopan et al., 1994). Several other downstream effectors have been identified: Notch1^{IC} also represses AP-1-mediated transactivation (Chu et al., 2002). AP-1 are Jun homodimers or Jun-Fos heterodimers that act as transcriptional modulators. AP-1 can

be activated by Ras-JNK or p38-Ser^{63+73} phosphorylation which then allow binding of CBP/p300 and its associated transcriptional complexes. Notch strongly represses (G12V)Hras-induced AP-1-mediated transactivation and this effect was highly dependent on an intact Notch RAM domain and nuclear localisation. The reversal of (G12V)Hras effect was not due to alteration of erk/jun/p38/mapk/jnk phosphorylation. In hepatocellular carcinoma Notch1^{IC} overexpression inhibits growth by reducing cyclinA, D and E, CDK2, phosphorylated retinoblastoma protein (Rb) and increasing p21cip1waf1, JNK and p53 (Qi et al., 2003). However, in another system Notch was seen to upregulate cyclinD1 (Ronchini et al., 2001). In T cells Notch1^{IC} inhibits apoptosis by increasing Bcl-2 and FLIP via a src-p56lck-PI3K-PKB pathway (Sade et al., 2004). Interestingly, in this system Notch1 was coimmunoprecipitated with PI3K and p56Lck suggesting direct interaction.

In Small Cell Lung Cancer Notch1 and 2 were overexpressed resulting in G1 arrest, p21cip1/waf1 and p27kip1 upregulation, Erk1 and 2 phosphorylation and reduced hASH though in this model these effects were Hes1-independent (Sriuranpong et al., 2001). In invasive cervical cancer Notch1 causes PKB phosphorylation and reduced anoikis (Rangarajan et al., 2001). In epithelial Mv1Lu cells Notch1 overcomes TGF-β-induced G1 arrest by upregulation of c-myc (Rao et al., 2003) and in K562 cells Notch1^{IC} is associated with YingYang1 via Notch ankyrin repeats (Yeh et al., 2003). YingYang1 has zinc finger domains and reduces Hes-1 luciferase expression via CBF-1.

A small number of other downstream Notch targets have been identified including Nrarp, an evolutionarily conserved small ankyrin repeat-containing protein that functions as a negative regulator of Notch signalling (Krebs et al., 2001),(Pirot et al., 2004), acid

α-glucosidase, a lysosomal enzyme that degrades glycogen [Yan et al., 2002a], mapk phosphatase, LIP-1, p21cip1waf1, cyclinD1/CDK2, MAMLs1-3 [Iso et al., 2003].

In addition, a number of other proteins have been shown to have RBPJκ binding sites similar to those seen in Hes/HERP but have not yet been shown to act directly as downstream Notch effectors (e.g. MHC-I, CD23, Il-6, erbB2, NFκB) [Iso et al., 2003].

3.1.1 Modulators of Notch

Delta/Notch levels do not correlate well with levels of proneural precursor proteins suggesting that post-translational signalling/feedback are important [Parks et al., 1997]. Several modulators of Notch function have been identified:

Fringe is a glycosyl transferase that glycosylates EGF repeats of Notch resulting in altered ligand binding affinity and increased signalling from Delta/reduced signalling via Serrate/Jagged [Blair, 2000]. Numb, a phosphotyrosine binding domain adaptor protein that binds Notch directly recruits Itch, an E3 ubiquitin ligase, which ubiquitinates Notch1 at the cell surface, increasing proteasomal degradation and reducing its translocation to the nucleus [McGill et al., 2003]. Ubiquitination involves three steps via ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). E3 provides selectivity and specificity. Ubiquitination can result in proteosomal degradation, receptor internalisation and trafficking to vesicle microorganelles. Mammalian Itch [Qiu et al., 2000] ubiquitinates at the cell membrane whilst the invertebrate homolog Sel-10 [Wu et al., 2001b] and Drosophila Suppressor of Deltex [Cornell et al., 1999] do so at the nucleus. $Notch1^{IC}$ increases antiapoptotic $Bcl_x(L)$ and FLICE-like inhibitor (FLIP) in T cells and these effects were antagonised by Numb [Sade et al., 2004]. Numb has also been implicated developmentally in "inherited"

Notch control [Bray, 1998]; i.e. when daughter cells are formed only one of the pair receives the Numb gene. In this setting the Notch daughter cell differentiates into a socket cell whilst Notch-depleted, Numb-expressing cells form hair cells. Numb knockout or $Notch^{IC}$ overexpressing cells both become socket cells [Rhyu et al., 1994),(Posakony, 1994]. Ubiquitination also modulates Notch ligand function: Neuralised and Mindbomb are ring-type E3 ubiquitin ligases that ubiquitinate the Notch ligand, Delta, causing internalisation of Delta from the membrane promoting its ability to activate Notch in neighbouring cells by virtue of increased transendocytosis [Itoh et al., 2003].

MINT binds to the intracellular portion of Notch and directly inhibits binding to RBPJ protein via its RAM domain to reduce Hes signalling [Kuroda et al., 2003].

Mastermind (a transcriptional activator recruited to the CSL complex) also stimulates phosphorylation and turnover of $Notch^{IC}$ resulting in cessation of Notch signal [Fryer et al., 2002].

Two groups have shown that different mammalian Notch subtypes produce different levels of Hes activation and may antagonise each other's activity. e.g. Notch2 antagonises Notch1 and Notch3 [Shimizu et al., 2002], Notch3 antagonises Notch1 [Beatus et al., 1999]. In embryonal brain tumour cell lines Notch1 reduces cell growth whilst Notch2 increases growth and is associated with a worse survival in medulloblastoma [Fan et al., 2004].

There is also evidence of rapid negative feedback loop involvement; Kuroda et al demonstrated a 10-fold reduction in Hes1-mRNA in cells that have intact translation machinery compared with those in which translation is inhibited [Kuroda et al., 1999].

3.1.2 CBF-independent Notch signalling

Although the majority of Notch-mediated signalling has been shown to dependent on S3 cleavage and nuclear localisation, several groups have reported cleavage-independent Notch signalling. Notably, genetic analysis of novel Notch alleles in Drosophila has clearly demonstrated that Notch can signal independently of Su(H) in a Deltex-dependent manner [(Ramain et al., 2001)]. Zecchini et al showed in Drosophila development that although dorsal closure was Notch-dependent, it was cleavage-independent [(Zecchini et al., 1999)] and acts via the JNK (c-Jun N-terminal kinase) signalling pathway. Two regions of the intracellular domain of Notch were critical for this function; the cdc10/ankyrin repeats and a region carboxy-terminal to this. Giniger demonstrated that Notch1 can directly regulate the actin cytoskeleton via the tyrosine kinase, abl [(Giniger, 1998)]. In mammals, Notch has been shown to inhibit mouse myogenesis without cleavage or interaction with CBF-1 [(Shawber et al., 1996),(Bush et al., 2001)].

3.1.3 Notch signalling in lower organism development

Notch signalling in development can be summarised as inductive, lateral and inherited.

The most well recognised example of (positive) inductive signalling is Drosophila wing development. Here a Notch ligand-bearing cell signals to a Notch receptor-bearing cell to induce wing margin growth via increased expression of vestigial. Loss of function mutants [(Kim et al., 1996)] produce the typical notched wing phenotype.

Lateral inhibition was first identified as the mechanism by which neural differentiation is controlled in Drosophila; Notch ligand-bearing cells signal to Notch receptor-bearing cells causing Hes-induced repression of neurogenic Achaete-Scute

Complex which results in inhibition of neural differentiation in the Notch receptor-bearing cells [Guillemot et al., 1993]. Using these signalling techniques Notch-mediated signalling has been shown to be crucial at multiple stages of Drosophila development [Bray, 1998].

The most well described example of inherited signalling involves Numb [Guo et al., 1996]; in early embryogenesis, during mitosis one daughter cell inherits Numb via assymetric segregation. This negatively regulates Notch in the Numb-bearing cell and downregulates the expression of Notch-dependent proteins. In contrast, the cell which does not inherit Numb expresses higher levels of Notch-dependent proteins. In this way cell fate is defined by the presence or absence of Notch signalling.

3.1.4 Notch – Evidence for signalling with Ras

In lower organisms

In C.elegans vulval development, 6 vulval precursor cells (VPCs P3.p-P8.p) are initially equivalent in developmental potential and equally capable of forming one of three primary fates [Wilkinson et al., 1994],[Christensen et al., 1996],[Eisenmann et al., 2000]. The anchor cell of the developing gonad stimulates the closest cell (P6.p) via a LIN-3 signal (an EGFR homolog) to induce a receptor tyrosine kinase (Let-23) → ras → mapk pathway which instructs the cell to adopt a primary cell fate. The P6.p cell then signals laterally to P5.p and P7.p instructing them to adopt an alternative, secondary, cell fate. This lateral signal is mediated by a Lin-12/Notch pathway that is dependent upon SUR-2-mediated Ras signalling in the P6.p cell [Singh et al., 1995]. Whereas Notch signalling is activated in P5.p and P7.p, the P6.p cell downregulates its own Notch signalling via a post-translational Notch modification which results in altered

endocytic routing dependent on a di-leucine intracellular domain situated between the transmembrane domain and the first ankyrin repeat [Shaye et al., 2002]. In addition, the lateral Notch signal to P5.p and P7.p results in upregulation of Lip-1 which is a mapk phosphatase, indirectly downregulating the primary cell fate-determining mapk signal in p5.p and p7.p [Berset et al., 2001]. C.elegans vulval development is one example of Notch and Ras interacting to influence cell development. In this setting Ras signalling from one cell results in serial Notch signalling in another.

A comparable system exists in Drosophila mesothorax development [Culi et al., 2001]. Here, each Drosophila mesothorax bristle is derived from a single Sensory Organ Mother Cell (SMC) which arises as a result of high cellular levels of the proneural gene product achaete-scute complex (AS-C) which is dependent upon an EGFR/Ras pathway. In order to limit the number of cells adopting bristle fate these SMCs direct Notch-mediated lateral inhibitory signals to adjacent cells which blocks AS-C via the repressive E(spl)-C bHLH mechanism described earlier.

In Drosophila dorsal mesoderm development, wingless and decapentaplegic (TGF-beta homolog) signals induce early mesodermal cells to become competent to respond to EGFR/FGF-mediated Ras activation which causes them to adopt Founder myoblast (FMC) status [Artero et al., 2001],[Carmena et al., 2002],[Dierick et al., 1999]. These cells express surface Delta to signal via Notch receptors to adjacent cells instructing them to adopt “Fusion-competent cell” , rather than FMC status. Notch further blocks EGF/FGF signalling within Fusion-competent cells via inhibition of pathway intermediates, heartless and rhomboid [Artero et al., 2003]. Interestingly, in this study a screen identified Hbs (Echinoid) to be downstream of Notch. In Drosophila eye development Echinoid antagonises the Ras pathway at the level of transcription of a

target gene tramtrack [Bai et al., 2001], representing a further possible mechanism for Notch antagonism of Ras signalling.

In Drosophila ommatidia development, Notch and Ras signalling converge in the same cell to define cell fate. The R7 ommatidial photoreceptor cell receives signals via both a Ras pathway (via Sevenless, a receptor tyrosine kinase, from R8) and a Notch pathway (Delta signal from R1+6) to define cell differentiation. i.e. Notch and Ras act here in parallel, exclusively but cooperatively [Tomlinson et al., 2001].

A further potential mechanism for Notch and Ras interaction may involve AF-6, a 180kDa mammalian protein (Drosophila homolog Canoe) which is the fusion partner of ALL-1/AF-6 which causes human leukaemia. Canoe acts downstream of Notch in Drosophila eye development and has a GLGF/DHR motif which is thought to function to localise a number of proteins to sites of cell-cell contact where it may regulate adhesive cell-cell interactions [Miyamoto et al., 1995],[Matsuo et al., 1999]. In addition to acting downstream of Notch, the C.elegans homolog Ce-AF-6 was also identified as a putative Ras effector which binds GTP-Hras but not GDP-Hras or inactivated A38-GTP-Hras [Watari et al., 1998],[Kuriyama et al., 1996]. Thus, Notch may influence Ras signalling via Canoe.

Warthog was found in a screen for modifiers of activated Notch phenotype and is a Ras-like GTPase (Drosophila homologue of rab6, Drab6) involved in vesicular trafficking [Purcell et al., 1999]. The authors postulate that delayed presentation of Notch or gamma secretase to the cell surface results in modified Notch signalling.

In higher organisms

Very few studies have investigated the interaction of Notch and Ras in higher organisms. In 3T3-L1 cells Hras causes adipogenesis only if Notch signalling is also present [(Ruiz-Hidalgo et al., 1999)]. Individually, Notch and Ras have well-described roles in oncogenesis: In a transgenic mouse model Notch4 is dependent upon downstream Ras signalling to sustain anchorage-independent growth [(Fitzgerald et al., 2000)]. Weijzen et al have demonstrated in human embryonic kidney cells and human foreskin fibroblasts that Notch1 is a downstream effector of oncogenic (G12V)Hras [(Weijzen et al., 2002)]. (G12V)Hras increased nuclear levels of $Notch1^{IC}$ and also increased delta1 and presenilin1 via a p38/MEKK6-mediated pathway.

Another potential level of Notch/Ras signalling overlap is ADAMs (a Disintegrin and Metalloprotease) [(Yan et al., 2002b)]. As well as being membrane metalloproteases that modulate Notch S2 cleavage and Notch ligand cleavage [(Schlondorff et al., 1999)] they are also involved in EGFR transactivation by G-proteins that can result in downstream Hras signalling.

3.1.5 Notch – Evidence for signalling with Integrins

In lower organisms

There is some indirect evidence that integrins and Notch may be involved in coordinated signalling in lower organisms. In Drosophila, Blister wing, where fluid accumulates between the dorsal and ventral wing surfaces due to a failure of integrin-mediated fusion is a common phenotype to both Notch and integrin loss-of-function mutants [(Brown et al., 2000),(Brabant et al., 1996)]. A screen for other autosomal mutations that cause the Blister wing phenotype also identified other key Notch pathway members,

Delta and Mastermind (a CSL activator) [Prout et al., 1997]. Additionally, data from Zebrafish somitogenesis suggests that mutations in the Notch pathway and integrin α5 subunit can produce a complementary disruption in somite formation possibly implicating Notch in integrin regulation [Julich et al., 2005],[Chong et al., 2005].

In neural stem cells the intracellular domain of Notch was coimmunoprecipitated with β1-integrin inferring a direct interaction [Campos et al., 2006].

In higher organisms

There is only one report of Notch-mediated modulation of integrin affinity in higher organism experiments. Leong at al showed that $Notch4^{IC}$ inhibits endothelial sprouting, VEGF-induced angiogenesis and migration of cells through collagen via increased β1-integrin-mediated adhesion [Leong et al., 2002] dependent upon the ankyrin repeats [MacKenzie et al., 2004].

Other evidence that Notch-mediated signals may influence integrin affinity is only indirect: e.g. Integrin beta cytoplasmic tails contain NPxY motifs that can form beta turns which recognise protein tyrosine binding domains e.g. Talin-PTB:NPxY-β1-integrin. Calderwood et al showed that Numb interacted directly with β1-integrin domains and coimmunoprecipitated with β3 and β5 integrin but not β1A, β2 or β7 [Calderwood et al., 2003].

ADAMs (a disintegrin and metalloprotease) offer a further potential pathway for Notch and integrin crosstalk. ADAMs such as TACE (TNFα-converting enzyme) and Kuzbanian are involved in ectodomain processing of proteins including the Notch ligand, Delta. In addition ADAMs contain snake venom-like domains capable of acting as integrin ligands and this feature may be important in adhesion events of

sperm-ova interactions [Schlondorff and Blobel, 1999),(Blobel et al., 1992)]. To date there is no evidence supporting a direct link between ADAMs, integrins and Notch signalling.

Del-1 (developmental endothelial locus-1) is a Delta-like protein which is a component of early endothelial cell extracellular matrix [Hidai et al., 1998]. It contains a RGD motif that binds αvβ3 integrins and it also has EGF-repeats that interact with other EGF-repeat-containing molecules such as Notch and Delta. Although there is no direct evidence of interaction, it presents a possible mechanism of Notch/integrin pathway crossover.

GABP-β is a protein with Notch homology that forms a heterotetrameric complex with GABP-α on the CD18 (β2-integrin) promoter to increase expression [Rosmarin et al., 1995]. This supports the possibility of Notch-mediated CBF-independent modulation of integrin transcription.

In view of this direct and indirect evidence supporting interactions of Notch with integrins and Ras, the following experiments were performed to investigate the role of Notch1 in integrin affinity modulation.

3.2 Confirmation of integrity of Notch1^{IC}V1744 construct used in experiments

Notch1^{IC}V1744 is a constitutively active Notch1 mutant that is amino-truncated at valine 1744 and thus lacks the transmembrane and extracellular domains. It is constitutively active and translocates to the nucleus where it activates transcription factors such as Hes1 [Schroeter et al., 1998]. The mouse Notch1^{IC}V1744 construct was provided with a carboxy-terminal myc-tag in a pEGFP1 vector (with a stop codon proximal to the GFP sequence). To standardise controls, Notch1^{IC}V1744 was cloned

from pEGFP1 into the HindIII/Apa1 sites of pcDNA3.1^{+}. The authenticity of all constructs used in the following experiments was confirmed by miniprep digest of the resultant construct, DNA sequencing analysis, immunoblotting of the protein product and immunofluorescence microscopy of the appropriate tag (myc or Flag). For coimmunoprecipitation and immunofluorescence experiments which employed cotransfection of Notch1^{IC}V1744 and myc-tagged Ras moieties, the myc-tag of Notch1^{IC}V1744 was removed by digestion with BamH1 and Sal1 prior to cloning into pCMV-Tag4A (which contains a Flag-tag at the 3' end). An untagged negative control, pcDNA3.1^{+}Notch^{IC}V1744(EcoRV), was produced by digesting pEGFPNotch1^{IC}V1744 with EcoRV to produce a truncated moiety which retains the amino terminal sequence but lacks some of the ankyrin repeats (ANK (6)), distal nuclear localising sequences and the carboxy-terminal myc tag (x6).

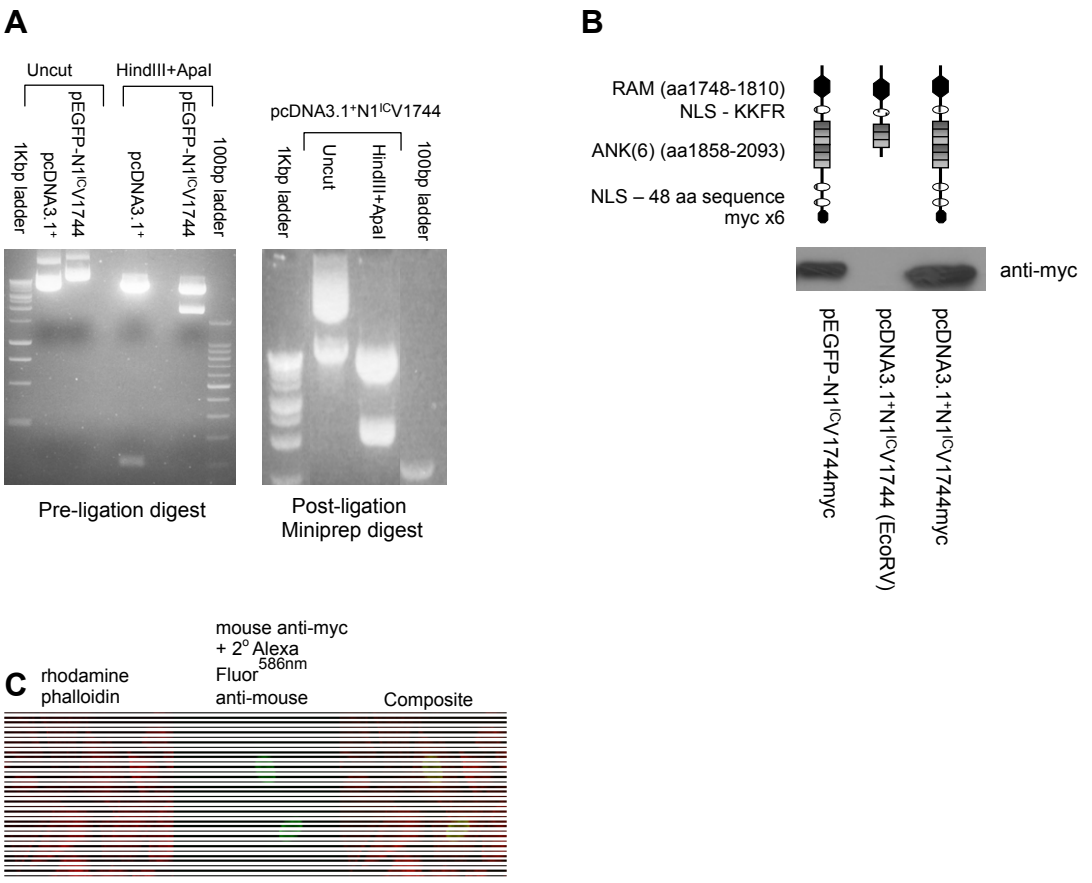


Figure 3.1 Production and authentication of Notch1 constructs used in experiments. **A** Ethidium bromide-stained 2% agarose gel electrophoresis demonstrating HindIII/Apa1 Notch1 cleavage product (left) from pEGFP-Notch^{IC}V1744 and confirmation of insertion of Notch^{IC}V1744 cleavage product in pcDNA3.1^{+} (right). **B** Western blot of Notch1 constructs probed with anti-myc antibody. **C** Immunofluorescence microscopy of Chinese hamster ovary (wt) cells transfected with Notch^{IC}V1744 stained with rhodamine-phalloidin (red) and nuclear Alexa-Fluor^{488} (green) staining of myc-tagged Notch1^{IC}V1744.

Results. DNA agarose gel electrophoresis (**Fig 3.1A**) and DNA sequencing confirmed the accurate cloning of Notch1 constructs into pcDNA3.1^{+} (and pCMVTag4A; not shown). After transfection into Chinese Hamster Ovary (CHO) cells, protein expression was confirmed by immunoblotting with anti-myc (9E10) antibody (**Fig 3.1B**) and appropriate nuclear localisation was demonstrated by immunofluorescence microscopy of -myc (and -Flag; not shown) staining (**Fig 3.1C**).

3.3 Notch1^{IC}V1744 increases integrin affinity and abrogates Hras-mediated integrin suppression

(G12V)Hras is a constitutively active Hras mutant which decreases integrin affinity (Sethi et al., 1999a). I tested the hypothesis that Notch1^{IC} increases integrin affinity and reverses (G12V)Hras-mediated integrin suppression.

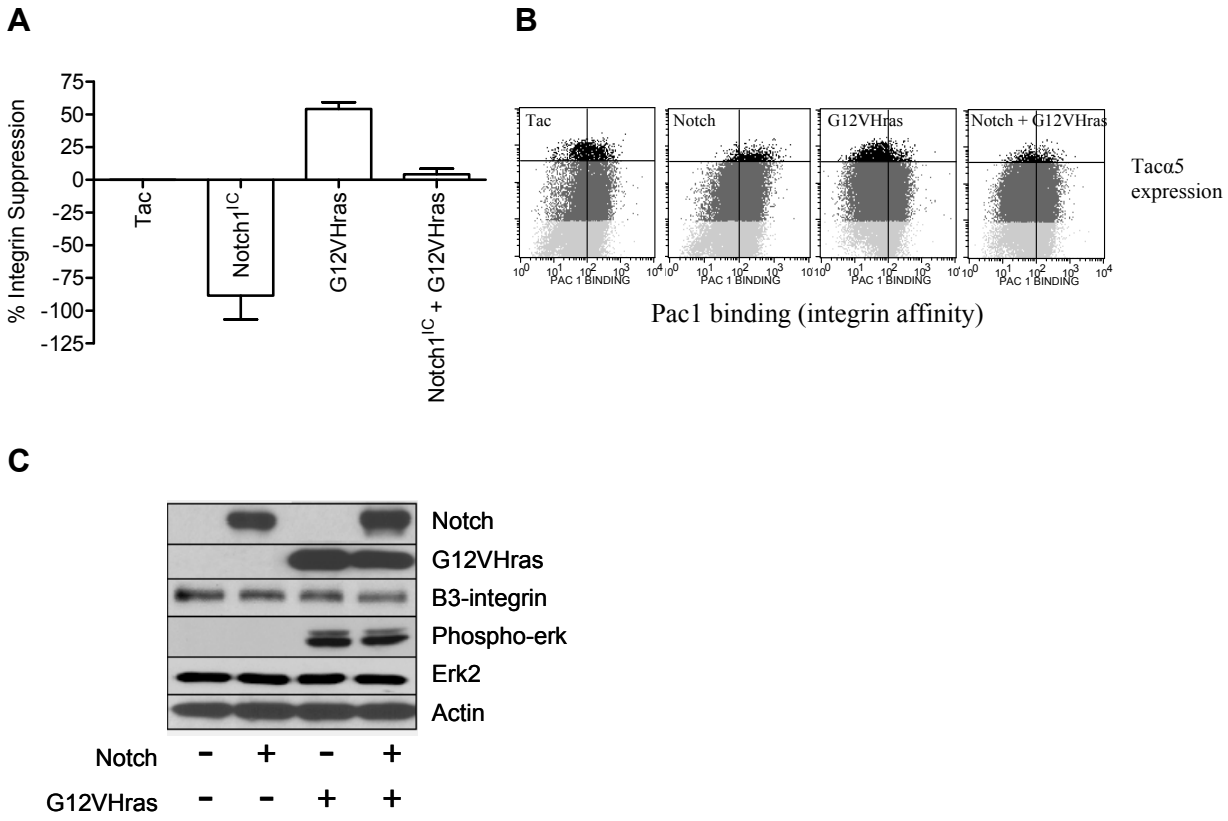


Figure 3.2 Notch1^{IC} increases integrin affinity and reverses Hras-mediated integrin suppression. Chinese hamster ovary cells stably expressing the chimaeric integrin αIIbβ3α6β1 were transiently transfected with interleukin-2 receptor (Tac-α5, 0.75μg) and Notch1^{IC}V1744 (1μg), (G12V)Hras (1μg) or Notch1^{IC}V1744 and (G12V)Hras. After 48 hours cells were stained with Pac-1 (specific for the active confirmation of αIIbβ3), anti-Tacα5 and Topro-3. Antibody binding on single, viable, cells was analysed by 3-colour flow cytometry. **A** αIIbβ3 integrin affinity was measured by flow cytometric assessment of Pac-1 binding and % integrin suppression normalised to Tac-alone values. Mean ± s.e. of n=16 experiments shown. **B** Representative dot-plots showing right-shift (increased Pac-1 binding due to increased integrin affinity) of highly transfected cells (black) transfected with Notch and reduced binding left shift by (G12V)Hras. **C** Western blot for transfected Notch1^{IC}V1744 and (G12V)Hras, β3-integrin, total/phosphorylated erk levels and beta-actin levels.

Results: The chimaeric integrin is in a relatively high affinity state and so (G12V)Hras was used to reduce integrin affinity (**Fig.3.2A+B**). Hras reduced integrin affinity by 54.2 ± 5.2% (s.e.) ($p<0.01$) demonstrated by a left shift of highly transfected cells (upper dark dots **Fig3.2B**) in this model as previously described (Hughes et al., 1997). Notch1^{IC}V1744 induced an increase in integrin affinity above vector control of 88.8 ± 18.3% (s.e.) ($p<0.001$) associated with a right shift of Pac binding and also reversed (G12V)Hras-mediated integrin suppression ($p<0.01$, **Fig 3.2A+B**). Western blots (**Fig 3.2C**) demonstrated that these effects were not produced by alterations in the expression of (G12V)Hras, Notch or αIIbβ3-integrin. (G12V)Hras increased erk phosphorylation as previously shown (Sethi et al., 1999a). Notch expression had no effect on erk phosphorylation when transfected alone or with (G12V)Hras.

3.4 Dominant-Negative Rras blocks Notch-mediated integrin activation

To date, few candidate proteins have been shown to mediate increases in integrin affinity and to reverse Hras-mediated suppression of integrin affinity. The best described is Rras (Zhang et al., 1996),(Sethi et al., 1999a) and thus I postulated that Notch1^{IC} mediates its effect on integrin affinity via activation of Rras. This postulate was initially tested by assessing the effect on integrin affinity of Rras inhibition in Notch1-transfected cells using transiently transfected Dominant-Negative (T43N)Rras.

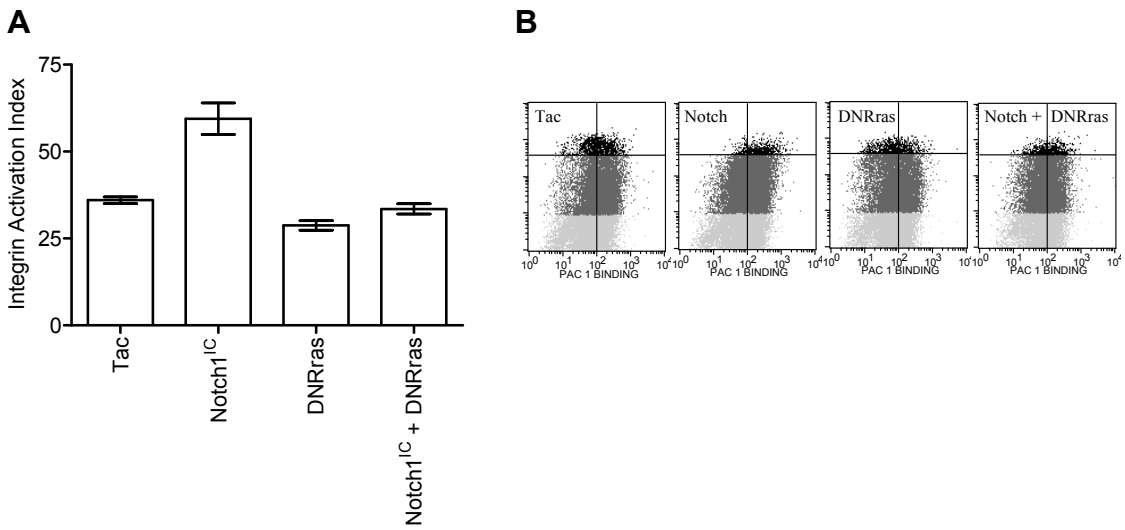


Figure 3.3 Dominant-Negative Rras blocks Notch-mediated integrin activation. Chinese hamster ovary cells stably expressing the chimaeric integrin αIIbβ3α6β1 were transiently transfected with interleukin-2 receptor (Tac-α5, 0.75μg) and Notch1^{IC}V1744 (1μg), Dominant-Negative (T43N)Rras (1μg) or Notch1^{IC}V1744 and (T43N)Rras. **A** αIIbβ3 integrin affinity was measured by 3-colour flow cytometric assessment of Pac-1 binding shown as % integrin activation. Mean ± s.e. of n=19 experiments shown. **B** Representative dot-plots showing right-shift of highly transfected cells (black) indicating increased integrin affinity of Notch-transfected cells and reversal of this by Dominant-Negative (T43N)Rras.

Results: Notch1^{IC} increased integrin activation above vector control from 36.0 ± 1.0% (s.e.) to 59.4 ± 4.5% (s.e.) ($p<0.001$). Whilst Dominant-Negative (T43N)Rras had no significant effect on integrin affinity alone ($p>0.05$), it completely abrogated Notch1^{IC}-mediated integrin activation (integrin activation index 33.5 ± 1.5% (s.e.), $p<0.001$).

3.5 Dominant-Negative Rras blocks Notch1^{IC} reversal of (G12V)Hras-mediated integrin suppression and this is independent of erk phosphorylation

Hras decreases integrin affinity (Hughes et al., 1997),(Hughes et al., 2002). Following the demonstration that DNRras abrogates Notch1^{IC}-mediated integrin activation I sought

to show that DNRras also abrogates Notch1^{IC}-mediated reversal of Hras-mediated integrin suppression. Since Hras-mediated integrin suppression has been shown to be dependent on erk activation, western blots of total and phosphorylated erk were performed to assess whether Notch modulates levels of this downstream effector.

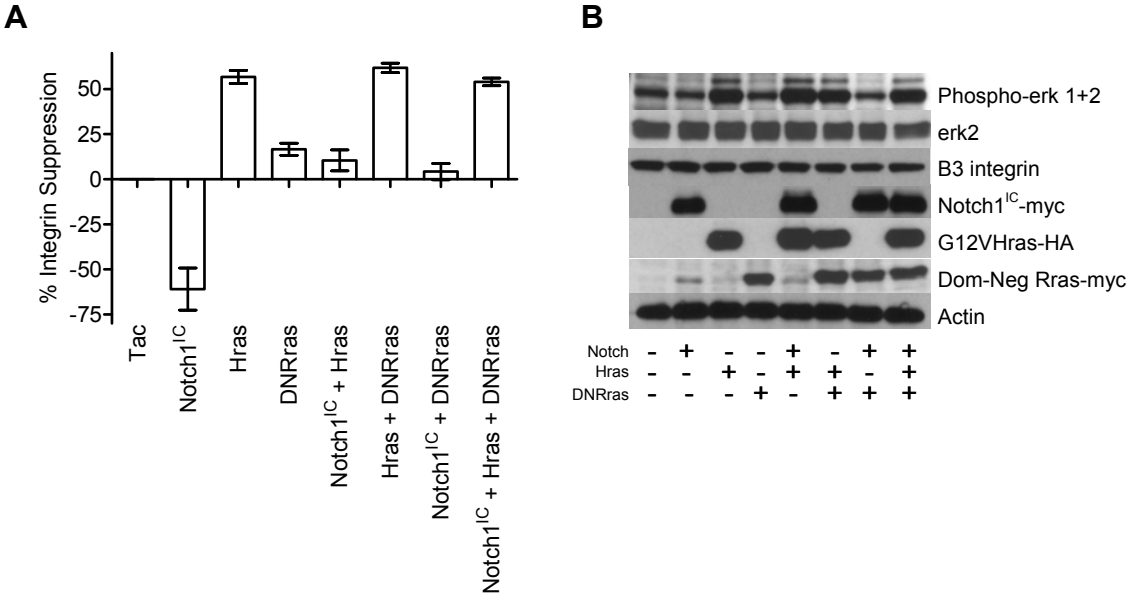


Figure 3.4 Dominant-Negative Rras blocks Notch1^{IC} reversal of (G12V)Hras-mediated integrin suppression and this is independent of erk phosphorylation. Chinese hamster ovary cells stably expressing the chimaeric integrin αIIbβ3α6β1 were transiently transfected with interleukin-2 (Tac-α5, 0.75μg) and combinations of Notch1^{IC}V1744 (1μg), (G12V)Hras (1μg) and Dominant-Negative (T43N)Rras (1μg). **A** αIIbβ3 integrin affinity was measured by 3-colour flow cytometric assessment of Pac-1 binding and % integrin suppression normalised to Tac-alone values. Mean ± s.e. of n=8 experiments shown. **B** Western blot confirming balanced expression of transfected constructs, equal β3-integrin expression and integrin suppression independent of erk phosphorylation.

Results: Notch1^{IC}V1744 increased integrin affinity compared with vector control by 60.9 ± 11.7% (s.e.) ($p<0.001$) and this was inhibited by DNRras (Notch1^{IC} +DNRras 4.3 ± 4.5% (s.e.) ($p<0.001$). (G12V)Hras reduced integrin affinity by 56.9 ± 3.6% (s.e.) ($p<0.001$) and Notch1^{IC} abrogated this effect (Notch1^{IC}+(G12V)Hras 10.5 ± 5.8% (s.e.), $p<0.01$). The effect of Notch1^{IC} on (G12V)Hras-mediated integrin

suppression was blocked by DNRras (Notch1^{IC}+(G12V)Hras vs Notch1^{IC}+(G12V)Hras+DNRras, $p<0.05$) supporting the hypothesis that the effects of Notch1^{IC} are mediated via Rras (**Fig 3.4A**). Expression of all constructs was confirmed by western blot (**Fig 3.4B**). Alterations in Pac-1 binding were not due to alterations in the expression of αIIbβ3 integrin assessed by western blot analysis (**Fig 3.4B**) Furthermore, as expected, although (G12V)Hras was shown to induce erk phosphorylation, this phosphorylation was independent of Notch and DNRras expression.

3.6 Dominant-Negative Rras blocks Notch1^{IC} reversal of Raf-mediated integrin suppression

Since Hras-mediated integrin suppression is dependent on downstream Raf activation (Hughes et al., 1997),(Hughes et al., 2002) the effect of (T43N)Rras and Notch1^{IC}V1744 on constitutively active Raf-CAAXBXB-mediated integrin suppression (Sethi et al., 1999a) as measured by Pac1 binding was assessed using similar cotransfection experiments in CHOαβpy cells.

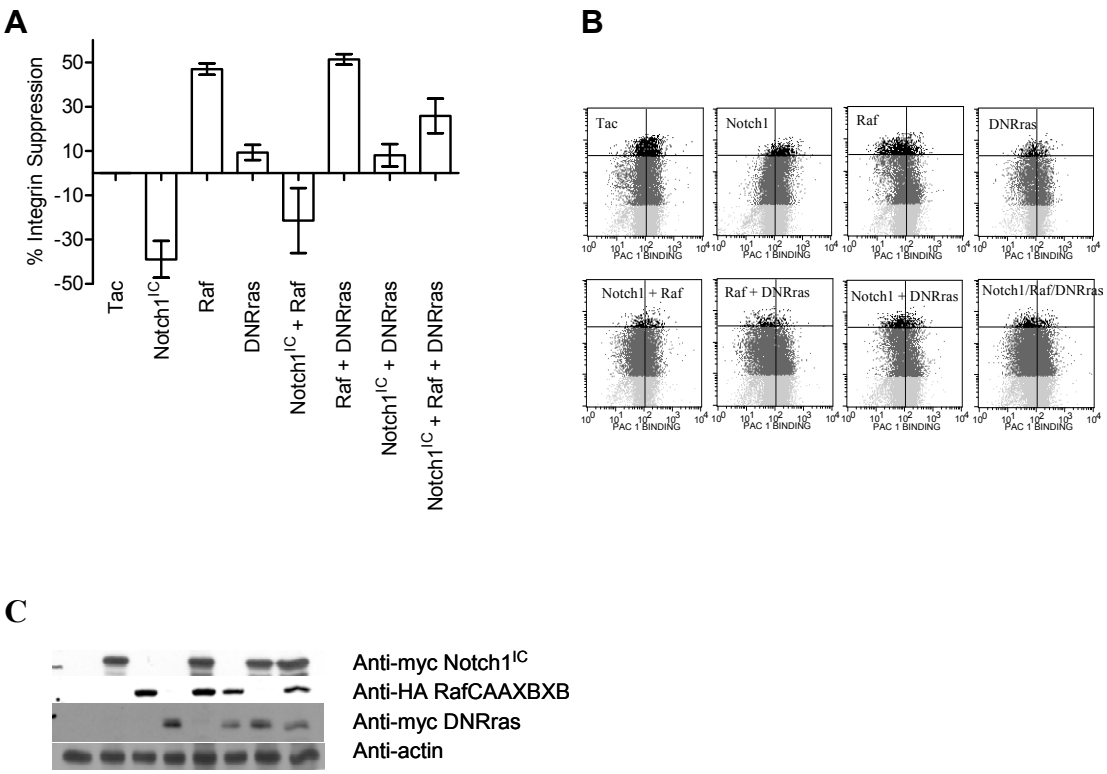


Figure 3.5 Dominant-Negative Rras blocks Notch1^{IC} reversal of Raf-mediated integrin suppression. Chinese hamster ovary cells stably expressing the chimaeric integrin αIIbβ3α6β1 were transiently transfected with interleukin-2 receptor (Tac-α5, 0.75μg) and combinations of Notch1^{IC}V1744 (1μg), Raf-CAAXBXB (1μg) and Dominant-Negative (T43N)Rras (1μg). **A** αIIbβ3 integrin affinity was measured by 3-colour flow cytometric assessment of Pac-1 binding and % integrin suppression normalised to Tac alone values. Mean ± s.e. of n=7 experiments shown. **B** Representative dot-plots demonstrating summed data in **A**. **C** Western blot confirming expression of transfected constructs.

Results: **Fig 3.5A+B**: Notch1^{IC}V1744 increased integrin affinity ($p<0.001$) and this was inhibited by DNRras ($p<0.001$). RafCAAXBXB reduced integrin affinity ($p<0.001$) and Notch1^{IC}V1744 completely abrogated this ($p<0.001$). The effect of Notch1^{IC}V1744 on Raf-CAAXBXB-mediated integrin suppression was blocked by DNRras ($p<0.001$). Western blotting confirmed expression of transfected constructs (**Fig 3.5C**)

3.7 Notch1^{IC}V1744 does not increase expression of β3-integrin

Most of the known cellular effects of Notch1 are a result of alterations in protein expression via CBF1-dependent transcriptional modulation. To assess whether increased Pac1 binding in the flow cytometry experiments was due to increased integrin expression rather than increased integrin affinity, highly transfected cells (Tac-PE FL2 signal intensity ≥600) were cell sorted using a FACSCalibur flow cytometer and western blot analysis of αIIb and β3 integrin expression performed.

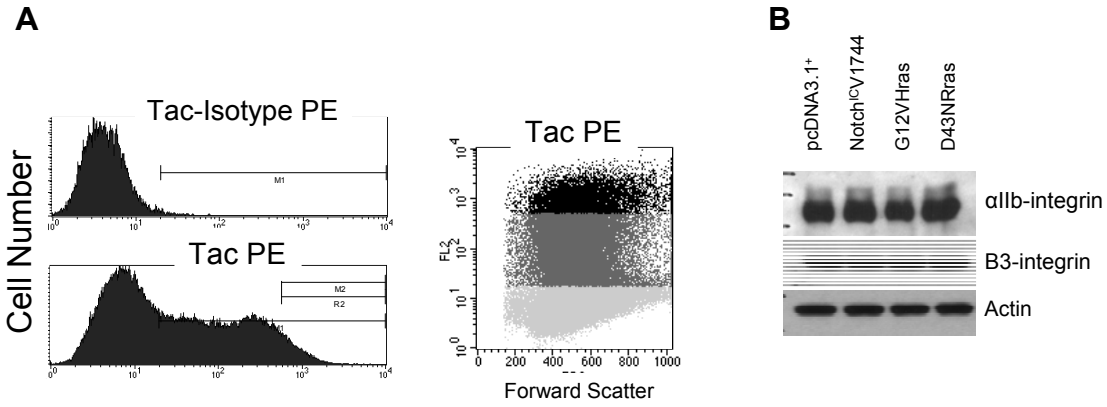


Figure 3.6 Notch1^{IC}V1744 does not increase expression of β3-integrin. Chinese hamster ovary (CHO) cells were transiently transfected with Tac (0.75μg) +/- pcDNA3.1^{+} (1μg), Notch1^{IC}V1744 (1μg), (G12V)Hras (1μg) or Dominant-Negative (T43N)Rras (1μg). After 48 hours cells exhibiting high levels (FL2 >600) of Tac-PE (a marker of transfection efficiency) were sorted using a FACS-Calibur flow cytometer. 0.5 million cells were lysed and total protein levels balanced. Western blots were probed for actin, αIIb and β3 integrin. **A** Representative histograms and dotblot of PE binding of sorted cells (Tac/Notch transfected cells shown). **B** Western blot probed for actin, αIIb and β3-integrin.

Results: Chinese hamster ovary cells highly transfected with Tac +/- pcDNA3.1^{+}, Notch1^{IC}V1744, (G12V)Hras and Dominant-Negative (T43N)Rras express equal amounts of αIIb and β3 integrin (**Fig 3.6B**). Thus, differences in flow cytometric analysis of Pac1 binding are due to alterations in integrin affinity and not integrin expression.

3.8 Notch1^{IC}V1744 induces Rras activation

Since the above data shows that Notch1^{IC} increases integrin affinity via Rras it was anticipated that Notch1^{IC} would produce an increase in the activated, GTP-bound, Rras moiety. The effect of Notch1^{IC}V1744 transfection on levels of total and GTP-bound Rras was assessed using an Rras activation assay (Poulson DF, 1945).

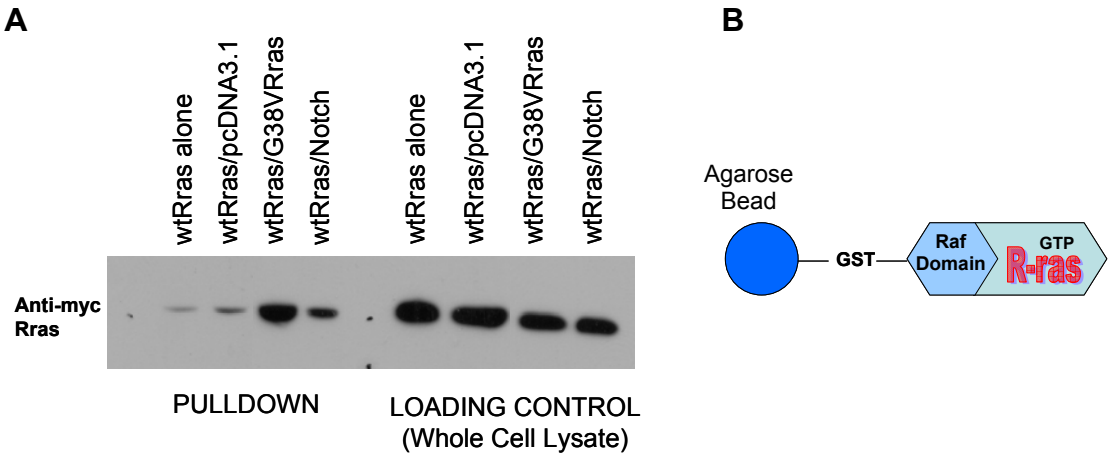


Figure 3.7 Notch1^{IC}V1744 induces Rras activation. Chinese hamster ovary cells stably expressing the chimaeric integrin αIIbβ3α6β1 were plated into 6 cm plates in DMEM complete media. Cells were transfected with wild type Rras (myc tagged) (0.5μg/well) plus pcDNA3.1^{+}, (G38V)Rras or Notch1^{IC}V1744, (all 1μg/well) as indicated. Cells were incubated for 36 hours in complete media and then quiesced for 12 hours prior to lysis. Cell lysates were incubated at 4^{0}C with Ras-Binding-Domain-Raf:GST agarose beads to bind active GTP-bound Rras. Washed beads were boiled at 95^{0}C for 5 minutes and Rras expression was determined by Western blot analysis. **A** Representative western blot of active Rras in RBD-Raf:GST pulldown and wildtype Rras in whole cell lysates probed with anti-myc(9E10) antibody. **B** Representation of GTP-Rras binding to agarose bead-GST-linked Raf domain.

Results: Equal levels of (wt)Rras were present in whole cell lysate loading controls. In the pulldown samples, the positive control (G38V)Rras, a constitutively active, GTP-bound mutant, demonstrated high levels of myc-tagged GTP-Rras bound to the RBD-Raf:GST-agarose beads. (wt)Rras alone did not induce high levels of GTP-bound Rras but Notch1^{IC}V1744 reproducibly produced an increase in activated GTP-

bound Rras levels further supporting the evidence that Notch1 mediates increased integrin affinity via activation of Rras (**Fig 3.7A**).

3.9 Functional assays of integrin affinity

In order to further substantiate the hypothesis that Notch1 increases integrin affinity it was necessary to demonstrate *functional* changes in cells transfected with Notch1 or treated with Notch ligands (Jagged or Delta) or inhibitors of the Notch signalling pathway (e.g. γ-secretase inhibitor MW167). K562 human leukaemic cells were chosen for assessment since i) they are highly transfectable using electroporation, ii) they express exclusively α5β1 integrins [(Jarvinen et al., 1993)] which bind fibronectin and iii) Many other cell lines have integrins in a maximally activated, high affinity state; however, under resting conditions, K562 cells are only weakly adherent to extracellular matrix and thus increases in integrin affinity were considered to be achievable and measurable with functional assays.

3.10 Characterisation of K562 transfection, viability and adhesion

Transfection of K562 cells was performed using an AMAXA electroporator. Transfection efficiency was assessed by flow cytometric analysis of Green Fluorescent Protein (GFP) expression. Viability of cells was assessed 24-72 hours later by acridine orange/ethidium bromide morphological assessment. Expression of transfected constructs was confirmed by western blotting. An adhesion dose response on fibronectin was performed to optimise extracellular matrix concentration in subsequent experiments.

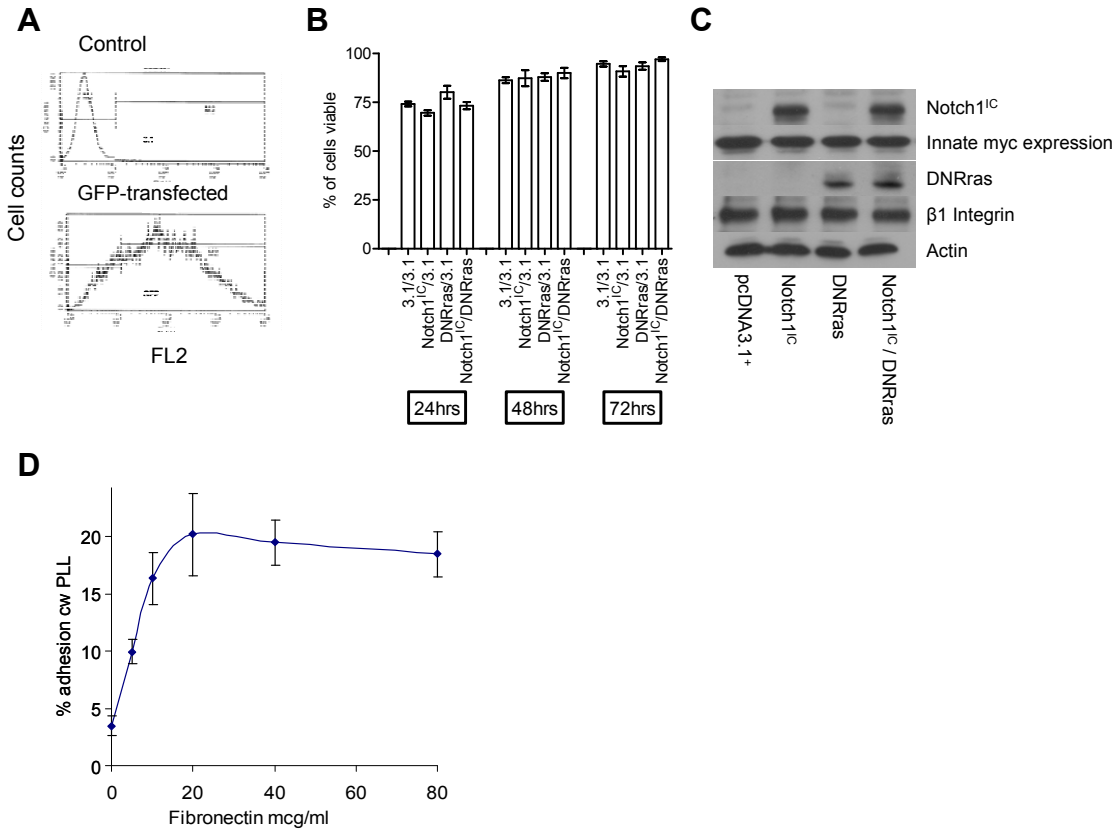


Figure 3.8 Characterisation of K562 transfection, viability and adhesion. A Using an AMAXA electroporator 5 million K562 cells were transfected with 5µg pEGFP3 or control plasmid and GFP expression assessed after 72 hours using a FACSCalibur flow cytometer. **B** 5 million K562 cells were transfected with 10µg of control pcDNA3.1^{+} or 5µg pcDNA3.1^{+}/5µg Notch1^{IC}V1744 or 5µg pcDNA3.1^{+}/5µg DNRras or 5µg Notch1^{IC}V1744/5µg DNRras using an AMAXA electroporation system. Viability of cells was assessed 24-72 hours after AMAXA electroporation by acridine orange/ethidium bromide morphological assessment. Mean ± s.e. of 3 experiments shown. **C** Concomitantly, expression of transfected constructs and β1-integrin was confirmed 72 hours after electroporation by Western blotting. **D** 96-well plates were precoated by incubation at 37^{0}C with fibronectin (0-80µg/ml) or poly-l-lysine (10µg/ml).10^{5} K562 cells were plated per well and cells were allowed to adhere for 30 min at 37°C. Non-adherent cells were then removed by gentle washing with PBS x2. Adhered cells were fixed with 3% paraformaldehyde (5 min) and stained with 0.4% methylene blue (5 min). After washing, intracellular methylene blue was eluted with 0.1M hydrochloric acid and the optical density (OD) (λ 560nM) of each sample was determined using an automated plate reader. Mean ± s.d. of n=3 experiments shown.

