Université de Strasbourg

Ecole Doctoral Science de la Vie et la Santé

Laboratoire de Biophotonique et de Pharmacologie, UMR7213 CNRS,

Faculté de Pharmacie

et

Université Charles de Prague

2ème Faculté de Médecine

Thèse en cotutelle

En vue d'obtenir le grade de :

Docteur de l'Université de Strasbourg

Discipline : Sciences du Vivant - Aspects moléculaire et cellulaires de la Biologie

Par

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Caractérisation de la voie de signalisation intégrine $\alpha_5\beta_1$ / protéine p53 dans la résistance à la chimiothérapie des gliomes et cancers du colon.

Soutenue publiquement le 09 décembre 2013

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2nd Faculty of Medicine

Bi-nationally supervised Doctoral Thesis

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Role of $\alpha_5\beta_1$ integrin / p53 pathway in the resistance of glioma and colon cancer to therapy

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ACKNOWLEDGEMENT

I would like to express my deepest appreciation to my supervisors, Dr. Monique Dontenwill and Ing. Jan Teisinger CSc. without whose guidance and persistent help this dissertation would not have been possible.

I am deeply grateful to Dr. Monique Dontenwill who accepted me in her "Integrin team" in France and who opened me the door to cancer research. I am grateful for the extra-hours that she sacrificed to me and for the scientific discussions that helped me understand the project. She probably changed my life by introducing me to this unique pleasure of research you can only feel when, after having formulated an audacious hypothesis, you are not really able to sleep until the moment your results confirmed it without the shadow of a doubt. She has always been with me, has shared with me the happy as well as the sad moments of my research project, encouraging me when I progressed, re-motivating me when I didn't. What is more, she patiently corrected my manuscript, and her councils have always been decisive.

I would like to express my deepest gratitude to Ing. Jan Teisinger CSc. He will probably never imagine to what extent his availability, encouragements and human qualities allowed me to progress. This work would never have been the same without his indefectible support; his faith in me and his immeasurable kindness have undoubtedly been a driving force without which I would certainly not have been able to achieve this project.

I owe enormous thanks to Fanny for her persistent help and kindness. I will never forget the numerous occasions when she proposed me to especially come to the laboratory at midnight in order to help me finish a manipulation, thus allowing me to sleep for a couple of hours...Her reliability and extreme rigor made her the ideal colleague to work with, and the person I could always count on.

I also owe a lot to my team colleagues who have always encouraged me and shared with me their ideas and enriching experiences. In alphabetical order:

- *Special thanks to you Anne-Marie for our never-ending discussions about science, but also for your encouragements and for your priceless help about so many things! I can't figure out these four years without her; I appreciated so much each time you announced me, late in the evening: "I am by car, I will drive you back home!"*

- *Florence, for her availability, her high scientific knowledge that she has always been willing to share, and for the kindness with which she proposed me to correct my numerous English mistakes.*

- *Isabelle, who shared with me her scientific knowledge with much kindness and simplicity, and who encouraged me a lot when I felt down.*

- *Laurence, for her availability to help, her personal qualities, and her smile.*

- *Maxime, for his unique way to broach the scientific questions, for his liberty of thinking, for the energy he brought in all our team, and of course for his ice creams in summer to refrigerate our ideas when they were a little confused by the lab's warmth!*

- *Sophie, who taught me so much about new experimental techniques, and has always been available for any question.*

Thanks to Anne-Florence and Marion for their kindness and friendship; I wish you good luck for your ongoing Thesis!

Thank you so much Tania, Ellen and Emily! It was a pleasure to work with you. I thank Jerome, Veronique and Lionel for their everyday smile and their good mood. Thanks to Marlyse for her kindness, and for her impeccable administrative work.

My sincere thanks belong to the institution that financially supported my work. Those are The French Embassy in Prague, EGIDE and ARC.

Last but certainly not least, I would like to particularly thank my family and Greg, who supported me during this Thesis, and who never ceased to believe in me. Greg, I thank you so much for your never-ending help and your permanent encouragements; but also for having stood me with such indulgence when I was discouraged and put out; and for the numerous journeys to lab and back home that allowed me to finish my project. Without you, this Thesis would never have been fulfilled.

ABSTRACT

Integrins are transmembrane proteins involved in various signaling events, including cell survival, proliferation and migration. Since these signaling pathways are usually altered in tumors, integrins seem to be attractive anti-cancer targets. In this work we investigated the role of integrin $\alpha_5\beta_1$ in glioma brain tumors and colon cancer. We were particularly interested in the role of integrin $\alpha_5\beta_1$/p53 pathway in resistance to therapy. We first focused on gliomas and found that $\alpha_5\beta_1$ integrin was overexpressed in aggressive malignant glioma tumors. Moreover, we showed that $\alpha_5\beta_1$ integrin upregulation was associated with a shorter patient survival. We also demonstrated that $\alpha_5\beta_1$ integrin expression in glioblastomas participates to the resistance to the chemotherapeutic agent Temozolomide, through a negative regulation of the tumor suppressor p53. A direct p53-activation by the non-genotoxic agent Nutlin-3a down-regulated α_5 integrin subunit and thus sensitized glioblastoma cells to Nutlin-3a. Furthermore, we demonstrated that the inhibition of $\alpha_5\beta_1$ integrin by a specific antagonist with a concomitant p53-activation enhanced the effects of p53-based therapy in glioblastoma cells. In the second part of this work, we showed that interfering with α_5 integrin expression or function decreased survival of colon cancer cells. We also found that the reactivation of p53 tumor suppressor by Nutlin-3a inhibited α_5 integrin subunit, and thus sensitized colon cancer cells to Nutlin-3a. Conversely, the repression of α_5 integrin increased p53 activity. These data were in accordance with those obtained in glioblastomas, confirming the existence of a negative cross-talk between $\alpha_5\beta_1$ integrin and p53 in both tumor types.

Altogether, our study demonstrated that $\alpha_5\beta_1$ integrin is a potential therapeutic target for malignant brain and colon cancers. Targeted therapies based on the repression of $\alpha_5\beta_1$ integrin function may be a useful strategy to sensitize these tumors to conventional chemotherapeutic drugs, or to another p53-based therapy.

Résumé

Les intégrines sont des protéines hétérodimériques transmembranaires de type αβ impliquées dans divers processus physiologiques, souvent dérégulés dans les cancers, tels que la prolifération/survie ou la migration des cellules. Les intégrines sont des cibles thérapeutiques pertinentes en oncologie. Dans cette thèse, nous avons exploré le rôle de l'intégrine $\alpha_5\beta_1$ dans les gliomes et les tumeurs du colon. Nous nous sommes particulièrement focalisés sur la voie intégrine-protéine p53 et son implication dans la résistance aux thérapies. Dans les gliomes, l'intégrine $\alpha_5\beta_1$ est surexprimée dans les glioblastomes (grade IV) et participe à un mauvais pronostic de survie des patients. Nous avons démontré que l'intégrine confère une résistance à la chimiothérapie par le Temozolomide en régulant négativement l'activité de la protéine suppresseur de tumeurs p53. L'activation directe de p53 par un agent non-génotoxique, la Nutlin-3a, entraine une inhibition de l'expression de l'intégrine suggérant ainsi une réaction croisée négative entre intégrine $\alpha_5\beta_1$ et p53. L'association de la Nutlin-3a avec un antagoniste spécifique de l'intégrine $\alpha_5\beta_1$ entraine une mort massive des cellules par apoptose. Nous avons confirmé l'existence d'une réaction croisée négative entre intégrine $\alpha_5\beta_1$ et protéine p53 dans les tumeurs du colon où l'intégrine représente également une cible thérapeutique.

Notre étude démontre la pertinence de l'intégrine $\alpha_5\beta_1$ comme cible thérapeutique dans les gliomes de haut grade et dans les tumeurs du colon. Sur la base de nos résultats, de nouvelles associations thérapeutiques peuvent être proposées pour une population de patients dont les tumeurs expriment l'intégrine $\alpha_5\beta_1$ et une protéine p53 fonctionnelle.

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ABBREVIATION

Ab	Antibody
ADMIDAS	Additional metal ion dependent adhesion site
ADP	Adenosine diphosphate
AKT	Serine/threonine-specific protein kinase
Apaf -1	Apoptotic protease-activating factor 1
ARF	Alternative reading frame
Arg	Arginine
ASPP	Apoptotic-stimulating protein of p53
ATM	Ataxia telangiectasia mutated protein
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
BAD	Bcl-2-associated death promoter protein
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-X_L	B-cell lymphoma-extra large
BH	Bcl-2 homology
BID	BH3-interacting death agonist
BIK	BCL2-interacting killer
BIM	B-cell lymphoma 2 interacting mediator of cell death
BSA	Bovine serum albumin
CBP	CREB-binding protein
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CHK1 (2)	CHK 1 (2) checkpoint homolog
COP-1	Constitutive photomorphogenic 1
CREB	C-AMP response element-binding protein

DEC1	Deleted in esophageal cancer 1
DISC	Death-inducing-signaling complex
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DR 4/5	Death receptor 4/5
DYRK2	Dual specificity tyrosine-phosphorylation-regulated kinase 2
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence assisted cell sorting/flow cytometry
FADD	Fas-associated protein with death domain
FAK	Focal adhesion kinase
FASL	FAS ligand
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FN	Fibronectin
GADD45	Growth arrest and DNA damage-inducible protein 45
GBM	Glioblastoma multiforme
GFR	Growth factor receptor
Gly	Glycine
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanosine triphosphate
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HIPK	Homeodomain-interacting protein kinase
IDH1	Isocitrate dehydrogenases
IL-6	Interleukin 6
ILK	Integrin-linked kinase
JMY	Junction-mediating and -regulatory protein
JNK	Jun amino-terminal kinase

Lys	Lysine
MAPK	Mitogen-activated protein kinase
MCL-1	Induced myeloid leukemia cell differentiation protein
MDM2	Mouse double minute 2
MDMX	MDM2 homolog
MGMT	Methylguanine DNA methyltransferase
MIDAS	Metal ion dependent adhesion site
MMP2	Matrix metalloproteinase 2
mRNA	Messenger RNA
MTIC	5-(3-methyltriazene-1-yl) imidazole-4-carboxamide
mTOR	Mammalian target of rapamycin
NES	Nuclear export signal sequence
NF1	Neurofibromin 1
NF-κB	Nuclear factor κB
NLS	Nuclear localization signal sequence
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
p53AIP1	p53-regulated apoptosis-inducing protein 1
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PEA-15	Phosphoprotein enriched in astrocytes 15
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIRH-2	p53-induced ubiquitin-protein ligase 2
PLD1	Phospholipase D1
PML	Promyelocytic leukaemia
PSI	Plexin-semaphorin-integrin
PTB	Phosphotyrosine-binding

PTEN	Phosphatase and tensin homolog
PUMA	p53-upregulated modulator of apoptosis
Raf	Serine/threonine-specific protein kinase
Ras	Small GTPase
RB	Retinoblastoma protein
RGD	Arginine-glycine-aspartate
Rho	Ras homolog
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-qPCR	Real time quantitative PCR
Ser	Serine
SFK	Src-family kinase
SH2	Src homology region 2
Shc	Shc-transforming protein 1
shRNA	Small hairpin RNA
siRNA	Small interfering
Src	Proto-oncogenic tyrosine kinase
STAT3	Signal transducer and activator of transcription 3
TBS	Tris buffered saline
Thr	Threonine
TMZ	Temozolomide
TNF	Tumor necrosis factor
TP53	Tumor protein 53
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
WB	Western blot
WHO	World Health Organization
Wt	Wild type
YY1	Yin Yang 1

INTRODUCTION

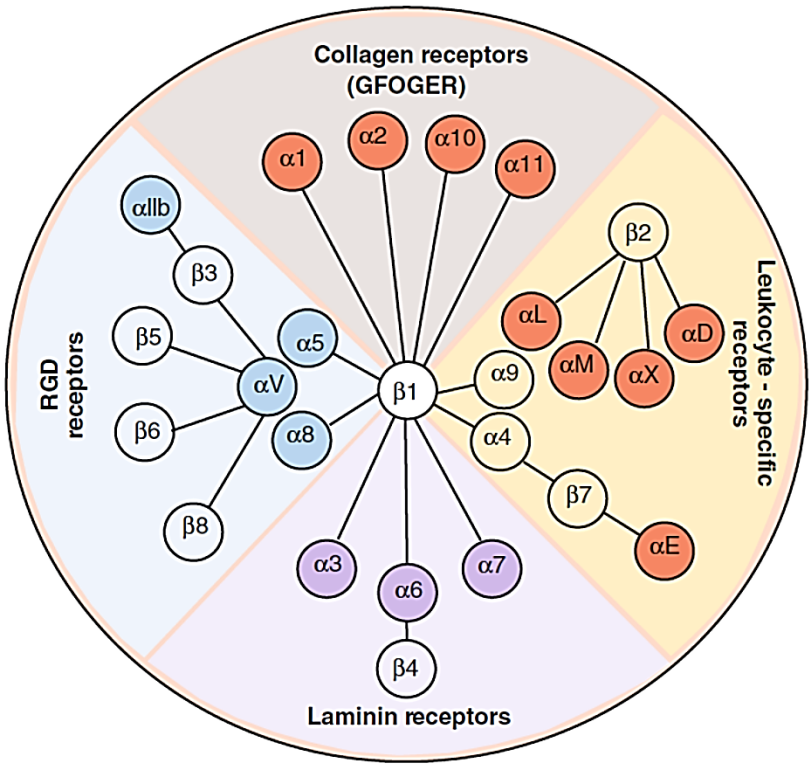

Figure 1: The members of the integrin family. In vertebrates, the integrin family contains 24 heterodimers with a distinct ligand-binding specificity and tissue distribution. Each integrin is generated from 18 α and 8 β subunits. *(Barczyk et al., 2010).*

Integrin

Integrins are evolutionary old cell adhesion transmembrane molecules and receptors for the extracellular matrix (ECM). The first integrin was identified almost 27 years ago *(Tamkun et al., 1986)*. It became rapidly clear that this membrane glycoprotein was only one member of large family of receptors with a similarity in structure and function. All integrins are heterodimers composed of an α and a β subunit *(Ruoslahti and Pierschbacher, 1987).* Nowadays, integrins are not only known as cell adhesion molecules, but also as true signaling receptors that play crucial roles in broad biological processes, such as cell migration, growth, differentiation and apoptosis. The de-regulation of integrins function often contributes to the development of various diseases, including cancer. Thus, integrins are recognized as promising therapeutic targets.

1. Integrin family

The integrin family consists of at least 24 different trans-membrane glycoproteins that are composed of a set non-covalently associated α and β subunits with various binding ligands and tissue specific distribution. In vertebrates, 18α and 8β subunits have already been described *(Danen and Yamada, 2001; Hu and Luo, 2013).*

The natural integrin ligands include important components of the ECM, such as fibronectin, laminin, collagen, vitronectin and others. The sequence arginine-glycine-aspartate, RGD, in fibronectin was originally identified as an integrin-binding pattern *(Pierschbacher and Ruoslathi, 1984).*

The integrin heterodimers are usually classified according to ligand specificity as RGD-binding, collagen-binding, leukocyte adhesion integrins and laminin-binding integrins *(Takada et al., 2007)* **(Figure 1)**. It is to note that some of the integrins can bind to the same ligands and some integrins can be promiscuous, with a single integrin binding to multiple ligands.

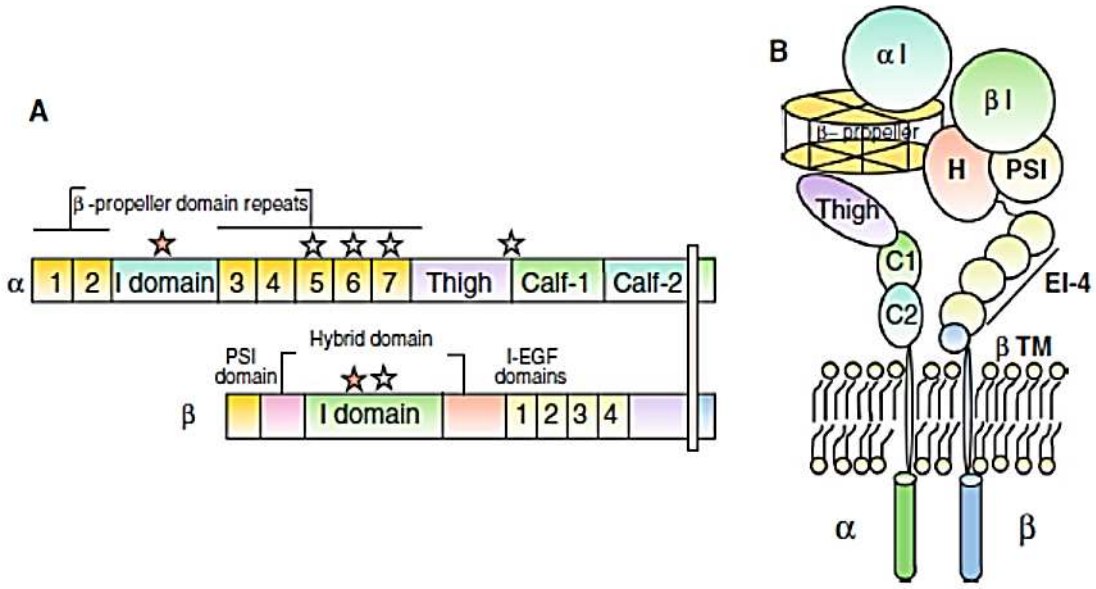

Figure 2: Integrin structure. **A.** The domain organization of α and β subunits. 9 out of the 18 integrins contain α-I domain. Stars denote divalent cation-binding sites. **B.** Schematic integrin structure *(Barczyk et al., 2010).*

The integrins can be further subdivided according to their structural similarity in α subunits. There are two groups of α subunits, α subunits that contain I domain, such as α_1, α_2, α_{10}, α_{11}, α_L, α_M, α_X, α_D and αE, or α subunits without I domain, such as α_3, α_4, α_5, α_6, α_7, α_8, α_9, α_V and α_{IIb}. I domains play a central role in ligand binding and intercellular adhesion *(Takada et al., 2007)*.

1.1. Integrin structure

Integrin α and β subunits are distinct to each other, with no homology between them. The sequence identity is about 30% for α subunits and about 45% for β subunits.

Each of the subunits has a single membrane-spanning helix, a large extracellular domain and a short cytoplasmic domain. The size of each subunit varies within the integrin members, but typically α subunit contains around 1000 amino acids and β subunit around 750 amino acids *(Luo et al., 2007).*

Extracellular domain of α subunit is composed of a 7-bladded β propeller connected to a thigh, a calf-1, and -2 domains. In addition, 9 of the integrin α chains have an I domain of around 200 amino acids, inserted between blades 2 and 3 of the β propeller *(Larson et al., 1989; Goswami, 2013).* This domain contains the metal ion-dependent adherent site (MIDAS) and is responsible for ligand binding *(Lee et al., 1995).* The integrins containing α-I domain have a high homology in their α-I domains, but α chain cytoplasmic domains are highly divergent *(Takada et al., 2007).*

The β subunit contains 7 domains, a plexin-semaphorin-integrin (PSI) domain, a hybrid domain, a β-I domain and 4 cysteine–rich epidermal growth factor (EGF) repeats *(Lee et al., 1995; Goswami, 2013).* The β-I domain has an Mg^{2+} coordinating MIDAS and a site adjacent to MIDAS (ADMIDAS) binding to an inhibitory Ca^{2+} ion. This ADMIDAS site binds Mn^{2+} ion, leading to a conformational change resulting in the integrin's activation *(Humphries et al., 2003; Campbell and Humphries, 2011).* In the cytoplasmic chain, the β integrins share homology in their NPX/Y motifs that allow them to bind proteins containing PTB domains *(Moser et al., 2008; Wegener and Campbell, 2008)* **(Figure 2)**.

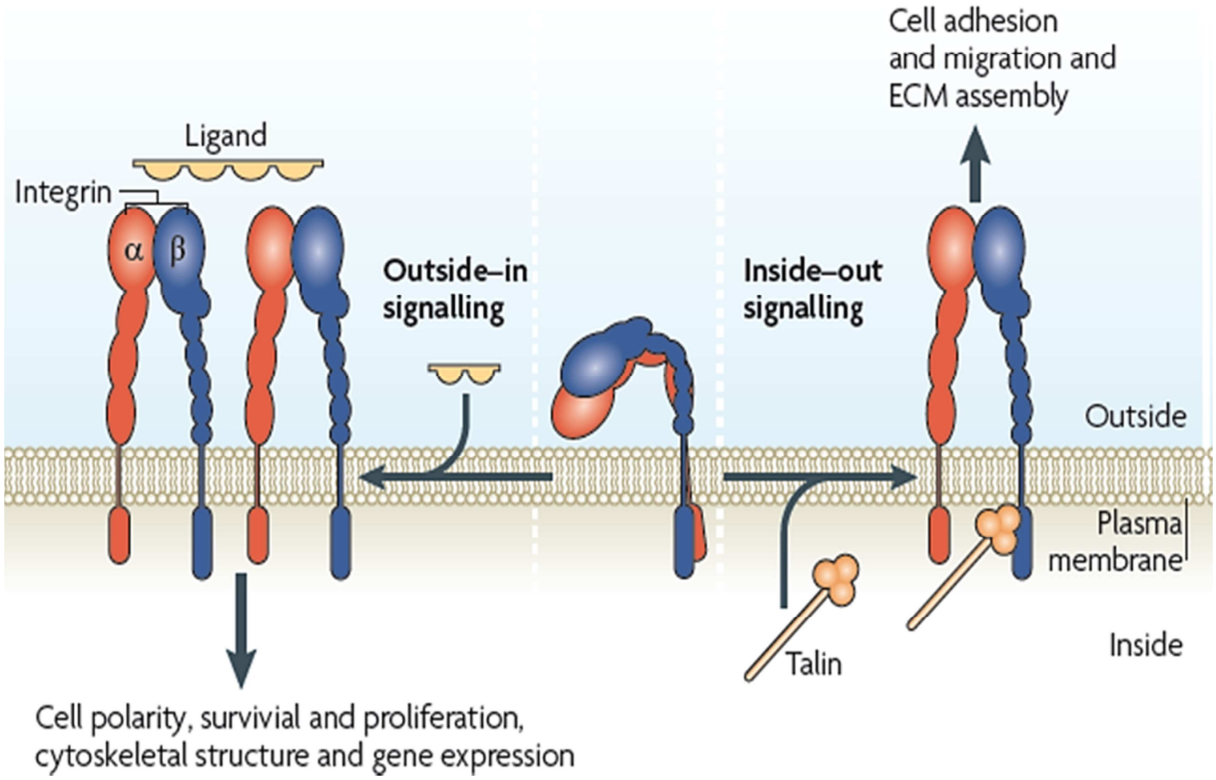

Figure 3: Bidirectional signaling of integrin. Integrin can be activated in two ways. Upon outside-in signaling, the binding of integrin to their extracellular ligands changes the conformation of the integrin and contributes to activation and integrin clustering. Upon inside-out signaling, intracellular activators, such as talin or kindlin, interact with β integrin cytoplasmic chain that lead to a conformation change and to an increase affinity for the extracellular ligand *(Shattil et al., 2010).*

The major role of the α chain is to determinate the ligand specificity. The ligand binds to the integrin through the β propeller domain of the α chain and the β-I domain. If the α-I domain is present, it forms the ligand-binding domain. The ligand binding to α-I domain causes its conformational changes, which in turn affect the conformation of the β subunit (*Barczyk et al., 2010; Goswami, 2013)*.

1.2. Integrin activation

Crystal structures have revealed that integrins display three distinct states, inactive bent conformation with a low affinity for the ligand, extended with closed headpiece and extended with an open headpiece (*Takagi and Springer, 2002; Xiao et al., 2004; Arnaout et al., 2007).* This 'switchblade' model is currently the most accepted, but another conformation change between these three states have been proposed (*Xiao et al., 2004; Wang and Luo, 2010).* The important step of integrin activation is the separation of α and β cytoplasmic tails and transmembrane regions *(Luo and Springer, 2006; Askari et al, 2009).*

The activation and priming of αβ heterodimers is achieved either upon ligand binding, outside-in signaling, or upon binding of cytoplasmic activator proteins, inside-out signaling, and results in conformation changes, from bent to an extended form of the integrin. Several key proteins containing PTB domains were shown to interact with the cytoplasmic chain of β subunit *(Legate and Fassler, 2009).* Especially the PTB proteins, talin and kindlin were shown to have an important role in integrin inside-out activation *(Hu and Luo, 2013).*

Talin interacts with the cytoplasmic tail of the β subunit through its amino terminus. Whereas the interaction between talin and the β tail is essential for integrin activation, the cooperation with another anchoring protein, kindlin, which binds also to β integrin's tail, is required *(Moser et al., 2008).* The effect of talin and kindlin can be further enhanced by the binding of vinculin to talin, resulting in the clustering of activated integrins *(Galbraith et al., 2002).* The activated integrins modulate various signaling cascades controlling cell attachment, proliferation, migration, differentiation and survival *(Hynes, 2002; Schwartz and Ginsberg, 2002; Hu and Luo, 2013)* **(Figure 3)**.

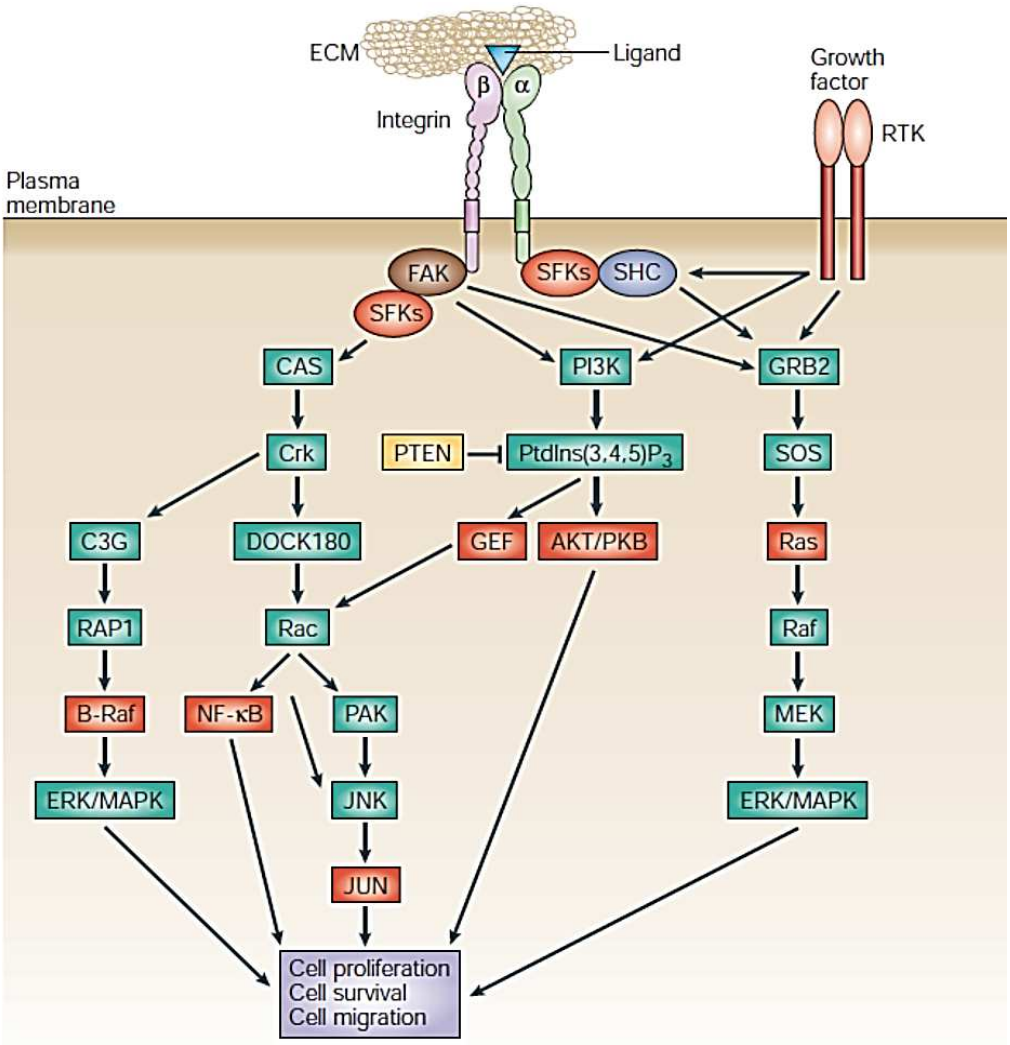

Figure 4: Overview of integrin-mediated signaling. The majority of integrins activates FAK as a main downstream component. FAK acts as a signaling scaffold to recruit Src to focal adhesion which activates several downstream partners, leading to the activation of JNK, NF-κB and PI3K. FAK also activates ERK/MAPK signaling pathway, either directly or by recruiting growth factor receptors. Integrin-mediated signaling controls cell migration, cell proliferation, and cell death *(Guo and Giancotti, 2004).*

1.3. Integrin signaling

Ligand binding to integrin promotes conformational changes and integrin clustering, which results in the activation of signaling cascades and in the recruitment of multi-protein complexes in areas termed focal adhesions. Integrins transmit the signals through a variety of cytoplasmic proteins and kinases, including Focal adhesion kinase (FAK), Integrin linked kinase (ILK), talin, paxillin, proto-oncogenic tyrosine kinase Src, Phosphoinositide-3-kinase (PI3K), Ras/Mitogen-activated protein kinase (Ras/MAPK). These signals in turn regulate mobility, cytoskeleton organization, adhesion, proliferation and survival, making the integrins true signaling molecules *(Hynes, 2002; Hehlgans et al., 2007; Legate and Fassler, 2009)*.

The most extensively studied kinase activated by integrin is a non-receptor protein tyrosine kinase FAK (Focal adhesion kinase) *(Hanks et al., 1992; Schaller et al., 1992)*. FAK is linked either directly to the β cytoplasmic tail or indirectly via talin or paxillin. Upon integrin binding, FAK is activated by auto-phosphorylation at Tyr397 *(Chan et al., 1994; Schaller et al., 1994).* Phosphorylated FAK acts as a scaffold, which serves as a binding site for SH2 domains of Src family kinases (SFKs) *(Schaller et al., 1994; Eide et al., 1995).* Src in turn phosphorylates further FAK and activates several downstream partners, leading to the activation of Jun amino-terminal kinase (JNK) and Nuclear factor κB (NF-κB) *(Guo and Giancotti, 2004; Brown et al., 2005).* FAK also activates Phosphoinositide 3-kinase (PI3K) and Extracellular signal-regulated kinase/ Mitogen-activated protein kinase (ERK/MAPK). FAK has been demonstrated to play an important role in integrin-mediated cellular events, such as cell migration, cell proliferation, and cell death (*Guo and Giancotti, 2004; Hu and Luo, 2013).*

SFKs, in addition to their role as cofactors of FAK, can be activated independently of FAK. Some SFKs interact directly with β cytoplasmic tail of integrins *(Arias-Salgado et al., 2003)*. Thereby, SFKs mediate the phosphorylation of the adaptor Shc, which activates the Ras-ERK/MAPK pathway. The signaling pathways activated by integrin-SFKs are sufficient to induce cell migration and protect the cells from apoptotic cell death *(Guo and Giancotti, 2004)*.

Integrin like kinase (ILK) is a serine / threonine kinase, another component that has been reported to be essential in integrin outside-in signaling *(Hannigan et al., 1996).* ILK directly binds to β cytoplasmic tail of integrin. ILK signaling up-regulates the activity of serine/threonine-specific protein kinase AKT, and thus promotes cell survival *(McDonald et al., 2008).*

Integrins can also mediate crosstalk with growth factor receptors (GFR). Both receptors can act separately, but more frequently they act synergistically *(Alam et al., 2007).* It has been proposed that integrins may control GFR activity through the recruitment of adaptors protein *(Goel et al., 2004).* In addition, *Baron and Swartz (2000)* demonstrated that cell adhesion alters the degradation rate of Platelet-derived growth factor receptor (PDGFR), suggesting that integrins modulate GFR internalization rate *(Baron and Schwartz, 2000).*

Figure 4 summarizes the integrin-mediated signaling.

2. Integrins and cancer

2.1. Integrin expression in cancer

As mentioned above, the integrins regulate signaling pathways involved in migration, proliferation, cell survival, and others. These signaling pathways are usually altered in cancer, and the deregulation of integrins' activity may thus contribute to a malignant phenotype.

Pathological studies of human cancer have provided evidences that tumor cells modulate the expression of their set of integrins. Although some neoplastic cells tend to lose integrins that promote their adhesion and differentiated state, others overexpress integrins, leading to the induction of survival, migration and proliferation during tumor invasion and metastasis. The changes in integrin expression and in integrin signaling are cell-type-dependent and it is therefore impossible to rigidly assign each of the integrins to the anti-neoplastic or to the pro-neoplastic category *(Desgrosellier and Cheresh, 2010)*.

Many solid tumors originate from epithelial cells. There is five integrins, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ and $\alpha_v\beta_5$, expressed by normal epithelial cells, as well as by transformed epithelial cells that give rise to solid tumors. However, the expression of these integrins is usually altered in tumors compared to normal epithelial cells *(Desgrosellier and Cheresh, 2010; Chamberlain et al., 2012).*

Interestingly, three integrins such as $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$ are expressed at very low or undetectable levels in normal tissue, and their expression is up-regulated in some tumors *(Desgrosellier and Cheresh, 2010).*

On the other hand, there are some integrins whose expression has been shown to be decreased in tumors. For example, the down-regulation of the integrin $\alpha_2\beta_1$ correlates with malignant properties of some tumor cells *(Zutter et al., 1995; Kren et al., 2007).* Re-expression of this integrin abrogates the malignant phenotype of breast carcinoma cells, suggesting that $\alpha_2\beta_1$ may act as a tumor suppressor *(Zutter et al., 1995).* Similarly, the overexpression of $\alpha_3\beta_1$ decreases the rate of carcinoma formation in the skin *(Owens and Watt, 2001).* In contrast with those data, there are other reports demonstrating that $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins might facilitate melanoma and pulmonary tumor progression *(Chan et al., 1991; Wang et al., 2004).* The role of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins is not clear, suggesting that their behavior is dependent on the cellular context and on the stage of the tumor progression.

The altered expression of integrins is not limited to tumor cells. For example $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are highly up-regulated on endothelium cells during tumor angiogenesis, whereas these integrins are only poorly expressed in resting endothelial cells *(Avraamides et al., 2008; Desgrosellier and Cheresh, 2010).*

Studies linking the expression of integrins with pathological outcomes have identified several integrins, including $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_6\beta_4$, $\alpha_v\beta_5$ and $\alpha_v\beta_6$, that play an important role in the tumor progression *(Chamberlain et al., 2012)* **(Table 1)**.

Tumor type	Integrins expressed
Breast	$\alpha_6\beta_4/\alpha_v\beta_3$
Cervical	$\alpha_v\beta_3/\alpha_v\beta_6$
Colon	$\alpha_v\beta_6$
Glioblastoma	$\alpha_v\beta_3/\alpha_v\beta_5$
Melanoma	$\alpha_v\beta_3/\alpha_5\beta_1$
Non-small-cell lung carcinoma	$\alpha_5\beta_1$
Ovarian	$\alpha_4\beta_1/\alpha_v\beta_3$
Pancreatic	$\alpha_v\beta_3$
Prostate	$\alpha_v\beta_3$

Table 1: The most studied integrins in cancer *(Chamberlain et al., 2012).*

2.2. Integrins and resistance to anticancer therapy

Integrins are implicated in promotion of tumor cell survival after both radiotherapy and chemotherapy *(Hehlgans et al., 2007).* The integrins may thus abrogate the therapeutic potential of these interventions. The understanding of how integrins induce survival and resistance to therapy attracted much interest, with the option to develop a more efficient therapy for the treatment of resistant tumors.

With a dependence on the environment, integrins have a capacity to modulate cell survival. Integrin ligation usually enhances cell survival through several mechanisms, including (1) the activation of PI3K/AKT and NF-κB pro-survival signaling pathways, (2) the up-regulation of BCL-2 or other pro-survival proteins, and (3) the modulation of p53 tumor suppressor activity.

PI3K/AKT signaling pathway is suggested to have a critical role in integrin-mediated cell death since the disruption of this pathway was shown to induce cell death. For instance, integrin $\alpha_5\beta_1$ was shown to protect intestinal epithelial cells against pro-apoptotic stimuli by enhancing the activity of PI3K/AKT (*Lee and Juliano, 2000).* AKT activation can then induce the expression of BCL-2 and BCL-X_L anti-apoptotic genes *(Leverrier et al., 1999; Gauthier et al., 2001; Matter and Ruoslahti, 2001).* This transcriptional up-regulation may be promoted by nuclear transcriptional factor NF-κB, however the activation of NF-κB by AKT seems to be cell-dependent *(Duriez et al., 2000; Stupack and Cheresh,*

2002). Furthermore, AKT phosphorylates and, thus inhibits the function of pro-apoptotic protein BAD *(Hayakawa et al., 2000).*

The protein p53 is the main regulator and activator of cell death (for an overview about p53 see below). There are few reports that investigated the role of integrins in the modulation of the p53 protein. Therefore, the role of integrin in p53 regulation is still not clear, and seems to be dependent on the cellular context as well as on the specific set of integrin. Both integrin-dependent activation *(Bachelder et al., 1999; Lewis et al., 2002)* and integrin-dependent inhibition *(Stromblad et al., 1996; de la Fuente et al., 2003; Bao and Stromblad, 2004; He et al., 2008)* of p53 protein was shown. For example, Bachelder and co-workers demonstrated that $\alpha_6\beta_4$ stimulated the activity of p53 and induced apoptosis in carcinoma cells (*Bachelder et al., 1999)*. On the other hand, $\alpha_v\beta_3$ integrin mediated the suppression of p53 in vascular and melanoma cells *(Stromblad et al., 1996; Bao and Stromblad, 2004)*. Similarly, the knockdown of β_4 integrin increased the level of p53 and induced cell death in lung adenocarcinoma cells (*He et al., 2008).*

It has been described that integrins influence the cellular response to genotoxic injury. Onoda (1992) reported that irradiation of melanoma cells resulted in enhanced surface expression of $\alpha_{IIB}\beta_3$ integrin that may alter surviving potential of tumor cells *(Onoda et al., 1992).* Similarly, ionizing radiation strongly induced the expression of functional β_1 integrin in lung cancer cell lines *(Cordes et al., 2002)*. Another study demonstrated that ionizing radiation modulated the cell surface expression of α_1, α_2, α_5, α_6 and β_1 integrins and enhanced resistance in colorectal tumor cell lines *(Meineke et al., 2002).* Cordes and co-workers investigated the way integrins regulate cell survival after radiation. They demonstrated that β_1 stimulated AKT, p130Cas and paxillin in a PI3K-dependent manner. In particular, they pointed out the β_1-mediated activation of AKT as a main actor of cell survival after radiation *(Cordes et al., 2006).* These data suggest that inhibition of β_1 integrin could improve the radiotherapy effects. This was investigated by Park (2008), who confirmed this hypothesis and demonstrated that β_1 inhibitory antibody enhanced radiotherapy in human breast cancer xenografts *(Park et al., 2008).*

The survival promoting effect of integrins has also been observed in the response of tumor to chemotherapeutic agents. It was reported that the adhesion of small cell lung cancer cells to fibronectin, collagen IV and laminin induced resistance to chemotherapeutic agents (etopoxide, cis-platinium and daunarubicin) and inhibited apoptosis through β_1 integrin, suggesting that the inhibition

of β_1 integrin could improve the response to chemotherapy in lung cancer cells *(Sethi et al., 1999).* Hodkinson and his co-workers demonstrated that the protective effect of β_1 integrin was mediated through PI3K/AKT signaling and subsequent inhibition of caspase-3 *(Hodkinson et al., 2006).* Similarly in breast cancer cells, ligation of β_1 integrin abrogated apoptosis induced by chemotherapeutic agents by activating PI3K/AKT pathway *(Aoudjit and Vuori, 2001).* In prostate cancer cell line, fibronectin inhibited ceramide- and docetaxel-induced apoptosis via β_1 integrin and insulin-like growth factor *(Thomas et al., 2010).*

Additionally, the work of de la Fuente (2003) discovered another possible link between integrins and chemoresistance. Here, integrin $\alpha_4\beta_1$ ligation inhibited p53 activation by fludarabine in chronic lymphocytic leukemia B-cells *(de la Fuente et al., 2003)*. These data suggest that integrin-dependent regulation of p53 may be involved in the resistance to chemotherapy in some tumor settings.

2.3. Integrins as targets for cancer therapy

The capacity of integrins to promote tumor progression and resistance to standard cancer therapy has made them appealing therapeutic targets. Preclinical studies demonstrated that integrin antagonists inhibited the tumor growth by affecting both tumor and endothelial cells. Moreover, inhibition of integrin was shown to improve the effects of radiotherapy *(Park et al., 2008).* At this time, three types of integrin inhibitors were developed: antibodies, peptides and small organic molecules *(Goswami, 2013).*

The first antagonist tested in clinical trials was a function-blocking monoclonal antibody, eratacizumab (the humanized version of LM609, MEDI-522, abegrin), which inhibits $\alpha_v\beta_3$ integrin. In preclinical studies, this $\alpha_v\beta_3$-directed antibody had anti-angiogenic effect, inhibited tumor growth by directly affecting tumor cells *(Brooks et al., 1995; Mulgrew et al., 2006).* Etaracizumab has been investigated in Phase II in metastatic prostate cancer and in metastatic melanoma. Phase I trials with the precursor of etaracizumab, vitaxin, showed low toxicity and disease stabilization in some patients with advanced solid tumors *(Delbaldo et al., 2008).* Randomized trial in metastatic melanoma showed that etaracizumab in combination with dacarbacine did not provide a relevant survival benefit compared to dacarbazine alone *(Hersey et al., 2010).*

Itetumumab (CNTO 95) is a human monoclonal antibody directed against both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. CNTO 95 reduced angiogenesis and tumor growth in human melanoma xenografts and showed no toxicity in preclinical studies *(Trikha et al., 2004; Martin et al., 2005).* In Phase I trial, itetumumab showed anti-tumor activity *(Mullamitha et al., 2007).* The results of a randomized Phase II trial with itetumumab suggest a potential benefit in stage IV melanoma *(O'Day et al., 2011).*

Cilengitide is a cyclic RGD pentapeptide which inhibits both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Cilengitide is being investigated as an anti-cancer treatment in high grade gliomas and other tumors. Cilengitide showed favorable safety and no dose-limiting toxicities *(Nabors et al., 2007).* Cilengitide is currently in Phase III trials for glioblastoma, and Phase II trials for other tumors *(Goodman and Picard, 2012).*

Integrin $\alpha_5\beta_1$ is implicated in both tumor development and tumor angiogenesis. Antagonists of $\alpha_5\beta_1$ integrin have undergone clinical testing. Volociximab is a function-blocking monoclonal antibody against $\alpha_5\beta_1$ integrin, currently in Phase II clinical trials for solid tumors *(Kuwada, 2007).* Another drug against $\alpha_5\beta_1$ integrin in clinical trials is a peptide inhibitor, ATN-161. ATN-161 inhibits $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. ATN-161 was tested in patients with advanced solid tumors and showed good tolerance and prolonged stable disease in one-third of the patients *(Cianfrocca et al., 2006).*

Table 2 summarizes anti-integrin agents under development for cancer therapy *(Chamberlain et al., 2012).*

Agent	Integrin target	Therapeutic target	CR phase
Peptidomimetics (RGD-based)			
Cilengitide	$\alpha_v\beta_3/\alpha_v\beta_6$	Glioblastoma, squamous cell carcinoma of the head and neck, non-small-cell lung cancer, other	III
ATN 161	$\alpha_5\beta_1$	Glioblastoma	II
HYD1	β_1	Multiple myeloma	PC
Antibodies			
Intetumumab (CNT095)	α_v	Hormone-refractory prostate cancer, melanoma, solid tumors	II
Volociximab	$\alpha_5\beta_1$	Renal cell carcinoma, melanoma, pancreatic cancer	II
Etaracizumab	$\alpha_v\beta_3$	Prostate	II
Natalizumab	α_v	Phase I/II terminated; marketed for MS	X
DI 17E6	$\alpha_v\beta_3$	Colorectal, prostate	II
PF-04605412	$\alpha_5\beta_1$	Solid tumors	I
IMGN388	α_v	Solid tumors	I
264RAD	$\alpha_v\beta_6$	Cancer	PC
Small organic molecules			
E7820	α_2	Colorectal, lymphoma	II
MK0429	$\alpha_v\beta_3$	Hormone-refractory prostate cancer	I
GLPG 0187	Five integrin receptors	Bone metastases in metastatic bone cancer	I
Celastrol	β_1 integrins	Prostate, pancreas	PC

CR: Clinical research; MS: Multiple sclerosis; PC: Preclinical; RGD: Arginine–glycine–aspartate; X: No longer in clinical trial.

Table 2: Anti-integrin agents under development for cancer therapy *(Chamberlain et al., 2012).*

3. Integrins in GBMs

3.1. Generalities about glioma

Primary central nervous system (CNS) tumors account for approximately 1.35% of all cancer in adults and 2.2% of all cancer-related deaths. The most common primary brain tumors are gliomas. The World Health Organization (WHO) classified and subtyped gliomas according to their histopathological features and clinical presentation as astrocytomas, oligodendrogliomas, mixed oligoastrocytomas and ependymomas. The astrocytomas are subdivided into four prognostic grades according to their degree of malignancy. Pilocytic astrocytomas (grade I) are biologically benign and can be cured by surgical resection. Diffuse astrocytomas (grade II) are low-grade tumors with a slow growth, but early diffuse infiltration into surrounding of the brain makes them incurable by surgery. Anaplastic astrocytomas (grade III) exhibit increased proliferation, mitotic activity and nuclear atypia. Glioblastomas (grade IV)

show even more advanced features of malignancy, including vascular proliferation and necrosis *(Louis et al., 2007)*. Grade III and grade IV tumors are considered as malignant gliomas.

Glioblastomas (GBMs) are histologically heterogeneous invasive tumors with the hallmark features of uncontrolled cell proliferation, necrosis, increased angiogenesis, infiltration, resistance to apoptosis, and extensive genomic instability *(Louis et al., 2007).* The origin of GBMs, as well as gliomas in general, is not defined. Originally, it was thought that GBMs arised from non-neuron glial cells, called astrocytes. Nowadays, the researches pointed out that a small subset of self-renewing cells, referred as glioma stem cells, could initiate the gliomagenesis *(Jiang and Uhrbom, 2012; Zong et al., 2012).* The most commonly GBMs appear in cerebral hemispheres, less often in the brain stem, cerebellum and spinal cord. Despite the current multimodal treatments, the median survival of GBM patients is about 15 months *(Stupp et al., 2005)*. Favorable prognostic factors are young age, absent or minimal neurological signs, maximal surgical resection and good performance status *(Behin et al., 2003).*

According to biologic and genetic differences, GBMs can be subdivided into primary or secondary GBMs subtypes. Most GBMs (90%) develop *de novo* and are termed as primary. Primary GBMs are typically detected in patients older than 50 years. In contrast, secondary GBMs that develop progressivelly from the lower grade astrocytomas are quite rare and occur in patients below the age of 45 years *(Maher et al., 2001; Furnari et al., 2007; Ohgaki and Kleihues, 2007).*

3.2. Oncogenic pathways in gliomagenesis

Numbers of intracellular events are frequently aberrant in glioma and attribute to their progression. Although glioma subtypes differ in their relative frequencies of genetic alterations, the most common aberrations that contribute to gliomagenesis affect signaling pathways regulating cellular proliferation, survival, migration/invasion and angiogenesis.

Growth signals are transmitted into the cells by a group of transmembrane proteins with tyrosine kinase activity, e.g. receptor tyrosine kinases (RTKs). Two of these RTKs best characterized in GBMs are Epidermal growth factor receptor (EGFR) and Platelet-derived growth factor receptor (PDGFR) *(Wong et al., 1987; Westermark and Nister, 1995).* After ligand binding, EGFR or PDGFR dimerize

and undergo auto-phosphorylation, thus activating various downstream effectors such as Ras/Raf/MAPK and/or PI3K/AKT/mTOR generally involved in cell proliferation and survival.

EGFR signaling is dysregulated through EGFR gene amplification or EGFR gene mutation. The most common mutant of EGFR gene is a constitutively active receptor variant EGFRvIII without a functional-binding domain. EGFR over-expression occurs in about 60% of primary GBMs, but is rare in secondary GBMs *(Watanabe et al., 1996; Heimberger et al., 2005).*

Increased PDGFR-α expression was found in astrocytic tumors of all malignancy stages, although highest levels were observed in GBMs. PDGFR-α gene amplification has only been detected in a limited subset of GBMs (16%), suggesting that the other mechanisms than gene amplification are present in glioma *(Hermanson et al., 1996).*

Phosphatase and tensin homolog (PTEN) mutation and loss of its gene expression is frequent in GBMs, and has been shown to occur almost exclusively in primary GBMs *(Fujisawa et al., 1999; Kleihues and Ohgaki, 2000)*. PTEN decrease can activate the RTKs/PI3K/AKT pathway since PTEN inhibits PI3K function.

The transition from G1 to S phase is often dysregulated in GBMs. The tumor supressor Retinoblastoma (RB) is a key regulator of this checkpoint. The Cyclin dependent kinase inhibitor 2A (CDKN2A) has been shown to inhibit Cyclin dependent kinase 4 (CDK4), which is not able to phosphorylate RB protein and to activate the E2F factors required for G1/S transition *(Ueki et al., 1996; Sherr and Roberts, 1999).* Mutations in CDKN2A/CDK4/RB are detected in 80% of GBMs and 50% of anaplastic astrocytomas. By contrast, these mutations are rare in low-grade astrocytomas *(Schmidt et al., 1994; Ueki et al., 1996).* CDKN2A deletions were significantly more frequent in primary than in secondary GBMs *(Biernat et al., 1997).*

Mutations of p53 were the first identified genetic alterations in astrocytic brain tumors *(Nigro et al., 1989).* p53 protein mutations were shown to be related to the development of secondary GBMs. High incidence of p53 mutations (more than 65%) was found in secondary GBMs and was less frequent in primary GBMs (about 30%) *(Ohgaki et al., 2004; Zheng et al., 2008).* However, in primary GBMs p53 signaling seems to be altered. For example, ARF ($p14^{ARF}$) mutations are common in primary (around 40%) and rare in secondary GBMs (around 10%) *(Biernat et al., 1997).* ARF stabilized p53 protein by

antagonizing MDM2, which targets p53 for degradation. Interestingly, MDM2 overexpression was observed in primary GBMs with intact p53 *(Biernat et al., 1997).*

3.3. Molecular sub-classification of GBMs

The analysis of multi-dimensional genomic data confirmed that RTK/RAS/PI3K and deregulation of RB and p53 are the core pathways required for GBM pathogenesis *(Parsons et al., 2008; TCGA, 2008).* In addition, new GBM-associated gene mutations were found and GBMs were subdivided in four subclasses: classical, mesenchymal, neural and proneural *(Verhaak et al., 2010)*.

The classical subgroup is characterized by an amplification of EGFR gene, loss of PTEN and CDKN2A gene. Notch and Sonic hedgehog signaling pathways are commonly activated. This subgroup show better response to the classical radiation and chemotherapies suggesting the positive effect of intact p53 pathway in this group of patients. Mesenchymal GBMs are characterized by frequent inactivation of NF1, TP53 and PTEN, and by overexpression of genes involved in TNF and NF-κB pathways. These tumors respond to aggressive chemoradiation therapy. The proneural subtype overexpresses PDGFR-α, and has frequent mutations in TP53, and IDH1 genes. In this group, patients are younger and this subtype seems to have very similar characteristics to secondary GBMs. The fourth subtype termed neural is less defined than the other types, and the gene expression signatures are most similar to those found in normal brain tissue *(Van Meir et al., 2010; Verhaak et al., 2010)*.

3.4. Current standard therapy

Current standard therapy for GBM patients includes gross total resection, radiotherapy and chemotherapy with temozolomide (TMZ). TMZ is a new promising drug that has been shown to increase response rates and patients‘ survival. Addition of TMZ to radiotherapy improved the median survival from 12 to 15 months and the five year overall survival from 1,9 to 9,8% *(Stupp et al., 2005; Stupp et al., 2007).*

TMZ (Temodar, Schering-Plough Corporation), an imidazole derivative, is an oral alkylating agent that undergoes spontaneous conversion to the active metabolite 5-(3-methyltriazene-1-yl) imidazole-4-carboxamide (MTIC). MTIC causes the methylation of DNA, most commonly at the N^7 position of guanine, followed by methylation at the O^3 position of adenine and O^6 position of guanine. Methylation of the O^6 position of guanine accounts for only minor methylation caused by TMZ, but seems to be responsible for the cytotoxic effect of TMZ *(Denny et al., 1994; Wedge et al., 1996).*

DNA repair enzyme O^6-methylguanine DNA methyltransferase (MGMT) repairs O^6-methyl adducts in DNA. In a one step alkyl transfer reaction, MGMT transfers the alkyl group from the oxygen in the DNA to a cystein residue in the catalytic pocket of MGMT. After each reaction, MGMT is inactivated and degradated by the proteasome. Both transcriptional silencing of the MGMT gene by methylation of its promoter as well as absence of MGMT protein have been associated with a good clinical response to alkylating agens, including TMZ, and enhanced survival of patients with an anaplastic astrocytoma or GBM *(Esteller et al., 2000; Hegi et al, 2005; Nagane et al., 2007; Sadones et al, 2009)*. MGMT promoter hypermethylation is associated with prolonged survival in GBM patients treated with TMZ and radiation compared to patients without promoter hypermethylation (*Hegi et al, 2005).*

3.5. Targeted therapy in GBMs

Although TMZ significantly prolongates the survival of GBM patients, the degree of benefit is very modest. There is a neverending effort to develop new treatment modalities that could overcome the resistance and cure these aggressive tumors. The discovery of new agents with the capacity to restore normal cell function through interaction with dysregulated targets is a hope for the treatment of GBMs. Numerous clinical trials are testing these molecular-based therapy approaches in GBMs.

EGFR has been an attractive target, since it can trigger downstream signaling pathways such as PI3K/AKT/mTOR and Ras/Raf/MAPK, and as it is abnormally activated in GBMs. Several strategies targeting EGFR have been proposed. Present efforts are focused on small-molecule tyrosine kinase inhibitors. These inhibitors block the receptor activation through competition with ATP binding in the receptor kinase pocket *(Taylor et al., 2012)*. Phase II clinical trials with two small-molecule tyrosine

kinase inhibitors, gefitinib (ZD-1839) and erlotinib (OSI-774), demonstrated that the overall efficacy in unselected patients is minimal compared with historical data *(Rich et al., 2004; Raizer et al., 2010).*

Imatinib has been studied as an inhibitor of the PDGFR. The results of Phase II demonstrated a limited efficacy in patients treated with single-agent or in neoadjuvant studies of imatinib *(Wen at al., 2006; Raymond et al., 2008; Razis et al., 2009).* In contrast, two studies using a combination of imatinib and hydroxyurea achieved better results compared with imatinib alone or historical control *(Dresemann, 2005; Reardon et al., 2005)*.

Mammalian target of rapamycin (mTOR) is another attractive target. mTOR inhibitor temsirolimus (CCI-779) was evaluated in two studies in Phase II clinical trials which demonstrated no improvement in response rates *(Galanis et al., 2005; Chang et al., 2004).*

Another therapeutic approach is represented by an anti-angiogenic therapy. GBMs have a high degree of vascularization, which is correlated with glioma malignancy, tumor aggressiveness and poor clinical prognosis. The inhibitors of angiogenesis include molecules mainly targeting Vascular endothelial growth factor (VEGF) (e.g. anti-VEGF antibody bevacizumab) and integrins (for instance cilengitide against integrin $\alpha_v\beta_3$).

3.6. Integrins as therapeutic targets in GBMs

3.6.1. Integrin expression and function in GBMs

The expression and function of integrins in glioma were investigated in several studies that underlined the importance of these proteins in glioma progression and glioma patients' survival.

The large-scale gene expression profiling showed that overexpression of ECM component such as fibronectin, known as a specific ligand of $\alpha_5\beta_1$ and $\alpha v\beta_3$ integrins, is associated with a more malignant phenotype and a poor survival of glioma patients *(Freije et al., 2004; Bredel et al., 2005; Colin et al., 2006; Tso et al., 2006).* Moreover, Bredel and his colleagues revealed that genes playing a role in integrin signaling, as fibronectin, and α_5 and α_v integrins are at the core of the most important GBM network, and contribute to gliomagenesis *(Bredel et al., 2005)*.