Results: K562 cells exhibit high GFP transfection efficiency (80-90%) 72 hours after AMAXA electroporation. >90% of these cells are viable at 72 hours after AMAXA electroporation (**Fig 3.8A+B**) and protein expression of transfected constructs was

confirmed (**Fig 3.8C**). Transfection of Notch1 and DNRras did not alter surface expression of β1-integrin (**Fig 3.8C**). K562 adhesion to fibronectin was concentration-dependent with maximal adhesion at 20μg/ml (**Fig 3.8D**).

3.11 Notch1^{IC} increases K562 adhesion in a β1-integrin dependent manner

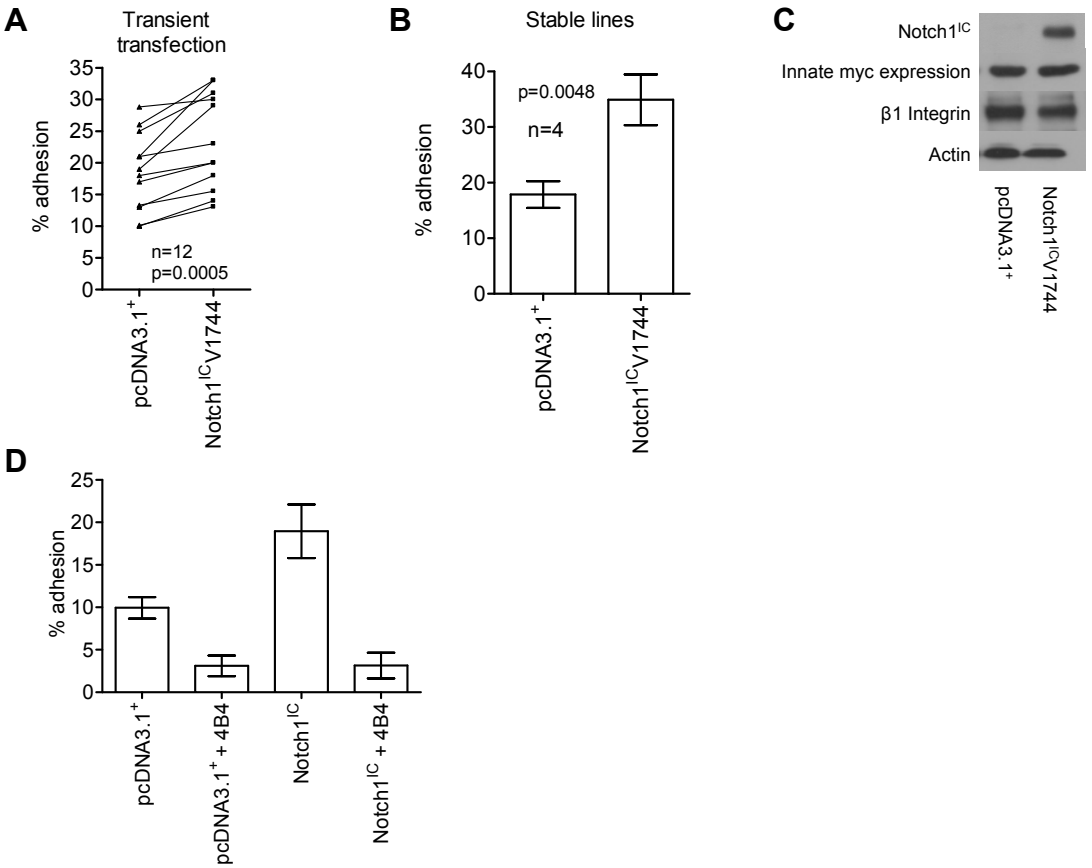


Figure 3.9 Notch1^{IC} increases K562 adhesion to fibronectin in a β1-integrin dependent manner. **A** K562 cells were transiently transfected with empty vector (pcDNA3.1^{+}) or Notch1^{IC}V1744 (1μg/10^{6} cells). Cells were harvested 72 hours later and assessment of adhesion to fibronectin performed. 96 well plates were precoated with 20μg/ml fibronectin. Harvested cells were washed with PBS and adhered for 1 hour at 37^{0}C in triplicate. Wells were gently agitated to dislodge non-adhered cells, washed twice with PBS, fixed with 3% PFA, stained with methylene blue, washed and lysed with 0.1% hydrochloric acid. Absorbance was measured on a plate-reader at 560nm and values normalised to poly-l-lysine values. **B** Stable cell lines expressing Notch1^{IC}V1744 were developed by G418-selection of K562 cells transfected with pcDNA3.1^{+}Notch1^{IC}V1744. Adhesion to fibronectin (20μg/ml) was compared with stable pcDNA3.1^{+} lines and values normalised to poly-l-lysine. Mean ± s.e. of n=4 experiments shown. **C** Western blot confirming Notch1 transfection and equal β1-integrin expression in stable lines. **D** K562 cells were transfected as indicated. 72 hours later cells were harvested and incubated with or without function-blocking β1-integrin antibody (4B4 10μg/ml 37^{0}C for 30 mins). A fibronectin adhesion assay was then performed as above. Results of mean ± s.e. n=5 experiments in triplicate shown.

Results: K562 cells transiently and stably transfected with Notch1^{IC}V1744 exhibit increased adhesion to fibronectin compared to vector-only control (**Fig 3.9A+B**). K562 cells pre-treated with function-blocking β1-integrin antibody (4B4) exhibited significantly reduced adhesion to fibronectin (**Fig 3.9C**) supporting previously published data showing that K562 cells express predominantly α4β1 and α5β1 integrins [(Turner et al., 1998),(Kikkawa et al., 2000)], the integrins responsible for adhesion to fibronectin. K562 cells transfected with Notch1^{IC}V1744 exhibited a significant increase in adhesion to fibronectin ($p<0.01$) from 9.9 ± 1.3% (s.e.) to 18.9 ± 3.2% (s.e.) of cells adherent compared with poly-l-lysine. This effect was completely abrogated by function-blocking β1-integrin antibody (4B4) (**Fig 3.9D**) but not isotype control (data not shown) confirming that this increase in adhesion was secondary to increased β1-integrin-mediated adhesion.

3.12 Dominant-Negative Rras inhibits Notch1^{IC}-mediated Adhesion of K562 cells to Extracellular Matrix

To corroborate the earlier FACS data showing that Dominant-Negative (T43N)Rras inhibited Notch-mediated increases in integrin affinity, assays of K562 adhesion to fibronectin were performed with cells cotransfected with Notch1^{IC}V1744 +/- (T43N)Rras.

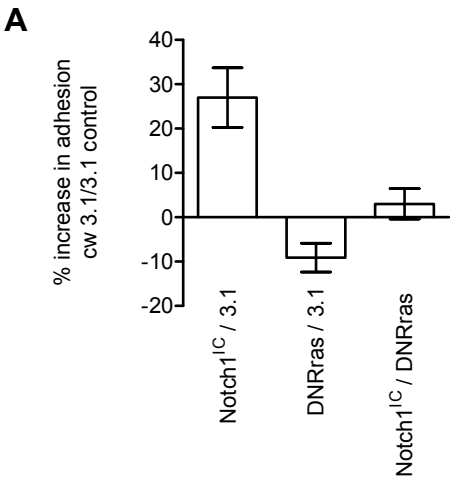


Figure 3.10 Dominant-Negative Rras inhibits Notch1^{IC}-mediated adhesion of K562 cells to fibronectin. A K562 cells were transiently transfected with empty vector (pcDNA3.1^{+}), Notch1^{IC}V1744, Dominant-Negative Rras or both (1µg/10^{6} cells). Cells were harvested 72 hours later and assessment of adhesion to fibronectin performed. Briefly, 96 well plates were precoated with 20µg/ml fibronectin. Harvested cells were washed with PBS and adhered for 1 hour at 37^{0}C in triplicate. Wells were gently agitated to dislodge non-adhered cells, washed twice with PBS, fixed with 3% PFA, stained with methylene blue, washed and lysed with 0.1% hydrochloric acid. Absorbance was measured at 560nm and values normalised to pcDNA3.1^{+} values. Mean ± s.e. of n=7 experiments shown.

Results: Compared with vector control, Notch1^{IC} increased adhesion of K562 cells to fibronectin by 26.99 ± 6.7% (s.e.) ($p<0.001$). Dominant-Negative Rras alone did not significantly affect adhesion compared with control vector but it did completely abrogate the Notch1^{IC}-mediated increase in adhesion ($p<0.01$).

3.13 Discussion

After confirmation that constitutively active mouse Notch1^{IC}V1744 constructs were accurately cloned into pcDNA3.1+ (**Fig 3.1**) it was demonstrated that Notch1^{IC}V1744 induced an increase in integrin affinity in CHO cells expressing an active chimeric integrin (αIIbβ3/α6β1) measured by increased binding of Pac1, an antibody specific for the active conformation of αIIbβ3 (**Fig 3.2A+B**). This integrin

has the ligand binding properties of αIIbβ3 but is activated through the α6Aβ1 cytoplasmic domains [Fenczik et al., 1997]. Furthermore, transfection of (G12V)Hras alone caused marked inhibition of PAC1 binding and cotransfection of Notch1^{IC}V1744 completely reversed this (G12V)Hras-mediated integrin suppression (**Fig3.2A+B**). Suppression of integrin activation by Hras is dependent on downstream erk1/2, though is not related to overall levels of erk phosphorylation (Hughes et al., 2002). Notch has previously been shown to have differing effects on erk phosphorylation in different model systems [Sriuranpong et al., 2001),(Eagar et al., 2004] and thus the effect of Notch on erk phosphorylation was assessed by western blotting for total and phosphorylated erk (**Fig 3.2C**). Whilst, as expected, Hras induced an increase in erk phosphorylation, this was unaffected by concomitant Notch expression. This supports data from genetic experiments in lower organisms which has previously provided indirect evidence of coordinated signalling between Notch and integrins.

To date, few intracellular signalling proteins have demonstrated the capability to increase integrin affinity and reverse (G12V)Hras-mediated integrin suppression; these include Rras and Rap (described previously), PEA-15 and CD98. The increase in integrin affinity produced by PEA-15 (Protein Enriched in Astrocytes) is blocked by Dominant-Negative Rras [Ramos et al., 1998] and PEA-15 may, in part, reverse Hras-mediated integrin suppression by sequestering mapk in the cytoplasm preventing erk/mapk-dependent transcription [Chou et al., 2003]. CD98 (heavy chain) mediates β1-integrin-mediated cell spreading and migration and is required for adhesion-induced activation of PKB-survival signalling [Fenczik et al., 1997),(Feral et al., 2005]. In lower organisms, Notch and Ras have been shown to exhibit multiple levels of interaction; in C.elegans vulval development Ras signalling from one cell results in upregulated

Notch signalling in the adjacent cell [Shaye and Greenwald, 2002]. A similar Ras-mediated lateral inhibition signal exists in Drosophila mesothorax development [Culi et al., 2001]. In Drosophila ommatidia development [Tomlinson and Struhl, 2001], Notch and Ras signalling converge in the same cell to define cellular differentiation, i.e. acting cooperatively rather than sequentially. AF-6 (also known as Canoe and Ce-AF-6) may provide a further mechanism for Notch/Ras interaction since it acts downstream of Notch in Drosophila eye development but also binds GTP-Hras and is thought to localise proteins to sites of cell-cell adhesion [Miyamoto et al., 1995),(Matsuo et al., 1999]. In addition to acting downstream of Notch, the C.elegans homolog Ce-AF-6 was also identified as a putative Ras effector [Watari et al., 1998] and Notch may alter Ras signalling via Canoe. Further evidence that Notch and Ras cooperate in lower organisms is provided by the demonstration that Notch and Drosophila Rras (Dras2/Ras64B) loss and gain-of-function mutants share similarities in phenotype including rough eye [Hori et al., 2005),(Schreiber et al., 2002),(Aplin et al., 1997].

It is perhaps not surprising that there has, as yet, been no demonstration of direct Notch to Rras signalling in Drosophila or C.elegans. Although Drosophila has developed discrete Hras and Rras entities, human Hras and Rras have limited homology with their invertebrate counterparts; for example human Rras has only ~63% amino acid homology with Drosophila Rras.. The fact that Notch and Ras interact at all in lower organisms makes it likely that more refined and complex systems exist in higher animals. Few studies have addressed the interaction of Notch with Ras in higher organisms. In 3T3-L1 cells Hras induces adipogenesis only if Notch signalling is also present [Garces et al., 1997]. Individually, Notch and Ras have well-described roles in oncogenesis: Fitzgerald et al [Fitzgerald et al., 2000] showed that

Notch4 is dependent upon Ras signalling to sustain anchorage independent growth. A further potential level of Notch/Ras signalling overlap is ADAMs (a Disintegrin and Metalloprotease) [(Yan et al., 2002b)]; membrane metalloproteases that modulate Notch S2 cleavage since, in addition, they are involved in EGFR transactivation by G-proteins that results in downstream Hras signalling. In view of this indirect evidence for Notch/Ras interaction and since Rras is the most well characterised positive modulator of integrin affinity it's role in Notch-mediated integrin affinity modulation was investigated further here.

(T43N)Rras is a mutant of Rras that has a much higher affinity for GDP than GTP and thus is constitutively inactive. Cotransfection of (T43N)Rras with $Notch1^{IC}V1744$ completely inhibited $Notch1^{IC}$-mediated integrin activation (**Fig 3.3**). It also abrogated (G12V)Hras-mediated (**Fig 3.4A**) and Raf-CAAXBXB-mediated integrin suppression (**Fig 3.5A**) inferring that Notch's effect on integrin affinity is mediated via Rras downstream of, or parallel to, the Hras-Raf pathway. Western blotting again confirmed that the influence of Notch did not relate to altered erk phosphorylation or levels of expression of β3-integrin.

An alternative explanation for the increase in Pac-1 binding caused by Notch1 is increased expression of the target for Pac-1, integrin αIIbβ3. Western blot analysis of whole cell lysates as shown above demonstrated no difference in overall expression but since these lysates contained all transfected cells (rather than only the highly transfected cells that exhibit these integrin affinity changes) they may not accurately reflect the expression of integrins on the surface of highly transfected cells. Therefore highly transfected cells (Tac-PE FL2 signal intensity ≥600) were cell sorted using a FACSCalibur flow cytometer and Western blot analysis of αIIb

and β3 integrin expression performed. This again confirmed no difference in integrin expression between the pcDNA3.1, Notch1^{IC}V1744, (G12V)Hras or Dominant-Negative (T43N)Rras-transfected cells (**Fig 3.6B**). (T43N)Rras can sequester GEFs when overexpressed. It is therefore possible that the effect of (T43N)Rras is a result of blocking GEF-dependent events shared by both Hras and Rras. However, previous data has shown that this mutant of Dominant-Negative Rras does not affect Hras signalling (Ramos et al., 1998).

If Notch activates integrins via a Rras-mediated mechanism then it was felt likely to be due to activation of Rras from GDP-bound to the GTP-bound form.

To determine whether Notch signalling could directly activate Rras to the GTP-bound form, CHO cells were transfected with myc-tagged wild-type Rras and either Notch1^{IC}V1744 or empty vector (pcDNA3.1+). After 48 hours the Ras-binding-domain (RBD) of Raf linked via GST to agarose beads, was used to pulldown GTP-bound Rras from cell lysates. Western blot assessment of GTP-Rras levels was then performed. Notch transfection reproducibly produced an increase in the amount of Rras pulled down in this assay confirming Notch-mediated Rras activation. (G38V)Rras, a constitutively active, GTP-bound mutant was transfected as a positive control. (Initial attempts, using this assay, were made to identify the increase in GTP-Rras of innate Rras in CHO cells by western blotting using anti-Rras antibody. However, as a result of poor antibody specificity and low overall levels of Rras no increase was identified).

After demonstrating a Notch-mediated increase in GTP-bound Rras, it was hypothesised that Notch may interact directly with Rras to produce this effect. However, using myc-tagged (wt)Rras/(G38V)Rras and Flag-tagged Notch1^{IC}V1744

(by subcloning into pCMV Tag4B – Stratagene) it was not possible to show a direct association using a coimmunoprecipitation technique or with confocal microscopy (data not shown). Thus, Notch1 increases integrin affinity via activation of Rras though it appears that Notch mediates Rras activation via an indirect mechanism. This may be via direct interaction with second messengers; for example Calderwood et al co-immunoprecipitated Numb, an inhibitor of Notch activity, with β3 and β5 integrin [(Calderwood et al., 2003)]. Another possible mechanism may involve the modulation of subcellular localisation of Rras.

CHOαβpy cells are a useful tool to investigate integrin affinity but express high levels of a non-physiological chimaeric integrin which is in a relatively high affinity state (and thus the use of Hras to initially reduce affinity in many experiments). In order to demonstrate a functional outcome from Notch-mediated integrin activation in a human cell line system with physiological levels of integrin expression, adhesion assays were performed following cotransfection with Notch1^{IC}V1744 and DNRras using human leukaemic K562 cells expressing β1-integrin in association with α4 and α5 subunits. 72 hours after transfection by electroporation, high levels of cell viability and protein expression of transfected products were confirmed by morphological assessment and western blotting respectively (**Fig 3.8A+B**). Furthermore, western blotting for β1-integrin showed equal expression in control, Notch1^{IC}V1744 and DNRras-transfected cells (**Fig 3.8C**) confirming that alterations in adhesion are not due to differences in surface expression of β1-integrin. In the absence of activation by TPA or hemin, K562 human myeloid leukaemia cells exist in a pluripotential resting state expressing only α4β1 and α5β1 integrins [(Jarvinen et al., 1993)]. 18.5% of cells transiently transfected with vector-only (pcDNA3.1+) adhered to

fibronectin and this increased to 23.2% (**Fig 3.9A**) in cells transiently transfected with Notch1^{IC}V1744 (paired "t" test, p=0.0006). A similar increase in adhesion was seen when comparing stable cell lines transfected with pcDNA3.1+ and Notch1^{IC}V1744 (**Fig 3.9B**). Confirmation that this adhesion to fibronectin occurred via β1-integrins was provided by experiments in which adhesion induced by Notch1^{IC}V1744 (and K562 adhesion per se) was completely inhibited by preincubation of K562 cells with function-blocking antibody 4B4 (**Fig 3.9D**) but not by 4B4 isotype antibody. Thus, the Notch-mediated increase in integrin affinity demonstrated in FACS experiments in CHO cells also results in a functionally measurable outcome in a human leukaemic cell line. Yeh at al previously demonstrated in K562 cells that Notch1^{IC} is associated with YingYang1 via Notch ankyrin repeats (Yeh et al., 2003). YingYang1 has zinc finger domains and reduces Hes-1 luciferase expression via CBF-1. Interestingly, in a human osteosarcoma cell line, de Nigris et al showed that myc and YingYang1 together bind to DNA to cooperate in reducing expression of α3-integrin (de Nigris et al., 2007). However, K562 cells do not express α3-integrin and expression levels of β1-integrin were unchanged in these experiments precluding the above YingYang1-mediated mechanism as a contributor to the above results.

Importantly, the Rras-dependent nature of this phenomenon was also reproduced in this human cell line model with the demonstration that Notch-mediated increase in adhesion was completely abrogated by cotransfection with DNRras (**Fig 3.10A**). This occurred without alterations in Notch or β1-integrin expression (data not shown).

In conclusion, the data here show that Notch1 activates integrins and reverses Hras-mediated suppression of integrins via activation of Rras. Interactions such as this have previously been suggested by indirect genetic data in invertebrates and mouse developmental models but this is the first direct evidence that Notch, integrins and Rras interact simultaneously to produce alterations in β1-integrin-mediated adhesion. The mechanisms underlying this may be important in cell-fate decision-making during embryogenesis and explain some of the overlap in phenotypes observed in Notch, integrin and Ras mutants in Drosophila and C.elegans. It implicates Notch in many integrin-mediated events that are critical in cellular behaviour related to adhesion and motility. Furthermore this data may provide a link to begin to explain the observation that Notch can act as a tumour suppressor: There is some evidence that integrins can become "blind" to the extracellular milieu and in doing so anchorage independence is promoted. By activating integrin affinity, Notch may inhibit this anchorage independence and via concomitant antagonism of Hras-mediated cell cycle promotion, prevent disordered cell growth.

[More recent data (not shown here) produced in this lab (Dr P.Hodkinson) has shown that:

1) Low dose Notch1^{IC}1744 (0.25μg/well) and low dose (wt)Rras (0.375μg/well) produce no significant effect on integrin activation/reversal of suppression when transfected alone but did produce complete reversal of (G12V)Hras suppression when both were transfected. This is consistent with increased levels of (wt)Rras being activated to GTP-Rras causing reversal of (G12V)Hras-mediated integrin

suppression, providing further support for a role for Rras activation downstream of Notch1 signalling

2) Although Notch-mediated effects on integrin affinity are dependent on γ-secretase intramembranous cleavage, they are not CBF-dependent; a Notch mutant which is S3-cleavable but lacks the ability to transactivate the CSL complex reversed (G12V)Hras integrin suppression without producing Hes activation. These experiments were supported by similar results using MINT, a Notch antagonist. Deletion mutants implicated Notch ankyrin repeats in Notch-mediated integrin activation.

3) The Notch ligand, Delta-like ligand-4 (Dll-4) significantly increased adhesion of K562 cells to fibronectin in a β1-integrin-dependent manner and MW167, a γ-secretase inhibitor, abrogated this effect completely].

Chapter 4

Extracellular Matrix overrides DNA-damage-induced cell cycle arrest in small cell lung cancer cells via β1-integrin dependent activation of PI3Kinase.

4.1 Introduction: Outside-In Integrin Signalling – Extracellular Matrix-mediated Anti-apoptotic Signalling

Over the last 50 years cytotoxic chemotherapy and radiotherapy has been the mainstay of cancer treatment for patients with haematological and metastatic tumours. In some solid tumour sites they have replaced surgery as the treatment modality for primary-site tumour control (e.g. anus, small cell lung cancer, some head and neck cancers and non-melanomatous skin cancers). In addition, adjuvant chemotherapy can also produce significant survival benefits to selected patients following the treatment of localised disease presumably by eliminating undetectable minimal or microscopic residual tumour. Complete, sustained tumour responses resulting in cure are frequently obtained in patients with haematological malignancies, trophoblastic tumours and germ cell tumours. Often, however, in haematological malignancies and ovarian/germ cell/small cell lung/breast cancer although impressive initial responses to chemotherapy are seen, in a significant number of patients residual and recurrent disease refractory to further therapy results in treatment failure, tumour relapse and ultimately death. Subpopulations of tumour

cells survive the initial drug treatment and become unresponsive to a wide spectrum of anti-cancer agents in a phenomenon known as *multi-drug resistance* or MDR (it is important to note that this is distinct from mechanisms of *single-drug resistance,* e.g. gene amplification of the methotrexate target-dihydrofolate reductase, which will not be considered further in this thesis). The molecular mechanisms underlying the development of this multi-drug chemotherapy resistance are the object of intense research activity and offer the potential to provide novel therapeutic targets to improve the response to chemotherapy and ultimately impact on survival in cancer patients.

Many forms of chemotherapy primarily exert their cytotoxic effect by inducing apoptosis (Airas et al., 2000). It has generally been considered that drug-resistant cells arise from genetic changes that either occur spontaneously because of inherent genetic instability in cancer cells or as a result of chemotherapy-induced genetic damage (Obara et al., 2002),(Duensing et al., 1996),(Struski et al., 2001). Factors that allow cell survival following acute cytotoxic drug exposure may differ from mechanisms selected for by chronic drug exposure. The inheritable nature of the drug resistant phenotype in transplantable mouse leukaemia models (Hayakawa et al., 2000),(Ader et al., 2002) together with the understanding of the biochemical action of certain anti-metabolites led to the early focus on genetic features of tumour cells as the determinant of drug sensitivity and resistance. Classically, investigations of drug resistance have focused on the single cell by selecting for drug resistant cells following exposure to cytotoxic agents. These studies have revealed a number of mechanisms such as a) Drug efflux via ATP binding cassette (ABC) cotransporters such as the P-glycoprotein (PgP) drug pump, b) Upregulation of drug detoxification mechanisms such as the

glutathione-S-transferase system and c) Topoisomerase II downregulation and mutation. Despite extensive research the clinical, in vivo relevance of these mechanisms is unclear and no significant clinical benefit has yet accrued. The search for novel mechanisms of MDR responsible for chemotherapy failure therefore continues. Recently it has become apparent that extracellular matrix-binding to integrins results in resistance against chemotherapy-induced apoptosis. Furthermore, this phenomenon has been demonstrated in a wide variety of cell types and results in resistance to anticancer agents that induce cell death via a variety of unrelated mechanisms. Phenomena which may explain this significant discovery include a) Increased resistance to tumour penetration resulting in reduced drug exposure, b) Reduced induction of DNA damage or upregulated DNA repair, c) Potentiation via autocrine loops and d) Modulation of cell survival and death signalling pathways.

4.1.1 Classical mechanisms of MDR

Drug Efflux Pumps

Reduced intracellular drug concentration can result from increased efflux of drug from resistant cells due to the presence of P-glycoprotein (PgP/MDR-1), multi-drug resistance-associated proteins (MRP2-7), breast cancer resistance protein and the lung cancer resistance protein drug pumps in the plasma membrane [(Biedler et al., 1970),(Kartner et al., 1983)]. The genes for P-glycoprotein and MRP have been cloned and it has been demonstrated that these ATP-driven pumps have broad substrate specificity [(Roninson et al., 1986),(Cole et al., 1992)]. As well as playing an important role in transport of endogenous substances (e.g. Leukotriene C4) they reduce intracellular drug accumulation thereby conferring resistance to a broad range of structurally and

mechanistically diverse chemotherapeutic agents [Gerlach et al., 1986]. P-glycoprotein-mediated drug resistance has been well characterised in haematological malignancies [Dalton et al., 1989] and P-glycoprotein expression has correlated with chemotherapy response and outcome in some clinical series [Jiang et al., 1998a),(Webb et al., 1998]. However, this mechanism alone cannot account for all drug resistance found in vivo or in vitro, nor is it likely to explain cell survival following acute cytotoxic drug exposure. The picture is less clear in solid tumours and though P-glycoprotein upregulation has been identified in many cell types e.g. bladder [Tada et al., 2002], breast [Takamura et al., 2001),(Faneyte et al., 2001] and colon cancer [Sinicrope et al., 1994] the relationship of expression to clinical chemoresistance and therefore the true clinical relevance of this phenomenon, remains unclear. In small cell lung cancer (SCLC) lines P-glycoprotein phenotype is easy to develop in vitro but it appears to be infrequently expressed in vivo [Lai et al., 1989]. P-glycoprotein specific mRNA expression was elevated in only 4 out of 23 SCLC cell lines and 3 out of 6 primary tumours. In no instance was elevation of P-glycoprotein expression to the very high levels sometimes seen in colon cancer [Duensing et al., 1994]. In addition there was no correlation between P-glycoprotein expression and response to chemotherapy or whether the tumour was derived from previously treated or untreated patients. This suggests that the expression of P-glycoprotein is not a dominant mechanism of drug resistance in SCLC.

Breast cancer resistance protein (BRCP) [Doyle et al., 1998),(Lage et al., 2000a], a 72kDa ATP binding cassette (ABC) cotransporter of the P-glycoprotein/MRP family has been shown to be associated with mitoxantrone, topotecan, methotrexate [Doyle et al., 1998),(Lage et al., 2000a),(Volk et al., 2000] and (in most studies) adriamycin resistance in breast [Volk et al.,

2000),(Faneyte et al., 2002),(Scheffer et al., 2000) colon, gastric and ovarian cancer, fibrosarcoma and myeloma (Ross et al., 1999). The major vault protein (MVP)/lung cancer resistance protein (LRP) is a further 110kDa ABC drug cotransporter (Scheffer et al., 1995) that is upregulated in chemoresistant cell lines in non-small cell lung cancer (NSCLC) (Berger et al., 2000),(Volm et al., 1997), acute myeloid leukaemia (List et al., 1996), prostate (van Brussel et al., 2001) and ovarian cancer (Arts et al., 1999). It localises to nuclear pore complexes and may influence intracellular drug transport and localisation, possibly diverting drugs from their nuclear target. We do not yet know the clinical importance of these more recently discovered drug transporters.

Glutathione-S-transferase

An alternative mechanism of drug resistance is upregulation of drug detoxifying enzymes such as the G-S-Transferases, a group of multigene isoenzymes that detoxify both xenobiotic and endobiotic compounds via conjugation with glutathione prior to further metabolism and excretion (Hall, 1999),(Salinas et al., 1999). Resistance to cis-platinum and doxorubicin is associated with increased G-S-T levels and this has been shown to result in protection against DNA damage (Goto et al., 2001). G-S-T levels are upregulated in a number of cancers including breast (Perquin et al., 2001),(Buser et al., 1997), colon (Redmond et al., 1991) and non-small cell lung cancer (NSCLC) (Nakanishi et al., 1999). Again, however, although this mechanism is important in many in vitro cancer cell lines it appears that it may only have minor clinical relevance (Buser et al., 1997),(Nakanishi et al., 1999),(Pratesi et al., 1995).

Topoisomerase II-associated Multi-Drug Resistance

Several important chemotherapeutic agents including etoposide, teniposide and doxorubicin induce apoptotic cancer cell death via interaction with topoisomerase-II (TopoII). TopoII regulates the three-dimensional structure of DNA by binding to a DNA molecule, cleaving both strands of that DNA and passing a second DNA duplex through the first before re-annealing the cleavage site [(Kaufmann, 1989)]. Etoposide blocks the re-annealing action of TopoII at a step in its catalytic cycle after the enzyme has created a double-strand break, the so-called "cleavage complex", and ultimately leads to activation of apoptosis [(Kaufmann, 1998)]. Most investigators show that cancer cell lines selected for resistance to TopoII poisons escape cell death by reducing the level of target TopoII enzyme [(Koshiyama et al., 2001),(Nielsen et al., 2000),(Lage et al., 2000b)]. This is usually associated with a concomitant reduction in TopoII activity (resistant cells are able to survive with a three to four-fold reduction in TopoII content [(Kobayashi et al., 2001)] though subsequent recovery in expression [(Matsumoto et al., 2001b)] or mutation [(Matsumoto et al., 2001a)] resulting in a maintained overall enzyme activity despite reduced enzyme concentration have been identified as mechanisms to counter this. NSCLC is intrinsically much more resistant to etoposide than SCLC and this may be due to the fact that NSCLC cells have significantly less TopoII target for etoposide to bind than SCLC [(de Jong et al., 1990)]. However, worse prognosis [(van Brussel et al., 2001),(Dingemans et al., 2001)] and lower chemotherapy response rates [(Dingemans et al., 1999)] have also been obtained in the presence of increased levels of TopoII enzyme and again the clinical importance of this well-researched phenomenon is unclear.

Several mechanisms have been described whereby cancer cells, upon exposure to one chemotherapeutic agent, can develop multi-drug resistance to several other

agents. It is not uncommon for these mechanisms to co-exist. For example a SCLC line made resistant in vitro by graded exposure to doxorubicin was found to show collateral resistance to vincristine, etoposide, cis-platinum and x-irradiation but not to melphalan, colchicine or actinomycin-D [(Zijlstra et al., 1987)]. This drug resistance was explained on the basis of a combined decrease in intracellular levels of doxorubicin, reduced TopoII activity and increased DNA repair.

Whereas these mechanisms may ultimately cause multi-drug resistance in previously chemosensitive tumours in vitro, both pre-clinical and clinical studies indicate that these mechanisms are unlikely to promote tumour cell survival in the initial aftermath of chemotherapy in chemosensitive tumours in vivo. Therefore the search has continued for alternative mechanisms of multi-drug resistance.

4.1.2 The role of integrins and extracellular matrix in chemoresistance

Most studies into multi-drug resistance have concentrated on the cancer cell in isolation without accounting for factors in the extracellular milieu. There is good evidence that soluble factors in the extracellular environment can modulate chemosensitivity, e.g. the cytotoxicity of vincristine, adriamycin and cyclophosphamide is inhibited by haemopoietic cytokines such as granulocyte macrophage colony stimulating factor, granulocyte colony stimulating factor or interleukin-6 [(Lotem et al., 1992)]. Etoposide-induced apoptosis in pancreatic carcinoma cell lines is increased by interleukin-1β [(Arlt et al., 2002)]. Interferon-alpha 2b sensitises HCT-15 cells to doxorubicin [(Lucero Gritti et al., 2001)] and type I interferon increases cell death in melanoma lines in response to cis-platinum [(Matarrese et al., 2002)].

Further support for the role of extracellular factors in multi-drug resistance is given by the finding that certain resistance mechanisms may only be functional in vivo [Raff, 1992]. For example mammary tumours made resistant to alkylating agents in vivo were sensitised to cytotoxic drugs once removed from the animal [Teicher et al., 1990]. In addition, studies have revealed differences in tumour cell response to drugs when assayed in monolayer versus spheroid culture [Kobayashi et al., 1993], a model that replicates some of the in vivo cellular and extracellular matrix architecture. The spheroid model has been shown to approximate the in vivo microenvironment with the formation of cell-cell junctions and activation of intracellular signalling cascades. Possible mechanisms of spheroid-associated resistance include; inhibition of apoptosis, a preponderance of quiescent cells, modulation of growth and repair enzymes, reduced drug permeability, central hypoxia/necrosis and alterations in subcellular localisation of TopoII [Desoize et al., 2000),(Oloumi et al., 2000).(Desoize et al., 1998]. The recent concept that factors in the extracellular environment may protect against chemotherapy, enabling cells to survive the primary chemotherapy insult and then go on to develop more random and chemotherapy-induced DNA damage which render the cells multi-drug resistant has been the object of significant study in recent years.

A finding of particular significance is that integrin-mediated adhesion to extracellular matrix protects cells from chemotherapy-induced apoptosis. In normal cells ligand binding to integrins is responsible for a multiplicity of cell functions including adhesion and motility [Hauser et al., 1993], angiogenesis [Carpizo et al., 2000),(Brooks et al., 1994], differentiation [Chenard et al., 2000], growth and cell survival [Frisch et al., 2001),(Zhang et al., 1995]. Integrins regulate many intracellular signalling pathways including tyrosine phosphorylation and inositol lipid metabolism that are central to regulation of

essential effectors of apoptosis [Meredith, Jr. et al., 1993),(Bates et al., 1994),(Frisch et al., 1994]. The potential role of integrin binding and signalling in cancer cell anchorage-independence, invasion, motility, metastasis and cancer cell survival has understandably provided the stimulus for many interrelated avenues of investigation.

In 1972 Durand and Sutherland showed that intercellular contact promotes tumour cell survival following cytotoxic damage such as radiation [Durand et al., 1972], an early clue to the role of extracellular protection-signalling. Two decades later Fridman [Fridman et al., 1990] demonstrated that adhesion of SCLC cells to laminin resulted in increased resistance to chemotherapy-induced apoptosis, the dominant form of cell death in response to chemotherapy [Hannun, 1997]. Further work in SCLC has confirmed that an extensive ECM that includes fibronectin, laminin and collagen IV surrounds SCLC cells in vivo. Adhesion of SCLC cells to these matrix proteins via β1-integrins confers resistance to apoptosis induced by etoposide, cis-platinum and doxorubicin and this protection can be completely abrogated by a function-blocking β1 integrin antibody [Damiano et al., 1999].

ECM-mediated protection against chemotherapy-induced apoptosis has been demonstrated in a number of haemopoietic cell lines. Direct in vitro evidence comes from the demonstration that myeloma cell lines expressing α4β1 and α5β1 integrin are resistant to doxorubicin and melphalan when adhered to fibronectin compared to cells grown in suspension. In these cells adherence to fibronectin resulted in no difference in intracellular doxorubicin concentration or expression of ABC cotransporters ruling out the induction of drug efflux pumps as a possible mechanism of cytoprotection [Damiano et al., 1999),(Damiano et al., 2000]. U937 human histiocytic lymphoma cells become resistant to doxorubicin, etoposide and mitoxantrone when adhered to

fibronectin [Hazlehurst et al., 2001b]. In a lymphoma model that mimics the germinal centre by provision of CD40 and interleukin-4, adherence to V-CAM1 (a ligand for α4β1 integrin) resulted in resistance to etoposide-induced apoptosis [Taylor et al., 2000),(Taylor et al., 1999]. Also, B-cell chronic lymphocytic leukaemia (CLL) cells in vitro were protected against fludarabine by α4β1-mediated binding to fibronectin [de la Fuente et al., 2002].

There is also evidence for the presence of this MDR pathway in several solid tumours. Adriamycin MCF-7 breast lines have increased expression of α6β1 integrin and adhesion to laminin compared with the parental line [Narita et al., 1998]. In addition α4β1 and α5β1 integrins are expressed on doxorubicin resistant lines but not on parental lines [Nista et al., 1997] and α5β1 expression enables these cells to derive a growth and survival advantage from fibronectin binding. MDA-MB-231 and 435 breast cancer lines in vitro demonstrated a significant reduction in paclitaxel and vincristine-induced apoptosis following ligation of β1 integrins by their ECM ligands [Aoudjit et al., 2001]. Breast cancer, fibrosarcoma and osteosarcoma lines were all protected against radiation and doxorubicin following incubation with basement membrane gel [Pogany et al., 2001]. Vitronectin, but not fibronectin, protects human glioma cell lines against etoposide-induced death [Uhm et al., 1999]. Similar protective effects have also been seen in colon cancer lines [Kouniavsky et al., 2002]. Indirect evidence of ECM-mediated chemoresistance is available for a broader spectrum of solid cancers. In head and neck cancer a melphalan-resistant nasal carcinoma cell line shows upregulation of integrin subunits α2, α5, α6, β1 and β2 compared with drug-sensitive parent lines. As well as chemoresistance phenotype this was associated with a significantly stronger binding to ECM and increased invasiveness [Liang et al., 2001]. A vinblastine-resistant subline of renal carcinoma cells showed increased expression of

α1β1/α2β1 and decreased expression of α6β1 integrin in association with increased attachment to collagen and fibronectin [Duensing et al., 1996]. Similar indirect evidence exists in drug-resistant sublines of glioma [Hikawa et al., 2000],[Gladson et al., 1995] and ovarian cancer [Sedlak et al., 1996],[Maubant et al., 2002].

Evidence is accumulating that ECM induces this important phenomenon of multidrug chemoresistance by a number of mechanisms which include:

4.1.3 Increased resistance to tumour penetration resulting in reduced drug exposure

There is some evidence to support the intuitive notion that ECM provides a physical barrier to chemotherapy thus blocking its delivery to cancer cells. In a multicellular layer model, mouse mammary and human bladder cancer cells were grown to a depth of 200μm. These cells developed an ECM of laminin and collagen and penetration of cis-platinum, etoposide, gemcitabine, paclitaxel and vinblastine through the matrix was diminished [Tannock et al., 2002]. A reduced macromolecule diffusion coefficient was noted in tumours with extensive collagen networks [Netti et al., 2000] and collagenase treatment of these tumours reversed this physical resistance. It is postulated that collagen influences macromolecule transport by binding to and stabilizing the glycosaminoglycans component of ECM. Early clinical trials using hyaluronidase have, however, failed to show synergy with chemo-irradiation [Baumgartner et al., 1998]. It is important to reflect that though this mechanism may genuinely occur in vivo there is good evidence*, in vivo*, that drug delivery is in fact adequate on many occasions (denoted by the occurrence of complete responses). Furthermore integrin-mediated MDR is present in *in vitro* models where drug intracellular drug delivery is

guaranteed [Damiano et al., 1999),(Damiano et al., 2000)]. It is therefore very unlikely that reduced drug penetration is responsible for all ECM-related chemoresistance *in vivo*.

4.1.4 Reduced induction of DNA damage or upregulated DNA repair

It is possible that ECM confers drug resistance by limiting the amount of DNA damage induced by chemotherapy. Although in a SCLC study [Sethi et al., 1999b] TopoII-induced DNA damage measured by a decatenation assay was similar in ECM and plastic-plated cells there have been other positive reports of diminished DNA damage in the presence of ECM. Dalton et al identified a 40-60% reduction in etoposide-induced DNA double-strand breaks measured by a neutral comet assay when U937 lymphoma cells were adhered to fibronectin [Hazlehurst et al., 2001a),(Hazlehurst et al., 2001b)]. It appears that this was due to a reduction in TopoII activity secondary to alterations in the nuclear distribution of enzyme. The mechanisms responsible for this are, as yet, unknown. In a study which used mouse lung endothelial cells adherence to collagen, laminin and soluble (or coated) α5, β1, β3 integrin antibodies resulted in integrin clustering and a reduction in bleomycin-induced DNA damage measured by DNA sedimentation and in-situ nick translation [Hoyt et al., 1997]. Further investigation showed that this DNA protection was PARP-dependent suggesting that an alteration in chromatin structure may be important [Jones et al., 2001].

4.1.5 Autocrine loops

It is well recognised that cancer cells are capable of production and secretion of ECM. Immunohistochemical staining of archival SCLC tissue demonstrated not only extensive ECM but also the presence of ECM proteins *within* cancer cells [Sethi et al,.

[1999b]. Vitronectin mRNA is increasingly expressed in glial cells as they progress from normal → low-grade → high-grade astrocytoma and vitronectin binding integrins were detected throughout these tumours [(Gladson et al., 1995)]. Human myeloma cell lines expressed fibronectin mRNA and fibronectin secretion was detectable by ELISA [(van, I et al., 1994)]. 7/10 of these myeloma lines showed subsequent adherence to this fibronectin via α4β1 integrins raising the possibility of an autocrine loop mediating increasing chemoresistance.

4.1.6 Integrin-mediated modulation of cell survival and death signalling pathways

Outside-in signalling

In this process intracellular biochemical pathways controlling cellular proliferation and apoptosis are modulated by integrin-mediated signals. Several intracellular signalling proteins are known to associate with the cytoplasmic domain of integrins. The most well characterised include FAK [(Schaller, 2001)], integrin-linked kinase (ILK) [(Wu et al., 2001a)] and integrin-associated protein (IAP) [(Juliano, 2002)] though increasing information is accruing on integrin cytoplasmic domain-associated protein-1 (ICAP-1), cytokesin-1, calcium and integrin-binding protein (CIB) and calreticulin [(Coppolino et al., 2000)]. These integrin-binding proteins then signal to second-messengers that include protein kinases, e.g. protein kinase B [(Nicholson et al., 2002)], protein kinase C, phosphoinositide-3-kinase [(Jones et al., 2000)], mitogen activated protein kinase [(Coppolino et al., 2000)], mapk kinase/MEK, glycogen synthase kinase, Jun-N-terminal kinase and GDP/GTP binding proteins (e.g. Ras, Rho [(Parise et al., 2000)]) sometimes in association

with adaptor/docking proteins such as Cas [O'Neill et al., 2000], Grb2/7. These cascades result in control of crucial enzyme systems such as the caspase system, Bax/Bcl-2 family of proteins and regulators of the cell cycle. Interestingly, until recently, unligated integrins were considered to be relatively inert and to contribute minimally to intracellular signalling. However, work by Stupack et al [Stupack et al., 2001] necessitates further consideration of this premise. They show, using carcinoma cells in a 3-dimensional matrix model, that unligated αvβ3 integrins actively recruit caspase-8 to the membrane and activate apoptosis in a death receptor/FADD independent manner.

It is postulated that ECM-binding to integrins results in upregulation of survival and downregulation of apoptotic pathways to prevent chemotherapy-induced apoptosis. To date very few studies have addressed the challenging task of identifying which of the above effectors are the key mediators in ECM-mediated drug-resistance. However, initial steps have been made.

Apoptotic second-messenger systems

There is an enormous literature describing intermediate effectors of apoptosis from cell surface to nucleus. Very few of these have addressed the specific pathways involved in ECM-dependent MDR. In small cell lung cancer ECM inhibits etoposide-induced caspase-3 cleavage and apoptosis [Sethi et al., 1999b],[Rintoul et al., 2002]. This mechanism was protein tyrosine kinase-dependent as shown by reversal by tyrphostin, a tyrosine kinase inhibitor. In breast cancer [Aoudjit et al., 2001] integrin-mediated protection from paclitaxel/vincristine was shown to be a result of inhibition of mitochondrial cytochrome-c release (a key activator of the caspase system) in a

mechanism dependent upon PI3K activation of Protein Kinase B. Interestingly the inhibition of PI3K in an in vitro pancreatic cancer cell line [Ng et al., 2000] and an in vivo (athymic mouse xenogeneic transplant) ovarian cancer model [Hu et al., 2002] resulted in significant chemosensitisation though neither of these studies addressed ECM binding.

Bax/Bcl-2 signalling

The Bax/Bcl-2 system is central to cell fate decision-making. Bax family members are pro-apoptotic acting via the mitochondrial caspase-activating pathway whilst Bcl-2 family members are anti-apoptotic [Inoue et al., 2001]. The ratio of these competing moieties determines the cell's fate (**Appendix: Fig 6**). Indirect evidence supports the role of Bax/Bcl-2 proteins in ECM-mediated MDR. In Chinese hamster ovary cells [Zhang et al., 1995] adhesion to fibronectin via the $\alpha5\beta1$ (but not $\alpha v\beta1$) integrin prevents cells from undergoing apoptosis on serum starvation. This requires the cytoplasmic domain of the $\alpha5\beta1$ integrin and was dependent on tyrosine phosphorylation of Focal Adhesion Kinase (FAK). Anti-$\beta1$ integrin antibodies and anti-sense oligonucleotides enhanced apoptosis as measured by nuclear condensation and nucleosomal laddering. In an in vitro melanoma cell line $Bcl_X(L)$ upregulation was shown to induce cis-platinum chemoresistance and antisense oligonucleotide treatment was shown to reverse this effect [Heere-Ress et al., 2002]. Studies in B lymphoma cells have shown that in a model mimicking conditions in a germinal centre activation of $\alpha4\beta1$ integrins resulted in elevated levels of $Bcl_X(L)$ gene transcription and protein levels in a NFκB-dependent manner. This resulted in etoposide resistance and ligand binding to $\alpha4\beta1$ diminished conformational changes in Bax proteins to prevent etoposide-

induced disruption of constitutive Bax/Bcl-2 binding [Taylor et al., 2000]. Similarly, fludarabine resistance in B-cell chronic lymphocytic leukaemia was associated with α4β1 integrin-induced upregulation of $Bcl_X(L)$ [de la Fuente et al., 2002]. Ligation of αvβ3 integrin in endothelial cells results in intracellular signals that leads to increased expression of Bcl-2 with a coordinate reduction in the levels of Bax. This stoichiometric change consequently increases the Bcl-2/Bax ratio thereby tipping the intracellular balance in favour of cell survival through the inhibition of apoptosis [Brooks et al., 1994]. The αvβ3 integrin, as well as αvβ5, the other receptor for vitronectin, is also expressed on the surface of malignant glioma cells in vivo [Gladson et al., 1995]. Ligation of either vitronectin receptor is associated with enhanced survival of glioblastoma cells. Moreover the enhanced survival conferred by these two vitronectin receptors translates into the phenotype of chemoresistance, a feature that typifies high-grade gliomas. Activation of vitronectin receptors in glioma cells is associated with increased expression of the anti-apoptotic proteins Bcl-2 and $Bcl_X(L)$. As levels of the pro-apoptotic protein Bax did not change, the net effect was to promote survival by apoptotic inhibition [Uhm et al.,1999]. However, in an in vitro myeloma model [Damiano et al., 1999], although a doxorubicin/melphalan-resistant subline was shown to express increased α4β1 receptors that rendered them chemoresistant when adhered to fibronectin, this drug resistance was not mediated via Bax/Bcl-2 changes suggesting cell-type specificity for this phenomenon.

Cell cycle effectors

The cell cycle is regulated by cascades of cyclins/cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs) that respectively promote and inhibit

the cell cycle. Manipulation of cell cycle activity represents another mechanism by which the ECM-mediated chemoprotective effect could be explained. For example, it may be hypothesised that promotion of the cell cycle through arrest points may avert DNA-damage checkpoint analysis and thereby prevent DNA damage-recognition and apoptotic decision-making. Integrin signalling has been shown to have multiple effects on the cell cycle: Integrin-mediated cyclin D1 upregulation, CDKI ($p21^{cip1}$ and $p27^{kip1}$) downregulation and hyperphosphorylation of the retinoblastoma protein results in progression of the cycle from G1 into S phase (Zhu et al., 1996). This mechanism is intimately linked to the activity of intracellular biochemical cascades and growth factor signalling. Most studies identify a positive influence from ECM on the cell cycle though there are reports of cell-type-specific cell cycle inhibition for αvβ8 (Cambier et al., 2000) and α2β1 (Coppolino et al., 2000). The evidence regarding cell-cycle effects in the setting of ECM chemoresistance is scarce and contradictory. In adriamycin-resistant MCF-7 breast cancer cells α5β1 binding to fibronectin promotes G1-S progression and prevents them from undergoing serum deprivation-induced apoptosis (Nista et al., 1997). However, upon adherence to fibronectin, myeloma cells develop drug-resistance but concomitantly undergo G1 cell cycle arrest associated with increased $p27^{kip1}$ levels and inhibition of cyclin A/E kinase activity (Hazlehurst et al., 2000).

This laboratory has previously shown that adhesion of small cell lung cancer (SCLC) cells to extracellular matrix (ECM) via β1-integrins (α2β1, α3β1, α6β1 and αvβ1 – **see Appendix: Table 6**) protects against chemotherapy-induced apoptosis (Sethi et al., 1999b) and this novel mechanism of drug resistance has been shown to exhibit a broad

cell type and chemotherapeutic agent specificity [Elliott et al., 2002]. However, the intracellular signalling responsible for this cell survival is poorly understood. Although small cell lung cancer primary site and metastatic tumours consist of closely packed cells, they are surrounded by an extensive stroma rich in ECM proteins and high levels of extracellular matrix expression around cells correlate with poorer patient prognosis [Sethi et al., 1999b),(Rintoul et al., 2001),(Rintoul et al., 2002].

In this body of work I initially confirmed reproducibility of the previous model used in SCLC in this laboratory for assessing ECM-mediated chemoprotection. Subsequently, experiments were performed to assess the hypotheses:

a "ECM mediates survival signalling in SCLC via β1-integrin-dependent modulation of DNA damage and repair".

b "ECM mediates β1-integrin-dependent survival signalling in SCLC by overriding cell cycle checkpoints and thus evades programmed cell death".

4.2 Extracellular matrix protects small cell lung cancer against etoposide and radiation-induced apoptosis

Experiments described here sought to reproduce previous results demonstrating matrix-mediated chemoprotection and further, since radiotherapy plays a central role in the clinical management of SCLC, to show that ECM-mediated antiapoptosis also protects against radiation-induced cell death.

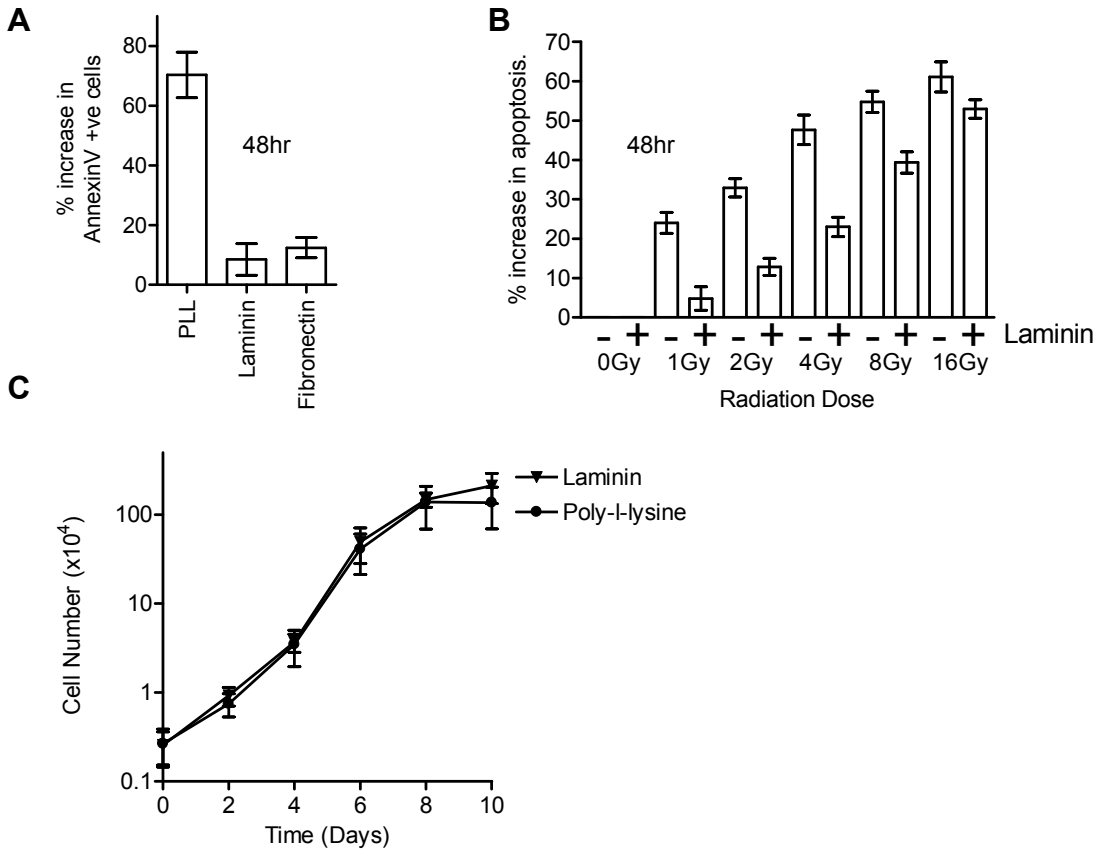


Figure 4.1 A Fibronectin and laminin protect against etoposide-induced apoptosis in SCLC cells. H345 SCLC cells were quiesced overnight and seeded into 96 well plates pre-coated with poly-l-lysine (10µg/ml), laminin (10µg/ml) or fibronectin (20µg/ml) and treated with or without etoposide (25µg/ml). The percentage of apoptotic cells was determined after 48 hours by flow cytometric assessment of AnnexinV binding. Results are presented as absolute % increase in apoptosis compared with non-treated cells (mean ± s.e. of n=4 experiments). Data analysis: matched 1-way ANOVA with Bonferroni post-test. **B Laminin protects against radiation-induced apoptosis in SCLC cells.** H345 SCLC cells were quiesced overnight and seeded into 96 well plates pre-coated with poly-l-lysine (10µg/ml) or laminin (10µg/ml) and treated with or without megavoltage radiotherapy (0-16Gy). The percentage of apoptotic cells was determined by morphological assessment of acridine orange/ethidium bromide-stained cells. Results are presented as absolute % increase in apoptosis compared with non-treated cells (mean ± s.e. of n=5 experiments). Data analysis: matched 1-way ANOVA with Bonferroni selected post-test. **C Extracellular matrix does not modulate SCLC growth rates.** $0.5x10^4$ H345 cells were plated into 24-well plates coated with poly-l-lysine (10µg/ml) or laminin (10µg/ml) and following disaggregation, cell numbers were counted at times indicated using a haemocytometer. Mean ± s.e. of n=3 experiments shown.

Results: After 48 hours of plating on poly-l-lysine, etoposide induced an increase in apoptosis in H345 cells measured by AnnexinV staining of 70.3 ± 7.6% (s.e.). This was significantly reduced by plating cells on laminin (8.5 ± 10.6%; $p<0.01$) and fibronectin (12.4 ± 6.8%, $p<0.01$) (**Fig 4.1A**). This protective effect was first seen at 36 hours and persisted to 72 hours and was confirmed by a) acridine orange/ethidium bromide staining assessment and b) a cell death detection ELISA kit which is based on the quantitative detection of histone-associated DNA fragments [(Hodkinson et al., 2006)]. This effect was not due to a direct physical interaction between etoposide and ECM, since etoposide-treated cell culture media incubated in wells plated with ECM for 48 hours was still able to induce apoptosis when transferred onto SCLC cells adhered to poly-l-lysine (data not shown [(Hodkinson et al., 2006)]).

Laminin (and fibronectin, data not shown) also protected H345 cells (and H69 cells, data not shown) against radiation-induced apoptosis (**Fig 4.1B**). This was statistically significant up to a dose of 8Gray (Gy) but was non-significant at higher radiation dose levels. Since radiation induces apoptosis via direct DNA damage this further supports the assumption that laminin-mediated protection against etoposide is not due to a direct physical interaction or reduction of entry of etoposide into SCLC cells.

Cells grown on laminin (and fibronectin, data not shown) proliferated at the same rate as cells grown on poly-l-lysine (**Fig 4.1C**) confirming that differences in apoptosis were not due to altered rates of proliferation, but to altered rates of apoptosis.

4.3 Extracellular matrix-mediated protection is β1-integrin-dependent

Previous experiments in this laboratory have shown that the β1-integrin is highly expressed in SCLC cells [(Sethi et al., 1999b)]. To test the hypothesis that ECM-mediated protection against etoposide-induced apoptosis in SCLC is due to β1-integrin-mediated signalling, experiments were performed using function-blocking β1-integrin antibodies (4B4) and function-activating antibodies (TS2/16).

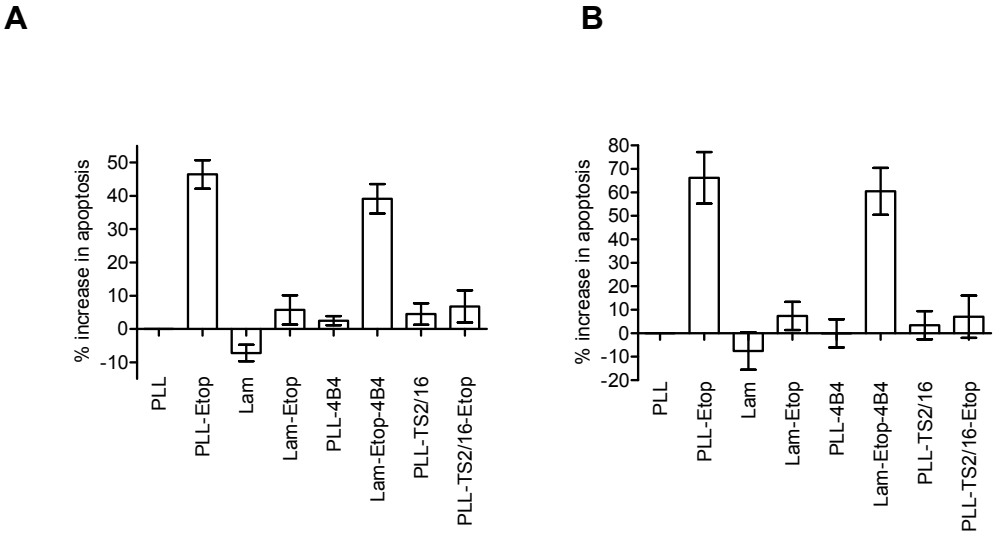


Figure 4.2 A Laminin protects against etoposide-induced apoptosis in a β1-integrin-dependent manner in SCLC cells. H345 SCLC cells were quiesced overnight, preincubated with or without function blocking β1-integrin antibody (10μg/ml 4B4) for ½ hour and seeded into 96 well plates pre-coated with poly-l-lysine (10μg/ml), laminin (10μg/ml). or function-activating antibody (10μg/ml TS2/16). They were treated with or without etoposide (25μg/ml) and the percentage of apoptotic cells was determined after 48 hours by morphological assessment of acridine orange/ethidium bromide-stained cells **(A)** and a cell death ELISA kit based on the quantitative detection of histone-associated DNA fragments (mean ± s.e. of n=4 experiments) **(B)**. Results are presented as % increase in apoptosis compared with non-treated cells on poly-l-lysine (mean ± s.e. of n=6 experiments). Data analysis: matched 1-way ANOVA with Bonferroni selected post-test.

Results: When H345 (and H69, data not shown) SCLC cells were plated on poly-l-lysine, etoposide induced an increase in morphologically assessed apoptosis of 46.44 ± 4.3% (s.e.). This was significantly reduced to 5.73 ± 4.4% ($p<0.001$) by adhesion to laminin (**Fig 4.2A**). However, although treatment of H345 SCLC cells with function-blocking β1-integrin antibody 4B4 did not induce a significant increase in apoptosis (PLL-4B4), it did abrogate laminin-mediated etoposide protection ($p<0.001$). 4B4-isotype antibody (anti-IgG1) did not produce this effect (data not shown). TS2/16, a function-activating β1-integrin antibody replicated the laminin-mediated protection against etoposide-induced apoptosis, further supporting the evidence that extracellular matrix protects against apoptosis via a β1-integrin-mediated mechanism. These results were confirmed in similar experiments where the technique of assessment of apoptosis was a cell death ELISA kit based on the quantitative detection of histone-associated DNA fragments (**Fig 4.2B**).

4.4 Extracellular matrix-mediated protection is Protein Tyrosine Kinase and Phospho-Inositol-3-Kinase-dependent

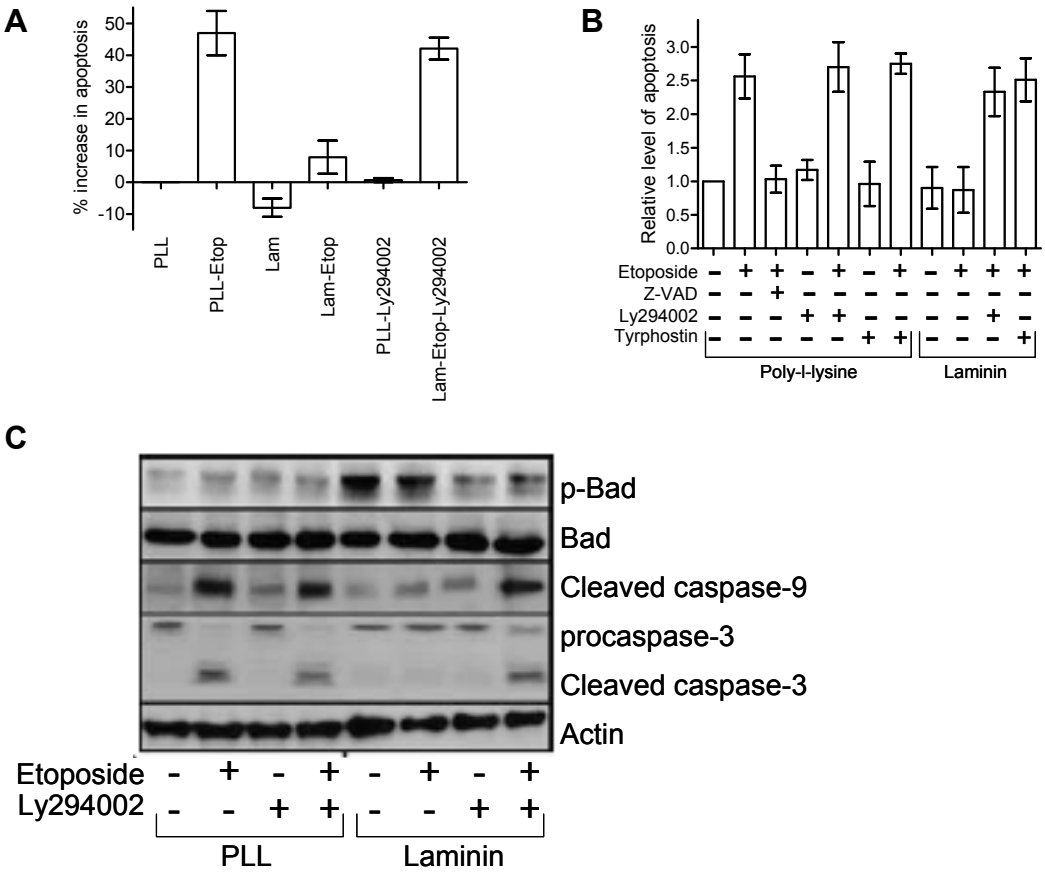


Figure 4.3 Etoposide-induced apoptosis in SCLC is reversed by laminin in a PI3Kinase and tyrosine kinase-dependent manner. Quiesced H345 cells were seeded into 96-well plates pre-coated with poly-l-lysine (10μg/ml) or laminin (10μg/ml) and treated with (+) or without (-) etoposide (25μg/ml), Ly294002 (30μM), Tyrphostin-25 (25mM) or z-VAD (100μM) as indicated. Apoptosis was measured after 48 hours using **A** morphological assessment of acridine orange/ethidium bromide stained cells and (mean ± s.e. of n=6 experiments shown). **B** Caspase-3 activity assay (as described in materials and methods), results normalised to untreated cells on poly-l-lysine (mean ± s.e. of n=6 experiments shown). Data analysis: matched 1-way ANOVA with Bonferroni selected post-test. **C** Quiesced H345 cells were seeded into six-well plates precoated with poly-l-lysine (10mg/ml) or laminin (10 mg/ml) and treated with (+) or without (-) etoposide (25 mg/ml) or Ly294002 (30mM) as indicated. After 24hrs the cells were lysed and the expression of phospho-Bad (p-Bad), Bad, cleaved caspase-9 (p10) and caspase-3 was determined by Western blot. Representative blots from are shown. Actin is shown as a loading control.

Results: Using morphological assessment (**Fig 4.3A**), laminin protected against etoposide-induced apoptosis. Treatment of H345 cells (and H69, data not shown) with the PI3Kinase inhibitor, Ly294002 alone (and wortmannin, data not shown) did not induce a significant increase in apoptosis but it did completely inhibit laminin-mediated etoposide protection ($p<0.001$). This effect of PI3Kinase blockade was confirmed using an ELISA assessment of apoptosis based on the quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes (**Fig 4.3B**). In addition, treatment of H345 cells (and H69, data not shown) with a broad spectrum tyrosine kinase inhibitor, Tyrphostin-25, alone did not induce a significant increase in apoptosis but did completely abrogate laminin-mediated protection against etoposide-induced apoptosis.

PKB is a downstream target of PI3Kinase which phosphorylates proapoptotic factors such as caspase-9 and Bad resulting in inhibition of programmed cell death [(Diehl et al., 1998),(Buss et al., 2004)]. Adhesion of H345 SCLC cells to laminin increased phosphorylation of Bad (without modulating total Bad levels) and reduced etoposide-induced cleavage of caspase-9 (**Fig 4.3C**). These effects were inhibited by treatment with the PI3Kinase inhibitor Ly294002. Caspase-9 activation results in cleavage of the 'effector' caspase-3, which stimulates apoptosis. Etoposide induced caspase-3 cleavage in H345 cells adhered to poly-l-lysine, but this cleavage was abrogated in cells adhered to laminin. Furthermore, Ly294002 did not affect caspase-3 cleavage in cells adhered to poly-l-lysine but did inhibit laminin-mediated blockade of caspase-3 cleavage. Coinvestigators in this laboratory confirmed this result by direct assay of caspase-3 activity [(Hodkinson et al., 2006)].

These data confirm that extracellular matrix-mediated protection against etoposide-induced apoptosis is mediated via a β1-integrin-dependent pathway that signals via second messenger systems involving tyrosine kinase and PI3Kinase signalling. Furthermore, PI3Kinase signalling is implicated in ECM-mediated resistance to etoposide-induced apoptosis in SCLC cells via phosphorylation of Bad and modulation of caspase activity.

4.5 Adhesion of SCLC cells to ECM proteins does not modulate initial levels of DNA damage or subsequent DNA repair

Etoposide and x-rays induce apoptosis via the initial production of double-strand DNA breaks [(Vincent, 1995),(Mathieu et al., 1999),(Verheij et al., 2000)]. Cells identify these sites of DNA damage, undergo cell cycle arrest at checkpoints and either repair the DNA breaks or undergo apoptosis [(Jackson, 2001)]. I hypothesised that ECM binding to β1-integrin cell surface receptors results in a) decreased levels of initial DNA damage or b) upregulation of DNA repair to reduce the number of cells instructed to undergo programmed cell death.

To determine the amount of DNA damage induced by etoposide and radiation the number of DNA double-strand breaks in cell nuclei were determined by immunofluorescence staining of phosphorylated H2Ax (γH2Ax). The foci of γH2Ax represent individual DNA double-strand breaks and have been shown to correlate closely with apoptosis in response to DNA damage [(Olive, 2004)]. Double-strand breaks were further confirmed by Western blotting for γH2Ax and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), another enzyme involved in double-strand break repair.

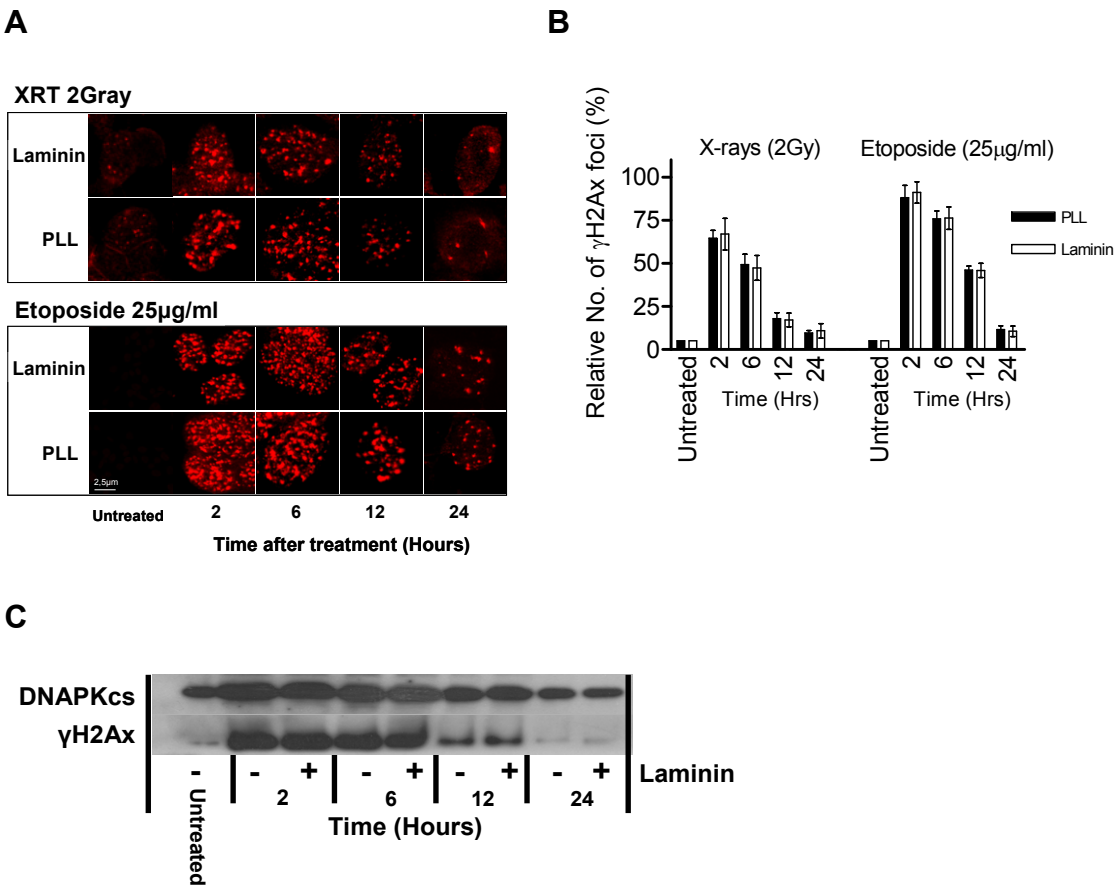


Figure 4.4 A Quiesced H345 cells were adhered to poly-l-lysine (10µg/ml) or laminin (10µg/ml) and treated with or without 6MV X-rays (2Gy) or 2 hours of etoposide (25µg/ml). Cells were washed twice and the media replaced with complete media containing 10% FCS. Cells were fixed 0-24 hours later, stained with anti-γH2Ax antibody and foci viewed by fluorescence microscopy. Representative images of γH2Ax foci from n=4 experiments. **B** The intensity of γH2Ax staining in 10 high-power fields was quantified with Openlab image analysis software and normalised to untreated cells (mean relative γH2Ax foci ± s.e. of n=3 experiments). **C** Concomitantly, parallel experiments in 6cm plates were submitted to western blot analysis for γH2Ax and $DNAPK_{CS}$. A representative blot from n=4 etoposide-treated plates is shown.

Results: In H345 cells, treatment with radiation and etoposide induced a marked increase in γH2Ax foci (**Fig 4.4A+B**) which was detectable 2 hours after etoposide treatment and immediately after radiotherapy (data not shown). Over the next 24 hours the number of γH2Ax foci decreased as double-strand break repair was

performed. Adherence to laminin (and fibronectin, data not shown) did not reduce the initial number of γH2Ax foci induced or the rate of double-strand break repair. It is notable that at 24 hours there were still a significant number of γH2Ax foci, suggesting persistence of unrepaired DNA damage. Western blot analysis for γH2Ax and DNA-PKcs (**Fig 4.4C**) demonstrated a parallel increase in expression of phosphorylated H2Ax and DNA-PKcs following etoposide exposure and subsequent reduction with time. Thus, extracellular matrix protection against etoposide and radiotherapy is not due to a reduction in initial DNA damage (and thus does not influence drug entry into small cell lung cancer cells) or subsequent modulation of DNA repair.

4.6 ECM proteins override etoposide and radiation-induced G2/M arrest via β1-integrin and PI3Kinase-dependent modulation of cell cycle regulatory proteins

When cells are exposed to DNA damage they characteristically undergo cell cycle arrest at either the G1/S or G2/M checkpoints [(Kastan et al., 2004),(Guadagno et al., 1993),(Fang et al., 1996),(Massague, 2004)]. This enables cells to repair damaged DNA or initiate programmed cell death to prevent the production of daughter cells with damaged DNA. Modulation of cell cycle checkpoints could therefore provide a mechanism for modifying a cell's response to chemotherapeutic or radiation insult.

It has previously been shown that protein ligand binding (e.g. Growth Factors) to cell surface receptors can regulate cell cycle progression via modulation of cyclins, cyclin dependent kinases and cyclin dependent kinase inhibitors [(Musgrove, 2006),(Danen et al., 2001)]. It was hypothesised that integrins induce comparable changes in cell cycle

signalling in small cell lung cancer to promote cell cycling and override G1/S and G2/M cell cycle checkpoints. By driving cells with DNA damage through these checkpoints, cells with treatment-induced DNA damage may circumvent apoptosis and go on to survive with additional DNA damage.

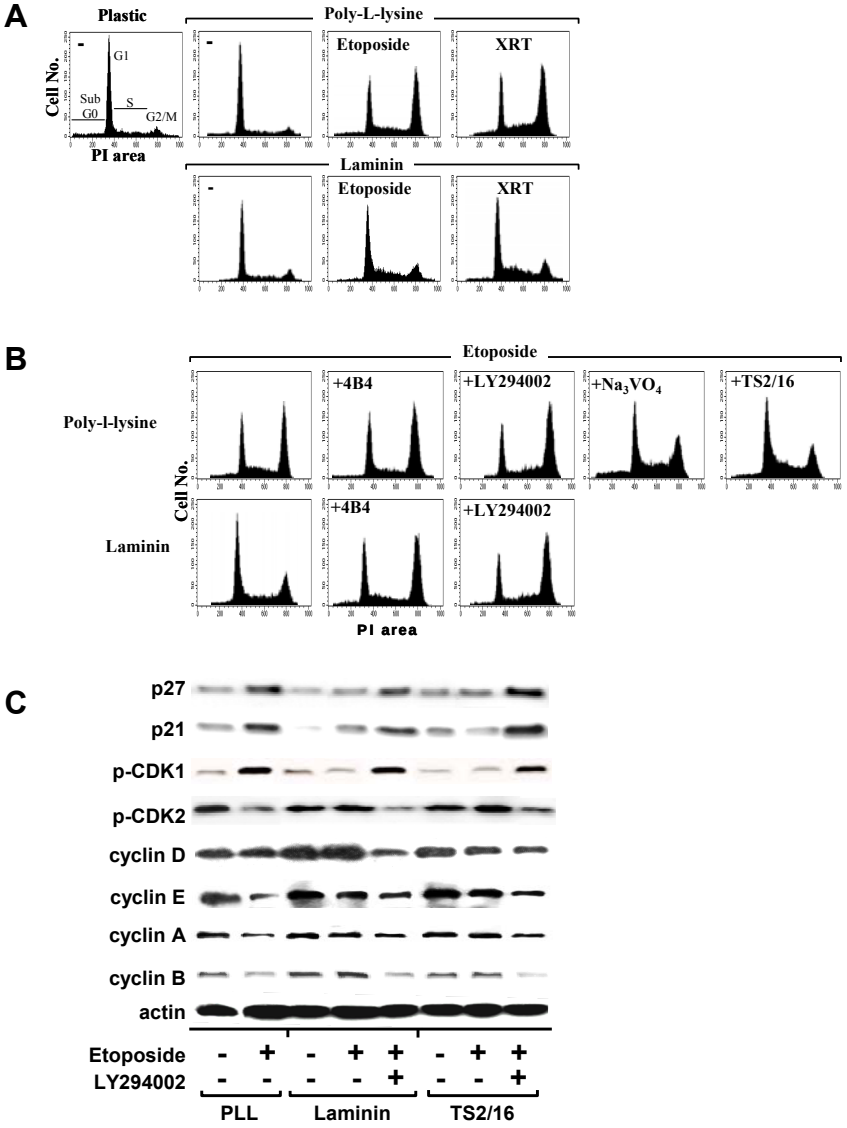


Figure 4.5 ECM proteins override etoposide- and radiation-induced G2/M arrest via β1-integrin and PI3Kinase-dependent modulation of cell cycle regulatory proteins. A H345 cells were quiesced overnight, washed twice and seeded into 6 well tissue culture plates uncoated (plastic) or pre-coated with poly-l-lysine (10μg/ml) or laminin (10μg/ml). Cells were treated as indicated: no further treatment (-), etoposide (25μg/ml) or ionising radiation (2 Gy) (XRT). Progression through the cell cycle was assessed 24 hours later by flow cytometric analysis of DNA content using CELL Quest software. Representative histograms are shown from n=5 independent experiments (table of actual percentage of cells in G1/S/G2-M phases of cell cycle included in **Appendix: Table 7)**. **B** Quiesced H345 SCLC cells were seeded into 6 well tissue culture plates pre-coated with poly-l-lysine (10 μg/ml) or laminin (10μg/ml) and treated with etoposide (25 μg/ml). In addition cells were treated as indicated: blockade of β1 integrin function with 4B4 (10 μg/ml), inhibition of PI3Kinase with Ly294002 (30μM), activation of tyrosine kinase with sodium orthovanadate (Na_3VO_4) (200μM) or stimulation of β1 integrins with TS2/16 (10 μg/ml). Progression through the cell cycle was assessed 24 hours later by flow cytometric analysis of DNA content using CELL Quest software. Representative histograms are shown from n=4 independent experiments (table of actual percentage of cells in G1/S/G2-M phases of cell cycle included in **Appendix: Table 8)**. **C** H345 SCLC cells were seeded into 6 well plates pre-coated with poly-l-lysine (10 μg/m l) (PLL), laminin (10 μg/ml) or TS2/16 (10 μg/ml). Cells were treated with (+) or without (-) etoposide (25 μg/ml), Ly294002 (30μM) or sodium orthovanadate (Na_3VO_4) (200μM) as indicated. Cells were lysed after 24 hours and the expression of $p21^{Cip1/WAF1}$, $p27^{Kip1}$, phosphorylated CDK1 (p-CDK1), phosphorylated CDK2 (p-CDK2) and cyclins D, E, A and B were determined by Western blot analysis.

Results: H345 cells demonstrated similar cell cycle profiles when grown in suspension or plated on poly-l-lysine or plastic. When treated with etoposide (25μg/ml) or radiotherapy (2Gy), cells plated on poly-l-lysine (or plastic – data not shown) underwent a significant change in DNA content profile. 24 hours after treatment with etoposide and radiotherapy 46.2 ± 1.5% (s.e.) and 47.8 ± 2.1% of cells respectively were in the G2/M phase of the cell cycle (with a reciprocal reduction in the percentage of cells in G1) compared with 8.8 ± 1.0% of untreated cells. However, when plated on laminin only 16.8 ± 2.0% (etoposide) and 16.1 ± 1.9% (XRT) of cells were arrested in the G2/M phase. Similar results were observed when plated on fibronectin (results not shown) (**Fig 4.5A**). Inhibition of β1-integrin signalling with 4B4 completely inhibited laminin-mediated protection from G2/M cell cycle arrest induced by etoposide in H345 SCLC cells without affecting cell cycle arrest in cells adhered to poly-l-lysine (**Fig 4.5B**). Also, laminin-mediated protection from etoposide-induced G2/M cell cycle arrest was blocked by the PI3Kinase inhibitor Ly294002. Stimulation of β1-integrins on H345 SCLC cells with TS2/16 or activation of tyrosine kinases with sodium orthovanadate (Na_3VO_4) reduced etoposide-induced G2/M cell cycle arrest and replicated the effects of ECM. These results indicate that protection of SCLC cells from etoposide-induced cell cycle arrest by ECM proteins is mediated via β1-integrin activation of PI3Kinase and tyrosine-kinase signalling.

After 24 h of etoposide treatment, H345 SCLC cells demonstrated a reduction in expression of cyclins E, A and B (**Fig 4.5C**). In parallel, the CDK inhibitors p21Cip1/WAF1 and p27Kip1 exhibited increased expression. Phosphorylated CDK1 (inactive form) was increased and expression of phosphorylated CDK2 (active form)

decreased. Adhesion of H345 SCLC cells to laminin both increased cyclin D expression and inhibited the etoposide-induced modulation of cyclins E, A and B, phosphorylated CDK1 and 2, p21Cip1/WAF1 and p27Kip1. The effects of ECM were reproduced by function-activating TS2/16 antibodies. These laminin and TS2/16-mediated effects on etoposide-induced changes in cell cycle regulators was blocked by the PI3Kinase inhibitor Ly294002. These results indicate that ECM proteins can override etoposide-induced G2/M cell arrest via β1-integrins by modulating expression of cyclins, CDKs and CDK inhibitors in a PI3Kinase dependent manner.

4.7 Discussion

4.7.1 Extracellular matrix protects small cell lung cancer against etoposide and radiation-induced apoptosis.

Lung cancer in the most common cause of death from malignancy in the developed world (Parkin et al., 2005). The small cell variety constitutes 25% of all lung cancers and has poor prognosis with 2-year survivals less than 5% in many series. Surgery plays no significant role in SCLC therapy and radiotherapy/chemotherapy are the mainstay of treatment. Although response rates of up to 80% are seen with initial etoposide-based chemotherapy, local and metastatic disease relapse characteristically develops several months later and this recurrent disease is frequently resistant to subsequent chemotherapeutic agents. This phenomenon is known as acquired multidrug resistance (MDR) and represents the major barrier to successful cure in the majority of cancer patients who present with overt or occult metastatic disease. Classical mechanisms of MDR such as overexpression of the multi-drug resistance gene

MDR1 are not implicated in SCLC MDR [Lai et al., 1989]. New therapeutic targets are urgently needed and a comprehensive understanding of the mechanisms of MDR will be required to enable the development of new anticancer agents based on plausible biological hypotheses.

This laboratory has previously demonstrated that SCLC is surrounded by a stroma rich in extracellular matrix proteins; laminin, fibronectin and collagen. SCLC cells bind to these proteins in a β1-integrin-dependent manner and this ECM-integrin interaction stimulates second messenger pathways that promote cell survival and protection against etoposide-induced cell death [Sethi et al., 1999b]. No previous data informs on the mechanisms involved in MDR in SCLC and the experiments presented here provide novel insights into SCLC biology and therapeutic resistance.

In vitro experiments using H345 and H69 SCLC cells plated into 96-well plates precoated with poly-l-lysine, laminin and fibronectin showed that ECM proteins protected SCLC cells against etoposide (**Fig 4.1A**) and radiation-induced (**Fig 4.1B**) apoptosis. Since morphological assessments of apoptosis can be subject to bias, results were confirmed by blinding and apoptosis was also measured by non-morphological techniques including Annexin V staining and an ELISA kit based on the quantitative detection of histone-associated DNA fragments. Etoposide and radiotherapy are the two most important therapeutic tools in SCLC treatment and this finding may have great significance in the mechanisms underlying treatment resistance. A possible explanation for ECM-mediated protection against apoptosis in vitro is a direct interaction of etoposide with ECM proteins (i.e. sequestration of active drug in the extracellular media). However two lines of evidence refute this: 1) etoposide-treated cell culture media incubated in wells plated with ECM for 48 hours

was able to induce apoptosis when transferred onto SCLC cells adhered to poly-l-lysine (data not shown [Hodkinson et al., 2006]) and 2) this criticism would not explain protection against radiation where the genetic insult occurs directly. Another possible mechanism by which ECM could modulate sensitivity to DNA-damaging agents is via modulation of proliferative rate; evidence shows that rapidly proliferating cells are more sensitive to DNA-damaging agents and thus an integrin-mediated reduction in proliferation would be expected to be protective against apoptotic insult. However, cells grown on laminin (and fibronectin, data not shown) proliferated at the same rate as cells grown on poly-l-lysine (**Fig 4.1C**) confirming that differences in apoptosis were not due to altered rates of proliferation, but to altered rates of apoptosis.

4.7.2 Extracellular matrix-mediated protection is β1-integrin-dependent

The dominant integrin expressed in H345 and H69 SCLC cell lines is β1-integrin (**see Appendix: Table 8**). Here, function-blocking β1-integrin antibodies (4B4), but not isotype antibodies, completely abrogated laminin and fibronectin's ability to prevent etoposide-induced apoptosis (**Fig 4.2**) and furthermore function-activating β1-integrin antibodies (TS2/16) replicated the ECM-mediated protective effect (**Fig 4.2**). Taken together this provides these results indicate that ECM proteins prevent apoptosis in SCLC cells via activation of β1-integrins.

4.7.3 Extracellular matrix-mediated protection is Protein Tyrosine Kinase and Phospho-Inositol-3-Kinase-dependent

It has previously shown that integrin-dependent adhesion induces an increase in tyrosine phosphorylation and PI3Kinase activation (Downward, 2004),(Kang et al., 2005). Activated PI3Kinase phosphorylates inositol lipids, resulting in the activation of downstream serine/threonine kinase PKB that can phosphorylate caspase-9 and BAD to promote cell survival [(Downward, 2004)]. PI3Kinase has also been shown to sustain ECM-mediated survival in epithelial cells [(Khwaja et al., 1997)] and so the effect of blockade of tyrosine kinase pathways with a broad-spectrum inhibitor (Tyrphostin-25) and the PI3Kinase pathway with Ly294002 was investigated. Acridine orange/ethidium bromide morphological assessment confirmed that laminin protects against of etoposide-induced apoptosis (**Fig 4.3A**). However, whilst Ly294002 alone caused only a minimal increase in apoptosis in SCLC cells at 48 hours, it completely abrogated laminin-mediated protection. This data was confirmed by a caspase-3 activity assay (**Fig 4.3B**). It is well established that Ly294002 inhibits other enzymes of the PI3Kinase family (e.g. DNA-PKcs ATM, ATR, and mTOR) at the concentrations used in these experiments [(Okayasu et al., 2003)] and more recently direct Ly294002-mediated inhibition of voltage-gated potassium channels has been demonstrated [(El Kholy et al., 2003)]. However, another PI3Kinase inhibitor, wortmannin, is much more selective (IC_{50} of 0.003, 3.6, 5.8 and 100μM for PI3K, DNA-PKcs ATM and ATR respectively [(Sak et al., 2002)]) and this also inhibited ECM-mediated protection against etoposide-induced apoptosis at a dose of 0.1μM (data not shown). Furthermore, additional experiments (see below) demonstrating modulation of downstream effectors of PI3Kinase, most notably the inhibition of ECM-mediated

protection by Dominant-Negative PKB [Hodkinson et al., 2006] provide further substantiation for the role of PI3Kinase in this process.

In this experiment, Tyrphostin-25, a broad spectrum tyrosine kinase inhibitor was also shown to abrogate laminin-mediated protection against etoposide-induced apoptosis implicating tyrosine kinases in the chemoprotective pathway. Similar results were obtained in H69 and H510 SCLC cells and results were also replicated by flow cytometric annexin V staining [Hodkinson et al., 2006]. z-valine-alanine-aspartate (z-VAD), a potent, broad spectrum, irreversible inhibitor of caspases was used as a negative control and effectively inhibited etoposide-induced apoptosis in these experiments.

A downstream effector of PI3Kinase, the serine-threonine kinase PKB, has been shown previously to phosphorylate proapoptotic effectors including caspase-9 and Bad and this phosphorylation prevents apoptosis [Diehl, 1998],[Buss et al., 2004],[Troussard et al., 1999]. During the apoptotic process a cascade of caspase cleavage events occurs: Caspases exist as latent precursors (procaspases) composed of heterotetramers of p10 and p20 subunits. Upstream caspases 2, 8, 9, and 10 are activated in an amplifying cleavage cascade resulting in activation of downstream "executioner" caspases 6, 7 and 3 which initiate destruction of key cellular substrates and degradation of DNA [Friedlander, 2003]. Using western blot analysis it was shown that in H345 cells, etoposide induced an expected increase in cleaved caspase-9 and cleaved caspase-3 (cleavage indicates apoptosis) when plated on poly-l-lysine (**Fig 4.3C**). However, these cleavages were inhibited by adhesion to laminin. This laminin-mediated anti-apoptotic effect was shown to be PI3Kinase dependent by virtue of its abrogation by

Ly294002. The Bcl-2 family of proteins consist of pro-apoptotic members (e.g. Bax, Bad, Bid) and anti-apoptotic members (e.g. Bcl-2, $Bcl_x(L)$) which exist in an equilibrium. The anti-apoptotic members maintain mitochondrial integrity to prevent cytochrome "c" release [(Yang et al., 1997)] and also inhibit the association of Apaf-1 with caspase-9 [(Hu et al., 1998)] to inhibit the initiation of apoptosis, whilst pro-apoptotic members do the reverse. Phosphorylation of Bad results in inability to heterodimerise with Bcl-2 members and sequestration in the cytoplasm shifting the Bcl equilibrium to a the anti-apoptotic state [(Hayakawa et al., 2000)]. Here, laminin adhesion of H345 SCLC cells resulted in anti-apoptotic phosphorylation of Bad. Again, this effect was shown to be PI3Kinase dependent by inhibition with Ly294002. These results indicate that ECM mediates Bad phosphorylation and inhibition of caspase cleavage via PI3Kinase and tyrosine kinases resulting in protection against etoposide-induced apoptosis.

[Further work performed in this laboratory by Dr P.Hodkinson has subsequently confirmed a direct laminin-mediated upregulation of PI3Kinase activity and downstream upregulation of PKB phosphorylation in SCLC cells in vitro, further supporting this data [(Hodkinson et al., 2006)]].

4.7.4 Adhesion of SCLC cells to ECM proteins does not modulate initial levels of DNA damage or subsequent DNA repair

A previously raised concern regarding the model of SCLC cells plated on ECM is that of direct sequestration of etoposide by matrix proteins resulting in lower intracellular doses of drug. In order to test whether the anti-apoptotic effects of ECM were due to this or to ECM-mediated alterations in DNA-damage repair, double-

strand break formation and repair was assessed directly by a) the detection of immunofluorescence stained foci of phosphorylated γH2Ax and b) western blotting for γH2Ax and DNA-repair enzymes such as DNA-protein kinase. H2Ax is a histone that is rapidly phosphorylated at Ser^{139} to form γH2Ax following DNA double-strand break formation. Within minutes of DNA damage, discrete foci of γH2Ax are formed and each focus of γH2Ax represents a single double-strand break. The number of γH2Ax foci correlate closely with cell death in response to DNA damage (Olive, 2004). The role of γH2Ax foci is to recruit double-strand break repair enzymes (such as BRCA1, DNA-PKcs) to form repair complexes and γH2Ax is removed from the complexes after repair following dephosphorylation by protein phosphatase 2A (PP2A) (Chowdhury et al., 2005). In eukaryotes double-strand breaks are repaired by two distinct pathways (for detailed reviews see Valerie and Povirk (Valerie et al., 2003) and Lisby and Rothstein (Lisby et al., 2004)); non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is error-prone and involves simple ligation of cut ends using the ku70/80 heterodimer, DNA-PKcs (DNA protein kinase catalytic subunit), the Artemis nuclease and LigaseIV/XRCC4 ligase. HR uses the homologous sister chromatid as a template for recovery of genetic information and is thus error-free though does result in loss of heterozygosity. HR uses replication protein A (RP-A), Rad52 group (Rad 51, 52 and 54) and the Mre11/Rad50/Nbs1 complex (Lisby et al., 2004). Mammalian cells utilise both pathways though for HR to occur a sister chromatid needs to be present and thus is not possible prior to S phase (Houtgraaf et al., 2006).

In the experiments shown in **Fig 4.4**, etoposide and radiation both induced multiple γH2Ax foci almost immediately after exposure (**Fig 4.4A+B**). There was no

difference in the number of initial double-strand breaks or the rate of double-strand break repair over the next 24 hours between poly-l-lysine and laminin-plated cells. Similarly, western blotting for DNA-PKcs and γH2Ax in nuclear extracts of radiotherapy-treated cells showed no differences when plated on poly-l-lysine or laminin (**Fig 4.4C**). The mechanisms by which DNA-PKcs are upregulated in response to double-strand breaks has not been fully elucidated but an increase in expression levels has previously been described (Shintani et al., 2003) (in view of the inability to demonstrate differences in double-strand break repair kinetics, formal assessment of DNA-PKcs enzymatic activity was not performed). Thus, extracellular matrix protection against etoposide and radiotherapy treatment is not due to a reduction in initial DNA damage (and thus does not influence drug entry into small cell lung cancer cells) or subsequent modulation of DNA repair. This supports previous data from this laboratory showing no difference in etoposide-induced DNA double-strand breaks when measured by ATP-dependent decatenation of high molecular weight DNA (Hodkinson et al., 2006).

4.7.5 ECM proteins override etoposide and radiation-induced G2/M arrest via β1-integrin and PI3Kinase-dependent modulation of cell cycle regulatory proteins

In eukaryotic cells, the cell cycle consists of four phases; gap1 (G1), synthesis (S), gap2 (G2) and mitosis (M). In G1, the cell increases in size and increases transcription and translation of many genes. In S phase, DNA is replicated. During G2 the cell grows and produces extra proteins in preparation for division in the M phase. When cells are exposed to DNA damage they characteristically undergo cell

cycle delay or arrest at cell type-specific checkpoints [Lisby et al., 2004),(Valerie et al., 2003),(Yoshida et al., 2004),(Samuel et al., 2002)]. The three major sites for checkpoint inhibition of the cell cycle are G1/S, inter-S-phase and G2/M. In all cases DNA damage sensor proteins transduce signals to effector proteins which result in cell cycle arrest. Sensors include the Rad50/Mre11/Nbs1 (which are also critically involved in DNA repair), Rad9/Hus1/Rad1 (proliferating cell nuclear antigen (PCNA)-like) and Rad17/RFC (replication factor C) protein complexes. These complexes enlist and phosphorylate transducers such as ATM and ATR, two PI3Kinase-like kinases closely related to DNA-PKcs. ATM is critically involved in repair of radiation-induced double-strand breaks downstream of Rad50/Mre11/Nbs1. ATR is more important in the response to UV damage and replication inhibitors such as hydroxyurea. ATM and ATR phosphorylate intermediate signalling proteins such as BRCA1, p53 binding protein (53BP) and mediator of DNA-damage checkpoint (MDC1) which signal to effector proteins such as Chk1 and Chk2. These are serine/threonine kinases which modulate key cell cycle proteins such as Cdc25A,B and C. For example, in the G1/S phase, non-phosphorylated Cdc25A promotes G1/S transition by dephosphorylation of CDK2. Phosphorylation of Cdc25A by Chk1 or Chk2 causes inactivation and ubiquitin-mediated degradation resulting in G1 arrest. Furthermore, ATM and ATR also phosphorylate p53 which inhibits CDK2 via p21 causing G1/S cell cycle arrest. A similar ATM/ATR-Chk1/2-Cdc25A-CDK2 pathway exists in the intra-S-phase and G2/M checkpoint. In addition, in the G2/M checkpoint Chk1 and Chk2 phosphorylate WEE1 to inactivate the Cdc2/CyclinB complex. Since Cdc2/CyclinB promotes the G2/M transition, this results in arrest in G2. Interestingly although the G1/S checkpoint is dependent upon intact p53, G2/M

arrest occurs despite p53 knockout suggesting a p53-independent mechanism (Lisby et al., 2004).

The paradigm of a soluble protein ligand binding to a cell surface receptor to regulate cell cycle progression has been well described for many growth factors. There is also an abundance of literature describing integrin-mediated alterations in cell cycle signalling: Most non-transformed cells need growth factor stimulation, integrin-mediated adhesion to ECM and cytoskeletal integrity for cell cycle progression (Walker et al., 2005). Cell-cell adhesion normally provides a stimulus to inhibit proliferation and dysfunction of this association results in loss of contact inhibition which contributes to tumorigenesis. Growth factors (GFs) alone are usually insufficient for the stimulation of cellular proliferation and concomitant binding to and signalling from integrin-mediated adhesion is needed. Loss of adhesion in non-transformed cells usually results in complete G1 phase cell cycle arrest and apoptosis (Giancotti et al., 1999). Dysregulation of this relationship results in anchorage-independent cell growth, an important step in cell transformation to a malignant phenotype. Mitogenic lipids and inflammatory cytokines provide further influencing controls in the biological management of the cell cycle (Schwartz et al., 2001a) and it is clear that complex interactions between multiple signalling pathways converge to define cell cycle activity. G1 phase regulation involves cyclins D and E), CDKs (2 and 4) and the CDKIs ($p21^{cip1}$ and $p27^{kip1}$). Integrins induce cyclinD1 which binds to and activates CDK4/6. Integrins also downregulate $p21^{cip1}$ and $p27^{kip1}$ blocking CDKI-mediated inhibition of cyclinE-CDK2 and cyclinA-CDK2 complexes (Roovers et al., 2000). Thus, activated CDK4/6 and CDK2 lead to phosphorylation of the Rb protein and the cell progresses through the G1 phase to the S phase.