The pattern of alteration in cell adhesion molecules expression that distinguished tumor from normal brain tissues was investigated by *Gingras et al. (1995).* The results of this work showed that GBMs overexpress α_2, α_3, α_5, $\alpha_6\beta_1$ and $\alpha_v\beta_3$ integrins. Neoplastic human astrocytes showed increased expressions of α_3 and β_1 integrins, and some also α_5, α_v, β_3 and β_4 integrins *(Paulus and Baur, 1993).* Integrins $\alpha_5\beta_1$ and β_8 were shown to be expressed in a perinecrotic and perivascular area *(Riemenschneider et al., 2005).* At mRNA level, higher levels of α_5 and β_3 integrins were demonstrated in GBMs via the comparison with normal or lower grade astrocytoma *(Kita et al., 2001).* The integrin $\alpha_v\beta_3$ expression was higher in GBM compared to low grade glioma *(Schnell et al., 2008)*. These findings were confirmed and extended by flow cytometry data for $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ expression levels in human glioma explants. Whereas $\alpha_v\beta_3$ integrin was expressed by a majority of the glioma cells, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ integrins levels were higher in human glioma explants compared with those of $\alpha_v\beta_3$ *(Mattern et al., 2005).* In a recent study, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_8$ were shown to be broadly expressed in GBMs tumor cells *(Roth et al., 2013)*. Data from our laboratory demonstrated a correlation between $\alpha_5\beta_1$ expression and tumor aggressiveness *(Cosset et al., 2012).* Schittenhelm (2013) recently demonstrated that up-regulation of parenchymal $\alpha_v\beta_3$ is a negative prognostic factor in GBMs *(Schittenhelm et al., 2013).*

The increase in integrin expression is not restricted to the tumor cells. In GBMs, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ integrins are upregulated on endothelial cells during tumor angiogenesis when compared to normal endothelial cells *(Avraamides et al., 2008)*. The process of angiogenesis plays a pivotal role in glioma development, similarly to tumor cell invasion and migration.

Glioma cells infiltrate in the surrounding of normal tissue as a consequence of an enhanced capacity for migration. Since integrins play a crucial role in cell adhesion and migration, these receptors have been often studied in glioma. The integrin β_1 was shown to be required for invasion of glioma cells *(Paulus et al., 1996).* Numerous studies investigated the role of $\alpha_3\beta_1$ integrin in migration and invasion of glioma cells. The $\alpha_3\beta_1$ integrin has been demonstrated to be a key regulator of glioma cell migration and invasion *(Fukushima et al., 1998; Mahesparan et al., 2003; Kawataki et al., 2007).* These data are supported by the fact that inhibition of this integrin reduced both migration and invasion *(Tysnes et al., 1996).* The $\alpha_v\beta_3$ integrin was shown to promote migration and adhesion in various glioma cells *(Gladson and Cheresh, 1991).* Moreover, neutralization of $\alpha_v\beta_3$ integrin with a specific antibody

inhibited migration and invasion selectively in glioma cell lines with high level of $\alpha_v\beta_3$ integrin *(Wild-Bode et al., 2001).* The expression of α_2, α_3, α_5 and β_1 integrins was enhanced in drug-resistant glioma cells and was responsible for their increased adhesive and invasive capacities *(Hikawa et al., 2000).* Recently, $\alpha_6\beta_1$ expression has been shown to increase the proliferation and to decrease apoptosis, as well as to increase migration and invasion of glioma *in vitro* and *in vivo (Delamarre et al., 2009).*

Monferran and co-workers demonstrated that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins controlled glioblastoma cell radio-resistance by activating ILK and RhoB signaling pathways *(Monferran et al., 2008).* Moreover, the inhibition of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins/FAK/RhoB/GSKβ pathway led to a reduction of glioma hypoxia, which was associated with a significant decrease in vessel density in xenografts *(Skuli et al., 2009).*

Our laboratory investigates the role of integrin $\alpha_5\beta_1$ in glioma. The $\alpha_5\beta_1$ integrin was shown to increase proliferation, clonogenic survival, adhesion, migration and invasion of various glioma cell lines *(Maglott et al., 2006; Bartik et al., 2008; Martin et al., 2009, Cosset et al., 2012).* The use of a specific non-peptidic antagonist of $\alpha_5\beta_1$ integrin has highlighted the role of this integrin in the resistance to chemotherapy in different glioma cell lines *(Martinkova et al., 2010).* Moreover, the results of our laboratory reported that the expression of $\alpha_5\beta_1$ integrins in glioma was controlled by caveolin-1 (*Martin et al., 2009, Cosset et al., 2012).* Kita and co-workers demonstrated that Est-1 contributed to glioma malignancy by up-regulating the expression of the α_5 subunit. Activation of $\alpha_5\beta_1$ integrin led to an acceleration of the invasive process, including cell adhesion and migration (*Kita et al., 2001)*. Recently, the interaction of $\alpha_5\beta_1$ integrin with MMP-2 was shown to regulate the IL-6/STAT3 survival signaling in glioma *(Kesanakurti et al., 2012).*

3.6.2. Anti-integrin therapy in GBMs

As mentioned above, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins are over-expressed on angiogenic blood vessels as well as on tumor cells, suggesting $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins as attractive therapeutic targets for glioblastoma.

Cilengitide, cyclic peptide inhibitor of integrin $\alpha_v\beta_3/\alpha_v\beta_5$, is currently in clinical trials for glioblastoma. In preclinical studies, cilengitide showed an effective inhibition of angiogenesis and growth of orthotopic glioblastoma *(MacDonald et al., 2001; Yamada et al., 2006).* Phase I clinical trials in recurrent glioblastoma patients showed that cilengitide was well tolerated. A Phase II trial demonstrated that

cilengitide had a modest anti-tumor efficacy in patients with recurrent glioblastoma *(Reardon et al., 2008).* Phase II clinical trials, cilengitide and standard therapy combination in patients with newly diagnosed glioblastoma demonstrated that patients with MGMT methylated promoter appeared to benefit more from cilengitide therapy than patients with unmetylated MGMT promoter. The encouraging results of early clinical trials insured the incoming of cilengitide to Phase III clinical trials *(Chamberlain et al., 2012).* However, the results of Phase III demonstrated that cillengitide in combination with chemoradiotherapy did not increase the overall survival in GBM patients with methylated MGMT. This was the first Phase III clinical trial carried out with an integrin antagonist.

Several preclinical studies have investigated the effects of small non-peptidic $\alpha_5\beta_1$ antagonists in glioma. The first selective non-peptidic $\alpha_5\beta_1$ antagonist described was SJ749, a RGD mimetic *(Smallheer et al., 2004).* The results of our laboratory demonstrated that SJ749 potently inhibited the proliferation, the clonogenic survival and the invasion of GBM cell lines *(Maglott et al., 2006; Martin et al., 2009).* Moreover, our laboratory reported that SJ749 sensitized glioma cells to chemotherapy *(Martinkova et al., 2010).* Another non-peptidic $\alpha_5\beta_1$ antagonist, termed K34c (IC_{50}=3.1nM for $\alpha_5\beta_1$ integrin, IC_{50}=1624nM for $\alpha_v\beta_3$ integrin), was synthetized by Pr. Kessler's team. The reported data of our laboratory showed that K34c was potent to decrease the survival of glioma cells *(Cosset et al., 2012),* and sensitized them to chemotherapy agents *(Martinkova et al., 2010).* JSM6427 non-peptidic $\alpha_5\beta_1$ antagonist was described by Jerini AG Pharma (Berlin, Germany). This coumpound was mainly reported to efficiently inhibit angiogenesis *(Umeda et al., 2006; Zahn et al., 2009; Zahn et al., 2010).* In glioma *in vivo* model, JSM6427 has been shown to significantly reduce the glioma tumor growth *(Farber et al., 2008).*

Human p53 is a nuclear phosphoprotein encoded by a gene located on the small arm of chromosome 17 *(Isobe et al., 1986).* Protein p53 belongs to a family including three members, p53, p63, p73 *(Schmale and Bamberger, 1997; Kaghad et al., 1997).* Although these proteins are structurally and functionally related, p53 is involved in the prevention of the tumor development, whereas p63 and p73 proteins play a crucial role in normal development biology *(Irwin and Kaelin, 2001).* Mutations of p53 are the most common alterations in human cancer *(Hollstein et al., 1991; Levine et al., 1991).* The protein p53 is a central player in the protection of the genome integrity by regulating the cell cycle and apoptosis. Under a variety of cellular stresses such as DNA damage, p53 accumulates and prevents the propagation of cells with DNA damage through the regulation of genes and proteins involved in cell cycle and/or apoptotic cell death. Because p53 is implicated in those processes, its role as a potential target to cure malignant cancer has been investigated over the past years.

1. Structural features of p53

Wild-type (wt) p53 contains 393 amino acids and is composed of three functional domains including N-terminus, Central core and C-terminus. The N-terminus part is subdivided into a transactivation domain (residues 1-42) and a proline-rich region (residues 61-94). The N-terminal domain is required for the transcriptional activity. Mouse double minute (MDM2), the main negative regulator of p53, interacts with the N-terminal transactivation domain *(Chen et al., 1995).* The central DNA-binding domain is required for sequence-specific DNA binding *(Kern et al., 1991).* This region is highly conserved and the majority of p53 mutations found in tumors are missense mutations mostly located in this domain (*Cho et al., 1994).* The Arg175, Gly245, Arg248, Arg249, Arg273 and Arg282 are hot spot mutations found in various human cancers *(Bouchet et al., 2006).* The C-terminal region contains a tetramerization (residues 324-355), a regulatory domain (residues 363-393), a nuclear localization signal sequence (NLS) and a nuclear export signal sequence (NES). The C-terminal tail regulates the ability of DNA core domain to induce transcriptional activity. The tetramerization domain facilitates the interactions of p53 monomers to form tetramer, which is essential for p53 regulating gene expression **(Figure 5)**.

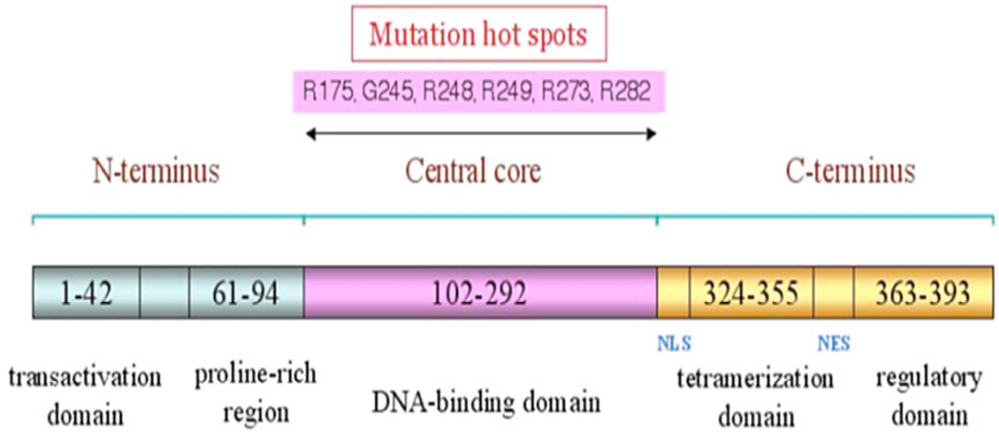

Figure 5: Functional domains of p53. Protein p53 is divided into three functional domains: N-terminus, Central core and C-terminus domains. DNA-binding domain is the most commonly mutated region *(Bai and Zhu, 2006).*

2. Regulation of p53 stabilization and activity

2.1. Posttranslational modifications

Under normal conditions, p53 is maintained at low levels in cells *(Levine, 1997).* After different stress stimuli, such as DNA damage (caused by ionizing radiation, UV radiation, chemotherapeutic and cytotoxic agents), heat shock, hypoxia, and oncogene overexpression, p53wt is stabilized and activated. According to the classical model, the first step of p53 stabilization is governed by its posttranslational modifications *(Oren, 1999).* Posttranslation modifications of p53 comprise phosphorylation, acetylation, ADP-ribosylation, ubiquitylation, sumoylation and neddylation *(Bode and Dong, 2004).* Phosphorylation and acetylation of p53 are the best understood modifications.

p53 has been shown to be phosphorylated on both N-terminal and C-terminal regulatory domains. Phosphorylation of p53 is controlled by a large variety of kinases. Different protein kinases phosphorylate several sites on p53, and in some conditions the same site can be phosphorylated by

more than one kinase. The phosphorylation at Ser15 and Ser20 after DNA damage was reported to stabilize p53 by inhibiting the interaction between p53 and MDM2 *(Shieh et al., 1997; Siliciano et al., 1997; Shieh et al., 1999; Unger et al., 1999)*. The phosphorylation of Ser15 and Ser20 is done mainly by Ataxia telangiectasia mutated protein (ATM), Ataxia telangiectasia and Rad3-related protein (ATR), DNA-dependent protein kinase (DNA-PK), and Checkpoint homolog kinases 1/2 (CHK1/2) *(Shieh et al., 1997; Canman et al., 1998; Khanna et al., 1998; Tibbetts et al., 1999; ; Shieh et al., 2000; Craig et al., 2003; Goudelock et al., 2003; Roos and Kaina, 2013).* Beyond these two most studied phosphorylation sites, other serine and threonine sites in the N-terminal region were shown to be phosphorylated, e.g. Ser6, Ser9, Thr18, Ser33, Ser37, Ser46, Thr55 and Thr81 *(Bode and Dong, 2004)*. For instance, phosphorylation of Ser46 by Homeodomain interacting protein kinase (HIPK) *(D'Orazi et al., 2002)* or Dual specificity tyrosine phosphorylation regulated kinase 2 (DYRK2) *(Taira et al., 2007)* was reported to mediate DNA binding selectivity of p53 and to specifically activate genes involved in apoptosis *(Oda et al., 2000).* In the C-terminal domain Ser315, Ser366, Ser371, Ser376, Ser378, Thr387 and Ser392 residues were shown to be phosphorylated. In addition, three phosphorylation sites Thr150, Thr155 and Ser149 in the central core were identified. There are some sites on p53 that are constitutively phosphorylated and that undergo de-phosphorylation in response to damage *(Bode and Dong, 2004).* This evidence gave rise to the possible importance of de-phosphorylation in p53 regulation *(Waterman et al., 1998)*, but further studies are required to fully understand this type of p53 regulation.

p53 protein is also acetylated in response to DNA damage (*Gu and Roeder, 1997; Luo et al., 2000; Brooks and Gu, 2011).* All of the acetylation sites on p53, involving Lys305, Lys320, Lys372, Lys373, Lys381, Lys382 and Lys386, are located in the C-terminus. p53 is acetylated by the histone acetyltransferases CBP/p300 and p300/CBP associated factor (PCAF) *(Brooks and Gu, 2011)*. It was shown that p53 acetylation is elevated in response to stress and correlates with p53 activation and stabilization (*Luo et al., 2000; Ito et al., 2001; Vaziri et al., 2001; Knights et al., 2006).* Moreover, the acetylation of p53 was demonstrated to modulate p53-sequence specific binding to DNA *(Gu and Roeder, 1997).* It is interesting to note that the lysine residues are also target sites for ubiquitination. Thus, acetylation may reduce p53 ubiquitination and extend p53 half-time *(Li et al., 2002).* In certain cases, phosphorylation is required for subsequent induction of acetylation. For instance, Ser15 and

Ser20 phosphorylation was shown to enhance the binding of CBP/p300 to p53, and to potentiate p53 acetylation *(Lambert et al., 1998; Sakaguchi et al., 1998).*

Although specific posttranslational modification patterns of p53 seem to be necessary to generate functional p53 protein, *Blattner et al. (1999)* demonstrated that p53 could be activated without any posttranslational modifications. Through mutation series of known stress-induced phosphorylation sites on p53, they found that p53 mutants could be stabilized. Mutations of the C-terminal amino acids known to be critical for the interaction between p300 and p53 also failed to disrupt p53 stabilization (*Blattner et al., 1999).* In accordance, the studies of *Ashcroft et al. (1999)* and *Wu et al. (2002)* also demonstrated that p53 was activated regardless of its phosphorylation status.

2.2. Ubiquitination and degradation of p53

The amount of p53 in unstressed cells is tightly controlled through ubiquitin-mediated proteasomal degradation *(Michael and Oren, 2003; Brooks and Gu, 2006).* MDM2 is the principal E3 ubiquitin ligase that mediates both ubiquitination and proteasomal degradation of p53 in cells *(Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997).* Other ubiquitin ligases were also described, including COP-1 *(Dornan et al., 2004)* and PIRH-2 *(Leng et al., 2003).* The importance of MDM2 as the main p53 regulator was shown with its silencing in mice. Indeed, the disruption of MDM2 gene is lethal in embryos at early development stages, whereas the double knockout mouse, with no MDM2 and p53 genes, is viable *(Jones et al., 1995).* MDM2 regulates p53 by promoting its degradation by the proteasome and by regulating its localization. p53 can be both mono- and poly-ubiquitinated by MDM2. Interestingly, MDM2-mediated p53 monoubiquitination is far more involved in p53 nuclear export and accumulation in cytoplasm than in degradation *(Lohrum et al., 2001; Li et al., 2003; Marchenko et al., 2007)*. Originally, the sequestration of p53 in cytoplasm was thought to be only a passive way to block the nuclear function of p53, but an increasing number of studies demonstrate a unique transcriptional independent function of p53 in the cytoplasm *(Marchenko and Moll, 2007).*

At high levels, MDM2 causes polyubiquitination and degradation of p53 protein. MDM2-dependent p53 degradation is not a simple way to reduce p53 levels in the cell, multiple layers regulate the connection between MDM2 and p53.

MDM2 activity is regulated by various inputs. MDM2 was shown to be phosphorylated by ATM. ATM is activated by DNA damage stress, and phosphorylates MDM2 on Ser395. The phosphorylation desactivates MDM2, which in turn increases p53 levels *(Maya et al., 2001).* MDM2 phosphorylation by AKT kinase has been demonstrated to increase MDM2 activity and to promote its transport to the nucleus *(Mayo and Donner, 2001; Zhou et al., 2001).* MDM2 activity is also regulated by CBP/p300 mediated acetylation. The acetylation inhibits MDM2, and consequently results in the stabilization of p53 *(Wang et al., 2004).* Moreover, MDM2 can trigger its own ubiquitination or its ubiquitination by other undefined E3 ligases *(Fang et al., 2000; Stommel and Wahl, 2004).*

One of the major regulators of MDM2 is the tumor suppressor ARF ($p14^{ARF}$). ARF induced by an oncogenic stress binds and sequesters MDM2 into the nucleus thereby resulting in p53 stabilization *(Weber et al., 1999).* In addition, ARF was shown to directly inhibit the ligase activity of MDM2 *(Honda and Yasuda, 1999; Llanos et al., 2001).* Another key regulator of MDM2 activity is MDMX (also known as MDM4). MDMX protein is structurally related to MDM2 *(Shvarts et al., 1996).* Despite this homology, MDMX does not have E3-ligase activity for p53. MDMX is able to stabilize MDM2 and to promote its E3-ligase activity *(Linares et al., 2003; Poyurovsky et al., 2007).* It is important to note, that MDMX also has the ability to repress the promoter of p53 target genes *(Kruse and Gu, 2009).*

To add more complexity, Herpesvirus associated ubiquitin specific protease (HAUSP) was described as a crucial actor in MDM2 stabilization. HAUSP deubiquitinates and stabilizes MDM2 in a p53-independent manner *(Cummins et al., 2004; Li et al., 2004).* Moreover, HAUSP specifically deubiquitinates and also stabilizes p53 protein *(Li et al., 2002).*

Several other proteins involved in the regulation of MDM2-p53 interaction have been identified. Promyelocytic leukaemia protein (PML) was shown to sequestrate MDM2 in nucleus and to impair p53 degradation *(Bernardi et al., 2004; de Stanchina et al., 2004).* Yin Yan protein (YY1) is a MDM2 cofactor that acts as a negative regulator of p53, inducing its ubiqutination and its degradation *(Sui et al., 2004).* Phospholipase D1 (PLD1) was shown to stimulate MDM2 expression and to suppress p53 activation *(Hui et al., 2004).*

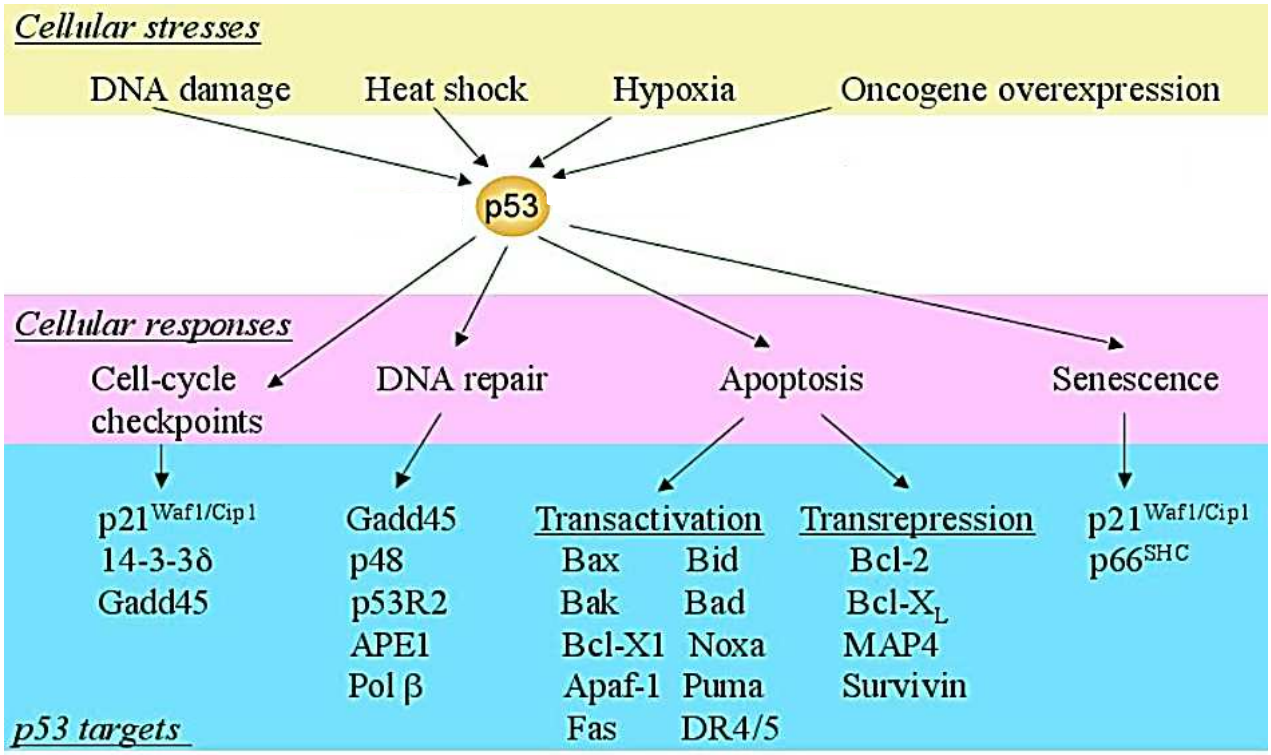

Figure 6: p53 functional network. Various stress signals activate p53, which leads to the induction of diverse p53 downstream target genes. These genes are involved in cell cycle arrest, DNA repair, apoptosis and senescence. The different gene expression is dependent on the cell type, the type of stress signals, and the cellular context. Besides gene transactivation, p53 is able to suppress gene expression *(*Adapted from *Bai and Zhu, 2006).*

It is important to note, that p53 transactivates MDM2 gene expression, thus creating a negative auto-regulatory feedback loop, in which MDM2 inhibits p53 stability, and in which p53 activates MDM2 expression *(Barak et al., 1993; Perry et al., 1993; Wu et al., 1993).*

Altogether, p53 stabilization is a very complex system of diverse posttranslational modifications of p53 and of protein binding members that regulates p53 stability. Only some modes of p53 regulation were mentioned here but many other events modulating p53 activation are, known or for some of them are still unknown.

3. Cellular responses induced by p53

In response to various stresses, p53 accumulates in the nucleus and binds in tetramer form to a specific DNA sequence, termed the p53-responsive element, composed of RRRCWWGYYY (a spacer of 0-21 nucleotides) RRRCWWGYYY (where R is purine, W is adenine or thymine and Y is a pyrimidine) *(Kern et al., 1991; Riley et al., 2008; Menendez et al., 2009).* Hundreds of p53-responsive genes have been reported. These genes are involved in the cell cycle arrest, DNA repair, senescence and apoptosis. Besides its transcriptional activity, p53 was shown to directly repress several genes, often involved in abrogation of apoptosis (*Mack et al., 1993; Vousden and Lu, 2002)* **(Figure 6)**. Among several responses that can be provoked by activated p53, the most understood are the induction of cell cycle arrest and apoptosis.

3.1. Regulation of the cell cycle

The protein p53 is able to induce cell cycle arrest in the G1, S and G2 phases. Since p53 deficient cells failed to undergo G1/S arrest after genotoxic stress, p53 was described as a critical regulator of G1/S transition *(Baker et al., 1990; Diller et al., 1990; Martinez et al., 1991).* The arrest of the cell cycle progression at G1/S checkpoint in response to DNA damage provides additional time to repair genomic damage and to prevent the replication of mutated DNA. A major player in the p53-mediated G1 arrest is a cyclin-dependent kinase inhibitor, p21 *(Harper at al., 1993; El-Deiry at el., 1993).* Upon p53 activation, the expression of p21 is up-regulated *(El-Deiry at el., 1993).* p21 binds and inactivates

cyclin-CDK (cyclin dependent kinase) complexes that in normal conditions promote G1 phase progression. This inhibition leads in turn to hypo-phosphorylation of Retinoblastoma (RB) protein. In its hypo-phosphorylated state, pRB binds and inhibits E2F transcription factor that is required for S phase entry *(Chen et al., 1996).* Deng and Brugarolas et al., demonstrated that embryonic fibroblasts lacking p21 were deficient in their ability to promote G1 arrest in response to DNA damage *(Deng et al., 1995; Brugarolas et al., 1995).* Moreover, the deletion of both p21 alleles in p53wt human colon cancer cell lines inhibited the ability of DNA damage to provoke G1/S arrest *(Waldman et al., 1995).* All these data demonstrate the importance of p21 in the p53-mediated G1/S cell cycle arrest.

p53 mediated induction of p21 also results in a S phase arrest. p21 was shown to bind to PCNA nuclear protein and to prevent PCNA dependent recognition of DNA primer-template complex, which results in the inhibition of the prolongation step during DNA replication *(Waga et al., 1994; Waga and Stillman, 1998).*

The p53-induced G2 arrest is mediated through transcriptional upregulation of the downstream target genes, including p21, 14-3-3σ and GADD45. Similarly to G1/S checkpoint, p21 binds and inhibits cyclin B1-CDK2 complex *(Harper at al., 1995; Innocente et al., 1999).* Protein 14-3-3σ modulates subcellular localization of CDK2 in a p53-dependent manner and retains it in the cytoplasm *(Chan et al., 1999).* The p53 dependent increase of GADD45 in primary fibroblasts resulted in a G2 arrest *(Wang et al., 1999).* Moreover, it was shown that GADD45 was able to directly inhibit the cyclin B1-CDK2 complex after UV irradiation *(Zhan et al., 1999).*

The protein p53 is also a pivotal player in the regulation of senescence. The cellular senescence is an irreversible form of cell cycle arrest *(Itahana et al., 2001).* Senescent cells remain metabolically active but they are characterized by morphological and physiological changes such as enlarging cell size, chromatin condensation, changes in gene expression, and high levels of senescence-associated β-galactosidase. They are unable to replicate DNA and consequently undergo permanent cell-cycle arrest. p53-driven senescence involves several p53-downstream proteins, such as p21, PML (Promyelocytic leukaemia), PAI-1 (Plasminogen activator inhibitor 1) and DEC1 (Deleted in esophageal cancer 1) *(Vousden and Prives, 2009; Zuckerman et al., 2009).* p53/p21 interplay is critical for the driving of senescence. The cellular choice to undergo transient or irreversible cell cycle arrest has still to be elucidated *(Vousden and Prives, 2009)*.

3.2. Apoptosis

One of the most extensively studied areas in p53 research surrounds its ability to control apoptosis. The apoptotic cell death is a very complex process regulated at multiple levels. Two different apoptotic signaling pathways leading to the activation of specific caspases have been described. The intrinsic apoptotic program is associated with mitochondrial depolarization and the release of the cytochrome c from the mitochondria into cytoplasm. Cytochrome c together with Apoptotic protease activating factor (APAF) and procaspase 9 form the complex termed apoptosome, by which initiator caspase 9 is activated and promotes the activation of effector caspases, caspase-3, -6 and -7. The extrinsic pathway requires particular cell surface death receptors that belong to the tumor necrosis receptor family. The death receptors induce the formation of the death inducing signaling complex (DISC), which results in the activation of initiator caspase 8 and effector caspase 3, and in the promotion of apoptosis *(Monian and Jiang, 2012).* Several studies suggest that the extrinsic and intrinsic pathways are more two converging than two distinct pathways *(Li et al., 1998; Gross et al., 1999).* p53 activates the transcription of key pro-apoptotic genes of both intrinsic and extrinsic pathways. Moreover, p53 represses the gene expression of several anti-apoptotic proteins. Nevertheless, p53 itself may translocate to mitochondria and activate apoptosis in a transcriptional independent manner.

3.2.1. The intrinsic apoptotic pathway

The intrinsic apoptotic pathway is mainly regulated by a family of proteins termed BCL-2, which governs the release of cytochrome c from mitochondria as the crucial step of the intrinsic apoptosis *(Cory and Adams, 2002; Monian and Jiang, 2012).* The BCL-2 family includes both anti- and pro-apoptotic members, and is divided into three groups on the basis of structural similarity to the BCL-2 homology domains (BH1, BH2, BH3 and BH4).

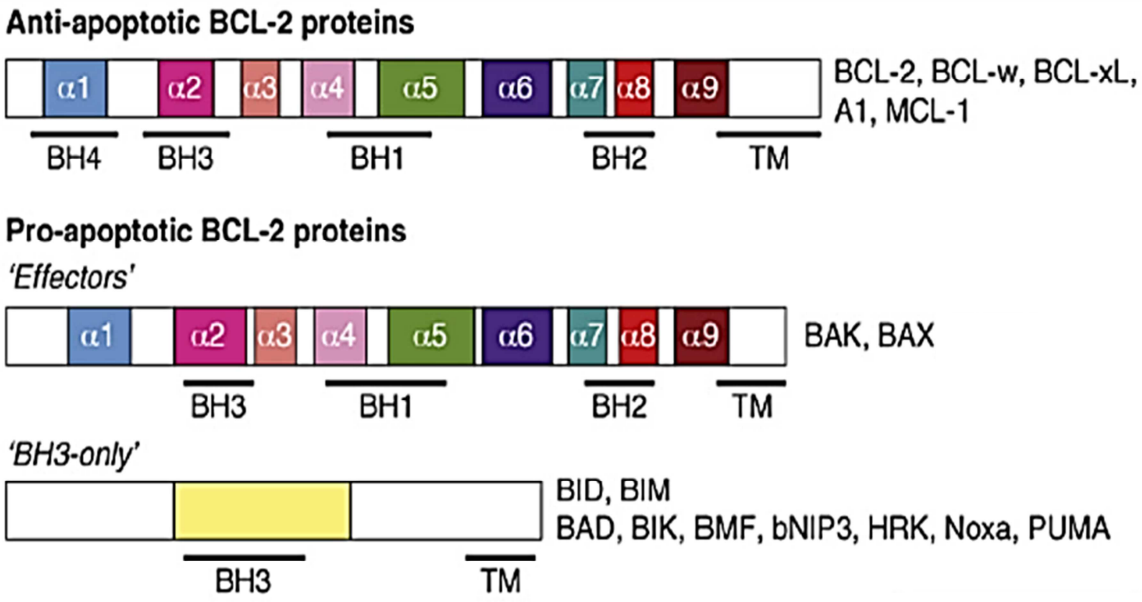

Figure 7: BCL-2 related proteins. The BCL-2 family is divided into three groups according to their homology in BCL-2 domains. Known α-helical and transmembrane regions are indicated. The anti-apoptotic members, such as BCL-2, BCL-X_L, BCL-w, MCL-1 and A1 comprise four BCL-2 homology domains (BH1, BH2, BH3 and BH4). The pro-apoptotic members termed as effectors including BAX and BAK, contain BH1, BH2 and BH3 domains. The BH3-only pro-apoptotic proteins, such as BID, BIM, BAD, BIK, NOXA, PUMA and others, include only BH3 domain (*Chipuk and Green, 2008*).

The BCL-2 family includes anti-apoptotic proteins such as BCL-2, BCL-X_L, BCL-w, MCL-1 and A1 with sequence homology at BH1, BH2, BH3, BH4 domains. The anti-apoptotic function is related with their ability to directly bind, and thus inhibit pro-apoptotic proteins.The pro-apoptotic proteins are divided into the effector proteins and into BH3-only proteins. Pro-apoptotic members termed as effectors are BAX and BAK with a sequence homology at BH1, BH2 and BH3. Upon activation, BAK and BAK homo-oligomerize into proteolipid pores, which results in the permeabilization of outer mitochondrial membrane, and to cell death. The pro-apoptotic proteins that only share homology at the BH3 domain are termed BH3-only proteins. These members are subdivided according to their ability to interact with the anti-apoptotic BCL-2 proteins or both anti-apoptotic proteins and the effectors. The BH3-only proteins binding only to the anti-apoptotic proteins are called sensitizer. BAD, BIK, NOXA are those sensitizers. BID and BIM bind to the anti-apoptotic members as well as to the effectors, and thus induce BAK and BAX oligomerization, leading to cell death. BID and BIM are referred to as direct activators *(Haupt et al., 2003; Chipuk and Green, 2008).* The assignment of PUMA is controversial. PUMA was at first classified as a sensitizer *(Kuwana et al., 2005; Certo et al., 2006)*. However, some studies have shown that PUMA interacts with BAX and causes its activation *(Cartron et al., 2004; Kim et al., 2006; Gallenne et al., 2009).* **Figure 7** summarizes three subfamilies of BCL-2-related proteins.

The p53-dependent apoptotic response involves transcriptional activation of multiple pro-apoptotic target genes. p53 transactivates key molecules of the intrinsic pathway, including pro-apoptotic BCL-2 related proteins such as BAX *(Miyashita and Reed, 1995),* NOXA *(Oda et al., 2000),* PUMA *(Nakano and Vousden, 2001)* and BID *(Sax et al., 2002).* Moreover, p53 can activate the expression of APAF, which acts as a co-activator of caspase 9 and helps to induce the activation of effector caspases *(Fortin et al., 2001; Moroni et al., 2001; Robles et al., 2001).* p53 was shown to have a capacity to trans-repress BCL-2 anti-apoptotic genes such as BCL-2 *(Wu et al., 2001)* and MCL-1 *(Pietrzak and Puzianowska-Kuznicka, 2008).* Interestingly, BCL-2 non-related anti-apoptotic proteins expression can also be inhibited by p53. For example, negative regulator of apoptosis Survivin may be repressed by p53 *(Hoffman et al., 2002).*

The first member shown to be induced by p53 was BAX *(Miyashita and Reed, 1995; Thornborrow et al., 2002).* BAX is normally distributed in the cytoplasm, or loosely associated with mitochondrial membrane. In response to stress, BAX undergoes conformational change followed by homo-

multimerization or hetero-multimerization with BAK. The oligomerization creates proteolipid pores leading to the outer mitochondrial membrane permeabilization and the release of cytochrome c from the mitochondrial intermembrane space to cytosol. Cytochrome c forms a complex with APAF-1 and caspase 9 termed the apoptosome. The apoptosome cleaves and activates at first initiator caspase 9 that recruits and activates the effector caspases, resulting in widespread proteolysis and cell death *(Monian and Jiang, 2012)*.

The pro-apoptotic BH3-only proteins including BID and BIM were shown to be involved in the direct induction of BAX and/or BAK oligomerization, leading to cell death *(Desagher et al., 1999; Wei et al., 2001; Letai et al., 2002; Marani et al., 2002).* The direct interaction helps BAX/BAK recruitment, so that BID and BIM have been termed as direct activators. The *in vitro* studies demonstrated the cooperation between effector molecules and the direct activators but it appears to have very weak affinity *(Korsmeyer et al., 2000; Kuwana et al., 2002)*. Moreover, a BIM mutant that fails to bind BAX was shown to have the ability to induce apoptosis at the same extent as wild type BIM does *(Willis et al., 2007)*. These results led to the speculation that the interaction of effector molecules with the direct activators is not essential for apoptosis, and that the crucial step is in fact the interaction between BH3-only proteins and anti-apoptotic proteins.

As mentioned above, BCL-2 family contains the anti-apoptotic proteins with BH 1-4 domains generally integrated within the outer mitochondrial membrane. These proteins have a critical role in apoptotic pathway since they were shown to directly bind and inhibit the pro-apoptotic BCL-2 proteins. Upon stress, all anti-apoptotic proteins are functionally neutralized by activated BH3-only proteins. This neutralization is driven by the direct binding of BH3-only proteins to anti-apoptotic proteins through BH3 domain. The inhibition of anti-apoptotic function promotes BAX and/or BAK liberation and cytochrome c release with subsequent induction of apoptosis. Differences in the ability of BH3-only proteins to bind anti-apoptotic have been described. BID, BIM and PUMA bind to all anti-apoptotic proteins. By contrast, BAD and NOXA selectively bind BCL-2, BCL-X_L, BCL-W and MCL-1, respectively *(Chipuk and Green et al., 2008).* BAX was shown to be sequestered by MCL-1 and BCL-X_L. The increase of PUMA expression levels liberate BAX through direct binding to the MCL-1 and/or BCL-X_L, which indirectly facilitates BAX oligomerization and cytochrome c release. BID can be also released from BCL-X_L by PUMA, which can result in transient interaction with BAX and induction of

apoptosis. Taken together, mitochondrial permeability is determined by the balance between the pro-apoptotic and anti-apoptotic proteins *(Chipuk and Green et al., 2008)*.

Besides the role of p53 to transactivate and to transrepress its target genes, several groups demonstrated the role of p53 independently on its transcriptional activity *(Speidel, 2010; Green and Kroemer, 2009)*. A mutant of p53 unable to induce transcription has been shown to effectively induce apoptosis *(Haupt et al., 1995; Haupt et al., 1997; Marchenko et al., 2000)*. After DNA damage or hypoxia, p53 was shown to be located in mitochondria, where it directly interacts with anti-apoptotic proteins such as BCL-2 and BCL-X_L *(Sansome et al., 2001; Mihara et al., 2003).* Moreover, it was demonstrated that p53 displaced pro-apoptotic proteins BID or BAX from BCL-X_L *(Chipuk et al., 2004; Green and Kroemer, 2009; Speidel, 2010).* Nevertheless, PUMA induced by p53-dependent genotoxic stimuli, displaced p53 from a cytosolic inhibitory p53/BCL-X_L complex and liberated p53. Liberated p53 subsequently bound to, and thus activated BAX, which in turn triggered mitochondria permeabilization *(Chipuk et al., 2004; Chipuk et al., 2005; Green and Kroemer, 2009; Speidel, 2010).* Although numerous research groups highlighted a transcription-independent role for p53, it is still unclear how much this function of p53 contributes to apoptosis, compared to the role of p53 in the transcriptional activation.

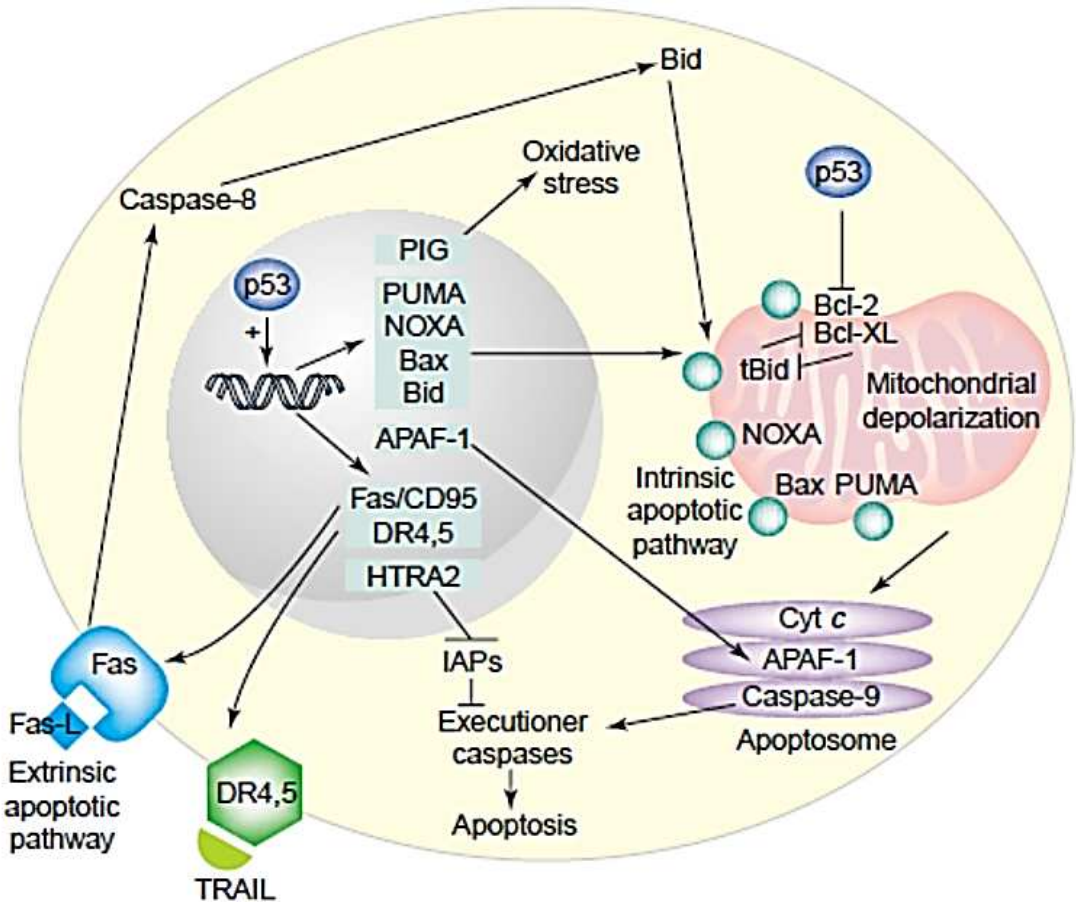

Figure 8: The complexity of p53-dependent apoptosis. The role of p53 in apoptosis is transcriptionally dependent and transcriptionally independent. After death stimuli, p53 activates gene transcription of key anti-apoptotic genes involved in both intrinsic and extrinsic apoptotic programs. The intrinsic apoptotic pathway is associated with p53 driven transcription of pro-apoptotic genes, including BAX, BID, NOXA and PUMA. Protein p53 also inhibits the transcription of BCL-2 anti-apoptotic genes, such as BCL-2 and BCL-X_L. In addition, p53 activates the expression of pro-apoptotic genes involved in death receptor extrinsic apoptosis, including FAS, DR4 and DR5. In transcriptional independent manner, p53 directly binds and inhibits BCL-2 and BCL-X_L in cytoplasm. These events result in activation of specific initiator caspases and recruitment of effector caspases leading to apoptotic cell death *(Hofseth et al., 2004).*

3.2.2. The extrinsic apoptotic pathway

The extrinsic apoptotic pathway is also regulated by p53, although the overall contribution of this regulation to p53-mediated cell death is poorly understood. The extrinsic pathway required particular death receptors that belong to the tumor necrosis factor receptor family.

Protein p53 activates extrinsic apoptotic pathway through the stimulation of genes transcription of three transmembranes proteins, such as FAS *(Muller et al., 1998)*, DR4 *(Liu et al., 2004)* and DR5 *(Takimoto and El-Deiry, 2000)*.

The cell-surface receptor FAS is a key component of the p53-dependent extrinsic death pathway *(Nagata and Golstein, 1995; Muller et al., 1998).* FAS is activated through the binding of its ligand FASL, that results in its trimerization. The adaptor proteins bind via their FAS-associated death domains (FADD) to cytosolic death domain of FAS trimer. Then procaspase 8 and 10 are recruited via their death effector domain motifs, which results in the formation of multi-protein death-inducing signaling complex (DISC). Active caspase 8 can cleave and activate caspase 3 or other effector caspases, leading to the apoptotic cell death *(Chinnaiyan et al., 1996; Kischkel et al., 2001).* Caspase 8 can also cleave BH3-only member, BID. Truncated BID translocates to the mitochondria and activates BAX and BAK. BID has a unique ability to link the activation of the extrinsic death receptor pathway with mitochondrial intrinsic pathway *(Fridman and Lowe, 2003).*

In addition to stimulating FAS transcription, p53 overexpression enhances the levels of FAS at the cell membrane by promoting trafficking of the FAS receptor from Golgi apparatus *(Bennett et al., 1998)*. The way p53 exactly promotes FAS trafficking remains to be elucidated.

Figure 8 summarizes the complexity of p53-dependent apoptosis.

3.3. The choice between cell cycle arrest and apoptosis

Although p53 can be a potent activator of apoptotic cell death, in reality it does not always induce apoptosis when it is up-regulated. The exact criteria by which p53 decides between cell cycle arrest and apoptosis is only partially understood and is under investigation. Many known and still unknown factors influence this decision, involving p53 expression levels, the type of stress signal, the cell type and the cellular context at the time of stress exposure *(Vousden and Lu, 2002; Vousden and Prives, 2009).*

It was suggested, that the choice between these two outcomes reflected distinctive affinities of p53 for the binding sites in different promoters. In the case of low levels of p53 protein, p53 promotes cell-cycle arrest through the high-affinity binding to cell cycle related genes. On the other hand, apoptotic cell death is only induced when p53 attains high levels, suggesting that the promoters of apoptotic genes have a lower affinity for p53 *(Chen et al., 1996).* Chromatin immunoprecipitation analysis confirmed the presence of high- and low-affinity p53 response elements. However, high-affinity p53 response elements have also been found in pro-apoptotic genes promoters, such as PUMA. The binding affinity of p53 to pro-apoptotic PUMA promoter is similar to the p21 and MDM2 promoters. These results show that all pro-apoptotic genes do not bind p53 with low affinity (*Kaeser and Iggo, 2002).* The limitations of these studies could be the fact that p53-dependent apoptosis and cell cycle arrest were usually caused by various DNA damage stimuli. The use of non-genotoxic agents overrides those limitations. In a recent study, Kracikova and co-workers showed that p53 activated by a non-genotoxic agent, Nutlin-3a, enhanced pro-arrest and pro-apoptotic genes proportionally to its expression levels. Low levels of p53 could easily initiate the cell cycle arrest, but low levels of pro-apoptotic genes failed to trigger apoptosis. They hypothetised that p53-mediated cell fate decision between arrest and apoptosis is determined by a higher execution threshold for the initiation of apoptosis, than for cell cycle arrest *(Kracikova et al., 2013).*

The protein p53 interacts with other regulators to activate its target, suggesting that the implication of p53-binding proteins may be important to modulate gene selection by activated p53. For instance, p53 forms a complex with p300/CBP. This interaction leads to the histones acetylation and opening up the surrounding chromatin, which allows access of the transcriptional machinery *(Espinosa and Emerson,*

2001). Junction mediating and regulatory protein (JMY), for example, was shown to cooperate with p300 and to enhance p53 induction of BAX gene, whereas p21 gene was not *(Shikama et al., 1999).*

The regulation of apoptotic cell death can be affected by p53-binding proteins that directly modulate the ability of p53 to interact with DNA. For example, Apoptotic stimulating proteins of p53, ASPP1 and ASPP2, were shown to interact directly with p53 and thus enhance its affinity to apoptotic genes *(Samuels-Lev et al., 2001).* The other members of p53 family, p63 and p73, were identified to regulate p53-induced cell death. In this example, p53 was not able to bind promoters of apoptotic targets in p63/p73 double-null cells *(Flores et al., 2002).*

In addition, posttranslational modifications might regulate the conformation of p53 and its interaction with different components modulating p53 transcriptional preference. For example, the phosphorylation of p53 on Ser46 has been suggested to be necessary to induce the expression of apoptotic target genes conversly to the mediators of the cell-cycle arrest *(Bulavin et al., 1999; Oda et al., 2000).* In addition to the phosphorylation, other posttranslational modifications can be involved in the determination of p53 response choice. The acetylation of p53 on C-terminal was shown to regulate the transcriptional activity of p53 in some circumstances *(Prives and Manley, 2001).* Interestingly, p73 has been shown to selectively enhance the activation of apoptotic target genes. Non-acetylated p73 was unable to activate the transcription of pro-apoptotic p53AIP1 gene, but retains an intact ability to transactivate the other targets such as p21 *(Costanzo et al., 2002)*. These results suggest and indicate that p53 could be modulated by a similar way.

The control of subcellular localization of p53 is also an important modulating component of the p53-response pathway. p53 shuttling from the cytoplasm to the nucleus is strictly controlled, and an impairment can cause the incapacity of p53 to induce apoptosis (*Vousden and Lu, 2002)*.

The choice between cell death and survival is also dependent on the activity of survival signals that can rescue cells from p53 induced apoptosis. The activation of AKT kinase was demonstrated to protect cells from apoptosis induced by p53 *(Sabbatini and McCormick, 1999).* AKT can phosphorylate and activate MDM2, which results in the deregulation of p53 through its degradation *(Mayo and Donner, 2001; Zhou et al., 2001).* AKT was also shown to phosphorylate BAD and thus inhibit its function through its interaction with 14-3-3 proteins *(Pommier et al., 2004).* Moreover, AKT phosphorylates and activates CREB transcription factor, which enhances the transcription of anti-

apoptotic BCL-2 gene and stimulates the cell survival *(Du and Montminy, 1998; Pugazhenthi et al., 2000).* Thus, AKT can protect the cell from p53-dependent apoptosis in various ways. Interestingly, AKT inhibition effect on p53-dependent apoptosis is counteracted by the p53-induced expression of PTEN gene that is a potent inhibitor of AKT signaling *(Stambolic et al., 2001).* Thus, besides the direct role of p53 to differentially regulate the expression of specific genes involved in the cell cycle arrest and the apoptosis, the response of cells is greatly depending on cellular context, which may render the cells resistant to apoptosis.

4. p53 and cancer

4.1. Inactivation of p53 in cancer

Wild type p53 is required to maintain the genomic integrity, and to protect cells from malignant transformation. On the other hand, some of the p53 mutants are considered to be oncogenes that can drive the tumor development. Patients with the rare Li-Fraumeni syndrome which have an inherited germline mutation in one of two p53 alleles are at very high risk of developing cancer (*Malkin et al., 1990; Srivastava et al., 1993).* Moreover, p53-deficient mice develop spontaneous cancers *(Donehower et al., 1992).* The function of p53 tumor suppressor in cancers can be lost by different mechanisms, including mutations within the p53 gene itself, mutations that impede activation of p53 or mutations of downstream factors of p53 pathway *(Vousden and Lu, 2002).*

Since the mutation of p53 has been found in 50% of human cancers, p53 gene is one of the most frequently inactivated genes in diverse human cancers *(Hollstein et al., 1991).* The prevalence of p53 mutations is variable and depending on the type of cancer. For instance, p53 is frequently mutated at about 70% in lung cancer, 60% in cancer of colon, head and neck, ovary and 45% in stomach cancer. The analysis of many tumors has shown that mutation of p53 gene results in a loss of the apoptotic function. Reported mutations are found mainly in the central region (95%) responsible for sequence-specific DNA binding and 5% in the regulatory domain. In the DNA binding domain, missence mutations have been found at almost all residues, but the most frequent mutations occur in six codons termed hotspots codons: 175, 245, 249, 248, 273 and 282 *(Vousden and Lu, 2002)*. The main consequence of p53 gene mutations is the loss of specific DNA-binding and transcriptional activity.

However, some p53 mutants retain transcriptional activity for a subset of target genes. Some tumor-derived p53 point mutants were characterized as transcriptionally active. These mutants retained the ability to activate the expression of p21 and to induce cell cycle arrest. However, these mutants failed to activate the expression of BAX gene, which correlated with the impaired apoptotic function displayed by those mutants *(Ludwig et al., 1996).* Moreover, most of the promoters shown to be activated by p53 mutant do not contain the sequences for p53 wt binding, suggesting that p53 mutant may regulate transcription of other genes without p53 wt response element *(Kim and Deppert, 2004).* These genes are generally implicated in the promotion of tumor cell growth and in the survival and the development of resistance to several chemotherapeutic agents *(Martinez-Rivera and Siddik, 2012)*. This 'gain of function' of p53 mutant that changes the selectivity of DNA targets was suggested to be the driving force of p53 mutant to act as an oncogenic factor.

p53 mutants also exhibit dominant-negative activities that shutdown p53 wt function through the direct binding to p53 wt. p53 mutant was shown to heterodimerize with p53 wt and to decrease the ability of wild type p53 to bind to its various specific DNA target sequences *(Shaulian et al., 1992; Unger et al., 1993; Chene, 1998; Srivastava et al., 1993; Kern et al., 1992).* There are as well evidences that the p53 mutant may also mediate its effects through inhibitory interactions with other transcriptional factors that normally bind to gene promoters. For example, p53 mutant was shown to bind p63 and p73 proteins and consequently to prevent them from activating their gene targets *(Brosh and Rotter, 2009).*

Tumors that retain wt p53 very often have defects, either in the pathways regulating the stabilization of p53 in response to stress, or in the effectors of p53's activity *(Woods and Vousden, 2001).* For example, elevated MDM2 expression that results in the suppression of p53 function was found in many tumor types. A 2-fold increase in MDM2 was reported to be sufficient to attenuate p53 activation, and thus to increase the risk of breast cancer *(Wade et al., 2010).* In parallel, the up-regulation of MDM2 reduced the therapeutic response of breast cancer. Patients presenting high MDM2 expression levels show significantly lower 10-year survival rate compared to survival rate of patients with normal expression levels of MDM2 *(Turbin et al., 2006).* Alternatively, p53 function can be attenuated by inactivating mutations in genes encoding positive regulators or effectors of p53. One such example is CHK2. CHK2 is a kinase that phosphorylates p53 at serine 20. This phosphorylation disrupts MDM2-p53 complex, which results in the stabilization of p53 protein. CHK2 was found to be inactivated in a

great variety of carcinoma. Another example is the downstream target of the p53 activation, APAF1. APAF1 was shown to be inactivated in chemoresistant malignant melanomas, tumors that usually retain intact p53. The result of this inactivation is that p53 is not able to initiate cell death *(Soengas et al., 2001).*

4.2. p53 as a therapeutic target

The indication that p53 restoration can suppress cancer came with studies showing that the reactivation of p53 in established tumors can stop tumor growth. The cellular response to p53 restoration is cancer type-specific. The re-expression of p53 induced apoptotic cell death in lymphomas, whereas sarcomas underwent cell cycle arrest, with signs of cellular senescence *(Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007)*. These promising results, associated to the already discussed importance of p53 in tumorigenesis, demonstrate that p53 protein is an attractive target for the anticancer therapies.

Several different approaches have been proposed with the aim of restoring p53 function. The most direct mechanism to induce p53 wild type is the disruption of p53 binding to its negative regulator MDM2. Several classes of drugs inhibiting MDM2-p53 interaction have been reported including analogs of spiro-oxindole, benzodiazepine, terphenyl, chalone and others. Two of the most potent and widely studied MDM2 small molecule inhibitors are spiro-oxindole inhibitor MI-219 *(Shangary et al., 2008)*, and cis-imidazoline analog Nutlin-3a *(Vassilev et al., 2004; Vassilev, 2004).*

Nutlin-3a is the best studied MDM2 antagonist. Nutlin-3a is a cis-imidazoline mimicking critical residues in the transactivation domain of p53, which are essential for MDM2 binding *(Vassilev et al., 2004; Vassilev, 2004).* Nutlin-3a potently displaces p53 from the p53-binding pocket on MDM2. This inhibition of MDM2-p53 interaction triggers p53 activation, which in turn induces cell cycle arrest, senescence or apoptotic cell death, depending on the cellular context. It is important to note, that these effects are observed strictly in cells with p53 wild type, but not in cells with p53 mutant *(Kojima et al., 2005)*. Interestingly, in normal cells, Nutlin-3a only induces a reversible growth arrest but not apoptosis, suggesting that the activation of p53 by a MDM2 inhibitor is nontoxic to normal cells, and is thus attractive for cancer therapy. In animal xenograft models, Nutlin-3a controlled tumor growth and

did not show toxicity in normal tissue *(Shangary et al., 2008; Shangary and Wang, 2009).* Importantly, Nutlin-3a shows synergism with conventional chemotherapeutic drugs, such as doxorubicin and cytarabine, radiation, TRAIL and inhibitors of JNK, PI3K, aurora kinase, pointing to the possibility of a combination therapy for the treatment of advanced stage blood, brain and bone cancers (*Stegh, 2012).* Nutlin-3a is currently in Phase I clinical trial for the treatment of retinoblastoma.

Besides the antagonists that inhibit MDM2-p53 interaction by binding to MDM2, small molecules binding to and thus directly activating p53 were reported. For example RITA interacts and thus stabilizes p53. RITA induces a conformational change in p53 that results in the prevention of MDM2 to bind and to degrade p53. RITA was shown to increase p53-dependent antitumor effect *in vitro* and *in vivo (Issaeva et al., 2004).* At this time, RITA is only in preclinical studies but it may be another attractive tool for the restoration of p53 function in the cancer therapy.

AIMS OF THE STUDY

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Glioblastomas (GBMs) are the most common and most aggressive brain tumors. The median survival for patients with GBMs is about one year. Despite several decades of ongoing neuro-physiopathology research as well as of clinical studies, an efficient therapy is still lacking. Previous results of our laboratory suggest that $\alpha_5\beta_1$ integrin may be a promising new therapeutic target for these aggressive tumors. The main aims of the first part of this work were:

- to investigate the role of $\alpha_5\beta_1$ integrin in the resistance of GBMs to temozolomide
- to study a combination therapy of an $\alpha_5\beta_1$ integrin antagonist with two different p53 activators in GBMs

In the second part of this work, we investigated the role of $\alpha_5\beta_1$ integrin in colorectal cancer, another solid tumor.

MATERIALS AND METHODS

Materials

1. CELLULAR MODELS

U87MG glioblastoma cell line (p53 wild type) was from American Type Culture Collection (LGC Standards Sarl, Molsheim, France).