When considering integrin-mediated effects on the cell cycle, transiently plating cells on ECM proteins has been considered analogous to acute stimulation of starved cells with Growth Factors (Schwartz et al., 2001b) and transiently stimulates many pathways including Rac, cdc42, tyrosine kinases and downstream effectors Src and Shc, Ras, JNK and PI3K (Giancotti and Ruoslahti, 1999). This transient stimulation is usually not sufficient to drive cells into S phase entry and more prolonged adhesion throughout the G1 phase is required (Bohmer et al., 1996). Prolonged adherence stimulates tyrosine phosphorylation of p130Cas and paxillin and PIP5-kinase activity (Schwartz et al., 1995) though there has been minimal direct evidence supporting links between many of these second messengers and cell cycle control. Integrins potentiate growth factor signalling at multiple levels to influence cell cycle progression; they can increase the density of surface growth factor receptors (GFRs) by reducing ubiquitin-dependent degradation (Baron et al., 2000) and directly associate with Growth Factors to increase Growth Factor-mediated signalling (Jones et al., 1997). In fact, Moro et al (Moro et al., 1998) showed that the EGFR intrinsic kinase activity is increased by association with β1-integrin even in the absence of EGF stimulation. Additionally integrins can modulate second messengers downstream of Growth Factor signalling e.g. integrin binding to fibronectin results in decreased association of Ras-GAP to PDGFR leading to increased Ras and Erk activation (DeMali et al., 1999). Growth factor stimulation of the Rac pathway is also dependent on integrin-mediated regulation of Rac subcellular localisation (del Pozo et al., 2000). EGF-mediated PI3Kinase and PKB activation (Khwaja et al., 1997), PDGF-mediated activation of PKC (McNamee et al., 1993) and growth factor activation of JNK signalling (Short et al., 1998) are all reduced in cells deprived of integrin-mediated signalling by detachment. ILK and two associated proteins PINCH and ILKBP

localise to sites of integrin adhesion to ECM [Guo et al., 2002] and inhibition of this interaction prevents progression into the S phase of the cell cycle. The most well studied area is integrin-mediated regulation of the G1 phase and G1/S checkpoint: Bill et al [Bill et al., 2004] demonstrated that integrin-dependent activation of EGFR results in upregulation of cyclinD1, Rb phosphorylation and activation of CDK4 . Integrins do transiently activate erk1 and erk2 [Morino et al., 1995]. However, induction of cyclinD1 mRNA, which drives cells through the G1/S checkpoint, occurs as a result of *sustained* erk1/2 activation [Weber et al., 1997]. It is the combination of sustained erk activation and adhesion-mediated signalling which drives the upregulation of cyclinD1 [Zhu et al., 1996] and G1 progression. Several potential mechanisms may explain this adhesion dependence: erk transport from cytoplasm to nucleus has been shown to require adhesion [Danilkovitch et al., 2000] and integrins may influence downstream events by alterations in the subcellular localisation of other proteins. A downstream effector of integrins, PI3Kinase potentiates cyclinD1 stability [Gille et al., 1999] since downstream PKB phosphorylates Glycogen synthase kinase3β (GSK3β) and this results in stabilization of cyclinD1 [Kang et al., 2005],[Su et al., 2002],[Phillips-Mason et al., 2000]. In endothelial cells integrin-mediated Rac-signalling is required for upregulation of cyclinD1 translation [Mettouchi et al., 2001]. FAK is another important downstream effector of integrin in outside-in signalling and there appear to be FAK-dependent and FAK-independent pathways regulating erk potentiation and cyclinD1 activation: Integrin-mediated FAK signalling has been shown to be essential for sustained erk activation, enhanced cyclin D1 expression and progression of the cell cycle from G1 to S phase [Zhao et al., 2001],[Buss et al., 2004]. FAK has been also shown to interact with growth regulatory pathways involving JNK [Oktay et al., 1999],[Lebrun et al., 2000]

and produces enhancement of GF signalling via Ras, Raf, MEK and Erk [(Renshaw et al., 1999)]. However, a FAK-independent mechanism involving caveolin and Fyn has also been demonstrated [(Wary et al., 1998)]. FAK has also been shown to influence levels of p27 (a CDKI) by altering degradation by ubiquitination [(Bond et al., 2004)]. The actin cytoskeleton and cell shape also appear to play important roles in regulation of cyclinD1 and the G1/S checkpoint [(Walker et al., 2005)]; cytoskeletal tension provided by cell adhesion is required for G1-S phase progression providing one explanation for cell cycle arrest in many cells in suspension. As well as effects on cyclinD1, integrins have been shown to be involved in the downregulation of CDKIs (p21/p27) in late G1, coincident with activation of cyclinE-CDK2 [(Roovers and Assoian, 2000)].

I hypothesised that integrins induce changes in cell cycle signalling in small cell lung cancer to promote cell cycling and override G1/S and G2/M cell cycle checkpoints. By driving cells with DNA damage through these checkpoints, cells with treatment-induced DNA damage may circumvent apoptosis and go on to survive with additional DNA damage.

In these experiments, quiesced H345 cells were adhered to 96-well plates uncoated or coated with poly-l-lysine, laminin (or fibronectin; data not shown). After treatment with or without etoposide (25μg/ml) or radiotherapy (2Gy) cell cycle profile was assessed by flow cytometric analysis of propidium iodide staining (whole cell and nuclear preparations were performed with similar results). When treated with etoposide or XRT, H345 cells adhered to poly-l-lysine showed a significant increase in the proportion of cells in the S and G2/M phases of the cell cycle (**Fig 4.5A**). This S phase and G2/M delay is characteristic of the effect of etoposide in other small cell lung cancer lines [(Soues et al., 2001)] and is predictable with the knowledge that etoposide

is a phase-dependent cytotoxic that acts during late S and early G2/M phase by stabilising the topoisomerase "cleaved complex" to produce a double-strand break. Similarly, XRT has well-documented effects on the cell cycle; G2 arrest is seen in almost all cell lines whilst G1 arrest and S phase delay are more variable [(Choudhury et al., 2006),(Maity et al., 1994)]. However, when H345 cells were plated on laminin and treated with etoposide or XRT there was only a small increase in the percentage of cells in S and G2/M. Furthermore, this etoposide-induced over-riding of etoposide/XRT-induced S phase delay and G2/M arrest is abrogated by concomitant treatment with 4B4 (function blocking β1-integrin antibody) and the PI3Kinase inhibitor Ly294002 and is replicated by function activating β1-integrin antibody implying that ECM-mediated protection from S phase delay and G2/M arrest is mediated by β1-integrin signalling via PI3Kinase (**Fig 4.5B**). Also sodium orthovanadate, a broad spectrum tyrosine phosphatase mimicked the effects of ECM indicating that this effect of ECM also involves tyrosine kinase activation.

Assessment of expression of cyclins (D, A, E and B), CDKs (1 and 2) and CDKIs (p21 and p27) by western blotting provided further insights into the mechanism of action of ECM signalling in cell cycle modulation in SCLC cells (**Fig 4.5C**). After 24 hours of etoposide treatment, H345 SCLC cells demonstrated a reduction in expression of cyclins E, A and B (**Fig 4.5C**). In parallel, the CDK inhibitors p21Cip1/WAF1 and p27Kip1 exhibited increased expression Phosphorylated CDK1 (inactive form) was increased and expression of phosphorylated CDK2 (active form) decreased. These results are consistent with the flow cytometric experiments described above where etoposide-induced increases in the S phase and G2/M fractions were identified. Plating H345 SCLC cells on laminin both increased cyclin

D expression and inhibited the etoposide-induced modulation of cyclins, CDKs and CDKIs, again consistent with the over-riding of etoposide-induced cell cycle delay/arrest identified in the flow cytometry experiments. These effects of ECM on cell cycle modulators were reproduced by function-activating TS2/16 antibodies, and the laminin and TS2/16-mediated effects on etoposide-induced changes in cell cycle regulators were abrogated by the PI3Kinase inhibitor Ly294002. Thus, ECM proteins override etoposide-induced S phase delay and G2/M cell arrest via β1-integrins by modulating expression of cyclins, CDKs and CDKIs in a PI3Kinase dependent manner.

[Activated PKB has been shown to regulate cellular proliferation by interaction with cell cycle regulators governing G1/S and G2/M checkpoints (Phillips-Mason et al., 2000) PI3Kinase has also previously been shown to induce expression of D-type cyclins in part by increasing the stability of cyclin-D1 through PKB-dependent phosphorylation of GSK3β (Diehl et al., 1998). Subsequent experiments performed in this laboratory have confirmed that the *ECM → β1-integrin → PI3K → Cell Cycle* modulation pathway in SCLC cells described here also involves PKB and GSK3β (an upstream regulator of cyclinD1). Constitutively active and Dominant-Negative PKB mutants were able to respectively replicate and inhibit ECM-mediated protection against apoptosis and ECM-mediated over-riding of cell cycle arrest (Hodkinson et al., 2006)].

4.8 Summary

These results show for the first time that ECM proteins activate β1-integrins to protect SCLC cells against the apoptotic effects of etoposide and ionizing radiation via PI3Kinase activation. This occurs in two ways: 1) PI3Kinase-dependent β1-

integrin signalling resulting in phosphorylation of Bad and reduced caspase-9 cleavage and 2) a β1-integrin-mediated over-riding of etoposide and radiotherapy-induced cell cycle S phase delay and G2/M arrest.

In concert these two processes promote cancer cell survival and proliferation. This may lead to the survival of clones with increased treatment-related DNA aberrations that could contribute to a multidrug-resistant phenotype. ECM-mediated resistance to pro-apoptotic DNA damaging agents has been shown to exist across a broad spectrum of cancers (see introduction) and this data describes an example of cell type-specific mechanisms by which this process occurs. Only by identifying the cancer-specific intracellular pathways responsible for treatment failure can we hope to develop new targeted cancer-specific agents to improve outcomes for patients.

Chapter 5

The Role of β1 Integrins and Protein Tyrosine Kinases *in vivo* in the Modulation of Extracellular Matrix-mediated Survival Signalling in Small Cell Lung Cancer.

5.1 Introduction

Earlier data presented in this thesis showed in SCLC cells that, in vitro, resistance against DNA-damaging agents is mediated via β1-integrin cell surface adhesion molecules [(Sethi et al, 1999b),(Hodkinson et al, 2006)]. ECM binding to β1-integrin activates a second messenger pathway involving protein tyrosine kinases, PI3Kinase, PKB and GSK-3β. This pathway inhibits cell cycle arrest by downregulating cell cycle-dependent kinase inhibitors p21Cip1/WAF1 and p27Kip1, reducing phosphorylation of CDK1 and maintaining expression of cyclins D/E/A/B and phosphorylation of CDK2. Concomitantly ECM, via PI3Kinase, results in phosphorylation of Bad and reduced caspase-9 cleavage preventing apoptosis. This in vitro data provided the impetus to strive towards identifying active *in vivo* biochemical inhibitors of this pathway to ultimately proceed to assessment in clinical Phase I/II trials. Initial experiments were devised to identify the presence of a similar, β1-integrin and protein tyrosine kinase-dependent survival signalling pathway in vivo. Small cell lung cancer xenografts were implanted into the flanks of nude mice and the effects of

a) β1-integrin blockade with function inhibiting antibody (4B4) and b) Tyrosine kinase inhibition, were investigated. The broad spectrum tyrosine kinase inhibitor genistein was chosen for investigation in vivo because: a) Previous dosing experiments in nu/nu nude mice were available in the literature obviating the need for dose finding experiments (Wietrzyk et al., 2000), b) It has been shown to have activity in human cancers in vitro; melanoma (Tamura et al., 2003),(Record et al., 1997), hepatoma (Chang et al., 2004), NSCLC (Wei et al., 2001), breast (Shao et al., 1998), prostate (Hillman et al., 2001) and in vivo; melanoma (Tamura et al., 2003), breast (Shao et al., 1998), bladder (Zhou et al., 1998), c) It is a naturally occurring, orally bioavailable soy bean extract that is well tolerated (in humans) and there is some in vitro and animal model evidence that genistein prevents cancer initiation, inhibits cancer progression (Arliss et al., 2002) and potentiates systemic therapy (Tamura., 2003),(Hillman et al., 2001),(El Rayes et al., 2006) d) Broad spectrum inhibitors may be superior to presently-available specific inhibitors in tumour growth inhibition either because multiple PTKs are implicated in carcinogenesis or the specific target/inhibitor combination is not achieved (Roussidis et al., 2002). In addition, genistein may also inhibit Multidrug Resistance (MDR) transporter pumps (Bogush et al., 2003) and topoisomerase-II (Bandele et al., 2007) which, if present in vivo, would be expected to increase efficacy.

Initial experiments aimed to demonstrate that in vivo, ECM ligands promote xenograft survival and growth per se in the absence of DNA-damaging agents. Matrigel was chosen for the initial experiments (shown above) since, a) it contains several extracellular matrix proteins that bind to β1-integrin (including laminin, collagen, entactin; **see Appendix: Table 9**) and produces ECM-mediated protection against apoptosis in vitro in an identical manner to laminin and fibronectin

experiments shown in chapter 4[(Buttery et al, 2004)] and b) it has previously been shown to support the growth of SCLC xenografts [(Yoshida et al., 1997)].

Subsequently, it was planned to test whether ECM proteins protect against the apoptotic effects of etoposide or radiotherapy in vivo. It was anticipated that this data may proceed into phase I/II trials assessing the combination of genistein with chemotherapy or radiotherapy.

The following hypotheses were initially tested:

1 In vivo, Matrigel^{TM}, a basement membrane preparation containing laminin, collagen, entactin and heparan sulphate proteoglycans promotes SCLC xenograft survival and growth.

2 This protective effect is β1-integrin dependent and replicates the cell cycle effects seen in vitro.

3 Tyrosine-kinase blockade with genistein inhibits ECM-mediated xenograft survival in vivo.

4 Individual matrix proteins (fibronectin, laminin and collagen) can replicate this protection.

5.2 In vivo, extracellular matrix promotes SCLC xenograft survival and growth

In order to identify whether ECM sustains and promotes xenograft survival in vivo, 200µl aliquots of H69 and H345 SCLC cells suspended in PBS or Matrigel (1:1 vol of cells:Matrigel/PBS) were implanted subcutaneously in contralateral flanks of Nude nu/nu:Balb(c) mice. Xenograft growth was assessed manually with callipers every 3-4 days.

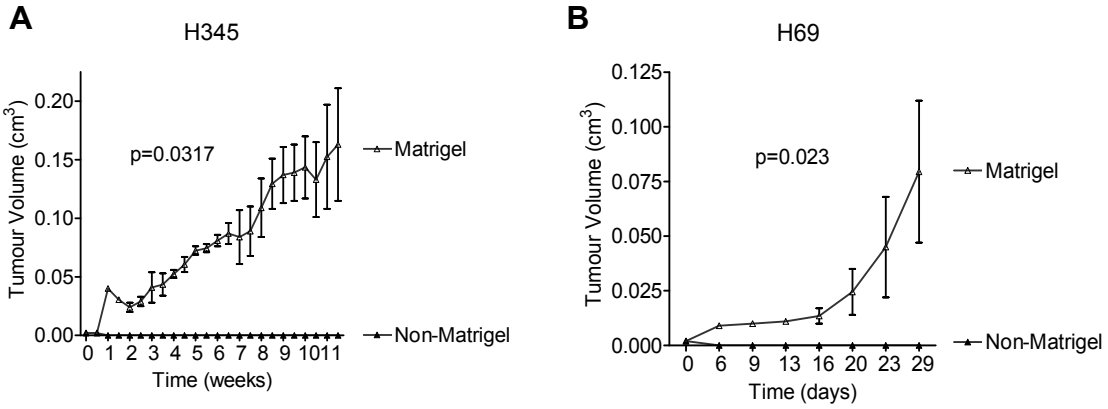


Figure 5.1 5x10^6 H345 (**A**) and H69 (**B**) SCLC cells suspended in PBS or Matrigel (1:1 vol of cells:Matrigel/PBS mixed at 4^{0}C, total volume 200µl) were implanted subcutaneously in contralateral flanks of Nude nu/nu:Balb(c) mice (10 mice per condition). Tumour size was assessed manually with calipers and volume calculated by: Volume = (Width^2 x Length)/2. Mean ± s.e. shown. Statistical analysis: 2-way Anova.

Results: H69 and H345 cells suspended in PBS did not result in measurable xenograft growth. When suspended in Matrigel both H69 and H345 implanted cells developed into measurable xenografts. H69 cells demonstrated significantly faster xenograft growth than H345 cells and for this reason were chosen to perform further experiments (**Fig 5.1A+B**).

5.3 Matrigel-mediated xenograft growth is β1-integrin dependent

Earlier, in vitro data confirmed that extracellular matrix-mediated protection against apoptosis was mediated via β1-integrin-dependent alterations in the activity of apoptotic 2nd messengers and cell-cycle modulators (**see Figs 4.2, 4.3 and 4.5**). This series of experiments sought to identify whether a similar, β1-integrin-dependent mechanism exists in the in vivo setting.

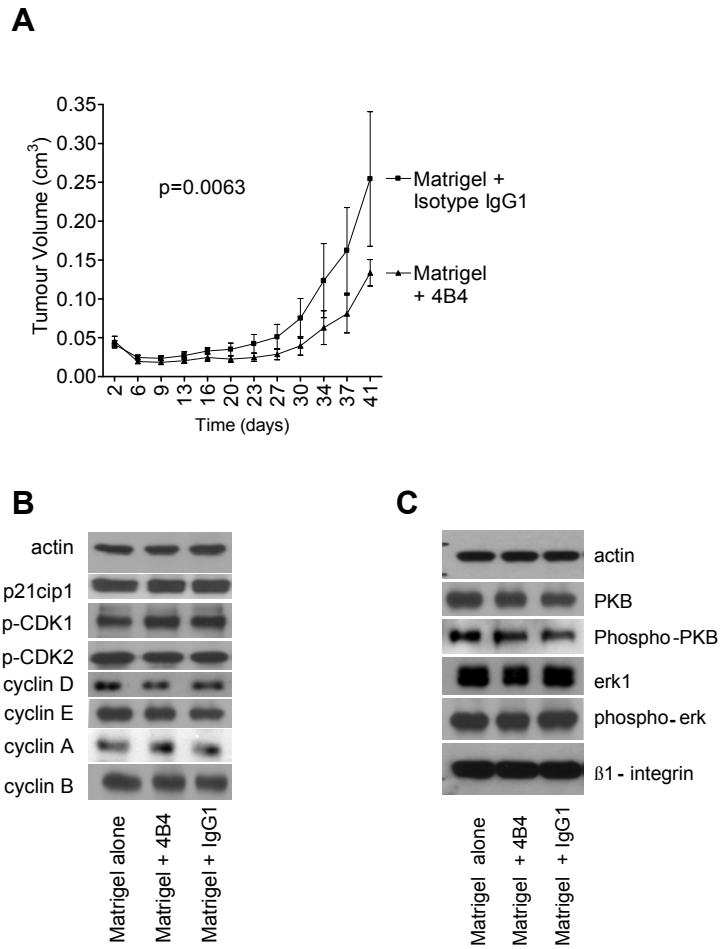


Figure 5.2 5x10^{6}H69 SCLC cells in PBS or Matrigel (1:1 vol of cells:Matrigel mixed at 4^{0}C) were implanted subcutaneously in flanks of Nude (nu/nu:Balb(c)) mice with or without the β1-integrin blocking antibody 4B4 or IgG1 isotype antibody (20μg/ml in matrigel with cells added 1 hour pre-implantation) (10 mice per condition). **A** Tumour size was measured with calipers and volume calculated by Volume = (Width^{2}xLength)/2. Mean ± s.e. shown. Statistical analysis: 2-way Anova. Xenograft lysates were protein balanced and submitted to western blot analysis for **B** cell cycle components and **C** PKB/erk phosphorylation and β1-integrin expression.

Results: H69 cell implants suspended in Matrigel with mouse IgG1 isotype antibody developed into measurable xenografts (**Fig 5.2A**). This rate of growth was not significantly different from xenografts grown with matrigel alone (data not shown). However, when H69 cells were implanted in matrigel treated with mouse anti-human β1-integrin antibody (4B4) xenograft growth was significantly reduced ($p=0.0063$). 4B4 treatment did not completely inhibit xenograft growth but induced tumour growth delay followed by exponential growth, consistent with a 4B4-mediated reduction in the initial number of surviving clones. Continuing xenograft growth despite initial 4B4 treatment may be a result of insufficient sustained 4B4 dose or, more likely, the presence of additional survival pathways. This confirms that Matrigel-mediated xenograft growth is at least partially dependent upon β1-integrin.

Western blot analysis of expression of proteins responsible for cell cycle control (**Fig 5.2B**) showed no difference between matrigel alone, 4B4 or isotype-treated xenografts. A possible explanation for this is that the β1-integrin-dependence may be a phenomenon which occurs very early in the development of xenografts in this model; immediately after implantation cells either survive or die and at this time β1-integrin signalling can inhibit apoptosis, thus defining the initial number of live cells capable of subsequently producing a measurable xenograft. In this way, it would not be unexpected for any subsequent xenograft to exhibit similar cell cycling protein expression. Similarly, by Western blot analysis, no differences were seen in levels of PKB or erk phosphorylation (kinases downstream of β1-integrin), again consistent with β1-integrin survival signalling being an early phenomenon. It was anticipated that Matrigel/Matrigel + IgG1-derived xenografts may be derived from sub-selected clone of cells with high β1-integrin expression and Matrigel + 4B4-derived from

clones with low β1-integrin expression. However, Western blot analysis did not confirm this (**Fig 5.2C**).

In order to identify any morphological differences between untreated, 4B4-treated and IgG1-treated xenografts, haematoxylin and eosin (H+E) staining was performed on paraffin-embedded sections of xenografts harvested in the above experiments. In addition, immunohistochemical staining of Ki67 (proliferation), CD34 (angiogenesis) and CD56 (neuroendocrine differentiation marker) was performed.

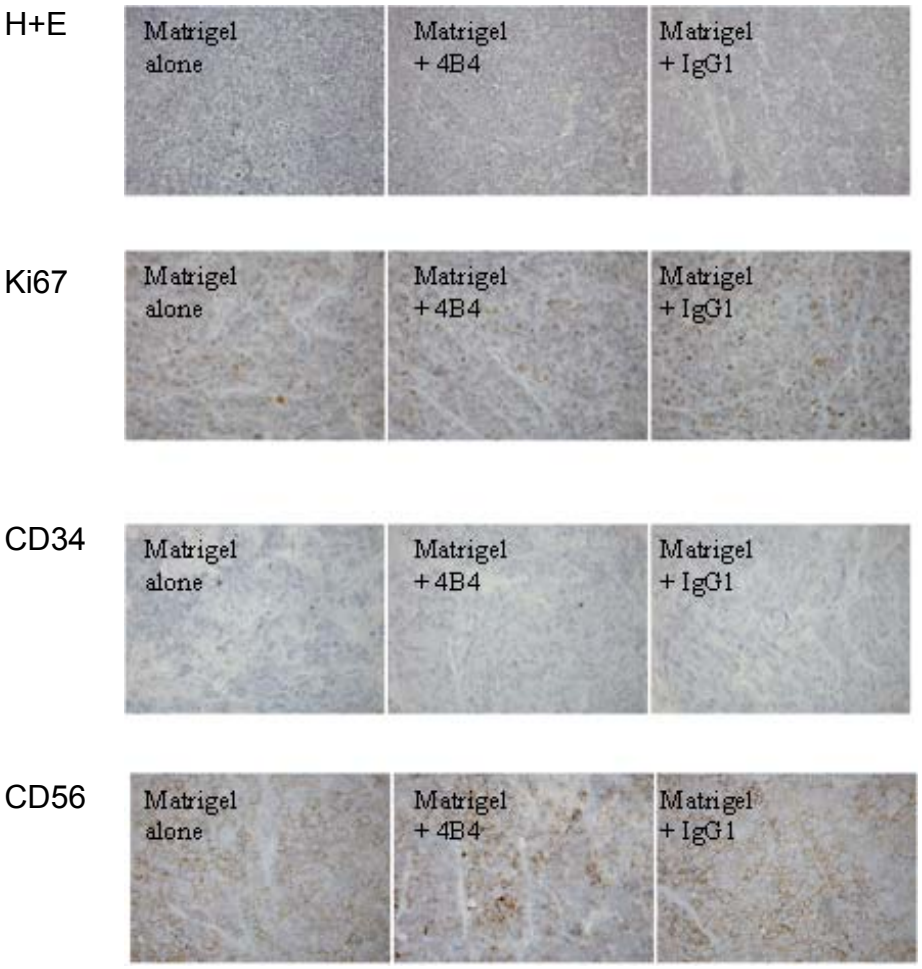


Figure 5.3 5x10^{6}H69 SCLC cells in PBS or Matrigel (1:1 vol of cells:Matrigel mixed at 4^{0}C) were implanted subcutaneously in flanks of Nude (nu/nu:Balb(c)) mice with or without the β1-integrin blocking antibody 4B4 or IgG1 isotype antibody (20μg/ml in matrigel with cells added 1 hour pre-implantation) (10 mice per condition). Xenografts were harvested after 41 days and paraffin-embedded sections submitted to H+E staining and immunohistochemical staining for Ki67, CD34 and CD56.

Results: No difference in the morphological H+E appearance of xenografts was seen (**Fig 5.3**). Sustained neuroendocrine differentiation of xenograft cells was demonstrated with high levels of CD56 staining in all sections viewed. No difference was observed between treatment conditions. As anticipated in SCLC, high levels of Ki67 staining indicating proliferation was observed. No difference was observed between treatment conditions. A low level of CD34 staining was identified in all treatment conditions indicating minimal angiogenesis (positive controls not shown). In summary, no morphological or immunohistochemical differences were seen between untreated, 4B4-treated and IgG1-treated xenografts. Again, this is consistent with the effect of β1-integrin blockade by 4B4 antibody being an early peri-implantation phenomenon.

5.4 Genistein inhibits ECM-mediated xenograft survival in vivo only when delivered pre-implantation.

Earlier experiments confirmed that protein tyrosine kinase inhibition with the broad-spectrum tyrosine kinase inhibitor tyrphostin-25 reversed the anti-apoptotic effects of laminin (**see Fig 4.3**). For the reasons described above genistein was the inhibitor of choice for these in vivo experiments and therefore initial in vitro experiments sought to confirm that genistein also abrogated extracellular matrix-mediated protection against etoposide-induced apoptosis.

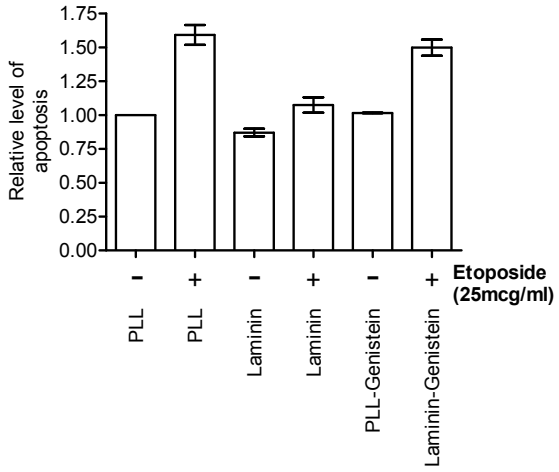


Figure 5.4 In vitro, genistein inhibits laminin-mediated protection against etoposide-induced apoptosis. Quiesced H345 cells were seeded into 96-well plates pre-coated with poly-l-lysine (10μg/ml) or laminin (10μg/ml) and treated with (+) or without (-) etoposide (25μg/ml) or genistein (50μM)as indicated. Apoptosis was measured after 48 hours using a Caspase-3 activity assay (as described in materials and methods). Results normalised to untreated cells on poly-l-lysine. Mean ± s.e. of n=5 experiments in triplicate. Data analysis: matched 1-way ANOVA with Bonferroni selected post-test.

Results: When SCLC cells were plated on poly-l-lysine, etoposide induced a significant increase in apoptosis of 59.3 ± 7.3% (s.e.). This was reduced to 7.4 ± 5.7% ($p<0.001$) when plated on laminin. Treatment of H345 cells (and H69, data not shown) with the broad spectrum tyrosine kinase inhibitor, genistein, alone did not induce a significant increase in apoptosis but did completely abrogate laminin-mediated protection against etoposide-induced apoptosis ($p<0.001$). This effect was confirmed using a Caspase-3 activity assay (**Fig 5.4**) and an ELISA-based assay based on the quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes (data not shown).

In order to confirm that a similar, tyrosine kinase-dependent signalling mechanism is present in vivo, xenograft experiments were carried out with Matrigel-implanted

SCLC cells treated with or without the broad-spectrum tyrosine kinase inhibitor, genistein. Since the β1-integrin/4B4 experiments above suggested that Matrigel's survival effect may occur at an early timepoint pre- or peri-implantation, genistein dosing was performed a) pre + post-implantation (pre = genistein 50μM with the Matrigel/cells at the time of implantation, post = by intraperitoneal injection 50mg/kg/day from days 2-8) or b) post implantation only. Vehicle (10% DMSO in PBS) controls were also performed.

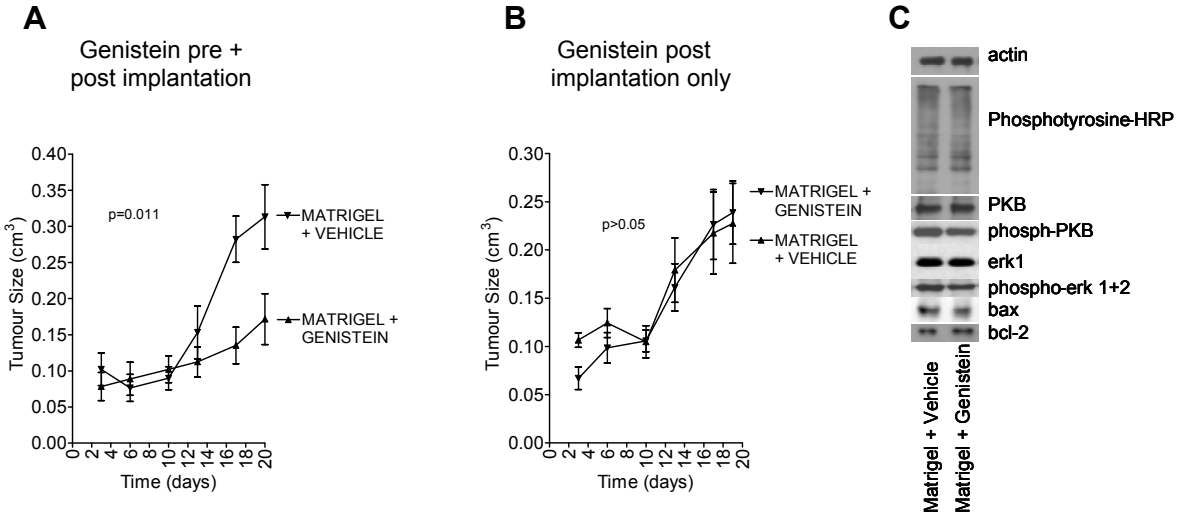


Figure 5.5 **A** 5x10^{6}H69 SCLC cells in Matrigel (1:1 vol of cells:Matrigel mixed at 4^{0}C) with or without genistein (50μM with implanted cells) or Vehicle (10% DMSO in PBS) were implanted subcutaneously in flanks of separate Nude (nu/nu:Balb(c)) mice (10 mice per condition). Mice subsequently received 50mg/kg/day i.p. genistein or Vehicle in 200μl injections day2-8. **B** 5x10^{6}H69 SCLC cells in Matrigel (1:1 vol of cells:Matrigel mixed at 4^{0}C) were implanted subcutaneously in flanks of separate Nude (nu/nu:Balb(c)) mice (10 mice per condition). Mice subsequently received 50mg/kg/day i.p. genistein or Vehicle in 200μl injections day2-8. Tumour size was assessed with callipers and volume calculated by Volume = (Width^2 x Length)/2. Statistical analysis: 2-way ANOVA. **C** Western blot analysis of xenografts harvested at end of experiment **A**. Probed for global tyrosine phosphorylation, PKB/erk phosphorylation, Bax and Bcl-2 expression.

Results: Xenografts implanted in Matrigel with vehicle (10% DMSO in PBS) grew at the same rate as cells implanted in Matrigel alone (data not shown) confirming that the DMSO vehicle is not significantly toxic to SCLC xenografts at the dose used in these experiments. When genistein was delivered solely by intraperitoneal injection from days 2 to 8 after implantation (**Fig 5.5B**) there was no significant difference in xenograft growth. Genistein only inhibited xenograft growth when delivered pre-implantation as well as on days 2 to 8 after implantation (**Fig 5.5A**), consistent with the earlier data suggesting that Matrigel-mediated xenograft survival is an early phenomenon. Western blot analysis of xenograft extracts harvested at the end of the experiment shown in **Fig A** demonstrated no difference in global tyrosine phosphorylation (**Fig 5.5C**). This is not unexpected since the final genistein dose was delivered 12 days prior to harvesting the xenografts. There was no difference in levels of PKB and erk phosphorylation or in expression of Bax (pro-apoptotic) or Bcl-2 (anti-apoptotic) proteins. These results are all consistent with genistein's action representing an early, peri-implantation phenomenon.

In order to identify any morphological differences between the vehicle-treated and genistein-treated xenografts, haematoxylin and eosin (H+E) staining was performed on paraffin-embedded sections of xenografts harvested after pre- and post-implantation delivery of vehicle/genistein. In addition, immunohistochemical staining of Ki67 (proliferation), CD34 (angiogenesis) and CD56 (neuroendocrine differentiation marker) was performed.

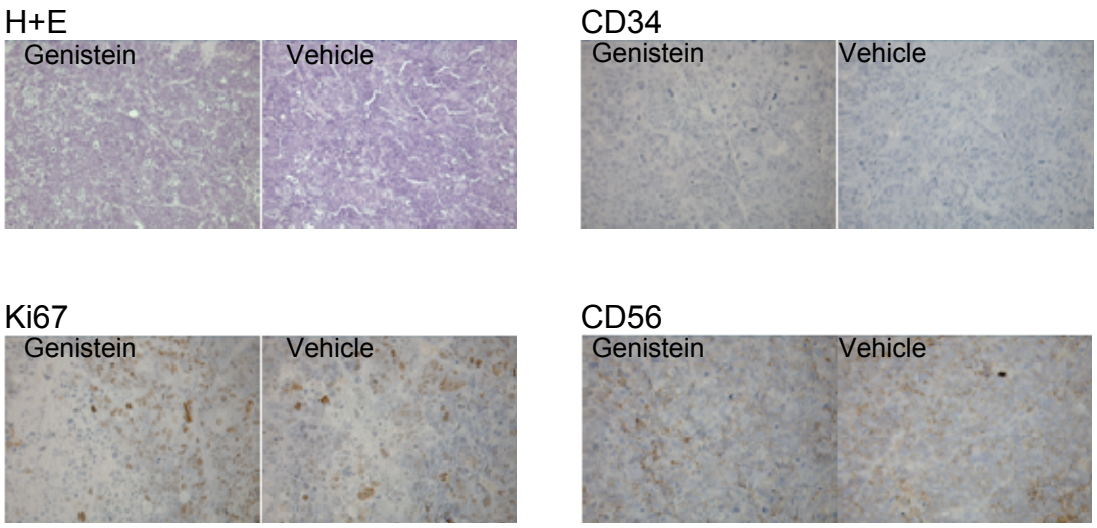


Figure 5.6 5x10^{6}H69 SCLC cells in Matrigel (1:1 vol of cells:Matrigel mixed at 4^{0}C) with or without genistein (50μM with implanted cells) or Vehicle (10% DMSO in PBS) were implanted subcutaneously in flanks of separate Nude (nu/nu:Balb(c)) mice (10 mice per condition). Mice subsequently received 50mg/kg/day i.p. genistein or Vehicle in 200μl injections day2-8. Xenografts were harvested after 20 days and paraffin-embedded sections submitted to H+E staining and immunohistochemical staining for Ki67, CD34 and CD56. Representative sections of xenografts from n=4 mice shown.

Results: No difference in the morphological H+E appearance of xenografts treated with genistein or vehicle was observed (**Fig 5.6**). Sustained neuroendocrine differentiation of xenograft cells was demonstrated with high levels of CD56 staining in all sections viewed. No difference was observed between treatment conditions. High levels of Ki67 staining indicating active proliferation was again demonstrated. No difference was observed between treatment conditions. A low level of CD34 staining was identified in all treatment conditions indicating minimal angiogenesis (positive control not shown). In summary, no morphological or immunohistochemical differences were seen between genistein- and vehicle-treated xenografts. This is consistent with the effect of β1-integrin blockade by 4B4 antibody being an early peri-implantation phenomenon.

5.5 Individual matrix proteins (fibronectin, laminin and collagen) do not replicate Matrigel-mediated growth

In order to identify whether individual matrix proteins were able to support xenograft growth, H69 SCLC cells suspended in Matrigel and laminin, fibronectin or collagen were implanted subcutaneously in contralateral flanks of Nude mice. Xenograft growth was measured with calipers.

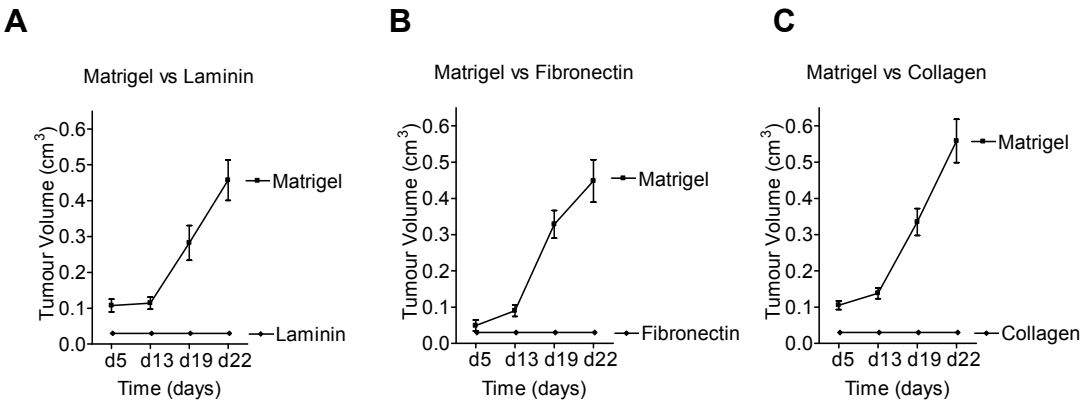


Figure 5.7 5x10^{6}H69 SCLC cells in Matrigel (1:1 vol of cells:Matrigel mixed at 4^{o}C) or (**A**) laminin (20μg/ml) or (**B**) fibronectin (40μg/ml) or (**C**) collagen (20μg/ml) were implanted subcutaneously in contralateral flanks of Nude (nu/nu:Balb(c)) mice (10 mice per condition). Tumour size was measured with calipers and volume calculated by Volume = (Width^{2}xLength)/2. Mean ± s.e. shown.

Results: H69 cells produced measurable xenograft growth in nude mice when implanted in Matrigel. However, the individual ECM proteins; laminin, collagen and fibronectin, did not support the growth of H69 xenografts (**Figure 5.7**).

5.6 Discussion

I hoped to demonstrate that the ECM-mediated, β1-integrin and PKB/PI3K-dependent cell survival pathway identified in vitro also exists in vivo in order that pathway inhibitors could be tested in vivo with a view to human anticancer

applications. This data does show that Matrigel, a basement membrane preparation containing extracellular matrix constituents laminin, collagen, entactin and heparan sulphate proteoglycans promotes the development of H69 and H345 SCLC xenografts in Nude nu/nu Balb/c mice (**Fig 5.1**). Tumour growth delay induced by function-blocking β1-integrin antibody (but not by IgG1 isotype antibody; **Fig 5.2**) supports the hypothesis that Matrigel-mediated survival signalling is, at least in part, β1-integrin-dependent. However, 4B4 treatment did not completely inhibit xenograft development. Furthermore, when xenografts were harvested at the end of the experiment Western blot and immunohistochemical analysis showed no difference in cell cycle modulator protein expression, PKB or erk phosphorylation, β1-integrin expression (**Fig 5.2 B+C**) or H+E, Ki67, CD34 or CD56 staining (**Fig 5.3**). This is consistent with the effect of 4B4 antibody taking place at an early timepoint in xenograft development. This may be an artefact of experimental design: in this in vivo experiment 4B4 was added exclusively with the cells at the time of xenograft implantation and any effect would therefore be likely to be short-lived due to time-dependent dilution and degradation of antibody. β1-integrin-mediated survival signalling may be important in maintaining cell viability at the time of implantation. 4B4 blockade of this signalling results in increased apoptosis and a reduction in the number of surviving cells capable of contributing to the developing xenograft. Thus, although 4B4-treated xenografts are initially lower in cell number, they go on to develop in an identical manner to untreated/IgG-treated xenografts. It is possible that β1-integrin-mediated survival signalling is also a more prolonged factor in xenograft development and further experiments utilising repeated dosing of 4B4 antibody would address this question.

In vitro, genistein replicated the earlier effects of tyrphostin-25 in abrogating extracellular matrix-mediated protection against apoptosis (**Fig 5.4**). In vivo the genistein experiments produced similar results to the 4B4 experiments. Broad spectrum tyrosine-kinase inhibition did inhibit xenograft development but only if delivered at the time of implantation admixed with the cells (**Fig 5.5 A+B**). When genistein was delivered exclusively from days 2 to 8 post-implantation no inhibition of xenograft growth was demonstrated. Possible explanations for this include insufficient dose or inadequate absorption of genistein into the structure of Matrigel implants when delivered via the intraperitoneal route. Western blot confirmation of reduced tyrosine phosphorylation of xenografts harvested during the period of genistein treatment would address this question though this may be complicated by sampling difficulties related to the small size of early implants. An alternative explanation is that broad spectrum tyrosine-kinase inhibition may only be an important factor in xenograft survival and growth at an early stage of development. i.e. analogous to the above explanation for β1-integrin inhibition. Inhibition by genistein may result in a reduced number of surviving cells which go on to form a smaller, though phenotypically identical, xenograft. Since the final genistein dose was delivered on day 8 and xenografts were not harvested until day 20 it is, perhaps, unsurprising that Western blot analysis of tyrosine phosphorylation and PKB/erk phosphorylation showed no differences between genistein and vehicle-treated xenografts (**Fig 5.5C**). Similarly this hypothesis would be expected to show no differences in Bax-Bcl2 levels or immunohistochemical staining for KI67, CD34 or CD56 (**Fig 5.6**). An alternative approach to the timescale of genistein's effect may be to utilise SCLC cells that have been passaged a number of times in vivo. This seems

to increase the tumorigenic potential of the cells and may remove the dependence on initial matrigel-mediated survival signalling and thus enable assessment of more longterm matrigel-mediated effects.

It appears that cessation of genistein dose at day 8 (**Fig 5.5A**) is followed several days later by exponential growth. Future experiments are intended to continue post-implant genistein over longer time-courses to confirm long-term inhibition of xenograft growth.

In vitro, individual matrix proteins (laminin, fibronectin and collagen) are capable of inducing ECM-mediated survival signalling with broad cell-type specificity. However, this was not replicated in vivo (**Fig 5.7**). Possible reasons for the failure of laminin, collagen and fibronectin to propagate xenograft growth in vivo include: i) Presence of and requirement for growth factors as an additional signal to survival in vivo. Although efforts have been made to eliminate growth factors from Matrigel, the Growth Factor-reduced Matrigel used in these studies does contain low levels of EGF, bFGF, NGF, PDGF, IGF-1, TGF-β (**see Appendix: Table 10**). It is possible that in the more demanding environs of the in vivo setting, dual (integrin and growth factor) signalling is required to sustain xenograft survival. Since xenograft growth was produced even in the presence of β1-integrin blockade by 4B4 antibody, GF signalling may be the dominant survival signal in vivo. ii) There is some evidence that the preservation of 3-dimensional structure produces additional survival signalling [(Chen et al., 2006),(Campos et al., 2006),(Oloumi., 2000),(Desoize et al., 1998)]. Matrigel suspended cells maintain a formed 3-dimensional mass after implantation. This does not occur with the individual extracellular matrices where the implant appears to be absorbed and dispersed in the initial hours and days. Further experiments in which individual

matrix components are first suspended in agar could address this issue. iii) Nude nu/nu Balb/c mice are athymic and thus are lymphocyte-deplete but they do retain an innate immune system. It is possible that the solid 3-dimensional structure produced by Matrigel acts to prevent entry of innate immune system cells e.g. neutrophils and macrophages and thus promotes survival in another β1-integrin-independent mechanism. iv) Matrigel contains entactin (also known as nidogen-1), which is a 150kDa multi-domain basement membrane glycoprotein that may be important in basement membrane organisation and assembly. Entactin may act as a bridge between the extracellular matrix molecules laminin-1 and type IV collagen. Thus, in vivo it may be critical per se (possibly related to the 3-dimensional integrity of Matrigel implants) or may act to converge multiple ECM-mediated cell survival signals by approximating individual ECM proteins (Fox et al., 1991). v) It is possible that the individual soluble matrix proteins are degraded post implant or are rapidly dissipated. The agar experiments described above may clarify this issue by sequestration of the ECM proteins.

In summary, this data demonstrates that Matrigel does induce survival pathways to promote the development of SCLC xenografts in Nude nu/nu Balb/c mice. It is likely that the mechanism of action of Matrigel is multifactorial. It appears that the β1-integrin-dependent and protein tyrosine kinase-dependent elements of this mechanism act early in the development of xenografts, around the time of implantation, since no differences in kinase activity, cell cycling protein expression, differentiation or angiogenesis can be seen immunohistochemically or by Western blot analysis at later timepoints. However, this may be due to the fact that the β1-

integrin function-blocking antibody (4B4) and genistein were applied several days before xenografts were harvested for analysis. Future experiments are planned to deliver multiple doses of antibody and to harvest xenograft tissue for immunohistochemical and Western blot analysis during the time of treatments to confirm ongoing activity at later timepoints. It is not clear why individual extracellular matrix proteins (laminin, collagen and fibronectin) did not replicate Matrigel in promoting xenograft survival and growth particularly since the earlier experiments above did confirm that Matrigel does induce a β1-integrin-dependent and protein tyrosine kinase-dependent survival signal. It suggests that, in vivo, additional factors are required for ECM-mediated survival signalling. These may relate to the presence of additional growth factor signalling in matrigel, the requirement for a 3-dimensional matrix, immune system effects, entactin and dilution/degradation of the individual, soluble extracellular matrix constituents.

Future experiments will develop upon this data which demonstrates the presence of a β1-integrin-dependent and protein tyrosine kinase-dependent survival pathway in xenograft development. Initial experiments will aim to confirm that longer term 4B4 and genistein inhibit xenograft growth. Subsequently, experiments assessing combinations of genistein and etoposide (and other chemotherapeutic agents used in SCLC) will be performed to identify whether in vivo synergy exists between these agents. Ultimately it is anticipated that combinations of chemotherapy and tyrosine kinase blockers will be tested in human phase I and II trials.

Chapter 6

Assessment of a GD25 Embryonic Murine Fibroblast Model to Investigate Extracellular Matrix-mediated Survival Signalling in vitro

6.1 Introduction

GD25 Null cells are embryonic murine fibroblasts which are stable deletion mutants that do not express β1-integrin at the cell membrane. GD25-β1 cells are GD25 Null cells stably transfected with mouse β1-integrin in association with a puromycin-resistance gene. These cell lines have been previously described [Wennerberg et al., 1996]. Using these cells in an in vitro model system I sought to investigate the identity of biochemical messengers involved in extracellular matrix-mediated chemoprotection. I hypothesised that GD25-β1 cells would exhibit β1-integrin-mediated outside-in survival signalling via adhesion to extracellular matrix proteins (fibronectin and laminin) whilst GD25 Null cells would not, due to the absence of β1-integrin expression. By confirming this β1-integrin-mediated resistance to chemotherapy-induced apoptosis it was hoped that further experiments would identify some of the intracellular signalling pathways responsible for this process. Initial experiments sought to confirm appropriate β1-integrin expression profiles of GD25-β1/GD25 Null cells and then to demonstrate the presence of extracellular matrix-mediated survival signalling in this model system.

6.2 GD25 Null and GD25 β1 cell lines: expression of mouse β1-integrin

To confirm that the GD25 β1 and GD25 Null cells used in these experiments respectively expressed high and absent levels of mouse β1-integrin at the cell surface, flow cytometric analysis of cells stained with 9EG7 rat anti-mouse β1-integrin antibody and isotype antibody was performed (**Fig 6.1**).

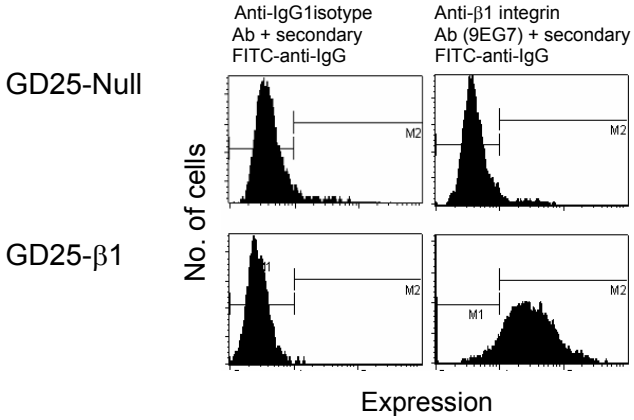


Figure 6.1 Confirmation of β1-integrin expression profile of GD25 Null and GD25 β1 lines. Flow cytometric analysis of β1-integrin expression on the surface of GD25 Null and GD25 β1 cells. $5x10^5$ cells were trypsinised, washed twice with PBS, resuspended in 100µl PBS and incubated with 1µg of rat anti-mouse β1-integrin (9EG7) or rat anti-mouse IgG1 at room temperature for 1 hour. After two washes with PBS, cells were incubated with species-specific fluorescein isothiocyanate-conjugated secondary antibody (1:50) for 30 min at 4°C and again washed twice with PBS. Samples were finally resuspended in PBS at 4°C and analyzed by flow cytometry using FACSCalibur™ (BD Biosciences). Typical flow cytometric profiles are shown.

Results: It was confirmed that the GD25 cell lines used for experiments exhibited appropriate cell surface expression of β1-integrin (**Fig 6.1**). This was repeated monthly during the timeframe of the following experiments.

6.3 Fibronectin and laminin do not mediate protection against etoposide-induced apoptosis in GD25 cells plated in DMEM with 10% FCS

6.3.1 Etoposide-induced apoptosis in GD25 cells plated in serum-containing media: time and dose response

In order to identify an optimum etoposide dose and treatment time for demonstration of extracellular matrix-mediated survival signalling, GD25 cells were treated with etoposide 0-10μg/ml for 0-48 hours and apoptosis and necrosis were assessed by i) morphology using fluorescent microscopy of acridine orange and ethidium bromide-treated cells and ii) flow cytometric analysis of cells labelled with Topro3 (necrosis) and AnnexinV-PE (apoptosis). GD25 cells did not tolerate prolonged periods of growth in serum-free media and so initial experiments were performed with cells in DMEM containing 10% foetal calf serum.

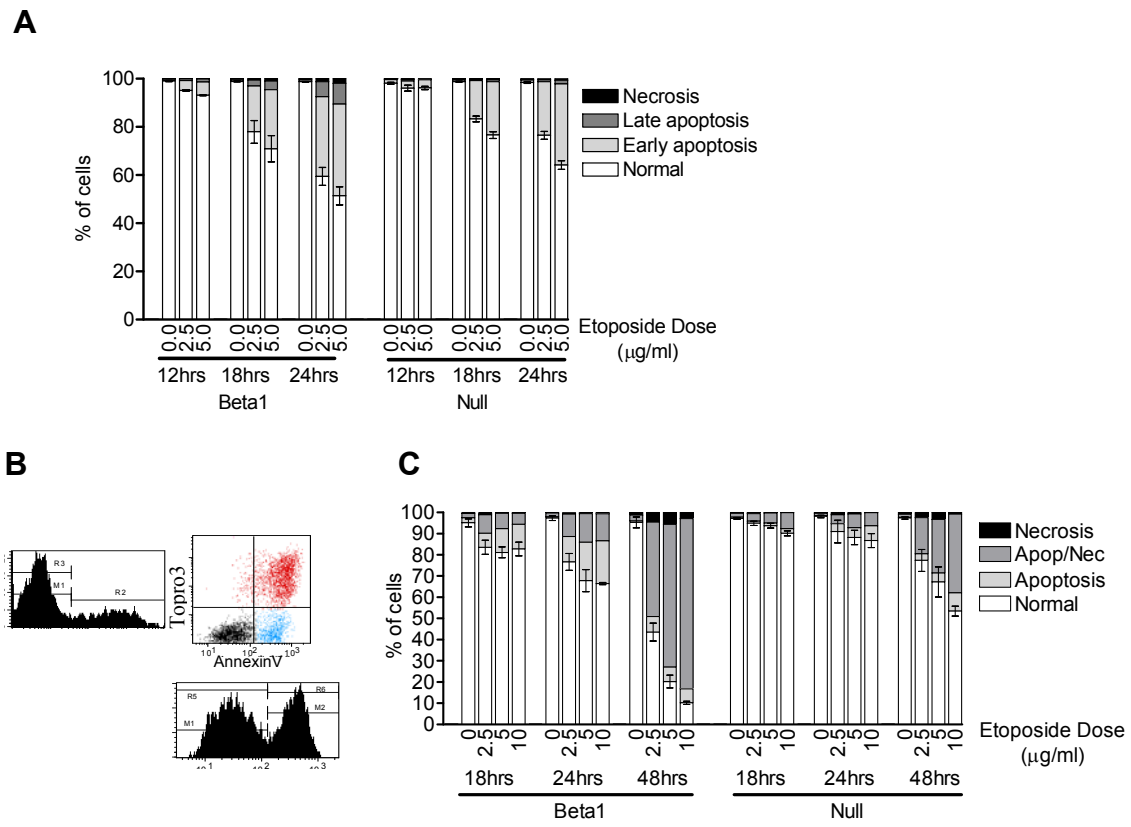


Figure 6.2 Etoposide-induced Apoptosis: Time and Dose-Response in GD25 cells cultured in DMEM with 10% FCS. $2x10^4$ GD25 cells were plated per well of 96 well plates and allowed to adhere for 6 hours in DMEM complete media followed by overnight quiescence in GD25 quiescent media. They were then treated with 0-10 μg/ml etoposide. The percentage of apoptotic and necrotic cells was assessed 12-48 hours later **A** morphologically by immunofluorescence microscopy after addition of 1μl of ethidium bromide (1 mg/ml) and 1μl of acridine orange (1mg/ml). Mean ± s.e. of n=4 experiments in triplicate shown. Apoptosis and necrosis was also assessed by flow cytometric analysis of AnnexinV and Topro3 staining: A typical example of flow cytometry data is shown in **B**. AnnexinV/Topro3 time and dose-response shown in **C**. Mean ± s.e. of n=4 experiments shown.

Results: AnnexinV/Topro3 flow cytometry, an objective technique for measurement of apoptosis, was performed in addition to the acridine orange/ethidium bromide assessment to obviate any potential subjective bias in morphological assessments. At equivalent time-points the morphological assessment (**Fig 6.2A**) and flow cytometry technique (**Fig 6.2C**) showed similar levels of cell death. A higher rate of necrosis and combined apoptosis (AnnexinV staining) with necrosis (Topro3 staining) was

demonstrated at all dose levels and timepoints with the flow cytometry technique and this may represent a degree of trauma to the cells during resuspension resulting in Topro3 breach of damaged cell membranes. At all dose and timepoints GD25 Null cells demonstrated lower levels of etoposide-induced apoptosis and necrosis. Using this data 18 and 24 hour timepoints and etoposide doses of 2.5 and 5μg/ml were selected for experiments assessing the presence of extracellular matrix-mediated survival signalling.

6.3.2 Fibronectin and laminin do not mediate protection against etoposide-induced apoptosis in GD25 cells cultured in DMEM with 10% FCS

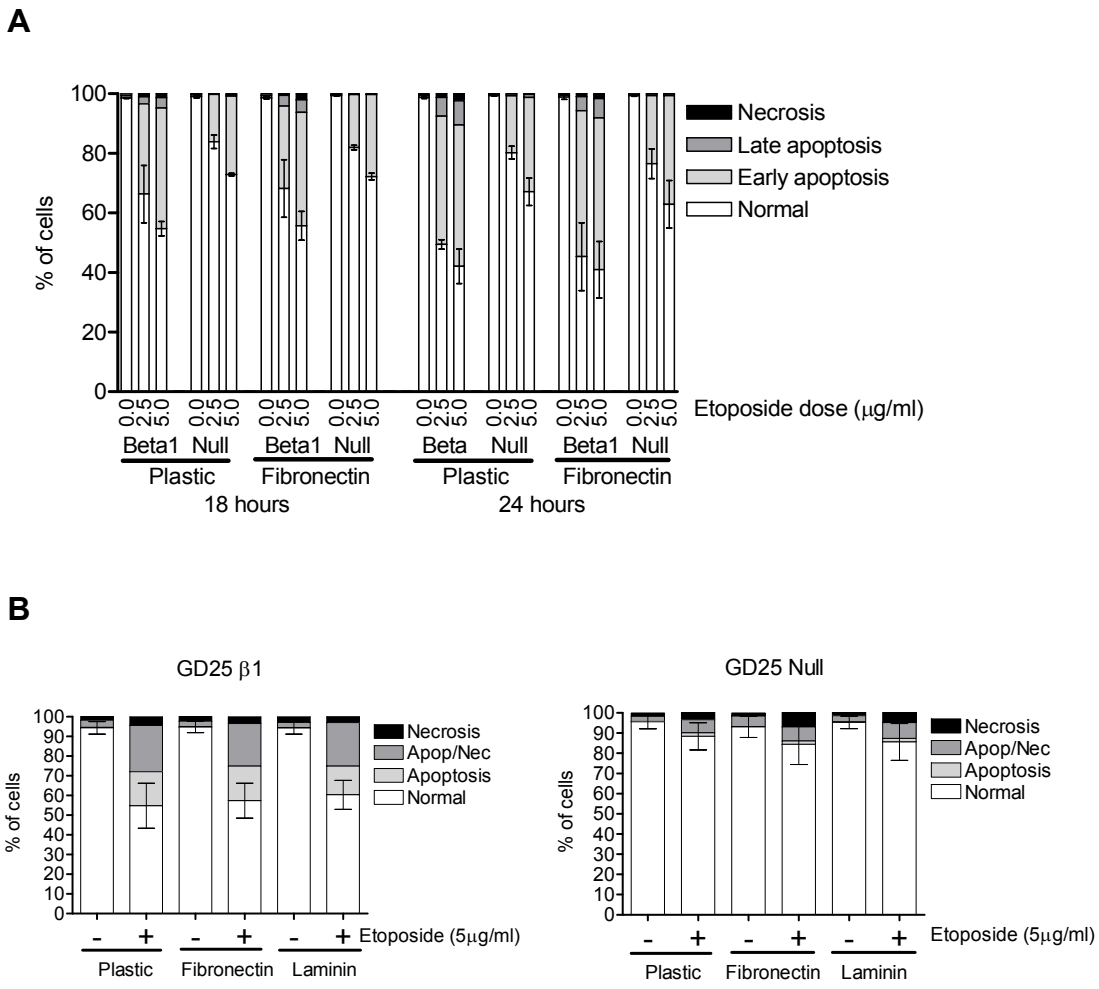


Figure 6.3 Fibronectin and laminin did not mediate protection against etoposide-induced apoptosis in GD25 cells cultured in DMEM with 10% FCS. 96 well plates were pre-coated by incubation at 37°C for 1 hour with or without 10μg/ml laminin or 20μg/ml fibronectin. $2x10^4$ GD25 cells were plated per well and allowed to adhere in GD25 complete media for 6 hours and quiesced overnight before treatment with etoposide 0-5μg/ml. The percentage of apoptotic and necrotic cells was assessed 0-48 hours later **A** morphologically by immunofluorescence microscopy after addition of 1μl of ethidium bromide (1 mg/ml) and 1μl of acridine orange (1mg/ml). 18 and 24 hour timepoints shown, mean ± s.e. of n=4 experiments, and **B** by flow cytometric analysis of AnnexinV and Topro3 staining. 24 hour timepoint shown, mean ± s.e. of n=4 experiments.

Results: Whether assessed morphologically (**Fig 6.3A**) or by flow cytometric analysis (**Fig 6.3B**), at 18 and 24hrs in both GD25 β1 and GD25 Null cells there was no statistically significant difference in apoptosis rates at either etoposide dose level (2.5 or 5μg/ml) when comparing plastic against fibronectin or laminin. i.e. fibronectin or laminin failed to protect GD25 cells against etoposide-induced apoptosis irrespective of the presence or absence of β1-integrin expression. Furthermore, GD25 β1 cells exhibited greater sensitivity to etoposide-induced apoptosis at all dose and time points than GD25 Null cells. A possible reason for the failure of ECM to produce chemoprotection in this model was the presence of multiple extracellular matrix components in GD25 complete media (containing foetal calf serum) that cells were cultured during the initial period of adhesion in these experiments. Foetal calf-containing media was used in these experiments since a) GD25 cells did not tolerate prolonged periods in quiescent media and b) adhesion did not occur in the presence of quiescent media.

In view of the above results, dose-response and extracellular matrix-mediated survival signalling experiments were repeated using SITA, a serum-free medium containing selenium, insulin, transferrin and bovine serum albumin(BSA).

6.4 Fibronectin and laminin do not mediate protection against etoposide-induced apoptosis in GD25 cells plated in SITA

6.4.1 Etoposide-induced apoptosis in GD25 cells plated in SITA: time and dose response

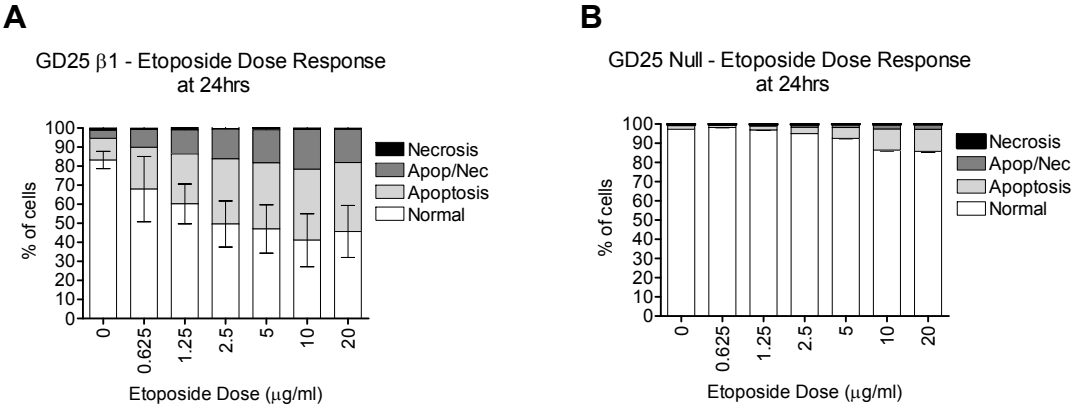


Figure 6.4 Etoposide-induced Apoptosis: Time and Dose-Response in GD25 cells cultured in SITA. $2x10^4$ GD25 cells were plated per well of 96 well plates in SITA and allowed to adhere overnight before treatment with 0-20 μg/ml etoposide. The percentage of apoptotic and necrotic cells was assessed 24 hours later by flow cytometric analysis of AnnexinV and Topro3 staining. Time and dose-response for GD25 β1 and GD25 Null cells are shown in **A** and **B** respectively. Mean ± s.e. of n=3 experiments shown.

Results: After 24 hours etoposide induced a dose-related increase in apoptosis and necrosis in GD25 cells (**Fig 6.4**). As previously demonstrated, GD25 β1 cells were more sensitive to etoposide-induced cell death than GD25 Null cells. Since the rate of etoposide-induced cell death in GD25 β1 cells was seen to plateau at 5μg/ml this dose was chosen for assessment of ECM-mediated chemoprotection.

6.4.2 Fibronectin and laminin do not mediate protection against etoposide-induced apoptosis in GD25 cells plated in SITA

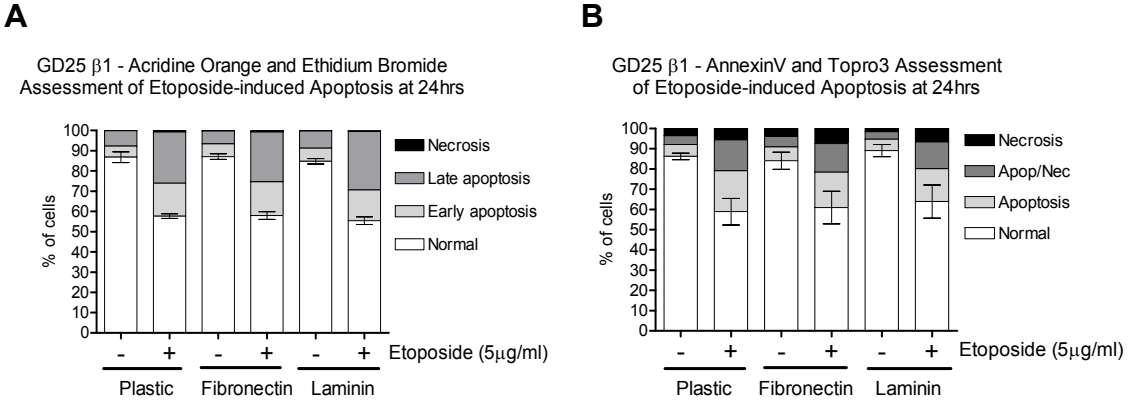


Figure 6.5 Fibronectin and laminin did not mediate protection against etoposide-induced apoptosis in GD25 cells cultured in SITA. 96 well plates were pre-coated by incubation at 37°C for 1 hour with or without 10μg/ml laminin or 20μg/ml fibronectin. $2x10^4$ GD25 cells were plated per well and allowed to adhere in SITA overnight before treatment with or without etoposide 5μg/ml. The percentage of apoptotic and necrotic cells was assessed 24 hours later **A** morphologically by immunofluorescence microscopy after addition of 1μl of ethidium bromide (1 mg/ml) and 1μl of acridine orange (1mg/ml) and **B** by flow cytometric analysis of AnnexinV and Topro3 staining. Mean ± s.e. of n=4 experiments shown.

Results: In serum-free media, laminin and fibronectin again failed to protect GD25 cells against etoposide-induced apoptosis (**Fig 6.5**).

6.5 GD25 β1 cells exhibit faster growth rates than GD25 Null cells

It is well recognised that cytotoxic agents which target DNA and induce DNA double-strand breaks are more effective at inducing apoptosis in rapidly cycling cells than slower growing cells [(Corvo et al., 2000),(Sharma et al., 2000),(Baguley et al., 1995)]. In clinical practice this is reflected in high initial response rates to chemotherapy in rapidly growing cancers such as lymphoma and small cell lung cancer. In order to investigate whether this could be a significant factor in the increased sensitivity of

GD25 β1 cells to etoposide-induced apoptosis, growth curves of GD25 β1 and GD25 Null cells were performed.

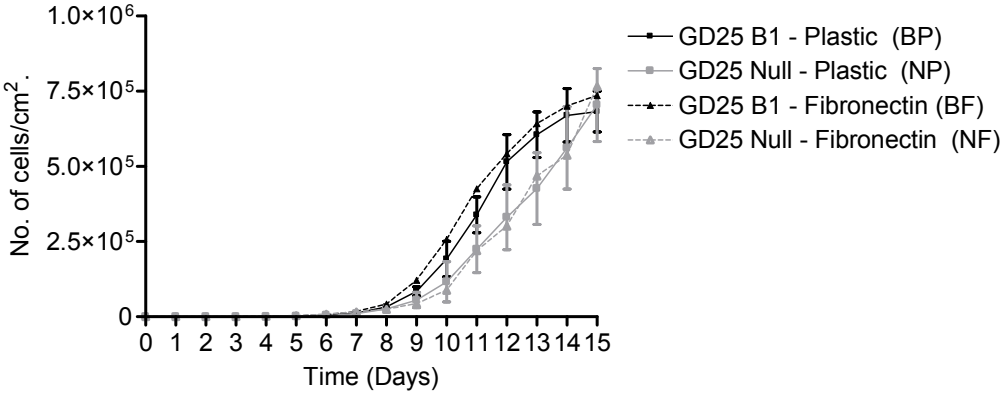


Figure 6.6 GD25 β1 and GD25 Null growth curves on plastic and fibronectin. 10,000 cells were plated per 10cm plate, suspended in 15mls of GD25 complete media and incubated at 37°C for 0-15 days. When counting, media was removed and the adherent cells were washed twice with filtered PBS. After resuspension in filtered PBS cells were counted using a Coulter counter. Mean ± s.e. of 4 experiments shown.

Results: GD25 β1 cells have a significantly faster rate of growth than GD25 Null cells ($p<0.0001$) (**Fig 6.6**). This is most marked at lower cell densities which is important since in the previous experiments (above) cells were initially plated at 10^5 cells per ml of media ($2x10^4$ cells per 200µl of media) when this difference in growth rates is most marked. Cell confluence occurs at approximately $5x10^5$ cells per cm^2. The reason that at later timepoints GD25 Null cell numbers approach GD25 β1 cell numbers is that GD25 Null cells showed a much greater tendency to overgrow and carpet the plates whilst GD25 β1 cells appeared to demonstrate a reduction in growth rates at high cell densities possibly related to a greater degree of contact inhibition. Fibronectin did not significantly alter growth rates of GD25 Null or GD25 β1 cells.

In view of these results it was evident that a comparison of etoposide sensitivity between the two cell types would not provide a useful model to assess extracellular matrix-mediated survival signalling and no further experiments were performed.

6.6 Discussion

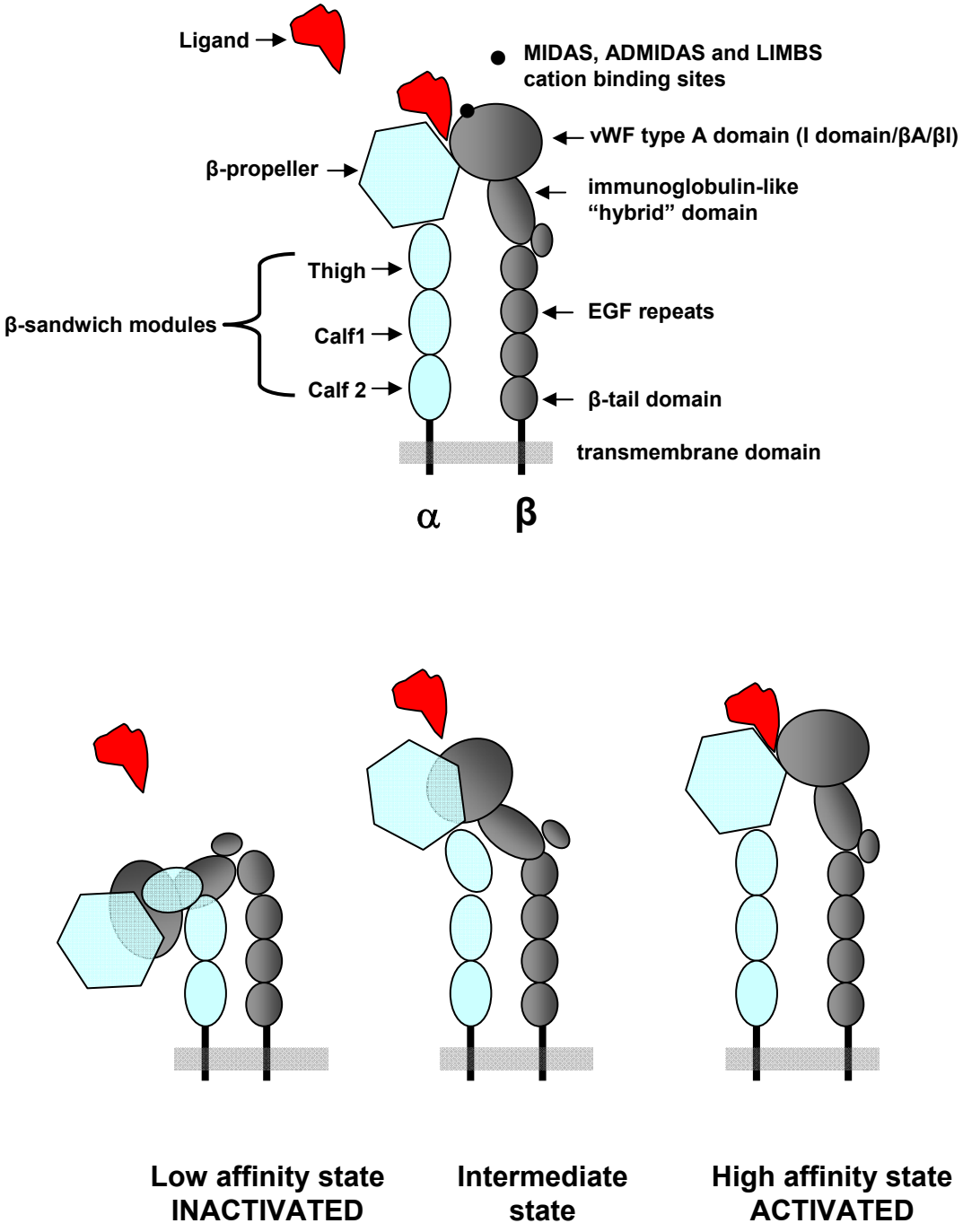
GD25 β1 and GD25 Null cells offered an appealing model for assessing the mechanisms underlying extracellular matrix-mediated survival signalling. Many model systems investigating β1-integrin signalling employ function-blocking antibodies as negative control arms. Null cells, with a stable deletion of β1-integrin, provide an alternative negative control and obviate the problems of antibody delivery, isotype controlling and difficulties in performing longer time-course experiments with interval antibody dosing. Furthermore, additional stable cell lines expressing mutants of β1-integrin have been developed in GD25 cells and these provide further opportunities for investigating the β1-integrin-mediated signalling involved in extracellular matrix-mediated survival signalling (e.g. GD25β1$A_{Y783/795F}$ cells have been shown to have a defect in β1-integrin-dependent FAK phosphorylation and activation (Wennerberg et al., 2000)). However, experimental assessment of this in vitro model system showed that it would not be useful as a tool to assess β1-integrin-mediated protection against chemotherapy-induced apoptosis. There was no evidence of extracellular matrix-mediated drug resistance using fibronectin or laminin at any dose level or timepoint. This may be a result of a) cell type specificity; GD25 cells may not possess the requisite intracellular survival signalling pathways, b) insufficient dose of extracellular matrix; however, in some

experiments, laminin and fibronectin were used at doses up to 40 and 80μg/ml respectively (four times the dose quoted in other published model systems) without any evidence of chemoprotection (data not shown), c) over-riding of the effect of chemoprotection by culture media: it is possible that insulin (present in SITA) and foetal calf serum (in GD25 DMEM complete media) provide survival signalling to these cells via β1-integrin-independent pathways (e.g. via PI3Kinase [(Gonzalez et al., 2006),(Su et al., 2006),(Alexia et al., 2006)]) and thus cells plated on plastic may already possess maximal anti-apoptotic signalling. It would have been preferable to plate GD25 cells onto ECM or plastic directly after overnight quiescence. However, cells plated in this way failed to adhere and experienced very high apoptotic rates after 24 hours, d) undefined survival signalling via other integrins expressed in GD25 cells (αvβ3, αvβ5, α6β4 [(Retta et al., 2001)]) though expected ligand specificity would not predict this.

In summary, though scientifically appealing, GD25 cells in practice were shown not to be a useful tool for the investigation of extracellular matrix-mediated survival signalling. No further experiments are planned.

APPENDIX

Figure 1 Integrin Structure and Affinity States



Adapted from Current Opinion in Cell Biology (2004),16; 544-551[Mould et al., 2004]