U373 glioblastoma cell line (p53 mutant) was from ECACC (Sigma, Lyon, France).

LN18 glioblastoma cell line (p53 mutant) was a generous gift from M. Hegi (University Hospital, Lausanne, Switzerland).

LNZ308 glioblastoma cell line (p53 knockout) was a generous gift from M. Hegi (University Hospital, Lausanne, Switzerland).

HCT116 p53+/+ colon carcinoma cell line (p53 wild type) was a kind gift from Pr. Vogelstein (Baltimore, USA).

HCT116 p53-/- colon carcinoma cell line (p53 knockout) was a kind gift from Pr. Vogelstein (Baltimore, USA).

The identity of cell lines was regularly checked by morphologic criteria, and importantly p53 status was routinely checked by Western blot quantification of p53 stability and phosphorylation, and by qPCR quantification of p53 target genes after treatment with Nutlin-3a.

2. TREATMENT AGENTS

Temozolomide (TMZ), 8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4-(3H)-one, was a kind gift from Schering-Plough. TMZ was prepared before use at 10mM in 50/50 ethanol/H_2O.

Nutlin-3a, (4-[4,5-bis-(4-chlorophenyl)-2-(2-isopropoxy-4-methoxy-phenyl)-4,5-dihydro-imidazole -1-carbonyl]-piperazin-2-one), the active enantiomer, was from Cayman chemical company (Interchim, France). Nutlin-3a was prepared as stock solution in ethanol at 10mM and was kept at -20℃ until use.

RITA, (5,5'-(2,5-furandiyl)bis-2-thiophenemethanol), was from Cayman chemical company (Interchim, France). RITA was prepared as stock solution in ethanol at 10mM and was kept at -20℃ until use.

K34c, (2-(S)-2,6 dimethylbenzamido)-3-[4-(3-pyridin-2-ylaminoprooxy)-phenyl]propionic acid) was synthesized in our laboratory according to the procedure described by *Heckmann et al. (2008)*. K34c was prepared as 10mM stock solution in DMSO and kept at 4˚C.

3. ANTIBODIES

Western Blot:

Antibody	Blocking solution	Antibody Dilution
anti-α_5 integrin H104 (Santa Cruz)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-α_5 integrin Ab1928 (Millipore)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-β_1 integrin Ab1952 (Millipore)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-p53 (BD Biosciences)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-p53Pser15 (Cell Signaling) *****	5% milk/1xTBS/0.1% Tween-20	1/500
anti-GAPDH (Millipore)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-caspase 3 cleaved (Cell Signaling)	5% milk/1xTBS/0.1% Tween-20	1/1000
anti-caspase 7 cleaved (Cell Signaling)	5% milk/1xTBS/0.1% Tween-20	1/1000
anti-caspase 8 (Cell Signaling)	3% BSA/1xTBS/0.1% Tween-20	1/500
anti-caspase 9 cleaved (Cell Signaling)	5% milk/1xTBS/0.1% Tween-20	1/500
anti-PARP (Millipore)	3% BSA/1xTBS/0.1% Tween-20	1/500
anti-AKT (Cell Signaling)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-AKTPser473 (Cell Signaling)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-GSKβser9 (Cell Signaling)	3% BSA/1xTBS/0.1% Tween-20	1/1000

anti-BCL-2 (Santa Cruz)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-MCL-1 (BD Biosciences)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-Survivin (Millipore)	3% BSA/1xTBS/0.1% Tween-20	1/1000
anti-PEA15 (Cell Signaling)	5% milk/1xTBS/0.1% Tween-20	1/500

anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (Promega)

Specific primary antibodies were diluted in the blocking solution specified above (3% BSA/1xTBS/0.1% Tween-20 or 5% nonfat dry milk/1xTBS/0.1% Tween-20). Secondary antibodies were diluted in the same blocking solution as for the primary antibody (usually 1/10 000). ***** With the exception of p53Pser15 antibody where: the blocking solution contained 5% nonfat dry milk/1xTBS/0.1% Tween-20, primary antibody was diluted in 3% BSA/1xTBS/0.1% Tween-20 (1/500) and secondary antibody was diluted in 5% nonfat dry milk/1xTBS/0.1% Tween-20 (1/10 000).

Immunofluorescence and Flow cytometry:

anti-α_5 IIAI (BD Biosciences)

anti-β_1 TS2/16 (Santa Cruz)

anti-β_1 mAB13 (BD Biosciences)

anti-β_1 9EG7 (BD Biosciences)

Alexa Fluor 488 labeled goat anti-mouse secondary antibody (JacksonimmunoResearch Laboratories)

FITC labeled goat anti-rat secondary antibody (JacksonimmunoResearch Laboratories)

Methods

1. CELL CULTURE

Cell lines were routinely grown in Eagle's MEM supplemented with 10% heat-inactivated FBS, 0.6 mg/mL, glutamine and 200 IU/mL penicillin/streptomycin. Cell cultures were maintained in a water saturated atmosphere at 37℃ under 5% CO_2/ 95% air. All experiments were performed in 2% FBS containing EMEM medium unless specified.

2. CELL TRANSFECTIONS

siRNA transfection

Specific siRNA for human α_5 and β_1 integrin subunits, p53 and non-targeting siRNA were obtained from Dharmacon (Thermo Scientific) and the transfection reagent jetPRIME™ was purchased from Polyplus Transfection. Manufacturer's instructions were followed to transiently supress the expression of the different proteins. The transfection efficiency was verified by immunoblotting.

pcDNA and shRNA transfections

Cells were stably transfected to overexpress (by transfecting a pcDNA3.1 plasmid containing the human α_5 integrin gene, provided by Dr. Ruoshlati, University of California, Santa Barbara, CA) or to repress (by transfecting a pSM2 plasmid coding for a short hairpin RNA (shRNA) targeting α_5 mRNA) the α_5 integrin subunit by using jetPRIME (Polyplus transfection) according to the manufacturer's instructions. The overexpression and the depletion of the α_5 subunit were detected at mRNA and protein levels. Flow cytometry analysis confirmed that only α_5 and neither β_1 nor $\alpha_v\beta_3$ integrin expression levels changed at the plasma membrane. Similarly, the cells were transiently transfected with empty pcDNA3.1 plasmid or p53wt gene-containing pcDNA3.1. The pcDNA3.1-p53wt vector was a kind gift from Dr. C. Blattner (Karlsruhe, Germany). The transfection efficiency was verified by immunoblotting.

3. CLONOGENIC ASSAY

Cells were plated (500 cells/well) into 6-well culture plates either uncoated or coated with human fibronectin (10 µg/ml) and treated for 72 hours with specific drugs or solvents in 2% FBS containing medium. Purified human fibronectin was a kind gift from Dr. F. Carreiras (Cergy-Pontoise, France). Medium was thereafter renewed with fresh 10% FBS containing medium and cells were allowed to grow further for 7 days. Colonies were fixed and stained with crystal violet/ethanol (0.1%, w/v) and counted. The surviving fraction was determined by the ratio of cells surviving to a specific drug treatment relative to their solvent-treated counterparts.

4. CELL VIABILITY ASSAY

To determine cell viability, Trypan Blue exclusion method was used. This method is based on the principle that live cells with intact cell membranes exclude Trypan blue dye, whereas dead cells do not. Cells were plated (100 000 cells/well) into 6-well plates and treated for 24 hours with specific drug or solvent in 2% FBS containing medium. The viable cells were then counted with the TC10 cell counter (Biorad).

5. WESTERN BLOTTING

Cells were lysed with Laemmli sample buffer (Biorad) on ice and lysates were heated at 90˚C for 10 minutes. Samples were loaded and run on precast 10% SDS PAGE gels (Biorad) and transferred to PVDF membranes (GE Healthcare). After blocking for 1 hour at room temperature, the blots were incubated overnight at 4˚C with specific primary antibodies. Membranes were subsequently incubated with a secondary antibody conjugated to horseradish peroxidase (Promega) and developed using a chemiluminescent (ECL) detection system followed by exposure to CL-Xposure films (Kodak). Quantification was done using ImageJ software. GAPDH was used as housekeeping protein to serve as a loading control for cell lysate samples.

6. REAL-TIME qPCR

RNA was extracted with RNeasy minikit from Qiagen according to manufacturer's instructions and was transcribed into cDNA using high capacity cDNA kit (Applied Biosystems). Real-time quantitative PCR was performed using the ABI7000 SYBRGreen PCR detector with the following probes (Invitrogen) **(Table 3)**. Relative levels of mRNA gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

Gene name	forward 5'- 3'	reverse 5'- 3'
α_5 subunit	TGCTGACTCCATTGGTTTCACAG	TCTCTCTGCAATCCTCTCGAGC
α_v subunit	AGGTGCCTACGAAGCTGAGC	AAGGCTTCATTGTTTCGGACA
β_1 subunit	TGTAACCAACCGTAGCAAAGGA	CCCTGATCTTAATCGCAAAACC
p21	GGCAGACCAGCATGACAGATT	TGTGGGCGGATTAGGGCT
Mdm2	AGACCCTGGTTAGACCAA	TGGCCAAGATAAAAAAGAACCTCT
Fas	CCCTCCTACCTCTGGTTCTTACG	AGTCTTCCTCAATTCCAATCCCTT
Bax	TGGAGCTGCAGAGGATGATTG	CCAGTTGAAGTTGCCGTCAGA
Noxa	GCAAGAACGCTCAACCGAG	AAGTTTCTGCCGGAAGTTCA
Bcl-2	TTGACAGAGGATCATGCTGTACTT	ATCTTTATTTCATGAGGCACGTT
Survivin	TGACGACCCCATAGAGGAACA	CGCACTTTCTCCGCAGTTTC
Pea-15	GCAGTGCCTGGTTTAGCTTC	TGCCGGATAATGTCTTTGTACTTC
cyclophilin	CAGGTCCTGGCATCTTGTCC	TTGCTGGTCTTGCCATTCCT

Table 3: Probes used for qPCR.

7. IMMUNOFLUORESCENCE

Cells (20 000/dish) were seeded onto IBIDI µ-dishes coated with 10µg/ml of poly-L-lysine and treated with Nutlin-3a or solvent for 24 hours. Cells were fixed with 4% paraformaldehyde for 10 minutes and incubated with a blocking solution (5%BSA in PBS) for 1h at room temperature. The cells were thereafter incubated with anti-α_5 IIAI antibody 1/300 (BD Biosciences) for 1 hour at room temperature, rinsed twice with PBS and exposed to Alexa Fluor-488 labeled goat anti-mouse secondary antibody 1/300 (JacksonimmunoResearch Laboratories) for 1 hour at room temperature followed by two washes with PBS. Cells were visualized with a confocal microscope (Biorad 1024) equipped with a water immersion 60x objective. Images were acquired using the Laser-Sharp 2000 software.

8. FLOW CYTOMETRY

IMMUNOSTAINING

Cells were detached with PBS/EDTA (0.53mM) and centrifuged for 5 minutes at 2000 rpm. Cells (200 000) were washed (5%BSA, 0.1% NaN3, TBS) and centrifuged for 5 minutes at 2000 rpm. The cell pellet was then incubated with a specific primary antibody at 4°C for 30 minutes. Primary antibodies used were 10µg/ml anti-α_5 IIAI (BD Biosciences), and 10µg/ml anti-β_1 TS2/16 (Santa Cruz), mAB13 (BD Biosciences) and 9EG7 (BD Biosciences), respectively. The cells were rinsed three times in washing buffer and exposed to either Alexa Fluor 488 labeled goat anti-mouse 1/200 or FITC labeled goat anti-rat 1/50 secondary antibody (JacksonimmunoResearch Laboratories) at 4°C for 30 minutes. After washing, cells were resuspended in 100µl of washing buffer and 200µl of TBS. A total of 20 000 cells were analyzed using a FACS Calibur flow cytometer (Becton-Dickinson). The mean fluorescence intensity characterizing surface expression of integrins was measured using the Cell Quest software.

ANNEXIN V/PI STAINING

Apoptosis was assessed using the Annexin V-FITC Apoptosis Detection kit (Calbiochem). Cells were plated onto 6-well plates. After treatment with specific drugs and solvents for 24 hours, the cells were detached with trypsin and centrifuged for 5 minutes at 1000 rpm. The cell pellet was then washed with cold PBS, centrifuged, resuspended in binding buffer and exposed to Annexin V-FITC for 15 minutes at room temperature. After washing, cells were resuspended in binding buffer with propidium iodide and immediately analyzed by FACS Calibur flow cytometer (Becton-Dickinson, San Diego, CA). The percentage of Annexin V positive cells was measured using the Cell Quest and FlowJo software.

9. HUMAN BIOPSIES

This study was conducted on a total of 110 adult brain biopsies collected retrospectively from archival material stored at the Centre de Ressources Biologiques et Tumorothèque (Hopitaux Universitaires de Strasbourg). Each sample was histologically analyzed by a pathologist to specify the tumor grade and the percentage of tumor cells. Control tissues were obtained from epileptic surgery. The study was

conducted in accordance with the Declaration of Helsinski and each patient was entered in the study after his/her consent.

10. HUMAN BRAIN TUMOR XENOGRAFTS

TCG4, TCG9, and TCG17 glioma xenograft models were obtained as previously described *(Leuraud et al., 2004).* Subcutaneous tumor growth was followed by measuring, 3 times per week, 2 perpendicular diameters with a caliper. Treatments began when tumors reached a volume of approximately 250 ± 50 mm^3. Temozolomide was administered orally at the dose of 50 mg/kg/d for 5 days. Mice were sacrificed when the tumor volumes reached 4 times their initial volume (V0). For each mouse, the time between the treatment onset and the animal sacrifice was defined as the "survival time." TP53 status of each xenograft was determined by the yeast functional assay *(Flaman et al., 1995).*

11. STATISTICAL ANALYSIS

Data are represented as mean ± SE. The values were obtained in at least three independent experiments (n). Statistical analyses were done by the Student's t test with the GraphPad Prism program. $p< 0.05$ was considered significant (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

RESULTS

AND DISCUSSION

Part I: $\alpha_5\beta_1$ integrin and its role in glioma

Glioblastoma (GBM) is the most common malignant primary intracranial tumor (*Ohgaki and Kleihues, 2005).* The standard therapy including surgical resection and radiotherapy with concomitant and adjuvant chemotherapy using temozolomide (TMZ) remains poorly efficient *(Stupp et al., 2005)*. The integrin $\alpha_5\beta_1$ was shown to be highly over-expressed in GBMs at mRNA *(Kita et al., 2001; Cosset et al., 2012)* and at protein levels compared to normal brain or low grade astrocytoma *(Gingrase et al., 1995).* The previous results of our laboratory demonstrated that integrin $\alpha_5\beta_1$ increases proliferation, clonogenic survival, adhesion, migration and invasion of various glioma cell lines *(Maglott et al., 2006; Bartik et al., 2008; Martin et al., 2009, Cosset et al., 2012).* These findings suggest that $\alpha_5\beta_1$ integrin is an attractive therapeutic target in GBMs. In the first part of this work, we studied the possible role of $\alpha_5\beta_1$ integrin in resistance of glioma to therapy with a particular focus on TMZ chemotherapy.

A. The role of $\alpha_5\beta_1$ integrin in resistance of glioma to therapy

1. $\alpha_5\beta_1$ integrin expression as a factor of glioma resistance

At first, we investigated whether $\alpha_5\beta_1$ integrin expression is associated with a particular grade of brain tumors. The expression of α_5 and β_1 subunits were examined by qPCR in 95 human brain tumors of different tumor grades and compared with 20 nontumor brain samples. The data showed that α_5 subunit gene expression was increased with increasing tumor grade, although the β_1 subunit was equally overexpressed amoung tumor grades compared to control tissue **(Figure 9A)**.

The subunit α_5 only dimerizes with β_1 subunit thus these data point toward a particular role of $\alpha_5\beta_1$ integrin in glioma progression.

Next, clinical data of grade III and grade IV patients were analyzed. Kaplan-Meier survival curves showed a significant survival advantage for patients with α_5 low expressing glioma compared to α_5 high expressing glioma **(Figure 9B)**. It is to note, all the patients were treated with standard therapy including surgery, radiotherapy and chemotherapy (TMZ).

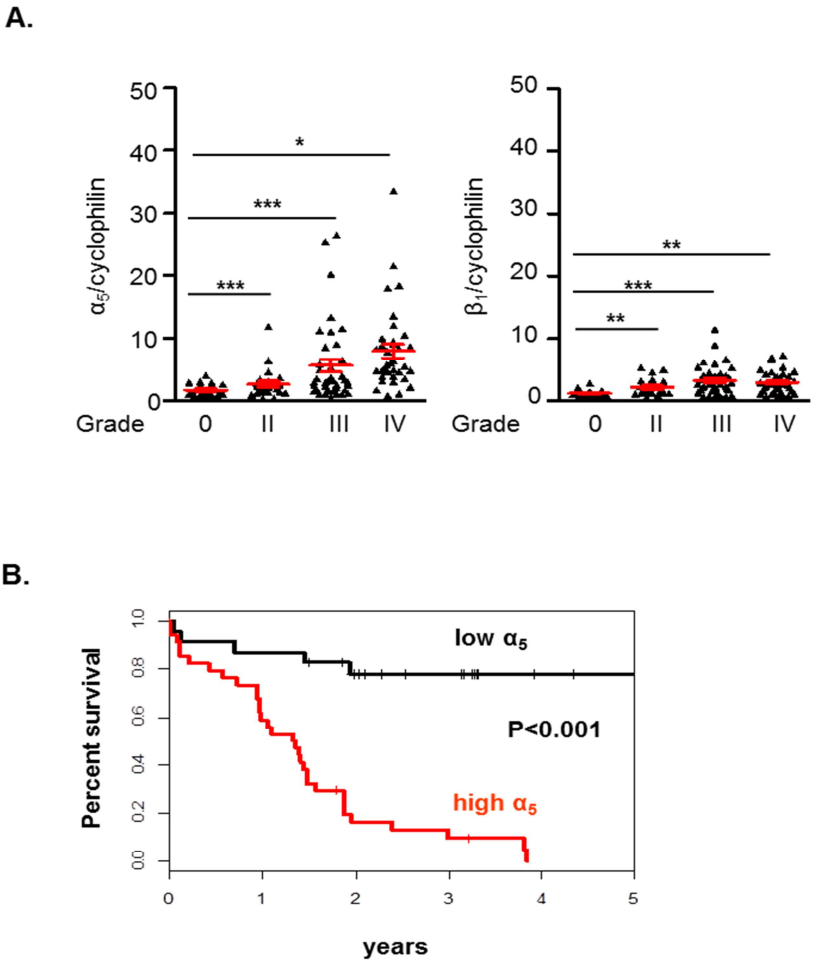


Figure 9: Elevated α_5-integrin gene expression is associated with high-grade glioma and predicts decreased survival rates. A. Gene expression levels of α_5 and β_1 integrin subunits were quantified by qPCR in 20 nontumor brain tissues (epileptic surgery) (G0), 22 grade 2 (GII), 38 grade 3 (GIII), and 35 glioblastoma (GIV) samples. Only the α_5 integrin subunit level is associated with the tumor grade. *, p<0.05; **, p<0.01; ***, p<0.001 as compared with nontumoral brain tissue (Mann–Whitney test). **B.** Kaplan-Meier survival analysis of α_5 low and α_5 high patients with high-grade glioma (23 α_5 low GIII and 13 α_5 high GIII; 7 α_5 low GIV and 28 α_5 high GIV).

Our data revealed an important role of $\alpha_5\beta_1$ integrin in tumor progression and in resistance to therapy, suggesting that $\alpha_5\beta_1$ integrin could be an attractive therapeutic target in high grade glioma.

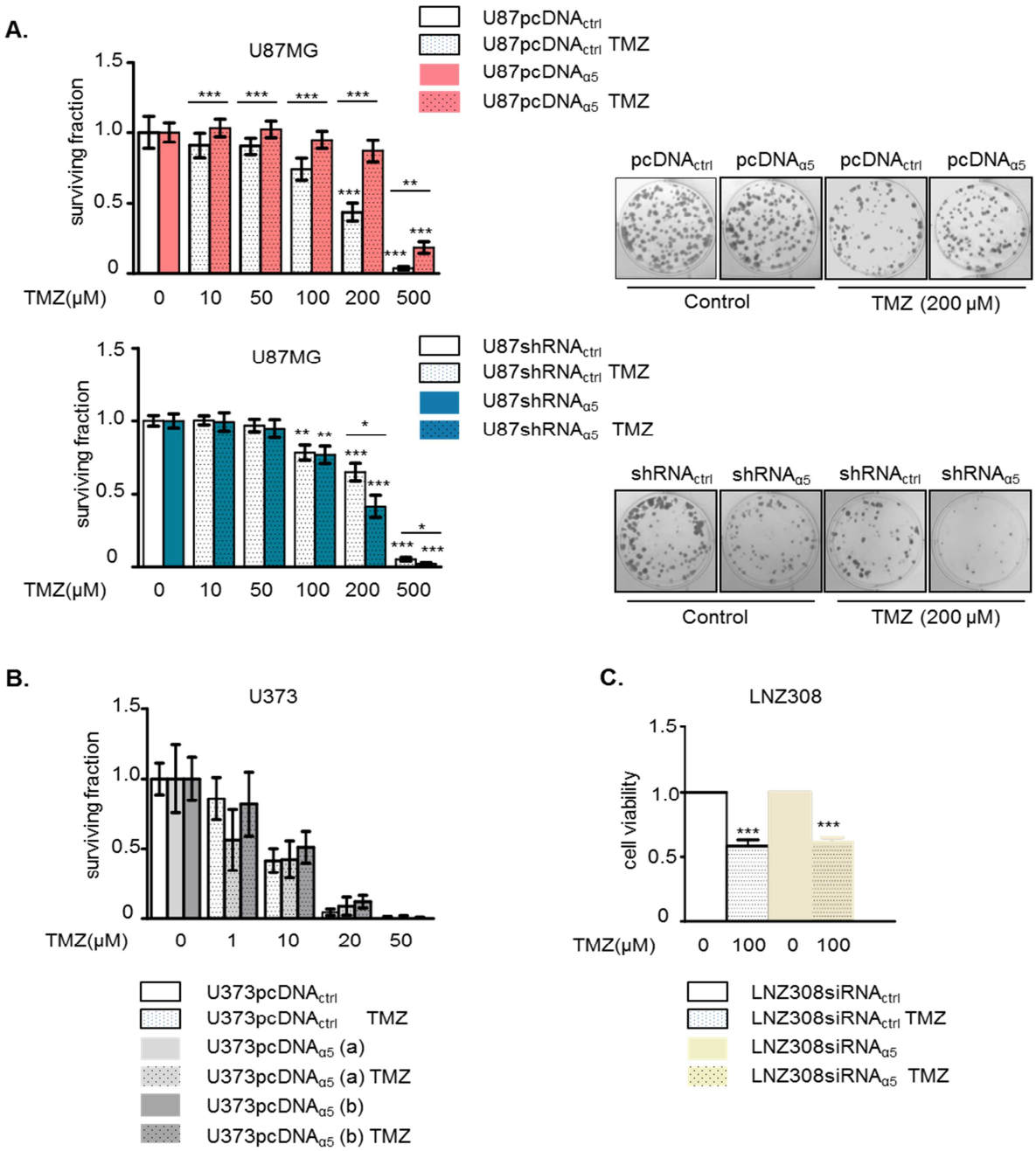


Figure 10: The effect of α_5 integrin expression on TMZ resistance in U87MG, U373 and LNZ308 cells. A. TMZ dose response of clonogenic survival in U87MG cells overexpressing α_5 integrin, U87pcDNA$_{\alpha5}$, compared to control cells, U87pcDNA$_{ctrl}$. U87DNA$_{\alpha5}$ cells are 2 times more resistant than control cells at the concentration of 200μM TMZ (top). Clonogenic survival of U87MG cells under-expressing α_5 integrin, U87shRNA$_{\alpha5}$, compared to control cells, U87shRNA$_{ctrl}$. U87shRNA$_{\alpha5}$ cells are 1.5 times more sensitive than control cells at 200μM TMZ (bottom). Representative images of colonies obtained with and without 200μM TMZ are shown. **B.** TMZ dose response of clonogenic survival in U373 cell clones overexpressing α_5 integrin, U373pcDNA$_{\alpha5}$ (a) and U373pcDNA$_{\alpha5}$ (b), compared to control cells, U373pcDNA$_{ctrl}$. **C.** LNZ308 cells with transiently depleted α_5 integrin, LNZ308siRNA$_{\alpha5}$, and control cells, LNZ308siRNA$_{ctrl}$, were treated with TMZ (100μM). Data represent cell viability (live cells were counted by using Trypan Blue) of TMZ treated compared to untreated cells. Histograms represent the mean±SEM of 3 to 4 independent experiments. Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001).

2. The role of $\alpha_5\beta_1$ integrin in resistance of glioma to chemotherapy

Next, we investigated whether $\alpha_5\beta_1$ integrin could be implicated in resistance of glioma to chemotherapeutic agent temozolomide (TMZ).

2.1. $\alpha_5\beta_1$ integrin effects on TMZ resistance of glioblastoma cell lines

First we studied the effect of α_5 subunit on TMZ resistance in three different glioblastoma cell lines, U87MG, U373 and LNZ308. The α_5 subunit was stably overexpressed in U87MG and U373, e.g. U87pcDNA$_{\alpha5,}$ U373pcDNA$_{\alpha5}$, and down-expressed in U87MG, e.g. U87shRNA$_{\alpha5}$ cells. LNZ308 were transiently depleted in α_5 subunit, LNZ308siRNA$_{\alpha5.}$ These cells were treated with increasing concentrations of TMZ and the cell survival fraction was quantified by clonogenic assays.

As shown in **Figure 10A**, TMZ dose dependently decreased the surviving fraction of U87pcDNA$_{ctrl}$, U87pcDNA$_{\alpha5,}$ U87shRNA$_{ctrl}$ and U87shRNA$_{\alpha5}$ cells. At high TMZ doses (200µM), U87pcDNA$_{\alpha5}$ cells were 2 times more resistant to TMZ treatment than control U87pcDNA$_{ctrl}$ cells **(Figure 10A, top)** and U87shRNA$_{\alpha5}$ cells were 1.5 times more sensitive than control cells at 200µM TMZ **(Figure 10B, bottom)**.

Similar experiments were performed with U373 cells. U373 control, U373pcDNA$_{ctrl}$, and α_5 overexpressing cells, U373pcDNA$_{\alpha5}$ clone a and clone b, were treated with increasing doses of TMZ. Although, two different clones overexpressing α_5 subunit were used, the overexpression of α_5 subunit did not affect the survival of U373 cells **(Figure 10B)**. To note, TMZ was more efficient to inhibit clonogenic survival in U373 cells (10µM TMZ caused 50% inhibition of survival) compared to U87MG cells (200µM TMZ caused 50% inhibition of survival) **(Figure 10A compared to Figure 10B)**.

Next, we examined the role of α_5 subunit on TMZ resistance in LNZ308 glioblastoma cell lines. LNZ308 transiently depleted in α_5 subunit, LNZ308siRNA$_{\alpha5,}$ and control cells, LNZ308siRNA$_{ctrl}$, were sensitive to TMZ, but α_5 depletion did not improve the TMZ induced effect on cell viability (live cells were counted by using Trypan Blue) **(Figure 10C)**. However, not exactly the same experiment performed for LNZ308 cells, 100µM TMZ was efficient to inhibit 50% of LNZ308 cell viability suggesting that TMZ has higher capacity to affect cell survival of U373, LNZ308 cells than of U87MG cells.

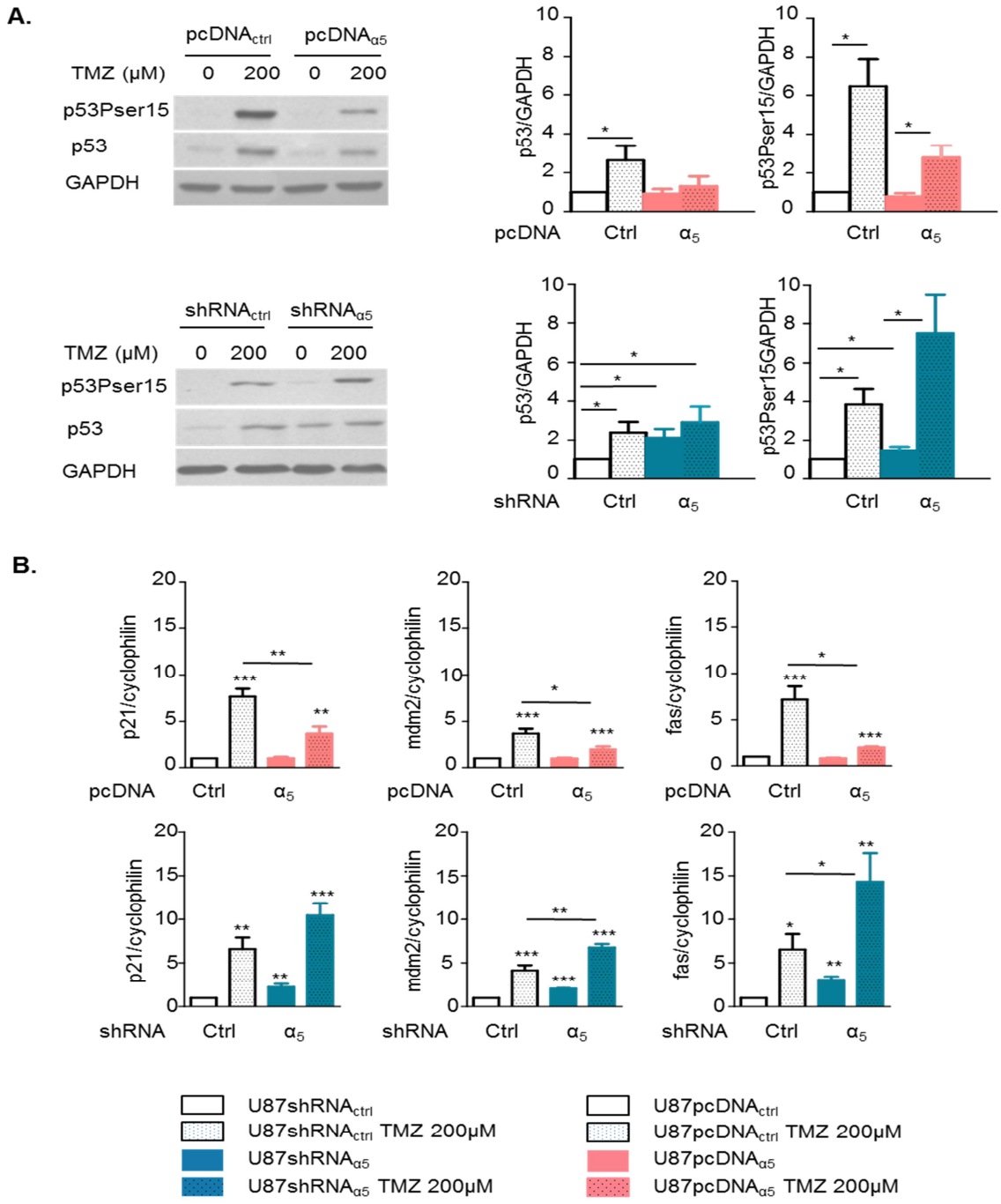


Figure 11: The effect of α_5 integrin on TMZ-induced p53 activation in U87MG. A. Stability and p53 phosphorylation on ser15 are affected by the α_5 integrin in U87MG cells. Western blot analysis for p53Pser15 and p53 from total cell lysates with and without TMZ 200μM treatment during 24 hours in control, U87pcDNA$_{ctrl}$, and α_5 integrin over-expressing, U87pcDNA$_{α5}$ cells (top), or control U87shRNA$_{ctrl}$, and α_5 integrin down-regulated, U87shRNA$_{α5}$ cells (bottom). GAPDH was used as a loading control. Histograms represent the mean±SEM of 6 to 8 independent experiments. **B.** qPCR quantification of p53 target genes. mRNA of target genes are differently affected by up-modulated (top) or down-modulated (bottom) α_5 integrin after TMZ treatment in U87MG cells. Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001).

Altogether, our results demonstrate that α_5 overexpression provokes TMZ chemoresistance of U87MG cells but does not change the sensitivity of U373 and LNZ308 cells to TMZ. Moreover, our data suggest a greater capacity of TMZ to inhibit cell survival in U373, LNZ308 cells compared to U87MG cells.

2.2. Effects of $\alpha_5\beta_1$ integrin on p53 activation by TMZ

Previous data of our laboratory showed that $\alpha_5\beta1$ integrin antagonists modulate p53 pathway *(Martinkova et al., 2010).* The data presented above showed that α_5 integrin was able to induce TMZ resistance only in p53 wt expressing U87MG cells but not in U373 expressing a p53 mutant and LNZ308 cells with no p53. These findings indicate the possible importance of functional p53 in mechanism of TMZ resistance driven by α_5 subunit.

We thus focused on α_5 subunit effects on TMZ-induced p53 activation. We examined both p53 stabilization and p53 phosphorylation on ser15, known as a key phosphorylation site leading to p53 transcriptional activation. We compared the effect of TMZ in U87MG cells overexpressing U87pcDNA$_{\alpha5}$ or down-expressing U87shRNA$_{\alpha5}$ integrin α_5 versus control cells, U87pcDNA$_{ctrl}$ and U87shRNA$_{ctrl}$, respectively. TMZ caused an increase in p53 protein in all cell lines but not significantly in U87pcDNA$_{\alpha5}$ cells **(Figure 11A, top)**. Interestingly, a significant increase in p53 protein was already observed in untreated U87shRNA$_{\alpha5}$ cells versus U87shRNA$_{ctrl}$ cells **(Figure 11A, bottom)**. After TMZ treatment, p53 activation measured by p53 phosphorylation on ser15 was induced in U87pcDNA$_{ctrl}$ and U87shRNA$_{ctrl}$ cells **(Figure 11A top and Figure 11A, bottom).** The increase in p53Pser15 was significantly more pronounced in U87shRNA$_{\alpha5}$ compared to control **(Figure 11A, bottom)**. In contrast, in U87pcDNA$_{\alpha5}$ cells, significantly less p53Pser15 was measured after TMZ treatment **(Figure 11A top)**. In accordance with phosphorylation of p53, transcriptional activity of p53 was enhanced by TMZ in the control cell lines as assessed by the increase in mRNA level of three p53 target genes (p21, mdm2, and fas). Transcriptional activity of p53 was higher in U87shRNA$_{\alpha5}$ cells and lower in U87pcDNA$_{\alpha5}$ cells than in TMZ treated control cells **(Figure 11B)**.

We made similar experiences with U373 and LN18 glioblastoma cells known to express a mutated p53. U373pcDNA$_{ctrl}$ and U373pcDNA$_{\alpha5}$ cells were treated with TMZ for 24h. TMZ had no effect on the

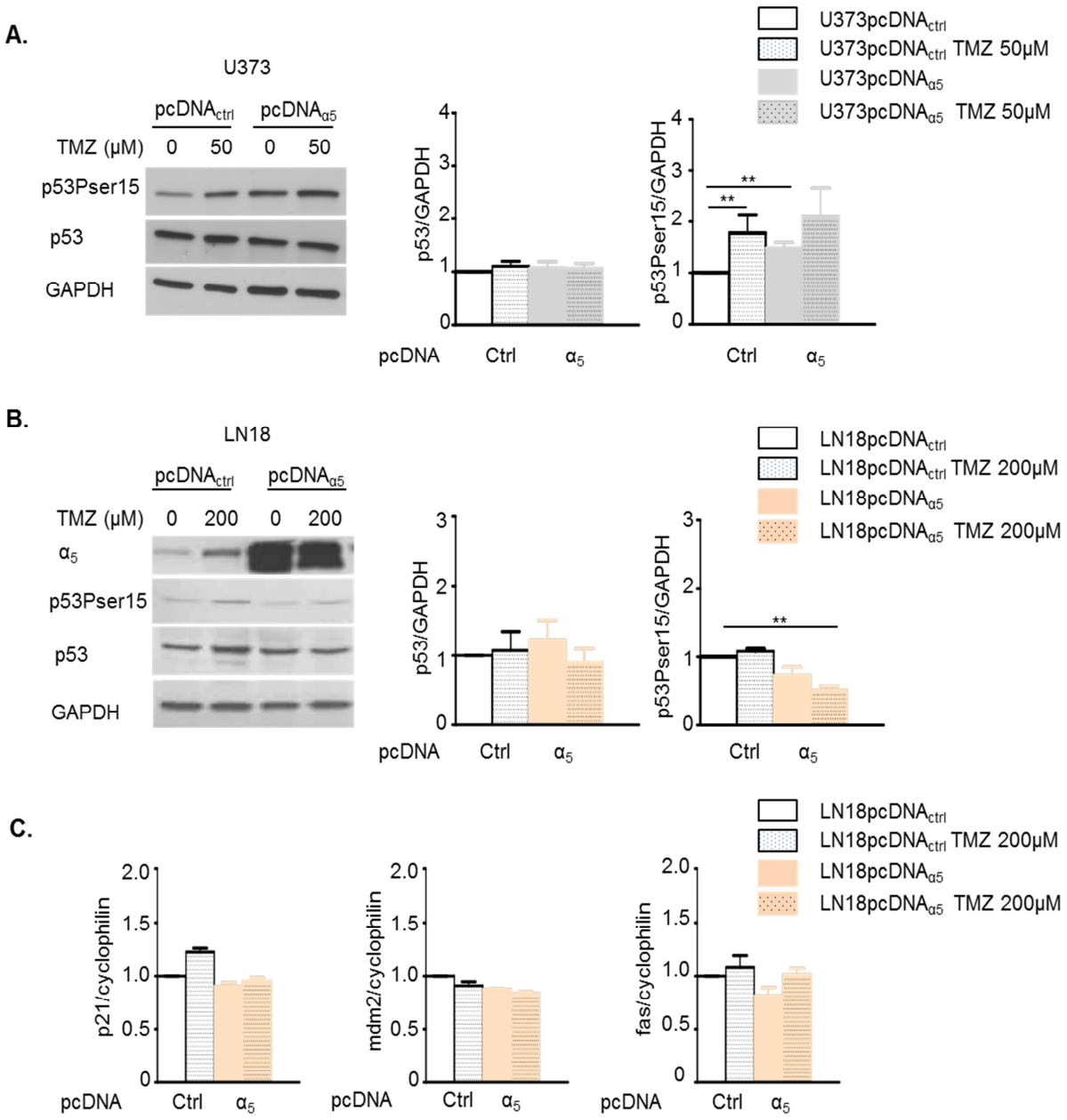
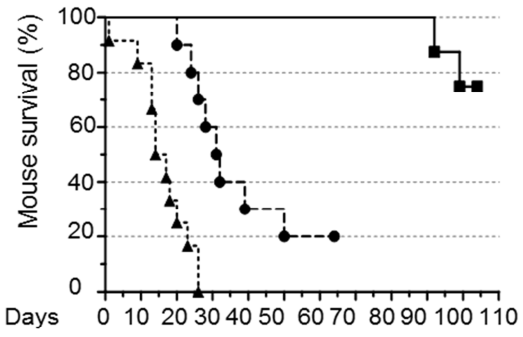


Figure 12: The effect of α_5 integrin on TMZ-induced p53 activation in U373 (p53mut) and LN18 (p53mut). A. Effect of TMZ on control, U373pcDNA$_{ctrl}$, and α_5 integrin overexpressing, U373pcDNA$_{\alpha5}$ cells. Western blots for p53Pser15 and p53 from total cell lysates after TMZ treatment (50μM, 24 hours). GAPDH was used as a loading control. Histograms represent the mean ± SEM of 3 independent experiments. **B.** LN18 cells transiently transfected (for 24 hours) with α_5-containing plasmids, LN18pcDNA$_{\alpha5}$, and control cells, LN18pcDNA$_{ctrl}$, were treated with TMZ (200μM, 24 hours) and α_5 integrin, p53Pser15 and p53 from total cell lysates were detected by Western blot. GAPDH was used as a loading control. **C.** Quantification of p53 target genes mRNA level in LN18 cells either control, LN18pcDNA$_{ctrl}$, or transiently transfected with α_5-expressing vector, LN18pcDNA$_{\alpha5}$, treated with TMZ (200 μM, 24 hours). Statistical analysis: Student t test (**, $p<0.01$).

stability of the protein p53 and slightly but significantly increased the phosphorylation on ser15 in these cells. The overexpression of α_5 integrin subunit did not affect p53 stability as well in presence as in absence of TMZ. The phosphorylation of p53 was rather increased than decreased even in non-treated cells **(Figure 12A)**. In LN18 glioma cells transiently modulated to overexpress α_5 subunit, TMZ had no effect on p53 stability. A significant decrease in p53Pser15 in α_5-overexpressing, LN18pcDNA$_{\alpha 5}$, and no effect on control, LN18pcDNA$_{ctrl}$, was observed in the presence of TMZ **(Figure 12B)**. As expected, we did not get any p53-dependent transcriptional activity in these cells **(Figure 12C)**.

We next explored with our collaborators (Dr. Pinel and Dr. Guérin) the role of $\alpha_5\beta_1$ integrin in TMZ chemoresistance *in vivo* by using subcutaneous xenografted human brain tumors in nude mice. Three xenografts expressing a wild type p53 and different levels of the α_5 subunit were selected and treated with TMZ. Kaplan–Meier analysis of mice survival suggests a relationship between α_5 integrin level and resistance to TMZ providing some evidence for a role of α_5 integrin in the chemoresistance of p53wt–expressing tumors *in vivo* **(Figure 13)**.



TCG4 (low α_5; 4 fold increase of α_5 levels vs non tumoral tissues)
TCG17 (medium α_5; 15 fold increase of α_5 levels vs non tumoral tissues)
TCG9 (high α_5; 33 fold increase of α_5 levels vs non tumoral tissues)

Figure 13: The role of α_5 integrin on TMZ resistance in xenograft model. TMZ antitumor effect on human malignant glioma xenografts in nude mice. Three glioma xenografts expressing p53wt were analyzed for α_5 mRNA expression (TCG9, TCG17, and TCG4 with 33-, 15-, and 4.5-fold more α_5 mRNA, respectively, compared with human nontumor brain tissue) and used to evaluate the tumor response to TMZ (orally daily 50mg/kg, 5 days). Results are expressed as Kaplan–Meier plots, considering the percentage of tumors that reached four times their initial volume as the survival end point.

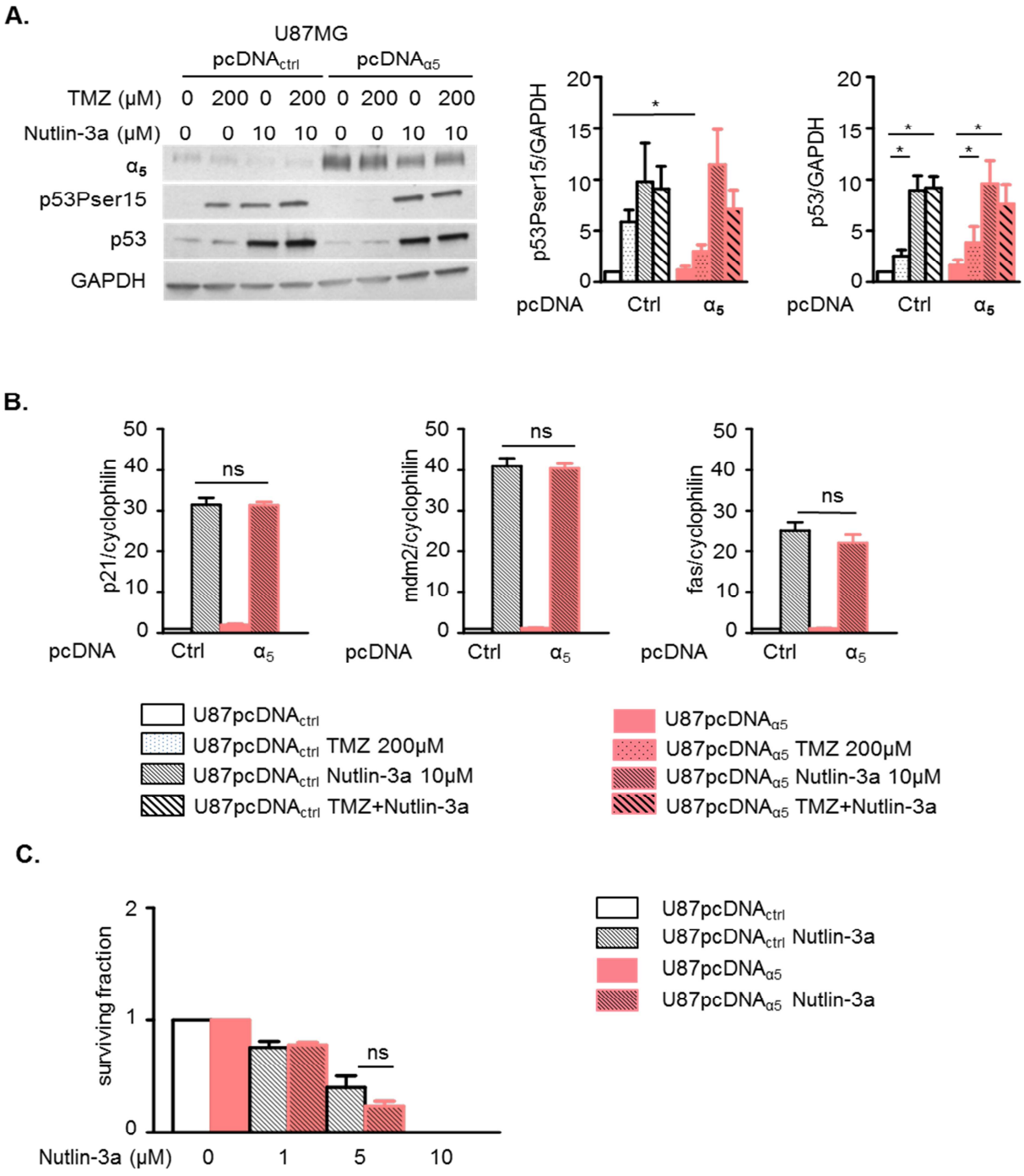


Figure 14: The effect of α_5 integrin on Nutlin-3a-induced p53 activation and cell survival. A. Western blot analysis of α_5 integrin, p53Pser15, p53 in U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ treated with Nutlin-3a (10μM), TMZ (200μM), or both drugs for 24 hours. Histograms display the mean ± SEM of 5 independent experiments. GAPDH was used as a loading control. **B.** qPCR analysis of p53 target genes. The histograms represent the fold increase of mRNA in U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ before and after Nutlin-3a (10μM) treatment over 24 hours. **C.** Nutlin-3a dose response of clonogenic survival in U87MG cells overexpressing α_5 integrin U87pcDNA$_{\alpha5}$ compared to control cells U87pcDNA$_{ctrl}$. Histograms display the mean ± SEM of 4 independent experiments. Statistical analysis: Student t test (*, $p<0.05$; ns, not significant).

Taken together, the results show that $\alpha_5\beta_1$ integrin plays an important role in the TMZ chemoresistance of GBM cells harboring a functional p53 through its negative modulation. The depletion of α_5 integrin subunit increases p53 activity and TMZ sensitivity. The $\alpha_5\beta_1$ integrin has no effect on the resistance to TMZ of p53 mutant and p53ko GBM cells.

2.3. Effects of $\alpha_5\beta_1$ integrin on p53 activation by Nutlin-3a

We next investigated whether a high α_5 subunit expression also had an impact on p53 activation by a non-genotoxic p53 activator, Nutlin-3a, in glioma cells. Nutlin-3a is a small-molecule inhibitor of the MDM2-p53 interaction that induces p53 stabilization *(Vassilev, 2004)*.

We treated U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells with TMZ (200µM), Nutlin-3a (10µM) and with both drugs for 24 hours. In contrast to the effects of TMZ, Nutlin-3a stabilized p53 and markedly increased the p53Pser15 in both cell lines. The addition of TMZ to Nutlin-3a does not further increase these effects **(Figure 14A).** Nutlin-3a also markedly increased the transactivation of p53-target genes in both cell lines with no differences in U87pcDNA$_{\alpha5}$ compared to U87pcDNA$_{ctrl}$ cells **(Figure 14B).**

Finally, clonogenic assays of U87pcDNA$_{ctrl}$, U87pcDNA$_{\alpha5}$ cells treated with Nutlin-3a showed that $\alpha_5\beta_1$ integrin did not protect efficiently these cells from death when p53 was activated by 10µM Nutlin-3a **(Figure 14C)**.

These data indicate that the overexpression of α_5 integrin has no effect on Nutlin-3a induced p53 activation and does not protect U87MG glioma cells from Nutlin-3a treatment as in the case of TMZ.

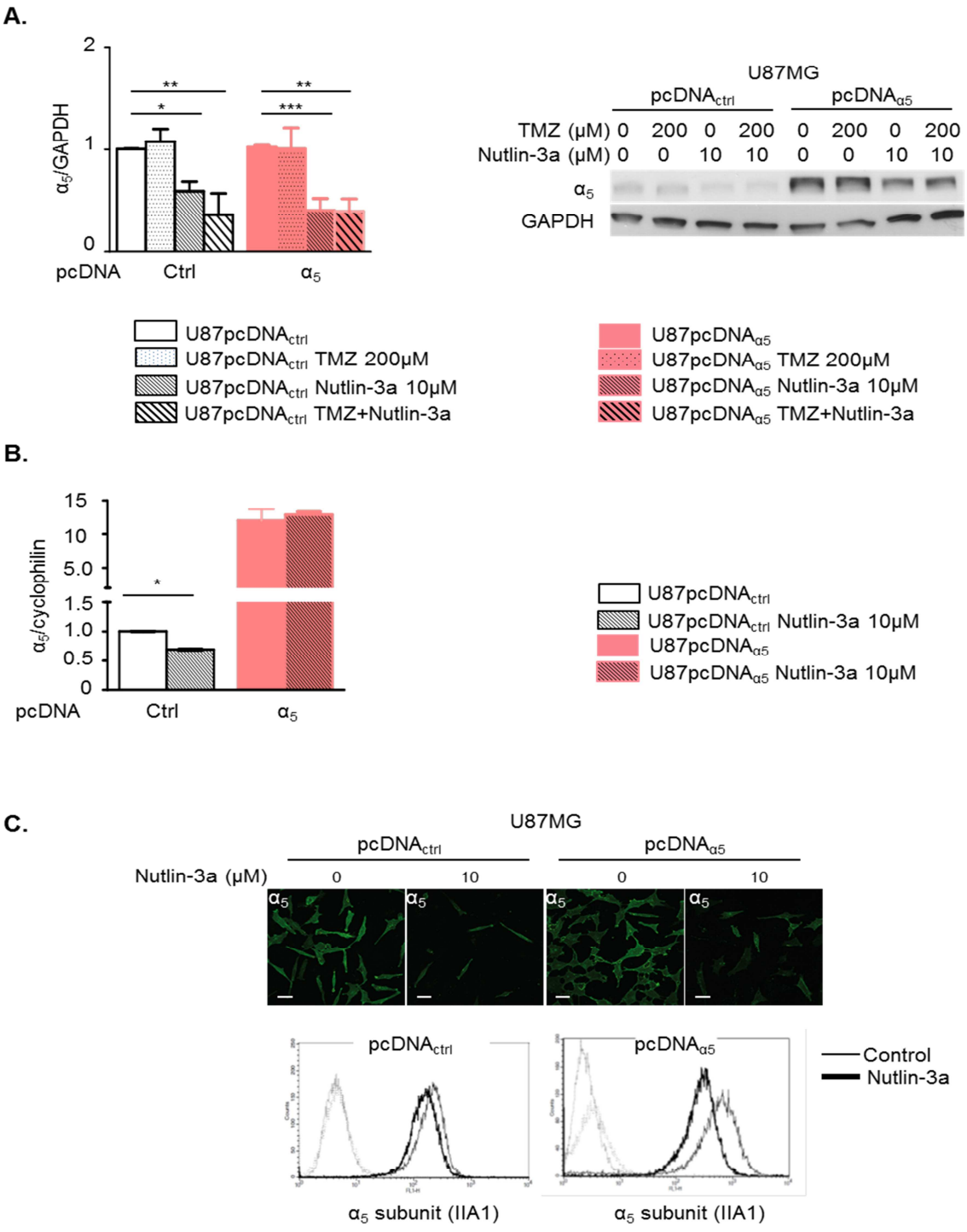


Figure 15: p53 activation effects on α_5 integrin expression in U87MG. A. Western blot analysis of α_5 integrin expression in U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells. Cells were treated with Nutlin-3a (10μM), TMZ (200μM), or both drugs for 24 hours. Histograms show the fold increase in protein expression normalized to GAPDH levels (mean±SEM). **B.** Histograms represent the fold increase of the α_5 mRNA in U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells after Nutlin-3a (10μM) treatment. **C.** Representative fluorescence images with specific anti-α_5 integrin antibodies of untreated and Nutlin-3a-treated U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells. Scale bars, 20 μm. Bottom, flow cytometry analysis of α_5 (IIA1 antibody) integrin subunit at the cell surface of U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells before and after Nutlin-3a (10μM) treatment for 24 hours. Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001).

2.4. Effects of p53 activation by Nutlin-3a on $\alpha_5\beta_1$ integrin expression

U87MG cells treated with Nutlin-3a showed some important morphological changes characterized by rounding up and detaching from the well. As integrin $\alpha_5\beta_1$ is a crucial adhesion molecule, we examined the expression of this integrin after p53 activation by Nutlin-3a.

Interestingly, treatment with Nutlin-3a decreased the expression of α_5 integrin at the protein level in U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells, an effect not observed after TMZ treatment **(Figure 15A)**.

A significant decrease in α_5 mRNA level was measured in U87MGpcDNA$_{ctrl}$, but not in pcDNA$_{\alpha5}$ cells, suggesting that Nutlin-3a affects the α_5 subunit at translational and posttranslational levels **(Figure 15B)**.

A specific immunostaining of α_5 integrin at the plasma membrane confirmed the decrease of the α_5 subunit after Nutlin-3a treatment, as shown in **Figure 15C** (fluorescence images and cytometry analysis with specific anti-α_5 integrin antibodies) **and Table 4.**

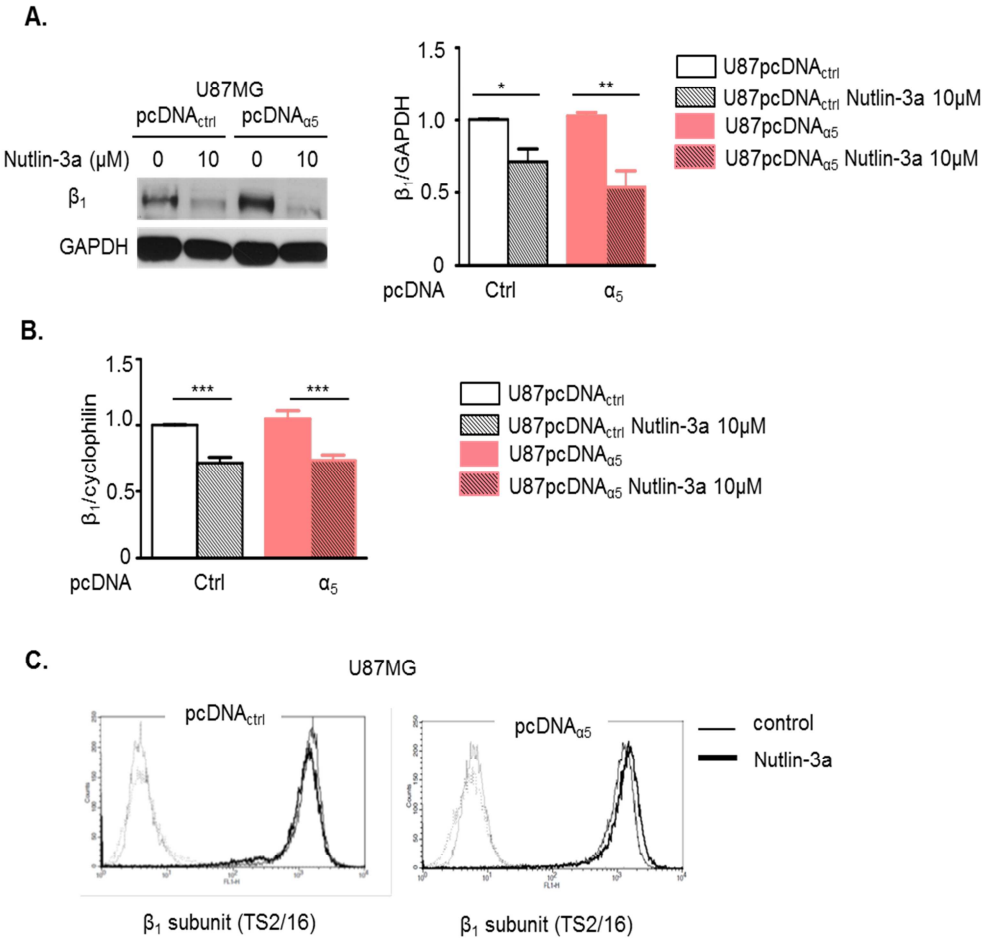


Figure 16: The effects of p53 activation by Nutlin-3a on β_1 integrin expression in U87MG. A. Western blot analysis of β_1 integrin expression in U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells. Cells were treated with Nutlin-3a (10μM) for 24 hours. Histograms show the fold increase in protein expression normalized to GAPDH levels (mean±SEM, 3 independent experiments). **B.** Histograms represent the fold increase of the β_1 mRNA in U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells after Nutlin-3a (10μM) treatment. **C.** Flow cytometry analysis of β_1 (TS2/16 antibody) integrin subunit at the cell membrane of U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells before and after Nutlin-3a (10μM) treatment for 24 hours. Statistical analysis: Student t test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

As shown for α_5 subunit, β_1 subunit was also decreased at protein level after Nutlin-3a treatment in U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells **(Figure 16A)**.