Figure 2 Conserved integrin cytoplasmic tail sequences

α subunit

```
α1      LALW KIGFFKRPLKKKMEK
α2      AILW KLGFFKRKYEKMTKNPDEIDETTELSS
α3A     LLLW KCGFFKRARTRALYEAKRQKAEMKSQPSETERLTDDY
α4      YVMW KAGFFKRQYKSILQEENRRDSWSYINSKSNDD
α5      YILY KLGFFKRSLPYGTAMEKAQLKPPATSDA
α6A     FILW KCGFFKRNKKDHYDATYHKAEIHAQPSDKERLTSDA
α7      LLLW KMGFFKRAKHPEATVPQYHAVKIPREDRQQFKEEKTGTILRNNWGSPRREGPDAHPILAADG
α8      LALW KCGFFDRARPPQEDMTDREQLTNDKTPEA
α9      VLLW KMGFFRRRYKEIIEAEKNRKENEDSWDWVQKNQ
α10     FCLW KLGFFAHKKIPEEEKREEKLEQ
α11     LALW KLGFFRSARRRREPGLDPTPKVLE
αV      FVMY RMGFFKRVRPPQEEQEREQLQPHENGEGNSET
αL      IVLY KVGFFKRNLKEKMEAGRGVPNGIPAEDSEQLASGQEAGDPGCLKPLHEKDSESGGGKD
αM      AALY KLGFFKRQYKDMMSEGGPPGAEPQ
αX      AVLY KVGFFKRQYKEMMEEANGQIAPENGTQTPSPPSEK
αD      ATLY KLGFFKRHYKEMLEDKPEDTATFSGDDFSCVAPNVPLS
αIIb    LAMW KVGFFKRNRPPLEEDDEEGE
αE      VILF KCGFFKRKYQQLNLESIRKAQLKSENLLEEEN
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β subunit

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β1A     LLIW KLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVV--------NPKYEGK
β2      LVIW KALIHLSDLREYRRFEKEKLKSQWNND-NPLFKSATTTVM--------NPKFAES
β3      LLIW KLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTFT--------NITYRGT
β5      LAIW KLLVTIHDRREFAKFQSERSRARYEMASNPLYRKPISTHTVDFTFNKFNKSYNGTVD
β6      LCIW KLLVSFHDRKEVAKFEAERSKAKWQTGTNPLYRGSTSTFK--------NVTYKHREKQKVDL
β7      VLAY RLSVEIYDRREYSRFEKEQQQLNWKQDSNPLYKSAITTTI--------NPRFQEADSPTL
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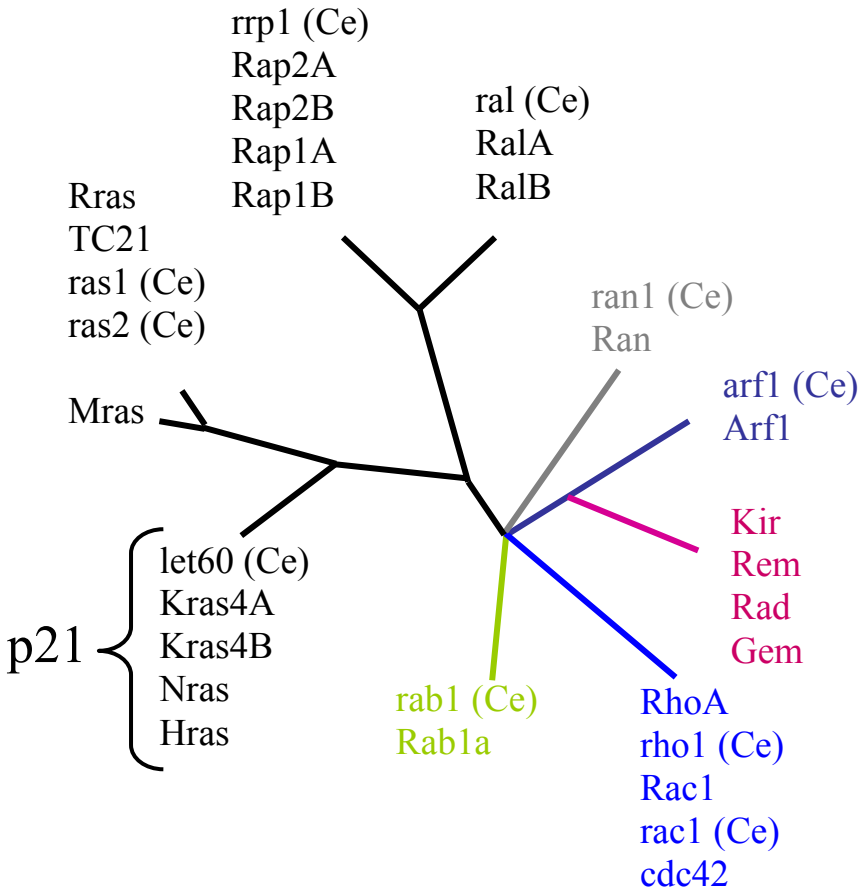
Adapted from Calderwood, D. A. J Cell Sci (2004),117; 657-666 (Calderwood, 2004)

Figure 3 Phylogenetic relationships of:

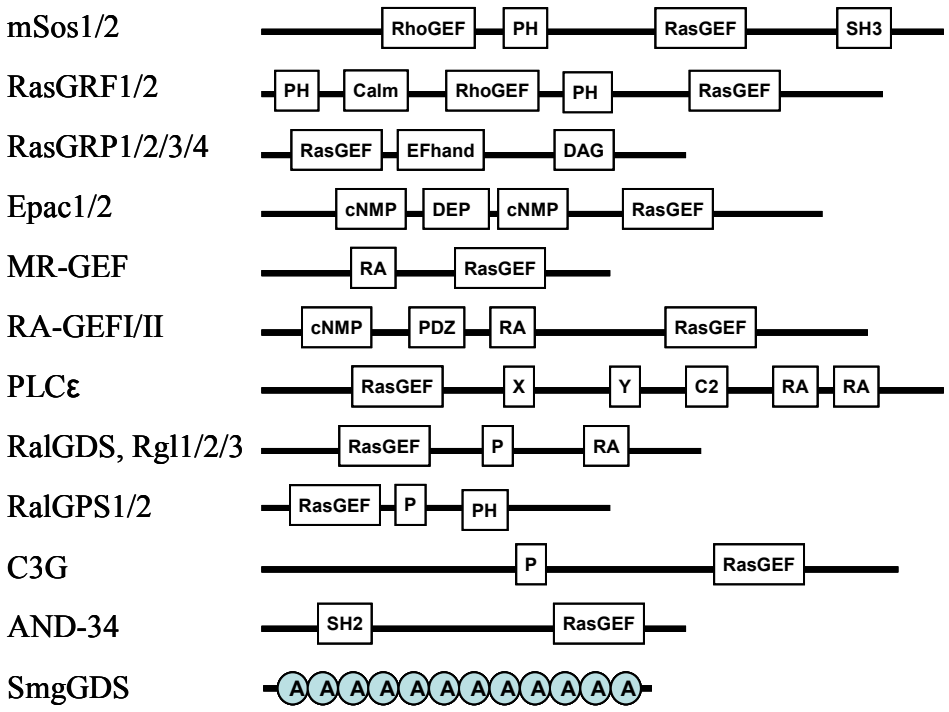
***Ras* superfamily, Ras family and ras subfamilies**



Ce: Caenorhabditis elegans

Adapted from Experimental Haematology (2002), 30; 1089-1106 (Ehrhardt et al., 2002)

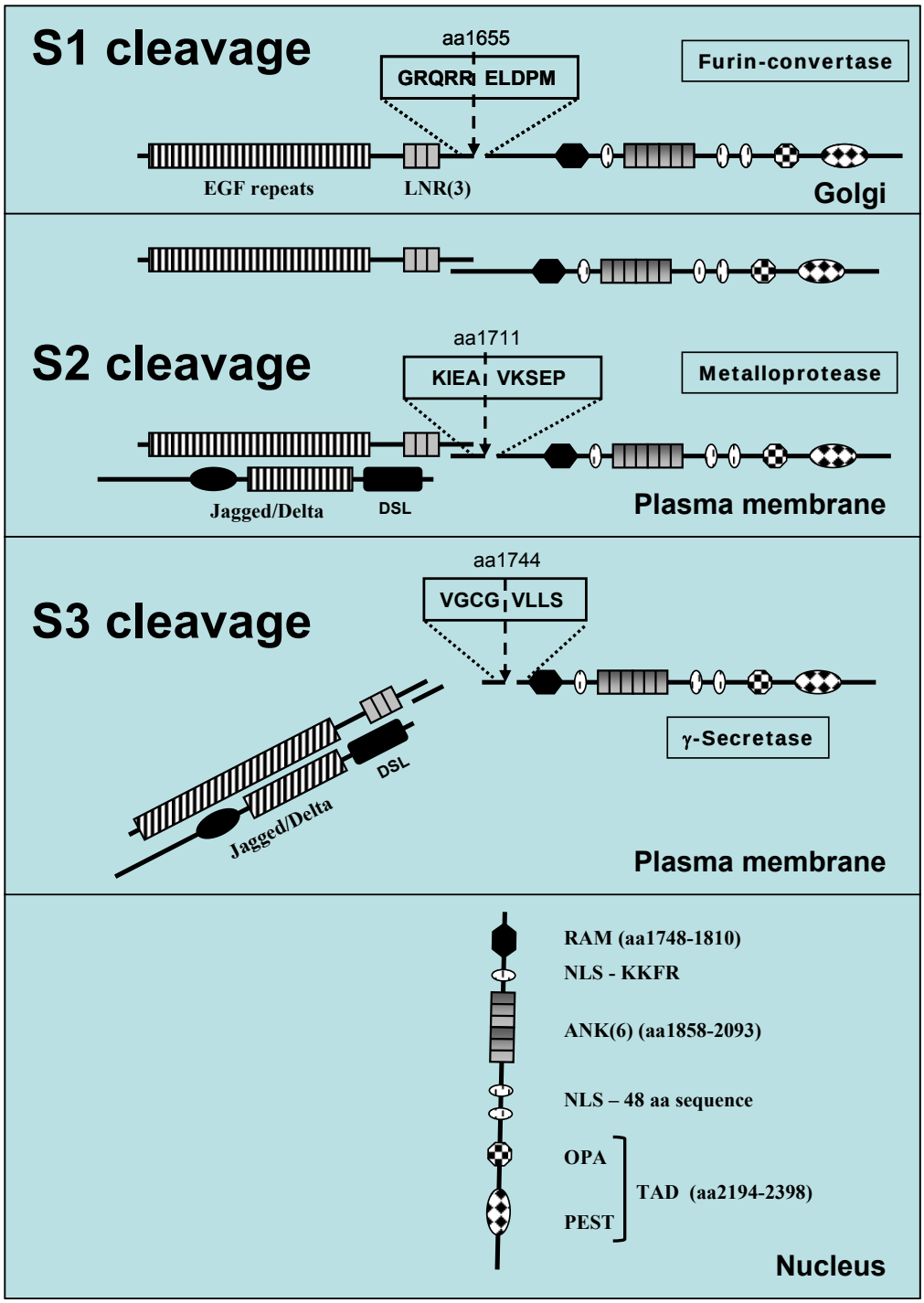
Figure 4 Sub-domains of Guanidine Exchange Factors



GEF – Guanidine Exchange Factor.
PH - pleckstrin homology.
P - poly-proline-rich sequences, representing SH3 (Src homology 3) domain binding sites
Calm - calmodulin binding.
DAG - diacylglycerol binding.
DEP - domain found in Dishevelled (Dvl) proteins.
RA - Ras association.
PDZ - domain found in PSD95, Dlg, ZO1 proteins.
SH2 - Src homology 2 domain, phosphotyrosine binding.
X,Y - phospholipase catalytic domains.
C2 - calcium-dependent lipid binding domain.
cNMP – cyclic nucleotide-binding domain
EF Hand – helix-turn-helix motif

Adapted from Experimental Haematology (2002), 30;1089-1106 (Ehrhardt et al., 2002)

Figure 5 Notch Structure and Cleavages



EGF - Epidermal Growth Factor
RAM - RBPJκ-associated molecule (binds csl proteins which bind cgtggggaa)
ANK - ankyrin repeats (mediate additional protein interractions and weak csl binding)
OPA - glutamine-rich sequence
PEST - proline, glutamate, serine, threonine-rich sequence
LNR - (Lin-12/Notch) Repeats
DSL - Delta, Serrate, Lag-2 conserved Notch-ligand sequence
NLS - Nuclear Localisation Sequence
TAD - Transactivation Domain
CSL - CBF1/RBPJκ, Su(H), Lag-1

Figure 6 Bax – Bcl-2 System in Cell Survival Signalling

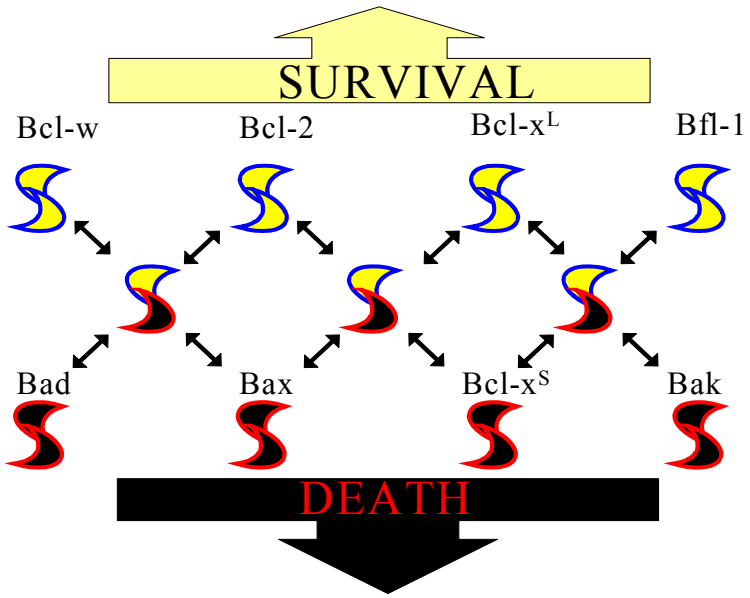


Table 1 Integrin heterodimers and Ligands

Integrin Subunit		Ligands			
		Laminin	**Fibronectin**	**Collagen**	**Other**
β1	α 1	X		X	
	α 2*	X	X	X	E-cadherin
	α 3*	X	X	X	Thrombospondin, entactin
	α 4		X		V-CAM1
	α 5		X		Fibrinogen,
	α 6*	X			
	α 7	X			
	α 8		X		Tenascin
	α 9				Tenascin
	α 10			X	
	α 11			X	
	α V*		X		Vitronectin
B 2	α L[1]				I-CAM (1-5), JAM-A
	α M[2]				I-CAM1,Fibrinogen, FactorX, C3bi
	α X				Fibrinogen, C3bi
	α D				V-CAM1
B 3	α IIb		X	X	Fibrinogen, vitronectin, vWF, ICAM4 disintegrins, plasminogen, prothrombin, thrombospondin
	α V	X	X	X	Fibrinogen, vitronectin, vWF, thrombospondin, tenascin, osteopontin, disintegrins, MMP-2, prothrombin
B 4	α 6	X			
B 5	α V		X		Vitronectin
B 6	α V		X		Tenascin
B 7	α 4		X		V-CAM1
B 7	α E				E-cadherin
B 8	α V		X		

[1]LFA-1
[2]Mac-1/CR3
* - dominant integrins expressed by Small Cell Lung Cancer(Sethi et al., 1999b).

Table 2 Inside-out modulators of integrin affinity

Integrin modulator	Integrin
Ancient Ubiquitous Protein1 (Aup1)	αIIb (Kato et al., 2002)
β3-endonexin	β3 (Kashiwagi et al., 1997)
Ca2+	αIIbβ3 (Hyduk et al., 2007)
Caspase 12	αIIbβ3 (Kerrigan et al., 2004)
CD151/Tetraspanins	α3β1 (Nishiuchi et al., 2005)
CD73	αLβ2 (Airas, Niemela, and Jalkanen, 2000)
CD98 heavy chain	β-integrin (Fenczik, Sethi, Ramos, Hughes, and Ginsberg, 1997),(Cai et al., 2005)
Chemokines	α4β1, αLβ2, αMβ2/Mac-1 (Johnston et al., 2002)
Calcium and Integrin Binding Protein (CIB)	αIIbβ3 (Tsuboi, 2002)
Csk	β1 (Li et al., 2002)
Cytohesin 1+3	αLβ2/LFA-1 (Mazerolles et al., 2002)
Epidermal Growth Factor	α2β1 (Genersch et al., 1998)
Ephrins	β1 (Davy et al., 2000)
FAK	β1 (Schaller, 2001)
FcRs	α2β1 (Watson et al., 2000)
14-3-3	αLβ2 (Nurmi et al., 2006)
Grb2/SH3	β3, αLβ2/LFA-1 (Saci et al., 2002)
Hras	αIIbβ3, αLβ2/LFA-1 (Hughes et al., 1997),(Hughes et al., 2002)
HSP60	α3β1 (Barazi et al., 2002)
Integrin Associated Protein/CD47	α2β1, αvβ3, αIIbβ3 (Chung et al., 1997)
IC1n	αIIbβ3 (Larkin et al., 2004)
IGF1	α3β1, αVβ3 (Maile et al., 2002)
ILK	β1, β2+β3 (Pasquet et al., 2002)
Integrins-transdominant inhibition	αIIbβ3, α2β1 (Calderwood et al., 2004)
Lck	αLβ2/LFA-1, α4β1 (Feigelson et al., 2001)
Lipid raft	αLβ2/LFA-1 (Hogg et al., 2002)
LPL/L-plastin	αMβ2/Mac-1 (Wang et al., 2001)
Mapk	α5 and β1 (Hughes et al., 2002)
Notch4	β1 (Leong et al., 2002)
PEA 15	β1 (Ramos et al., 1998)
Phosphorylation	β-integrins (Hogervorst et al., 1993)
PI3K	αLβ2/LFA-1, α4β1, α5β1, αIIbβ3, αVβ3 (Cai et al., 2005)
PKC	αIIbβ3, αLβ2/LFA-1 (Hauss et al., 1993)
PLC	αIIbβ3, α2β1, α5β1, α6β1, αLβ2/LFA-1 (Hyduk et al., 2007)
Post-translation	α7β1, α2-6, αVβ3, β1 (Berthet et al., 2000)
Rap	β-integrin, αIIbβ3, αLβ2/LFA-1 (Bertoni et al., 2002)
Redox potential	β2, αIIbβ3 (Yan et al., 2000)
Reducing agents	α4β1, αIIbβ3 (Chigaev et al., 2004)
Rras	β1 (Keely et al., 1999)
selectin	αMβ2/Mac-1, αLβ2/LFA-1 (Giblin et al., 1997)
Shc	αIIbβ3 (Saci et al., 2002)
Src	αIIbβ3, α4β1 (Saci et al., 2002)
Syk	αIIbβ3, α4β1 (Woodside et al., 2002)
Talin	β1, αIIbβ3 (Calderwood et al., 1999)
Thrombopoietin	αIIbβ3 (Van Os et al., 2003)
uPA/uPAR	αMβ2/Mac-1 (Czekay et al., 2003)

Table 3 Specificity of GEFs and GAPs for Hras, Rras and Rap.

Hras		Rras		Rap	
GEFs	GAPs	GEFs	GAPs	GEFs	GAPs
RasGRF1 RasGRF2 RasGRP1 RasGRP3 Sos1/2	NF-1 GAP1m GAPIII P120RasGAP GAP^{1P4BP}	RasGRF1 RasGRP1 RasGRP3 CalDAG-GEF1	NF-1 GAP1m GAPIII P120RasGAP GAP^{1P4BP}	RasGRP2 RasGRP3 MR-GEF RA-GEFI/II Epac1/2 C3G PLCε	SPA1 GAP^{1P4BP} Rap1GAP

Adapted from Molecular Cell Biology. (2003), 4; 767-776 (Kinbara et al., 2003).

Table 4 Proteins that interact with Ras

PROTEIN INTERACTORS	PROTEIN INTERACTORS
AF-6	Ral guanine nucleotide dissociation stimulator
Angiotensin receptor 1	cGMP 3`,5`-cyclic phosphodiesterase delta subunit
B-Raf	NMDAR2D
Bcl 2	Ras binding protein Sur 8
CDC 25A	Phosphatidylinositol 3 kinase, catalytic subunit, delta
Galectin 1	IRAK2
Grb2	RAS guanyl nucleotide releasing protein 1
Insulin receptor	Rab acceptor 1
MEK1	Ras association domain family protein 1
Neurofibromatosis type I	RAS inhibitor 1
Phosphatidylinositol 3 kinase regulatory subunit, alpha	Zinc finger protein, subfamily 1A, member 3
Phosphatidylinositol 3 kinase, catalytic subunit alpha	Ras association (RalGDS/AF-6) domain family 5
Phospholipase C, epsilon 1	LGN protein
Protein kinase C, iota type	Isoprenylcysteine carboxyl methyltransferase
RAF1	RASGRP4
RHOD	Ral GDS related protein Rgr
Ras-GAP	RAP1 GTP GDP dissociation stimulator 1
SOS1	Tetratricopeptide repeat protein 1
Toll like receptor 2	Ral guanine nucleotide dissociation stimulator like 1
Toll like receptor 9	Ras interacting protein 1
VAV1	Ras association (RalGDS/AF-6) domain family 2
Intersectin 1	RIN
PDGF beta	Zinc finger and BTB domain containing 12
c-src	Glutamate receptor, ionotropic, N-methyl D-aspartate 1
Interleukin 1 receptor associated kinase 1	BRAP2
A_Raf	Protein farnesyltransferase alpha subunit
Guanine nucleotide releasing factor 2	Protein farnesyltransferase beta subunit
T cell lymphoma invasion and metastasis 1	Protein kinase C, zeta
Caveolin 1	Interleukin 3
Mitogen-activated protein kinase 8	Fyn
Phosphoinositide 3 kinase, catalytic subunit, gamma	p73
SOS2	Retinoic acid receptor responder 3
Diacylglycerol kinase, zeta	

Obtained from: Human Protein Reference Database by searching for Hras – Interactions. http://www.hprd.org.browse/interactions?protein=01813&isoform_id=01813_1&isoform_name=Isoform_1

Table 5 Components of Notch Pathway

	Caenorhabditis. elegans	**Drosophila. melanogaster**	**Mammals**
Ligand	Lag-2 APX-1 ARG-2F16 B12.2	Delta Serrate	DLL1 DLL3 DLL4 Jagged1 Jagged2 F3/Contactin[291]
Receptor	Lin-12 GLP-1	Notch	Notch1 Notch2 Notch3 Notch4
Transcription Factor	Lag-1	Su(H)	CSL CBF/RBPJκ
S1 cleavage	NK	NK	Furin convertase
S2 cleavage	SUP-17	Kuzbanian/ADAM10	TACE
S3 cleavage			γ-secretase (presenilin, nicastrin, Aph1 and Pen2)

Lag - Lin and GLP
DLL - Delta-like ligand
GLP-1 - Germline proliferation
Su(H) - Suppressor of Hairless
CSL - Core Binding Factor, Su(H), Lag-1
CBF - Core Binding Factor
RBPJκ - recombination signal binding protein for immunoglobulin kappa J region
TACE - Tumor necrosis factor-alpha-converting enzyme
Aph1 - Artemia POU-Homeoprotein
Pen-2 - presenilin enhancer-2
NK - Not known

Table 6 Integrin expression in SCLC cell lines

Integrin Subunit	H69	H345	H510
α1	4.3 ± 0.2	6.1 ± 0.5	4.9 ± 0.8
α2	2.3 ± 0.3	7.1 ± 0.6	17.4 ± 2.2
α3	**36.4 ± 2.5**	**38.0 ± 2.2**	**30.0 ± 1.8**
α4	5.7 ± 0.4	6.5 ± 0.3	3.2 ± 0.1
α5	3.0 ± 0.2	2.4 ± 0.4	4.4 ± 0.4
α6	**32.4 ± 2.8**	**22.5 ± 2.5**	**17.1 ± 2.9**
αV	**20.6 ± 3.1**	**14.7 ± 2.8**	**26.3 ± 1.2**
β1	**30.8 ± 3.5**	**17.1 ± 1.4**	**42.3 ± 3.9**
β2	5.0 ± 0.3	1.4 ± 0.2	4.2 ± 0.4
β3	6.8 ± 0.5	6.8 ± 0.6	4.5 ± 0.7
β4	6.7 ± 0.9	4.6 ± 0.5	6.0 ± 0.1
β5	5.6 ± 0.8	3.8 ± 0.2	6.8 ± 0.6
β6	5.6 ± 0.6	5.9 ± 0.4	2.5 ± 0.1

Integrin expression was determined by flow cytometric analysis using monoclonal antibodies against integrin subunits.

Table 7 % of SCLC cells in cell cycle phases

	Plastic	Poly-L-lysine			Laminin		
Cell cycle phase	-	-	Etoposide	XRT	-	Etoposide	XRT
Sub-G0	5.6±1.6	6.1±1.3	4.9±2.4	5.4±1.4	6.3±1.7	5.1±1.1	3.5±1.7
G1	64.8±2.5	65.3±2.8	23.2±2.3	22.1±1.8	67.1±3.1	53.2±1.9	60.1±2.6
S	17.3±2.0	18.2±1.7	25.4±1.6	24.5±2.1	16.7±2.3	22.2±1.7	20.1±1.8
G2/M	8.8±1.0	9.1±0.6	46.2±1.5	47.8±2.1	8.4±2.1	16.8±2.0	16.1±1.9

Corresponds to Fig 4.5A

Table 8 % of SCLC cells in cell cycle phases

	Poly-L-lysine			
Cell cycle phase	-	4B4	Ly294002	Na3V04
Sub-G0	3.6±1.7	4.9±1.1	4.3±0.9	4.5±0.7
G1	24.2±3.9	23.2±2.2	22.1±2.5	39.8±1.8
S	22.6±2.1	20.8±2.8	19.3±1.3	24.5±2.1
G2/M	48.2±3.1	46.9±2.8	52.5±3.0	28.6±2.0
	Laminin			TS2/16
Cell cycle phase	-	4B4	Ly294002	
Sub-G0	4.2±2.1	4.3±1.1	3.2±0.9	4.1±2.1
G1	52.3±3.1	22.8±2.0	20.9±1.7	44.2±1.8
S	20.2±1.9	19.7±3.0	16.1±2.4	28.7±2.2
G2/M	18.4±2.5	51.2±2.2	58.8±3.1	21.7±2.6

Corresponds to Fig 4.5B

Tables 9 and 10 Extracellular Matrix and Growth Factor composition of Growth Factor-reduced Matrigel™

Table 9

ECM Composition of Growth Factor-reduced Matrigel Matrix	
Basement Membrane Matrigel Component	Percent in GFR BD Matrigel ™ Matrix
Laminin	61%
Collagen IV	30%
Entactin	7%

Table 10

Concentration of Growth Factors (GF) Present in Growth Factor-reduced BD Matrigel™ Matrix	
Growth Factor	Typical GF Concentration
EGF	< 0.5 ng/ml
bFGF	n.d.*
NGF	< 0.2 ng/ml
PDGF	< 5 pg/ml
IGF-1	5 ng/ml
TGF-β	1.7 ng/ml
**n.d. - not determined*	

Data obtained from manufacturer's datasheet.

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MAMMALIAN NOTCH-1 ACTIVATES β1 INTEGRINS VIA THE SMALL GTPASE R-RAS.

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Running title: Notch-1 activates β1 integrins via R-Ras

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Notch is a central regulator of important cell fate decisions. Notch activation produces diverse cellular effects suggesting the presence of context-dependent control mechanisms. Genetic studies have demonstrated that Notch and integrin mutations have related phenotypes in key developmental processes such as vascular development and somitogenesis. We show that the intracellular domain of mammalian Notch-1 activates integrins without affecting integrin expression. Integrin activation is dependent on γ-secretase-mediated intra-membranous cleavage of membrane bound Notch releasing intracellular Notch which activates R-Ras, independent of CSL-transcription. Notch also reverses H-Ras and Raf-mediated integrin suppression without affecting ERK phosphorylation. Membrane bound Notch mutants that are inefficiently cleaved or intracellular Notch mutants lacking the ankyrin repeat sequence do not activate R-Ras or integrins. Co-expression of Msx2-Interacting Nuclear Target (MINT) protein with Notch or expression of intracellular Notch-1 truncation mutants lacking the C-terminal transactivation/PEST domain suppresses Notch transcriptional activity without affecting integrin activation. Notch ligand, Delta-like ligand-4 stimulates R-Ras-dependent α5 β1 integrin -mediated adhesion, demonstrating the physiological relevance of this pathway. This new CSL-independent Notch/R-Ras pathway provides a molecular mechanism to explain Notch, integrin and Ras cross-talk during the development of multi-cellular organisms.

The diverse biological processes intrinsic to the development of multi-cellular organisms are co-ordinated by communication between adjacent cells involving a small number of evolutionarily conserved signalling pathways. The Notch signalling pathway is an important mechanism for mediating these intercellular signalling events to direct cell fate decisions (1). The components of the Notch pathway have been identified in a broad range of metazoans and have been extensively studied in insects, nematodes and mammals (2-4). Consequently, Notch has been shown to be a key regulator of many developmental processes including somitogenesis, vasculogenesis and neurogenesis (4-6). In addition, the Notch pathway plays a critical role in mammalian immune development and carcinogenesis (7,8).

The molecular components of the Notch pathway have been extensively studied and are highly conserved between species. Notch encodes a single-pass heterodimeric transmembrane receptor with an extracellular domain that contains epidermal growth factor (EGF)-like repeats (9). Four Notch homologs (Notch-1-4) and two groups of ligands (Delta-like (Dll -1, -3 and -4) and Serrate-like (Jagged 1, 2)) have been identified in mammals (10,11). Notch-ligand interaction triggers two distinct proteolytic cleavage events (S2 and S3) that release the intracellular portion of Notch (NIC) from the plasma membrane (12-14). NIC translocates to the nucleus where it binds to a transcriptional regulator CSL (CBF-1/Su(H)/LAG-1), displacing co-repressors and recruiting co-activators, thus inducing expression of Hairy-Enhancer of Split (HES) and HES-related proteins (HERP) genes (15-18). Data from several groups suggest that Notch may also signal without cleavage at S3 or CSL-dependent transcription (19-21). The molecular components of this "non-classical" Notch signalling pathway are not yet fully understood.

Recent genetic studies have indicated important parallels between the developmental processes controlled by Notch and integrin-mediated adhesion. Integrins are heterodimeric transmembrane glycoproteins that mediate cell-

cell and cell-matrix interactions and have been identified in insects, nematodes and vertebrates (22). A key feature of integrins is their ability to modulate ligand-binding affinity in response to intracellular signals, a process called activation (23). They are essential for embryogenesis and are involved in neurogenesis, myogenesis and angiogenesis, processes also controlled by Notch (24-26). □6 □1 and □5 □1 integrins are highly expressed on stem cells and regulate survival, migration and differentiation (27,28). Notch has been shown to associate with □1 integrins in neural stem cells and expression of Notch 4 in endothelial cells increases adhesion to collagen (29,30). Additionally, data from Zebrafish somitogenesis suggests that mutations in the Notch pathway and integrin □5 subunit can produce a complementary disruption in somite formation (31). This suggests the possibility that Notch may affect integrin activation, modulating important developmental processes by alterating cell-matrix interactions (32). We therefore sought to investigate whether Notch signalling could activate integrins and define the mechanisms of this important interaction using an *in vitro* model of integrin affinity.

EXPERIMENTAL PROCEDURES

DNA constructs - Mouse Notch-1 constructs: NIC, N□E, N□E(V1774K) and NLNG in pCS2 (C-terminal myc-tag) from R. Kopan (Washington University School of Medicine, St Louis, USA) (14). Human Notch-1 constructs: ICN1, □TADP, □EN1 and □ANK in pcDNA3.1 from J. C. Aster (Department of Pathology, Harvard Medical School, USA) (33), subcloned into pCMV Tag 4A (Stratagene) to incorporate a C-terminal Flag-tag. MINT (vector pEF-Bos-Neo, N-terminal Myc tag) (T. Honjo, Kyoto University, Japan) (34). Tac-□5, pDCR-H-Ras(G12V) (HA-tagged), pSG5 R-Ras(WT), R-Ras(T43N) (myc-tagged) and pSG5 R-Ras(G38V) (myc-tagged) as previously described (35,36). pCDNA3-Raf-CAAX (Flag tagged) from Dr. C. K. Weber (University of Ulm, Germany). pGL2 4xCSL (A. Israel, Institute Pasteur, France) (37). pcDNA3.1 LacZ (Invitrogen).

Cell Lines/Transfection - CHO(□□-py) cells were maintained in DMEM (Sigma) with 10% (v/v) foetal bovine serum (FBS), 1% L-Glutamine (LG), 1% Penicillin/Streptomycin (PS), 1% non-essential amino acids and G418 antibiotic at 400□g/ml (Invitrogen). Transient transfection of □□-py cells was performed with JetPEI™ (Polyplus Transfection Agents, UK) as per manufacturers instructions. For □-secretase inhibition, cells were treated with MW167 (50mM) at 36 and 12 hours prior to analysis. K562 cells (ATCC) were maintained in RPMI (Sigma) with 10% (v/v) FBS, 1% LG and 1% PS. K562 cells were transfected by Nucleofector electroporation (Amaxa, Germany) according to the manufacturers instructions. K562 cells were seeded onto human recombinant Delta-like ligand-4 (DLL-4) (R+D) coated to plastic (10□g/ml in PBS for 60 min at 37°C) for 12 hours with or without MW167 (50 □M) or DMSO (Vehicle) (Calbiochem) as indicated.

Flow Cytometry - CHO(□ □-py) cells were transfected with test DNA together with 0.75□g Tac-□5 transfection reporter construct. After 48 hours, cells were sequentially labelled with PAC1 antibody (BD Biosciences, UK) (5□g/ml) +/- 5mM EDTA or 100□M $MnCl_2$ in HEPES/NaCl buffer (20mM HEPES, 140mM NaCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$ and 2mg/ml Glucose, pH 7.4), 1:25 (v/v) anti-mouse IgM-FITC (Biosource) and 1:50 (v/v) anti-Tac-RPE (ACT-1) (Dako, UK). ToPro3 (Molecular Probes) was added to each sample (1□M) and integrin affinity was analysed by three-colour flow cytometry. PAC1 binding was determined by gating for live and highly transfected cells. Integrin activation index (AI) was calculated ($AI = ((F_N-F_I)/(F_A-F_I))*100$) where F_N is geometric mean (GM) fluorescence intensity (MFI) of PAC-1 binding of the native integrin, F_I is MFI of PAC-1 binding in the presence of 5mM EDTA and F_A is MFI of PAC-1 binding in the presence of 100□M Mn^{2+}. AI was used to calculate percentage integrin suppression ($(AI_0-AI)/(AI_0)*100$). AI_0 is the Activation index with the control vector and AI is the Activation index with DNA under test. For analysis of □3 integrin surface expression (independent of integrin affinity), anti-CD61(□3) antibody (Serotec) was used in the above conditions.

Gel Electrophoresis and Western Blotting - Cells were lysed in RIPA buffer, protein balanced by BCA protein assay (Pierce, USA) and resuspended in Laemmli sample buffer. Samples were resolved on 8-12% SDS-PAGE gels (10mg protein per lane) and transferred onto Hybond C nitrocellulose (Amersham Pharmacia Biotech). Immunoblotting: anti-HA (Y-11), anti-Myc (9E10), anti-ERK2 (C-14), anti-□3-integrin (N-20) (Santa Cruz Biotech, USA), anti-phospho-ERK1/2 (ERK-PT115), anti-□-actin (AC-40), anti-FLAG (M2) (Sigma, UK) in 5% non-fat milk, detected with species specific horseradish peroxidase-

conjugated antibodies (DAKO, UK) and chemiluminescence (ECL) (Amersham Pharmacia Biotech).

CSL-luciferase activity - CSL-luciferase activity was assayed as previously described (14). In brief, ab-py cells transfected with test DNA plus pGl2 4xCSL-luc (0.2□g) and pcDNA3.1 LacZ (0.1mg) were lysed after 48 hours and luciferase activity was determined using Steady-Glo (Promega) according to the manufacturers instructions. In parallel samples, galactosidase activity was determined using Galacto-Plus (Tropix). Luciferase activity for each transfection condition was normalised for galactosidase activity and expressed as a fold change from that observed with empty vector.

R-Ras activation assay - R-Ras activity was determined by binding to the Ras binding domain (RBD) of Raf as previously described (38). In brief, □□-py cells were transfected with R-Ras(WT) or R-Ras(G38V) (0.25 □g) and test DNA. Cells were quiesced in serum free media and lysed at 48 hours in buffer (200mM NaCl, 2.5mM $MgCl_2$, 50mM Tris-HCl pH 7.4, 15% glycerol, 1% NP40 and Complete protease inhibitor (Boehringer Mannheim, Sussex, UK)) at 4°C for 20 min. The clarified lysate was incubated with GST-Raf-RBD coupled to glutathione agarose beads for 2 hours at 4°C. Beads were washed and eluted protein separated by SDS-PAGE. Bound R-Ras was detected by western blotting for myc.

Cell Adhesion Assay - $2x10^5$ K562 cells were resuspended in HEPES/NaCl buffer and incubated with 4B4 (Beckman Coulter) (10□g/ml 45 min 37 °C), EDTA (5mM final concentration) or Mn^{2+} (100□M final concentration) as indicated. Cells were seeded into plates coated with fibronectin or poly-l-lysine (10□g/ml in PBS for 60 min 37°C) and incubated for 30 min at 37°C. Non-adherent cells were removed by washing with PBS. Remaining cells were fixed with 3% paraformaldehyde (5 min) and stained with 0.4% methylene blue (5 min). Intracellular methylene blue was eluted with 0.1M HCl and the optical density (630 nM) of each sample was determined using an automated plate reader. Cell adhesion per condition was expressed as a percentage of total cellular adhesion to poly-l-lysine.

HES-1 expression - Total RNA was extracted from $1x10^6$ K562 cells using RNeasy kit (Qiagen), contaminating DNA was removed with RQ1 DNase (Promega) and cDNA was generated by reverse transcription of 400ng RNA using TaqMan reverse transcription reagents as per manufacturer's instructions (Applied Biosystems). cDNA quality was verified by PCR amplification of □-actin. Real time quantitative RT-PCR analysis was performed with TaqMan reagents and an ABI 7900HT machine as per manufacturer instructions (Applied Biosystems). HES-1 for: CATTCTGGAAATGACAGTGAAGCA; HES-1 rev: CAGCGCAGCCGTCATCT; HES-1 probe: CTCCGGAACCTGCAGCGGGC fam labelled. Triplicate measurements were performed and analyzed with ABI sequence detector software (version 2.1) using the $2^{-\Delta\Delta Ct}$ method. HES-1 expression for each condition was normalised for 18S expression and represented as a fold change from control cells.

Statistical analysis - Data were analysed by one-way analysis of variance and the appropriate post-test analyses were applied. p values < 0.05 were considered to be significant.

RESULTS

Notch-1 activates integrins and reverses H-Ras/Raf-mediated integrin suppression - To investigate whether Notch signalling could modulate integrin activation, we used a well-established model of integrin affinity (39). This utilises a CHO cell line (□□-py) that stably expresses an active chimeric integrin (□IIb □6 □3 □1), which has the ligand binding properties of □IIb □3 but is activated through the □6 □1 cytoplasmic domains. We assessed the activation status of the chimeric integrin expressed on the □□-py cells using flow cytometry to detect binding of a monoclonal antibody specific for the active confirmation of □IIb □3 (PAC-1). A cell surface marker encoding the extracellular domain of the IL-2 receptor, termed Tac, and the intracellular domain of the □5 integrin (Tac-□5) was used as a marker for DNA transfection. Transfection of □□-py cells was detected by flow cytometry using an antibody against the IL-2 receptor, Tac-R-PE (R-phycoerythrin). We routinely observed transfection efficiencies of 70-80%. Using this model we were able to determine integrin activation status (geometric mean fluorescence intensity of FITC-labelled PAC-1 binding) in highly transfected cells (Tac-R-PE positive cells) allowing accurate assessment of the effects of transfected test DNA on integrin affinity. For each DNA transfection we compared PAC1 binding under 'native' conditions to those in the presence of EDTA (maximally inhibited integrins) or manganese (maximally activated

integrins) allowing calculation of an integrin activation index as described in experimental procedures.

We found that expression of the intracellular domain of mouse Notch 1 (designated NIC), which constitutively activates the Notch signalling pathway (14), increased integrin activation in □□-py cells compared to vector control. This was demonstrated by a rightward shift in PAC-1 binding in highly transfected cells (vector 49.8%, NIC 75% cells in right upper quadrant) (Fig.1a). There was no change in PAC-1 binding in the untransfected cells. Correspondingly, NIC expression increased integrin activation index (88.4 +/- 5.7%) compared to expression of vector alone (67.3 +/- 2.2%) (Fig.1b).

The chimeric integrin in □□-py cells is relatively activated in the resting state. Therefore, we sought to suppress integrin affinity and determine whether Notch signalling would reverse integrin suppression. The small GTP-binding protein H-Ras has been shown to suppress integrin activation (39). We found that transfection of constitutively active H-Ras(G12V) alone caused marked inhibition of PAC1 binding; however co-transfection with NIC completely reversed suppression of integrin activation (Fig.1 a and b). These effects were not produced by alterations in H-Ras(G12V) or NIC expression when the constructs were co-transfected, nor was expression of the chimeric integrin in the □□-py cells affected (Fig.1c).

Hughes et al. (1997) have previously shown that H-Ras-mediated suppression of the chimeric integrin in □□-py CHO-K1 cells is not a consequence of a decrease in integrin expression levels (39). To confirm that Notch expression did not alter surface integrin expression we used flow cytometry to detect surface expression of □3 integrins. Using an anti-□3 integrin antibody whose binding was not dependent on integrin activation status we found that neither H-Ras(G12V), NIC or R-Ras(G38V) expression altered surface expression of □3 integrins in □□-py cells (Fig.1d). Thus changes in PAC1 binding are due to alterations in integrin activation not expression.

H-Ras suppresses integrin activation via its downstream effector kinase Raf-1 (39). We therefore tested the ability of active Notch to reverse integrin suppression by an activated membrane-targeted variant of Raf, Raf-CAAX. Similar results were observed to those found with H-Ras(G12V) (Fig.2a-c). Thus NIC reversed the suppressive effect of activated H-Ras or Raf-1 on integrin affinity, suggesting that Notch signalling can over-ride integrin inactivation caused by the H-Ras dependent suppression pathway.

Previous work has suggested that suppression of integrin activation by H-Ras and Raf-1 is dependent upon ERK1/2 function but does not correlate with bulk phosphorylation of ERK (40). However, reversal of integrin suppression by the small death effector domain-containing protein PEA-15 depends upon its capacity to bind ERK1/2 (40,41). Notch signalling has previously been shown to have differing effects on ERK phosphorylation depending upon cellular context (42,43). Therefore, we investigated whether Notch signalling could reverse suppression of integrin activation by H-Ras through effects on ERK1/2. We found that transfection with NIC alone did not affect ERK phosphorylation. Furthermore NIC did not affect phosphorylation of ERK induced by H-Ras(G12V) or Raf CAAX (Fig. 1c and 2c). In addition, we were unable to demonstrate a physical association between NIC and ERK1/2 by co-immunoprecipitaion (data not shown). This suggests that Notch modulation of integrin affinity is distinct from the Ras/Raf pathway.

Activation of □1 integrins by Notch-1 requires intramembrane cleavage at S3 to release the intracellular domain - Classically, Notch activation requires ligand-induced cleavage of the Notch receptor approximately 12 amino acids N-terminal to the transmembrane domain (S2), which allows intramembrane cleavage (S3) by the protease complex □-secretase (13,14,44). However analysis of the role of Notch in patterning of the *Drosophila* embryo dorsal epidermis suggest that Notch may signal without membrane cleavage (45). We therefore determined whether activation of □1 integrins by Notch was dependent on intra-membrane cleavage.

Mutants of mouse Notch-1 that are membrane bound and show differing efficiencies of processing by □-secretase were used (14) (summarised in Figure 3) to assess rescue of H-Ras(G12V)-induced integrin suppression. All subsequent □□-py assays are represented as levels of integrin suppression. The Notch mutant N□E consists of the transmembrane and intracellular domains of mouse Notch-1 with a short extracellular segment and is cleaved by the □-secretase complex to yield active intracellular Notch. Transfection of □□-py cells with N□E yielded a fragment of similar molecular weight to NIC and reversed H-Ras-mediated integrin

suppression without affecting H-Ras expression or ERK1/2 activation (Fig.4 a and b). The effect on integrin activity was less pronounced than observed with NIC, presumably as a result of a lower yield of intracellular Notch from NDE (Fig.4a). To investigate this, two Notch mutants were used which are inefficiently processed by γ-secretase and do not yield detectable levels of intracellular Notch; an NΔE variant with a point mutation at amino acid 1774, NΔE(V1774K); and NLNG which has identical transmembrane /intracellular domains to NΔE but an extracellular domain containing LNG repeats. Transfection of ΔΔ-py cells with NΔE(V1774K) or NLNG did not yield detectable intracellular Notch and did not significantly reverse H-Ras suppression of integrins (Fig.4a). Furthermore, pre-treatment of ΔΔ-py cells with the γ-secretase inhibitor MW167 blocked the processing of NDE to intracellular Notch and prevented integrin activation without altering H-Ras(G12V) expression or ERK1/2 phosphorylation (Fig.4b).

We confirmed the transcriptional activity of the Notch mutants by measuring CSL-dependent luciferase activity (14). CSL-luciferase activity was significantly stimulated by NIC and NΔE (Fig.4c). However, transfection of the Notch mutants NΔE(V1774K) or NLNG, which are inefficiently cleaved, stimulated CSL-luciferase activity significantly less than NIC (Fig.4c). Furthermore, inhibiting γ-secretase with MW167 significantly reduced the capacity of NΔE to activate CSL (Fig.4d). Co-transfection of H-Ras(G12V) with the Notch mutants did not affect CSL-luciferase activity (data not shown). Taken together this data indicates that S3 cleavage is essential for both classical Notch activation and Notch-mediated integrin activation.

Activation of β1 integrins by Notch-1 is not dependent on CSL-mediated transcription - S3 cleavage releases the intracellular portion of the Notch receptor, which associates with CSL (46). In the resting state CSL binds to DNA acting as a transcriptional repressor, but upon interaction with Notch, co-repressors are displaced and CSL activates transcription leading to up-regulation of downstream target genes (e.g. HES-1) (47,48). However, Notch may also signal independently from CSL-mediated transcription (49). We therefore sought to determine the role of transcription in Notch activation of integrins using two approaches: inhibition of Notch-CSL interaction and deletion of the transactivation domain of Notch-1.

We used Msx2-interacting nuclear target protein (MINT), which has been shown to compete with the intracellular region of Notch for binding to CSL, suppressing the transcriptional activity of Notch (34). Transfection of MINT into ΔΔ-py cells did not affect integrin affinity or the ability of H-Ras to suppress integrins/phosphorylate ERK1/2 (Fig.5 a and b). Available MINT antibodies are ineffective for Western blot analysis, therefore expression of MINT was confirmed by immunofluorescence microscopy (Fig.5c-j) (34). Importantly, MINT expression did not block NIC activation of integrins despite significantly reducing CSL-luciferase activity (Fig.5k). This suggests that Notch activation of integrins is not dependent upon transcriptional activity.

To confirm our transcriptional activity data we used intracellular Notch-1 truncation mutants (Fig.3). The C-terminal transactivation/PEST (TADP) domain of Notch-1 is important for Notch transactivation and CSL-dependent transcription (33). Transfection of human intracellular Notch-1 (ICN1) into ΔΔ-py cells reversed H-Ras-mediated integrin suppression in a similar manner to mouse NIC (Fig.5l). Furthermore, transfection of an ICN1 truncation mutant, lacking the TADP domain (ΔTADP) also reversed H-Ras-mediated integrin suppression to a similar degree as ICN1 (Fig.5l). We confirmed that removal of the TADP region from the intracellular domain of Notch-1 significantly reduced CSL-dependent transcription (Fig.5m). This data confirms that integrin activation by Notch is not dependent on CSL-dependent transcription.

Activation of β1 integrins by Notch-1 requires the ankyrin repeat domain - The intracellular domain of the Notch receptor consists of the RAM domain, two nuclear localisation sequences, TAD, PEST and ankyrin repeat regions (48,50). We sought to investigate whether the integrin activating function of Notch could be mapped to one of these regions using deletion mutants (Fig.3) (33). We used a membrane bound Notch mutant (ΔEN1) that consists of a short extracellular segment, the transmembrane region and intracellular domain of human Notch-1 (Fig.3), which is processed by γ-secretase and is constitutively active. We found that this mutant reversed H-Ras suppression of integrins in ΔΔ-py cells (Fig.6a). In addition a ΔEN1 mutant (ΔRAM) lacking the 23RAM domain and the ΔTADP mutant (lacking TAD, PEST or NLS domains) were able to activate integrins (Fig.6 a and b). However a mutant of ΔEN1 (ΔANK), which was

lacking the ankyrin repeats, was unable to reverse H-Ras suppression of integrins (Fig.6a). We confirmed the previously described transcriptional activity of these mutants in □□-py cells (Fig.6c) (33). This data specifically implicates the ankyrin repeats in Notch activation of integrins.

Activation of □1 integrins by Notch is mediated by R-Ras - The CSL-independent effectors of Notch are not fully defined. However, our data suggests that Notch may regulate integrin activity by activating effectors that antagonise H-Ras signalling to integrins. R-Ras is a small GTP-binding protein homologous to H-Ras that can activate integrins and reverse H-Ras/Raf-initiated integrin suppression without affecting bulk ERK phosphorylation (36,51). We found that transfection of low doses of NIC (0.25□g) had a minimal effect on integrin suppression mediated by H-Ras(G12V) (Fig.7a left). Importantly, whilst transfection of wild type R-Ras alone did not affect integrin affinity, co-transfection of wild type R-Ras with 0.25 □g of NIC reversed H-Ras-mediated integrin suppression, without affecting H-Ras expression or ERK1/2 phosphorylation (Fig.7a left, right). This suggests that activation of integrins by Notch is potentiated by R-Ras.

To demonstrate a specific role for R-Ras in Notch-mediated integrin activation we used a dominant-negative R-Ras mutant, R-Ras(T43N) (52). Transfection of R-Ras(T43N) alone had a minimal suppressive effect on integrin affinity; however co-transfection of R-Ras(T43N) with NIC blocked the ability of active Notch to increase integrin affinity (Fig.7b left). Furthermore R-Ras(T43N) prevented NIC from reversing H-Ras(G12V)-mediated integrin suppression but did not affect integrin suppression when co-transfected with H-Ras(G12V) alone (Fig.7b left). These effects occurred without alterations in expression of H-Ras, Notch or dominant-negative R-Ras (Fig.7b right). In addition transfection of R-Ras(T43N) did not alter integrin expression in the ab-py cells or H-Ras stimulation of ERK1/2 phosphorylation (Fig.7b right).

To determine whether Notch signalling can directly activate R-Ras, we used the Ras-binding domain (RBD) of Raf, linked to GST, to pulldown GTP-bound (active) R-Ras from CHO cell lysates (38). We found that transfection of NIC or N□E stimulated an increase in active GTP-bound R-Ras in □□-py cells, in comparison to wild type R-Ras alone, without affecting R-Ras expression levels (Fig.7c left). Furthermore, □TADP, which did not activate transcription, was also able to activate R-Ras (Fig.7c right). However, the Notch mutant N□E(V1744K), which did not yield detectable intracellular Notch, or □ANK, which lacked the ankyrin repeats, were not able to stimulate an increase in active R-Ras (Fig.7c left, right). These results indicate that the intracellular domain of Notch can specifically activate R-Ras in CHO cells and this requires the ankyrin repeats but not CSL-dependent transcription. These results demonstrate that the ability of Notch signalling to regulate integrin affinity is mediated through R-Ras.

Notch-1 activates native □5 □1 integrins in an R-Ras dependent manner to increase cellular adhesion to fibronectin - To investigate the physiological relevance of the above findings, we examined the effect of Notch on the fibronectin receptor □5 □1 integrin, in the human myeloid cell line K562 (53). Transfection of K562 cells with NIC significantly increased adhesion to fibronectin in comparison to empty vector (Fig.8a). Similar increases in cellular adhesion were observed with ICN1 and □TADP (results not shown). Adhesion of K562 cells transfected with either Notch or vector was significantly blocked by prior incubation with the □1 integrin-blocking antibody 4B4 (Fig.8a). Furthermore, co-expression of dominant-negative R-Ras blocked the effect of NIC on adhesion of K562 cells to fibronectin (Fig.8b). This occurred without alterations in Notch or □1 integrin expression. We confirmed that NIC activated Notch signalling in K562 by realtime PCR measurement of HES-1 expression and that HES-1 expression was unaffected by co-transfection of dominant-negative R-Ras (Fig.8c). These results indicate that active Notch signalling can regulate natively expressed integrin affinity through R-Ras activation.

K562 cells express Notch-1 receptors in the undifferentiated state and previous data has shown that Notch signalling can be activated *in vitro* by recombinant Notch ligand coated to tissue culture plastic (54,55). Human recombinant Delta-like ligand-4 (DLL-4) significantly increased adhesion of K562 cells to fibronectin (Fig.7d right). This adhesion was blocked by EDTA and increased by Mn^{2+} (Fig.8d). DLL-4 increased HES-1 expression in K562 cells and this was prevented by MW167 (Fig.8e). Crucially, pre-treatment of K562 cells with MW167, or pre-incubation with the □1 integrin blocking antibody 4B4, prevented DLL-4 from increasing adhesion to fibronectin (Fig.8f). Furthermore, DLL-4 induced activation of □5 □1 integrins was blocked by transfection of

dominant-negative R-Ras (Fig.8g). These data indicate that ligation of Notch receptors by naturally occurring Notch ligands can activate β1 integrins increasing cellular adhesion to ECM through R-Ras.

DISCUSSION

We show here that Notch-1 signalling in mammalian cells activates β1 integrins. Furthermore we demonstrate the physiological relevance of our findings by showing that ligation of native Notch-1 receptors by Notch ligand increases cellular adhesion to fibronectin through α5 β1 integrins. Crucially our data provides a mechanism for previous observations demonstrating key interactions between Notch and integrins in somitogenesis and vascular development.

Somitogenesis is the process whereby segmented precursors of the skeletal muscle and vertebral column are generated during vertebrate embryogenesis (32). Mutations in zebrafish integrin α5 disrupt anterior somite formation, giving a complementary phenotype to the posterior defects seen in Notch pathway mutants (31). Analysis of integrin:notch double mutants revealed redundancy between integrin and Notch pathways in promoting somite border morphogenesis, suggests a regulatory relationship between Notch and integrins (32). Our data demonstrates that Notch regulates integrin affinity and provides a mechanism for this interaction that is crucial to vertebrate development.

Genetic studies in mice have suggested that Notch signalling is important for formation of the vasculature. Mutations in Jagged-1, Notch-1 and Notch-1 / Notch-4 in mice result in embryonic lethality with severe vascular defects (56). Genetic analyses also indicate a central role for integrins in vascular development - antagonists of β1 integrins inhibit angiogenesis in the chick chorioallantoic membrane, and Notch 4 increases endothelial cell adhesion preventing endothelial sprouting (30). Notch may therefore regulate vascular development through interaction with integrins. Interestingly, R-Ras knockout mice demonstrate defects in angiogenesis in response to vascular injury and tumour growth (57). We provide direct evidence of a link between Notch and β1 integrins involving R-Ras activation that may represent a central mechanism for regulation of vascular development and angiogenesis. Notch-1 and DLL-4 expression on vascular endothelial cells is regulated by vascular endothelial growth factor and thus our findings of integrin activation by DLL-4 may be particularly relevant (5).

We showed that Notch-1 overrides H-Ras-mediated integrin suppression via R-Ras activation. Studies of vulval development in *Caenorhabditis elegans* and the *Drosophila* eye have indicated important interactions between Notch and Ras (58,59). The Notch pathway can antagonise Ras signalling (60,61). Inhibitory cross-talk between Notch and H-Ras regulates *Drosophila* bristle patterning, *C. elegans* vulva formation and may be important in T-cell positive selection (62-64). These interactions occur in part because each pathway can affect the expression or activities of components of the other pathway (65). Our data provides a new mechanism for Notch antagonism of H-Ras through activation of R-Ras. This does not require CSL/transcription and may therefore allow for rapid changes in cellular signalling in response to interaction with Notch ligands expressed on adjacent cells. The importance of this signalling cascade in worms and insects remains to be demonstrated as these organisms have a simpler repertoire of small GTP-binding proteins and therefore may lack the essential signalling components (i.e. R-Ras) for this interaction (66).

The mechanisms by which R-Ras might antagonise H-Ras functions have not yet been defined. Previous work has demonstrated that reversal of H-Ras-mediated integrin suppression by R-Ras is not due to competition for downstream effectors or Raf-induced MAP kinase activation (36). Furthermore, specific mutations in the effector binding loop of R-Ras impaired the ability of R-Ras to reverse H-Ras-mediated integrin suppression but did not correlate with their ability to activate known R-Ras effectors (67). Recent data has indicated that targeting of R-Ras to focal adhesions at the cell surface is critical for its ability to regulate integrin activation (68). Interestingly H-Ras has also been found to be associated with focal adhesions and data has suggested that Notch can interact with β1 integrins involving lipid rafts (29,69). Therefore, a better understanding of the interaction between Notch, the small GTPases and integrins at the cell surface may help to explain crucial processes in integrin activation and Notch antagonism of H-Ras.

Our data indicates that Notch-1-mediated integrin activation requires cleavage of the Notch receptor at S3 to release the intracellular domain but is independent of CSL-transcription. Importantly we showed that integrin activation by Notch is not inhibited by MINT, which prevents Notch binding

to CSL (34). CSL-independent Notch signalling has been suggested by previous analysis of Notch mutant phenotypes in *Drosophila* - two gain of function classes of Notch alleles were defined which prevent development of sensory bristles, one of which is not rescued by removing Su(H) (19). *In vivo* evidence for CSL-independent signalling in vertebrates was provided by experiments examining Delta-1 activated signals in the developing avian neural crest (70). *In vitro* studies have shown that Notch can prevent differentiation of C2C12 mouse myoblasts into myotubes. Myoblast differentiation is not blocked by a dominant negative CSL protein and can be mimicked by truncated forms of Notch, which cannot activate a CBF-1-dependent promoter (20). The mediators of these pathways are not well defined. We provide direct evidence of CSL-independent Notch signalling and demonstrate that R-Ras is a key signalling moiety in this pathway. We show that intracellular Notch specifically activates R-Ras and that dominant-negative R-Ras blocks Notch integrin activation. R-Ras has important cellular effects including survival, transcription and adhesion but the physiological activators are poorly understood (66). Thus our data defines a novel Notch signalling pathway and describes a new R-Ras activator.

We show here that Notch activation of integrins requires the ankyrin repeat domain of intracellular Notch. Recent studies indicated that this region is essential for all known Notch functions (50). Genetic analyses in *Drosophila* utilising Notch deletion mutants have indicated that deletion of the RAM-ANK region abolishes the ability of full length Notch to transduce a Su(H)-dependent signal (71). Point mutations within the ankyrin repeats of full length Notch produce strong dominant negative phenotypes, whereas mutations that eliminate RAM activity do not abolish Notch signalling (3,71). Studies of mouse Notch-1 have shown that the ankyrin repeats are responsible for binding to CSL and transactivation (48); Notch mutants consisting of the TAD/PEST region were unable to activate transcription but this could be rescued by co-expression of the ankyrin repeat domain. Our data concurs with these genetic and *in vitro* studies. In addition we show that the ankyrin repeats have a previously undefined function not related to CSL binding. Therefore analysis of ankyrin repeat point mutations should enable dissection of Notch CSL -dependent and –independent signalling pathways.

In conclusion, the data presented in this paper demonstrates clear evidence for a new Notch signalling pathway. We show that ligand-induced cleavage of the Notch-1 receptor at S3 releases the Notch intracellular domain. This activates R-Ras, in a CSL-independent manner involving the ankyrin repeats, anatgonises H-Ras-mediated integrin suppression and increases integrin affinity. Activation of Notch could enable a cell to respond to its environmental context, which may be particularly important in the control of stem cell fate decisions, as these cells express high levels of Notch-1, 1 integrins and are frequently found in an ECM rich niche (28). Thus, our data defining an interaction between Notch, the Ras family of GTPases and 1 integrins adds a new level of complexity to the Notch signalling pathway and has important implications for the future understanding of development and disease.

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FOOTNOTES

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[1]The abbreviations used are: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagles medium; ECM, extracellular matrix; ERK, extracellular regulated kinase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; LG, L-glutamine; PBS, phosphate buffered saline; PS, penicillin/streptomycin.

FIGURE LEGENDS

FIGURE 1: Notch activates integrins and reverses H-Ras-mediated suppression of integrin affinity.

(a) CHO(□□-py) cells transfected with Tac-□5 (0.75□g), intracellular Notch-1 (NIC) (1 □g) and H-Ras(G12V) (0.375□g). Integrin activation state was analysed by flow cytometry (n=7 independent experiments). The representative dot blots display PAC1-FITC binding (integrin activation status) on the x-axis and Tac-R-PE antibody binding (transfection efficiency) on the y-axis. The quadrant marker on each dot blot differentiates on the x-axis, cells with high and low integrin affinity status and on the y-axis, highly transfected cells (upper quadrants) against cells transfected to a lesser extent (lower quadrants). The quadrant marker separating highly transfected cells was set for individual experiments to contain 20-25% of Tac-□5 positive cells. The figure in the right upper quadrant of each dot blot represents the percentage of highly transfected cells present in that quadrant (i.e. cells with high integrin affinity). Representative dot blots are shown. H-Ras(G12V) induced a left shift in PAC1 binding in transfected cells indicating integrin suppression. Co-transfection of NIC increased PAC1 binding and reversed the left shift, indicating integrin activation. (b) Mean percentage integrin activation +/-SEM is shown. Inset: Dose-dependent effect of NIC on H-Ras(G12V) (0.375□g)-mediated integrin suppression. (c) Representative immunoblot of NIC, H-Ras, □3 integrin, ERK2, phospho-ERK and □ actin expression. (d) CHO(□□-py) cells were transfected with Tac-a5 (0.75□g) and either intracellular Notch-1 (NIC) (1□g), H-Ras(G12V) (0.375□g) or R-Ras(G38V) (0.5□g). Surface expression of □3 integrins was determined by flow cytometry. Representative overlay histograms of anti-□3 integrin (white) or isotype control (black) staining on highly transfected cells are shown. Statistically significant differences between results are indicated by * ($p<0.05$) or ** ($p<0.01$).

FIGURE 2: Notch reverses Raf-mediated suppression of integrin affinity.

(a) PAC1 binding in □□-py cells transfected with Tac-□5 (0.75□g), NIC (1 □g) and Raf-CAAX (0.5 □g) (n=5 independent experiments, representative dot blots shown). (b) Mean percentage integrin activation +/-SEM. (c) Representative immunoblot analysis of NIC, Raf-CAAX, □3 integrin, ERK2, phospho-ERK and □-actin expression. Statistically significant differences between results are indicated by * ($p<0.05$) or ** ($p<0.01$).

FIGURE 3: Description of Notch constructs.

Mouse (a) and Human (b) Notch-1 constructs used in this study. Key defines important structural components and mutations/deletions in the intracellular domain of each construct.

FIGURE 4: Notch activation of integrins requires intramembranous (S3) cleavage.

(a) The effect of Notch mutants NIC, N□E, N□E(V1744K) and NLNG (1□g) on H-Ras(G12V) (0.375□g)-mediated integrin suppression (mean+/-SEM, n=4 independent experiments). Representative immunoblot of Notch, H-Ras(G12V) and □ actin expression. (b) N□E (1□g) reversal of H-Ras(G12V) (0.375□g)-mediated integrin suppression in the presence of MW167 (50 □M) (□) or vehicle (■) (mean+/-SEM, n=4 independent experiments). Representative immunoblot of Notch, H-Ras(G12V), ERK2, phospho-ERK and □ actin expression. (c) The effect of NIC, N□E, N□E(V1744K) or NLNG (1□g) on CSL-luciferase activity in □□-py cells (mean fold increase compared to empty vector +/-SEM, n=4 independent experiments). (d) The effect of NIC or N□E (1□g) in the presence of MW167 (50 □M) (□) or vehicle (■) on CSL-luciferase activity (mean fold increase compared to empty vector +/-SEM, n=3 experiments). Statistically significant differences between results are indicated by * ($p<0.05$).

FIGURE 5: Notch activation of integrins is independent of CSL-transactivation.

(a) The effect of NIC (1□g) and MINT (2□g) on H-Ras(G12V) (0.375□g)-mediated integrin suppression (mean+/-SEM, n=3 independent experiments). (b) Representative immunoblot of NIC, H-Ras(G12V), phospho-ERK and ERK2 expression. (c-j) Expression of MINT (2□g) and NIC (1□g) in □□-py cells shown by immunofluoresence microscopy. (c-d) □□-py cells expressing NIC-GFP (green), and phase contrast; (e-f) □□-py cells expressing myc-MINT (labelled with alexafluor 568) (red), and phase contrast; (g-h) □□-py cells expressing NIC-GFP (green) and myc-MINT (alexafluor 568) (red); (i) Merge of green (NIC) and red (MINT) channels; (j) Phase contrast of (g-i). (k) The effect of NIC (1□g) and MINT (2□g) on CSL-luciferase activity (mean fold increase compared to empty vector +/-SEM, n=3 independent experiments). (l) The effect of Notch mutants, ICN1 and □TADP (1□g), on H-Ras(G12V) (0.375□g)-mediated integrin suppression (mean +/-SEM, n=3). Representative immunoblot of Notch, H-Ras(G12V) and □ actin expression. (m) The effect of ICN1 and □TADP (1□g) on CSL-luciferase activity (mean fold increase compared to empty vector +/-SEM, n=4 independent experiments). Statistically significant differences between results are indicated by * ($p<0.05$).

FIGURE 6: Notch activation of integrins requires the ankyrin repeats.

(a) The effect of Notch mutants □EN1 and □ANK (1□g) on H-Ras(G12V) (0.375□g)-mediated integrin suppression (mean+/-SEM, n=4 independent experiments). Representative immunoblot of Notch, H-Ras(G12V) and □ actin expression. (b) The effect of Notch mutants ICN1, □TADP and □RAM (1□g) on H-Ras(G12V) (0.375□g)-mediated integrin suppression (mean+/-SEM, n=5 independent experiments). Representative immunoblot of Notch, H-Ras(G12V) and □ actin expression. (c) The effect of ICN1, □EN1, □RAM, □TADP and □ANK (1□g) on CSL-luciferase activity (mean fold increase compared to empty vector +/-SEM, n=3 independent experiments). Statistically significant differences between results are indicated by * ($p<0.05$).

FIGURE 7: Notch activation of integrins is mediated by R-Ras.

(a) Left: The effect of NIC (0.25□g) and R-Ras(WT) (0.5□g) on H-Ras(G12V) (0.375□g)-mediated integrin suppression in □□-py cells (mean +/-SEM). Right: Representative immunoblot of NIC, H-Ras(G12V), R-Ras(WT), □3 integrin, ERK2, phospho-ERK (p-ERK) and □ actin expressiom (n=4 independent experiments). (b) Left: The effect of R-Ras(T43N) (1□g) on NIC (1□g) reversal of H-Ras(G12V) (0.375□g)-mediated integrin suppression in □□-py cells (mean +/-SEM). Right: Representative immunoblot of NIC, H-Ras(G12V), R-Ras(T43N), □3 integrin, ERK2, phospho-ERK (p-ERK) and □ actin expression (n=4 independent experiments). (c) Left: The effect of Notch mutants, NIC, N□E and N□E(V-K) (1□g), on R-Ras activation in □□-py cells. Right: The effect of Notch mutants, □TADP and □ANK (1□g), on R-Ras activation in □□-py cells. Representative immunoblots of myc-tagged active R-Ras (from Ras binding domain of Raf), total R-Ras (from whole cell lysates) and Notch (n=4 independent experiments). Statistically significant differences between results are indicated by * ($p<0.05$). NS indicates no significant difference.

FIGURE 8: Notch increases adhesion in a □1 integrin/R-Ras dependent manner.

(a) The effect of NIC (1□g) on K562 cell adhesion to fibronectin with (□) or without (■) 4B4 (mean percentage adhesion compared to poly-l-lysine +/-SEM, n=4 independent experiments). (b) The effect of NIC and R-Ras(T43N) on K562 cell adhesion to fibronectin (mean percentage adhesion compared to poly-l-lysine +/-SEM). Representative immunoblot of NIC, R-Ras(T43N), □1 integrin and □-actin expression (n=5 independent experiments). (c) The effect of NIC and R-Ras(T43N) (1□g) on HES-1 expression in K562 cells (mean fold increase in HES-1+/-SEM, normalised for 18S and compared to empty vector, n=3 independent experiments). (d-g) K562 cells incubated for 12 hours with recombinant human Delta ligand 4 (Dll-4) with or without MW167 (50 mM). (d) Dll-4 effect on K562

cell adhesion to fibronectin with or without EDTA (5mM) or Mn2+ (100□M) (mean percentage adhesion +/-SEM compared to poly-l-lysine, n=4 independent experiments). (e) The effect of Dll-4 +/- MW167 or 4B4 (10 □g/ml) on K562 cell adhesion to fibronectin (mean percentage adhesion +/-SEM compared to poly-l-lysine, n=3 independent experiments). (f) The effect of Dll-4 +/- MW167 on HES-1 expression in K562 cells (mean fold increase in HES-1+/-SEM, normalised for 18S and compared to empty vector, n=3 independent experiments). (g) The effect on K562 cell adhesion to fibronectin of cells transfected with R-Ras(T43N) or empty vector, and +/-Dll-4 (mean percentage adhesion +/-SEM compared to poly-l-lysine, n=3 independent experiments). Statistically significant differences between results are indicated by * ($p<0.05$). NS indicates no significant difference.

FIGURE 1

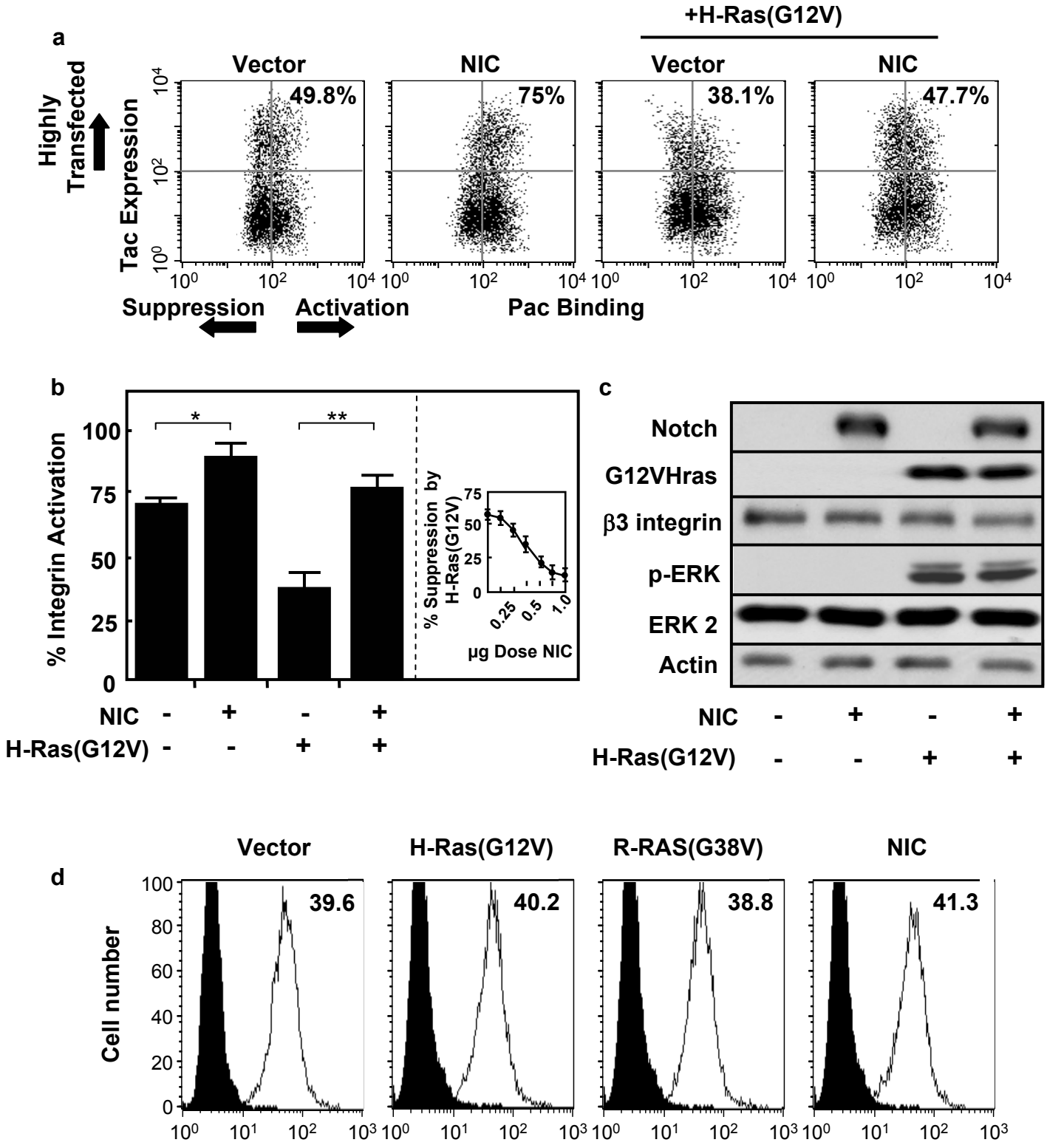

a
+H-Ras(G12V)
Vector
NIC
Vector
NIC
49.8%
75%
38.1%
47.7%
Highly Transfected
Tac Expression
Suppression
Activation
Pac Binding
b
% Integrin Activation
% Suppression by H-Ras(G12V)
µg Dose NIC
NIC
H-Ras(G12V)
c
Notch
G12VHras
β3 integrin
p-ERK
ERK 2
Actin
NIC
H-Ras(G12V)
d
Vector
H-Ras(G12V)
R-RAS(G38V)
NIC
39.6
40.2
38.8
41.3
Cell number

FIGURE 2

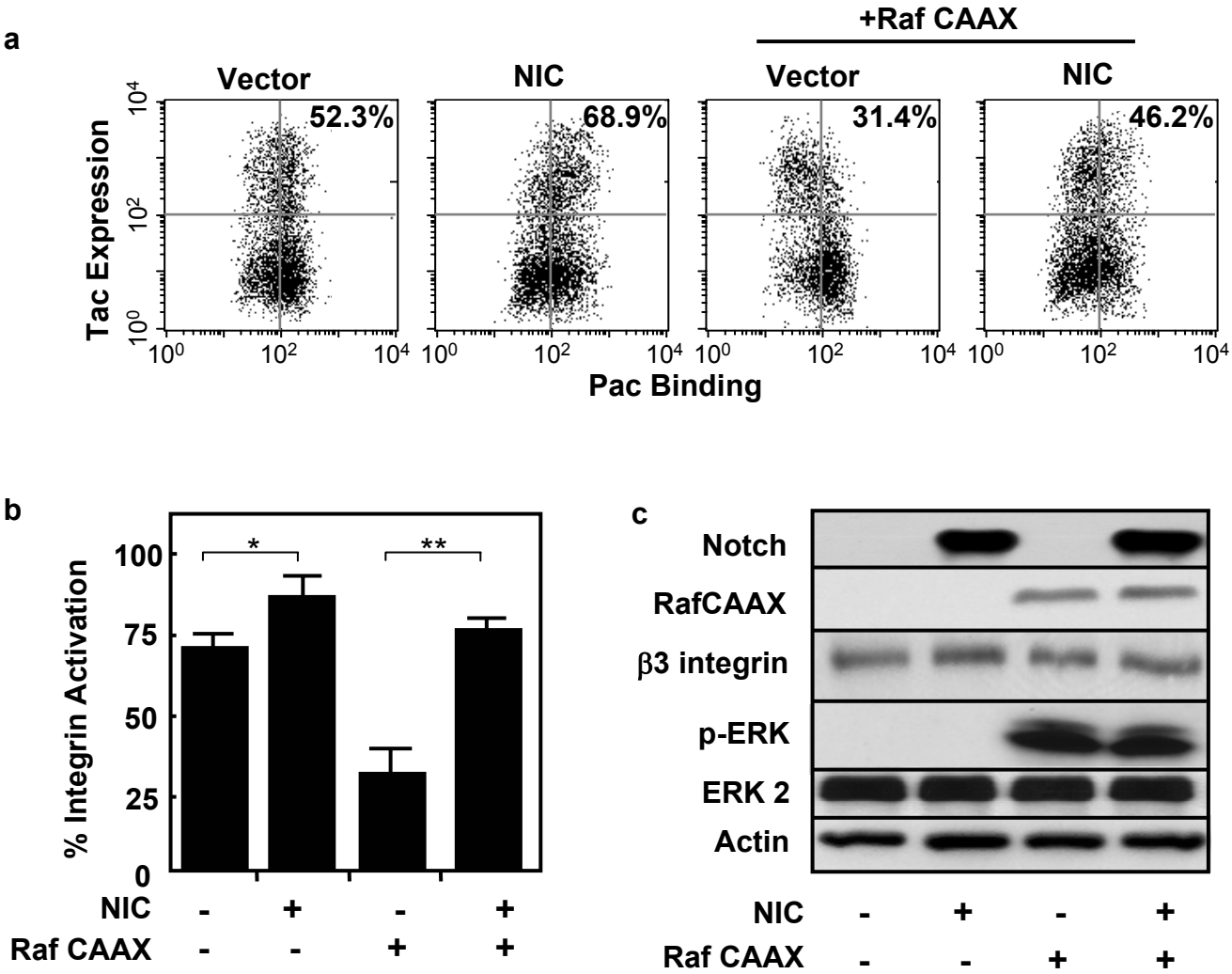
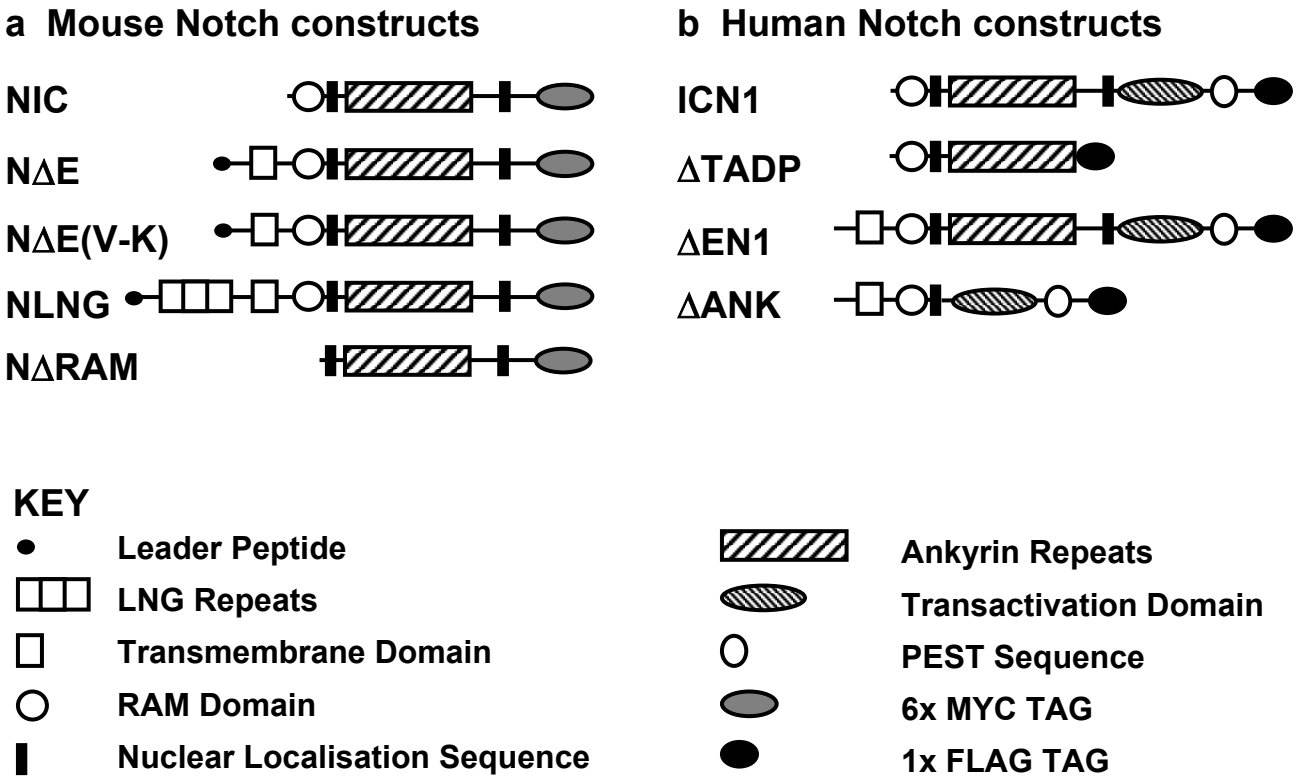

a
+Raf CAAX
Vector
NIC
Vector
NIC
52.3%
68.9%
31.4%
46.2%
Tac Expression
Pac Binding
b
% Integrin Activation
100
75
50
25
0
*
**
NIC - + - +
Raf CAAX - - + +
c
Notch
RafCAAX
β3 integrin
p-ERK
ERK 2
Actin
NIC - + - +
Raf CAAX - - + +

FIGURE 3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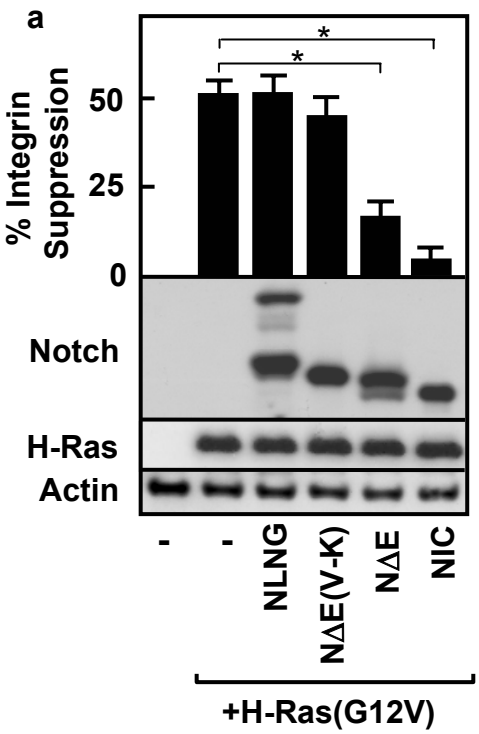
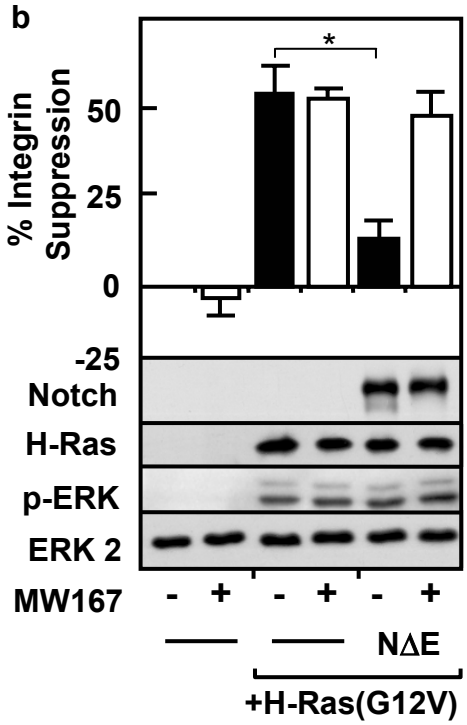
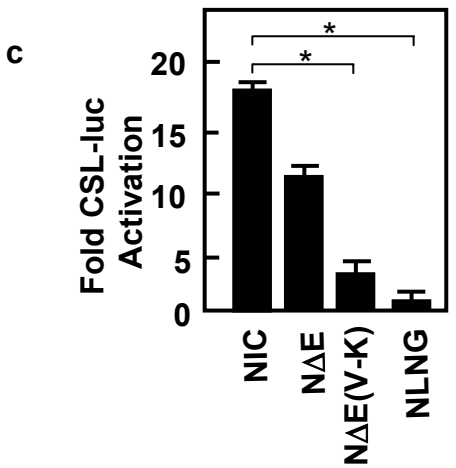
NIC
NΔE
NΔE(V-K)
NLNG
NΔRAM
b Human Notch constructs
ICN1
ΔTADP
ΔEN1
ΔANK
KEY
Leader Peptide
LNG Repeats
Transmembrane Domain
RAM Domain
Nuclear Localisation Sequence
Ankyrin Repeats
Transactivation Domain
PEST Sequence
6x MYC TAG
1x FLAG TAG

FIGURE 4


a
% Integrin Suppression
50
25
0
Notch
H-Ras
Actin
*
*
-
-
NLNG
NΔE(V-K)
NΔE
NIC
+H-Ras(G12V)


b
% Integrin Suppression
50
25
0
-25
Notch
H-Ras
p-ERK
ERK 2
MW167
- + - + - +
NΔE
+H-Ras(G12V)
*


c
Fold CSL-luc Activation
20
15
10
5
0
*
*
NIC
NΔE
NΔE(V-K)
NLNG

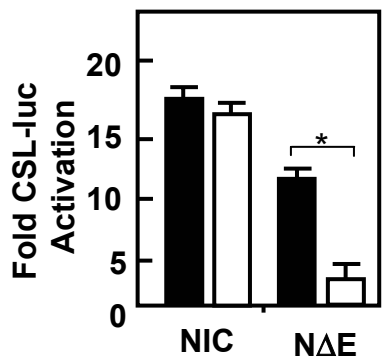

d
Fold CSL-luc Activation
20
15
10
5
0
*
NIC
NΔE

FIGURE 5

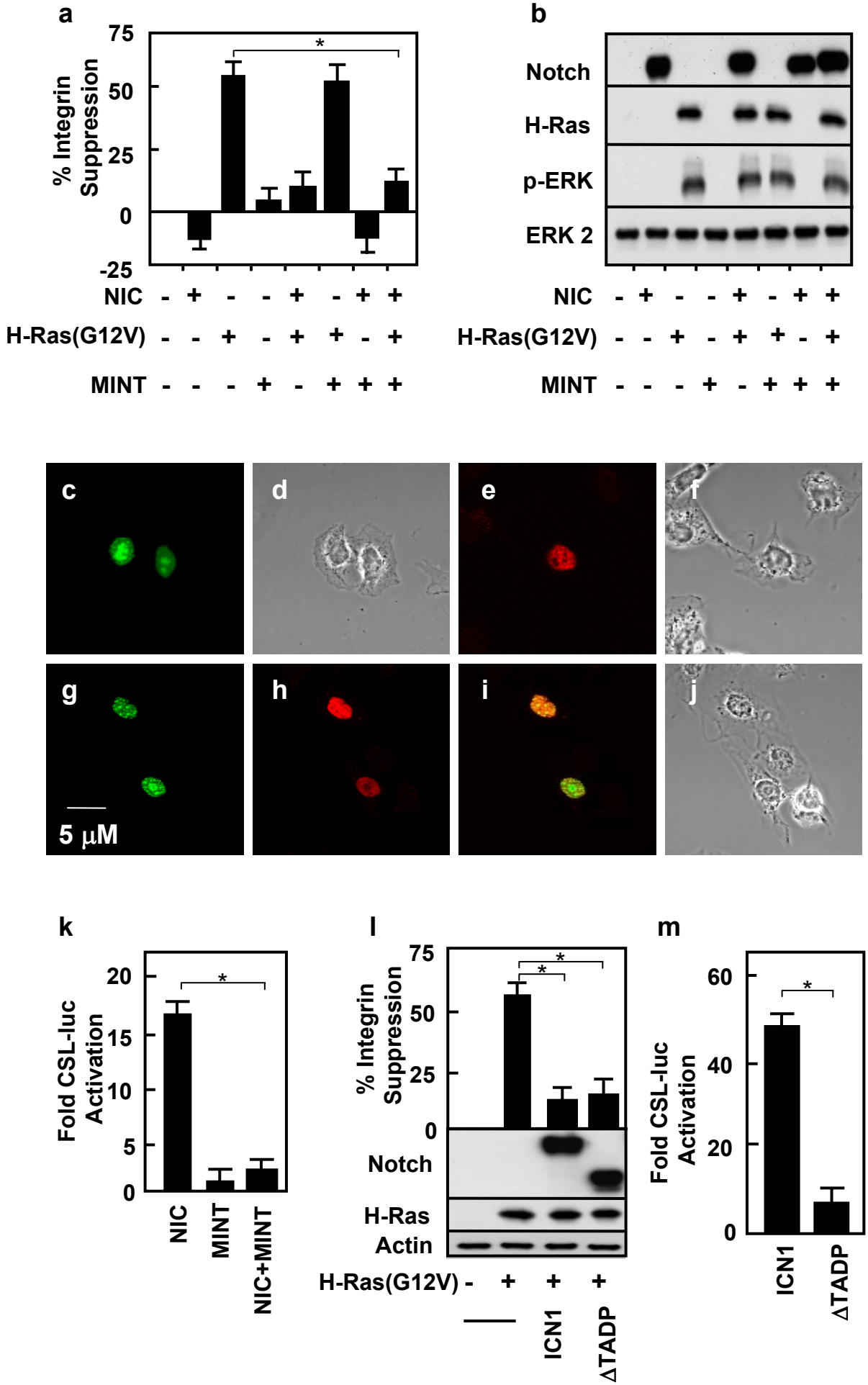

a
% Integrin Suppression
75
50
25
0
-25
*
NIC - + - - + - + +
H-Ras(G12V) - - + - + + - +
MINT - - - + - + + +
b
Notch
H-Ras
p-ERK
ERK 2
NIC - + - - + - + +
H-Ras(G12V) - - + - + + - +
MINT - - - + - + + +
c
d
e
f
g
h
i
j
5 μM
k
Fold CSL-luc Activation
20
15
10
5
0
*
NIC
MINT
NIC+MINT
l
% Integrin Suppression
75
50
25
0
*
*
Notch
H-Ras
Actin
H-Ras(G12V) - + + +
ICN1
ΔTADP
m
Fold CSL-luc Activation
60
40
20
0
*
ICN1
ΔTADP

FIGURE 6

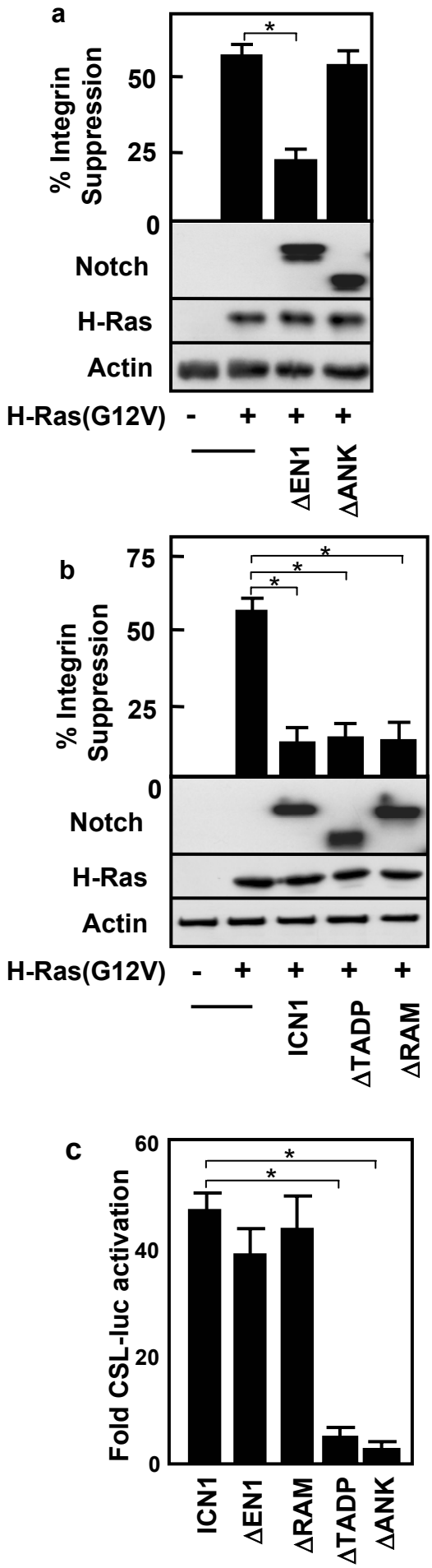

a
% Integrin Suppression
50
25
0
*
Notch
H-Ras
Actin
H-Ras(G12V) - + + +
ΔEN1
ΔANK
b
75
50
25
0
*
*
*
% Integrin Suppression
Notch
H-Ras
Actin
H-Ras(G12V) - + + + +
ICN1
ΔTADP
ΔRAM
c
60
40
20
0
*
*
Fold CSL-luc activation
ICN1
ΔEN1
ΔRAM
ΔTADP
ΔANK

FIGURE 7

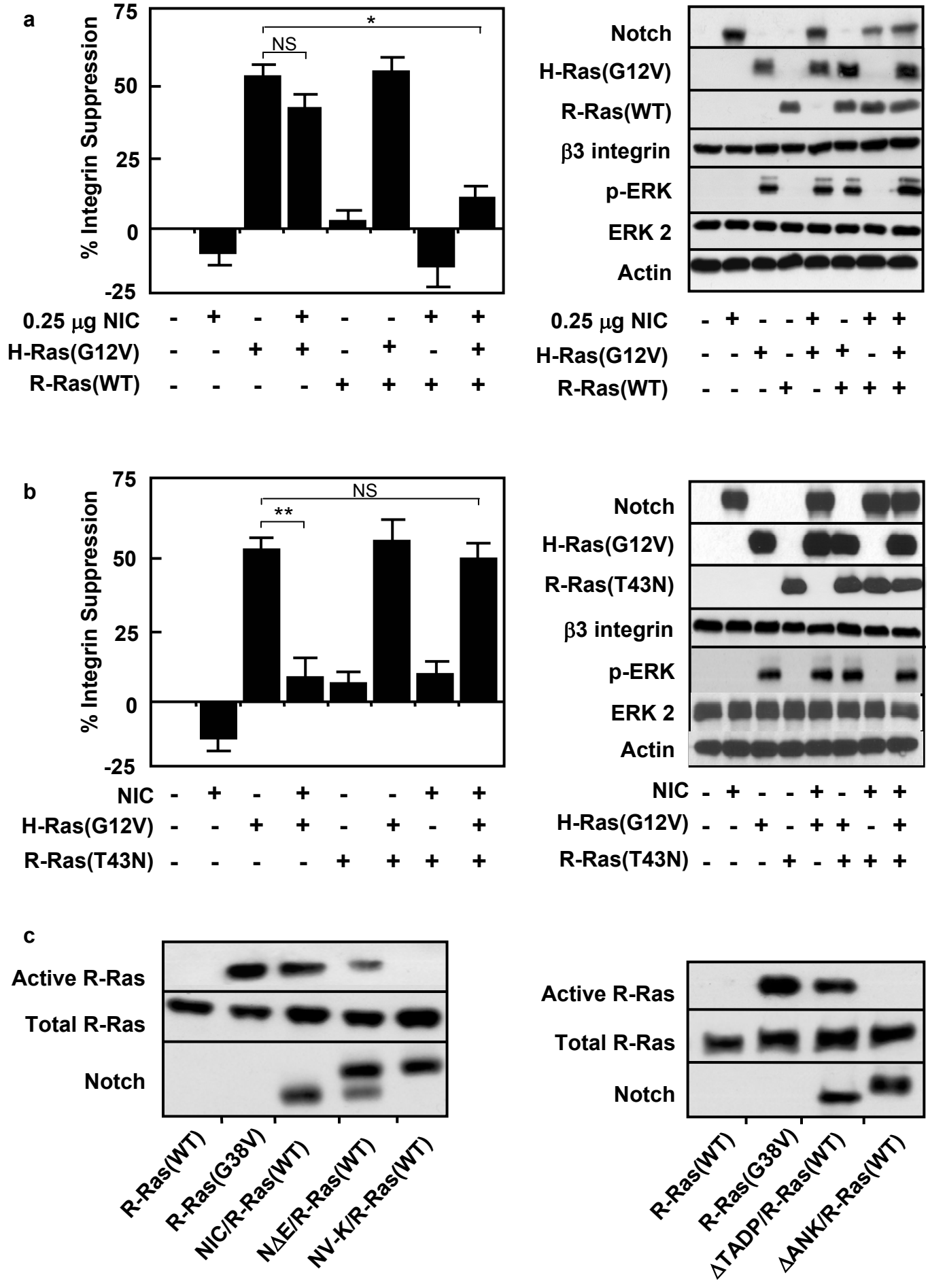
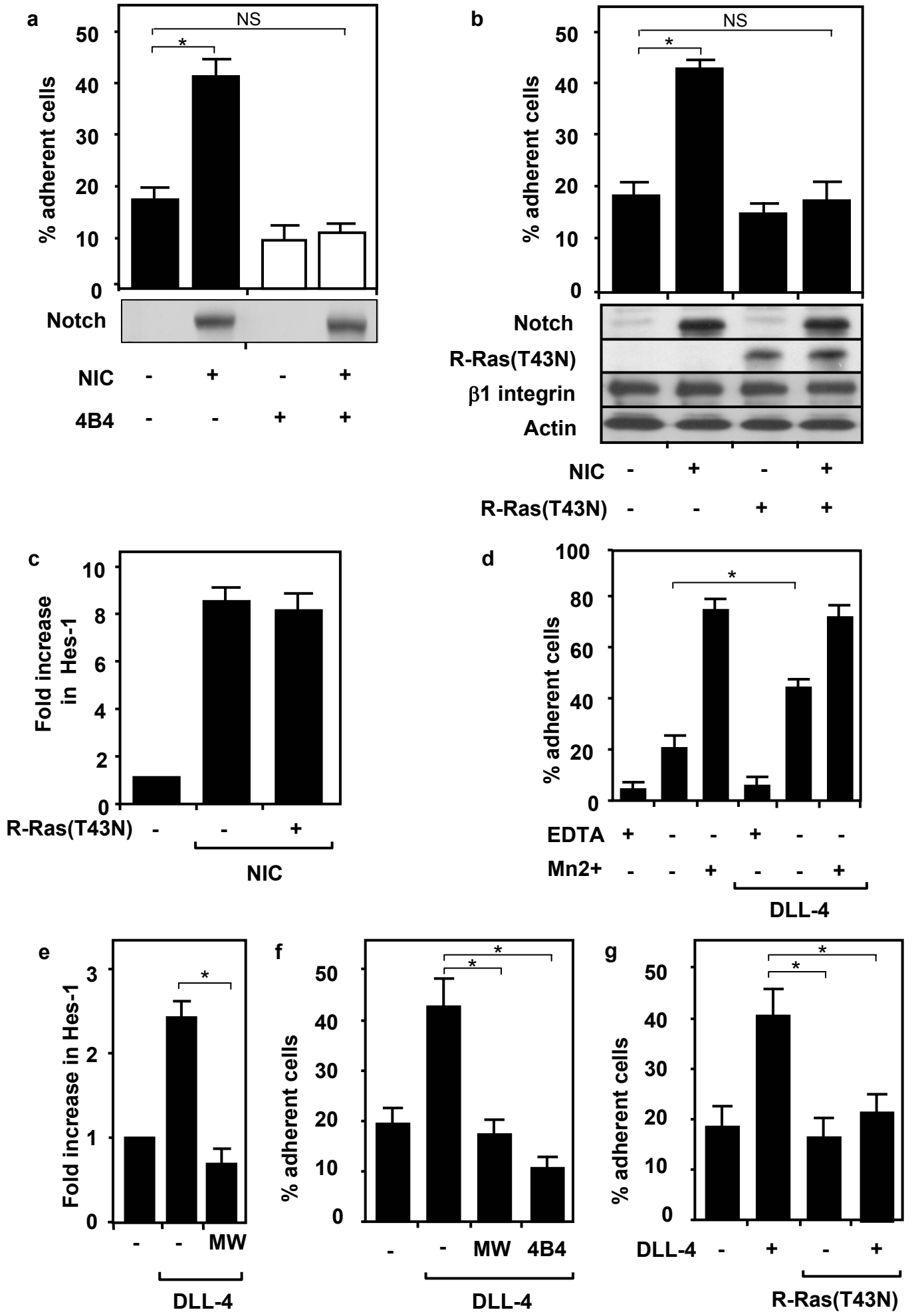

a
% Integrin Suppression
75
50
25
0
-25
NS
*
0.25 μg NIC - + - + - - + +
H-Ras(G12V) - - + + - + - +
R-Ras(WT) - - - - + + + +
Notch
H-Ras(G12V)
R-Ras(WT)
β3 integrin
p-ERK
ERK 2
Actin
0.25 μg NIC - + - - + - + +
H-Ras(G12V) - - + - + + - +
R-Ras(WT) - - - + - + + +
b
% Integrin Suppression
75
50
25
0
-25
**
NS
NIC - + - + - - + +
H-Ras(G12V) - - + + - + - +
R-Ras(T43N) - - - - + + + +
Notch
H-Ras(G12V)
R-Ras(T43N)
β3 integrin
p-ERK
ERK 2
Actin
NIC - + - - + - + +
H-Ras(G12V) - - + - + + - +
R-Ras(T43N) - - - + - + + +
c
Active R-Ras
Total R-Ras
Notch
R-Ras(WT)
R-Ras(G38V)
NIC/R-Ras(WT)
NΔE/R-Ras(WT)
NV-K/R-Ras(WT)
Active R-Ras
Total R-Ras
Notch
R-Ras(WT)
R-Ras(G38V)
ΔTADP/R-Ras(WT)
ΔANK/R-Ras(WT)

FIGURE 8


a
NS
*
% adherent cells
50
40
30
20
10
0
Notch
NIC - + - +
4B4 - - + +
b
NS
*
% adherent cells
50
40
30
20
10
0
Notch
R-Ras(T43N)
β1 integrin
Actin
NIC - + - +
R-Ras(T43N) - - + +
c
Fold increase in Hes-1
10
8
6
4
2
0
R-Ras(T43N) - - +
NIC
d
100
80
60
40
20
0
*
% adherent cells
EDTA + - - + - -
Mn2+ - - + - - +
DLL-4
e
Fold increase in Hes-1
3
2
1
0
*
- - MW
DLL-4
f
*
*
% adherent cells
50
40
30
20
10
0
- - MW 4B4
DLL-4
g
*
*
% adherent cells
50
40
30
20
10
0
DLL-4 - + - +
R-Ras(T43N)

Cell Death and Differentiation (2006) 13, 1776–1788

www.nature.com/cdd

ECM overrides DNA damage-induced cell cycle arrest and apoptosis in small-cell lung cancer cells through β1 integrin-dependent activation of PI3-kinase

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Edited by M Oren

Abstract

The emergence of resistance to chemotherapy remains a principle problem in the treatment of small-cell lung cancer (SCLC). We demonstrate that extracellular matrix (ECM) activates phosphatidyl inositol 3-kinase (PI3-kinase) signaling in SCLC cells and prevents etoposide-induced caspase-3 activation and subsequent apoptosis in a β1 integrin/PI3-kinase-dependent manner. Crucially we show that etoposide and radiation induce G2/M cell cycle arrest in SCLC cells prior to apoptosis and that ECM prevents this by overriding the upregulation of $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ and the down-regulation of cyclins E, A and B. These effects are abrogated by pharmacological and genetic inhibition of PI3-kinase signaling. Importantly we show that chemoprotection is not mediated by altered SCLC cell proliferation or DNA repair. Thus, ECM via β1 integrin-mediated PI3-kinase activation overrides treatment-induced cell cycle arrest and apoptosis, allowing SCLC cells to survive with persistent DNA damage, providing a model to account for the emergence of acquired drug resistance.

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Keywords: cancer; extracellular matrix; cell cycle; apoptosis; chemoresistance

Abbreviations: CDK, cyclin-dependent kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; ECM, extracellular matrix; FCS, fetal calf serum; GSK-3, glycogen synthase kinase-3; Gy, Gray; MDR, multi-drug resistance; Na_3VO_4, sodium orthovanadate; PI3-kinase, phosphatidyl inositol 3-kinase; PKB, protein kinase B; Rb, retinoblastoma protein; SCLC, small-cell lung cancer; Topo II, topoisomerase II; XRT, radiation; z-VAD, z-valine–alanine–aspartate

Introduction

Lung cancer is the most common fatal malignancy in the developed world.[1] Small-cell lung cancer (SCLC), which constitutes 25% of all lung tumors, has a very poor prognosis (2-year survival less than 5%). Etoposide-based chemotherapeutic regimens and radiotherapy are the main stay of treatment.[2] Although impressive initial responses are seen, in the majority of patients, recurrent disease refractory to further treatment results in tumor relapse and ultimately death. This phenomenon, known as multi-drug resistance (MDR), is the major cause of cancer treatment failure.[2] New therapeutic strategies are urgently needed, which will require an in-depth understanding of the mechanisms of MDR in SCLC.

We have previously shown that SCLC is surrounded by an extensive stroma of extracellular matrix (ECM) at both primary and metastatic sites and that high levels of expression correlate with poor patient prognosis.[3] Cells interact with ECM components via heterodimeric cell surface proteins called integrins.[4] ECM–integrin interaction stimulates signal transduction pathways that regulate a number of cellular processes important in cancer growth, including cell cycle transition and protection from apoptosis.[4] We have shown that adhesion of SCLC cells via β1 integrins to ECM components promotes cell survival and confers resistance to chemotherapeutic agents.[3] This has now been demonstrated to be a general mechanism for acquired drug resistance in a number of malignancies including myeloma, breast and colon cancer.[3,5–7] However, the intracellular signals activated by ECM components in cancer cells, which influence response to chemotherapy and radiotherapy, have not been fully elucidated. A clear understanding of these mechanisms is essential to the development of rational therapeutic strategies to overcome acquired MDR and enhance the effects of chemotherapy and radiotherapy.

Central to the cellular response to chemotherapy and radiation-induced DNA damage is cell cycle arrest, predominantly at the G1/S and G2/M checkpoints.[8] Alterations in expression of genes that control progression through the cell cycle have been demonstrated to affect chemosensitivity. The best described in cancer cells are alterations in expression of p53 and the retinoblastoma protein (Rb). Mutated forms of p53 and/or Rb have been shown to be associated with increased resistance of tumor cells to various anticancer drugs and irradiation, mainly because of disruption of cell cycle checkpoints.[9] Alterations in other cell cycle regulators such as the cyclins, cyclin-dependent kinases (CDKs) and their inhibitors, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$, may also play an important role in the regulation of drug sensitivity.[10]

Anchorage to ECM is required for proliferation of all untransformed cells and the development of anchorage independence is an important stage in transformation.[11] Regulation of the cell cycle by mitogens and cell adhesion in

untransformed cells is restricted to the G1/S transition.[12,13] Progression through the G1 phase is mediated by CDK 2, 4 and 6, whose activities are controlled by their associated cyclins and CDK inhibitors, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$.[14] Accumulation of cyclin D, which associates with and activates CDK 4 and 6, initiates progression through G1.[15] The cyclin D–CDK 4 and 6 complex also prevents transcriptional repression of cyclin E and the de-repression of cyclin A, promoting the onset of S phase.[16] Cells in suspension have higher levels of CDK 2 inhibitors than do attached cells, leading to impaired cyclin E–CDK 2 activity with resultant cell cycle arrest in late G1.[14,17] These results suggest that the regulation of cyclin E–CDK 2 activity is an important mechanism by which anchorage to ECM controls progression through the G1/S transition in untransformed cells. Regulation of cyclin E–CDK 2 activity may also represent how ECM protects cancer cells from chemotherapy-induced cell cycle arrest and subsequent apoptosis.

The phosphatidyl inositol 3-kinase (PI3-kinase) signaling pathway has been demonstrated to be an essential survival signal in SCLC cells, and is a key regulator of cell cycle progression.[18,19] Integrin activation by ECM couples integrin cytoplasmic tails to focal adhesion kinase and activates PI3-kinase, which phosphorylates inositol lipids, leading to activation of protein kinase B (PKB) and phosphorylation of glycogen synthase kinase 3β (GSK3β).[19,20] PKB phosphorylates and inactivates proapoptotic factors such as caspase 9 and Bad, preventing apoptosis.[19] Phosphorylation of GSK3β stabilizes cyclin D1, promoting progression through G1 phase of the cell cycle, and prevents apoptosis by altering gene expression through NF-κB and AP-1.[21–23] Therefore, prevention of cell cycle arrest and subsequent apoptosis through integrin activation of PI3-kinase may represent an important mechanism by which ECM proteins protect SCLC cells from chemotherapy and radiation.

In this paper, we demonstrate that ECM proteins can override etoposide-induced G2/M cell cycle arrest and apoptosis in SCLC cells through β1 integrin activation of tyrosine kinase/PI3-kinase signaling. Furthermore, we show that ECM-mediated protection from etoposide occurs despite topoisomerase II (Topo II) inhibition and DNA double-strand break formation allowing the survival of DNA damaged cells. Thus, integrin activation by ECM proteins may provide a central mechanism for the emergence of MDR in cancer.

Results

ECM proteins protect SCLC cells from chemotherapy- and radiation-induced apoptosis via β1 integrin activation

We have previously shown that SCLC cesIls are surrounded by the ECM proteins laminin and fibronectin *in vivo*, and that the degree of expression of ECM adversely affects patient prognosis.[3] To elucidate the mechanism by which ECM promotes SCLC cell survival, we investigated the effect of specific ECM proteins *in vitro* on apoptosis induced in SCLC cells by etoposide and ionizing radiation. H345 SCLC cells were adhered to poly-L-lysine, fibronectin or laminin and treated with etoposide (25 μg/ml). The percentage of apoptotic cells was assessed by acridine orange/ethidium bromide staining at time points over a period of 72 h. Apoptosis induced by etoposide was first evident at 24 h in H345 SCLC cells adhered to poly-L-lysine, reaching a maximum at 48–72 h (Figure 1a). No significant difference was observed whether cells were plated onto tissue culture plastic or adhered to poly-L-lysine (data not shown). However, adhesion to both laminin and fibronectin significantly protected H345 SCLC cells from apoptosis at all time points (Figure 1a). This effect was not due to a physical interaction between etoposide and ECM, as medium that had been treated with etoposide and incubated with laminin-coated plastic for 72 h was still able to induce apoptosis when added to SCLC cells adhered to poly-L-lysine (Figure 1b). Furthermore, adhesion of H345 SCLC cells to ECM proteins protected SCLC cells against the proapoptotic effect of ionizing radiation (Figure 1c and d), further confirming that ECM protection is not caused by a physical effect on chemotherapeutic agents. Similar results were seen with H69 and H510 cells (data not shown). These results were confirmed using two other methods for assessing apoptosis: a cell death detection ELISA kit which is based on the quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes (Figure 1e) and annexin V expression (data not shown). To determine whether this survival benefit was due to an increase in the growth rate of cells on ECM, we compared the growth of H345 SCLC cells adhered to laminin or poly-L-lysine (Figure 1f). ECM proteins stimulated a small increase in cellular growth over 9 days. However, there was no significant difference in cell number between H345 cells grown on ECM or on poly-L-lysine at the early time points corresponding to when apoptosis was being assessed. These results indicate that the ECM-mediated protection of SCLC cells from chemotherapy is due to an antiapoptotic effect and not increased growth.

We have previously characterized integrin expression on SCLC cells and the β1 integrin subunit is highly expressed.[3] To investigate whether the antiapoptotic effect of ECM proteins was mediated by the activation of β1 integrins on SCLC cells, we used specific β1 integrin function blocking and stimulating antibodies to manipulate ECM chemoprotection. H345 SCLC cells were adhered to poly-L-lysine or laminin, treated with etoposide (25 μg/ml), and apoptosis was assessed at 48 h by acridine orange/ethidium bromide staining (Figure 1g) and annexin V (Figure 1h). The basal level of apoptosis in H345 SCLC cells on poly-L-lysine was 8 ± 2%, and this was increased to 55 ± 4% in the presence of etoposide ($n = 3$ independent experiments in quadruplicate $\pm$ S.E.M.). Adhesion of H345 SCLC cells to the ECM proteins laminin or fibronectin significantly reduced apoptosis caused by etoposide at 48 h from 55 ± 4 to 7 ± 1.7 and 8 ± 1.4%, respectively ($n = 3$ independent experiments in quadruplicate $\pm$ S.E.M.). ECM-mediated protection from etoposide-induced apoptosis was completely abolished by preincubation of H345 SCLC cells with the β1 integrin function-blocking antibody (4B4 10 μg/ml). Furthermore, the β1-stimulating antibody TS2/16 (10 μg/ml) and the broad-spectrum phosphatase inhibitor sodium orthovanadate (Na_3VO_4) both reproduced the effects of ECM proteins. Taken together, these results indicate that ECM proteins

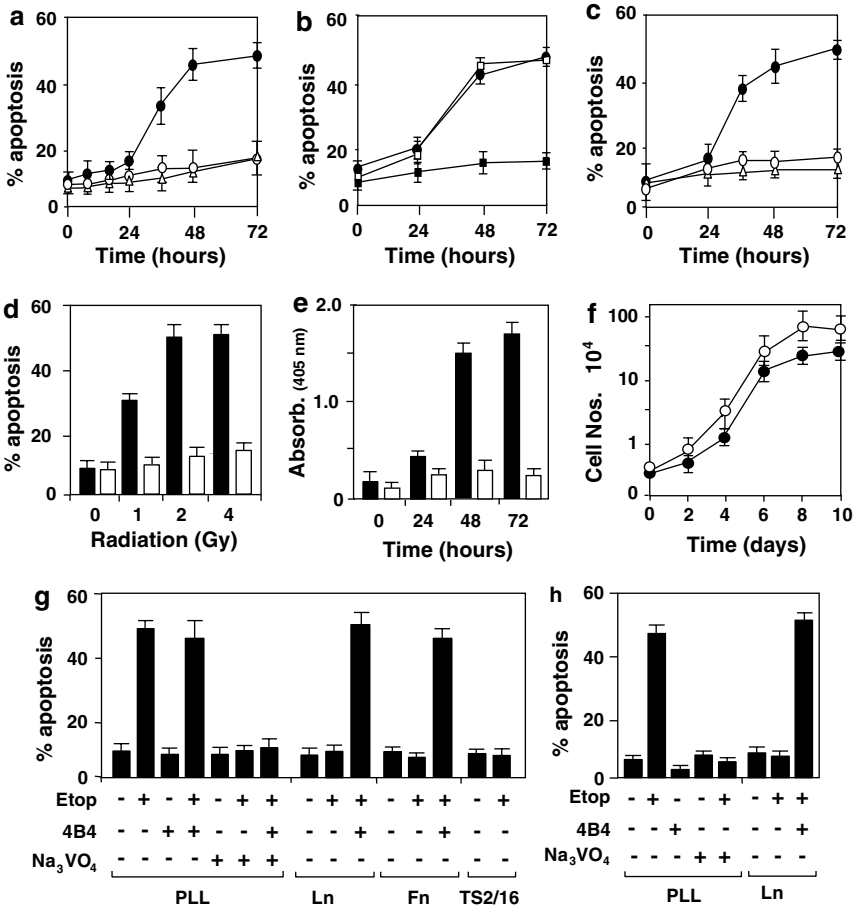

Figure 1 ECM-mediated protection from etoposide- and radiation-induced apoptosis in SCLC cells is β1 integrin-dependent. **(a)** H345 SCLC cells were seeded into 96-well plates precoated with poly-L-lysine (10 μg/ml) (●), laminin (10 μg/ml) (○) or fibronectin (20 μg/ml) (△), and treated with etoposide (25 μg/ml). The percentage of apoptotic cells was determined at the times indicated by staining cells with acridine orange/ethidium bromide and subsequent fluorescent microscopy (mean of $n=3$ independent experiments in quadruplicate $\pm$ S.E.M.). **(b)** Quiesced H345 SCLC cells were seeded into 96-well plates precoated with poly-L-lysine (10 μg/ml) and treated with etoposide (25 μg/ml) (●) or seeded on poly-L-lysine in a medium that had been conditioned by incubation for 72 h in laminin-coated (10 μg/ml) six-well plates with (□) or without (■) etoposide (25 μg/ml). The percentage of apoptotic cells was determined as above (mean of $n=3$ independent experiments in quadruplicate $\pm$ S.E.M.). **(c)** Quiesced H345 SCLC cells were seeded into 96-well plates precoated with poly-L-lysine (10 μg/ml) (●), laminin (10 μg/ml) (○) or fibronectin (20 μg/ml) (△), and treated with ionizing radiation (2 Gy). The percentage of apoptotic cells was determined as above (mean of $n=3$ independent experiments in quadruplicate $\pm$ S.E.M.). **(d)** Quiesced H345 cells were adhered to poly-L-lysine (10 μg/ml) (black bar) or laminin (10 μg/ml) (white bar), treated with ionizing radiation (0–4 Gy) as indicated, and the percentage of apoptotic cells was determined at 48 h (mean of $n=3$ independent experiments in quadruplicate $\pm$ S.E.M.). **(e)** H345 cells were adhered to poly-L-lysine (black bar) or laminin (white bar) and treated with etoposide (25 μg/ml). Apoptosis was assessed at the time indicated using a cell death ELISA kit. The graph represents the mean absorbance at 405 nm from $n=3$ experiments $\pm$ S.E.M. Increase in absorbance indicates increased apoptosis. **(f)** H345 SCLC cells were plated into 24-well plates coated with poly-L-lysine (●) or laminin (○) and cell numbers were counted at times indicated (mean $n=4$ independent experiments $\pm$ S.E.M.). (**g**) Quiesced H345 cells were incubated with (+) or without (−) the function blocking anti-β1 integrin antibody 4B4 (10 μg/ml) for 30 min at 37°C as indicated, and then seeded into 96-well plates precoated with poly-L-lysine (PLL) (10 μg/ml), laminin (Ln) (10 μg/ml), fibronectin (Fn) (20 μg/ml) or the integrin-activating antibody TS2/16 (10 μg/ml). Cells were treated with (+) or without (−) etoposide (Etop) (25 μg/ml) or Na_3VO_4 (200 μm) as indicated. The percentage of apoptotic cells was determined at 48 h as above (mean of $n=3$ independent experiments in quadruplicate $\pm$ S.E.M.). **(h)** H345 cells were treated as above and the percentage of apoptotic cells was assessed by surface expression of annexin V using flow cytometry (mean of $n=3$ experiments $\pm$ S.E.M.)

prevent apoptosis in SCLC cells through activation of β1 integrins and tyrosine kinase signaling pathways.

Laminin and Na_3VO_4 stimulate PI3-kinase activity, PKB phosphorylation and activity, and increase glycogen synthase kinase-3 (GSK-3)β phosphorylation

Integrin-dependent cell adhesion triggers an increase in tyrosine phosphorylation and PI3-kinase activation.[19] PI3-kinase has also been shown to mediate matrix-induced survival in normal epithelial cells.[24] Therefore, we examined if ECM proteins activated PI3-kinase signaling pathways in SCLC cells. Adhesion of H345 cells to ECM proteins laminin and fibronectin stimulated a marked increase in PI3-kinase activation (average 3.1 × and 2.9 × increases in PI3-kinase activity, respectively, $n=3$ independent experiments in quadruplicate) (Figure 2a). Furthermore, the β1 integrin-stimulating antibody TS2/16 (10 μg/ml) and Na_3VO_4 (200 μM) increased PI3-kinase activity by a similar amount to ECM (average 2.5 × and 2.8 × increases, respectively, $n=3$

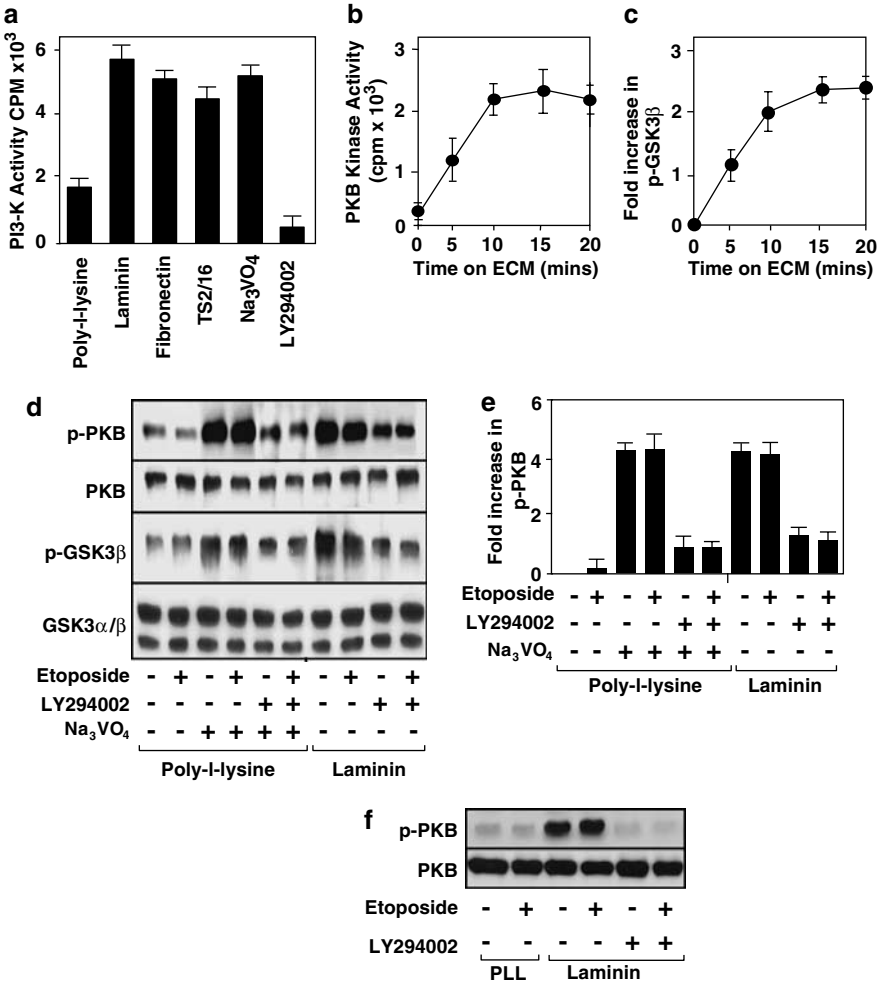

Figure 2 ECM activates PI3-kinase signaling in SCLC cells. **(a)** H345 cells were adhered to 6-well plates precoated with poly-L-lysine (10 μg/ml), laminin (10 μg/ml), fibronectin (20 μg/ml) or TS2/16 (10 μg/ml). In addition, cells on poly-L-lysine were treated with Na_3VO_4 (200 μm) or LY294002 (30 μM) as indicated. PI3-kinase activity was assayed after 10 min as described in Materials and Methods. **(b)** Quiesced H345 cells were adhered to laminin for the times indicated and PKB activity was determined as described in Materials and Methods. **(c)** Quiesced H345 cells were adhered to laminin for the times indicated and expression of phosphorylated GSK3β was determined by Western blot analysis. Relative protein expression was quantified using Image J software (mean of $n=3$ experiments $\pm$ S.E.M.). **(d)** H345 cells were adhered to six-well plates precoated with poly-L-lysine (10 μg/ml) or laminin (10 μg/ml) and treated with (+) or without (−) etoposide (25 μg/ml), Na_3VO_4 (200 μM) or LY294002 (30 μM) as indicated. Cells were lysed after 10 min, and expression of phosphorylated protein kinase B (p-PKB), total PKB, phosphorylated glycogen synthase kinase 3β (p-GSK3β) and glycogen synthase kinase 3α/β (GSK 3α/β) was determined by Western blot analysis. Representative blots from $n=3$ experiments. **(e)** Relative expression of phospho-PKB from cells treated as above. Graph represents the mean fold increase in phospho-PKB expression normalized to untreated controls ($n=3$ experiments $\pm$ S.E.M.). **(f)** H345 cells were quiesced, plated onto laminin or poly-L-lysine, and treated as indicated. Cells were lysed after 48 h and phospho-PKB (p-PKB) and total PKB expression was determined by Western blot. Representative blot from $n=3$ experiments

independent experiments in quadruplicate). As predicted, LY294002 reduced basal levels of PI3-kinase activity. PKB and GSK-3β, which are downstream effectors of PI3-kinase and regulate cell survival, may also participate in overriding chemotherapy- and radiation-induced apoptosis in SCLC cells. Adhesion of H345 SCLC cells to laminin caused a time-dependent increase in PKB activity, noted first at 5 min with maximum activity at 10 min (Figure 2b). An increase in GSK3β phosphorylation was also noted at 5 min, rising to a plateau at 15 min (Figure 2c). Similar results were seen with fibronectin and TS2/16 (results not shown). Furthermore, Na_3VO_4 (200 μM) stimulated PKB and GSK3β phosphorylation in H345 cells adhered to poly-L-lysine (Figure 2d and e). The ECM-mediated increase in phosphorylation of PKB and GSK3β was sensitive to PI3-kinase inhibition with LY294002 (10 μM) and was not affected by etoposide treatment (25 μg/ml) (Figure 2d and e). In addition, the inhibition of PKB phosphorylation by LY294002 was prolonged in H345 cells, being observed up to 48 h after treatment (Figure 2f). Similar effects were seen in H69 SCLC cells (data not shown).

ECM protection against etoposide-induced apoptosis is dependent on tyrosine kinase and phosphotidyl inositol 3-kinase activation

To specifically investigate the role of PI3-kinase signaling in ECM-mediated chemoprotection, we initially used a pharmacological inhibitor of PI3-kinase activity, LY294002. Analysis of annexin V staining at 48 h in H345 SCLC cells adhered to

poly-L-lysine revealed that inhibition of PI3-kinase with LY294002 produced a small increase in the degree of apoptosis both with and without etoposide (Figure 3a). Furthermore, the protection of H345 SCLC cells against etoposide-induced apoptosis mediated by ECM was completely reversed by the addition of LY294002 (Figure 3a). In addition, chemoprotection resulting from specific β1 integrin stimulation with TS2/16 or stimulation of the tyrosine kinase pathway with Na_3VO_4 was abrogated by LY294002. Similar results were seen in H69 and H510 SCLC cells. As expected, z-valine–alanine–aspartate (z-VAD), which inhibits caspase activity, prevented apoptosis induced by etoposide (Figure 3a).

PI3-kinase activates downstream PKB, which phosphorylates proapoptotic factors such as caspase-9 and Bad, preventing cell death.[21–23] Thus, we investigated whether ECM could alter Bad phosphorylation and etoposide-induced caspase-9 activation in a PI3-kinase-dependent manner. We found that adhesion of H345 SCLC cells to ECM stimulated phosphorylation of Bad and prevented cleavage of caspase-9 induced by etoposide after 24 h (Figure 3b). These effects were prevented by treatment with LY294002. Activation of caspase-9 initiates cleavage of the 'effector' caspase-3, which ultimately results in apoptosis. We found that etoposide could induce caspase-3 cleavage in H345 cells adhered to poly-L-lysine, but not in cells adhered to laminin. However, treatment with LY294002 prevented laminin from blocking caspase-3 cleavage without affecting caspase-3 activation in cells adhered to poly-L-lysine (Figure 3b). In parallel experiments, caspase-3 activity was measured (Figure 3c). Etoposide induced an increase in caspase-3 activity in H345 SCLC cells, which, as expected, was blocked by z-VAD. Adhesion to laminin and stimulation of the tyrosine kinase pathway with Na_3VO_4 overcame etoposide-induced caspase-3 activation, preventing cells undergoing apoptosis. Furthermore, the ECM- and Na_3VO_4-mediated inhibition of etoposide-induced caspase-3 activation was prevented by the tyrosine kinase inhibitor tyrophostin-25 and the PI3-kinase inhibitor LY294002 (Figure 3c). These results indicate that PI3-kinase signaling, through phosphorylation of Bad and modulation of caspase activity, is critical for ECM-mediated resistance to etoposide-induced apoptosis in SCLC cells.