The decrease of β_1 integrin protein was accompanied by a down-regulation of its mRNA in both cell lines **(Figure 16B)**.

Intriguingly, the level of β_1 integrin measured by TS2/16 antibody at the cell membrane was not affected by the treatment with Nutlin-3a, although the global expression of the protein was decreased. We confirmed these data with 2 other specific anti-β1 integrin antibodies, 9EG7 and mAb13 **(Figure 16C and Table 4).**

cells \ antibodies	Ig control (mouse)	Ig control (rabbit)	α_5 (IIa1)	β_1 (9EG7)	β_1 (TS2/16)	β_1 (mAb13)
U87-pcDNA$_{ctrl}$	4.8±0.2	4.3±0.	219±15	37±2	1317±151	482±53
U87-pcDNA$_{ctrl}$ + nutlin	5.3±0.7	4.0±0.	165±15 * (p=0.04)	33±2	1246±73	455±35
U87-pcDNA$_{\alpha5}$	5.3±0.4	4.6±0.3	621±35	40±6	1207±67	430±123
U87-pcDNA$_{\alpha5}$ + nutlin	5.8±0.5	4.4±0.6	452±42* (p=0.01)	43±6	1322±62	425±66

Table 4: Mean fluorescence intensities of α_5 and β_1 integrin subunit expression at the cell membrane. U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ cells were treated with solvent or Nutlin-3a (10µM, 24 hours) and processed for flow cytometry analysis. Data report mean±SEM of 3 independent experiments. Student's t test, * indicates a statistically significant difference between cells treated with Nutlin-3a and cells treated with solvent.

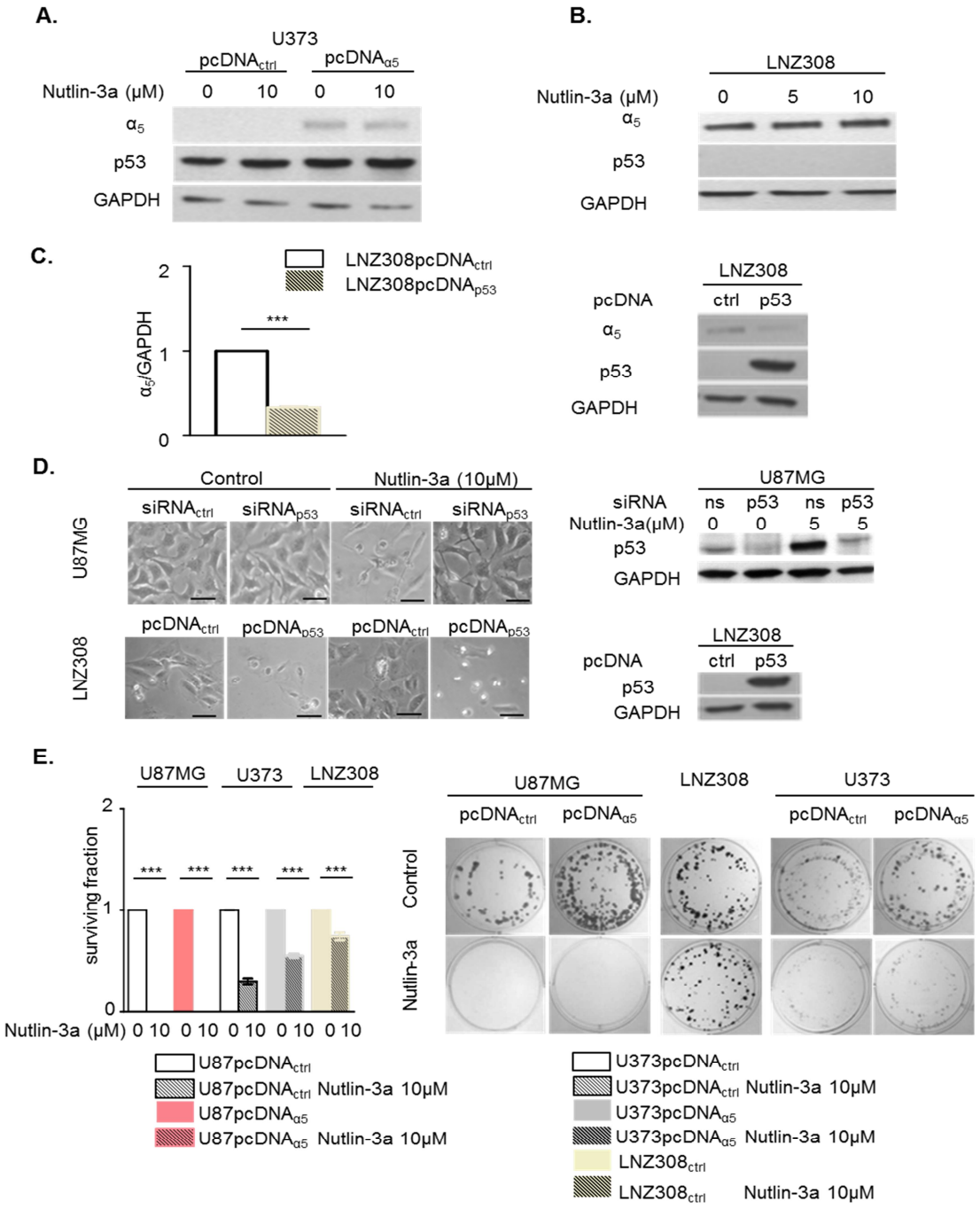


Figure 17: p53 activation effects on α_5 integrin expression in U373 and LNZ308. A. Western blot analysis of α_5 subunit and p53 expression in U373pcDNA$_{ctrl}$ and U373pcDNA$_{\alpha5}$ cells after treatment with 10μM Nutlin-3a. GAPDH was used as a loading control. A representative blot out of three is shown. **B.** Western blot analysis of α_5 and p53 protein expression in LNZ308 cells treated with Nutlin-3a (5 and 10μM) for 24 hours. GAPDH was used as a loading control. A representative blot out of three is shown. **C.** LNZ308 cells were transfected with p53wt and α_5 integrin expression was detected by Western blot analysis (right). Histograms show the fold increase in α_5 expression normalized to GAPDH levels (mean±SEM, 3 independent experiments). **D.** U87MG and LNZ308 cell morphology after 24 hours of Nutlin-3a treatment (Scale bars, 50μm). U87MG cells were transfected either with control siRNA$_{ctrl}$ or with siRNA$_{p53}$ and treated with Nutlin-3a (5μM) for 24 hours. Silencing of p53 was analyzed by immunoblotting (right up). LNZ308 cells were transfected with pcDNA$_{ctrl}$ or pcDNA$_{p53}$ and treated with Nutlin-3a (10μM). Expression of p53 was verified by immunoblotting (right down). **E.** Clonogenic survival of U87pcDNA$_{ctrl}$ and U87pcDNA$_{\alpha5}$ (left), LNZ308 (middle), and U373pcDNA$_{ctrl}$ and U373pcDNA$_{\alpha5}$ (right) cells after Nutlin-3a (10μM) treatment. Histograms represent the mean±SEM of 4 independent experiments. Representative images of colonies obtained with and without Nutlin-3a (10μM) are shown. Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001).

Next we wanted to clarify if p53 was required for down-modulation of α_5 subunit. In this purpose, we analysed the expression of α_5 subunit in U373 (p53 mutant) and LNZ308 (p53 knockout) glioma cells after Nutlin-3a treatment.

Nutlin-3a had no effect on α_5 expression in U373pcDNA$_{ctrl}$ cells and in α_5-transfected U373pcDNA$_{\alpha5}$ cells **(Figure 17A)**.

In LNZ308, Nutlin-3a did not affect the endogenous α_5 protein **(Figure 17B)** unless p53 was re-expressed in those cells **(Figure 17C)**.

As mentioned above, U87MG cells treated with Nutlin-3a rounded up and detached from the wells. This effect was lost when p53 expression was inhibited with specific siRNA **(Figure17D, top)**. In contrast, LNZ308 cells did not exhibit any morphologic alterations after Nutlin-3a treatment unless p53wt was re-expressed in the cells **(Figure 17D, bottom)**.

In clonogenic assay as summarized in **Figure 17E**, data show that U87MG cells expressing a p53wt are highly sensitive to Nutlin-3a, although U373 cells expressing a mutant p53 or LNZ308 with no p53 are clearly less sensitive to this drug. Interestingly, no decrease in α_5 integrin expression could be observed in those last two cell lines. Altogether, these data suggest that Nutlin-3a required a functional p53 to decrease the expression of α_5, which in turn makes the cells susceptible to this drug.

Taken together, p53wt induced by Nutlin-3a has a capacity to decrease α_5 subunit at the plasma membrane. The decrease in α_5 subunit is strictly p53wt dependent since the down-regulation of α_5 subunit is not observed in p53 mutant or p53ko cells. Down-regulation of α_5 subunit by Nutlin-3a-induced p53 results in sensitization of p53 intact glioblastoma cell lines to Nutlin-3a treatment.

α_5 RNA levels	low α_5 RNA levels		high α_5 RNA levels	
grade \ p53 status	p53 wt	p53 mutant	p53 wt	p53 mutant
grade III	11 (18)	7 (18)	8 (10)	2 (10)
grade IV	2 (7)	5 (7)	15 (22)	7 (22)
total	13	12	23	9
%	52	48	72	28

Table 5: The α_5 mRNA expression level and p53 status in human brain biopsies. In human biopsies of 28 patients grade III and 29 patients grade IV, the expression of α_5 mRNA and p53 status were studied The α_5 low expressing tumor group contained 52% of tumors with p53wt and 48% of tumors with p53 mutant, whereas in α_5 high expressing tumor group 72% of tumors expressed p53wt.

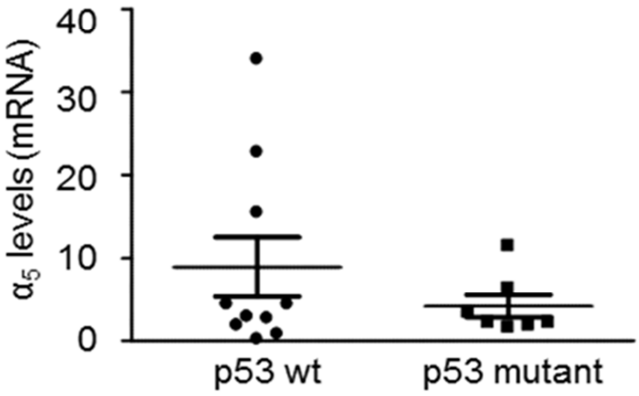


Figure 18: The α_5 mRNA expression level and p53 status in human brain tumor xenografts in nude mice. Ten xenografts expressed a wild type p53 and 7 xenografts had a mutant p53. The mRNA levels were determined in at least 3 different grafts of the same tumor, and the mean levels were plotted according to p53 status. The mean values $\pm$SEM of the α_5 mRNA level in p53wt and p53 mutant tumors were 8.9$\pm$3.6 and 4.1$\pm$1.3, respectively. Although not significant, this difference shows a trend toward an increased level of in α_5 p53wt tumors.

2.5. The α_5 mRNA expression level and p53 status in human brain tumors

We next evaluated with our collaborators (Pr. Plenat, Dr. Pinel and Pr. Entz-Werle) the relationship between the expression level of α_5 integrin and the status of p53 in 56 human biopsies (grade III and IV) and in 17 human tumor xenografts.

In human biopsies, α_5 low expressing tumor group contained 52% of tumors with p53wt and 48% of tumors with p53 mutant, whereas in α_5 high expressing tumor group 72% of tumors expressed p53wt **(Table 5)**.

In human tumor xenografts, the mean values $\pm$SEM of the α_5 mRNA level in p53wt and p53 mutant tumors were 8.9$\pm$3.6 and 4.1$\pm$1.3, respectively. Although not significant, this difference shows a trend toward an increased level of α_5 subunit in p53wt tumors **(Figure 18)**.

These data point out the trend toward an increase of the α_5 subunit in tumors with functional p53.

The key results of the first part of this study show for the first time that $\alpha_5\beta_1$ integrin negatively modulates the activation of p53 that in turn leads to TMZ chemoresistance in glioma tumors with functional p53. Nutlin-3a overrides the α_5 inhibitory effect on p53 signaling by its capacity to decrease the expression of α_5. Taken together, our findings indicate the existence of a negative cross-talk between $\alpha_5\beta_1$ integrin and p53 protein that may support glioma resistance to chemotherapeutic drugs. These results also support the hypothesis that the inhibition of $\alpha_5\beta_1$ integrin in GBMs harboring a p53wt may sensitize tumors to therapies.

These results led to the publication *Janouskova H. et al., Cancer Research (2012)*.

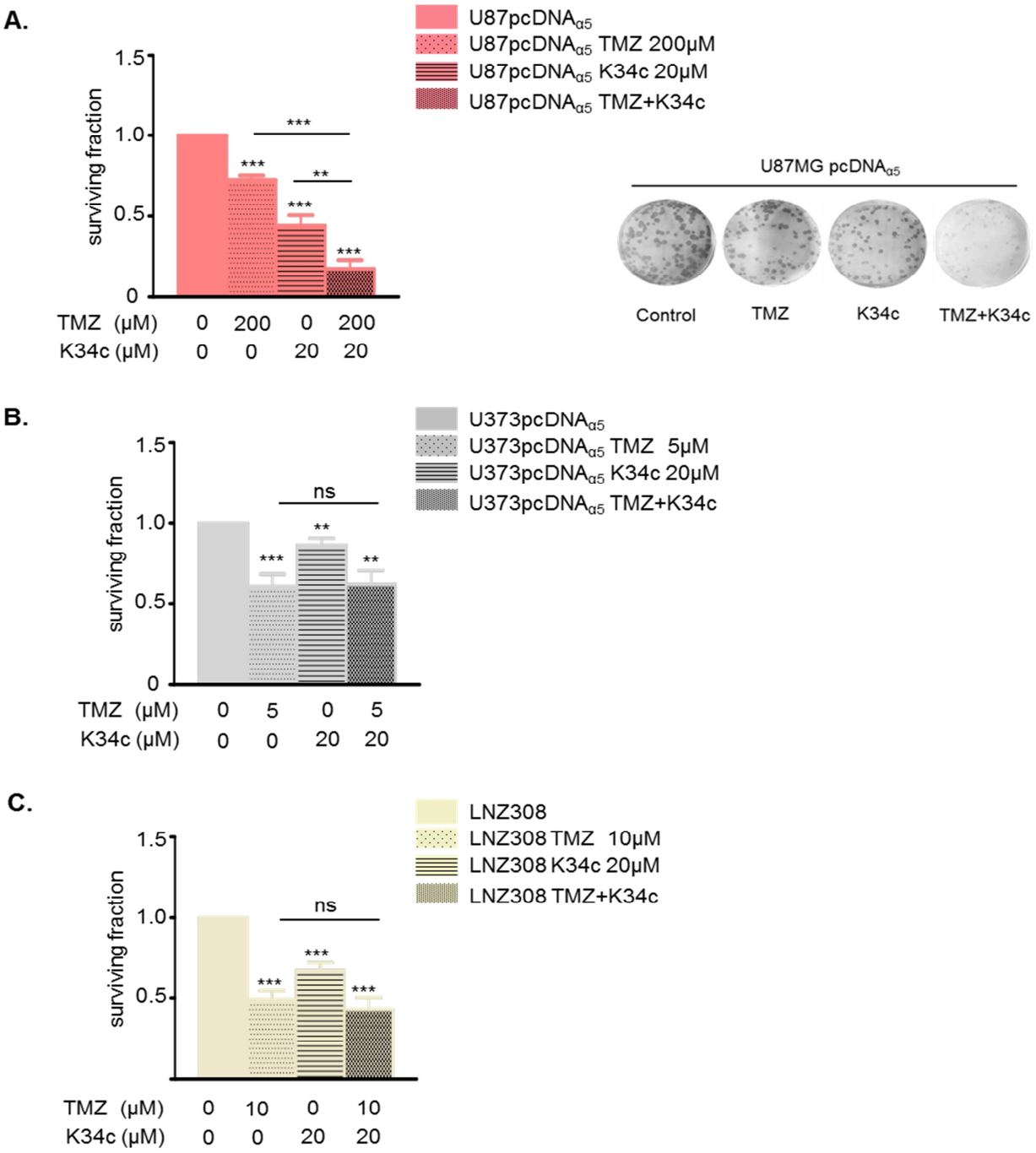


Figure 19: The effects of $\alpha_5\beta_1$ integrin antagonist, TMZ and their combination on cell survival of U87MG, U373 and LNZ308 cells. A. Long term survival assay of U87pcDNA$_{\alpha5}$ after TMZ (200μM), K34c (20μM) or combination treatments. Representative images of colonies with or without drugs are shown. Histograms represent the mean±SEM of 4 independent experiments. **B.** Clonogenic survival of U373pcDNA$_{\alpha5}$ cells treated with TMZ (5μM), K34c (20μM) or two drugs. Histograms represent the mean±SEM of 6 independent experiments. **C.** Clonogenic survival of LNZ308 cells treated with TMZ (10μM), K34c (20μM) or two drugs. Histograms represent the mean±SEM of 4 independent experiments. Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001, ns, not significant).

B. The association of $\alpha_5\beta_1$ antagonist with p53 activators in GBM cells

As stated above, integrin $\alpha_5\beta_1$ plays a crucial role in TMZ chemoresistance of glioblastoma cells with functional p53, suggesting that the inhibition of α_5 integrin could improve the effects of TMZ in these cells. We have also shown that Nutlin-3a at 10µM activates p53 protein and decreases the expression of α_5 subunit. Since p53 induced by Nutlin-3a does not inhibit total amount of α_5 subunit in U87pcDNA$_{\alpha5}$, we investigated whether blocking the residual α_5 subunit might synergize with the effects of Nutlin-3a.

In the second part of this work, we studied the effects of the combination therapy (integrin $\alpha_5\beta_1$ antagonist: K34c, and p53 activators: TMZ or Nutlin-3a) in different glioma cell lines.

1. The effects of combination therapy on cell survival and apoptosis

1.1. The effects of combination therapy on cell survival

1.1.1. The association of integrin $\alpha_5\beta_1$ antagonist and TMZ

At first, we examined the survival of U87pcDNA$_{\alpha5}$ (p53wt), U373 pcDNA$_{\alpha5}$ (p53 mutant) and LNZ308 (no p53) glioblastoma cell lines by long term survival assays after TMZ, $\alpha_5\beta_1$ integrin antagonist K34c, and both drugs combination treatments. For each cell line, cells were treated with the required concentration of TMZ to inhibit the clonogenic cell survival at about 50% as previously indicated in **Figure 10**.

As already shown above, U87pcDNA$_{\alpha5}$ cells were slightly sensitive to TMZ at high concentration (200µM) **(Figure 10A and Figure 19A)**. By contrast, already at low concentration TMZ decreased the survival of about 40% (5µM TMZ) and 51% (10µM TMZ) in U373pcDNA$_{\alpha5}$ **(Figure 10B and Figure 19B)** and in LNZ308 cells **(Figure 19C)**, respectively**.** These data confirmed our previous results **(Figure 10)**, indicating that glioblastoma cells with mutant p53 or no p53 are more sensitive to TMZ than p53wt cells.

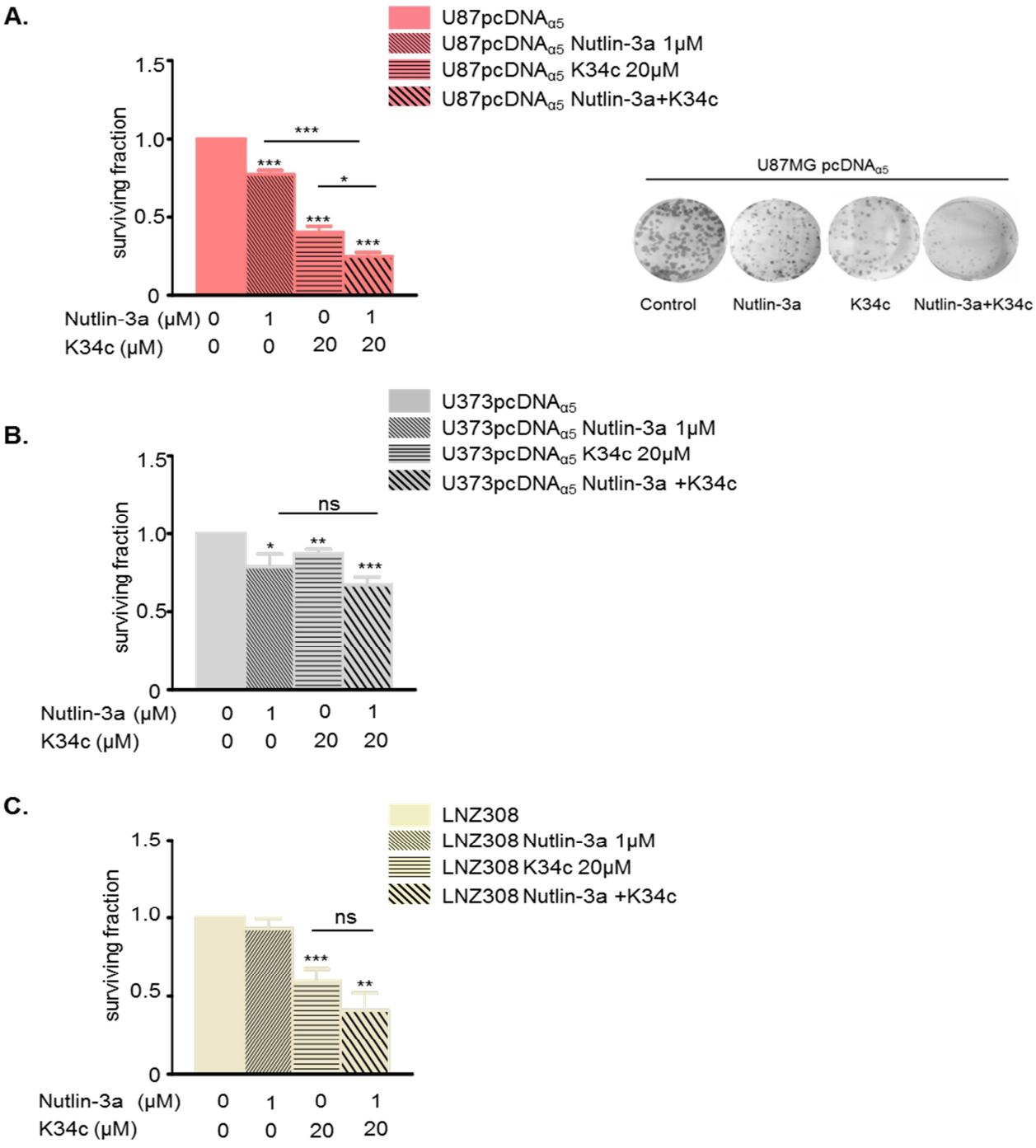


Figure 20: The effects of $\alpha_5\beta_1$ integrin antagonist, Nutlin-3a and their combination on cell survival of U87MG, U373 and LNZ308 cells. A. Long term survival assay of U87MGpcDNA$_{\alpha5}$ after Nutlin-3a (1μM), K34c (20μM) or combination treatments. Representative images of colonies with or without drugs are shown. Histograms represent the mean±SEM of 4 independent experiments. **B.** Clonogenic survival of U373pcDNA$_{\alpha5}$ cells treated with Nutlin-3a (1μM), K34c (20μM) or two drugs. Histograms represent the mean±SEM of 6 independent experiments. **C.** Clonogenic survival of LNZ308 cells treated with Nutlin-3a (1μM), K34c (20μM) or two drugs. Histograms represent the mean±SEM of 4 independent experiments. Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001, ns, not significant).

The $\alpha_5\beta_1$ integrin antagonist K34c significantly decreased the surviving fraction of about 56%, 14% and 33% in U87pcDNA$_{\alpha5}$ **(Figures 19A)**, U373pcDNA$_{\alpha5}$ **(Figure 19B)** and LNZ308 **(Figure 19C)** glioblastoma cell lines, respectively.

The combination of K34c with TMZ led to a significantly improved inhibition of survival in U87pcDNA$_{\alpha5}$ cells compared to any of the agents used separately **(Figure 19A)**. In contrast, the combination of K34c with TMZ has no improved effect on U373pcDNA$_{\alpha5}$ **(Figure 19B)** and LNZ308 **(Figure 19C)** survival compared to each drug alone.

1.1.2. The association of integrin $\alpha_5\beta_1$ antagonist and Nutlin-3a

We next investigated whether K34c was able to improve the Nutlin-3a induced effect on survival of U87pcDNA$_{\alpha5,}$ U373pcDNA$_{\alpha5}$ and LNZ308 cells. Since Nutlin-3a at a final concentration of 10µM caused a total inhibition of clonogenic survival, we have used a lower concentration (1µM). As shown in **Figure 20**, Nutlin-3a at low concentration had a slight effect on the clonogenic potential of U87pcDNA$_{\alpha5}$ **(Figure 20A)** and of U373pcDNA$_{\alpha5}$ cells **(Figure 20B)**, and no effect on the clonogenic potential of LNZ308 cells **(Figure 20C)**.

Nutlin-3a in combination with K34c resulted in a significant reduction of the clonogenic capacity, compared to each treatment alone in U87pcDNA$_{\alpha5}$ cells **(Figure 20A)**. Whereas no additional effect of combination therapy was observed in U373pcDNA$_{\alpha5}$ **(Figure 20B)** and LNZ308 cells **(Figure 20C)**.

Altogether, our data show that $\alpha_5\beta_1$ integrin antagonist inhibits the clonogenic survival regardless of p53 status. Conversely, genotoxic agent TMZ is more effective to affect the clonogenic survival in p53 inactive cells. Nutlin-3a at low concentrations only slightly affected the clonogenic survival in cells harboring p53wt and p53 mutant. The concomitant treatment with $\alpha_5\beta_1$ integrin antagonist and p53 activators (TMZ and Nutlin-3a) only sensitizes the cells with intact p53.

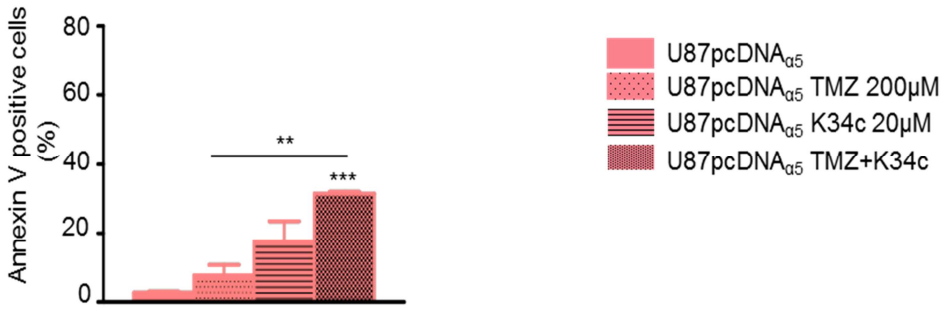


Figure 21: The effects of $\alpha_5\beta_1$ integrin antagonist, TMZ and their combination on apoptosis of U87MG cells. U87pcDNA$_{\alpha5}$ cells were treated for 24 hours with solvent, TMZ (200µM), K34c (20µM) or combination, stained with annexin V-FITC/PI and annexin V-FITC positive population was detected by FACS analysis. Data represent the percentage of cells positively stained for annexin V (3 independent experiments). Statistical analysis: Student t test (**, p<0.01; ***, p<0.001).

1.2. The effects of combination therapy on apoptosis

The previous data of our laboratory already demonstrated that TMZ was not able to induce apoptosis in U87MG cells but rather provoked a premature senescence, and that $\alpha_5\beta_1$ integrin antagonist alone preferentially induced the cell cycle arrest in cells with intact p53. However, the association of both drugs led to apoptosis in U87MG cells *(Martinkova et al., 2010).* Nutlin-3a is known to promote cell cycle arrest and senescence but hardly apoptosis in U87MG cells *(Villalonga-Planells et al., 2011).* We therefore investigated whether the concomitant inhibition of $\alpha_5\beta_1$ integrin and p53 activation by TMZ or Nutlin-3a could induce apoptosis in α_5 high expressing cells, which would explain the strong reduction of colony formation in U87pcDNA$_{\alpha5}$ cells.

U87pcDNA$_{\alpha5}$ cells were treated with TMZ (200µM) in combination with K34c (20µM) for 24 hours and apoptosis was analyzed by flow cytometry (annexin V/PI staining). As shown in **Figure 21**, TMZ or K34c as a single treatment did not significantly increase apoptosis in U87pcDNA$_{\alpha5}$ cells. However, the concomitant treatment resulted in a significant increase of apoptotic cell death (31% of apoptotic cells).

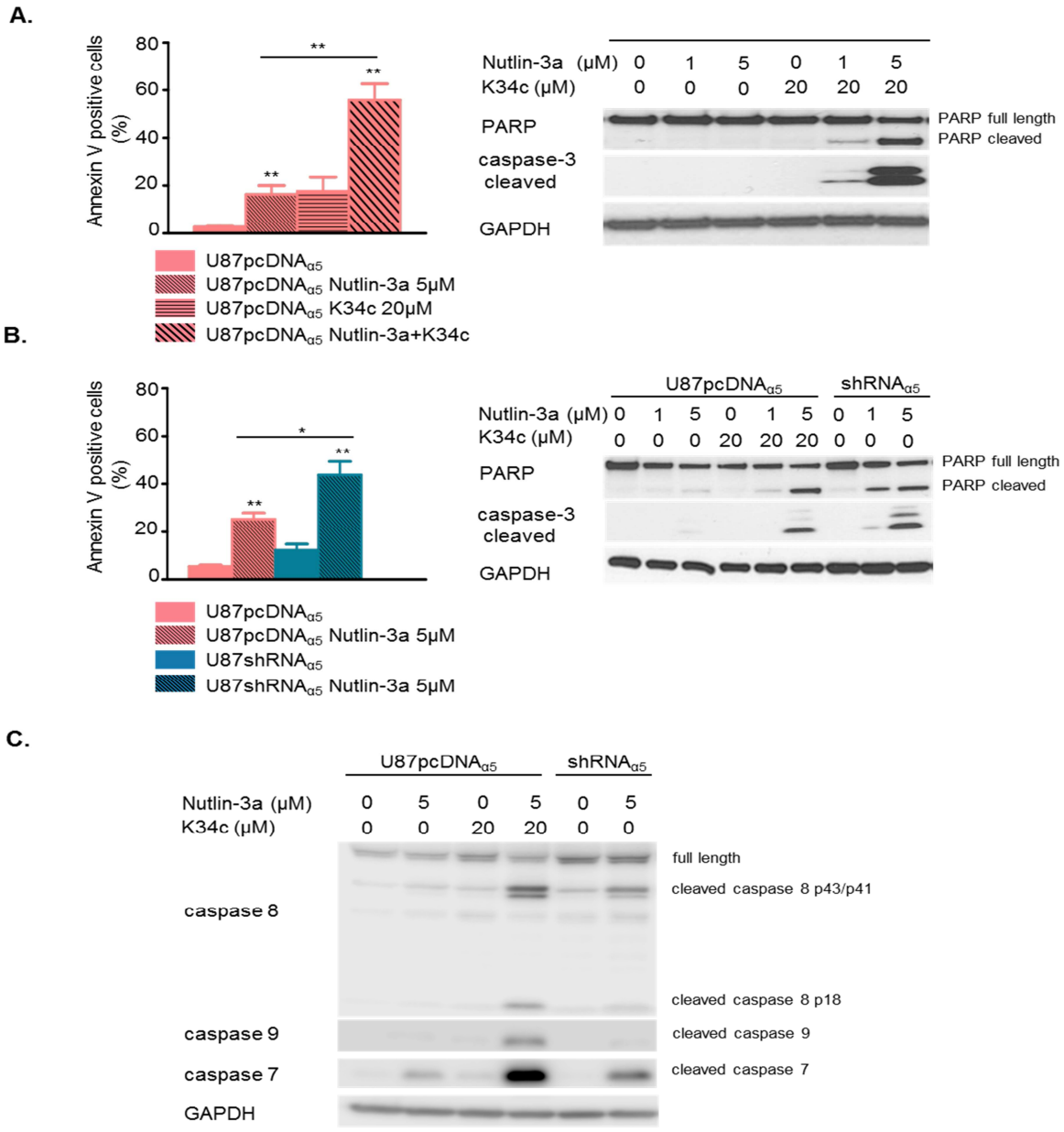


Figure 22: The effects of $\alpha_5\beta_1$ integrin antagonist, Nutlin-3a and their combination on apoptosis of U87MG cells. A. U87pcDNA$_{\alpha5}$ cells were treated for 24 hours with solvent, Nutlin-3a (5μM), K34c (20μM) or both, stained with annexin V-FITC and annexin V-FITC positive population detected by FACS analysis. Data represent the percentage of cells positively stained for annexin V (3 independent experiments) (left). Representative Western blot of cleaved PARP and caspase 3 in U87pcDNA$_{\alpha5}$ treated with Nutlin-3a (1 and 5μM), K34c (20μM) or combinations (right). GAPDH was used as a loading control. **B.** Histogram represents the percentage of annexin V positive U87pcDNA$_{\alpha5}$ and U87shRNA$_{\alpha5}$ cells after Nutlin-3a (5μM) treatment (24 hours) (left). A representative Western blot of cleaved PARP and caspase 3 in U87pcDNA$_{\alpha5}$ treated with Nutlin-3a (1 and 5μM), K34c (20μM) or combinations and U87shRNA$_{\alpha5}$ treated with Nutlin-3a alone (1 and 5μM) (12 hours) is shown (right). GAPDH was used as a loading control. **C.** U87pcDNA$_{\alpha5}$ cells were treated with K34c (20μM) and/or Nutlin-3a (5μM) and U87shRNA$_{\alpha5}$ were treated with Nutlin-3a (5μM) alone. Representative Western blots showing levels of active caspase 8, caspase 9 and caspase 7. GAPDH was used as a loading control. Statistical analysis: Student t test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

We next investigated the effects of the Nutlin-3a and K34c combination therapy on apoptosis of U87pcDNA$_{\alpha5}$ cells. Apoptosis was assessed by Western blot, using the anti-cleaved PARP and anti-cleaved caspase 3 antibodies, and by FACS analysis (annexin V/propidium iodide staining). U87pcDNA$_{\alpha5}$ cells treated with K34c or Nutlin-3a (1µM and 5µM) alone did not induce the cleavage of PARP or caspase 3. In contrast, K34c and Nutlin-3a co-treatment promoted apoptosis with high levels of cleaved PARP and caspase 3 **(Figure 22A, right)**. FACS analysis confirmed these results and showed a slight induction of apoptosis in U87pcDNA$_{\alpha5}$ cells (16% of apoptotic population) after Nutlin-3a treatment, and a huge activation of apoptotic cell death reaching 59% in U87pcDNA$_{\alpha5}$ cells treated concomitantly with K34c and Nutlin-3a **(Figure 22A, left)**.

Based on our results with α_5 high expressing U87pcDNA$_{\alpha5}$ cells, we expected a higher sensitivity of α_5 low expressing U87shRNA$_{\alpha5}$ cells to Nutlin-3a. As hypothethized, Nutlin-3a alone promoted an apoptotic cell death and multiplied by 1.7 the percentage of apoptotic population in α_5 low expressing U87shRNA$_{\alpha5}$ cells compared to Nutlin-3a-treated U87pcDNA$_{\alpha5}$ cells **(Figure 22B, left)**. These results were further confirmed by Western blot where the Nutlin-3a single treatment showed to effectively cleave PARP and caspase 3 **(Figure 22B, right)**. Our results indicate that only a concomitant inhibition of $\alpha_5\beta_1$ integrin with p53 activation can fully induce apoptosis in U87MG cells.

Two different apoptotic signaling pathways leading to the activation of specific caspases are described. The intrinsic mitochondrial apoptotic cell death is associated with the activation of initiator caspase 9, whereas the extrinsic apoptosis results in the activation of initiator caspase 8. These two initiators caspases activate in turn effector caspases, including caspase 3 and caspase 7. To determine which type of apoptotic program is activated after the combination therapy, we treated U87pcDNA$_{\alpha5}$ cells with Nutlin-3a and/or K34c and the cleavage of caspase 8 and caspase 9 was assessed by Western blot. As seen in **Figure 22C**, the activation of caspases 8 as well as caspase 9 is observed after the combination treatment of U87pcDNA$_{\alpha5}$ cells, suggesting the involvement of both apoptotic programs. In addition, caspase 7 activity is highly enhanced after Nutlin-3a and K34c co-treatment in U87pcDNA$_{\alpha5}$ cells. At the time of examination (12h), caspase 8 and caspase 9 seemed to be less cleaved than caspase 3 and caspase 7 **(Figure 22A and Figure 22C)**.

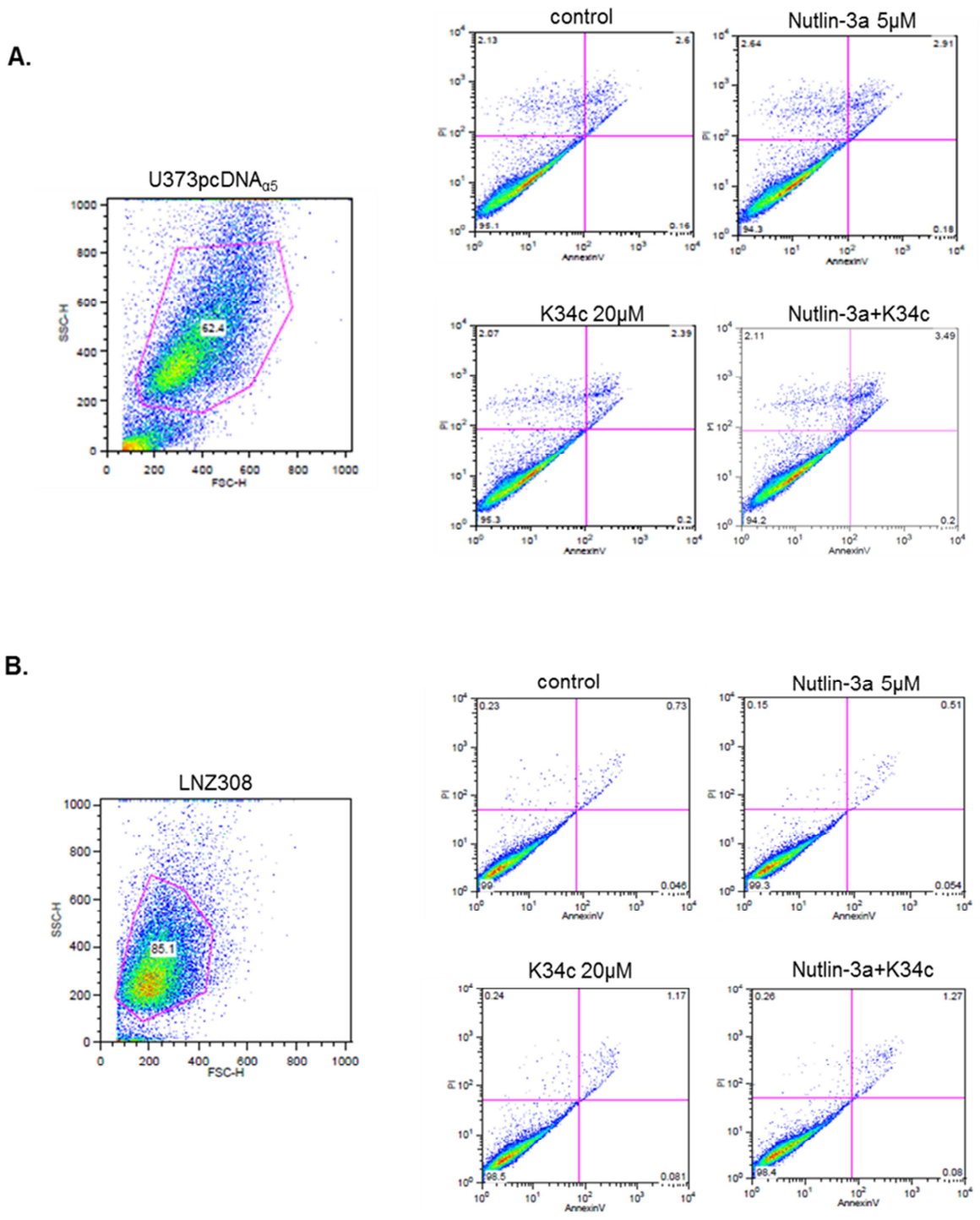


Figure 23: The effects of $\alpha_5\beta_1$ integrin antagonist, Nutlin-3a and their combination on the apoptosis level of U373 and LNZ308 cells. A. U373pcDNA$_{\alpha5}$ cells were labeled with annexin V-FITC/PI after Nutlin-3a (5μM), K34c (20μM) or both drugs treatments (24h). **B.** LNZ308 cells were labeled with annexin V-FITC/PI after Nutlin-3a (5μM), K34c (20μM) or both drugs treatments (24h). Flow cytometry scatter plots for green and red fluorescence channels were used to measure annexin V and PI positivity.

It is important to note, that a single Nutlin-3a treatment showed the capacity to activate caspase 8, caspase 9 and caspase 7 in α_5 low expressing U87shRNA$_{\alpha5}$ cells but to a lesser extent than in U87pcDNA$_{\alpha5}$ cells exposed to combination therapy **(Figure 22C)**.

Next, we evaluated the possible induction of apoptotic cell death in U373pcDNA$_{\alpha5}$ and LNZ308 cells after Nutlin-3a and/or K34c treatment. In line with previously shown data, neither a concomitant treatment nor each treatment separately induced apoptosis, as shown on representative flow cytometry scatter plots for U373pcDNA$_{\alpha5}$ **(Figure 23A)** and LNZ308 **(Figure 23B)**.

To summarize the results obtained, $\alpha_5\beta_1$ integrin antagonist and TMZ as single treatments are not potent to induce apoptosis in GBM cells. Nutlin-3a has a capacity to slightly induce apoptosis in p53wt GBM cells. However, blocking $\alpha_5\beta_1$ integrin function or expression with simultaneous p53wt activation leads to a significant increase of apoptosis in GBM cells.

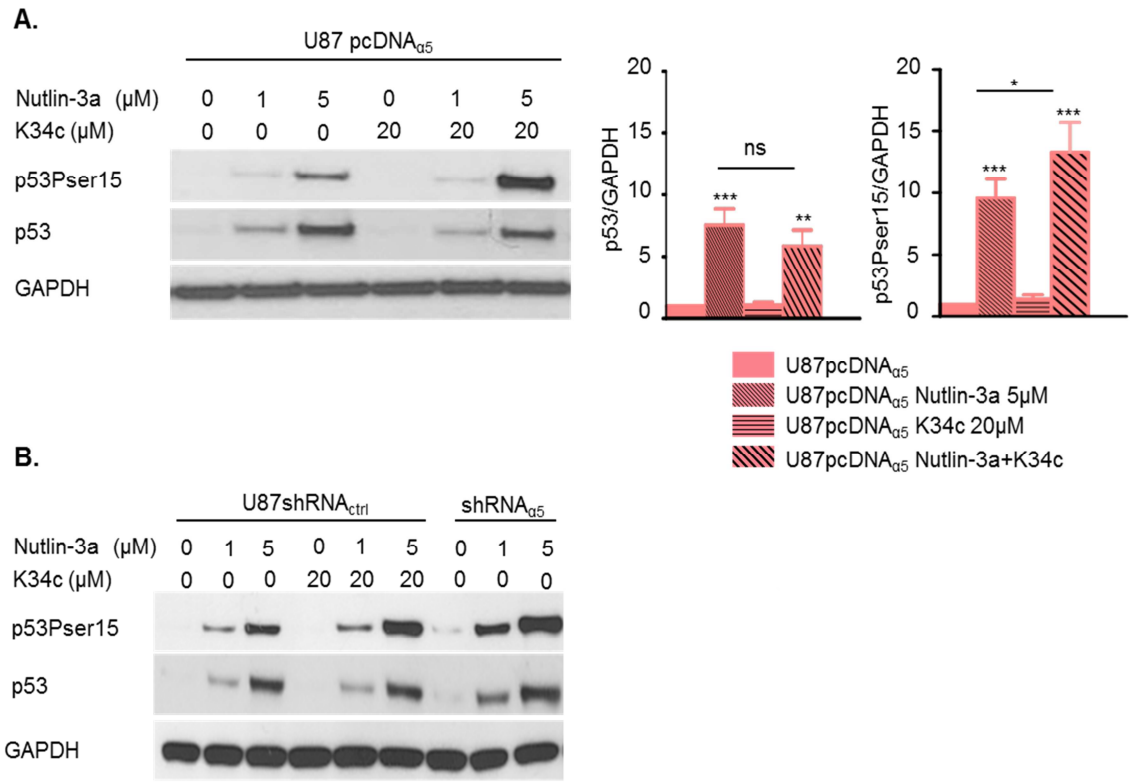


Figure 24: The inhibition of $\alpha_5\beta_1$ integrin potentiates p53 activation by Nutlin-3a in U87MG cells. A. Western blot analysis of p53Pser15 and p53 protein expression of U87pcDNA$_{\alpha5}$ cells treated with K34c (20μM) and/or Nutlin-3a (1 and 5μM) for 12 hours. GAPDH was used as a loading control. The graphs display mean ± SEM of the p53Pser15/GAPDH and p53/GAPDH ratios (5 independent experiments). **B.** Representative Western blot analysis of p53Pser15 and p53 proteins expression in U87shRNA$_{ctrl}$ treated with K34c (20μM) and/or Nutlin-3a (1 and 5μM) and U87shRNA$_{\alpha5}$ cells treated with Nutlin-3a (1 and 5μM) for 12 hours. GAPDH was used as a loading control. Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001, ns, not significant).

2. Analysis of signaling pathways involved in apoptosis

2.1. p53 signaling pathway

2.1.1. p53 protein

We investigated the effects of combination therapy on regulatory proteins and genes implicated in apoptosis. Protein p53 is a key regulator of apoptotic events. Since apoptosis induced by combinatory treatment is strictly dependent on functional p53 in glioblastoma cell lines, we focused on p53 stabilization and activation.

We treated α_5 high expressing U87pcDNA$_{\alpha5}$ cells with Nutlin-3a (1 and 5 µM) and/or K34c (20 µM) for 12 hours. We examined p53 stabilization and p53 activating phosphorylation on ser15. Nutlin-3a strongly stabilized p53 and induced phosphorylation of p53 on ser15 **(Figure 24A)**. The inhibition of $\alpha_5\beta_1$ integrin by K34c affected neither p53 total protein level nor p53 phosphorylation **(Figure 24A)**. When Nutlin-3a was associated with K34c, a significant increase of p53Pser15 was observed, whereas total p53 level was not altered when compared to Nutlin-3a alone **(Figure 24A).**

In line with these results, α_5 low expressing U87shRNA$_{\alpha5}$ cells treated with Nutlin-3a alone increased p53 phosphorylation more than U87pcDNA$_{\alpha5}$ cells exposed to single Nutlin-3a treatment. The level of phosphorylated p53 in U87shRNA$_{\alpha5}$ cells was comparable to those observed in U87pcDNA$_{\alpha5}$ exposed to combination treatment **(Figure 24B)**.

Although Nutlin-3a alone is able to stabilize and activate p53, the blocking of $\alpha_5\beta_1$ integrin function or expression potentiates the p53 activation induced by Nutlin-3a in U87MG cells.

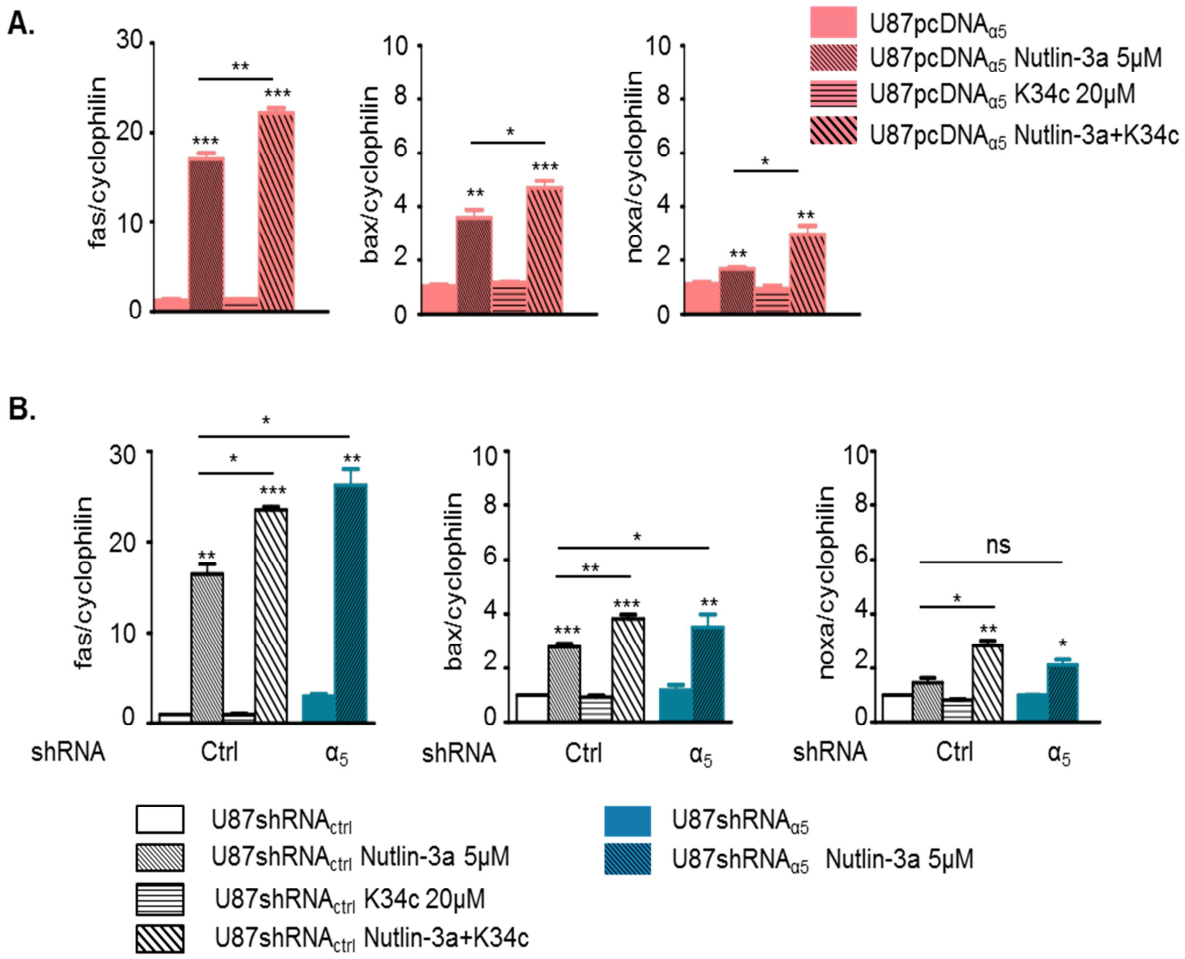


Figure 25: The inhibition of $\alpha_5\beta_1$ integrin potentiates the activation of pro-apoptotic p53 target genes induced by Nutlin-3a. A. qPCR analysis of p53 pro-apoptotic target genes fas (extrinsic apoptotic program), bax and noxa (intrinsic apoptotic program) in U87pcDNA$_{\alpha5}$ cells after 5μM of Nutlin-3a, 20μM of K34c and both drugs treatments for 12 hours. **B.** qPCR analysis of p53 target genes fas (extrinsic apoptotic program), bax and noxa (intrinsic apoptotic program) in U87shRNA$_{ctrl}$ cells treated with Nutlin-3a (1 and 5μM) and/or K34c (20μM) and U87shRNA$_{\alpha5}$ cells treated with Nutlin-3a (1 and 5μM) for 12 hours. Statistical analysis: Student t test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ns, not significant).

2.1.2. p53-dependent activation of pro-apoptotic genes

The protein p53 mainly induces apoptosis through the activation of its target genes, including the pro-apoptotic genes implicated in both extrinsic and intrinsic apoptotic programs. We investigated the effect of combination therapy on the expression of these genes in U87pcDNA$_{\alpha5}$ cells. In particular, we wondered whether the association of K34c to Nutlin-3a enhances the p53-dependent activation of three selected genes: fas, bax and noxa. Fas is a key component of p53-dependent extrinsic death pathway, whereas bax and noxa are associated with intrinsic apoptotic program.

As shown in **Figure 25**, Nutlin-3a strongly increased fas, bax and noxa mRNA levels, whereas K34c was inefficient to enhance any of the pro-apoptotic genes studied. A combination of Nutlin-3a and K34c significantly enhanced the expression of tested genes involved in both extrinsic and intrinsic apoptosis **(Figure 25A)**.

Consistent with our data, Nutlin-3a induced a stronger activation of two pro-apoptotic genes in U87shRNA$_{\alpha5}$ than in Nutlin-3a treated control cells. The mRNA levels of fas and bax in U87shRNA$_{\alpha5}$ cells treated with Nutlin-3a were comparable to those observed in U87shRNA$_{ctrl}$ exposed to the combination treatment **(Figure 25B)**. To note, Nutlin-3a did not significantly up-regulate the transcription of noxa in U87shRNA$_{\alpha5}$ compared to concomitant treatment in U87shRNA$_{ctrl}$ cells.

These data show that the inhibition of $\alpha_5\beta_1$ integrin function or expression enhances Nutlin-3a induced activation of pro-apoptotic genes involved in both extrinsic and intrinsic apoptotic programs, presumably due to the activation of p53. The solely inhibition of $\alpha_5\beta_1$ integrin has no effect on expression of pro-apoptotic genes.

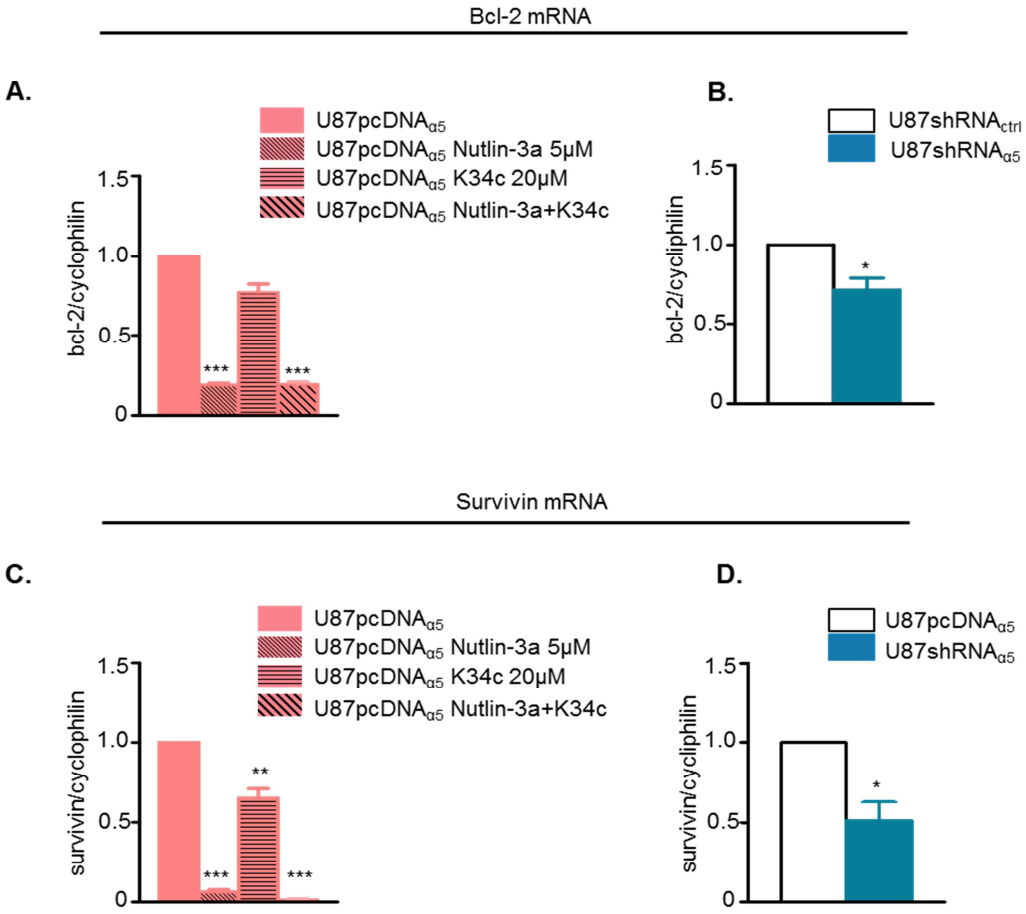


Figure 26: Effects of the combination therapy on the expression of anti-apoptotic genes bcl-2 and survivin. A. qPCR analysis of bcl-2 gene in U87pcDNA$_{\alpha5}$ cells after Nutlin-3a (1 and 5μM) and/or K34c (20μM) treatment (24 hours). **B.** qPCR analysis of bcl-2 gene in U87shRNA$_{\alpha5}$ compared to U87shRNA$_{ctrl}$. **C.** qPCR analysis of survivin gene in U87pcDNA$_{\alpha5}$ treated with Nutlin-3a (5μM) and/or K34c (20μM) for 12 hours. **D.** qPCR analysis of survivin gene in U87shRNA$_{\alpha5}$ compared to U87pcDNA$_{\alpha5}$. Statistical analysis: Student test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

2.1.3. p53-dependent repression of anti-apoptotic genes

In addition to activate pro-apoptotic genes p53 was reported to trans-repress anti-apoptotic genes, including bcl-2 *(Wu et al., 2001)* and survivin *(Hoffman et al., 2002).* Thus, we next investigated the effect of the combination therapy on the expression of bcl-2 and survivin anti-apoptotic genes in U87MG cells.

At first, we measured bcl-2 and survivin gene expression after Nutlin-3a and/or K34c treatment in U87pcDNA$_{\alpha 5}$ cells. As shown in **Figure 26**, a single Nutlin-3a treatment caused a strong inhibition of bcl-2 **(Figure 26A)** and survivin **(Figure 26C)** at mRNA level, suggesting that a highly activated p53 trans-repressed bcl-2 and survivin genes.

The inhibition of $\alpha_5\beta_1$ integrin by K34c slightly reduced, but not significantly, the expression of bcl-2 gene after 12 hours of treatment **(Figure 26A),** and significantly inhibited the expression of survivin gene **(Figure 26C)**. The results with K34c were confirmed in α_5 low U87shRNA$_{\alpha 5}$ cells, where the basal expression levels of both bcl-2 **(Figure 26B)** and survivin **(Figure 26D)** genes were lower than in control cells, indicating the potential of $\alpha_5\beta_1$ integrin to modulate the transcription of anti-apoptotic genes, such as bcl-2 and survivin. The $\alpha_5\beta_1$ integrin antagonist regulated the transcription of anti-apoptotic genes in a p53-independent way, since the blocking of $\alpha_5\beta_1$ integrin was not associated with an elevated level of p53.

In the combination treatment, K34c did not further inhibit the strong reduction of bcl-2 **(Figure 26A)** and survivin **(Figure 26C)** in the presence of Nutlin-3a.

After having evaluated bcl-2 and survivin gene expression, we verified their protein expression levels after a combinatory treatment in U87MG cells. Moreover, we examined the expression of another anti-apoptotic protein MCL-1, recently shown to be repressed by p53 *(Pietrzak and Puzianowska-Kuznicka, 2008).*

As seen in **Figure 27**, p53 induced by Nutlin-3a significantly decreased the protein level of BCL-2 **(Figure 27A)**, survivin **(Figure 27C)** and MCL-1 **(Figure 27E)**.

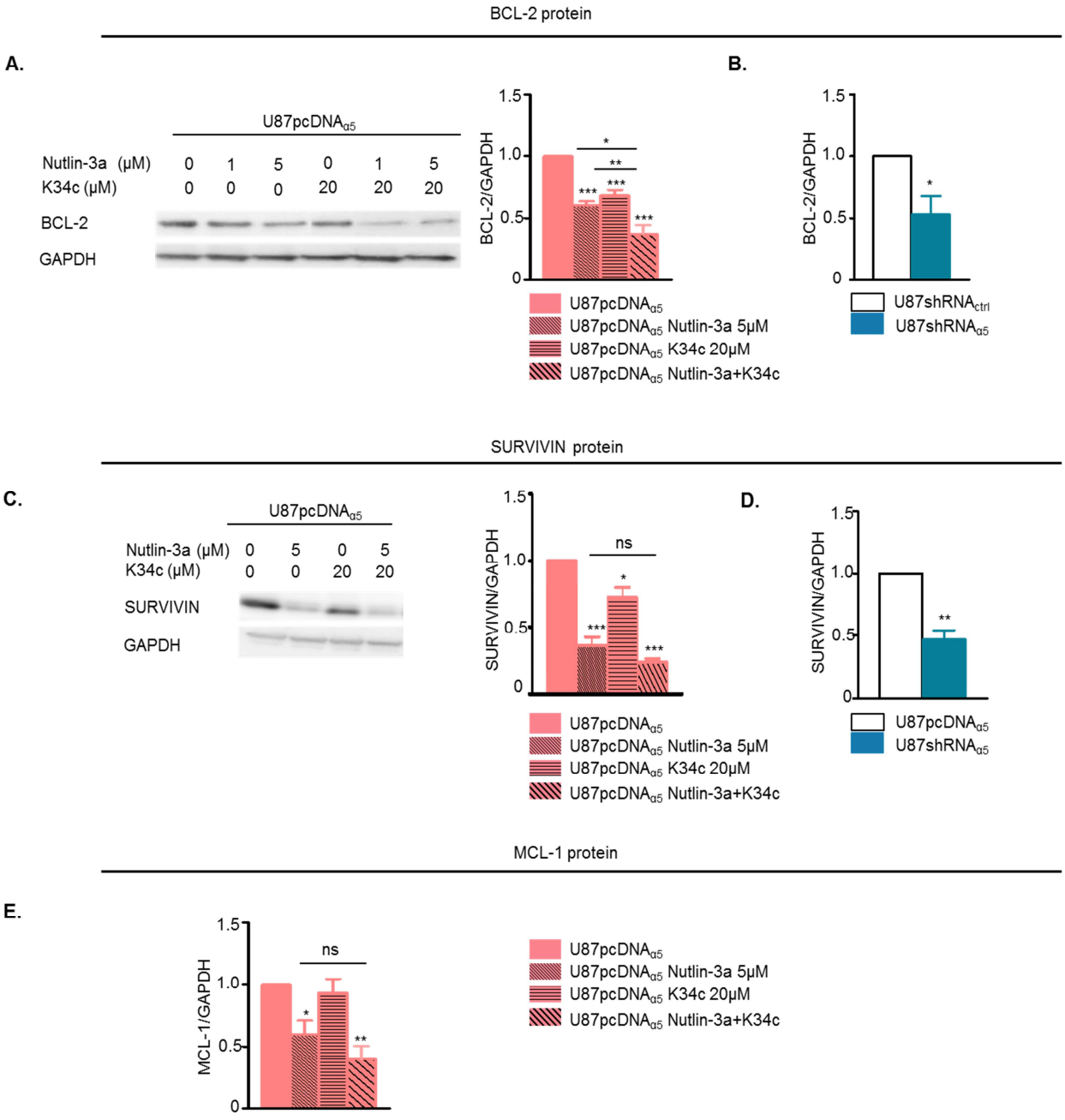


Figure 27: Effects of the combination therapy on protein expression of anti-apoptotic genes BCL-2, Survivin and MCL-1. A. Representative Western blot of BCL-2 protein expression in U87pcDNA$_{\alpha5}$ cells after Nutlin-3a (1μM and 5μM) and/or K34c (20μM) treatment (24 hours). Histograms show the fold increase in BCL-2 protein expression normalized to GAPDH levels (mean ± SEM of 5 independent experiments). **B.** BCL-2 basal protein expression in U87shRNA$_{\alpha5}$ compared to U87shRNA$_{ctrl}$. **C.** Representative Western blot of Survivin protein expression in U87pcDNA$_{\alpha5}$ treated with Nutlin-3a (5μM) and/or K34c (20μM) for 12 hours. Histograms show the fold increase in Survivin protein normalized to GAPDH levels treated U87pcDNA$_{\alpha5}$ versus non-treated U87pcDNA$_{\alpha5}$ cells (mean ± SEM of 3 independent experiments). **D.** Survivin basal protein expression in U87shRNA$_{\alpha5}$ compared to U87pcDNA$_{\alpha5}$. **E.** Histograms show the fold increase in the MCL-1 protein expression normalized to GAPDH levels in U87pcDNA$_{\alpha5}$ cells after Nutlin-3a (5μM) and/or K34c (20μM) treatment (24 hours) (mean ± SEM of 4 independent experiments). Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001, ns, not significant).

K34c efficiently blocked the expression of BCL-2 **(Figure 27A)** and survivin **(Figure 27C)** at protein level, although no inhibition of MCL-1 protein **(Figure 27E)** has been observed. The results with K34c were confirmed in α_5 low U87shRNA$_{\alpha 5}$ cells. The basal expressions of BCL-2 **(Figure 27B)** and survivin **(Figure 28D)** were lower in α_5 low U87shRNA$_{\alpha 5}$ than in control cells.

Finally, a combination of K34c and Nutlin-3a further only blocked the expression of BCL-2 protein **(Figure 28A)**, whereas no further changes of Survivin **(Figure 27C)** and MCL-1 proteins **(Figure 27E)** were detected.

Our results show that Nutlin-3a mediates the activation of pro-apoptotic and the inhibition of anti-proapoptotic genes, whereas $\alpha_5\beta_1$ integrin inhibition is efficient to slightly reduce only anti-apoptotic genes, and has no effect on pro-apoptotic genes. The association of both drugs further enhances the activation of pro-apoptotic genes, although anti-apoptotic genes are not further altered. The changes in anti-apoptotic bcl-2 and survivin genes correspond to changes observed at the protein level. Moreover, the reduction of BCL-2 protein, but not of Survivin protein, is further enhanced by the concomitant treatment. Nutlin-3a causes an inhibition of MCL-1 protein expression, whereas K34c has no effect on MCL-1 expression. The association treatment does not further increase the effect of Nutlin-3a on MCL-1 expression.

Altogether, we suggest that two convergent ($\alpha_5\beta_1$ integrin-based and Nutlin-3a-based), but dissociable pathways induce apoptosis after a combination treatment in glioblastoma cells with functional p53.

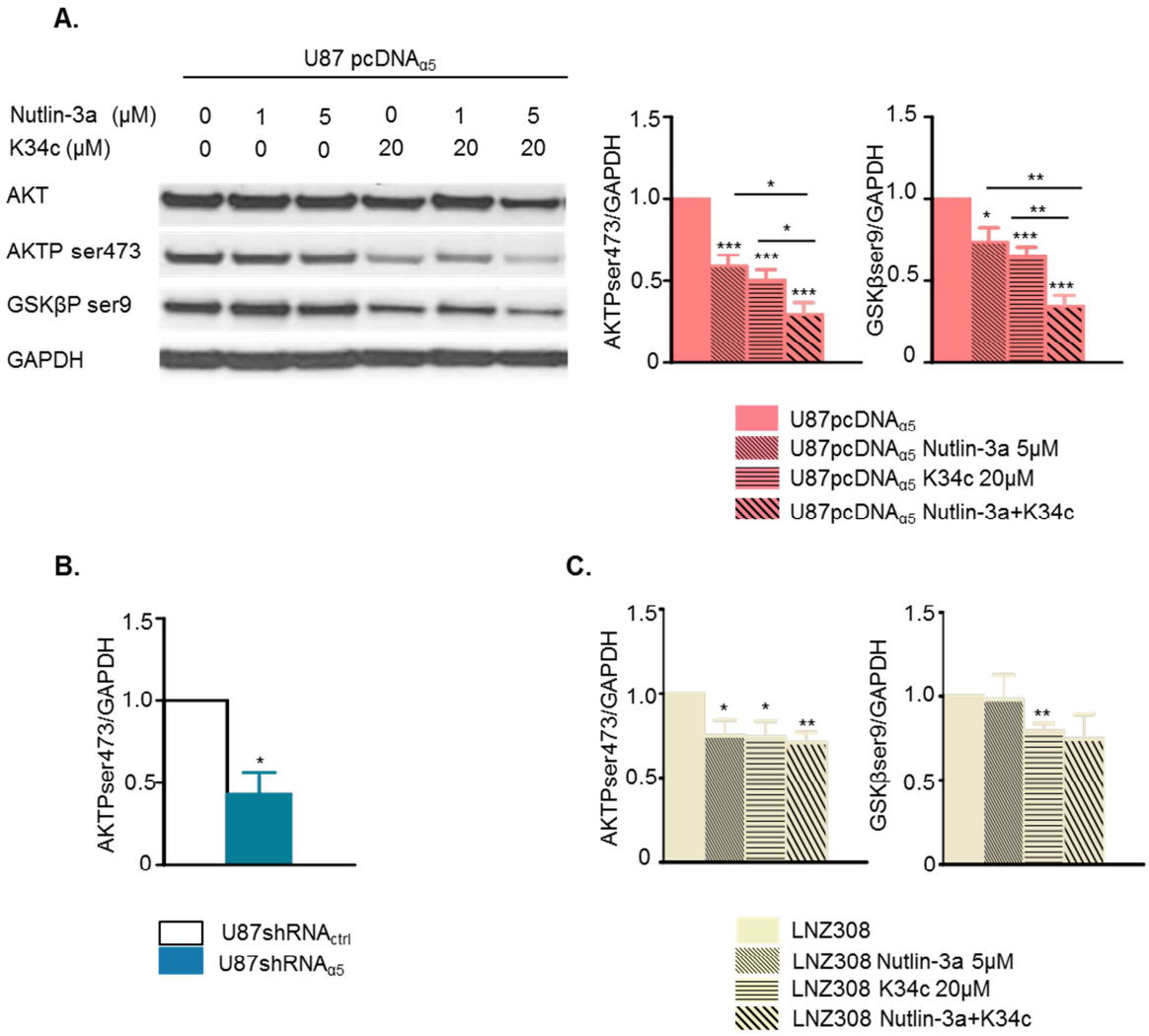


Figure 28: Effects of the combination therapy on AKT activity. A. Western blot analysis of AKTPser473, AKT and GSKβPser9 proteins in U87pcDNA$_{α5}$ cells treated with Nutlin-3a (1 and 5μM), K34c (20μM), or with a combination of both drugs for 12 hours. Histograms show the fold increase in the AKTPser473 (left) and GSKβPser9 (right) protein normalized to GAPDH levels (mean ± SEM of 6 independent experiments). **B.** AKTPser473 protein expression in U87shRNA$_{α5}$ compared to U87shRNA$_{ctrl}$ at the basal level. **C.** LNZ308 were treated with Nutlin-3a (1 and 5μM), K34c (20μM), or with a combination of both drugs for 12 hours. Histograms represent AKTPser473/GAPDH and GSKβPser9/GAPDH fold increase in treated compared to non-treated LNZ308 cells (mean ± SEM of 4 independent experiments). Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001).

2.2. AKT signaling pathway

2.2.1. AKT

Integrin pro-survival signaling is mostly mediated by AKT. Moreover, AKT signaling pathway is known to counteract p53-mediated apoptosis. Thus, we sought to determine whether AKT is down-regulated in our conditions, which could facilitate apoptosis in U87MG cells.

We studied the efficacy of Nutlin-3a (1 and 5µM) and/or K34c (20µM) to modulate AKT activation. U87MG cells were incubated with each treatment or with their combination, and subsequently subjected to Western blot analysis for AKTPser473 (active phosphorylation site of AKT) and GSKβser9 (target of active AKT). As illustrated in **Figure 28**, Nutlin-3a at 5µM potently decreased AKT phosphorylation on ser473. Similarly, the capacity of AKT to phosphorylate its target GSKβ on ser9 was attenuated, indicating that Nutlin-3a efficiently blocked AKT activation **(Figure 28A)**. Antagonist of $\alpha_5\beta_1$ integrin also caused a significant inhibition of AKT activation, as indicated by decreased levels in AKT phosphorylation and GSKβ phosphorylation **(Figure 28A)**. These results were confirmed by shRNAα_5 in U87MG cells. The inhibition of $\alpha_5\beta_1$ integrin expression resulted in a decreased AKT phosphorylation **(Figure 28B)**. Interestingly, the co-treatment with $\alpha_5\beta_1$ integrin antagonist and Nutlin-3a enhanced the inhibition of AKT activation caused by each drug **(Figure 28A)**. The enhanced effect of $\alpha_5\beta_1$ integrin antagonist in combination with p53 activator Nutlin-3a on AKT inhibition seemed to be additive (AKT activity decreased of about 26%, 35% and 66% after Nutlin-3a, K34c and both drugs treatments, respectively) and indicates two signaling pathways converging to trigger apoptosis. Since Nutlin-3a mediated a high p53 activation and K34c did not affect p53, we supposed that AKT inhibition was driven through both p53-dependent and p53-independent signaling pathways.

To confirm our hypothesis, we treated non-apoptotic LNZ308 (p53 null) cells with Nutlin-3a (5µM) and/or K34c (20µM). Nutlin-3a at 5µM slightly decreased AKT phosphorylation in LNZ308 cells **(Figure 28C, left)**. This modest effect on AKT phosphorylation was insufficient to decrease the phosphorylation of GSKβ **(Figure 28C, right)**, indicating that AKT activity was not affected by Nutlin-3a in LNZ308 cells. Thus, Nutlin-3a efficiently decreased AKT activation in U87pcDNA$_{\alpha5}$ cells with intact p53 and not in p53 null LNZ308 cells. Our results indicate the requirement of a functional

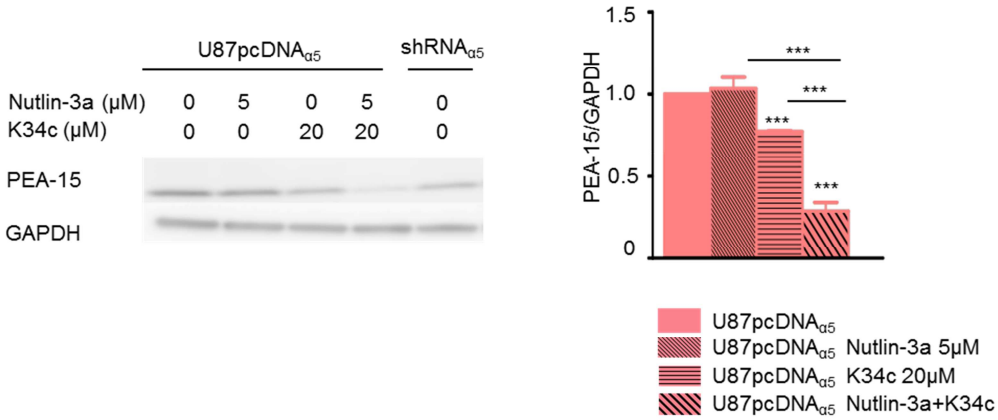


Figure 29: Effects of the combination therapy on PEA-15 expression. Western blot analysis of PEA-15 expression in U87pcDNA$_{\alpha 5}$ treated with K34c (20μM) and/or Nutlin-3a (5μM) (12h) and non-treated U87shRNA$_{\alpha 5}$ cells. Histograms show the fold increase in the PEA-15 protein normalized to GAPDH levels of treated versus non-treated U87pcDNA$_{\alpha 5}$ cells (mean ± SEM of 3 independent experiments). Statistical analysis: Student t test (***, $p<0.001$).

p53 to inhibit AKT activity after Nutlin-3a treatment. On the other hand, $\alpha_5\beta_1$ integrin antagonist repressed AKT activity in LNZ308 cells, as demonstrated by a decreased AKT phosphorylation **(Figure 28C, left)** and GSKβ phosphorylation **(Figure 28C, right)**. The effects of $\alpha_5\beta_1$ integrin antagonist on AKT activation were p53-independent, since $\alpha_5\beta_1$ integrin antagonist decreased AKT phosphorylation in both p53 wild type U87pcDNA$_{\alpha5}$ cells and p53 knockout LNZ308 cells **(Figure 28C)**. The combination treatment with Nutlin-3a and K34c did not improve the reduction of AKT activity caused by K34c **(Figure 28C)**. These data confirmed the p53-dependent (driven by Nutlin-3a) and the p53-independent pathways (driven by $\alpha_5\beta_1$ integrin) converging to inhibit AKT activity. We suggest that these two pathways are necessary to facilitate apoptosis in p53wt glioblastoma cells after combination therapy.

2.2.2. PEA-15

Our preliminary results with Human Apoptosis gene arrays revealed that PEA-15 mRNA was affected by a combination treatment in U87pcDNA$_{\alpha5}$. It has been reported that AKT kinase phosphorylated PEA-15, and thus increased its stability and its anti-apoptotic function *(Trencia et al., 2003).* Thus, PEA-15 may link AKT pathway to apoptosis.

As shown in **Figure 29**, PEA-15 was highly expressed in U87pcDNA$_{\alpha5}$ cells as compared to U87shRNA$_{\alpha5}$ cells. The $\alpha_5\beta_1$ integrin antagonist significantly reduced PEA-15 expression, confirming the impact of $\alpha_5\beta_1$ integrin on PEA-15. Interestingly, Nutlin-3a had no effect on PEA-15 expression**.** However, the combination of both drugs enhanced the reduction of PEA-15 expression compared to $\alpha_5\beta_1$ antagonist single treatment. Since PEA-15 was highly reduced by the combination treatment, we suggest, that PEA-15 inhibition might be involved in promotion of apoptosis in U87MG cells.

Although the modulation of AKT pathway seems to be under the control of Nutlin-3a (through p53 activation) and of $\alpha_5\beta_1$ integrin, PEA-15 appears uniquely modulated by the integrin $\alpha_5\beta_1$. However, the co-treatment leads to a strong inhibition of PEA-15 protein expression.

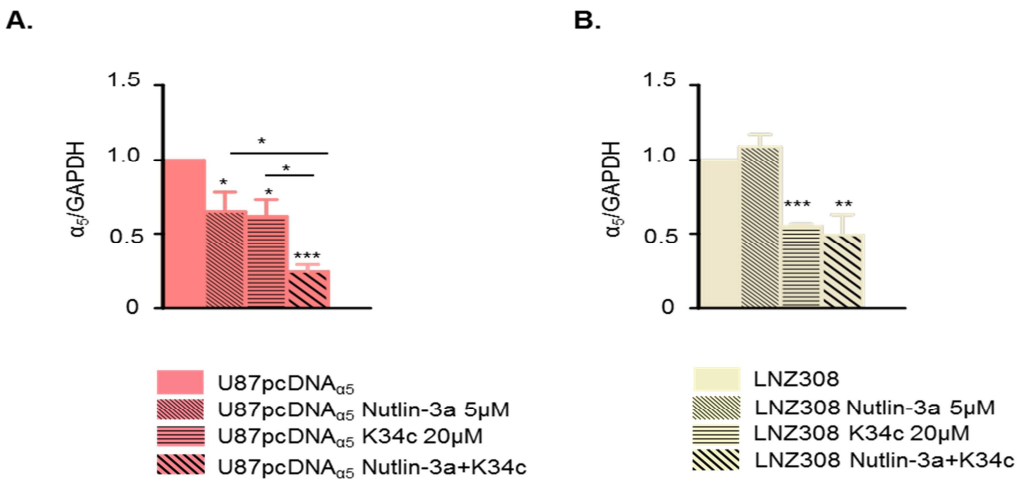


Figure 30: Effects of combined treatment with Nutlin-3a and $\alpha_5\beta_1$ integrin antagonist on α_5 expression. A. Western blot analysis of the α_5 protein expression normalized to GAPDH levels in U87pcDNA$_{\alpha5}$ treated with Nutlin-3a (5μM) and/or K34c (20μM) for 24h (mean±SEM, 4 independent experiments). **B.** Western blot analysis of the α_5 protein expression normalized to GAPDH levels in LNZ308 treated with Nutlin-3a (5μM) and/or K34c (20μM) for 24h (mean±SEM, 4 independent experiments). Statistical analysis: Student t test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

2.2.3. α_5 integrin

Blocking $\alpha_5\beta_1$ integrin affected the expression of PEA-15 protein. The combinatory treatment was very efficient to inhibit PEA-15. Thus, we wondered whether the expression of α_5 subunit was not affected by the combination treatment.

We studied the expression of α_5 subunit in U87MG cells after a treatment with Nutlin-3a (5µM) and/or K34c (20µM). As already demonstrated by our previous data, p53wt induced by Nutlin-3a decreased the expression of α_5 integrin subunit. Interestingly, $\alpha_5\beta_1$ integrin antagonist not only blocked $\alpha_5\beta_1$ integrin, but also reduced α_5 integrin subunit at the protein level. Moreover, the combination treatment enhanced the reduction of α_5 subunit compared to single drug therapy **(Figure 30A)**. The α_5 subunit expression level was decreased by Nutlin-3a, K34c and both drugs together of about 35%, 39% and 76%, respectively. As in the case of AKT, the reduction in α_5 subunit after a concomitant treatment is additive, suggesting two signaling pathways converging to a reduced α_5 expression level in U87pcDNA$_{\alpha5}$ cells.

To confirm this hypothesis, we performed the same experiment with LNZ308 cells (p53 null) shown to be resistant to apoptosis after a concomitant treatment with Nutlin-3a and K34c. In accordance with our previous data, Nutlin-3a did not alter the α_5 expression in these cells, confirming the requirement of a functional p53 to inhibit the α_5 expression by Nutlin-3a. The $\alpha_5\beta_1$ integrin antagonist was efficient to decrease α_5 protein expression in LNZ308 cells, indicating that a p53-independent pathway was involved **(Figure 30B)**. The concomitant blocking of $\alpha_5\beta_1$ integrin in Nutlin-3a-treated LNZ308 cells did not improve the α_5 reduction obtained by K34c **(Figure 30B)**.

We suggest that the inhibition of α_5 subunit is driven by two distinct converging p53-dependent and p53-independent pathways. The additive effect of both drugs on α_5 expression could be essential in the promotion of apoptosis in p53wt U87MG cells. This is also supported by the fact that p53 knockout LNZ308 cells did not undergo apoptosis after the combined therapy.

GBMs are the most aggressive and the most common brain tumors. Despite the current standard therapy, including surgery, radiotherapy and chemotherapy, the median survival of GBM patients is only about 15 months *(Stupp et al., 2005).* Thus, new therapies are urgently needed. In the recent years, certain studies pointed out the $\alpha_5\beta_1$ integrin as a potential target for the development of cancer therapies. The $\alpha_5\beta_1$ integrin has been shown to play an important role in tumor progression *(Roman et al., 2010)*, metastasis *(Sawada et al., 2008; Mitra et al., 2011)* and/or resistance to therapies *(Nam et al., 2010)* in lung, ovarian and breast cancer, respectively (reviewed by *Schaffner et al., 2013*).

1. $\alpha_5\beta_1$ integrin in GBM patients

In the first part of this work, we investigated the role of $\alpha_5\beta_1$ integrin in the resistance to therapy in glioma. The analysis of 105 brain tumor biopsies revealed that the gene expression of α_5 subunit correlated with tumor grades, making α_5 expression a marker of glioma aggressiveness. Moreover, our results demonstrated for the first time that the expression of α_5 subunit was negatively associated with patient's survival, suggesting a role for $\alpha_5\beta_1$ integrin in resistance to standard therapy. Our data present $\alpha_5\beta_1$ integrin as an attractive target for the treatment of glioma. In agreement with our data, it was reported that α_5 integrin was overexpressed at both mRNA *(Kita et al., 2001)* and protein levels in GBMs *(Gingras et al., 1995).* Holmes and his coworkers confirmed our findings, and showed that $\alpha_5\beta_1$ integrin expression in glioma patients was associated with a poor prognosis *(Holmes et al., 2012).*

2. $\alpha_5\beta_1$ integrin and resistance to chemotherapy

GBMs are highly resistant tumors to standard therapy and resistance to chemotherapeutic drug TMZ has become a major obstacle to GBM therapy. Here, our data brought the first evidence demonstrating that $\alpha_5\beta_1$ integrin compromises the TMZ-induced p53 activation, and thus provokes a resistance to TMZ chemotherapy of GBM cells harboring a functional p53.

A p53 mutation has been found in about 50% of human cancers. The most current primary GBMs harbor a p53 gene mutation in about 30% of cases. In secondary GBMs, p53 mutations are more frequent with an incidence of about 65% *(Ohgaki et al., 2004; Zheng et al., 2008).* The analysis of multi-dimensional genomic data identified four subclasses of GBMs: proneural, mesenchymal, neural and classical *(Verhaak et al., 2010)*. The p53 mutations occur in about 54, 32, 21 and 0% in glioblastoma subtypes proneural, mesenchymal, neural and classical, respectively *(Verhaak et al., 2010).*

We found that GBMs with intact p53 were more resistant to TMZ than p53 mutant or p53 null GBM cell lines. Several studies already addressed the role of p53 status in response to TMZ. Some studies demonstrated an enhanced response to TMZ in GBMs with intact p53 *(Hermisson et al., 2006; Roos et al., 2007).* Conversely, others have reported that p53 wild type inhibition improved TMZ capacity in GBM cell lines (*Hirose et al., 2001; Xu et al., 2005, Dinca et al., 2008; Blough et al., 2011).* Since contradictory results have been reported, the relation between p53 status and chemotherapy with TMZ in GBMs is not clear. Previous data of our laboratory and others suggested that p53 wild type glioma cells impaired their apoptotic function through preferential mediation of cell cycle arrest and DNA repair, whereas the disruption of p53 functions were associated with transient cell cycle arrest and apoptotic cell death *(Hirose et al., 2001; Martinkova et al., 2010).*

We demonstrated here that $\alpha_5\beta_1$ integrin counteracts p53 activity and thus provokes TMZ resistance. A few studies investigated the role of integrins in p53 regulation. They reported both integrin-dependent activation *(Bachalder et al., 1999; Lewis et al., 2002)* and inhibition *(Stromblad et al., 1996; Bao and Stromblad, 2004; He et al., 2008)* of p53 signaling in various tumors. However, the exact mechanism of p53 regulation by the integrin is not clear. The $\alpha_5\beta_1$ integrin may be directly or indirectly implicated in p53 activation. For instance, it was demonstrated that AKT (activated mainly by integrins) directly regulated the family of stress-activated kinases known to enhance p53 activity *(She et al., 2000; Widenmaier et al., 2009; Saha et al., 2012)*. AKT was also shown to increase MDM2 activity, and thus to counteract p53 activity *(Zhou et al., 2001).*

We showed that p53 wild type glioma tumors increased their expression of α_5 subunit. Altogether, our findings suggest that p53 wild type GBMs overexpress $\alpha_5\beta_1$ integrin, and thus attenuate p53 signaling, which in turn make them resistant to TMZ therapy.

3. A negative crosstalk between $\alpha_5\beta_1$ integrin and p53

Nutlin-3a is a small inhibitor of the MDM2-p53 interaction *(Vassilev, 2004).* Nutlin-3a binds to the p53-binding pocket of MDM2, and thus disrupts MDM2-p53 complex, which results in p53 activation. We demonstrated that p53 activation by Nutlin-3a down-regulated the expression of functional α_5 subunit. We suggest that Nutlin-3a overrides the α_5 inhibitory effect on p53 activation by its capacity to decrease α_5 subunit. The mechanism by which p53 activator Nutlin-3 represses α_5 expression in p53 wild type GBM cells remains to be elucidated. Since the promoter of α_5 gene does not contain p53-binding consensus, p53-driven repression of α_5 gene does not seem to be likely. However, p53 was reported to bind SP1 transcriptional factor, one of the regulators of α_5 gene, suggesting a possible impact of p53 on the regulation of α_5 gene transcription *(Pietrzak and Puzianowska- Kuznicka, 2008; Gingras et al., 2009)*. At protein level, $\alpha_5\beta_1$ integrin can be degraded by proteasome or lysosome *(Margadant et al., 2011).*

It has been demonstrated that the reactivation of p53 *in vivo* stopped tumor growth *(Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007).* A small p53 activator, Nutlin-3a, was tested in several xenograft models of human cancer with p53wt. In these models, Nutlin-3a efficiently inhibited the tumor growth with no toxicity for the normal tissue *(Vassilev et al., 2004; Sarek et al., 2007; Shangary et al., 2008; Shangary and Wang, 2009).* Nutlin-3a is currently in Phase I clinical trial for the treatment of retinoblastoma. These data indicate the importance of p53 protein in tumorigenesis. Thus, the restoration of p53 through $\alpha_5\beta_1$ integrin inhibition may be a new strategy to fight these aggressive tumors.

4. The activation of p53 is not sufficient to trigger a full apoptotic response

The protein p53 is a potent activator of apoptotic cell death. However, cells harboring p53wt usually promote cell cycle arrest and/or senescence, rather than apoptosis. These three outputs have already been shown to promote tumor regression; however, the therapeutic impact is higher in the case of apoptotic cell death. Moreover, senescent cells were shown to secrete cytokines that stimulate the proliferation of surrounding tumor cells *(Jackson et al., 2012).* Previous data of our laboratory showed that TMZ was potent to induce a premature senescence in p53wt GBM cells rather than apoptosis *(Martinkova et al., 2010)*. Different p53wt tumor cells, including GBMs treated with Nutlin-3a

preferentially underwent cell cycle arrest or senescence rather than apoptosis *(Villalonga-Planells et al., 2011).* These data indicate that p53wt activated by genotoxic or non-genotoxic agents is not effective to induce apoptosis in GBMs with a functional p53.

Tumor cells can acquired an apoptotic resistance through several strategies, including overexpression of oncogenes counteracting with cellular death *(Vousden and Lu, 2002; Slee et al., 2004)*. Here, we found that $\alpha_5\beta_1$ integrin blocking both enhanced TMZ and Nutlin-3a inhibitory effects on survival of GBM cells with intact p53. The enhanced inhibitory effect of the combination therapy on the cell survival is caused by promotion of apoptosis in GBM cells with an intact p53. Thus, we underscored here the importance of $\alpha_5\beta_1$ integrin in resistance to p53-dependent apoptosis in GBM cells.

5. The convergent signaling pathways involved in apoptosis

The balance between pro-apoptotic and pro-survival pathways determines whether the cells undergo or not apoptosis. The protein p53 is the main actor in the induction of apoptosis through its ability to activate pro-apoptotic genes and to repress anti-apoptotic genes. We found that Nutlin-3a efficiently activated pro-apoptotic genes and repressed anti-apoptotic genes. However, Nutlin-3a was not efficient to induce a full apoptotic response in p53wt GBMs. *Grinkevich et al. (2009)* demonstrated that reactivated p53 by RITA up-regulated pro-apoptotic genes and down-regulated survival factors. They suggested that a simultaneous activation of pro-apoptotic genes and repression of pro-survival factors result in a robust apoptosis. In our model, a simultaneous activation of apoptotic genes, and an inhibition of pro-survival proteins by p53 activator Nutlin-3a did not lead to a full apoptotic response. Only an additional inhibition of $\alpha_5\beta_1$ integrin in p53-activated GBM cells promoted the apoptotic cell death. We showed that the concomitant treatment modestly enhanced the pro-apoptotic genes induced by Nutlin-3a. However, we suggested that the slightly elevated level of apoptotic genes could not fully explain apoptosis of GBM cells after a combination therapy.

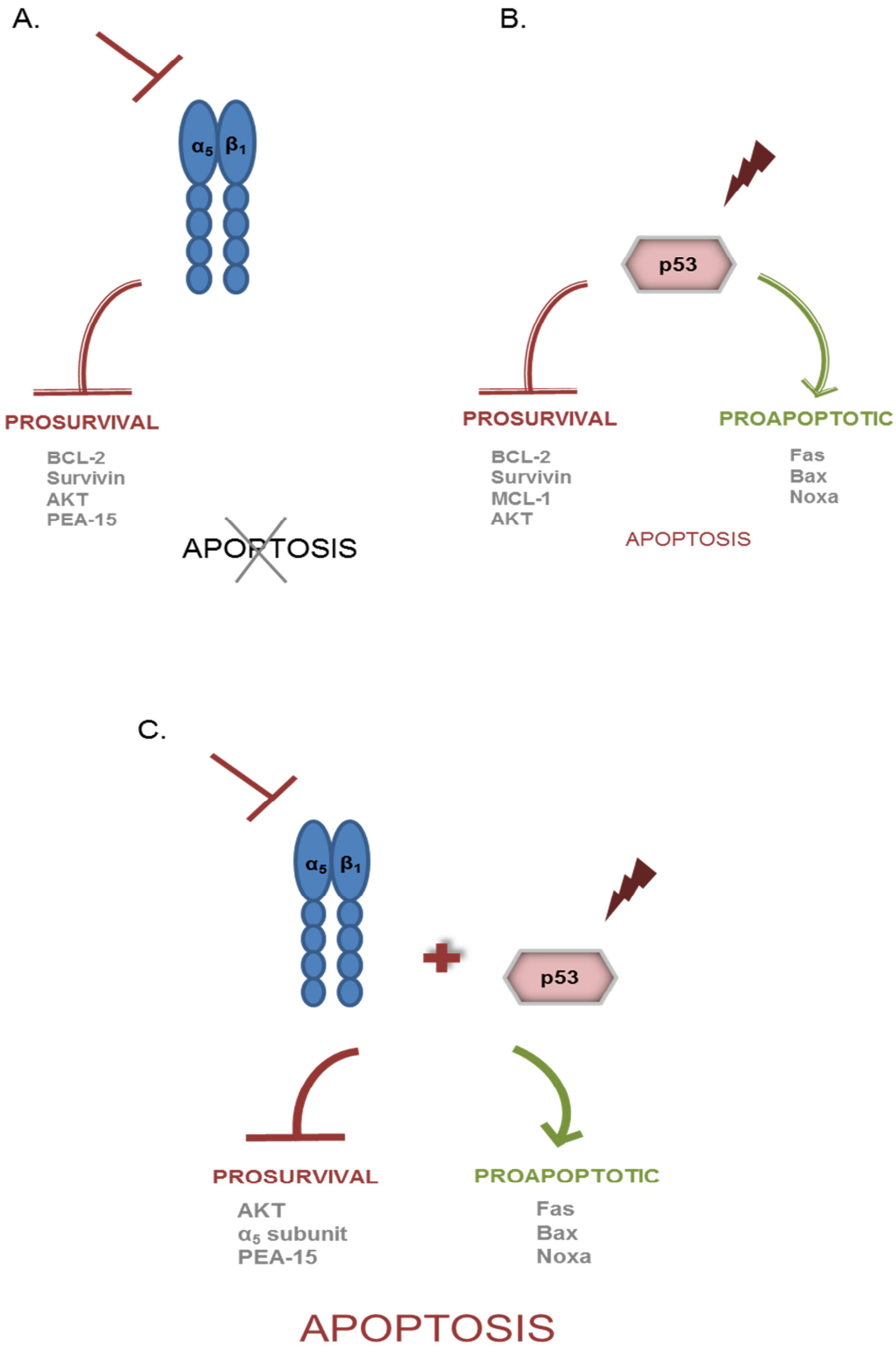


Figure 31: The effects of single and combinatory treatments on the expression pattern of pro-apoptotic and pro-survival components.

Whereas p53 is a key component in the apoptotic cell death, the presence of survival signals can render cells resistant to p53-driven apoptosis. We hypothesize that $\alpha_5\beta_1$ integrin enhanced a pro-survival threshold and thus inhibited p53-dependent apoptosis. The most studied integrin-dependent pro-survival signaling pathway is PI3K/AKT. We demonstrated that two distinct converging p53-dependent (through p53) and p53-independent (through $\alpha_5\beta_1$ integrin) signaling pathways inhibited AKT activity after concomitant treatment. Interestingly, we found that α_5 integrin subunit was repressed in the same way as AKT. Thus, we presume that the additive effects of both drugs in AKT and α_5 integrin subunit inhibitions may be key events to facilitate apoptosis in GBM cells. In accordance with our findings, it has already been reported that the inhibition of AKT synergized with Nutlin-3a and enhanced apoptosis in acute lymphoblastic leukemia cells *(Zhu et al., 2008).* Moreover, we discovered another key candidate, PEA-15, that could play a role in an efficient apoptotic response of GBM cells. PEA-15 is a pro-survival protein, and its stability is known to be affected by AKT *(Trencia et al., 2003; Fiory et al., 2009).* PEA-15 is over-expressed in high-grade astrocytomas *(Watanabe et al., 2010)*. We showed here that PEA-15 stability was only affected by $\alpha_5\beta_1$ integrin antagonist, and not by p53 activator Nutlin-3a. We observed a dramatic decrease in PEA-15 protein after combination treatment. PEA-15 has been demonstrated by several studies to counteract apoptotic cell death (*Condorelli et al., 1999; Estelles et al., 1999; Hao et al., 2001; Song et al., 2006; Peacock et al., 2009; Hayashi et al., 2012).*

The **Figure 31** summarizes the effects of Nutlin-3a and K34c single and combinatory treatments on the expression pattern of pro-apoptotic and pro-survival components in GBM cells.

6. Conclusions

Altogether, we demonstrated for the first time that the expression of $\alpha_5\beta_1$ integrin is a prognostic and therapeutic marker in GBMs. We found that $\alpha_5\beta_1$ integrin provokes a resistance to TMZ chemotherapy via the down-regulation of p53 activation. Non-genotoxic p53 activator Nutlin-3a down-regulates $\alpha_5\beta_1$ integrin, and thus makes GBM cells susceptible to p53 activation. The inhibition of $\alpha_5\beta_1$ integrin sensitized GBM cells to a p53-based therapy via the induction of p53-dependent apoptosis. We suggest that the down-regulation of AKT, α_5 integrin subunit and PEA-15 pro-survival factors may be a

crucial step in the induction of apoptosis in GBM cells. The detailed mechanism by which the inhibition of $\alpha_5\beta_1$ integrin induces apoptosis in p53-activated GBM cells is under investigation.

We believe that a concomitant $\alpha_5\beta_1$ integrin inhibition with p53-activation could increase the patients' response to p53-based therapies. This preclinical study proposes a new potential therapy for a subpopulation of GBM patients with a high $\alpha_5\beta_1$ integrin expression and an intact p53. Since the most frequent GBMs harbor p53wt, our data are of important value, and open the door to a new strategy against these aggressive brain tumors.

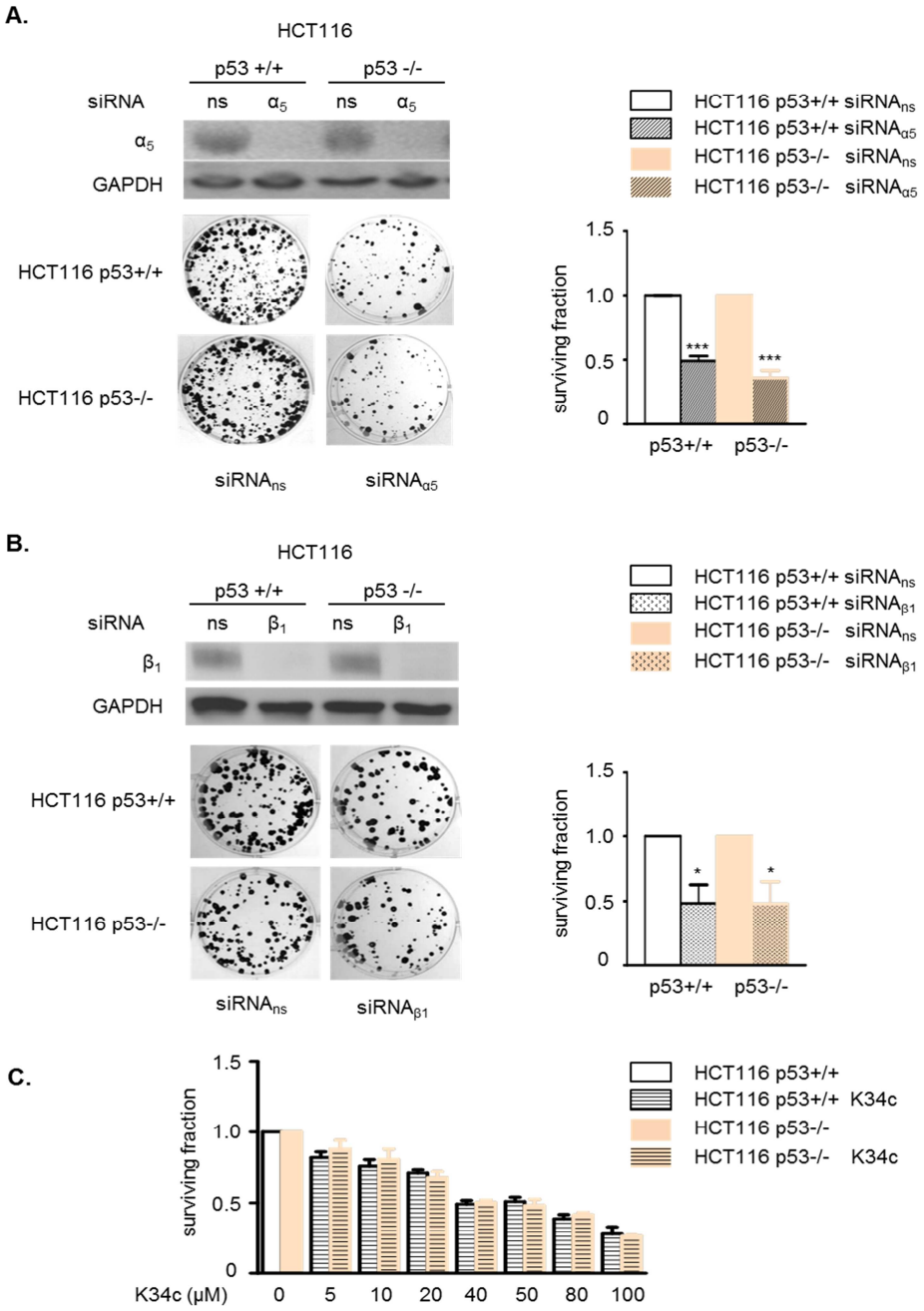


Figure 32: Integrin $\alpha_5\beta_1$ effects on HCT116 cell survival. A. Upper panel: HCT116 p53+/+ and HCT116 p53-/- cells were transfected with non-silencing siRNA (siRNA-ns) or specific siRNA targeting α_5 integrin (siRNA-α_5). The repression of the α_5 subunit was verified by Western blot. GAPDH was used as a loading control. Lower panel: Clonogenic survival assays were performed with transfected cells. Histograms display the mean ± SEM of 3 independent experiments. **B.** Upper panel: HCT116 p53+/+ and HCT116 p53-/- cells were transfected with non-silencing siRNAs (siRNA-ns) or specific siRNA targeting β_1 integrin (siRNA-β_1). The repression of the β_1 subunit was verified by Western blot. GAPDH was used as a loading control. Lower panel: Clonogenic survival assays were performed with transfected cells. Histograms display the mean ± SEM of 3 independentexperiments. **C.** The clonogenic survival of HCT116 p53+/+ and HCT116 p53-/- cells treated with increasing concentrations of an antagonist of $\alpha_5\beta_1$ integrin, K34c. Histograms represent the mean ± SEM of treated versus non-treated cells (n=4).

Part II: $\alpha_5\beta_1$ integrin and its role in colon cancer

In the first part we demonstrated the negative cross-talk between $\alpha_5\beta_1$ integrin and p53 that participate to the resistance to therapy in glioma. In the second part of this work we investigated whether the results obtained in glioblastoma could be extrapolated to other solid tumors, in particular to colorectal cancer. Colorectal cancer is one of the most common cancer. Most deaths due to colon cancer are caused by tumor metastatic dissemination to lungs and liver *(Kanwar et al., 2012)*. Cell adherence, migration and motility are the key characteristics of metastatic cancer and involve families of proteins such as integrins. Previously, it was shown that $\alpha_5\beta_1$ integrin is highly expressed in highly invasive colon cancer cell lines compared to poorly invasive cells *(Gong et al., 1997).* Moreover, the inhibition of $\alpha_5\beta_1$ integrin function sensitizes colon tumors to chemotherapeutic drug 5-fluorouracil *(Stoeltzing et al., 2003).* These findings suggest that $\alpha_5\beta_1$ integrin may be a therapeutic target in colon cancer. In this part of our work, we investigated the role of integrin $\alpha_5\beta_1$ in highly invasive colon cell line HCT116.

1. $\alpha_5\beta_1$ integrin effects on HCT116 cell survival

To determine the implication of integrin $\alpha_5\beta_1$ in colon cancer cell survival, the clonogenic survival assay was performed. We used HCT116 colon cancer cells expressing p53 wild type protein or their isogenic counterparts with no p53 expression (respectively named HCT116 p53+/+ and p53 -/- cells). These two cell lines express similar level of α_5 integrin **(Figure 32A)**.

In these cellular models, we decreased the expression of the α_5 subunit by using specific siRNA. 72 hours after siRNA transfection, α_5 protein expression was decreased to about 81% with the α_5-targeting siRNA compared to the non-silencing siRNA **(Figure 32A)**. Long term survival assays showed that loss of α_5 expression led to a decreased number of colonies formed either by HCT116 p53+/+ or HCT116 p53-/- cells **(Figure 32A)**.

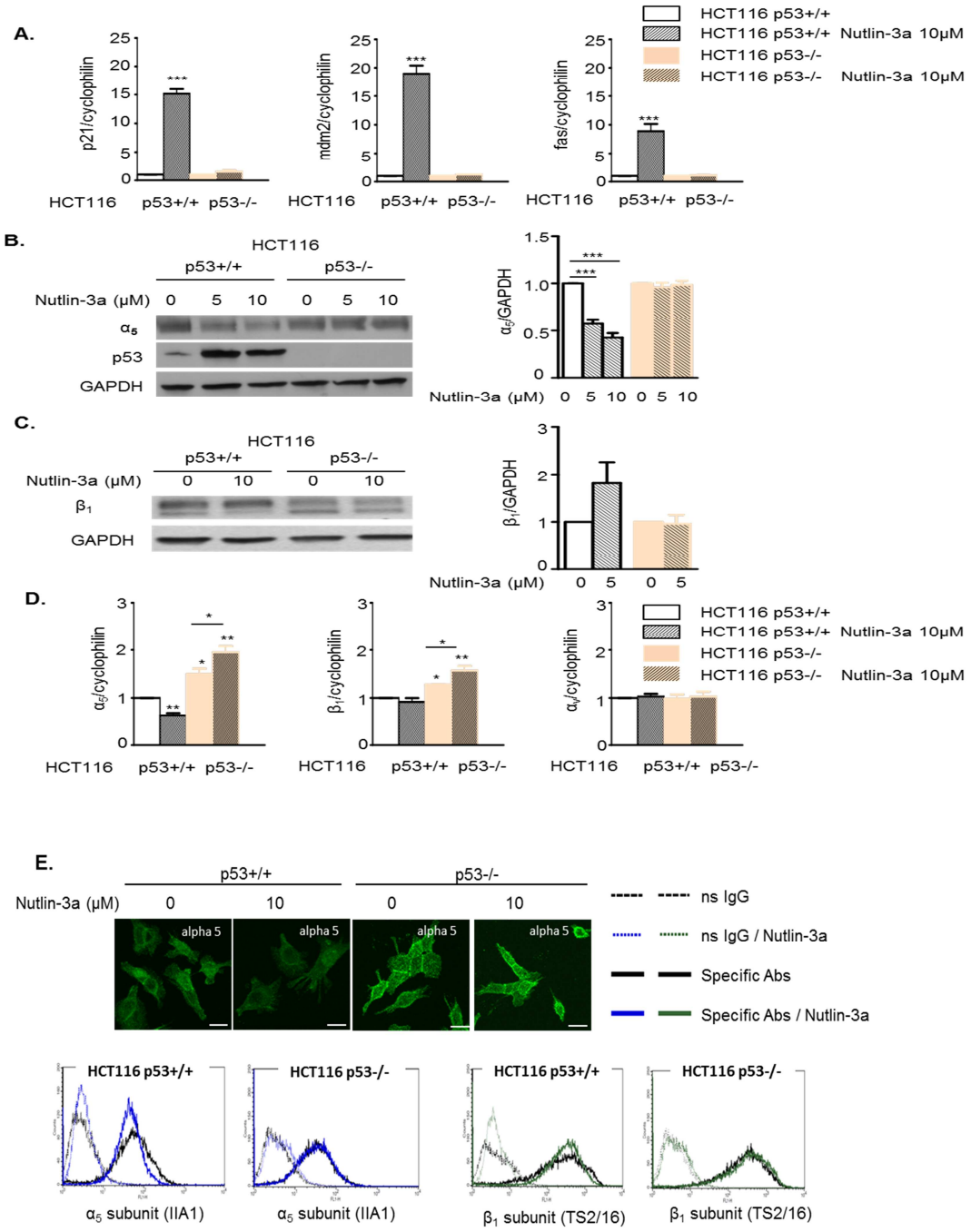


Figure 33: p53 activation by Nutlin-3a affects α_5 integrin expression in HCT116. A. qPCR analysis of p53 target genes p21, mdm2 and Fas in HCT116 p53+/+ and HCT116 p53-/- cells after 10μM of Nutlin-3a treatment for 24h. **B.** Western blot analysis of α_5 and p53 protein expression in HCT116 p53+/+ and HCT116 p53-/- cells after 5μM and 10μM Nutlin-3a treatment for 24h. Histograms show the fold increase in the protein expression normalized to GAPDH levels (mean ± SEM of 3-4 independent experiments). **C.** Western blot analysis of β_1 protein expression in HCT116 p53+/+ and HCT116 p53-/- cells treated with 10μM Nutlin-3a for 24h. Histograms show the fold increase in the protein expression normalized to GAPDH levels (mean ± SEM of 3 independent experiments). The anti-β_1 antibody (AB1952, Chemicon) detects two bands corresponding to the premature (90kDa) and mature (110kDa) proteins. Only the 110kDa band was considered in the histograms. **D.** qPCR analysis of the α_5, β_1 and α_v subunits mRNA in HCT116 p53+/+ and HCT116 p53-/- cells after 10μM of Nutlin-3a treatment for 24h. **E.** Upper panel: Representative fluorescence confocal microscopy images of the α_5 subunit expression in the HCT116 p53+/+ and HCT116 p53-/- cells after 10μM of Nutlin-3a treatment for 24h. Scale bars: 20μm. Lower panel: Flow cytometry analysis of the α_5 (IIA1 antibody) and β_1 (TS2/16 antibody) subunit expression at the cell membrane in HCT116 p53+/+ and HCT116 p53-/- cells after 10μM Nutlin-3a for 24h. A representative experiment is shown in the Figure and mean values are shown in Table 6. *** p<0.005.

Similar results were obtained when β_1 integrin subunit expression was decreased with specific siRNA **(Figure 32B)**.

In order to confirm the impact of $\alpha_5\beta_1$ integrin on HCT116 cell survival, we treated the cells with the specific non-peptidic small antagonist of $\alpha_5\beta_1$ integrin, K34c. K34c dose-dependently inhibited clonogenic survival at similar levels in HCT116 p53+/+ and p53-/- cells **(Figure 32C)**.

The data show that the clonogenic potential of HCT116 cells was altered either when integrin functions were blocked by K34c or when integrin expression was inhibited by siRNA interference. It appears that the inhibition of cell survival by depletion of α_5 and β_1 integrin subunits or by functional inhibition of the integrin by K34c is independent of p53 expression, suggesting some p53 independent survival pathways triggered by the integrin. These results confirm that $\alpha_5\beta_1$ integrin is a therapeutic target in colon cancer cells, and suggest that therapies capable of affecting the integrin expression/function may prove to be a valuable strategy for colon carcinoma eradication.

2. p53 activation by Nutlin-3a: effects on $\alpha_5\beta_1$ integrin expression

We have previously shown that the activation of p53 by Nutlin-3a decreases the α_5 integrin expression in glioma cells. To investigate if activation of p53 modulates the expression of $\alpha_5\beta_1$ integrin in colon tumor cells, we treated HCT116 cells with Nutlin-3a. Nutlin-3a activated p53 as shown by an increased level of p53 target genes **(Figure 33A)** and stabilized p53 protein **(Figure 33B)** in HCT116 p53+/+ cells but not, as expected, in HCT116 p53 -/- cells.

Treatment of HCT116 p53+/+ cells by Nutlin-3a decreased the expression level of α_5 integrin subunit in a dose dependent manner, but not in p53 negative counterparts as shown by Western blot analysis of total protein extracts **(Figure 33B)**. Importantly, Nutlin-3a had no impact on α_5 in p53 null cells. This effect was specific to α_5 integrin since treatment with Nutlin-3a resulted in no significant effect on the β_1 subunit protein expression **(Figure 33C)**.

We next investigated if Nutlin-3a affects α_5 subunit expression at the transcriptional level. Integrin α_5 mRNA was decreased in HCT116 p53+/+ cells after Nutlin-3a treatment **(Figure 33D).** This effect was specific for the α_5 integrin subunit since neither β_1 nor α_v integrin subunit mRNAs were affected by Nutlin-3a in HCT116 p53+/+ cells **(Figure 33D)**. Interestingly, α_5 and β_1 mRNA levels were significantly increased in HCT116 p53-/- cells compared to HCT116 p53+/+ cells. This suggests that p53 may exert a negative control at the transcriptional level on the two genes, which does not translate to an increase at the protein level **(Figure 33B and Figure 33C)**. Interestingly, both α_5 and β_1 mRNA levels were significantly further increased after Nutlin-3a treatment in HCT116 p53-/- cells, suggesting a p53-independent effect of Nutlin-3a in these cells **(Figure 33D)**.

Immunofluorescence analysis of Nutlin-treated cells with specific anti-α_5 integrin antibodies confirmed the previous Western blot data. A clear decrease of the specific labeling was observed after Nutlin-3a treatment in HCT116 p53+/+, but not in HCT116 p53-/- **(Figure 33E, top)**. To determine if functional integrin level may be affected, we analysed the expression of $\alpha_5\beta_1$ integrin at the cell surface by FACS analysis. As reported in **Figure 33E**, the cell surface expression of α_5 subunit was decreased after 10µM Nutlin-3a treatment to about 55% of the control non-treated cell (MFI value) in HCT116 p53+/+, but not in HCT116 p53-/- cells. No effect on β_1 subunit cell surface expression (recorded by TS2/16 antibody) was observed **(Figure 33E, bottom)**, confirming the Western blot results.

We also confirmed, using different integrin conformation specific antibodies (mAb13, inactive β_1 and 9EG7, active β_1) by FACS analysis, that active or inactive conformations of β_1 subunit were insensitive to Nutlin-3a treatment in both HCT116 cell lines **(Table 6)**.