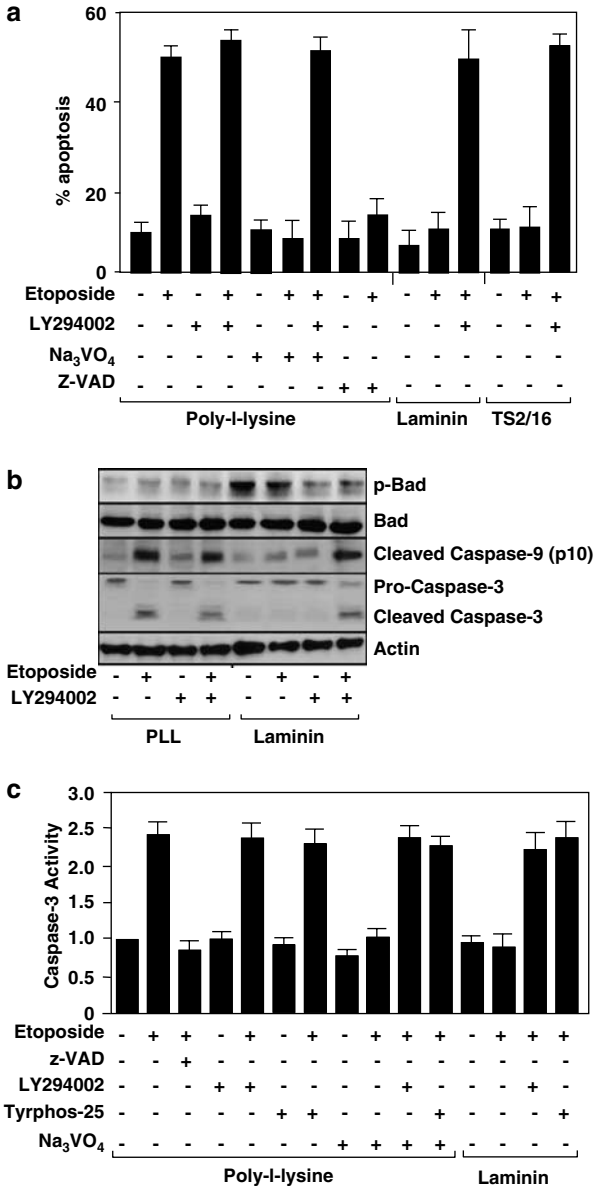

Figure 3 ECM-mediated protection from caspase-3 activation and apoptosis requires PI3-kinase signaling. **(a)** Quiesced H345 cells were seeded into 96-well plates precoated with poly-L-lysine (10 μg/ml), laminin (10 μg/ml) or TS2/16 (10 μg/ml), allowed to adhere for 1 h and then treated with (+) or without (−) etoposide (25 μg/ml), Na_3VO_4 (200 μM) or LY294002 (30 μM) as indicated. The percentage of apoptotic cells was assessed at 48 h by flow-cytometric analysis of surface annexin V staining (mean of $n=3$ independent experiments $\pm$ S.E.M.). **(b)** Quiesced H345 cells were seeded into six-well plates precoated with poly-L-lysine (10 μg/ml) or laminin (10 μg/ml) and treated with (+) or without (−) etoposide (25 μg/ml) or LY294002 (30 μM) as indicated. After 24 h the cells were lysed and the expression of phospho-Bad (p-Bad), Bad, cleaved caspase-9 (p10) and caspase-3 was determined by Western blot. Representative blots from $n=3$ experiments are shown. Actin is shown as a loading control. **(c)** Quiesced H345 cells were seeded into six-well plates precoated with poly-L-lysine (10 μg/ml) or laminin (10 μg/ml) and treated with (+) or without (−) etoposide (25 μg/ml), z-VAD (100 μM), LY294002 (30 μM), tyrphostin-25 (tyrpho-25) (25 μM) or Na_3VO_4 (200 μM) as indicated. Caspase-3 activity was measured after 48 h as described in Materials and Methods, and normalized to untreated cells on poly-L-lysine (mean of $n=3$ experiments $\pm$ S.E.M.)

ECM proteins override etoposide- and radiation-induced G2/M cell cycle arrest via PI3-kinase modulation of cell cycle regulators

DNA damage is central to the cellular response to chemotherapy and radiation and results in cell cycle arrest predominantly at the G1/S and G2/M checkpoints.[8,14] Subsequent, activation of checkpoint signaling cascades can result in apoptotic cell death preventing accumulation of genetic damage. Modulation of cell cycle checkpoints may therefore play an important role in sensitivity to radiation and chemotherapy. We investigated whether ECM proteins could override cell cycle arrest induced in SCLC cells by etoposide and radiation. H345 SCLC cells were adhered to poly-L-lysine or laminin, treated with etoposide (25 μg/ml) or ionizing radiation (2 Gray (Gy)), and nuclear DNA content was assessed after 24 h by

flow-cytometric analysis of propidium iodide staining. H345 SCLC cells showed similar cell cycle profiles whether grown in suspension or adhered to poly-L-lysine or laminin (Figure 4a). At 24 h after treatment with etoposide (25 μg/ml) or ionizing radiation (2 Gy), H345 SCLC cells adhered to poly-L-lysine showed a significant increase in the percentage of cells in the S and G2/M phases of the cell cycle, with a reciprocal reduction in the percentage of cells in G1 (Figure 4a and Table 1). In contrast, H345 cells adhered to laminin and treated with etoposide or ionizing radiation showed only a small increase in the percentage of cells in S and G2/M phase, with a minimal reciprocal reduction in the percentage of cells in G1. Similar results were observed with fibronectin (data not shown).

To investigate whether ECM proteins protect against cell cycle arrest via activation of β1 integrin/PI3-kinase signalling, we treated H345 SCLC cells adhered to poly-L-lysine or laminin with etoposide for 24 h in the presence of the β1 integrin function-blocking antibody 4B4, the β1 integrin-

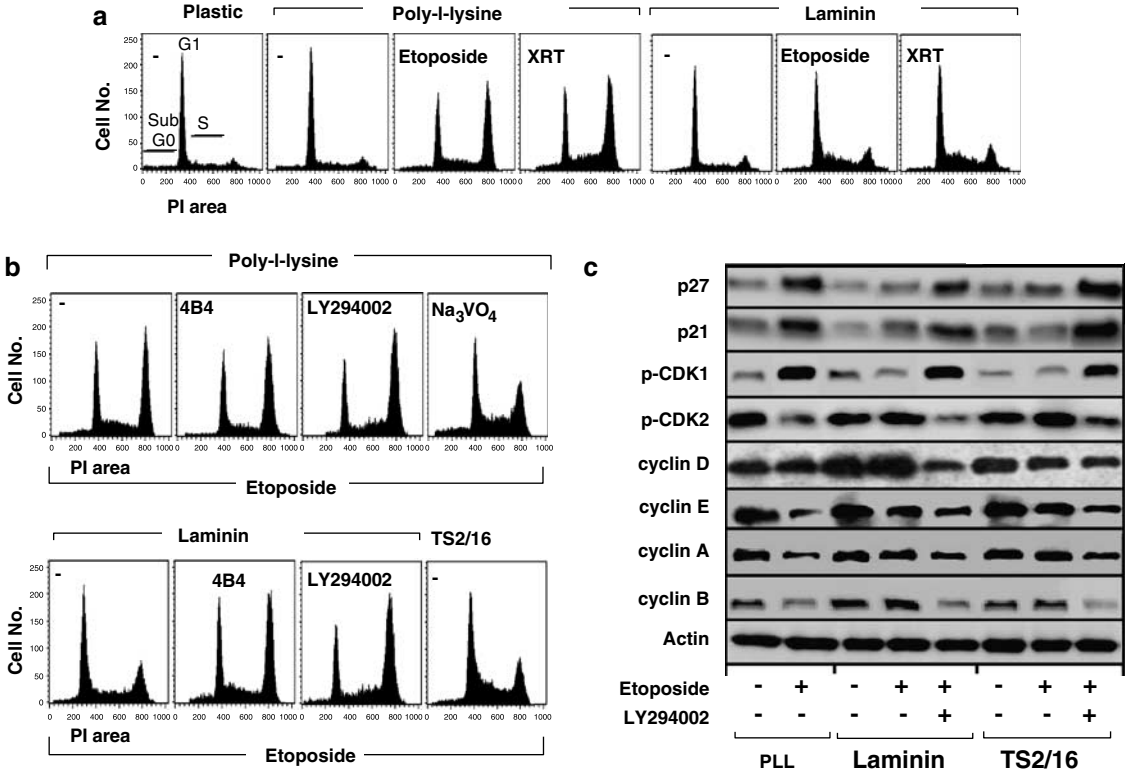

Figure 4 ECM overrides etoposide- and radiation-induced cell cycle arrest via β1 integrin-mediated PI3-kinase activation. **(a)** H345 cells were quiesced overnight, washed twice and seeded into a six-well tissue culture plate, uncoated (plastic) or precoated with poly-L-lysine (10 μg/ml) or laminin (10 μg/ml). Cells were treated as indicated: no further treatment (−), etoposide (25 μg/ml) or ionizing radiation (2 Gy) (XRT). Progression through the cell cycle was assessed 24 h later by flow-cytometric analysis of DNA content using CELL Quest software. Representative histograms are shown from $n=5$ independent experiments. **(b)** Quiesced H345 SCLC cells were seeded into six-well tissue culture plates precoated with poly-L-lysine (10 μg/ml) or laminin (10 μg/ml) and treated with etoposide (25 μg/ml). In addition, cells were treated as indicated: blockade of β1 integrin function with 4B4 (10 μg/ml), stimulation of β1 integrins with TS2/16 (10 μg/ml), inhibition of PI3-kinase with LY294002 (30 μM) or activation of tyrosine kinase with Na_3VO_4 (200 μM). Progression through the cell cycle was assessed 24 h later by flow-cytometric analysis of DNA content using CELL Quest software. Representative histograms are shown from $n=5$ independent experiments. **(c)** H345 SCLC cells were seeded into six-well plates precoated with poly-L-lysine (10 μg/ml) (PLL), laminin (10 μg/ml) or TS2/16 (10 μg/ml). Cells were treated with (+) or without (−) etoposide (25 μg/ml), LY294002 (30 μM) or Na_3VO_4 (200 μM) as indicated. Cells were lysed after 24 h and the expressions of $p21^{Cip1/WAF1}$, $p27^{Kip1}$, phosphorylated CDK1 (p-CDK1), phosphorylated CDK2 (p-CDK2) and cyclins D, E, A and B were determined by Western blot analysis. Representative blot from $n=3$ experiments. Actin is shown as a loading control

Table 1 ECM protects SCLC cells from etoposide- and radiation-induced cell cycle arrest

	Plastic	Poly-L-lysine			Laminin		
Cell cycle phase	—	—	Etoposide	XRT	—	Etoposide	XRT
Sub-G0	5.6±1.6	6.1±1.3	4.9±2.4	5.4±1.4	6.3±1.7	5.1±1.1	3.5±1.7
G1	64.8±2.5	65.3±2.8	23.2±2.3	22.1±1.8	67.1±3.1	53.2±1.9	60.1±2.6
S	17.3±2.0	18.2±1.7	25.4±1.6	24.5±2.1	16.7±2.3	22.2±1.7	20.1±1.8
G2/M	8.8±1.0	9.1±0.6	46.2±1.5	47.8±2.1	8.4±2.1	16.8±2.0	16.1±1.9

H345 cells were quiesced overnight, washed twice and seeded into a six-well tissue culture plate, uncoated (plastic) or precoated with poly-L-lysine or laminin. Cells were treated as indicated: no further treatment (−), etoposide or ionizing radiation (XRT). Progression through the cell cycle was assessed 24 h later by flow cytometric analysis of DNA content using CELL Quest software. Mean percentage of total cells in each phase of the cell cycle is shown from $n=5$ experiments ±S.E.M.

Table 2 ECM overrides etoposide- and radiation-induced cell cycle arrest via β1 integrin-mediated PI3-kinase activation

	Poly-L-lysine			
Cell cycle phase	**—**	**4B4**	**LY294002**	**Na_3VO_4**
Sub-G0	3.6±1.7	4.9±1.1	4.3±0.9	4.5±0.7
G1	24.2±3.9	23.2±2.2	22.1±2.5	39.8±1.8
S	22.6±2.1	20.8±2.8	19.3±1.3	24.5±2.1
G2/M	48.2±3.1	46.9±2.8	52.5±3.0	28.6±2.0
	Laminin			**TS2/16**
Cell cycle phase	**—**	**4B4**	**LY294002**	**—**
Sub-G0	4.2±2.1	4.3±1.1	3.2±0.9	4.1±2.1
G1	52.3±3.1	22.8±2.0	20.9±1.7	44.2±1.8
S	20.2±1.9	19.7±3.0	16.1±2.4	28.7±2.2
G2/M	18.4±2.5	51.2±2.2	58.8±3.1	21.7±2.6

H345 SCLC cells were seeded into six-well tissue culture plates precoated with poly-L-lysine, laminin or TS2/16 and treated with etoposide. In addition, cells were treated as indicated: blockade of β1 integrins with 4B4, inhibition of PI3-kinase with LY294002 or activation of tyrosine kinase with sodium orthovanadate (Na_3VO_4). Progression through the cell cycle was assessed 24 h later by flow cytometric analysis of DNA content using CELL Quest software. Mean percentage total cells in each phase of the cell cycle is shown from $n=5$ independent experiments ±S.E.M.

stimulating antibody TS2/16, Na_3VO_4 or LY294002, and assessed progression through the cell cycle (Figure 4b and Table 2). Blockade of β1 integrins with 4B4 completely abrogated laminin-mediated protection from G2/M cell cycle arrest induced by etoposide in H345 SCLC cells without affecting cell cycle arrest in cells adhered to poly-L-lysine. Furthermore, laminin-mediated protection from etoposide-induced G2/M cell cycle arrest was blocked by inhibition of PI3-kinase with LY294002. In addition, stimulation of β1 integrins on H345 SCLC cells with TS2/16 or activation of tyrosine kinase with Na_3VO_4 reduced etoposide-induced G2/M cell cycle arrest, simulating the effects of ECM. These results indicate that protection of SCLC cells from etoposide-induced cell cycle arrest by ECM proteins is mediated via β1 integrin activation of PI3-kinase signaling.

DNA damage can activate $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ and inhibit Cdc25s (activator of CDK1) to cause G1/S and G2/M arrest.[8] PI3-kinase has been shown to induce expression of D-type cyclins and to increase stability of cyclin-D1 through PKB-dependent phosphorylation of GSK3β.[21] Therefore, we investigated whether ECM proteins could modulate cell cycle regulators through PI3-kinase and thus prevent G2/M cell cycle arrest. After 24 h of etoposide treatment, H345 SCLC cells showed an increase in expression of the CDK inhibitors $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ and phosphorylated CDK1 (inactive form) with decreased expression of phosphorylated CDK2 (active form) and cyclins E, A and B (Figure 4d). Adhesion of H345 SCLC cells to laminin increased cyclin D expression and blocked the etoposide-induced changes in $p21^{Cip1/WAF1}$, $p27^{Kip1}$, phosphorylated CDK1 and 2, and cyclins E, A and B. Similar results were seen with fibronectin (data not shown). Furthermore, the effects of ECM were simulated by TS2/16 antibodies, but with less pronounced effects due to the bivalent nature of antibody binding, which will produce less integrin clustering and three-dimensional changes than laminin. The ability of laminin or TS2/16 to overcome etoposide-induced changes in cell cycle regulators was blocked by the PI3-kinase inhibitor LY294002. These results indicate that ECM proteins via β1 integrins can override etoposide-induced G2/M cell arrest by modulating expression of cyclins, CDKs and CDK inhibitors in a PI3-kinase-dependent manner.

Expression of a dominant-negative PKB mutant prevents, and a constitutively active PKB mutant mimics, ECM-mediated protection from apoptosis and cell cycle arrest

Although LY294002 is accepted as a specific inhibitor of PI3-kinase/PKB signaling at the doses used, it can inhibit other pathways that may affect cell survival. Therefore, we sought to genetically interfere with PI3-kinase signaling in SCLC cells to confirm the effects of LY294002 on ECM-mediated chemoprotection. We have shown that binding of SCLC cells to ECM leads to activation of PI3-kinase, which activates PKB. Thus, to specifically examine downstream PKB signaling, we interfered with the activity of PKB through the expression of both constitutively active (PKB CA) and dominant-negative (PKB DN) PKB mutants. We found that transient expression of PKB DN in H345 SCLC cells markedly inhibited phosphorylation of downstream GSK3β, whereas expression of PKB CA increased phospho-GSK3β levels, in accordance with the expected activity of these mutants on PKB signaling (Figure 5a). Importantly, expression of PKB CA protected H345 cells adhered to poly-L-lysine from etoposide-induced apoptosis (Figure 5b). Expression of PKB DN produced a small increase in apoptosis both with and without etoposide treatment in cells adhered to poly-L-lysine. However, PKB DN markedly increased etoposide-induced apoptosis in cells adhered to laminin (Figure 5b). Similar results were obtained with H69 cells (data not shown).

To confirm the results obtained with LY294002 on ECM-mediated protection from G2/M arrest, we transiently expressed PKB DN and PKB CA in H345 cells adhered to poly-L-lysine or laminin and analyzed progression through the cell cycle. In cells adhered to poly-L-lysine, transient expression of PKB CA reversed etoposide-induced G2/M arrest (Figure 5c and Table 3). Transient expression of PKB DN in cells adhered to poly-L-lysine caused a modest increase in the G2/M population with and without etoposide treatment. Importantly, expression of PKB DN in cells adhered to laminin significantly reversed protection from etoposide-induced G2/M arrest and caused a small, but significant, increase in G2/M arrest without etoposide treatment (Figure 5c and Table 3). Taken together, the results observed with LY294002 and the PKB mutants suggest a central role for PI3-kinase/PKB signaling in ECM-mediated chemoprotection in SCLC cells.

Adhesion to ECM proteins does not modulate ongoing etoposide- and radiotherapy-induced DNA damage or repair

In order to confirm that the antiapoptotic and cell cycle effects of ECM were not due to a reduction in etoposide activity or altered repair of etoposide- and radiation-induced DNA

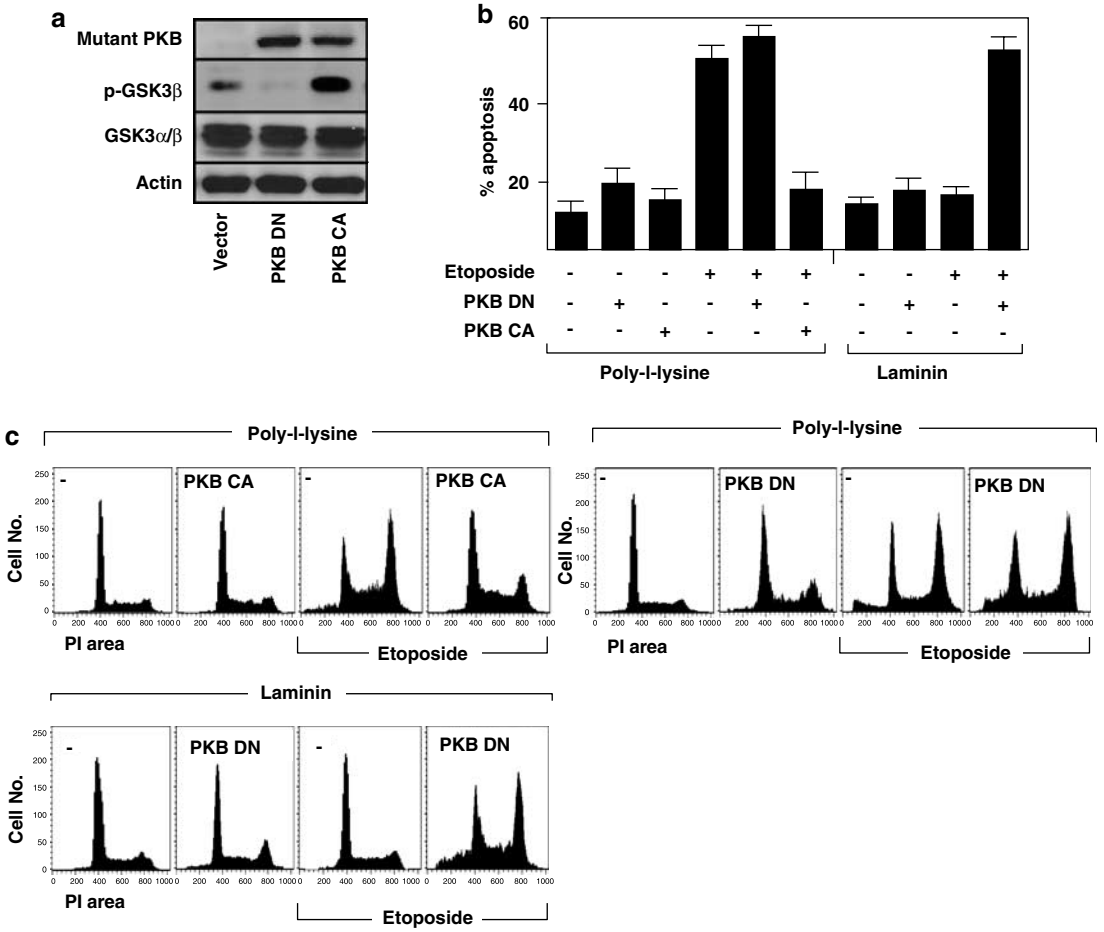

Figure 5 Expression of a dominant-negative PKB mutant prevents, and a constitutively active PKB mutant mimics, ECM-mediated protection from apoptosis and cell cycle arrest. **(a)** H345 SCLC cells were transfected with equal amounts of empty vector (pUSEamp), dominant-negative PKB (PKB DN) or constitutively active PKB (PKB CA). After 48 h, cells were lysed and expression of the myc-tagged PKB mutants, GSK3 α/β and phospho-GSK3α (p-GSK3α) was determined by Western blot. **(b)** H345 cells were transfected with equal amounts of PKB DN (+) PKB CA (+) or empty pUSEamp (−) as indicated. After 24 h cells were quiesced, resuspended in SITA, plated onto poly-L-lysine or laminin and treated with etoposide as indicated. After 48 h the percentage of apoptotic cells was determined by flow-cytometric analysis of annexin V. **(c)** H345 cells were transfected with equal amounts of dominant-negative PKB (PKB DN) (+), constitutively active PKB (PKB CA) (+) or pUSEamp (−). After 24 h the cells were quiesced, seeded into six-well plates precoated with poly-L-lysine or laminin and treated with etoposide as indicated. Progression through the cell cycle was assessed 24 h later by flow-cytometric analysis of DNA content using CELL Quest software. Representative histograms are shown from $n = 5$ independent experiments

damage, we assessed Topo II activity and DNA double-strand break formation and repair. Topo II resolves sister chromatid entanglement by binding to DNA, cleaving both strands and passing a second duplex through the first before reannealing the cleavage site.[25] Etoposide blocks the reannealing action of Topo II after the enzyme has created a double-strand break leading to activation of DNA damage signaling pathways and ultimately apoptosis.[25] We measured Topo II activity by the ATP-dependent de-catenation of high-molecular-weight DNA. While etoposide was able to inhibit Topo II activity, adhesion to the ECM protein laminin did not affect etoposide inhibition of Topo II activity or Topo II expression. The PI3-kinase inhibitor LY294002 had no effect on either Topo II expression or activity (Figure 6a).

To determine the amount of DNA damage induced by etoposide and radiation, H345 cells were seeded onto plastic precoated with poly-L-lysine or laminin and then treated as specified. The cells were washed, allowed to recover, and the amount of DNA double-strand breaks in cell nuclei was determined by immunofluorescence staining of phosphorylated H2AX (γH2AX). The foci of γH2AX represent actual DNA double-strand breaks and correlate closely with cell death in response to DNA damage (Figure 6b and c).[26] DNA double-strand breaks were also confirmed by Western blotting for γH2Ax and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Figure 6d). A very marked and equal increase in γH2Ax foci was seen 2 h after etoposide (30 min after radiotherapy – data not shown). Over the following 24-h period, there was a decrease in the number of double-strand breaks as DNA damage was repaired (Figure 6b–d). Crucially there was no difference in the amount of DNA damage or rate of repair in response to etoposide or radiation between cells plated on laminin or nonspecifically adhered to plastic by poly-L-lysine. Interestingly, while nearly all of the DNA damage was repaired, there were still a significant number of γH2Ax foci present at 24 h, suggesting persistence of DNA damage.

Table 3 Expression of dominant-negative PKB prevents, and constitutively active PKB mimics, ECM-mediated protection from G2/M cell cycle arrest

	Poly-L-lysine		Etoposide treatment	
Cell cycle phase	**—**	**PKB CA**	**—**	**PKB CA**
Sub-G0	4.3±0.7	4.1±0.8	8.3±1.3	3.6±0.9
G1	63.2±2.9	60.1±3.3	19.6±2.1	49.8±1.8
S	15.6±1.8	17.3±1.6	27.3±1.4	21.5±3.1
G2/M	12.2±1.1	14.2±1.1	53.5±3.0	22.6±2.3
	Poly-L-lysine		**Etoposide treatment**	
Cell cycle phase	**—**	**PKB DN**	**—**	**PKB DN**
Sub-G0	3.2±0.7	4.1±0.8	7.5±2.3	8.9±1.7
G1	61.3±3.9	51.6±3.3	24.6±1.9	27.6±1.9
S	17.3±2.1	20.4±1.9	26.8±2.4	26.8±2.7
G2/M	14.2±2.1	23.2±3.1	47.5±3.1	43.6± 3.8
	Laminin		**Etoposide treatment**	
Cell cycle phase	**—**	**PKB DN**	**—**	**PKB DN**
Sub-G0	4.0±1.8	4.2±1.9	3.1±0.7	8.1±1.1
G1	64.5±3.0	47.6±1.5	55.3±2.2	20.8±2.2
S	16.2±1.7	24.5±2.9	20.6±1.1	27.7±1.7
G2/M	11.6±2.1	21.2±2.0	15.2±1.0	49.2±2.1

H345 cells were transfected with equal amounts of dominant-negative PKB (PKB DN), constitutively active PKB (PKB CA) or empty vector (−). After 24 h, the cells were quiesced, seeded into six-well plates precoated with poly-L-lysine or laminin and treated with etoposide as indicated. Progression through the cell cycle was assessed 24 h later by flow cytometric analysis of DNA content using CELL Quest software. Mean percentage of total cells in each phase of the cell cycle is shown from $n=5$ independent experiments ±S.E.M.

Discussion

Our results show for the first time that ECM proteins activate β1 integrins to protect SCLC cells against the proapoptotic effects of the DNA damaging agents, etoposide and ionizing radiation through tyrosine kinase-mediated PI3-kinase activation. Etoposide, which inhibits the re-annealing activity of Topo II causing DNA damage, upregulates CDK inhibitors $p21^{Cip1/WAF1}$ and $p27^{Kip1}$, increases CDK 1 phosphorylation and downregulates cyclins D, E, A and B and CDK 2 phosphorylation, causing G2/M cell cycle arrest. In SCLC cells β1 integrin-mediated PI3-kinase activation via phosphorylation of PKB and GSK-3β leads to maintenance of cyclins D, E, A and B, increases expression of phosphorylated CDK2 and downregulates CDK inhibitors, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ despite the presence of etoposide. This leads to continued progression through the cell cycle, overriding etoposide-induced G2/M arrest. Furthermore, adhesion of SCLC cells to ECM via β1 integrin/PI3-kinase signaling results in phosphorylation of Bad and reduced caspase-9 cleavage, in response to etoposide, preventing apoptosis. The interrelationship of ECM effects on G2/M progression and apoptosis is not clear, but it is likely that through PI3-kinase ECM overrides these processes in parallel, to effect cell survival and proliferation. Importantly, the effects of ECM occur despite etoposide-induced Topo II inhibition and extensive DNA damage. As a consequence, SCLC cells in the presence of ECM are resistant to chemotherapeutic agents and continue to cycle despite genetic abberations, possibly leading to the

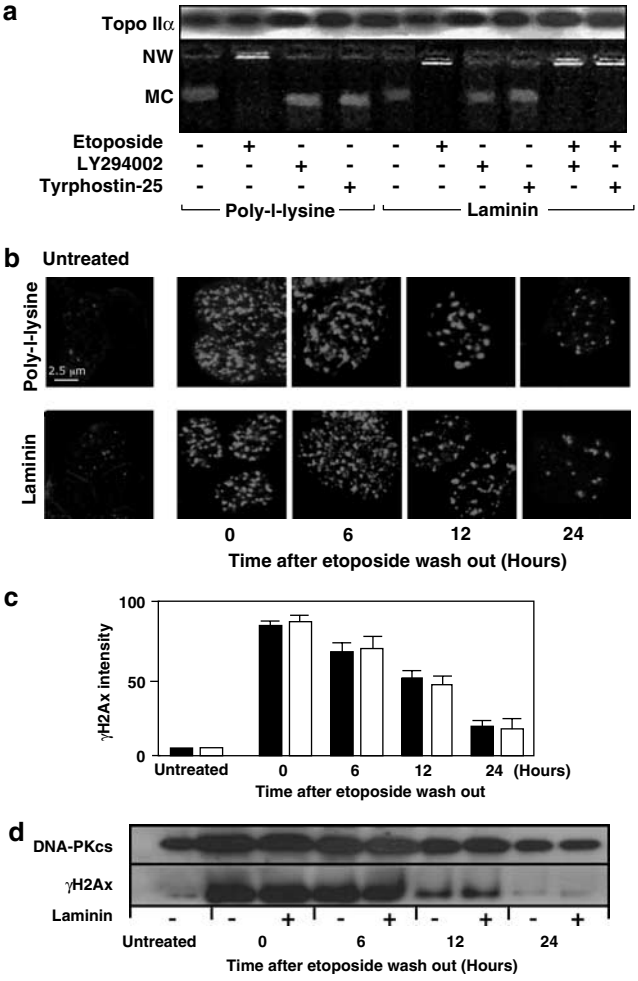

Figure 6 Adhesion to ECM does not affect etoposide-induced Topo II inhibition or DNA double-strand break formation and repair. **(a)** H345 cells were adhered to poly-L-lysine or laminin and treated with (+) or without (−) etoposide (25 μg/ml), LY294002 (30 mM) or tyrphostin-25 (25 mM) as indicated. Nuclear extracts were prepared and Topo II activity was determined by ATP-dependent decatenation of kinetoplast DNA (kDNA) as described in Materials and Methods. A representative UV gel is shown (from $n=3$ independent experiments): MC – decatenated individual DNA mini-circles (active Topo II), NW – high-molecular-weight interlocking kDNA network (inactive Topo II)). In parallel samples, Topo II expression was determined by Western blot analysis. **(b)** Quiesced H345 SCLC cells were adhered for 1 h to eight-well coverslides precoated with poly-L-lysine (10 μg/ml) or laminin (10 μg/ml) and treated with etoposide (25 μg/ml) for 2 h. Cells were washed twice and the medium was replaced with RPMI containing 10% FCS. Cells were fixed at 0–24 h after removal of etoposide as indicated, stained with anti-γH2Ax antibody and foci viewed by fluorescence microscopy. Representative images of γH2Ax foci from $n=4$ independent experiments. Untreated cells are shown for comparison. **(c)** H345 SCLC cells were seeded into eight-well coverslips precoated and treated with poly-L-lysine (closed bars) or laminin (open bars) and allowed to adhere for 1 h. The cells were treated with etoposide (25 μg/ml) for 2 h, washed and allowed to recover in RPMI with FCS. Cells were fixed at 0–24 h after removal of etoposide as indicated, stained with anti-γH2Ax antibody and foci viewed by fluorescence microscopy The intensity of γH2Ax staining in 10 high-power fields was quantified with Openlab image analysis software for each condition and normalized to untreated cells (mean relative γH2Ax foci of $n=4$ experiments). **(d)** H345 cells were adhered to poly-L-lysine (−) or laminin (+), treated with etoposide (25 μg/ml) and lysed at the times indicated. Expression of DNA-PKcs and γH2Ax was determined by Western blot analysis. Representative blot from $n=3$ independent experiments

emergence of drug-resistant clones. As the DNA damage is random, genetic drug resistance can be achieved as a result of a wide variety of insults, explaining why no unifying mechanism for the emergence of MDR in SCLC has been defined.

The development of MDR is a major cause of treatment failure in cancer.[2] Central to the cellular response to DNA damage is cell cycle arrest at regulated checkpoints with subsequent activation of cell death by apoptosis.[8] MDR is a multi-factorial phenomenon, and therefore it may be more effective to target the processes which enhance survival and prevent apoptosis induced by initial drug treatment in cancer cells, preventing the emergence of acquired drug resistance.[27] Resistance mechanisms *in vitro* differ from those *in vivo*, suggesting that the local cellular environment plays a significant role in selection for *in vivo* drug resistance. Our previous results in SCLC suggest that *in vivo* interactions between tumor cells and ECM proteins in the microenvironment are involved in the early stages of drug selection, providing a survival advantage to ECM adherent SCLC cells, and promote the acquisition of the classical forms of drug resistance.

Detachment of anchorage-dependent cells results in apoptotic cell death.[28] This can be prevented by constitutive activation of integrin-dependent signaling pathways such as PI3-kinase and PKB.[29] While anchorage-independent growth is a major step in cellular transformation leading to cancer and metastases, cancer cells may still use integrin survival signals. Our work and results from other groups show that a number of cancers, including SCLC, breast, prostate, colon cancer and hematological malignancies, all use ECM adhesion for survival to evade the cytotoxic effects of chemotherapy or radiotherapy.[3,5–7] While this appears to be a general and important phenomenon, the actual intracellular mechanisms coupling integrin activation to protection from chemotherapy- and radiotherapy-induced apoptosis may be cancer cell type specific. The mechanisms described here for SCLC differ from those reported previously, particularly myeloma cells. This may reflect cell type specificity potentially relating to differing integrin behavior between hematological malignancies and solid tumors. Previous studies in hemapoeitic tumor cells have demonstrated that adhesion to fibronectin protected against chemotherapeutic agents through increased expression of $p27^{Kip1}$ and G1 cell cycle arrest.[30] Furthermore, integrin-mediated adhesion in hemapoeitic cancer cells reduced DNA double-strand breaks and apoptosis caused by chemotherapeutic agents such as mitoxantrone and etoposide.[31] This primarily $\alpha5\beta1$-mediated reduction in DNA damage correlated with decreased topoisomerase-II enzymatic activity. However, the $\alpha5\beta1$ integrin is not found in SCLC cells and is reduced in other carcinomas.[3,32] Furthermore, our results show that, in SCLC cells grown in suspension or adhered to ECM proteins such as laminin and fibronectin, the enzymatic activity of topoisomerase-II and the amount of etoposide- or radiation-induced DNA double-strand breaks remain unchanged. In addition, the rate of proliferation is also unaffected by adhesion to ECM proteins. These results demonstrate that decreased proliferation and changes in Topo II activity are not the mechanisms of integrin-mediated drug resistance in SCLC cells.

The PI3-kinase/PKB pathway contributes to the pathogenesis of cancer and confers resistance to apoptosis.[19,33] Activated PI3-kinase phosphorylates inositol lipids, resulting in the activation of the serine/threonine kinase PKB that phosphorylates caspase-9 and BAD, promoting cell survival.[19] Activated PKB can also regulate cellular proliferation by interaction in the nucleus with cell cycle regulators governing G1/S and G2/M checkpoints.[34] In this study, we have shown that inhibition of PI3-kinase prevents $\beta1$ integrins from overriding etoposide-induced G2/M cell cycle arrest and apoptosis, indicating that PI3-kinase is crucial for ECM-mediated chemoprotection in SCLC cells. Previous work has indicated that inhibition of growth factor stimulated PI3-kinase activity, promotes apoptosis and enhances sensitivity of SCLC cells to chemotherapy.[18] Primarily, we found that pharmacological and genetic inhibition of PI3-kinase/PKB signaling dramatically reversed ECM-mediated chemoprotection. However, we did observe an increase in apoptosis measured by annexin V staining at 48 h with inhibiton of PI3-kinase signaling both with and without etoposide. These results confirm previous data by Krystal *et al*. (2002), but the effects on apoptosis were less pronounced in our system, possibly due to differences in experimental design and cell types examined. This small effect on apoptosis is in keeping with previous data published on the use of LY294002.[18,35]

Integrins and growth factors in untransformed cells only regulate cell cycle progression at the G1/S checkpoint.[11] Current evidence suggests a central role for PI3-kinase signaling pathway in regulating cell cycle progression. PI3-kinase activation can be sufficient to induce G1 transit in fibroblasts.[34] Thus, downstream targets of PI3-kinase/PKB-dependent pathways that regulate normal cell cycle progression may also participate in overriding etoposide- and radiation-induced cell cycle checkpoints. PI3-kinase activity has been shown to induce expression of D-type cyclins and to increase cyclin D1 stability through PKB-dependent phosphorylation of GSK-3β.[21] G1 progression depends on the sustained expression of D type cyclins. The cyclin D–CDK 4 and 6 complex sequesters the CDK inhibitors $p21^{Cip1/WAF1}$ and $p27^{Kip1}$, which allows cyclin E–CDK 2 to drive the cell cycle through late G1, allowing the derepression of cyclin A at the onset of S phase.[16] Deletion of the *p27^{Kip1}* gene restores the growth phenotype of cyclin D1 null mice.[36] Furthermore cells, which overexpress cyclin E, still depend on cyclin D1 accumulation to sequester the enhanced $p21^{Cip1/WAF1}$ levels, suggesting that a major function of cyclin D1 in cell cycle control is the redistribution of CDK inhibitors.[37] PI3-kinase effects on G1-phase progression may also be mediated through the downregulation of expression and function of $p27^{Kip1}$.[38] Thus, adhesion to ECM via integrin-mediated activation of PI3-kinase may reduce levels of $p21^{Cip1/WAF1}$ and $p27^{Kip1}$, allowing cells to progress through the G1/S phase of the cell cycle.

SCLC cells have previously been shown to have a defective G1/S checkpoint, which correlates with mutant inactive p53.[39] We confirm the reports of others that etoposide and ionizing radiation lead to G2/M cell cycle arrest in SCLC cells. Furthermore, our data clearly demonstrate that the $\beta1$ integrin/PI3-kinase pathway in SCLC cells regulates progression through the G2/M checkpoint. The ability of $\beta1$ integrins to

override etoposide-induced G2/M arrest in SCLC cells could result from increased DNA repair, thus removing signals for cell cycle arrest. Importantly, we found no difference in the degree of DNA damage induced by etoposide or XRT in cells adhered to either laminin or poly-L-lysine. Furthermore, when cells were allowed to recover, we observed identical rates of DNA repair. This suggests that ECM-mediated protection from G2/M arrest is not caused by alterations in the amount of DNA damage or the kinetics of DNA repair. The finding of identical DNA repair is interesting because laminin, by driving cells through the G2/M cell cycle checkpoint, might reduce the time scale for effective DNA repair, resulting in increased amounts of damaged DNA. However, previous data have demonstrated that DNA repair can occur at all stages of the cell cycle and cells have mechanisms for tolerating damaged DNA, allowing replication of the genome.[40] Therefore, it is possible that, in cells driven through the G2 checkpoint by laminin, damaged DNA is tolerated, to be repaired during other phases of the cell cycle. This could result in similar apparent levels of DNA double-strand break repair between cells adhered to laminin and poly-L-lysine. Importantly, tolerating mechanisms are more likely to introduce point mutations, increasing the likelihood of long-term genetic damage.

Crucially, what we have demonstrated in this paper is that etoposide treatment of SCLC cells adhered to poly-L-lysine induces DNA double-strand breaks causing G2/M cell cycle arrest and ultimately apoptotic cell death via caspase activation. However, although etoposide induces identical DNA damage in cells adhered to ECM, β1 integrin/PI3-kinase activation by ECM proteins prevents prolonged G2/M arrest by downregulating $p21^{Cip1/WAF1}$ and $p27^{Kip1}$, reduces phosphorylation of CDK1, maintains expression of cyclin D, E, A and B and phosphorylates CDK2. Thus, adhesion to ECM allows SCLC cells to survive following significant treatment-induced DNA damage, increasing the likelihood of propagation of genetic mutations, providing an explanation for the acquisition of MDR following initial therapy.

β1 integrin-mediated adhesion between tumor cells and the ECM in their environment confers a survival advantage to SCLC cells, protecting from apoptosis and allowing cells with damaged DNA to drive through the cell cycle. This may lead to the propagation of further transforming genetic mutations, which may ultimately cause acquired MDR in what were initially chemo-sensitive cells. Thus, the environmental context and specificity of cancer cells may have important consequences on the acquisition of MDR and therapies designed to disrupt specific interactions between cells, and their environment may be important targets in the circumvention of MDR. This has important implications for the role of dose intensification and adjuvant treatment by inhibiting β1 integrin/PI3 kinase signaling to enhance the cytotoxic effects of chemotherapy. However, integrins play a key role particularly in the immune system and protection against infection; thus targeting β1 integrins may have deleterious side effects. Our initial observations on cell-adhesion mediated drug resistance have been shown to be widely applicable in a number of cancers including breast, colon and hematological cancers. We believe the mechanisms outlined in this article will also have broad implications, particularly in cancers that are initially sensitive to cytotoxic treatment and then become resistant. A better understanding of the β1-specific mechanisms mediating integrin protection against the proapoptotic effect of DNA damaging drugs may lead to the development of novel therapeutic strategies in the circumvention of MDR.

Materials and Methods

Cell culture

All experiments used NCI-H69, NCI-H345 and NCI-H510 cell lines from ATCC (Rockville, MD, USA) which were grown in HEPES buffered RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Labtec), 5 μg/ml L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Ltd, UK). For experiments, cells were cultured overnight in quiescent media (RPMI supplemented with 0.25% (w/v) BSA, 100 U/ml penicillin and 100 μg/ml streptomycin), and then resuspended in SITA media (RPMI supplemented with 30 nM selenium, 5 μg/ml insulin, 10 μg/ml transferrin, 0.25% (w/v) BSA, 100 U/ml penicillin and 100 μg/ml streptomycin). FCS contains fibronectin (25 μg/ml), which will stimulate β1 integrin/PI3-kinase signaling, and therefore it was necessary to remove FCS prior to and during experiments examining the effects of ECM on SCLC cells.

Transfection of cell lines

Plasmids encoding myc-tagged constitutively active PKB-pUSEamp myr-*Akt1/PKB*α and myc-tagged dominant-negative PKB-pUSEamp *Akt1/PKB*α (K179M) were obtained from Upstate Biotechnology. pUSEamp was used as an empty vector control. Plasmid inserts were sequenced prior to use. For transfection H345 and H69 cells were washed twice, resuspended in antibiotic free culture RPMI (0.5×10^6 cells/ml) and transfected with 1 μg of DNA/10^6 cells using Lipofectamine 2000 (1 : 5 ratio) according to the manufacturer's instructions.

Apoptosis

SCLC cells were quiesced by overnight incubation at 37°C in quiescent medium. Cells were washed twice and seeded (4×10^4 cells in 200 μl of SITA per well) into 96-well plates which had been precoated by incubation at 37°C for 1 h with poly-L-lysine (10 μg/ml) (Sigma), laminin (10 μg/ml) (Sigma), fibronectin (20 μg/ml) (Sigma) or TS2/16 (10 μg/ml) (BioLegend) as specified. Cells were allowed to settle for 1 h at 37°C and then treated with Na_3VO_4 (200 μM) (Sigma), LY294002 (30 μM) (Calbiochem), tyrphostin-25 (25 μM) (Calbiochem) or etoposide (25 μg/ml) (Sigma) as specified. Cells were treated with ionizing radiation (1–4 Gy) as specified using a 6 MV linear accelerator. In experiments using 4B4 to block β1 integrin function, cells were incubated with 4B4 (10 μg/ml) (Beckman Coulter) at 37°C for 30 min prior to seeding onto ECM. Apoptosis was assessed 0–72 h later by the addition of 1 μl of ethidium bromide (1 mg/ml) (Sigma) and 1 μl acridine orange (1 mg/ml) (Sigma) and the percentage of apoptotic cells was determined by fluorescent microscopy as described previously.[5] Apoptosis was also determined using a cell death detection ELISA™ kit (based on the quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes) according to the manufacturer's instructions (Roche Diagnostics) and by flow-cytometric analysis of Annexin V staining according to the manufacturer's instructions (Annexin V-PE BD Bioscience).

PI3-kinase activity assay

PI3-K activity was measured as described previously.[29] Briefly, cells were spun onto six-well plates precoated with poly-L-lysine (10 μg/ml) (Sigma), laminin (10 μg/ml) (Sigma), fibronectin (20 μg/ml) (Sigma) or TS2/16 (10 μg/ml) (BioLegend) as specified and lysed. PI3-kinase was immunoprecipitated from protein-equilibrated cell lysates using a 10 μg 1 : 1 mixture of anti-phosphotyrosine antibodies PY20 and PY99 (Upstate Biotechnology, Lake Placid, USA) and assayed for activity using [γ-^{32}P]ATP and a mixture of phosphatidyl inositol and phosphatidyl serine as substrate. 3-Phosphorylated lipids were resolved using thin-layer chromatography after Folch extraction. PIP_3 was identified by autoradiography and quantified by liquid scintillation counting.

PKB activity

PKB activity was measured as described previously.[29] In brief, cells were quiesced overnight, gently disaggregated and then treated as specified. Cells were lysed, PKB was immunoprecipitated with anti-PKBα conjugated to protein G-sepharose beads (4°C for 90 min) and assayed for activity using [γ-^{32}P]ATP and crosstide substrate (Upstate Biotechnology). Radioactive incorporation was quantified by liquid scintillation counting.

Caspase-3 activity

Quiesced SCLC cells were adhered to poly-L-lysine (10 μg/ml) (Sigma), laminin (10 μg/ml) (Sigma), fibronectin (20 μg/ml) (Sigma), or TS2/16 (10 μg/ml) (BioLegend) as specified and treated with Na_3VO_4 (200 μM) (Sigma), LY294002 (30 μM) (Calbiochem), tyrphostin-25 (25 μM) (Calbiochem), etoposide (25 μg/ml) (Sigma) or z-VAD (100 μM) (Promega) as indicated. Cells were lysed at 48 h and caspase-3 activity was assessed using a caspase-3 cellular activity kit according to the manufacturer's instructions (Cat. no. 235419 Calbiochem). Specific caspase-3 activity (pmol/min/mg protein) in each experiment was normalized to untreated cells.

Western blotting

Cells were treated as specified, lysed in RIPA buffer and protein balanced by BCA protein assay (Pierce). Samples were resolved on 10, 12 or 14% acrylamide gels (10 μg protein/lane) and separated proteins were transferred onto nitrocellulose membranes. Blots were probed with the following antibodies overnight at 4°C in either 5% milk or 3% BSA in TBS Tween: rabbit anti-actin (Sigma); rabbit anti-phospho-PKB (Sigma), mouse anti-GSK-3α/β (Biosource International), rabbit anti-phospho-GSK-3β (pS9) (Biosource International); mouse anti-p21$^{Cip1/WAF1}$ (clone CP74) (Sigma); mouse anti-p27^{Kip1} (clone DCS-72) (Sigma); mouse anti-cyclin A (Oncogene Research Products) mouse anti-cyclin B (BD Transduction Labs, USA); mouse anti-cyclin D1 (clone AM29) (Zymed); mouse anti-cyclin E (clone HE12) (Upstate); rabbit anti-phospho-CDK2 (Thr160), rabbit anti-phospho-CDK1 (Tyr15) (both Cell Signaling Technology, USA); mouse anti-γH2Ax (Upstate); mouse anti-DNA-PKcs (p350 clone 6) (BD Biosciences); rabbit anti-phospho-Bad (Ser136); rabbit anti-Bad (9292) (Cell Signaling Technology); rabbit anti-cleavage site-specific caspase-9 antibody (CCSA 315/316) (Biosource); mouse anti-myc (9E10) (Abcam); and rabbit anti-caspase-3 (Asp179) (5A1). Species-specific horseradish peroxidase-conjugated antibodies (Dako) were used as secondary labels and bands were visualized using ECL (Amersham) according to manufacturer's instructions. Relative protein expression was quantified using Image J software.

Cell cycle analysis

Cells were quiesced overnight and seeded into six-well plates precoated with laminin, fibronectin or TS2/16 and treated as specified. Cells were fixed 0–24 h later in 70% ice-cold ethanol (for at least 2 h), stained with propidium iodide and subjected to flow-cytometric analysis (pulse processing) with a FACS Calibur (BD Biosciences) flow cytometer. Cell cycle parameters from 10 000 events were analyzed using CELLQuest software.

Topo II activity

Topo II activity was measured as described previously.[3] Briefly, nuclei were extracted from SCLC cells treated as specified and lysed. The supernatant was used in a Topo II assay, which measures Topo II activity by the ATP-dependent de-catenation of high-molecular-weight kinetoplast DNA (kDNA). Each sample was incubated for 30 min at 37°C in reaction buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 0.5 mM EDTA, 30 μg/ml BSA, 1 mM ATP, 0.5 mM dithiothreitol and 100 μg/ml kDNA (TopoGEN)). The reaction products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide and photographed under UV light.

Visualization of DNA double-strand breaks

Cells were quiesced overnight and seeded into eight-well coverslides (Nalge NUNC) at a density of 2.5×10^5 cells/cm^2 precoated with laminin or poly-L-lysine. Cells were allowed to adhere for 1 h at 37°C and then treated with etoposide (25 μg/ml) for 2 h. Cells were then washed and allowed to recover in RPMI with FCS. Cells were fixed at 0–24 h after etoposide treatment with 3% paraformaldehyde for 5 min, washed twice with PBS, permeabilized with 0.1% Triton and blocked with 0.2% fish skin gelatin. Cells were incubated with 1 : 500 dilution of anti-γH2Ax (Upstate) for 2 h at room temperature and washed with PBS. Secondary antibody labeled with ALEXAflour 488 (1 : 500) was applied for 1 h at room temperature. The slides were viewed using a Zeiss Axioskop 2 microscope. Image analysis was performed using Openlab software from Improvision.

Statistical analysis

Data were analyzed by one-way analysis of variance and the appropriate post-test analyses were applied. *P*-values < 0.05 were considered to be significant.

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