Abs cells	Ig control (mouse)	Ig control (rat)	α5 (IIA1)	β1 (9EG7)	β1 (TS2/16)	β1 (mAb13)
HCT116 p53+/+	4.4 ±0.6	4.2 ±0.3	68 ±9	47 ±9	614 ±206	246 ±47
+ Nutlin-3a	4.2 ±0.5	4.1 ±0.1	38 ±7 * (p=0.05)	51 ±7	631 ±206	252 ±53
HCT116 p53-/-	4.4 ±0.2	4.4 ±0.3	60 ±9	32 ±2	561 ±129	250 ±36
+ Nutlin-3a	4.8 ±0.3	4.7 ±0.2	64 ±11	38 ±5	625 ±145	272 ±32

Table 6: The mean of fluorescence intensities of α_5 and β_1 integrin subunit expression at the cell membrane of HCT116 cells. Cells were treated with solvent or Nutlin-3a (10µM, 24h), and processed for flow cytometry analysis with specific antibody labeling. Data report the mean ± SEM of 3 independent experiments. * indicates statistically significant difference between the cells treated with Nutlin-3a and the cells treated with the solvent.

The results show that a non-genotoxic p53 activator Nutlin-3a selectively decreased, the α_5 subunit expression at the mRNA and protein levels only in HCT116 p53+/+ cells, without affecting the β_1 subunit. These data are in accordance with those obtained in glioma cells in the first part of this work.

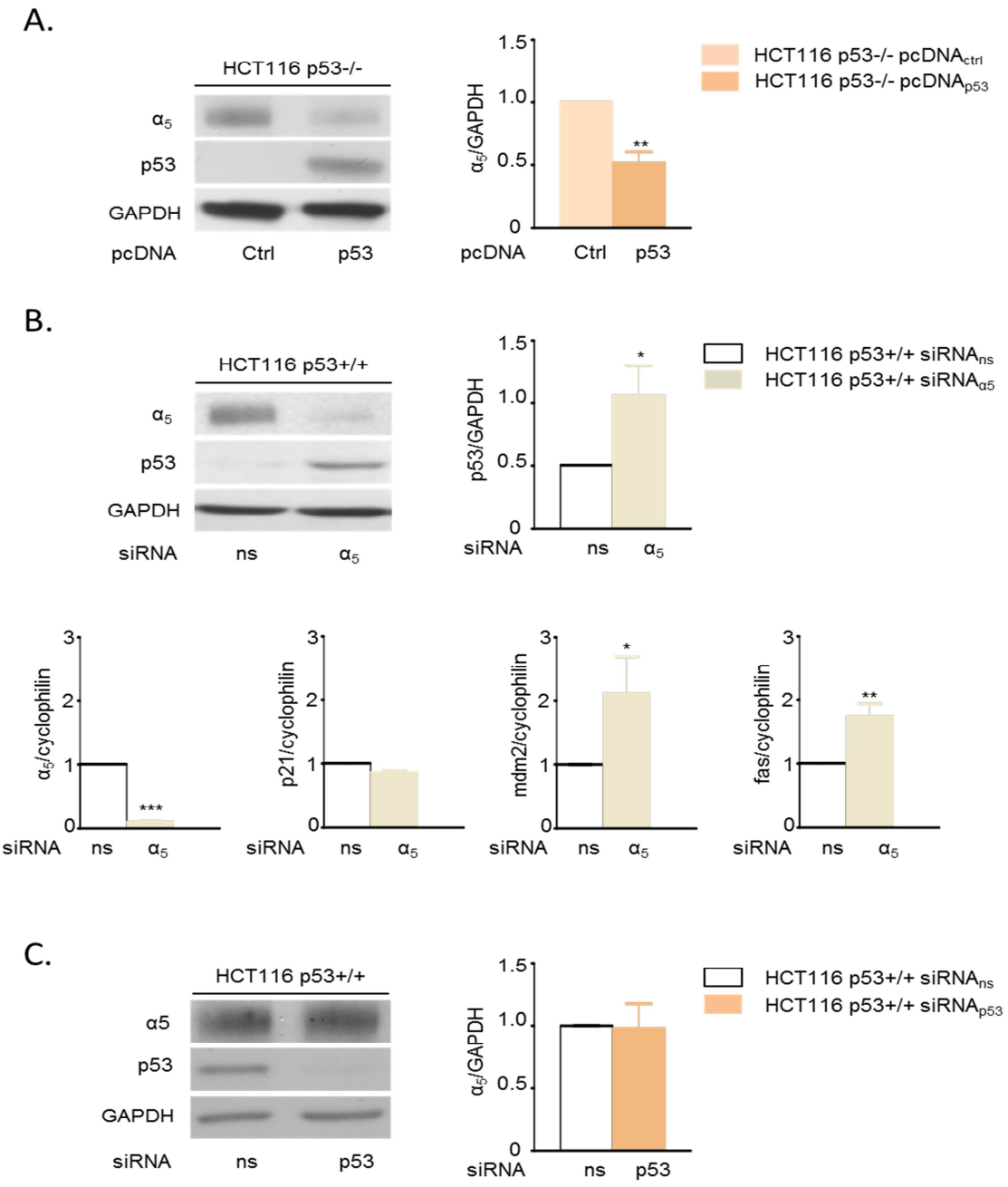


Figure 34: p53 and α_5 integrin expression are inversely related. A. Western blot analysis of α_5 integrin and p53 proteins in HCT116 p53-/- cells transfected with empty control- (pcDNA-control) or p53 gene–containing (pcDNA-p53) vectors. GAPDH was used as a loading control**.** Histograms represent the fold increase in α_5 expression in pcDNA-p53 transfected cells compared to pcDNA-control cells (n=3). **B.** (top) Western blot analysis of α_5 and p53 proteins in HCT116 p53+/+ cells transfected with non-silencing siRNA (siRNA-ns) or specific siRNA targeting α_5 integrin (siRNA-α_5). GAPDH was used as a loading control. Histograms represent the fold increase in p53 expression in siRNA-α_5 transfected cells compared to siRNA-ns cells. (bottom) qPCR analysis of α_5, p21, mdm2, fas gene mRNA in HCT116 p53+/+ transfected with non-silencing siRNA (siRNA-ns) or specific siRNA targeting α_5 integrin (siRNA-α_5) (n=3). **C.** Western blot analysis of α_5 integrin and p53 proteins in HCT116 p53+/+ cells transfected with non-silencing siRNA (siRNA-ns) or specific siRNA targeting p53 (siRNA-p53). Histograms represent the fold increase in α_5 expression in siRNA-p53 transfected cells compared to siRNA-ns cells (n=3). Statistical analysis: Student t test (*, p<0.05; **, p<0.01).

3. An inverse relationship between p53 and α_5 integrin

To provide clearer evidences that p53 and α_5 integrin expressions were linked, three different approaches were used.

At first, we transfected HCT116 p53-/- cells with a plasmid containing p53 gene. The expression of p53 activated p53 target genes **(Table 7)** and clearly decreased the α_5 integrin protein level **(Figure 34A)**. Conversely, repression of α_5 integrin expression by specific siRNA in HCT116 p53+/+, increased p53 protein level **(Figure 34B, upper panel)** and activity **(Figure 34B, lower panel)** in HCT116 p53+/+ cells. However, when p53 expression was decreased by specific siRNA in HCT116 p53+/+ cells, no effect on α_5 integrin expression was observed **(Figure 34C)**.

These data are summarized in **Table 7**.

p53 targets / p53	p21 (mRNA)	Mdm2 (mRNA)	Fas (mRNA)	α5 (mRNA)	α5 (protein)
increase					
p53-/- with p53	2.5 ±0.3	2.1 ±0.2	2.2 ±0.5	0.58 ±0.03	0.51 ±0.08
p53+/+ with Nutlin-3a (10µM)	15 ±0.8	19 ±1.4	8.9 ±1.2	0.62 ±0.06	0.42 ±0.04
p53+/+ with RITA (0.5 µM)	2.7 ±0.3	1.9 ±0.4	2.8 ±0.3	1.07 ±0.06	0.48 ±0.09
decrease					
p53+/+ with sip53	0.36 ±0.04	0.69 ±0.01	0.62 ±0.08	1.09 ±0.04	0.97 ±0.19
p53-/- vs p53+/+	0.31 ±0.002	0.92 ±0.04	0.47 ±0.02	1.5 ±0.11	1.2 ±0.02

Table 7: Summary of p53 activation in the different experimental settings and effects on α_5 integrin mRNA and protein levels. The activity of p53 is reported as the increase in p53-target gene mRNA level (determined by qPCR). Effects on α_5 integrin expression were recorded in each condition at the transcriptional level (by qPCR) and at the protein level (by Western blot). Data are shown as mean ± SEM of 3 independent experiments.

Altogether, our results indicate that the expression and the activation of p53 either by transfection or by Nutlin-3a actually affects α_5 integrin expression, but also that knocking down p53 expression has no effect on α_5 integrin in our experimental conditions.

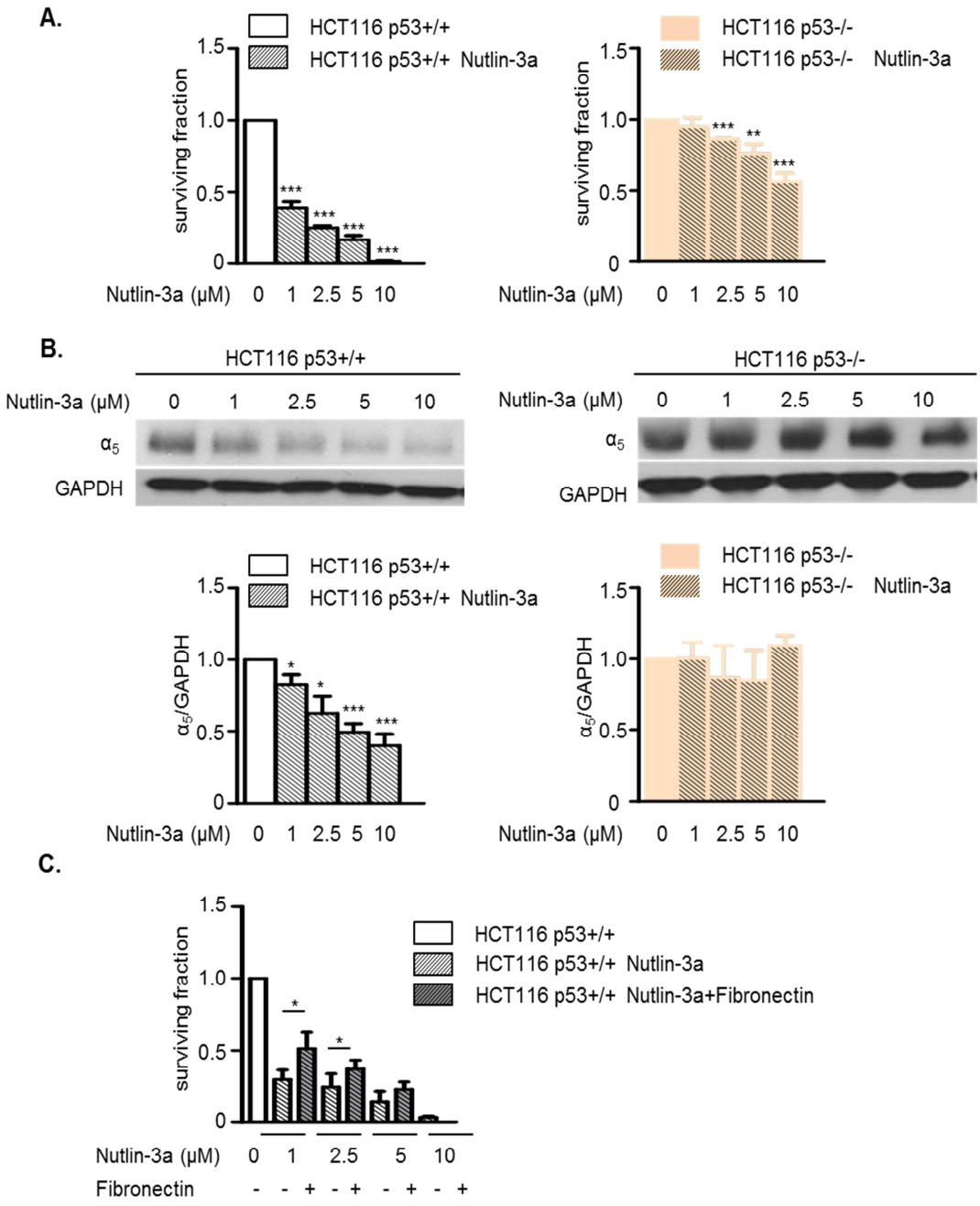


Figure 35: Nutlin-3a decreases α_5 subunit and reduces the survival of HCT116 p53+/+ cells. A. Clonogenic survival of HCT116 p53+/+ and HCT116 p53-/- cells treated with 1, 2.5, 5 and 10μM Nutlin-3a. Histograms represent the mean ± SEM of 4 independent experiments. **B.** Western Blot analysis of the α_5 protein in HCT116 p53+/+ (left) and HCT116 p53-/- (right) cells treated with 1, 2.5, 5 and 10μM Nutlin-3a for 24h. Histograms show the fold decrease in the α_5 protein expression normalized to GAPDH levels (mean ± SEM of 4 independent experiments). **C.** Clonogenic survival of HCT116 p53+/+ cells treated by increasing concentrations of Nutlin-3a plated either on fibronectin-coated wells or on non-coated wells. Statistical analysis: Student t test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

4. Impact of the reduction of α_5 subunit expression by Nutlin-3a on cell survival

We next investigated if the decrease in α_5 integrin expression observed after p53 activation by Nutlin-3a may impact cell survival.

Cells were treated with increasing concentration of Nutlin-3a (0 to 10µM) and clonogenic survival as well as α_5 integrin expression were analysed **(Figure 35)**. In a long-term growth assay, HCT116 p53+/+ cells were more sensitive to Nutlin-3a (at all concentrations used) than HCT116 p53-/- cells, as expected **(Figure 35A)**. For example, at the highest dose of 10µM, HCT116 p53+/+ cell survival was decreased to 1% of the control cells whereas HCT116 p53-/- cells showed a 56% reduction of cell survival **(Figure 35A)**. Concomitantly, α_5 integrin expression at the protein level was dose dependently inhibited by Nutlin-3a in HCT116 p53+/+ and not in HCT116 p53-/- **(Figure 35B)**, suggesting a correlation between the survival potential and the expression of α_5.

This is in agreement with the data from **Figure 32**, which showed that the repression of α_5 subunit by siRNA decreased HCT116 cell survival. In addition, the Nutlin-3a-dependent survival inhibition in HCT116 p53+/+ cells was decreased when $\alpha_5\beta_1$ integrins were activated by their specific ligand fibronectin **(Figure 35C)**.

Taken together, these data support the concept that α_5 inhibition may be involved in the Nutlin-3a-dependent p53-mediated biological response in colon cancer cells.

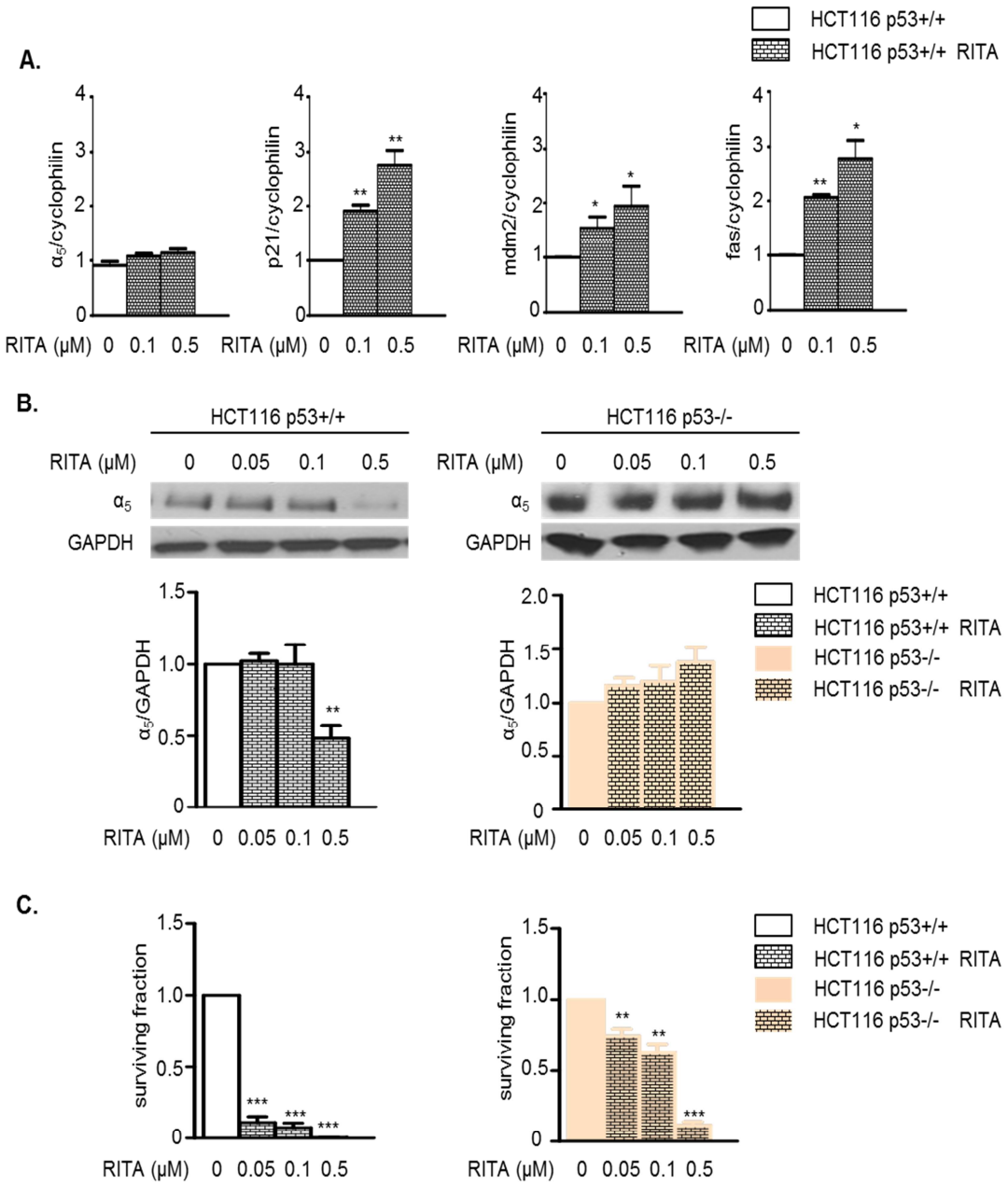


Figure 36: Effect of RITA on α_5 subunit expression and on the clonogenic survival. A. qPCR analysis of p53 target genes (p21, mdm2 and fas) and α_5 integrin in HCT116 p53+/+ cells after RITA treatment (0.1 or 0.5μM) for 24h. Data are shown as mean ± SEM of 3 independent experiments. **B.** α_5 protein expression in HCT116 p53+/+ (left) and HCT116 p53-/- (right) cells after 0.05, 0.1 and 0.5μM RITA treatment for 24h (n=4). Histograms represent α_5/GAPDH fold increase in RITA treated HCT116 p53+/+ (left) and HCT116 p53-/- (right) cells. **C.** Clonogenic survival of HCT116 p53+/+ and HCT116 p53-/- cells treated with 0.05, 0.1 and 0.5μM RITA (n=4). Histograms represent the mean ± SEM of HCT116 p53+/+ (left) and HCT116 p53-/- cells (right) surviving fraction of cells treated with RITA versus cells treated with solvent (n=4). Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001).

5. The effect of p53 activation by RITA on α_5 subunit expression and on cell survival

Other p53 reactivating drugs have been described. For example, RITA, another non-genotoxic activator, binds to p53, impairs the mdm2-p53 interaction leading to p53 stabilisation and activation *(Issaeva et al., 2004*). It was already shown that the rescue of p53 by Nutlin-3a or by RITA did not have the same impact on the transcriptional program of p53 in HCT116 cells. Nutlin-3a induced a greater fraction of p53 target genes involved in cell cycle arrest, whereas induction of pro-apoptotic p53 target genes was a hallmark of RITA *(Enge et al., 2009)*.

In order to compare Nutlin-3a and RITA on α_5 integrin expression level, we treated HCT116 cells with RITA. RITA dose dependently activated p53 in HCT116 p53+/+ cells as shown by the increase in p53 target genes but without affecting mRNA level of α_5 integrin **(Figure 36A)**. In addition, no effect on the α_5 integrin expression was observed in any cell lines except when the highest concentration of RITA (0.5µM) was used in HCT116 p53+/+ cells **(Figure 36B)**.

These data confirmed that the activation of p53 is required to affect α_5 integrin expression. However, RITA strongly inhibited the formation of HCT116 p53+/+ cell colonies at all concentrations tested, but was less efficient in HCT116 p53-/- cells **(Figure 36C)**.

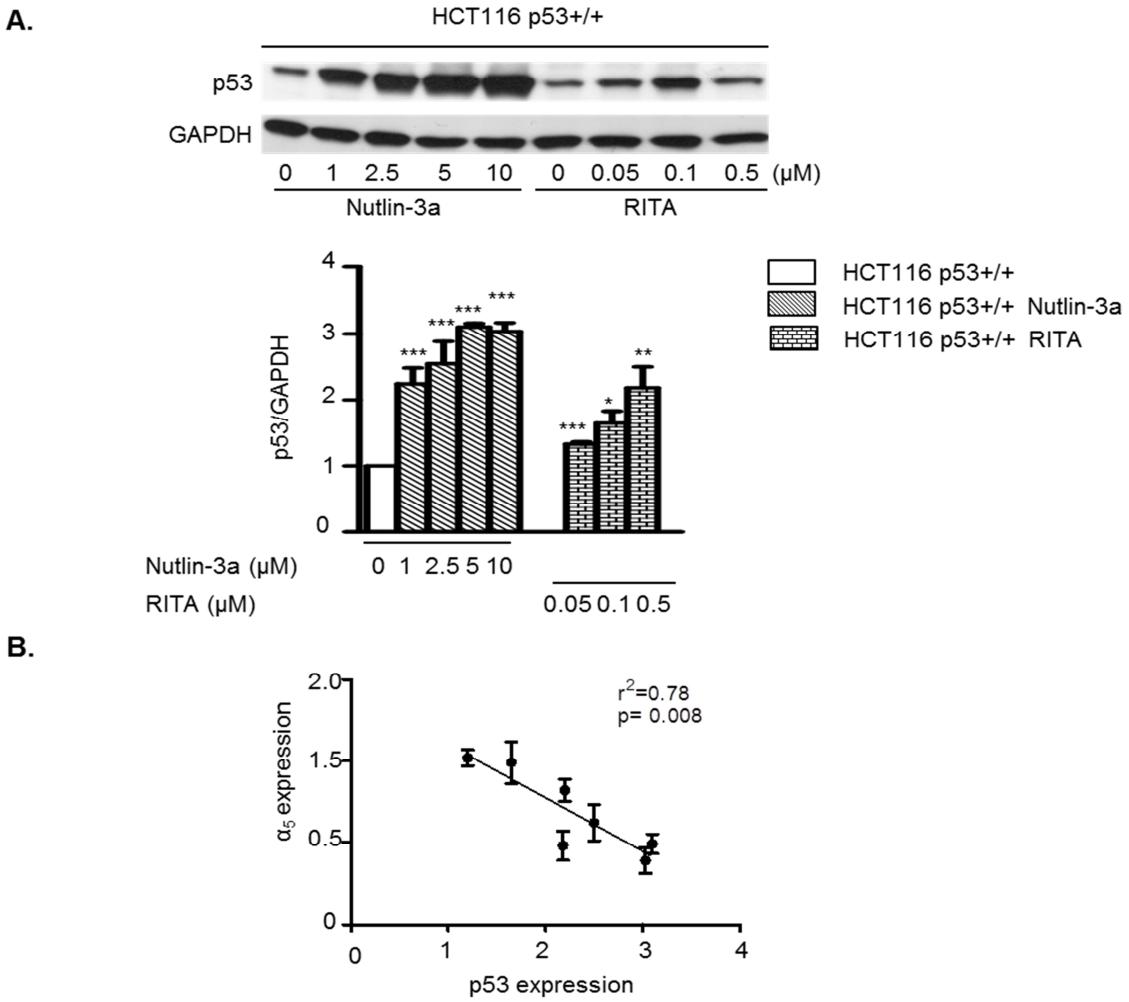


Figure 37: Comparison of p53 activation by Nutlin-3a and by RITA in HCT116 p53+/+ cells. A. Representative Western Blot of p53 stabilization in HCT116 p53+/+ cells after Nutlin-3a and RITA treatment (increasing concentrations - 24h). GAPDH was used as a loading control. Histograms represent the mean ± SEM of 3 independent experiments. **B.** Values of p53 expression (Figure 37A) were plotted versus values of α_5 protein expression (from Figures 35B and 36B), both determined by Western blot analysis. The plot shows the reverse correlation obtained between the 2 proteins of interest. a.u.: arbitrary units (densitometric data normalized to GAPDH levels). Statistical analysis: Student t test (*, p<0.05; **, p<0.01; ***, p<0.001).

We compared the respective effect of Nutlin-3a and of RITA on p53 expression in HCT116 p53+/+ cells. Nutlin-3a was clearly more efficient than RITA to increase p53 expression at all concentrations tested **(Figure 37A)**, as well as to activate p53 target genes **(Figure 36A in comparison with Figure 33A)** in HCT116 p53+/+ cells, suggesting that an activation of p53 above a certain threshold is needed to affect the α_5 integrin expression.

To confirm this hypothesis, we evaluated the relationship between p53 **(Figure 37A)** and α_5 integrin **(Figure 35B** for Nutlin-3a **and Figure 36B** for RITA**)** expression. A significant inverse correlation was obtained as shown in the **Figure 37B**.

These results show that RITA has a strong capacity to inhibit the survival of HCT116 p53+/+ cells. In contrast to Nutlin-3a, RITA was not able to decrease the expression of α_5 subunit at the low concentration tested. RITA thus behaves differently than Nutlin-3a as RITA-dependent clonogenic inhibition seems unrelated to α_5 integrin repression at the mRNA and protein levels.

Altogether, we demonstrate here the existence of an integrin/p53 negative loop in the HCT116 colon cancer cells, and thus we extend this new concept from glioma to other solid tumors.

These results led to the publication *Janouskova H. et al., Cancer Letters (2013).*

The integrin $\alpha_5\beta_1$ was shown to be implicated in tumorigenesis and resistance to therapies in various solid tumors, such as lung *(Adachi et al., 2000; Roman et al., 2010)*, ovarian *(Sawada et al., 2008; Mitra et al., 2011)* and breast cancer *(Nam et al., 2010).* In the first part of this work, we reported integrin $\alpha_5\beta_1$ integrin as a therapeutic target in another solid tumor, glioblastoma. Here, we investigated the role of $\alpha_5\beta_1$ integrin in colon cancer. Previously, high $\alpha_5\beta_1$ integrin expression has been shown to contribute to a malignant progression in colon carcinoma *(Gong et al., 1997).* Several studies demonstrated that $\alpha_5\beta_1$ integrin protected human colon cancer cells against pro-apoptotic stimuli *(Lee and Juliano, 2000; Murillo et al., 2004).* Moreover, the inhibition of $\alpha_5\beta_1$ integrin by RGD peptide Lunasin or by ATN-161 enhanced the chemotherapeutic drug potential in colon cancer cells *(Stoeltzing et al., 2003; Dia and Mejia, 2011).* However, few studies have reported that $\alpha_5\beta_1$ integrin overexpression was associated with reduced proliferation and tumorigenicity *(Varner et al., 1995; Schirner et al., 1998).*

1. $\alpha_5\beta_1$ integrin as a therapeutic target in colon cancer cells

We investigated the impact of $\alpha_5\beta_1$ integrin on HCT116 cell survival. We demonstrated that the clonogenic potential of HCT116 cells was reduced either by $\alpha_5\beta_1$ integrin non-peptidic antagonist, or by inhibition of integrin $\alpha_5\beta_1$ expression. We found that direct inhibition of α_5 function or expression decreased the cell survival of HCT116 colon cell lines independently of p53 status. Our data are in line with findings demonstrating that $\alpha_5\beta_1$ integrin enhanced survival via the PI3K/AKT signaling pathway, and that $\alpha_5\beta_1$ integrin inhibition by α_5 integrin directed antibody decreased AKT activation, and induced apoptotic cell death *(Lee and Juliano, 2000; Murillo et al., 2004)*. Our results demonstrate that $\alpha_5\beta_1$ integrin may be a pertinent therapeutic target for colon tumors regardless of p53 status, and suggest that the therapies capable of affecting integrin expression or function may be a valuable strategy for colon carcinoma eradication.

2. Negative cross-talk between $\alpha_5\beta_1$ integrin and p53 in colon cancer cells

In the first part of this work, we showed that the reactivation of p53 by non-genotoxic agent Nutlin-3a repressed $\alpha_5\beta_1$ integrin expression in glioma cells. We unrevealed an original negative crosstalk between $\alpha_5\beta_1$ integrin and p53, as we demonstrated that a high level of α_5 subunit impaired p53 activation, and conversely that integrin antagonist restored glioma cell sensitivity to p53-based therapy. In the present study, we extended these findings in colon cancer cells through the use of isogenic HCT116 cell lines, expressing or not wild type p53. We could show that, Nutlin-3a-mediated p53 activation decreased selectively, only in HCT116 p53+/+ cells, the expression of α_5 subunit at mRNA and protein level without affecting β_1 subunit. The promoter of α_5 gene lacks a p53 response element, thus p53 direct modulation of α_5 gene transcription appeared unlikely. The expression of α_5 gene is affected by different transcription factors, including Sp1, AP-1 and NFI *(Gingras et al., 2009)*. ZEB2 was shown to interact with SP1 and to modulate α_5 gene expression *(Nam et al., 2012).* Interestingly, p53 is known to interfere with some of transcription factors, including SP1 *(Pietrzak and Puzianowska- Kuznicka, 2008)*. Thus, p53 could be indirectly involved in α_5 gene expression through binding to transcription factors. Whether Nutlin-3a-mediated p53 activation has an impact on function of α_5 gene transcription factors remains to be elucidated. Our results further suggest that α_5 integrin is down-regulated in a p53-dependent way, and that in turn α_5 integrin decrease sustains p53 activation. Conversely, the repression of α_5 integrin increased p53 activity. We demonstrate here the existence of a negative integrin/p53 loop in HCT116 colon cancer cells. Thus, we extend this new concept from glioma to another solid tumor.

3. The activation of p53 by RITA is not sufficient to repress α_5 integrin

Finally, we investigated whether another p53 activator, RITA, may affect the expression of α_5 subunit. RITA is a small molecule that binds directly to p53, and thus inhibits MDM2-p53 complex, leading to p53 activation *(Issaeva et al., 2004).* In contrast to Nutlin-3a, RITA at low concentrations was inefficient to modulate the expression of α_5 subunit. At high concentration, RITA only decreased α_5 subunit in HCT116 p53-expressing cells, confirming the requirement of p53 to inhibit α_5 expression. Intriguingly, RITA to the contrary of Nutlin-3a did not inhibit α_5 expression at mRNA level in our

conditions, indicating the involvement of non-transcriptional mechanisms. Additionally, a less efficient induction of p53 was observed with RITA compared to Nutlin-3a, even at concentrations that inhibited the cell clonogenic potential. Our data suggest that p53 activation below a threshold value, as obtained with RITA, is not sufficient to affect α_5 integrin expression. Such a threshold mechanism has recently been proposed for p53-mediated growth arrest or apoptosis *(Kracikova et al., 2013).* Although both RITA and Nutlin-3a increase p53 activity via a disruption of MDM2-p53 complex, different outcomes on p53, including its transcriptional activity and its impact on cell survival, have been described. RITA preferentially induces apoptosis, whereas Nutlin-3a is more potent to promote cell cycle arrest *(Endge et al., 2009).* Whether differences in p53 transcriptional pattern are implicated in RITA or Nutlin-3a driven α_5 repression, remain to be determined.

In our experimental conditions, Nutlin-3a and RITA at high concentration were shown to affect the survival of cells lacking p53. In fact, an increasing amount of data point to a p53-independent role of these agents, including the initiation of DNA damage response, or the activation of p73 and E2F1 instead of p53 by Nutlin-3a *(Ray et al., 2011; Valentine et al., 2011)*, or enhancement of MDM2-dependent oncogene degradation by RITA *(Di Conza et al., 2012).* Moreover, it has been recently reported that Nutlin-3a can bind to anti-apoptotic BCL-2 members, which provides new evidence about p53 independent effects of Nutlin-3a *(Shin et al., 2012).*

4. Conclusions

Altogether, we demonstrated that $\alpha_5\beta_1$ integrin has a crucial role in colon cancer survival. Targeted therapies based on the repression of $\alpha_5\beta_1$ integrin function or of its expression may be useful to sensitize these tumors to conventional chemotherapeutic drugs, as it had already been shown in the first part about glioma. The characterization of a negative crosstalk between $\alpha_5\beta_1$ integrin and p53 protein in colon cancer cells might be a potential approach for the improvement of the drug efficiency in a specific subpopulation of colon cancer patients with a high expression of $\alpha_5\beta_1$ integrin and a functional p53.

GENERAL CONCLUSIONS AND PERSPECTIVES

GENERAL CONCLUSIONS AND PERSPECTIVES

Current anti-cancer therapies are often inefficient to cure highly aggressive tumors. The main interest of researches is to develop new therapy interventions to fight these incurable tumors in order to improve the patient's survival. The various intracellular events are frequently aberrant in specific tumor types, and thus contribute to cancer progression. The strategies targeting a specific altered signaling pathway became the key to tailored therapies.

We demonstrated that $\alpha_5\beta_1$ integrin was a potential therapeutic target in brain and colon cancers. We found an original crosstalk between $\alpha_5\beta_1$ integrin and p53 protein, which contributes to the resistance to chemotherapy or to other p53-based therapies in both cancers. We demonstrated that $\alpha_5\beta_1$ integrin counteracted p53 effects on apoptosis. A full apoptotic response is only achieved through inhibition of $\alpha_5\beta_1$ integrin in p53 activated cells. Apoptosis is the result of two converging p53-dependent and $\alpha_5\beta_1$ integrin-dependent signaling pathways. Currently, the effects of combinatory therapy in human xenograft model *in vivo* are under investigation.

Targeted therapies alone are not very efficient. However, the combination of targeted therapies together with radio- and chemo-therapy became a successful approach to fight cancer. We propose here a new personalized therapeutic approach through $\alpha_5\beta_1$ integrin inhibition and p53wt activation for the improvement of chemotherapy or other p53-based therapies in brain and colon cancers. In this perspective, a screening of $\alpha_5\beta_1$ integrin expression and p53 status needs to be performed in order to identify the subpopulation of patients who would benefit from this therapeutic intervention. Recently, genomic data identified four types of GBMs. GBM subtype termed as classical harbors intact p53 protein. These GBMs may benefit from a therapy based on blocking $\alpha_5\beta_1$ integrin. However, the characterization of $\alpha_5\beta_1$ integrin expression has not been involved it these studies.

In summary, we discovered a potential therapeutic approach for the improvement of drug efficiency in the subpopulation of GBM and colon cancer patients with a high expression of $\alpha_5\beta_1$ integrin and a functional p53. The concomitant inhibition of $\alpha_5\beta_1$ integrin with p53 activation may be an effective anti-cancer therapy against these aggressive tumors.

LIST OF PUBLICATIONS AND COMMUNICATIONS

PUBLICATIONS

Janouskova, H., Ray, A.M., Noulet, F., Lelong-Rebel, I., Choulier, L., Schaffner, F., Lehmann, M., Martin, S., Teisinger, J., & Dontenwill, M. 2013. *Activation of p53 pathway by Nutlin-3a inhibits the expression of the therapeutic target alpha5 integrin in colon cancer cells*. Cancer Lett, [Epub ahead of print].

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ORAL COMMUNICATIONS

Janouskova H, Maglott A, Guenot D, Pinel S, Chastagner P, Plenat F, Entz-Werle N, Godet J, Martin S, Dontenwill M. *Integrin α5β1 plays a critical role in resistance to chemotherapy by interfering with the p53 pathway in high grade glioma.* EUCC, Strasbourg, France, 2013.

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POSTERS

Janouskova H, Maglott A, Guenot D, Pinel S, Chastagner P, Plenat F, Entz-Werle N, Godet J, Martin S, Dontenwill M.*Integrin α5β1 plays a critical role in resistance to chemotherapy by interfering with the p53 pathway in high grade glioma.* Le Cancéropôle du Grand-Est, Strasbourg, France, 2012.

Janouskova H, Maglott A, Guenot D, Pinel S, Chastagner P, Plenat F, Entz-Werle N, Godet J, Martin S, Dontenwill M.*Integrin α5β1 plays a critical role in resistance to chemotherapy by interfering with the p53 pathway in high grade glioma.* EACR, Barcelona, Spain 2012.

Ray AM, **Janouskova H**, Lehmann M, Bonnet D and Dontenwill M. *Use of an α5β1 small non-peptidic antagonist to inhibit glioblastoma migration fostered by integrin expression.* EACR, Barcelona, Spain 2012.

Ray AM, **Janouskova H**, Lehmann M, Bonnet D, Villa P, Brino L,Martin S, Lelong Rebel I, Rognan D, Dontenwill M. *Use of an α5β1 small non peptidic antagonist to inhibit glioblastoma migration fostered by integrin expression.* Journées Campus Illkirch, Strasbourg, France 2012.

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RESUME DE LA THESE DE DOCTORAT

I. Introduction

Les tumeurs cérébrales représentent un défi en oncologie car malgré des avancées significatives dans la compréhension des mécanismes moléculaires impliqués dans leur développement et leur progression, les progrès thérapeutiques restent mineurs. La durée moyenne de survie de patients atteints de glioblastomes (GBM - tumeurs cérébrales les plus agressives) est de 12 à 14 mois malgré une thérapie combinant une résection chirurgicale, une radiothérapie et une chimiothérapie (la drogue de référence étant le Temozolomide ; TMZ) *(Stupp et al., 2005).* Ces tumeurs se caractérisent par une forte hétérogénéité. Les analyses moléculaires à grande échelle ont permis des progrès significatifs de classification en sous-populations (proneural, neural, classical, mesenchymal) mettant en évidence des marqueurs pronostiques de survie *(Verhaak et al., 2010)*. Un enjeu majeur de ces prochaines années sera de caractériser parmi les marqueurs pronostiques, des marqueurs prédictifs de la réponse à un traitement donné (marqueurs théranostiques) pour orienter de façon rationnelle le choix de thérapies personnalisées. Ainsi la méthylation du promoteur de la MGMT est apparu comme un marqueur de meilleure réponse des patients à la chimiothérapie au Temozolomide *(Hegi et al., 2004; Hegi et al., 2008)*. De nouvelles cibles thérapeutiques sont caractérisées régulièrement mais les thérapies ciblées n'ont pas pour le moment atteint leurs objectifs car testées vraisemblablement sur des populations trop larges de patients.

La compilation des données d'études transcriptomiques à grande échelle met en évidence la surexpression de composantes de la matrice extracellulaire telles que la fibronectine, ligand préférentiel des intégrines αvβ3/5 et α5β1, dans les GBM *(Freije et al., 2004; Tso et al., 2006)*. Les intégrines sont apparues récemment

comme cibles thérapeutiques anti-tumorales. Cet intérêt a été basé dans un premier temps sur la démonstration de leur implication dans la néo-angiogénèse tumorale ; les intégrines αvβ3 et α5β1 sont surexprimées dans les cellules endothéliales des néo-vaisseaux tumoraux *(Brooks et al.,1994; Kim et al., 2000; Desgrosellier and Cheresh, 2010).* Plus récemment, l'implication des intégrines dans l'agressivité des cellules tumorales elles-mêmes (prolifération/survie, migration/invasion, résistance aux traitements) a été démontrée *(Schaffner et al., 2013)*. L'inhibition fonctionnelle des intégrines permettrait ainsi de bloquer à la fois la croissance de la masse tumorale mais également l'angiogenèse associée. Les travaux antérieurs du laboratoire ainsi que les données de la littérature nous permettent de proposer l'intégrine α5β1 comme une cible thérapeutique pertinente dans les tumeurs cérébrales de haut grade. Au contraire de l'intégrine αvβ3, dont le rôle dans l'angiogénèse tumorale a été remis régulièrement en question *(Hodivala-Dilke, 2008; Robinson and Hodivala-Dilke, 2011)*. l'intégrine α5β1 est pro-angiogénique sans ambigüité *(Kim et al., 2000)*. De plus, la démonstration de sa pertinence en tant que marqueur pronostique et cible thérapeutique a été faite récemment dans différentes tumeurs solides (colon, poumon, ovaire, sein) où elle apparaît particulièrement surexprimée dans les grades les plus agressifs et participe à la résistance aux traitements, à la dissémination tumorale et la formation de métastases *(Schaffner et al., 2013)*.

La transformation d'une cellule normale en cellule tumorale se fait en plusieurs étapes qui demandent l'activation d'oncogènes et l'inactivation de gènes suppresseurs de tumeurs. L'accumulation de mutations dans ces deux types de gènes participe au maintien de la tumeur en favorisant les fonctions soit activatrices soit inhibitrices des protéines correspondantes à ces gènes. Une protéine

particulièrement explorée dans ce domaine est la protéine p53 qui apparaît mutée (et donc inactive) dans plus de la moitié des cancers. P53 est un suppresseur de tumeurs qui restreint la prolifération cellulaire en réponse à des dommages de l'ADN ou à une dérégulation d'oncogènes en induisant des points de contrôle du cycle cellulaire, de l'apoptose ou de la sénescence prématurée mais également en régulant d'autres processus tels que l'autophagie, le métabolisme cellulaire, la réponse immunitaire et le microenvironnement tumoral *(Vousden and Prives, 2009)*. De ce fait, les mutations de p53 favorisent la prolifération et la survie cellulaire, peuvent promouvoir une instabilité génomique et une résistance aux thérapies anti-cancéreuses. Lorsque p53 n'est pas mutée, on retrouve dans la tumeur des altérations génétiques de protéines impliquées dans sa régulation comme par exemple une amplification de mdm2 (régulateur négatif) ou une délétion de ARF (régulateur positif). La restauration des fonctions de p53 est donc devenue ces dernières années un enjeu thérapeutique pour un grand nombre de tumeurs *(Ventura et al., 2007 ; Junttila et al., 2010)*. Deux types d'approches sont actuellement à l'étude : la restauration de l'activité de p53 mutée et la réactivation de p53 sauvage. Pour chacune des deux approches, différentes molécules ont été décrites dont les mécanismes d'action ne sont pas encore complètement élucidés *(Selivanova, 2010 ; Wang and Sun, 2010).* Le développement de petites molécules capables d'interférer avec la liaison mdm2-p53 et inhibant ainsi la dégradation de p53 par le protéasome semble être une démarche prometteuse. Ces molécules se fixent soit sur mdm2 (Nutlin-3a) soit directement sur p53 (RITA), induisent l'accumulation de p53, son activation, la transcription de gènes cibles de p53 impliqués dans l'arrêt du cycle cellulaire et/ou dans l'apoptose mais également dans la répression de l'expression de certains oncogènes *(Grinkevich et al., 2009)*.

Les travaux antérieurs du laboratoire ont suggéré un impact de l'intégrine α5β1 sur l'activité de la protéine p53 induite par des agents chimiothérapeutiques *(Martinkova et al., 2010)*. Ainsi, il a été montré que l'inhibition des fonctions de cette intégrine par des antagonistes spécifiques favorisait la mort des cellules par apoptose au détriment de l'induction de sénescence dépendante de la protéine p53 activée par l'ellipticine ou le Témozolomide.

Dans ce contexte, les objectifs de cette thèse ont été les suivants :

1. **Caractériser le rôle de l'intégrine α5β1 dans la résistance à la chimiothérapie des glioblastomes humains en relation avec le statut de p53.**

2. **Evaluer l'impact d'une nouvelle approche thérapeutique combinant une réactivation de p53 avec la thérapie ciblée sur l`intégrine α5β1 pour les glioblastomes humains.**

3. **Confirmer une relation inverse entre protéine p53 et intégrine α5β1 dans des cellules tumorales de colon.**

II. Matériel et méthodes

Pour atteindre ces objectifs, nous avons utilisé des lignées cellulaires de glioblastomes humains et de tumeurs du colon ayant différents statuts de p53 et différents taux d'expression de l'intégrine α5β1 :

- les cellules U87MG, p53 sauvage (lignée manipulée pour avoir une expression élevée et faible de l'intégrine)

- les cellules U373, p53 mutée (lignée manipulée pour avoir une expression élevée et faible de l'intégrine)
- les cellules LNZ308, p53 KO
- Les cellules de colon HCT116, p53+/+ et p53 -/- (don de Pr B. Vogelstein)
-

Nous avons également eu accès à une cohorte de biopsies de patients atteints de tumeurs cérébrales (Pr Entz-Werlé, CHU Strasbourg, Pr Plénat, CHU Nancy).
En collaboration avec les équipes de la plateforme de xénogreffes du Cancéropôle Grand Est (Dr Guénot/ Dr Guérin, Strasbourg ; Pr Plénat/Dr Pinel, Nancy), des biopsies de tumeurs humaines xénogreffées dans la souris Nude ont été caractérisées.

Les drogues utilisées comme activateurs de p53 sont : le Témozolomide, la Nutlin-3a et RITA. Pour antagoniser l`intégrine α5β1, nous avons utilisé la molécule K34c décrite par l`équipe du Prof Kessler *(Heckmann et al., 2008)*, qui mime la séquence peptidique RGD de la fibronectine.

Les techniques de Biologie cellulaire et moléculaire sont disponibles au laboratoire et incluent :

- des expériences de Western blot
- des expériences de cytométrie de flux
- des expériences de marquages immunohistochimiques et de microscopie confocale
- des expériences de transfection de plasmides ou de si/shRNA d'intérêt
- des quantifications d'ARNm par RT-qPCR
- des essais de viabilité cellulaire

III. Résultats et perspectives

III.1. Objectif 1 : Caractériser le rôle de l'intégrine α5β1 dans la résistance à la chimiothérapie des glioblastomes humains en relation avec le statut de p53.

L'analyse du taux d'expression de l'intégrine α5β1 dans des biopsies de tumeurs cérébrales humaines de différents grades et la mise en relation avec les données cliniques des patients a confirmé son rôle dans la progression tumorale et dans la résistance aux thérapies standards. Ces données ont été confirmées par des études de xénogreffes de tumeurs humaines traitées au Témozolomide qui montrent une réponse en fonction du taux d'expression de l'intégrine.

Enfin les études in vitro sur des lignées cellulaires établissent une résistance au Témozolomide des cellules exprimant fortement l'intégrine uniquement si elles expriment une protéine p53 sauvage. Dans ces cellules, la surexpression de l'intégrine induit une inhibition de l'activation de p53 par le Temozolomide et inversement la protéine p53 est suractivée lorsque l'intégrine est réprimée. Lorsque la protéine p53 est activée par un agent non génotoxique, la Nutlin-3a, l'intégrine α5β1 ne joue plus son rôle protecteur. Dans ces conditions, nous avons pu montrer que le traitement par la Nutlin-3a entrainait une diminution de l'expression de l'intégrine (au niveau protéique) de façon dépendante de l'activité de p53. Ces résultats sont corroborés par les données des tumeurs de patients où nous avons mis en évidence une surexpression de l'intégrine α5β1 dans les tumeurs ayant une protéine p53 sauvage.

Ainsi, nos résultats montrent pour la première fois que l'intégrine α5β1 exerce un effet négatif sur l'activité de la protéine suppresseur de tumeurs p53 et qu'inversement l'activation de p53 peut diminuer l'expression de l'intégrine. Cette

réaction croisée négative est impliquée dans la résistance des glioblastomes au Témozolomide. L'ensemble de ces résultats a été publié dans Cancer Research *(Janouskova et al., Cancer Research, 2012).* Un article de revue sur l'impact du statut de p53 sur la résistance des glioblastomes au Témozolomide a également été publié *(Martin et al., Frontiers in Oncology, 2012).*

III.2. Objectif 2 : Evaluer l'impact d'une nouvelle approche thérapeutique combinant une réactivation de p53 avec la thérapie ciblée sur l`intégrine α5β1 pour les glioblastomes humains.

L'ensemble des résultats de la partie III.1. suggère que l'inhibition de l'intégrine α5β1 par des antagonistes sélectifs pourrait améliorer la sensibilité des glioblastomes à la chimiothérapie. Nous avons donc traité les cellules à fort taux d'intégrine par une association antagoniste d'intégrine/ Témozolomide. Ce traitement s'est avéré très efficace puisqu'il diminue la survie des cellules (essai de clonogénicité) et augmente la mort par apoptose des cellules (30%). Aucun des traitements individuels ne donne de tels effets. Le traitement des cellules par l'association antagoniste d'intégrine/Nutlin 3a est encore plus drastique puisque 60% des cellules entrent en apoptose (montré par marquage à l'Annexine V, par clivage de PARP et de caspase 3). Même si la Nutlin-3a seule est capable de stabiliser et activer p53, l'addition de l'antagoniste d'intégrine potentialise cette activation. La Nutlin-3a est capable d'initier la transcription de gènes cibles de p53 impliqués dans l'apoptose (Fas, bax, Noxa). L'antagoniste seul n'a pas d'effet sur ces gènes mais potentialise les effets de la Nutlin-3a. De plus, des protéines anti-apoptotiques (survivine, bcl2, mcl-1) sont réprimées soit par la Nutlin-3a et l'antagoniste, soit uniquement par la Nutlin-3a. Ces résultats suggèrent l'existence de deux voies de signalisation soit dépendantes de

l'intégrine soit dépendantes de l'activité de p53 qui convergent pour aboutir a un effet pro-apoptotique optimal.

L'activité pro-survie des intégrines est largement médiée par la kinase AKT. Nous avons évalué l'activité de AKT (phosphorylation de la ser473) lors des traitements individuels ou en association et montré qu'elle était largement diminuée par le cotraitement antagoniste/Nutlin3a et partiellement diminuée par chaque traitement seul. Cet effet additif apparaît p53 dépendant car non retrouvé dans des cellules n'exprimant pas p53 (LNZ308). Les mêmes résultats ont été obtenus pour l'expression de l'intégrine elle-même qui apparaît réprimée de façon drastique par le cotraitement antagoniste/Nutlin3a et partiellement réprimée par chaque traitement seul.

Ainsi nos résultats montrent pour la première fois que l'association d'un antagoniste de l'intégrine α5β1 avec un activateur de p53 entraine une mort des cellules de glioblastome par apoptose certainement par la convergence de l'inhibition de la signalisation de l'intégrine et de l'activation de p53. L'effet additif de chaque drogue sur la répression de l'expression de l'intégrine elle-même joue certainement un rôle prépondérant. L'ensemble de ces résultats fera l'objet d'une publication en cours de rédaction.

III.3. Objectif 3 : Confirmer une relation inverse entre protéine p53 et intégrine α5β1 dans des cellules tumorales de colon.

Les données de la littérature indiquent déjà un rôle prépondérant de l'intégrine α5β1 dans la progression de plusieurs types de tumeurs solides. Pour conforter nos résultats, nous avons exploré son implication dans les tumeurs du colon et en particulier son interférence avec la protéine p53. Pour cela des cellules HCT116

exprimant ou non p53 sauvage ont été utilisées. Nous avons tout d'abord démontré que la déplétion de l'intégrine ou son blocage fonctionnel par un antagoniste affectaient la survie des cellules tumorales HCT116 quelque soit leur statut pour p53. Ainsi les voies de survie dépendantes de l'intégrine apparaissent indépendantes de p53. Ces données suggéraient que des drogues susceptibles de diminuer l'expression de l'intégrine seraient efficaces pour éradiquer ces tumeurs. De façon similaire aux données obtenues dans le glioblastome, la Nutlin-3a réprime l'expression de l'intégrine α5β1 dans les cellules p53 +/+ et pas dans les cellules p53-/-. La réexpression de p53 par transfection dans ces dernières cellules entraine une diminution du taux d'intégrine confirmant le rôle de p53. Inversement la répression de α5 dans les cellules p53 +/+, entraine une activation de p53 confirmant ainsi également la réaction croisée entre les 2 intervenants dans les cellules tumorales de colon. L'utilisation d'un autre activateur de p53 non génotoxique, RITA, nous a permis de montrer une relation entre le taux d'activation de p53 et l'inhibition de l'expression de l'intégrine suggérant que tous les agents capables d'activer p53 ne seront pas forcément capables 1) de diminuer l'expression de l'intégrine et donc 2) d'inhiber ses fonctions pro-tumorales.

Ces résultats ont donné lieu à une publication dans Cancer Letters *(Janouskova et al., Cancer Letters, 2013).*

III.4. Conclusions et perspectives

Les strategies thérapeutiques anti-cancéreuses sont souvent inefficaces pour éradiquer les tumeurs les plus agressives telles que les glioblastomes. Actuellement les thérapies ciblées suscitent beaucoup d'espoir mais sont encore peu efficaces lorsque testées sur des populations larges de patients.

Ce travail de thèse a permis de mettre en évidence une cible thérapeutique originale pour les glioblastomes mais également pour les tumeurs du colon : l'intégrine α5β1. Cette intégrine semble être particulièrement importante pour contrecarrer les effets d'un suppresseur de tumeurs, la protéine p53. Nous avons démontré pour la première fois une réaction croisée négative entre intégrine α5β1 et protéine p53 sauvage. Cette relation pourra être exploitée pour la proposition d'une thérapie personnalisée qui sera efficace pour les patients à fort taux d'intégrine et à p53 sauvage. La combinaison de thérapies activant p53 avec une thérapie ciblant l'intégrine α5β1 pourra provoquer une apoptose massive des cellules tumorales dans ces patients. La confirmation de cette hypothèse devra être faite dans des modèles précliniques in vivo (xénogreffes de tumeurs humaines dans la souris) avant de pouvoir être proposée en clinique humaine. De plus la caractérisation plus approfondie des paramètres moléculaires impliqués dans l'induction de l'apoptose par blocage de l'intégrine α5β1 et par activation de p53 permettra potentiellement de proposer de nouvelles cibles thérapeutiques efficaces dans une sous-population bien définie de patients.

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PUBLICATIONS

APPENDIX 1:

Janouskova, H., Maglott, A., Leger, D.Y., Bossert, C., Noulet, F., Guerin, E., Guenot, D., Pinel, S., Chastagner, P., Plenat, F., Entz-Werle, N., Lehmann-Che, J., Godet, J., Martin, S., Teisinger, J., & Dontenwill, M. 2012. *Integrin alpha5beta1 plays a critical role in resistance to temozolomide by interfering with the p53 pathway in high-grade glioma.* Cancer Res*,* 72(14), 3463-3470.

Published OnlineFirst May 16, 2012; DOI:10.1158/0008-5472.CAN-11-4199

Cancer Research

Integrin α5β1 Plays a Critical Role in Resistance to Temozolomide by Interfering with the p53 Pathway in High-Grade Glioma

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Cancer Res 2012;72:3463-3470. Published OnlineFirst May 16, 2012.

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Priority Report

Cancer Research

Integrin α5β1 Plays a Critical Role in Resistance to Temozolomide by Interfering with the p53 Pathway in High-Grade Glioma

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Abstract

Integrins play a role in the resistance of advanced cancers to radiotherapy and chemotherapy. In this study, we show that high expression of the α5 integrin subunit compromises temozolomide-induced tumor suppressor p53 activity in human glioblastoma cells. We found that depletion of the α5 integrin subunit increased p53 activity and temozolomide sensitivity. However, when cells were treated with the p53 activator nutlin-3a, the protective effect of α5 integrin on p53 activation and cell survival was lost. In a functional p53 background, nutlin-3a down-regulated the α5 integrin subunit, thereby increasing the cytotoxic effect of temozolomide. Clinically, α5β1 integrin expression was associated with a more aggressive phenotype in brain tumors, and high α5 integrin gene expression was associated with decreased survival of patients with high-grade glioma. Taken together, our findings indicate that negative cross-talk between α5β1 integrin and p53 supports glioma resistance to temozolomide, providing preclinical proof-of-concept that α5β1 integrin represents a therapeutic target for high-grade brain tumors. Direct activation of p53 may remain a therapeutic option in the subset of patients with high-grade gliomas that express both functional p53 and a high level of α5β1 integrin. *Cancer Res; 72(14); 3463–70.* ©*2012 AACR.*

Introduction

Glioblastoma multiforme (GBM) are the most aggressive brain tumors and remain a challenge for oncologists. New therapies are urgently needed. Gene expression profiling of high-grade glioma revealed that genes of extracellular matrix components and their regulators are often affected in the patients. Fibronectin is overexpressed in glioblastoma versus normal brain (1) and belongs to the cluster of genes associated with a more malignant phenotype (2, 3). It has recently been shown that fibronectin knockdown delays tumor growth in a mouse glioma model (4). The α5β1 integrin is a fibronectin receptor that was recently shown to have an important role in tumor progression (5), metastasis (6), and/or resistance to therapies (7) in lung, ovarian, and breast cancer, respectively. Few works addressed directly the issue of α5β1 integrin in glioma. Through the use of non-peptidic α5β1 integrin antagonists and GBM cell lines, we previously showed that α5β1 integrin may be a therapeutic target for these tumors (8, 9) and that concomitant addition of α5β1 antagonists sensitizes p53 wild-type (p53-wt) glioma cells to chemotherapeutic drugs (10). The presence of p53 mutations in high-grade glioma varied across GBM subtypes with 0%, 21%, 32%, and 54% in classical, neural, mesenchymal, and proneural subtypes, respectively (11). There is increasing evidence that gliomas harboring a p53-wt resist to therapies through inhibitory pathways upstream of p53. Nutlin-3 belongs to the family of small-molecule inhibitors of the MDM2–p53 interaction (12). Nutlin-3 has been shown, alone or in combination with chemotherapeutic agents, to increase the degree of apoptosis in hematologic malignancies (13). Recent studies extended its therapeutic window for use in solid tumors (14, 15).

The aim of this study was to investigate the role of α5β1 integrin in glioma resistance to temozolomide chemotherapy using *in vitro* and *in vivo* models. We found that a high expression of α5 subunit inhibited the temozolomide-induced p53 pathway and that reactivation of p53 by nutlin-3a restores the sensitivity to temozolomide by decreasing the expression of the α5β1 integrin. Finally, we found that high α5 integrin gene expression is associated with a more aggressive phenotype in brain tumors and a decrease in survival of patients. Our results provide a clinical rationale for including α5β1 integrin–targeted therapy in a subpopulation of patients with glioma.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-11-4199

Published OnlineFirst May 16, 2012; DOI:10.1158/0008-5472.CAN-11-4199

Materials and Methods

Reagents

Temozolomide was a kind gift from Schering-Plough. Nutlin-3a, the active enantiomer of nutlin, was from Cayman. Temozolomide was prepared before use at 10 mmol/L in 50/50 ethanol/H_2O. Other drugs were prepared as stock solutions in ethanol at 10 mmol/L and were kept at −20°C until use.

Cell culture and transfection

The U87MG cells (p53-wt) was from American Type Culture Collection; the U373 cells (p53-mutated) from ECACC (Sigma) and not authenticated in the laboratory. The LN18 (p53-mutated) and LNZ308 [p53 knockout (KO)] cells were kindly provided by M. Hegi (University Hospital, Lausanne, Switzerland). Cells were cultured as described elsewhere (10). The identity of cell lines was regularly checked by morphologic criteria, and importantly p53 status was routinely checked by the yeast functional assay (16), Western blot quantification of p53 stability and phosphorylation, and by quantitative PCR (qPCR) quantification of p53 target genes after treatment with ellipticine. Cells were stably transfected to overexpress (by transfecting a pcDNA3.1 plasmid containing the human α5 integrin gene; provided by Dr. Ruoshlati, University of California, Santa Barbara, CA) or to repress [by transfecting a pSM2 plasmid coding for a short hairpin RNA (shRNA) targeting the α5 mRNA; Open Biosystems] the α5 integrin subunit by using jetPRIME (Polyplus transfection) according to the manufacturer's instructions. The vector for the p53-wt transfection was a kind gift from Dr. C. Blattner (Karlsruhe Institute of Technology, Karlsruhe, Germany). Cells were transfected with specific siRNA for human p53, the α5 integrin subunit, or nontargeting siRNA (Thermo Scientific Dharmacon) with jetPRIME (Polyplus transfection) according to the manufacturer's instructions.

Western blot

Western blotting was carried out as previously described (10). Antibodies used were against α5 integrin Ab1928 (Millipore) or H104 (Santa-Cruz), β1 integrin Ab1952, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Millipore,), p53 (BD Biosciences), or $p53^{pser15}$ (Cell Signaling).

Flow cytometry

After detachment with EDTA, cells were incubated for 30 minutes at 4°C under agitation in the presence of primary antibodies: anti-α5 integrin antibody IIA1 (BD Biosciences) and anti-β1 integrin antibodies (TS2/16 from Santa Cruz; 9EG7 and mab13 from BD Biosciences). After washing, cells were incubated for 30 minutes with secondary antibody (Alexa488-conjugated goat anti-mouse or rat; Jackson ImmunoResearch). After washing, cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson), and the mean fluorescence intensity characterizing surface expression of integrins was measured using the CellQuest software.

Clonogenic assay

Clonogenic survival was determined as previously described (9).

Immunofluorescence

A total of 20,000 cells were seeded onto IBIDI μ-dishes coated with 10 μg/mL of poly-L-lysine. Cells were treated with nutlin-3a (10 μmol/L) or with solvent during 24 hours before fixation with 4% paraformaldehyde (10 minutes at room temperature) and then processed for α5 immunodetection (IIA1 antibody; 1:300). Confocal images were taken with a confocal microscope (BioRad 1024) equipped with a water immersion ×60 objective. Images were collected using the Laser-Sharp 2000 software.

Human biopsies

This study was conducted on 115 adult brain biopsies, 95 brain tumors (22 grade II, 38 grade III, and 35 grade IV) and 20 nontumoral brain tissues collected retrospectively from archival material stored at the Centre de Ressources Biologiques et Tumorothèque (Hopitaux Universitaires de Strasbourg, Strasbourg, France). The patient characteristics have been described elsewhere (17). Each sample was histologically analyzed by a pathologist to specify the tumor grade and the percentage of tumor cells. Only samples with at least 50% of tumoral cells (>50% of samples were >70% tumoral cells) have been included in the study. Control tissues were obtained from epileptic surgery. The study was conducted in accordance with the Declaration of Helsinki. Real-time qPCR was carried out as described previously (17). The threshold cycle (C_t) values for each gene were normalized to expression level of *cyclophilin* used as the housekeeping gene. Values were normalized relatively to the value obtained for one nontumoral control brain tissue, which was included in each qPCR run. Immunologic analysis of α5 protein expression was conducted as shown previously (17).

Human brain tumor data sets

Glioma gene expression data sets from 2 other cohorts were downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession numbers GSE4271 and GSE4412). Microarray raw data were processed using R (version 2.10.1; http://cran.r-project.org/), implemented with the BioConductor package (http://www.bioconductor.org). Estimates of the survival curves were computed using the Kaplan–Meier method. Univariate survival comparisons between the patients, according to low or high α5 integrin expression levels, were conducted using a log-rank test.

Human brain tumor xenografts

TCG4, TCG9, and TCG17 glioma xenograft models were obtained as previously described (18). Subcutaneous tumor growth was followed by measuring, 3 times per week, 2 perpendicular diameters with a caliper. Treatments began when tumors reached a volume of approximately 250 ± 50 mm^3. Temozolomide was administered orally at the dose of 50 mg/kg/d for 5 days. Mice were sacrificed when the tumor volumes reached 4 times their initial volume (V_0). For each mouse, the time between the treatment onset and the animal sacrifice was defined as the "survival time." *TP53* status of each xenograft was determined by the yeast functional assay (16).

Published OnlineFirst May 16, 2012; DOI:10.1158/0008-5472.CAN-11-4199

Statistical analysis

Data are represented as the mean ± SEM, and n is the number of independent experiments. Statistical analyses were conducted using the Student t test or the Mann–Whitney test with the GraphPad Prism program. $P < 0.05$ was considered significant.

Results

α5β1 integrin impedes temozolomide-induced p53-wt activity

We compared the effect of temozolomide in U87MG cells depleted in ($shRNA_{\alpha5}$) or overexpressing ($pcDNA_{\alpha5}$) α5 integrin versus control cells ($shRNA_{ns}$ and $pcDNA_{ctrl}$, respectively). Because p53 is largely involved in chemotherapeutic drug effects and we showed previously that α5β1 integrin antagonists modulate the p53 pathway (10), we focused on temozolomide-induced p53 activation. Temozolomide caused an increase in p53 protein in all cell lines but not significantly in $pcDNA_{\alpha5}$ cells (when normalized to GAPDH: Fig. 1A). Interestingly, a significant increase in p53 protein was already observed in untreated $shRNA_{\alpha5}$ cells versus $shRNA_{ctrl}$ cells (Fig. 1A). After temozolomide treatment, an increase in $p53^{pser15}$ was detectable in $pcDNA_{ctrl}$ and $shRNA_{ns}$ cells, which was significantly more pronounced in $shRNA_{\alpha5}$ (Fig. 1A). In contrast, in $pcDNA_{\alpha5}$ cells, significantly less $p53^{pser15}$ was measured after temozolomide treatment. Transcriptional activity of p53 was higher in $shRNA_{\alpha5}$ cells and lower in $pcDNA_{\alpha5}$ cells than in temozolomide-treated control cells (Fig. 1B). Taken together, these results indicate that α5β1 integrin modulates p53 activity and that high expression of this integrin inhibits temozolomide-induced p53 stimulation. Modulation of p53 activity was related to cell survival, as $pcDNA_{\alpha5}$ cells are significantly more resistant at high temozolomide concentration whereas $shRNA_{\alpha5}$ cells appear more sensitive than their control counterparts (Fig. 1C). α5 integrin overexpression did not modulate p53, nor clonogenic survival in U373 and LN18 cells expressing a p53-mutant (Supplementary Fig. S1). In addition, repression of α5 integrin in p53-deficient LNZ308 cells did not sensitize cells to temozolomide (Supplementary Fig. S1). From these data, we concluded that α5β1 integrin–induced temozolomide resistance requires a functional p53.

As a first approach to confirm the role of the α5β1 integrin in temozolomide chemoresistance *in vivo*, we used subcutaneous xenografted human brain tumors in nude mice. We selected 3 xenografts that exhibited a wild-type p53 and different levels of the α5 subunit. Kaplan–Meier analysis of the mouse survival suggests a relationship between α5 integrin level and resistance to temozolomide (Fig. 1D), providing some evidence for a role of the α5 integrin in the chemoresistance of p53-wt–expressing tumors *in vivo*.

Activation of p53 by nutlin-3a overrides the α5 integrin effects

We next investigated whether high α5 also impacts on p53 activation by a non-genotoxic p53 activator in glioma cells. U87MG cells were treated with nutlin-3a. In contrast to the effects of temozolomide, nutlin-3a stabilized p53, markedly increased the $p53^{pser15}$ and the transactivation of p53 target genes in $pcDNA_{ctrl}$ and in $pcDNA_{\alpha5}$ cells (Fig. 2A and B). Addition of temozolomide to nutlin-3a does not further increase these effects (Fig. 2A). In clonogenic assays, the α5β1 integrin did not efficiently protect the cells from death when p53 was activated by 10 μmol/L nutlin-3a (Fig. 2C). Survival of LNZ308 cells (p53 KO) or U373 cells (p53-mutant) was less affected after treatment with nutlin-3a (Fig. 2C).

Activation of p53 by nutlin-3a markedly decreases the α5 expression level in glioma cells

U87MG cells treated with nutlin-3a rounded up and detached from the wells. This effect was lost when p53 expression was inhibited with specific siRNA (Fig. 3A, left). In contrast, LNZ308 cells did not exhibit any morphologic alterations after a 10 μmol/L nutlin-3a treatment unless p53 was reexpressed in the cells (Fig. 3A, right). Interestingly, cell treatment with nutlin-3a decreased the expression of the α5 integrin at the protein level in U87MG-$pcDNA_{ctrl}$ and U87MG-$pcDNA_{\alpha5}$ cells, an effect not observed after treatment with temozolomide (Fig. 3B). The decrease in α5 protein expression after nutlin-3a treatment was confirmed by specific immunostaining of the α5 subunit in U87MG-$pcDNA_{ctrl}$ and U87MG-$pcDNA_{\alpha5}$ cells and by flow cytometric analysis of the α5 subunit at the cell membrane (Fig. 3B). A significant decrease in the α5 mRNA level was measured in U87MG $pcDNA_{ctrl}$ but not in $pcDNA_{\alpha5}$ cells, suggesting that nutlin-3a affected the α5 subunit at translational and posttranslational levels (Fig. 3C). Nutlin-3a also decreased β1 at the protein and mRNA level in the U87MG cells, suggesting that both subunits of the α5β1 integrin are processed similarly after nutlin-3a treatment (Supplementary Fig. S2). However, no effect on the β1 subunit expressed at the cell membrane could be detected after nutlin-3a treatment (Supplementary Fig. S2 and Supplementary Table S1).

Nutlin-3a did not affect the endogenous α5 protein in p53-knockout LNZ308 cells unless p53 was reexpressed (Fig. 3D). Nutlin-3a had no effect on α5 expression in U373 cells (Supplementary Fig. S3). Altogether, these data suggest that nutlin-3a requires a functional p53 to decrease α5 expression, which in turn make the cells susceptible to the nutlin-3a–induced cell death.

High α5β1 integrin expression is associated with worse clinical outcome in high-grade glioma

To our knowledge, no studies have associated α5β1 integrin with clinical outcome in patients with glioma. To investigate first whether integrin expression is associated with the grade of brain tumors, gene expression of the α5 and β1 subunits were examined by qPCR in 95 human brain tumors of different grades and compared with 20 nontumor brain samples. The data revealed that α5 subunit gene expression was increased with increasing tumor grade, although the β1 subunit was equally overexpressed in the 3 tumoral grades compared with control tissue (Fig. 4A). Data were confirmed at the protein level (Fig. 4B). Because the α5 subunit only dimerizes with β1,

Published OnlineFirst May 16, 2012; DOI:10.1158/0008-5472.CAN-11-4199

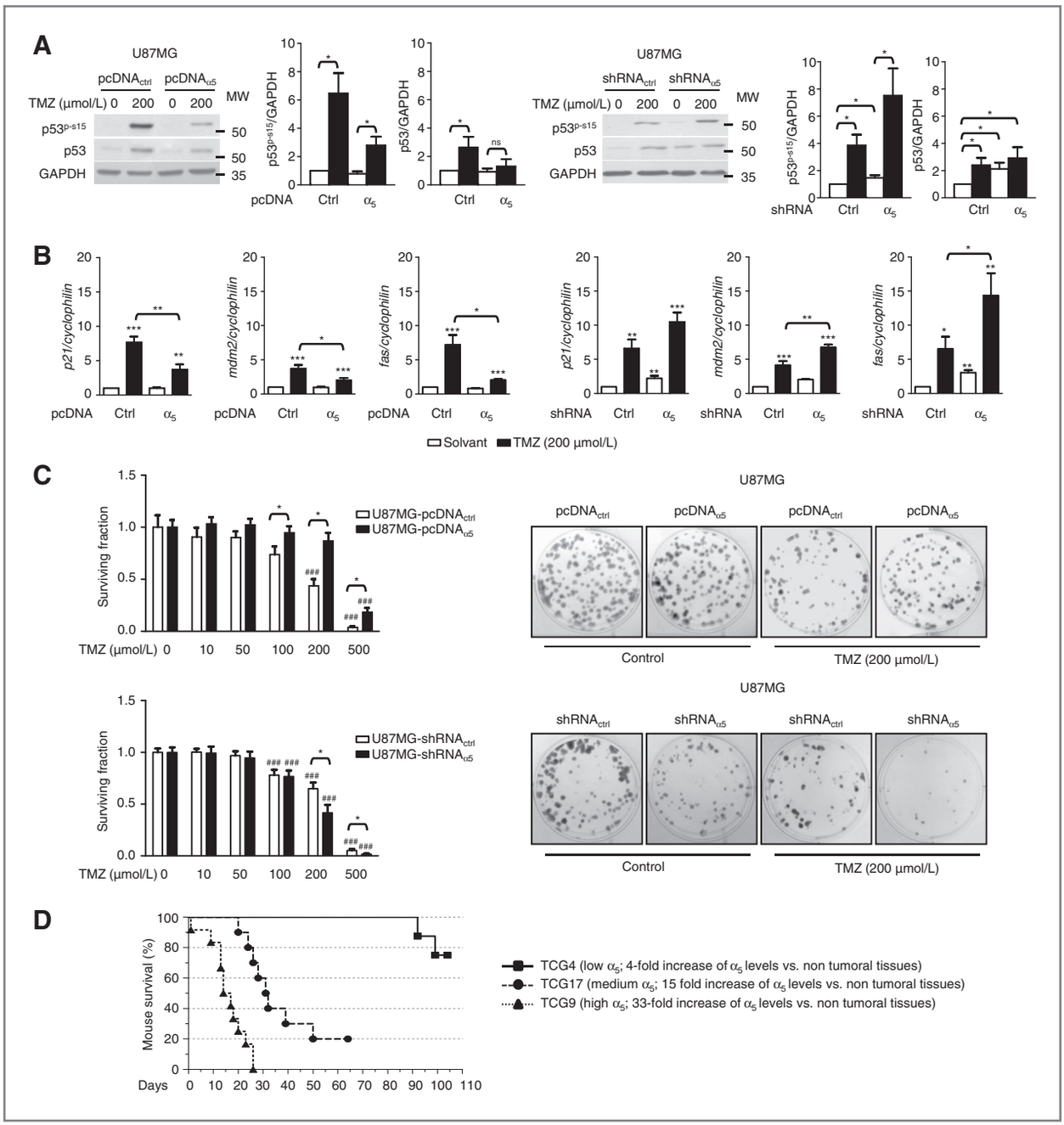

Figure 1. Elevated α5 integrin expression impairs temozolomide (TMZ)-induced p53-wt signalling and triggers TMZ resistance. A, stability and p53 phosphorylation on Ser15 are affected by the α5 integrin expression level. Western blot analysis for p53 and p53^{pser15} from total cell lysates with and without TMZ (200 μmol/L) treatment during 24 hours in control (pcDNA$_{ctrl}$) and α5 integrin–overexpressing (pcDNA$_{\alpha5}$) U87MG cells (top) or control (shRNA$_{ns}$) and α5 integrin–downregulated (shRNA$_{\alpha5}$) U87MG cells (bottom). Histograms represent the mean ± SEM of 6 to 8 independent experiments. B, qPCR quantification of p53 target genes. mRNA of target genes are differentially affected by upmodulated (top) or downmodulated (bottom) α5 integrin after TMZ treatment in U87MG cells. C, TMZ dose response of clonogenic survival in U87MG cells overexpressing α5 integrin (U87-pcDNA$_{\alpha5}$) compared with control cells (U87-pcDNA$_{ctrl}$). pcDNA$_{\alpha5}$ cells are 2 times more resistant than control cells at 200 μmol/L TMZ (top) or U87MG cells underexpressing α5 integrin (U87-shRNA$_{\alpha5}$) compared with control cells (U87-shRNAns). shRNA$_{\alpha5}$ are 1.5 times more sensitive than control cells at 200 μmol/L TMZ (bottom). Representative images of colonies obtained with and without 200 μmol/L TMZ are shown. Statistical analysis: ##, $P < 0.01$; ###, $P < 0.001$ for treated cells versus nontreated cells; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for genetically manipulated cells versus corresponding control cells. D, TMZ antitumor effect on human malignant glioma xenografts in nude mice. Three glioma xenografts expressing p53-wt were analyzed for α5 mRNA expression (TCG9, TCG17, and TCG4 with 33-, 15-, and 4.5-fold more α5 mRNA, respectively, compared with human nontumor brain tissue) and used to evaluate the tumor response to TMZ (orally daily 50 mg/kg × 5 days). Results are expressed as Kaplan–Meier plots, considering the percentage of tumors that reached four V_0 as the survival end point. Ctrl, control; ns, not significant.

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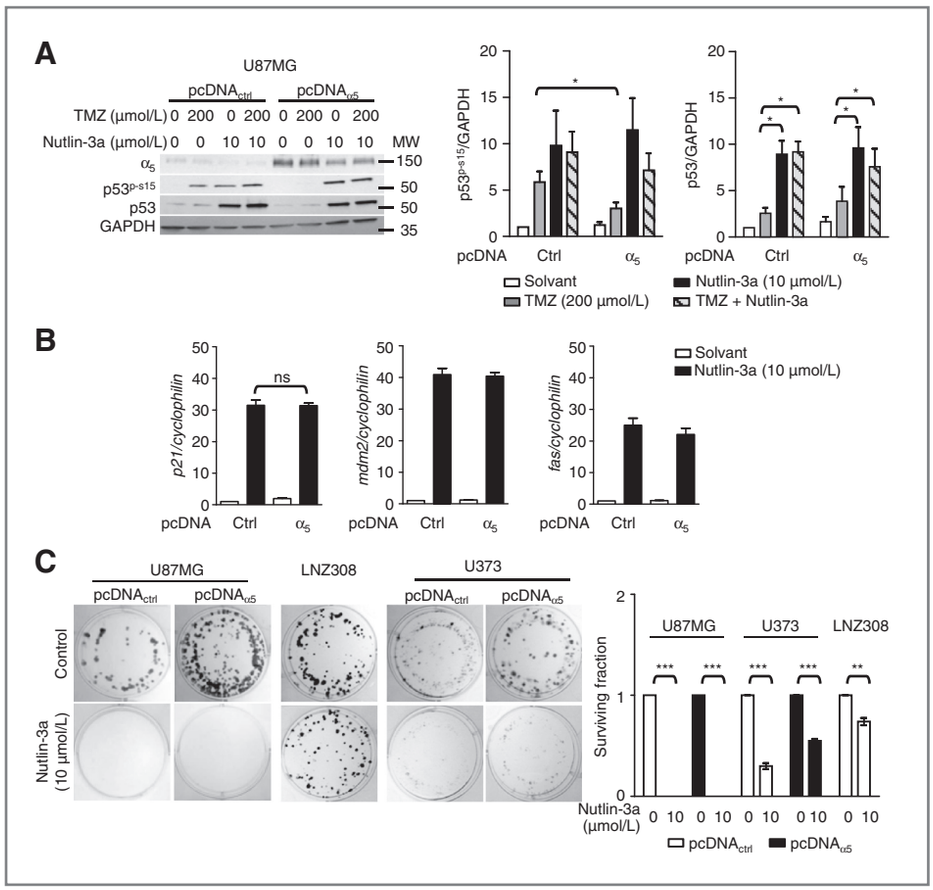

Figure 2. Activation of p53 by nutlin-3a overrides the inhibitory effect of the α5 integrin. A, Western blot analysis of the α5 integrin, p53, and p53^{pser15} in pcDNA$_{ctrl}$ and pcDNA$_{α5}$ -transfected U87MG cells. U87MG cells were treated with nutlin-3a (10 μmol/L), temozolomide (TMZ; 200 μmol/L), or both for 24 hours. The histograms display the mean ± SEM of 5 independent experiments. GAPDH was used as the loading control. B, qPCR analysis of p53 target genes. The histograms represent the fold increase of mRNA in pcDNA$_{ctrl}$ and pcDNA$_{α5}$ -transfected U87MG cells before and after nutlin-3a (10 μmol/L) treatment over 24 hours. ns, nonsignificant. C, clonogenic survival of pcDNA$_{ctrl}$ -U87MG and pcDNA$_{α5}$-U87MG cells (left), LNZ308 cells (middle), and pcDNA$_{ctrl}$-U373 and pcDNA$_{α5}$ -U373 cells (right) after nutlin-3a treatment (10 μmol/L). Histograms represent the mean ± SEM. *, $P < 0.05$ of 3 independent experiments. **, $P < 0.01$; ***, $P < 0.001$ in nutlin-3a–treated versus nontreated cells.

the data point toward a particular role for α5β1 integrin in glioma.

We analyzed the clinical data of grade III and grade IV patients. Log-rank analysis of the Kaplan–Meier survival curves showed a significant survival advantage for patients with low α5-expressing glioma compared with high α5-expressing glioma. These results were validated in 2 independent public data sets (2, 19). Considering all 3 cohorts together, the group of high α5-expressing tumors included 39% of grade III and 81% of grade IV tumors (Fig. 4C; Supplementary Table S2). Finally, we evaluated the relationship between α5 integrin level and the status of p53 in 56 human biopsies (grade III and IV) and in 17 human tumor xenografts. A clear tendency toward a higher level of α5 in p53-wt versus p53-mutant tumors was found in biopsies and in xenografts (Fig. 4D; Supplementary Table S3).

Discussion

The data summarized here document the impact of the α5β1 integrin on the high-grade glioma resistance to temozolomide therapy. When the α5 integrin subunit is overexpressed, the p53-mediated responses to genotoxic damage are compromised. When the α5 integrin level is low or suppressed, p53 is stabilized and fully functional. An inverse relationship between the α5 integrin level and p53 has been revealed through the use of the p53 activator, nutlin-3a. These results may have clinical relevance in light of the clear advantage reported here for prolonged survival of patients with high-grade glioma with low α5 integrin subunit expression.

In agreement with our data, it was reported that the α5β1 integrin is overexpressed at the protein level in a significant proportion of human glioblastoma biopsies (20). Here, we show for the first time that in glioma, the α5 mRNA level is negatively correlated to survival in 3 different cohorts of patients, which adds brain tumors to the growing list of cancers in which the α5β1 integrin should be considered as a therapeutic target. The role of p53 in temozolomide resistance is far from being understood. Although several groups reported that p53 status is not predictive of response to chemotherapy with alkylating agents (18, 21), more recent works suggest that the absence of a functional p53 increases temozolomide sensitivity in glioma cell lines (22) and in intracranial glioblastoma xenografts (23). A trend toward an increased temozolomide sensitivity in patients with p53 mutations was also suggested (24). We propose that overexpression of the α5β1 integrin in GBM represents an alternative mechanism, aside from p53 deletion/mutation, to inactivate the tumor-suppressive function of the p53 pathway. We are currently investigating the molecular mechanisms involved in the integrin–p53 cross-talk by addressing the role of α5β1 integrin in transcriptional and nontranscriptional effects of p53. Activation of the p53 pathway by nutlin-3a led to downregulation of

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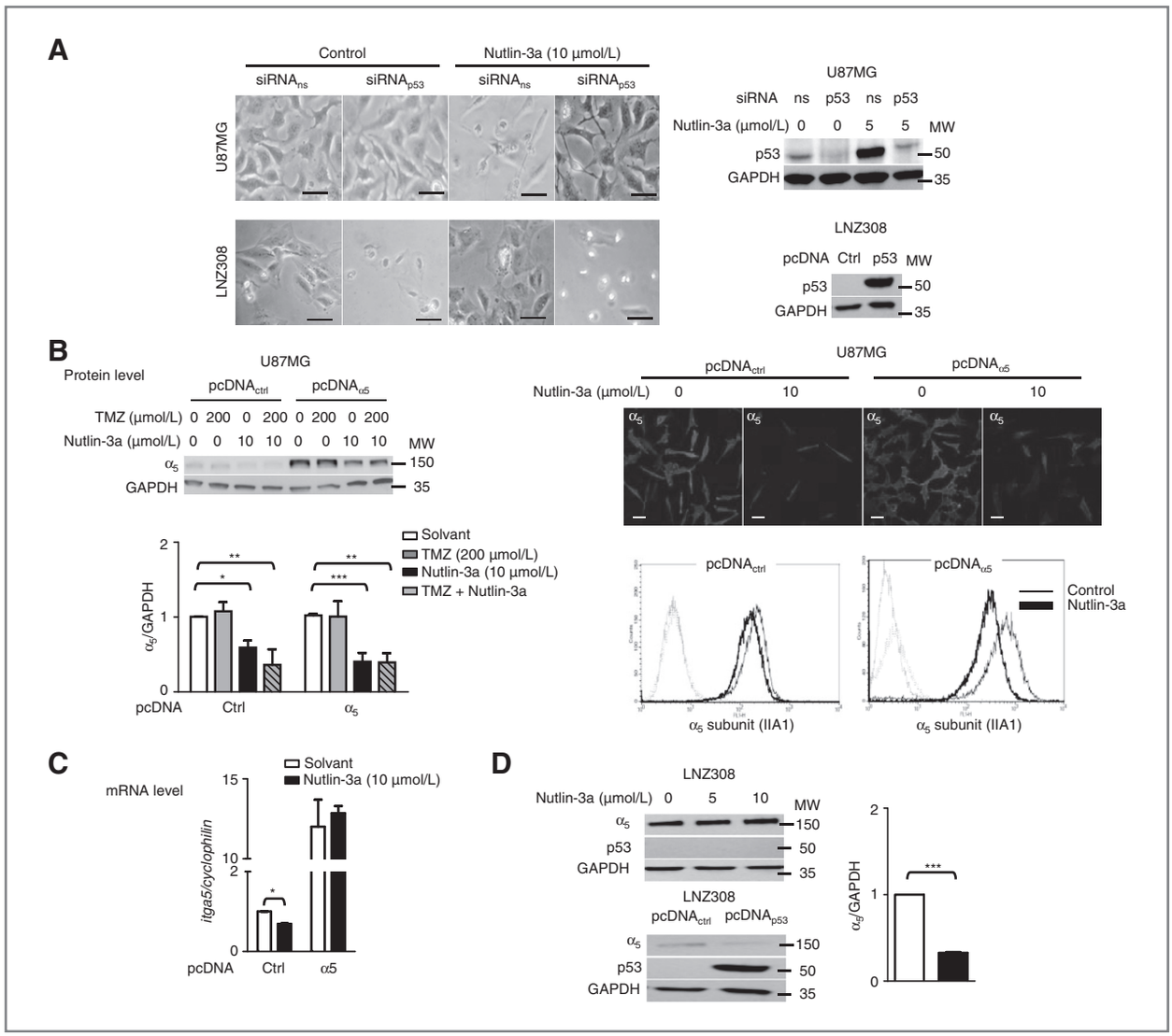

Figure 3. Activation of p53 by nutlin-3a affects the α5β1 integrin expression in U87MG cells. A, U87MG and LNZ308 cell morphology after 24 hours of nutlin-3a treatment. U87MG cells were transfected either with control $siRNA_{ns}$ or with $siRNA_{p53}$ and treated with nutlin-3a (5 μmol/L) for 24 hours. Silencing of the p53 protein was verified by immunoblotting. LNZ308 cells were transfected with $pcDNA_{ctrl}$ or $pcDNA_{p53}$ and treated with nutlin-3a (10 μmol/L). Expression of p53 was verified by immunoblotting. Scale bars, 50 μm. B, top, Western blot analysis of the α5 integrin protein expression in $pcDNA_{ctrl}$- and $pcDNA_{α5}$-transfected U87MG cells. Cells were treated with nutlin-3a (10 μmol/L), TMZ (200 μmol/L), or both drugs for 24 hours. Histograms show the fold increase in the protein expression normalized to GAPDH levels (mean ± SEM of 3–4 independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for treated cells versus nontreated cells. Middle, representative fluorescence images with specific anti-α5 integrin antibodies of untreated and nutlin-3a-treated $pcDNA_{ctrl}$- and $pcDNA_{α5}$-transfected U87MG cells. Scale bars, 20 μm. Bottom, flow cytometric analysis of the α5 (IIA1 antibody) integrin subunit at the cell membrane of $pcDNA_{ctrl}$- and $pcDNA_{α5}$-transfected U87MG cells before and after nutlin-3a (10 μmol/L) treatment for 24 hours. C, histograms represent the fold increase of the α5 mRNA in $pcDNA_{ctrl}$- and $pcDNA_{α5}$-transfected U87MG cells after nutlin-3a (10 μmol/L) treatment. *, $P < 0.05$ for treated cells versus nontreated cells. D, left, Western blot analysis of α5 and p53 protein expression in p53-null LNZ308 cells treated with nutlin-3a (5 and 10 μmol/L) for 24 hours. A representative blot of 3 is shown. Right, LNZ308 cells were transfected with p53-wt, and α5 integrin expression was detected by Western blot analysis. Histogram represents the mean ± SEM of 3 independent experiments. GAPDH was used as the loading control. Ctrl, control; ns, not significant.

the α5 integrin subunit in glioma cells. On the basis of our data, there seems to be a cross-antagonistic interaction between the α5 integrin and p53 that was only revealed by nutlin-3a, which may explain why this drug overcomes the prosurvival activity of the integrin. Our results are similar to recent data showing that nutlin-3a downregulates the oncogene DEK or overcomes the antiapoptotic Bcl2 overexpression, thus leading to cell apoptosis (25, 26).

In summary, we have shown for the first time that α5β1 integrin plays a critical role in resistance to temozolomide therapy by interfering with the p53 pathway in high-grade glioma. In addition, we have shown that activation of p53 by

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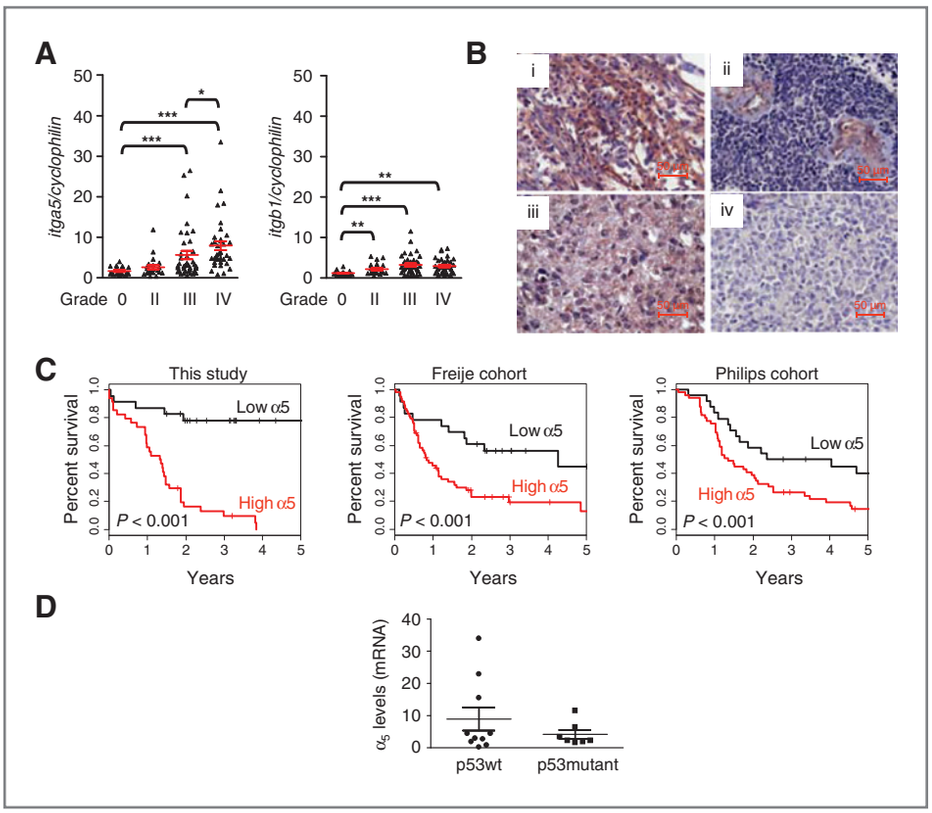

Figure 4. Elevated α5-integrin gene expression is associated with high-grade glioma and predicts decreased survival rates. A, gene expression levels of α5 and β1 integrin subunits were quantified with specific primers by qPCR in 20 nontumor brain tissues (G0), 22 grade 2 (GII), 38 grade 3 (GIII), and 35 glioblastoma (GIV) samples. Only the α5 integrin subunit level is associated with the tumor grade. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared with nontumoral brain tissue (Mann–Whitney test). B, immunohistochemical analysis of α5 protein in tumoral cells of human high-grade glioma. Representative slides of α5 high intensity staining in GBM (i), α5-negative staining in GBM (ii), α5 high intensity staining in grade III tumor (iii), and α5-negative staining in grade III tumors (iv). Scale bars, 50μm. C, elevated α5 integrin gene expression is associated with significantly decreased long-term survival in patients with high-grade glioma. Kaplan–Meier survival analysis of 3 patient cohorts is shown. Left, cohort from this study; middle, data from Freije and colleagues (2); right, data from Phillips and colleagues (19). D, the α5 mRNA expression level and p53 status in human brain tumor xenografts in nude mice. Ten xenografts expressed a wild-type p53 and 7 xenografts had a mutant p53, as determined by the FASAY assay. The α5 mRNA levels were determined in at least 3 different grafts of the same tumor, and the mean levels were plotted according to p53 status. The mean values ± SEM of the α5 mRNA level in p53-wt and mutant p53 tumors were 8.9 ± 3.6 and 4.1 ± 1.3, respectively. Although not significant, this difference shows a trend toward an increased level of α5 in p53-wt tumors.

nutlin-3a represses the α5β1 integrin, and we propose that such downregulation is an important mediator of nutlin-3a cytotoxic activity. The relevance of our results is emphasized by the finding that α5 integrin gene overexpression is associated with decreased survival in patients with high-grade glioma. Our data provide the rationale for a preclinical evaluation of p53 activators and/or α5β1 integrin antagonists in a subset of high-grade glioma that expresses a functional p53 and high levels of α5β1 integrin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Janouskova, S. Pinel, P. Chastagner, M. Dontenwill
Development of methodology: H. Janouskova, A. Maglott, D.Y. Leger, S. Pinel, P. Chastagner, S. Martin, J. Teisinger
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Maglott, D.Y. Leger, C. Bossert, F. Noulet, E. Guerin, S. Pinel, F. Plenat, N. Entz-Werle, J. Lehmann-Che
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Janouskova, A. Maglott, F. Noulet, P. Chastagner, F. Plenat, J. Godet, M. Dontenwill
Writing, review, and/or revision of the manuscript: H. Janouskova, D. Guenot, P. Chastagner, F. Plenat, J. Lehmann-Che, J. Godet, S. Martin, M. Dontenwill
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Guenot, N. Entz-Werle, S. Martin
Study supervision: D. Guenot, P. Chastagner, M. Dontenwill

Acknowledgments

The authors thank M. Hegi for the LNZ308 and LN18 human glioblastoma cell lines and Dr. C. Blattner for the p53-wt containing plasmid.

Grant Support

This work was supported by grants from the Ligue contre le Cancer (comité du Grand-Est), the Institut National du Cancer, and the Association pour la Recherche contre le Cancer. H. Janouskova is a recipient of the Ministère des Affaires Etrangères PhD student fellowship, and D.Y. Leger is a recipient of an Institut National du Cancer postdoctoral fellowship.

Received December 30, 2011; revised May 7, 2012; accepted May 9, 2012; published OnlineFirst May 16, 2012.

Published OnlineFirst May 16, 2012; DOI:10.1158/0008-5472.CAN-11-4199

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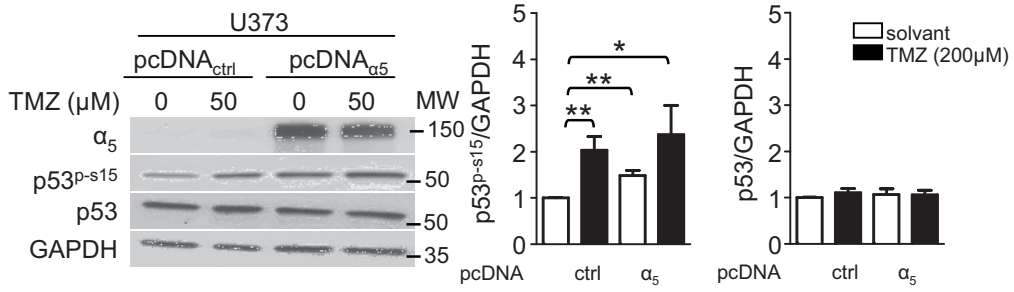
Supplementary Figure S1

a.



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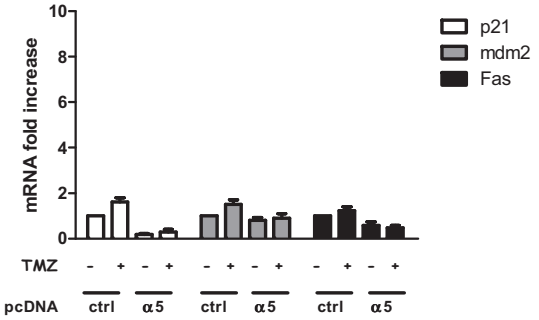

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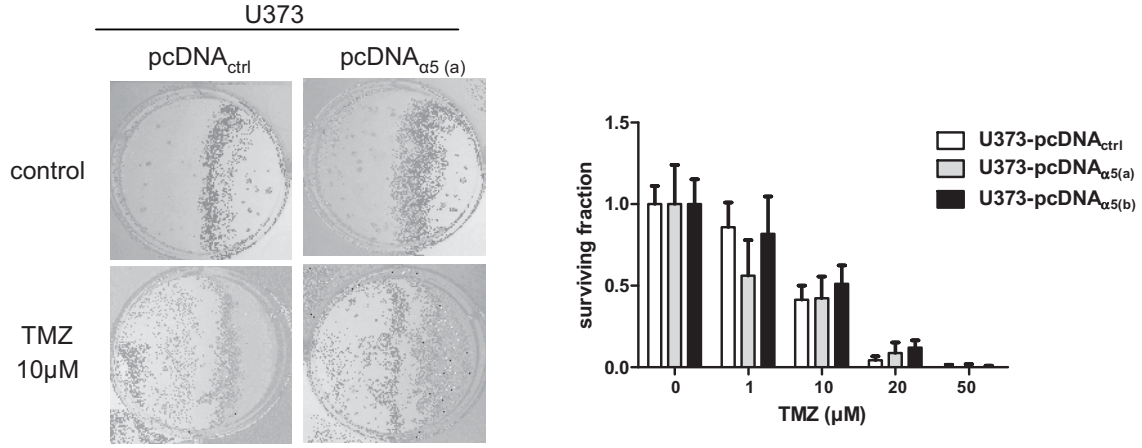

Supplementary Figure S1.

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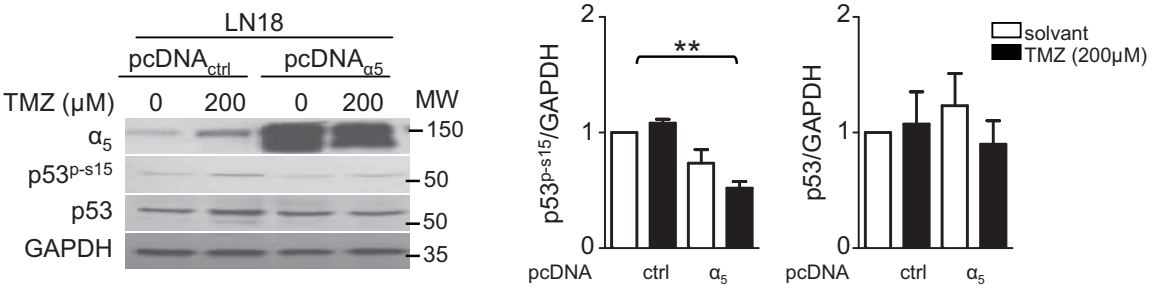

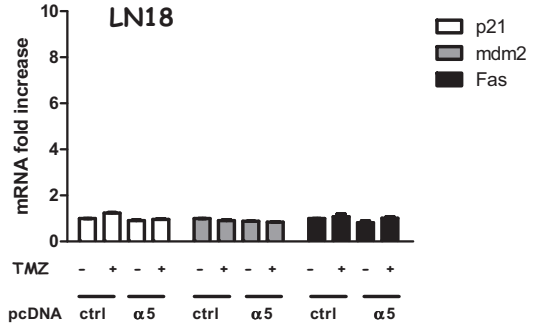

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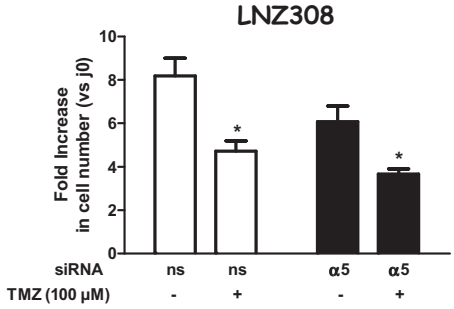
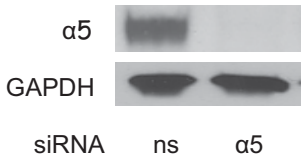
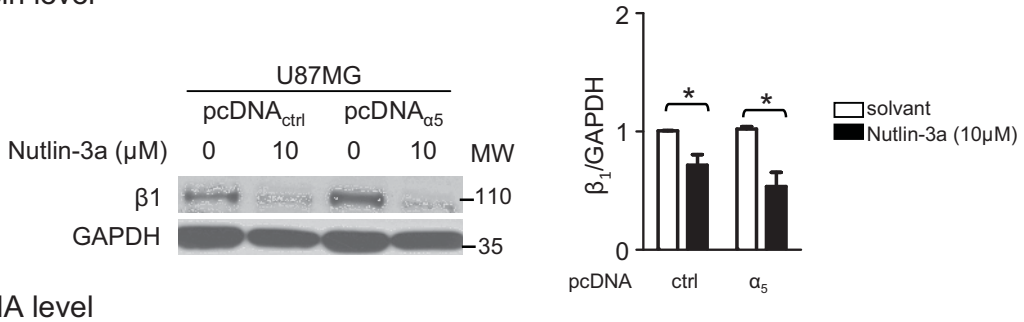




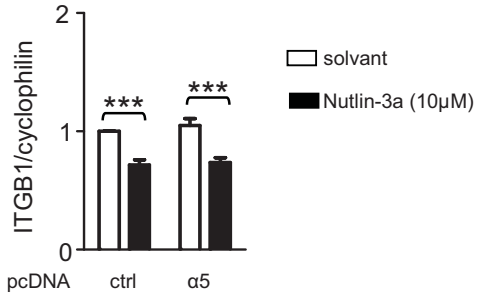
Supplementary Figure S2.

U87MG cells

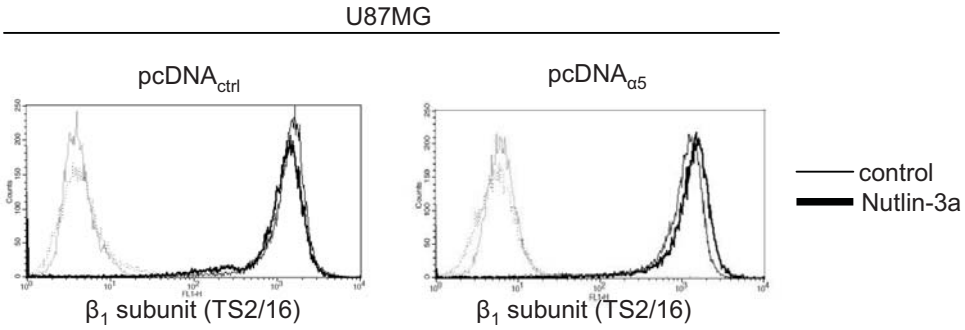
a. protein level



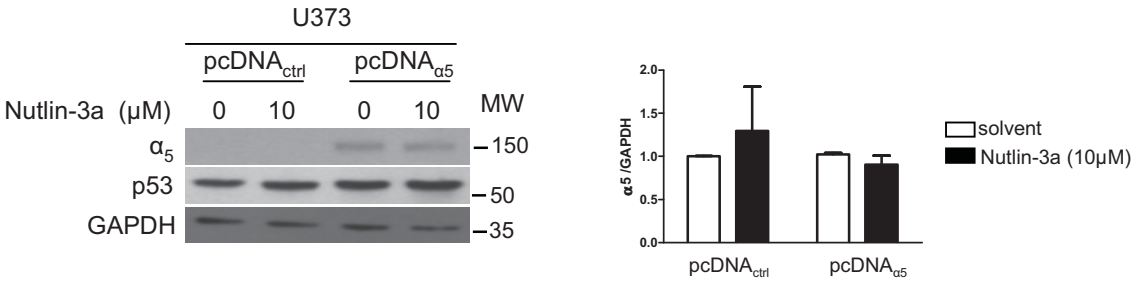
b. mRNA level



c. flow cytometry analysis



Supplementary Figure S3.



Legends to Supplementary figures

FIGURE S1. The effect of α5 integrin on p53 activation is restricted to p53wt-expressing cells.

(a) Effect of TMZ on U373 cells stably transfected with $pcDNA_{ctrl}$ or $pcDNA_{\alpha5}$. Western blot analysis of α5 integrin, p53, $p53^{pser15}$ from total cell lysates with and without TMZ treatment (50 μM, 24 hours). GAPDH was used as the loading control. Histograms represent the mean ± sem of 3 independent experiments. (b) qPCR quantification of p53 target genes in U373 cells. mRNA of target genes are not affected after TMZ treatment in cells overexpressing the α5 integrin. (c) The TMZ dose response of clonogenic survival in U373 cell clones overexpressing α5 integrin (U373-$pcDNA_{\alpha5a}$ and U373-$pcDNA_{\alpha5b}$) compared to control cells (U373-$pcDNA_{ctrl}$). (d) The effect of TMZ on LN18 cells transiently transfected with $pcDNA_{ctrl}$ or $pcDNA_{\alpha5}$. Top, twenty-four hours post-transfection, cells were treated with TMZ (24 hours, 200 μM), and α5 integrin, p53, $p53^{pser15}$ were detected by western blot analysis. Bottom, qPCR quantification of p53 target genes in LN18 cells. mRNA of target genes are not affected after TMZ treatment by overexpression of α5 integrin. (e) Effect of TMZ on LNZ308 cells (p53 deficient) either transfected with siRNA non silencing (siRNAns) or siRNA targeting α5 integrin (siRNAα5). Cell proliferation after 72 hours treatment with solvent or TMZ (100 μM) is represented. TMZ decreased cell proliferation to 58 and 59% of control non treated cells in siRNAns cells and siRNAα5 cells respectively. Repression of α5 integrin subunit was confirmed by western blot.

Figure S2. Effects of Nutlin-3a on β1 integrin subunit in U87MG cells.

(a) Western blot analysis of the β1 integrin subunit expression in $pcDNA_{ctrl}$- and $pcDNA_{\alpha5}$-transfected U87MG cells. Cells were treated with nutlin-3a (10 μM) for 24

hours. Histograms show the fold increase in the protein expression normalized to GAPDH levels in treated cells versus non treated cells (mean ± sem of 3 - 4 independent experiments). (b) Histograms represent the fold increase of the β1 mRNA in $pcDNA_{ctrl}$- and $pcDNA_{\alpha5}$-transfected U87MG cells after nutlin-3a (10 μM) treatment compared to non-treated cells (mean ± sem of 3 independent experiments). (c) Flow cytometry analysis of the β1 integrin subunit (TS2/16 antibody) at the cell membrane of $pcDNA_{ctrl}$- and $pcDNA_{\alpha5}$-transfected U87MG cells before and after nutlin-3a (10μM) treatment for 24 hours.

Figure S3. A functional p53 is required for nutlin-3a modulation of α5β1 integrin expression in glioma cells. Western blot analysis of α5 and p53 expression in p53 mutant U373 cells after treatment with 10 μM nutlin-3a. $pcDNA_{ctrl}$- and $pcDNA_{\alpha5}$-transfected U373 cells were used. GAPDH was used as the loading control. Histograms represent the mean ± sem of three independent experiments.

Supplementary Table 1.

cells \ antibodies	Ig control (mouse)	Ig control (rabbit)	α_5 (IIa1)	β_1 (9EG7)	β_1 (TS2/16)	β_1 (mAb13)
U87-pcDNA$_{ctrl}$	4.8±0.2	4.3±0.	219±15	37±2	1317±151	482±53
U87-pcDNA$_{ctrl}$ + nutlin	5.3±0.7	4.0±0.	165±15 * (p=0.04)	33±2	1246±73	455±35
U87-pcDNA$_{\alpha5}$	5.3±0.4	4.6±0.3	621±35	40±6	1207±67	430±123
U87-pcDNA$_{\alpha5}$ + nutlin	5.8±0.5	4.4±0.6	452±42* (p=0.01)	43±6	1322±62	425±66

Mean fluorescence intensities of α5 and β1 integrin subunit expression at the cell membrane. Cells were treated with solvent or Nutlin-3a (10 μM, 24 hours) and proccessed for flow cytometry analysis as described in the material and methods section. Data report mean ± sem of 3 independent experiments. * indicates a statistically significant difference between cells treated with Nutlin-3a and cells treated with solvent. Integrin β1 expression at the cell membrane is not affected by nutlin-3a.

Supplementary Table 2.

tumor grade	III		IV	
integrin level / database	low α_5 integrin	high α_5 integrin	low α_5 integrin	high α_5 integrin
This study	23	13	7	28
Freije	14	11	9	47
Philips	13	8	11	41
Σ / grade	50	32	27	116
% / grade	61	39	19	81

Proportion of grade III and grade IV glioma in the subgroups of patients with high and low α5 integrin mRNA expression in the three cohorts of patients included in this study.

Supplementary Table 3.

A

α_5 RNA levels	low α_5 RNA levels		high α_5 RNA levels	
p53 status / grade	p53 wt	p53 mutant	p53 wt	p53 mutant
grade III	11 (18)	7 (18)	8 (10)	2 (10)
grade IV	2 (7)	5 (7)	15 (22)	7 (22)
total	13	12	23	9
%	52	48	72	28

B

	p53 wt		p53 mutant	
	Tumor identification	α_5 RNA levels	Tumor identification	α_5 RNA levels
	TCG1	0.2	TCG2	3.4
	TCG4	4.4	TCG3	2.2
	TCG7	1.9	TCG13	1.6
	TCG9	33.9	TCG14	6.4
	TCG11	22.9	TCG15	2.2
	TCG12	4.4	TC20	2
	TCG17	15.5	TC22	11.5
	TC10	0.85		
	TC14	2.7		
	TC24	2.9		
mean ± SEM		8.9 ± 3.6		4.2± 1.4

Relationship between α5 mRNA level and p53 status.

A. in human biopsies; The loss of heterozygosity (LOH) on chromosome 17pll.2-pter, containing the p53 gene and occurring together with p53 mutation, was assessed by allelotyping according to Guenot et al, (J Pathol,2006). Results were available for 28/36 patients grade III and 29/35 patients grade IV included in the survival curves of Figure 4c (our cohort). In the low α5 expressing tumor group, 52% and 48% of tumors were p53 wt and p53 mutant respectively whereas 72% of tumors with high α5 expression express a p53wt.

B. In human xenografts in nude mice. p53 status was assessed by the Fasay assay on 17 xenografted tumors. Values of α5 mRNA level for each tumor relative to non tumoral brain tissue is given and correspond to the values represented in the figure 4d.

APPENDIX 2:

Janouskova, H., Ray, A.M., Noulet, F., Lelong-Rebel, I., Choulier, L., Schaffner, F., Lehmann, M., Martin, S., Teisinger, J., & Dontenwill, M. 2013. *Activation of p53 pathway by Nutlin-3a inhibits the expression of the therapeutic target alpha5 integrin in colon cancer cells*. Cancer Lett, [Epub ahead of print].

Cancer Letters xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Activation of p53 pathway by Nutlin-3a inhibits the expression of the therapeutic target α5 integrin in colon cancer cells

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ARTICLE INFO

Article history:
Received 5 November 2012
Received in revised form 1 March 2013
Accepted 15 March 2013
Available online xxxx

Keywords:
Colon cancer
Integrin α5β1
p53
Nutlin-3a
RITA

ABSTRACT

Integrins emerge nowadays as crucial actors of tumor aggressiveness and resistance to therapies. Integrin α5β1, the fibronectin receptor, determines malignant properties of colon carcinoma which is one of the most important causes of cancer-related deaths in the world. Here we show that inhibition of α5 integrin subunit expression by siRNA or α5β1 integrin function by specific antagonist affects the survival of HCT116 colon cancer cells. We also evidence that pharmacological reactivation of the tumor suppressor p53 by Nutlin-3a inhibits specifically the expression of the α5 integrin subunit both at the transcriptional and protein level. Inversely repression of α5 integrin modulates p53 activity. A clear relationship between p53 activation by Nutlin-3a, α5 repression and cell survival is shown. No such effects are obtained in cells lacking p53 or when another non-genotoxic activator of p53, RITA, is used. Our results emphasize the crucial role of α5β1 integrin in colon tumors. Data also suggest that interfering with the integrin α5β1 through the reactivation of p53 by Nutlin-3a may be of valuable interest as a new therapeutic option for colon tumors expressing high level of the integrin and a wild type p53.

1. Introduction

Colorectal cancer is the third and second most common cancer in males and females, respectively. This progressive disease arises from genetic/epigenetic changes in adenoma considered as premalignant lesion. Most deaths by colon cancer are due to their systemic dissemination and metastasis to lung and liver rather than from the primary tumor which may be eradicated by surgery and standard chemotherapy including 5-FU and oxaliplatin [1]. Cell adherence, migration and motility are key characteristics of metastatic cancer and involve families of proteins such as integrins.

Integrins are transmembrane αβ heterodimeric proteins regulating fundamental processes often dysregulated in tumoral processes. They link cells to the surrounding extracellular matrix (ECM) and modulate numerous signaling pathways implicated in proliferation/survival, migration, invasion and differentiation [2]. Integrins are also involved in tumor resistance to therapies [2]. In colon tumor cells, altered expression of ECM-binding integrins during tumor progression has been observed since several years [3,4]. In particular, α5β1 integrin, the fibronectin receptor, determines malignant properties of colon carcinoma cells. Highly invasive colon cell lines (such as HCT116 cells) were shown to express higher levels of α5β1 integrin mRNA and protein compared to poorly invasive cells [5]. Recently, it was shown that transcription of α5β1 integrin increased during colon tumor progression either in association with a loss of ADAM15 [6] or under hypoxic conditions [7]. Transcriptional upregulation of α5β1 integrin can also be triggered by PTHrP [8] or ZEB2 through cooperation with SP1 [9]. Increase in α5β1 integrin expression enhances cell adhesion to fibronectin expressed by stromal elements which in turn participates to cell dissemination and metastasis.

Blockade of cell surface α5 integrin by specific antibodies lead to a decrease in α5 expression and colon cancer cell adhesion, to PI3K/AKT pathway downregulation and to apoptosis induction [10]. Inhibition of α5β1 integrin function with a small peptide (ATN161) sensitized colon tumors to 5-FU while reducing liver metastases and improving mice survival [11]. Lunasin, a naturally occurring peptide isolated from soybean, inhibits human colon cancer liver metastasis by direct binding to α5β1 integrin and impairment of integrin signaling. Lunasin also potentiates the effect of oxaliplatin in preventing the outgrowth of metastasis [12]. Taken together, these findings suggest that α5β1 integrin can be considered as a therapeutic target in colon cancer. Therefore blocking α5β1 integrin expression and functions may be an

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http://dx.doi.org/10.1016/j.canlet.2013.03.018

interesting adjuvant treatment to enhance chemotherapeutic agent effects.

The TP53 tumor suppressor gene encodes the transcription factor p53, which is activated by several cellular stresses including DNA damage. Activated p53 participates in a program that includes cell cycle arrest, DNA repair, apoptosis, senescence, or autophagy [13]. Loss of p53 function is a critical event in tumorigenesis and resistance to therapies. Inactivating TP53 mutations are found in about 29% of colorectal cancers but were not associated with poor survival in colon cancer [14] to the exception of patients with Dukes' stage D tumors [15]. Functional wild type p53 is often inactivated by altered upstream pathways [16]. The pharmacological reactivation of p53 appeared these last years as an effective therapeutic strategy in different tumors [17,18]. Mdm2 is the main regulator of p53 by binding and targeting p53 to ubiquitin-dependent proteolysis. Modulating p53 by mdm2 inhibitors, such as Nutlin-3a, has been successfully used to promote growth arrest in mouse colon tumors and human colon cancer cells [19]. Nutlin-3a enhances TRAIL-induced apoptosis through upregulation of death receptor DR5 in human colon cancer cell HCT116 [20].

We have previously reported that Nutlin-3a decreases α5β1 integrin expression in glioma cell lines through p53 activation [21]. In this work, we extend these findings to a colon cancer cell line and we show that (1) α5β1 integrin is a survival factor independently of p53 status and (2) that reactivation of a functional p53 by Nutlin-3a modulates selectively the expression of the α5 subunit. These results may have therapeutic implications in the management of colon cancer.

2. Materials and methods

2.1. Reagents

Nutlin-3a (4-[4,5-bis-(4-chlorophenyl)-2-(2-isopropoxy-4-methoxy-phenyl)-4,5-dihydro-imidazole-1-carbonyl]-piperazin-2-one) and RITA (5,5′-(2,5-furandiyl)bis-2-thiophenemethanol) were from Cayman chemical company (Interchim, France). The compound K34c (2-(S)-2,6 dimethylbenzamido)-3-[4-(3-pyridin-2-ylaminoprooxy)-phenyl]propionic acid) was synthesized in our laboratory according to the procedure described by Heckmann et al. [22]. Purified human fibronectin was a kind gift from Dr. F. Carreiras (Cergy-Pontoise, France).

Nutlin-3a and RITA were prepared as 10 mM stock solution in ethanol and were kept at −20 °C until use. K34c was prepared as 10 mM stock solution in DMSO and kept at 4 °C.

2.2. Cell lines and culture conditions

HCT116 p53+/+ (p53 wild type) and HCT116 p53−/− (p53 null) cells were a kind gift from Pr. Vogelstein (Baltimore, USA). Cell lines were routinely grown in Eagle's MEM supplemented with 10% heat-inactivated FBS, 0.6 mg/mL, glutamine and 200 IU/mL penicillin/streptomycin. Cell cultures were maintained in a water saturated atmosphere at 37 °C under 5% CO_2/95% air. All experiments were performed in 2% FBS containing EMEM medium unless specified.

2.3. Cell transfections

Specific siRNA for human α5 and β1 integrin subunits, p53 and non targeting siRNA were obtained from Dharmacon (Thermo Scientific) and the transfection reagent jetPRIME™ was purchased from Polyplus Transfection. Manufacturer's instructions were followed to transiently inhibit the expression of the different proteins in HCT116 p53+/+ and HCT116 p53−/− cells. The transfection efficacy was verified by immunoblotting. HCT116 p53−/− cells were similarly transiently transfected with empty pcDNA3.1 plasmid or p53wt gene-containing pcDNA3.1. The pcDNA3.1–p53 vector was a kind gift from Dr. C. Blattner (Karlsruhe, Germany).

2.4. Clonogenic assay

Cells were plated (500 cells/well) into six-well culture plates either uncoated or coated with human fibronectin (10 μg/ml) and treated for 72 h with specific drugs or solvents in 2% FBS containing medium. Medium was thereafter renewed with fresh 10% FBS containing medium and cells allowed to grow further for 7 days. Colonies were fixed and stained with crystal violet/ethanol (0.1%, w/v) and counted. The surviving fraction was determined by the ratio of cells surviving to a specific drug treatment relative to their solvent-treated counterparts.

2.5. Western blotting

Cells were lysed with Laemmli sample buffer (Biorad) on ice and lysates were heated at 90 °C for 10 min. Samples were loaded and run on precast 10% SDS PAGE gels (Biorad) and transferred to PVDF membranes (GE Healthcare). After blocking for 1 h at room temperature, the blots were incubated overnight at 4 °C with specific primary antibodies. Primary antibodies used were anti-α5 integrin H104 1/1000 (Santa Cruz), anti-β1 integrin Ab1952 1/1000 (Millipore), anti-p53 1/1000 (BD Biosciences) and anti-GAPDH 1/50,000 (Millipore). Membranes were subsequently incubated with a secondary antibody conjugated to horseradish peroxidase 1/10,000 (Promega) and developed using a chemiluminescent (ECL) detection system followed by exposure to CL-Xposure films (Kodak). Quantification was done using ImageJ software. GAPDH was used as housekeeping protein to serve as the loading control for cell lysate samples.

2.6. Real-time quantitative PCR

RNA was extracted with RNeasy minikit from Qiagen according to manufacturer's instructions and was transcribed into cDNA using high capacity cDNA kit (Applied Biosystems). Real-time quantitative PCR were performed using the ABI7000 SYBRGreen PCR detector with the following probes (Invitrogen):

cyclophilin forward 5′-CAGGTCCTGGCATCTTGTCC-3′
and reverse 5′-TTGCTGGTCTTGCCATTCCT-3′
p21 forward 5′GGCAGACCAGCATGACAGATT3′ and reverse
5′TGTGGGCGGATTAGGGCT3′;
mdm2 forward 5′AGACCCTGGTTAGACCAA3′ and reverse
5′TGGCCAAGATAAAAAAGAACCTCT3′;
fas forward 5′CCCTCCTACCTCTGGTTCTTACG3′ and reverse
5′AGTCTTCCTCAATTCCAATCCCTT3′;
α5 forward 5′TGCTGACTCCATTGGTTTCACAG3′ and reverse
5′TCTCTCTGCAATCCTCTCGAGC3′;
αv forward 5′AGGTGCCTACGAAGCTGAGC3′ and reverse
5′AAGGCTTCATTGTTTCGGACA3′
β1 forward 5′TGTAACCAACCGTAGCAAAGGA3′ and reverse
5′CCCTGATCTTAATCGCAAAACC3′.

Relative levels of mRNA gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Immunofluorescence

Cells (20,000/dish) were seeded onto IBIDI μ-dishes coated with 10 μg/ml of poly-L-lysine and treated with nutlin-3a or solvent for 24 h. Cells were fixed with 4% paraformaldehyde for 10 min and incubated with a blocking solution (5% BSA in PBS) for 1 h at room temperature. The cells were thereafter incubated with anti-α5 IIAI antibody 1/300 (BD Biosciences) for 1 h at room temperature, rinsed twice with PBS and exposed to Alexa Fluor-488 labeled goat anti-mouse secondary antibody 1/300 (Jackson ImmunoResearch Laboratories) for 1 h at room temperature followed by two washes with PBS. Cells were visualized with a fluorescent microscope (Biorad 1024) equipped with a water immersion 60× objective. Images were acquired using the Laser-Sharp 2000 software.

2.8. Flow cytometry

Cells were detached with PBS/EDTA (0.53 mM) and centrifuged for 5 min at 2000 rpm. Cells (200,000) were washed (5% BSA, 0.1% NaN3, TBS) and centrifuged for 5 min at 2000 rpm. The cell pellet was then incubated with a specific primary antibody at 4 °C for 30 min. Primary antibodies used were 10 μg/ml anti-α5 IIAI (BD Biosciences), and 10 μg/ml anti-β1 TS2/16 (Santa Cruz), mAB13 (BD Biosciences) and 9EG7 (BD Biosciences) respectively. The cells were rinsed three times in washing buffer and exposed to either Alexa Fluor 488 labeled goat anti-mouse 1/200 or FITC labeled goat anti-rat 1/50 secondary antibody (Jackson ImmunoResearch Laboratories) at 4 °C for 30 min. After washing, cells were resuspended in 100 μl of washing buffer and 200 μl of TBS. A total of 20,000 cells were analyzed using a FACS Calibur flow cytometer (Becton–Dickinson, San Diego, CA). The mean fluorescence intensity characterizing surface expression of integrins was measured using the Cell Quest software.

2.9. Statistical analysis

Data are represented as mean ± SE. The values were obtained in at least three independent experiments (n). Statistical analyses were done by the Student's t test with the GraphPad Prism program where $p < 0.05$ was considered significant.

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3. Results

3.1. Integrin α5β1 is involved in colon cancer cell survival

We used HCT116 colon cancer cells expressing p53 wild type protein or their isogenic counterparts with no p53 expression (respectively named HCT116 p53+/+ and p53−/− cells). These two cell lines express similar level of α5 integrin (Fig. 1A). To determine the implication of integrin α5β1 in colon cancer cell survival, expression of the α5 subunit was decreased in HCT116 cells by using specific siRNA. 72 h after siRNA transfection, α5 protein expression was decreased by about 81% with the α5-targeting siRNA compared to the non-silencing siRNA (Fig. 1A). Long term survival assays showed that loss of α5 expression led to a decrease in the number of colonies formed either by HCT116 p53+/+ or HCT116 p53−/− cells (Fig. 1A). Similar results were obtained when β1 integrin subunit expression was decreased with specific siRNA (Fig. 1B). In order to confirm the impact of α5β1 integrin on HCT116 cell survival, we treated the cells with a specific non-peptidic small antagonist of α5β1 integrin, K34c [23]. K34c dose-dependently inhibited clonogenic survival at similar levels in HCT116 p53+/+ and p53−/− cells (Fig. 1C). Data show that clonogenic potential of HCT116 cells was altered either when integrin functions were blocked by K34c or when integrin expression was

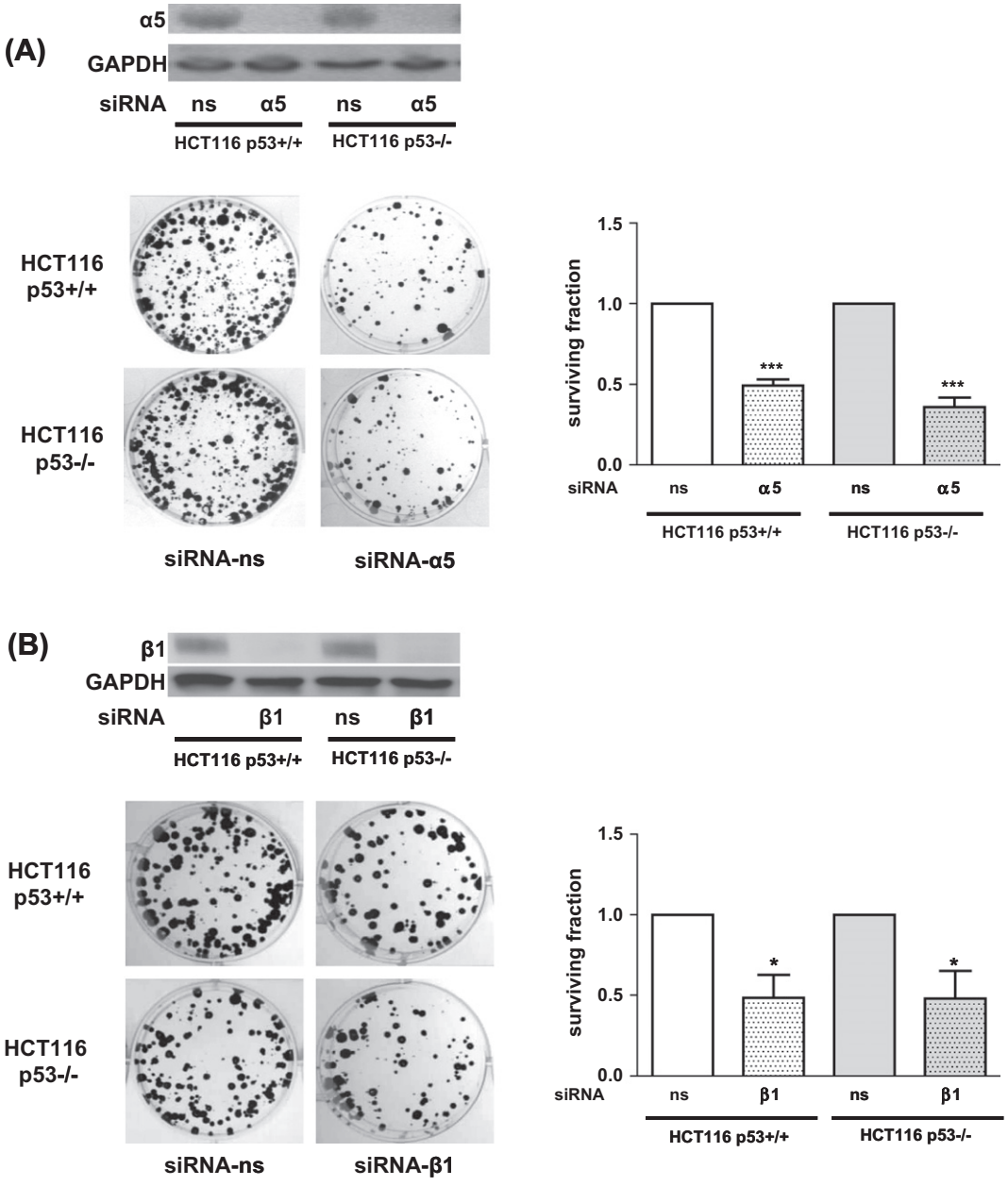

Fig. 1. α5β1 integrin promotes the cell survival of HCT116 colon carcinoma cells. (A) Upper panel: HCT116 p53+/+ and HCT116 p53−/− cells were transfected with non-silencing siRNA (siRNA-ns) or specific siRNA targeting α5 integrin (siRNA-α5). The repression of the α5 subunit was verified by Western blot. GAPDH was used as the loading control. Lower panel: clonogenic survival assays were performed with transfected cells. Histograms display the mean ± SEM of three independent experiments. (B) Upper panel: HCT116 p53+/+ and HCT116 p53−/− cells were transfected with non-silencing siRNAs (siRNA-ns) or specific siRNA targeting β1 integrin (siRNA-β1). The repression of the β1 subunit was verified by Western blot. GAPDH was used as the loading control. Lower panel: clonogenic survival assays were performed with transfected cells. Histograms display the mean ± SEM of three independent experiments. (C) The clonogenic survival of HCT116 p53+/+ and HCT116 p53−/− cells treated with increasing concentrations of an antagonist of α5β1 integrin, K34c . Histograms represent the mean ± SEM of treated versus non-treated cells ($n = 4$). $^{*}p < 0.05$ and $^{***}p < 0.005$.

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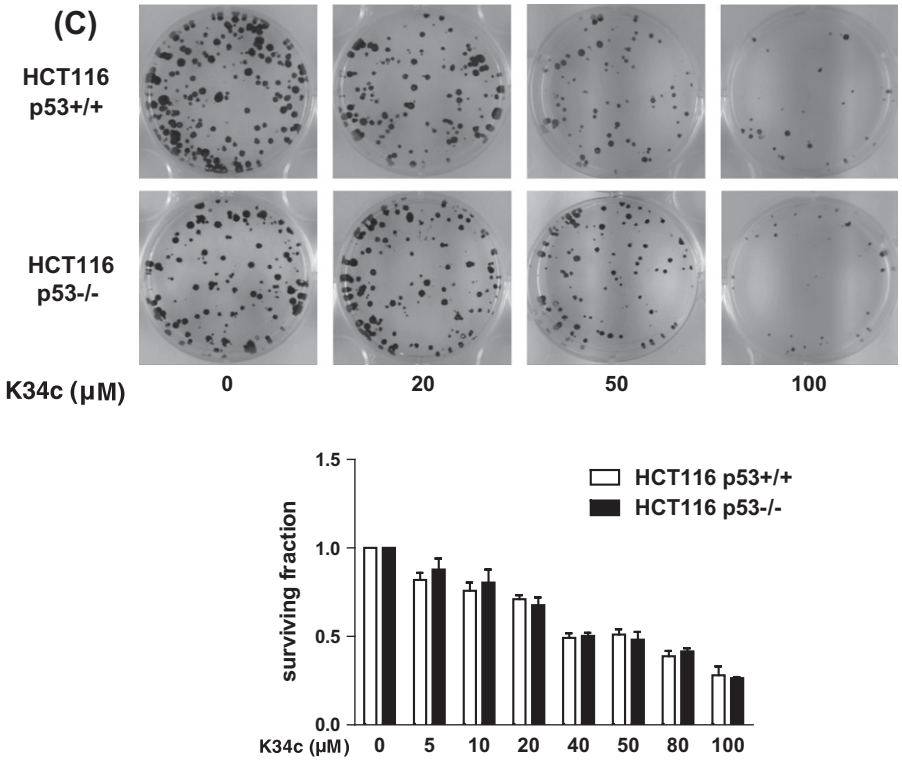

Fig. 1. (*continued*)

inhibited by siRNA interference. It appears that inhibition of cell survival by depletion of α5 and β1 integrin subunits or by functional inhibition of the integrin by K34c is independent of p53 expression suggesting some p53 independent survival pathways triggered by the integrin. These results confirm that α5β1 integrin is a therapeutic target in colon cancer cells and suggest that treatments capable of affecting the integrin expression/function may prove to be a valuable strategy for colon carcinoma eradication.

3.2. Nutlin-3a, a non-genotoxic drug, represses α5β1 integrin expression in a p53-dependent way

Nutlin-3a is known to inhibit the mdm2–p53 regulatory loop by binding to mdm2 thus impairing mdm2-dependent p53 degradation and leading to direct p53 activation. We have recently shown that activation of p53 by Nutlin-3a decreases α5 integrin expression in glioma cells [21]. To investigate if activation of p53 modulates the expression of α5β1 integrin in colon tumor cells, we treated the HCT116 cells with Nutlin-3a. Nutlin-3a activated p53 as shown by an increased level of p53 target genes (Fig. 2A) and stabilized p53 protein (Fig. 2B) in HCT116 p53+/+ cells but not, as expected, in HCT116 p53−/− cells. Treatment of HCT116 p53+/+ cells by Nutlin-3a decreased the expression level of α5 integrin subunit in a dose dependent manner, but not in p53 negative counterparts as shown by Western blot analysis of total protein extracts (Fig. 2B). Importantly, Nutlin-3a had no impact on α5 in p53 null cells. This effect was specific to α5 integrin since treatment with Nutlin-3a resulted in no significant effect on the β1 subunit protein expression (Fig. 2C).

Immunofluorescence analysis of Nutlin-treated cells with specific anti-α5 integrin antibodies confirmed the previous Western blot data. A clear decrease of the specific labeling was observed after Nutlin-3a treatment in HCT116 p53+/+ but not in HCT116 p53−/− (Fig. 2D). To determine if functional integrin level may be affected, we analyzed the expression of α5β1 integrin at the cell surface by FACS analysis. As reported in Fig. 2E, cell surface expression of α5 subunit was decreased after 10 μM Nutlin-3a treatment at about 55% of the control non-treated cell (MFI value) in HCT116 p53+/+ but not in HCT116 p53−/− cells. No effect on β1 subunit cell surface expression (recorded by TS2/16 antibodies) was observed (Fig. 2E) confirming the Western blot results. We also confirmed using different integrin conformation specific antibodies (mAb13, inactive β1 and 9EG7, active β1) by FACS analysis that active or inactive conformations of β1 subunit were insensitive to Nutlin-3A treatment in both HCT116 cell lines (Table 1).

We next investigated if Nutlin-3a affects α5 subunit expression at the transcriptional level. Integrin α5 mRNA was decreased in HCT116 p53+/+ cells after Nutlin-3a treatment (Fig. 2F). This effect was specific for the α5 integrin subunit since neither β1 nor αv integrin subunit mRNAs were affected by Nutlin-3a in HCT116 p53+/+ cells (Fig. 2F). Interestingly, α5 and β1 mRNA levels were significantly increased in HCT116 p53−/− cells compared to HCT116 p53+/+ cells suggesting that p53 may exert a negative control at the transcriptional level on the two genes which does not translate to an increase in the protein levels (Fig. 2 B and C). Curiously, both α5 and β1 mRNA levels were significantly further increased after Nutlin-3a treatment in HCT116 p53−/− cells suggesting a p53-independent effect of Nutlin-3a in these cells (Fig. 2F). Taken together, these results show that a non-genotoxic p53 activator induces the down regulation at the mRNA and protein level of endogenous pro-survival α5 integrin in a p53 dependent manner in colon cancer cells.

3.3. An inverse relationship exists between p53 and α5 integrin

We transfected HCT116 p53−/− cells with a plasmid containing the p53 gene to provide clearer evidences that p53 and α5 integrin

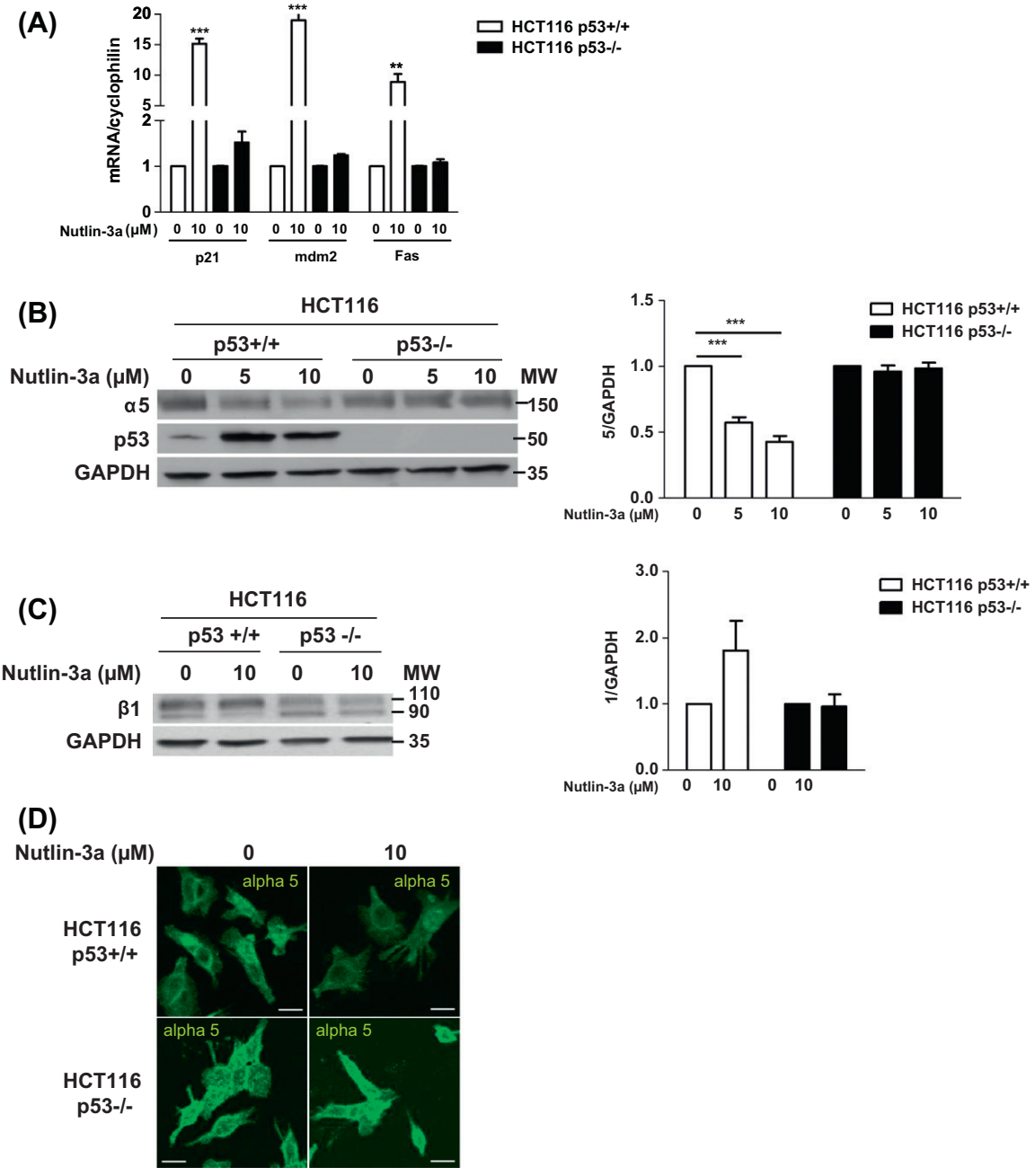

Fig. 2. Nutlin-3a activates the expression of p53 target genes and affects the α5 integrin expression in HCT116 only if p53 is functional. (A) qPCR analysis of p53 target genes p21, mdm2 and Fas in HCT116 p53+/+ and HCT116 p53−/− cells after 10 μM of Nutlin-3a treatment for 24 h. (B) Western blot analysis of α5 and p53 protein expression in HCT116 p53+/+ and HCT116 p53−/− cells after 5 μM and 10 μM Nutlin-3a treatment for 24 h. Histograms show the fold increase in the protein expression normalized to GAPDH levels (mean ± SEM of 3–4 independent experiments). (C) Western blot analysis of β1 protein expression in HCT116 p53+/+ and HCT116 p53−/− cells treated with 10 μM Nutlin-3a for 24 h. Histograms show the fold increase in the protein expression normalized to GAPDH levels (mean ± SEM of three independent experiments). The anti-β1 antibody (AB1952, Chemicon) detects two bands corresponding to the premature (90 kDa) and mature (110 kDa) proteins. Only the 110 kDa band was considered in the histograms. (D) Representative fluorescence confocal microscopy images of the α5 subunit expression in the HCT116 p53+/+ and HCT116 p53−/− cells after 10 μM of Nutlin-3a treatment for 24 h. Scale bars: 20 μm. (E) Flow cytometry analysis of the α5 (IIA1 antibody) and β1 (TS2/16 antibody) subunit expression at the cell membrane in HCT116 p53+/+ and HCT116 p53−/− cells after 10 μM Nutlin-3a for 24 h. A representative experiment is shown in the figure and mean values are shown in Table 1. (F) qPCR analysis of the α5, β1 and αv subunits mRNA in HCT116 p53+/+ and HCT116 p53−/− cells after 10 μM of Nutlin-3a treatment for 24 h. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$.

expressions were linked. Expression of p53 activated the p53 target genes (Table 2) and clearly decreased the α5 integrin protein level (Fig. 3A). Inversely, repression of α5 integrin expression by specific siRNA, increased p53 protein level (Fig. 3B, upper panel) and activity (Fig. 3B, lower panel) in HCT116 p53+/+ cells. However, when p53 expression was decreased by specific siRNA in HCT116 p53+/+ cells, no effect on α5 integrin expression was observed (Fig. 3C). These data, summarized in Table 2, suggested that expression and activation of p53 either by transfection or by Nutlin actually affects α5 integrin expression but also that knocking down p53 expression had no effect on α5 integrin in our experimental conditions.

3.4. Nutlin-3a dose-dependently inhibits the clonogenic survival of HCT116 cells in relation with a decrease in α5 integrin expression

We next investigated if the decrease in α5 integrin expression observed after p53 activation by Nutlin-3a may impact cell

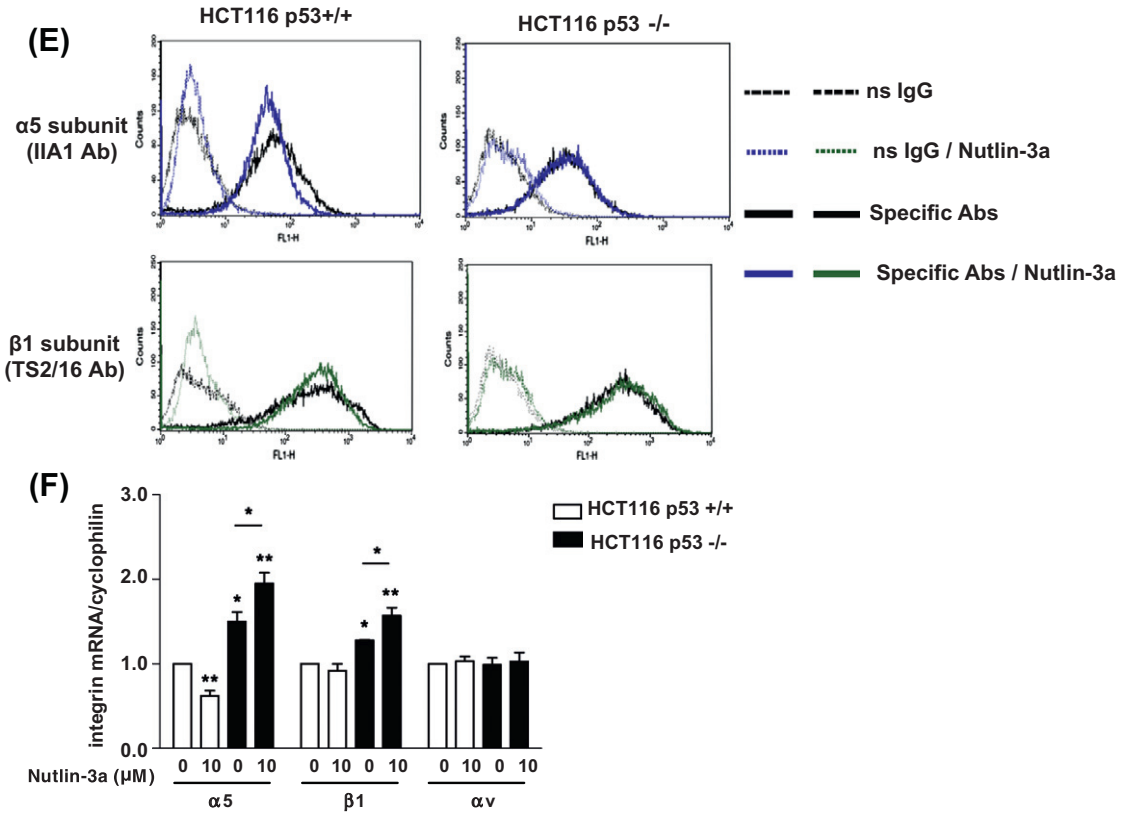

Fig. 2. (*continued*)

Table 1
The mean of fluorescence intensities of the α5 and β1 integrin subunit expression at the cell membrane of HCT116 cells. Cells were treated with solvent or Nutlin-3a (10 μM, 24 h), and processed for flow cytometry analysis with specific antibody labeling. Data report the mean ± SEM of three independent experiments. * indicates a statistically significant difference between the cells treated with Nutlin-3a and the cells treated with the solvent.

Abs cells	Ig control (mouse)	Ig control (rat)	α5 (IIA1)	β1 (9EG7)	β1 (TS2/16)	β1 (mAb13)
HCT116 p53+/+	4.4 ± 0.6	4.2 ± 0.3	68 ± 9	47 ± 9	614 ± 206	246 ± 47
+Nutlin-3a	4.2 ± 0.5	4.1 ± 0.1	38 ± 7* ($p = 0.05$)	51 ± 7	631 ± 206	252 ± 53
HCT116 p53−/−	4.4 ± 0.2	4.4 ± 0.3	60 ± 9	32 ± 2	561 ± 129	250 ± 36
+Nutlin-3a	4.8 ± 0.3	4.7 ± 0.2	64 ± 11	38 ± 5	625 ± 145	272 ± 32

Table 2
Summary of p53 activation in the different experimental settings and effects on α5 integrin mRNA and protein levels. The activity of p53 is reported as the increase in p53-target gene mRNA level (determined by qPCR). Effects on α5 integrin expression were recorded in each condition at the transcriptional level (by qPCR) and at the protein level (by Westren blot). Data are shown as mean SEM of three independent experiments.

	p53 targets				
p53	p21 (mRNA)	Mdm2 (mRNA)	Fas (mRNA)	α5 (mRNA)	α5 (protein)
Increase					
p53−/− with p53	2.5 ± 0.3	2.1 ± 0.2	2.2 ± 0.5	0.58 ± 0.03	0.51 ± 0.08
p53+/+ with Nutlin-3a (10 μM)	15 ± 0.8	19 ± 1.4	8.9 ± 1.2	0.62 ± 0.06	0.42 ± 0.04
p53+/+ with RITA (0.5 μM)	2.7 ± 0.3	1.9 ± 0.4	2.8 ± 0.3	1.07 ± 0.06	0.48 ± 0.09
Decrease					
p53+/+ with sip53	0.36 ± 0.04	0.69 ± 0.01	0.62 ± 0.08	1.09 ± 0.04	0.97 ± 0.19
p53−/− vs p53+/+	0.31 ± 0.002	0.92 ± 0.04	0.47 ± 0.02	1.5 ± 0.11	1.2 ± 0.02

survival. Cells were treated with increasing concentrations of Nutlin-3a (0–10 μM) and clonogenic survival as well as α5 integrin expression were analyzed (Fig. 4). In the long term growth assay, HCT116 p53+/+ cells were more sensitive to Nutlin-3a (at all concentrations used) than HCT116 p53−/− cells, as expected (Fig. 4A). For example, at the highest dose of 10 μM, HCT116 p53+/+ cell survival was decreased to 1% of the control cells whereas HCT116 p53−/− cells showed a 56% reduction of cell survival (Fig. 4A). Concomitantly, α5 integrin expression at the protein level was dose dependently inhibited by Nutlin-3a in HCT116 p53+/+ and not in HCT116 p53−/− (Fig. 4B) suggesting a correlation between survival potential and α5 expression. This is in agreement with data from Fig. 1 showing that repression of α5 by siRNA decreased HCT116 cell survival. In addition, Nutlin-3a-dependent survival

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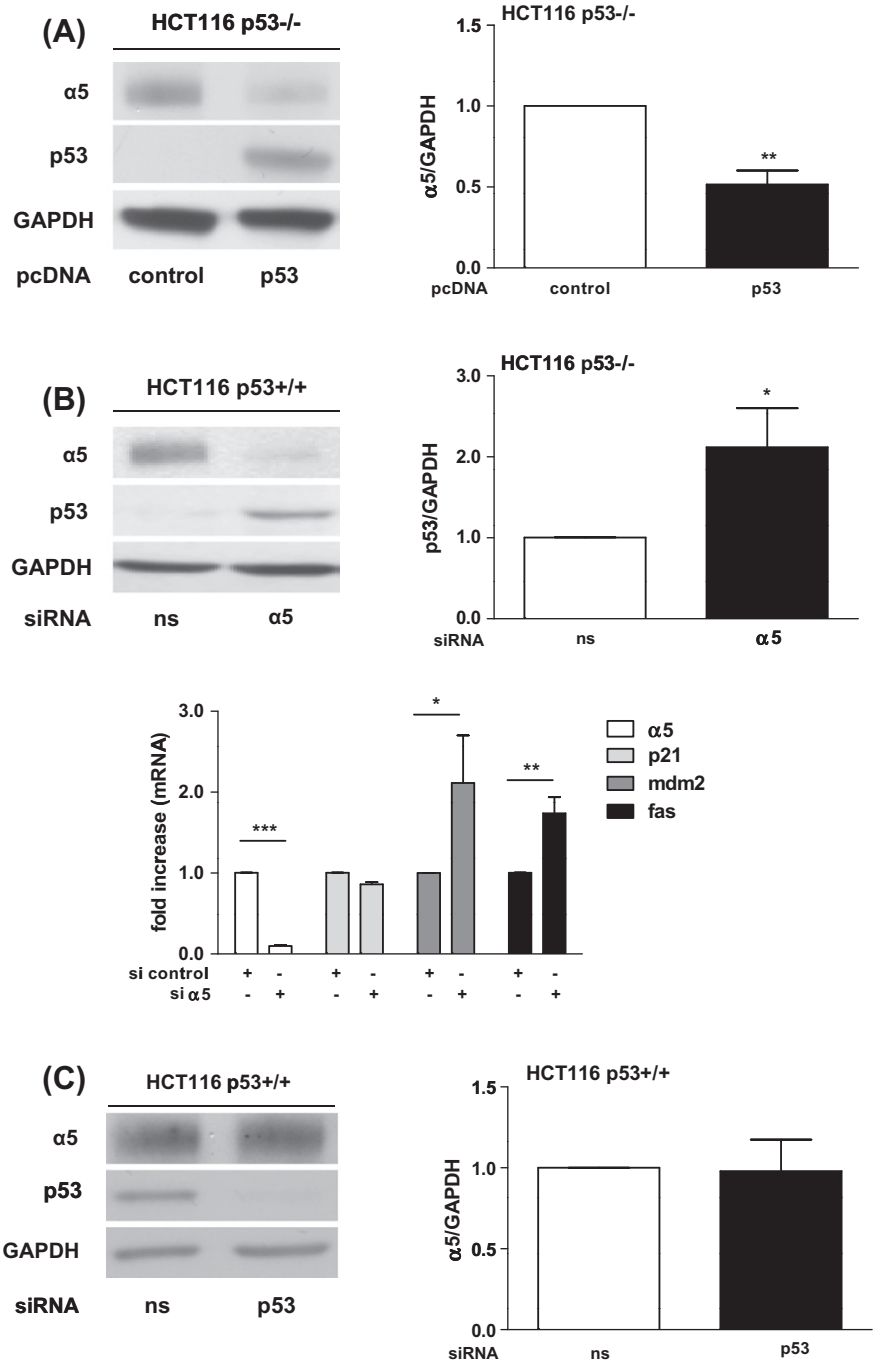

Fig. 3. p53 and α5 integrin expressions are inversely related. (A) Western blot analysis of α5 integrin and p53 proteins in HCT116 p53−/− cells transfected with empty control- (pcDNA-control) or p53 gene-containing (pcDNA–p53) vectors. GAPDH was used as the loading control. Histograms represent the fold increase in α5 expression in pcDNA–p53 transfected cells compared to pcDNA-control cells ($n = 3$). (B) (top) Western blot analysis of α5 and p53 proteins in HCT116 p53+/+ cells transfected with non-silencing siRNA (siRNA-ns) or specific siRNA targeting α5 integrin (siRNA-α5). GAPDH was used as the loading control. Histograms represent the fold increase in p53 expression in siRNA-α5 transfected cells compared to siRNA-ns cells. (bottom) qPCR analysis of α5, p21, mdm2, fas gene mRNA in HCT116 p53+/+ transfected with non-silencing siRNA (siRNA-ns) or specific siRNA targeting α5 integrin (siRNA-α5) ($n = 3$). (C) Western blot analysis of α5 integrin and p53 proteins in HCT116 p53+/+ cells transfected with non-silencing siRNA (siRNA-ns) or specific siRNA targeting p53 (siRNA–p53). Histograms represent the fold increase in α5 expression in siRNA–p53 transfected cells compared to siRNA-ns cells ($n = 3$).

inhibition in HCT116 p53+/+ cells was decreased when α5β1 integrins were activated by their specific ligand fibronectin (Fig. 4C). Together, these data support that α5 inhibition may be involved in the Nutlin-3a-dependent p53-mediated biological response in colon cancer cells.

3.5. Activation of p53 by RITA inhibits clonogenic survival but does not inhibit α5 expression at low concentrations

As shown previously in glioma cells [21], activation of p53 by Nutlin-3a appeared as the key event for the modulation of the α5

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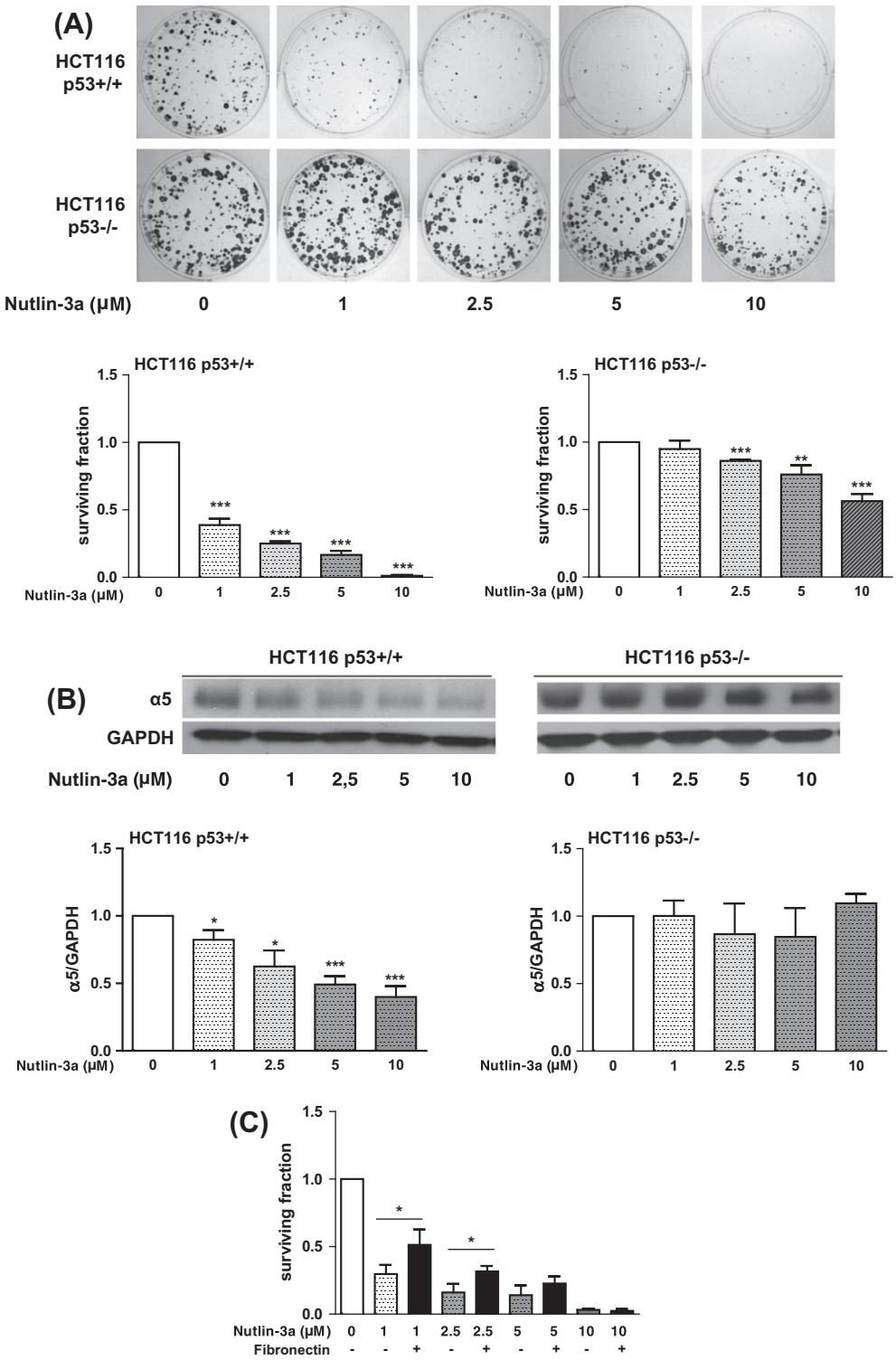

Fig. 4. Nutlin-3a decreases the α5 subunit and reduces survival of HCT116 p53+/+ cells. (A) Clonogenic survival of HCT116 p53+/+ and HCT116 p53−/− cells treated with 1, 2.5, 5 and 10 μM Nutlin-3a. Histograms represent the mean ± SEM of four independent experiments. (B) Western blot analysis of the α5 protein in HCT 116 p53+/+ (left) and HCT116 p53−/− (right) cells treated with 1, 2.5, 5 and 10 μM Nutlin-3a for 24 h. Histograms show the fold decrease in the α5 protein expression normalized to GAPDH levels (mean ± SEM of four independent experiments). (C) Clonogenic survival of HCT116 p53+/+ cells treated by increasing concentrations of Nutlin-3a plated either on fibronectin-coated wells or on non-coated wells. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$.

integrin expression. Other p53 reactivating drugs have been described. For example, RITA, another non-genotoxic activator, binds to p53, impairs the mdm2–p53 interaction leading to p53 stabilization and activation [24]. It was already shown that p53 rescue either by Nutlin-3a or by RITA differentially affects the transcriptional program of p53 in HCT116 cells. Nutlin-3a induced a greater fraction of p53 target genes involved in cell cycle arrest whereas induction of pro-apoptotic p53 target genes is a hallmark of RITA [25]. In order to compare Nutlin-3a and RITA on α5 integrin expression level, we treated HCT116 cells with RITA. RITA, dose

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dependently activated the p53 pathway in HCT116 p53+/+ cells as shown by the increase in p53 target genes but without affecting the mRNA level of α5 integrin (Fig. 5A). In addition, no effect on α5 integrin expression was observed in either cell lines unless when the highest concentration of RITA (0.5 μM) was used in HCT116 p53+/+ cells (Fig. 5B). Data confirm that p53 activation is required to affect α5 integrin expression. However, RITA strongly inhibited the formation of HCT116 p53+/+ cell colonies at all concentrations tested but was less efficient in HCT116 p53−/− cells (Fig. 5C).

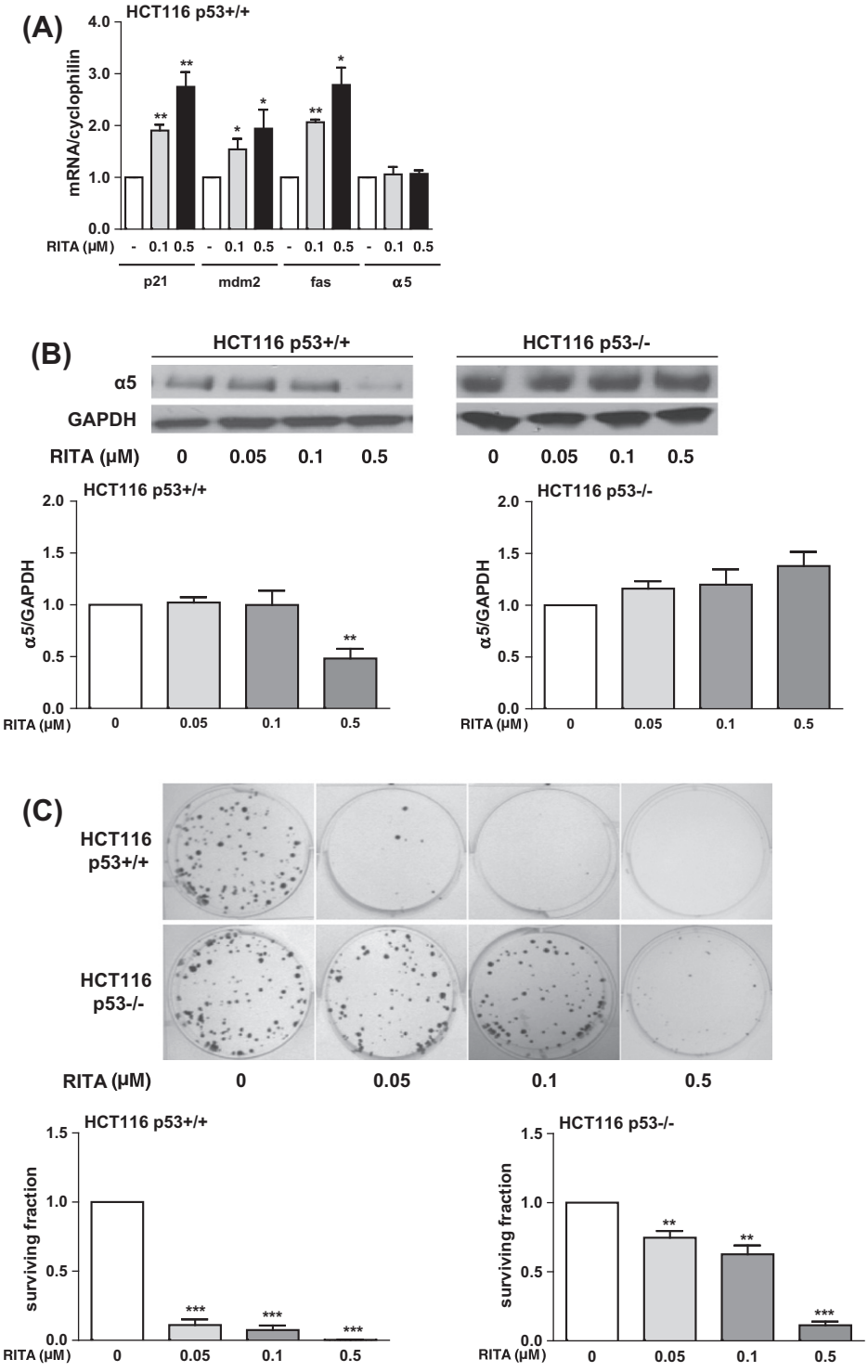

Fig. 5. RITA activates p53 and inhibits clonogenic survival regardless of the α5 expression. (A) qPCR analysis of p53 target genes (p21, mdm2 and Fas) and α5 integrin in HCT116 p53+/+ cells after RITA treatment (0.1 or 0.5 μM) for 24 h. Data are shown as mean ± SEM of three experiments. (B) α5 protein expression in HCT116 p53+/+ (left) and HCT116 p53−/− (right) cells after 0.05, 0.1 and 0.5 μM RITA treatment for 24 h ($n = 4$). Histograms represent α5/GAPDH fold increase in RITA treated HCT116 p53+/+ (left) and HCT116 p53−/− (right) cells. (C) Clonogenic assay of HCT116 p53+/+ and HCT116 p53−/− cells treated with 0.05, 0.1 and 0.5 μM RITA ($n = 4$). Histograms represent the mean ± SEM of HCT116 p53+/+ (left) and HCT116 p53−/− cell (right) surviving fraction of cells treated with RITA versus cells treated with solvent ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$.

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We compared the respective effect of Nutlin-3a and RITA on p53 expression in HCT116 p53+/+ cells. Nutlin-3a was clearly more efficient than RITA to increase the p53 expression at all concentrations tested (Fig. 6A) as well as to activate p53 target genes (Fig. 5A in comparison with Fig. 2A) in HCT116 p53+/+ cells suggesting that an activation of p53 above a threshold is needed to affect α5 integrin expression. To confirm this hypothesis, we evaluated the relationship between p53 (Fig. 6A) and α5 integrin (Fig. 4B for Nutlin-3a and Fig. 5B for RITA) expressions. A significant inverse correlation was obtained as shown in Fig. 6B. RITA thus behaves differently than Nutlin-3a as RITA-dependent clonogenic inhibition seems unrelated to α5 integrin repression at the mRNA and protein levels.

4. Discussion

Integrins have a role in regulating proliferation, differentiation, migration, invasion, metastasis and neo-angiogenesis in tumor [2]. Due to their participation in different aspects of tumor progression, integrins appear as pertinent targets in new therapeutic schedules [26,27]. Among integrins, the fibronectin receptor, integrin α5β1, is moving in the forefront of research for anticancer drugs. Its implication in tumorigenesis and resistance to treatment is increasingly recognized in various solid tumors including lung [28], ovarian [29], high grade glioma [21] and, as confirmed in this work, in colon cancer. The α5 integrin subunit only dimerizes with the β1 subunit to form the α5β1 fibronectin receptor. Modulation of the α5 subunit expression thus directly affects the heterodimer function.

4.1. Integrin α5β1 impacts on HCT116 cell survival

Our results show for the first time that repression of the α5 subunit expression in the HCT116 cell line impacted cell survival. Functional inhibition of integrin α5β1 by a small non-peptidic antagonist, K34c, confirmed these data. Our results are in line with those described by Murillo et al. [10] in which blockade of α5 integrin by specific antibodies decreased PI3Kinase activation and increased cell apoptosis in three colon cancer cell lines. Preferential adhesion of colon carcinoma cells to lung cells was shown to be blocked either by anti-fibronectin or by anti-α5 subunit specific antibodies [3]. We propose that the α5β1 integrin may be a pertinent therapeutic target for colon tumors and that specific integrin antagonist may control both survival and ability to form metastases of colon cancer cells. We found that direct inhibition of α5 expression or function decreased cell survival independently of p53 status. These data are in line with the fact that α5 integrin protects cells from apoptosis by activating the PI3K/AKT pathway even in colon cells with a non-functional p53 [30–32]. As p53-independent pathways are also implicated in the survival effect of the integrin, specific antagonists may be useful in p53wt colon tumors as well as in those expressing non-functional p53.

4.2. Selective repression of α5 integrin subunit by Nutlin-3a-induced p53 activation

Targeted therapies have got disappointing results in the human clinic despite encouraging pre-clinical investigations.

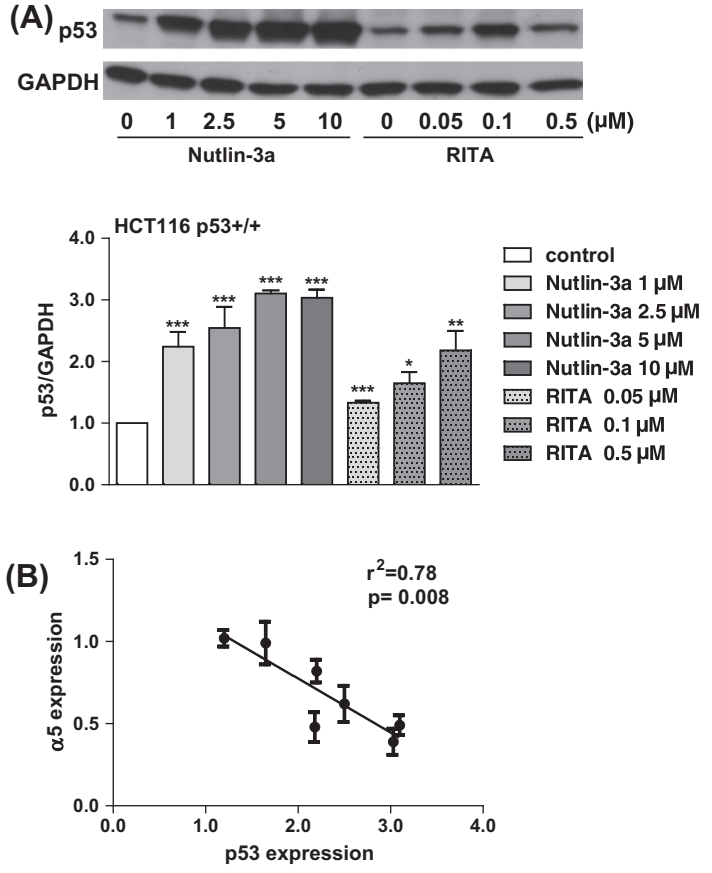

Fig. 6. Comparison of p53 activation by Nutlin-3a and RITA in HCT116 p53+/+ cells. (A) Representative Western blot of p53 stabilization in HCT116 p53+/+ cells after Nutlin-3a and RITA treatment (increasing concentrations – 24 h). GAPDH was used as the loading control. Histograms represent the mean ± SEM of three independent experiments. (B) Values of p53 expression (A) were plotted versus values of α5 protein expression (from Figs. 4B and 5B), both determined by Western blot analysis. Plot shows the reverse correlation obtained between the two proteins of interest. a.u.: arbitrary units (densitometric data normalized to GAPDH levels). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$.

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Therapy-induced elimination of key drivers of tumor aggressiveness may be another way to eradicate tumors. Our results suggest that agents able to selectively target α5 integrin subunit expression may provide novel therapeutic options for selected colon cancers. We showed recently that reactivation of p53 by non-genotoxic drugs such as Nutlin-3a affected α5β1 integrin expression in glioma cells [21]. We unravelled an original negative crosstalk between the integrin and p53 as we demonstrated that high level of α5 subunit impaired the p53 activation [21] and conversely that integrin antagonists restored glioma cell sensitivity to chemotherapeutic drugs [23]. We extended these findings in colon cancer cells and took advantage of the availability of HCT116 cell lines expressing or not a wild type p53. Within these cells, effects directly related to p53 could be investigated in an isogenic background. As described here, Nutlin-3a treatment decreased selectively, only in HCT116 p53+/+ cells, the α5 subunit expression at the mRNA and protein levels without affecting the β1 subunit. This selective effect is reminiscent of data showing that PI3K inhibitors affected α5 and not β1 expression in human colon cancers [10]. A direct effect of p53 on the transcription of α5 gene seems unlikely as α5 gene promoter lack consensus p53 response elements. However, p53 is known to interfere with the binding of other transcription factors into DNA such as Sp1 [33]. Binding of different transcription factors to the promoter of the α5 gene is implicated to its transcriptional activity; Sp1, AP-1, NFI transcription factors belong to this list as well as coactivators SRC-1 and zeb2 [9,34,35]. Whether Nutlin-3a-dependent p53 activation has an impact on expression or function of these factors remains to be determined. Remarkably, repression of α5 integrin subunit alone was sufficient to impact on cell survival and apoptosis (this work and [10]) and as suggested in our study this repression can be triggered in some way by p53 activation. Our results suggest that α5 integrin down-regulation may be dependent on p53 activation and this decrease in turn sustains p53 activation as shown in Fig. 3B thus enhancing the Nutlin-3a effects. Thus, we demonstrate here the existence of an integrin/p53 negative loop in the HCT116 colon cancer cells; by the way, we extend this new concept from glioma [21] to other solid tumors.

4.3. Differences between p53 reactivation by Nutlin-3a and RITA

In contrast to Nutlin-3a, RITA did not decrease α5 expression at low concentrations but did at the highest concentration tested. This effect was only obtained in p53-expressing cells thus confirming a role of p53 in the regulation of α5 expression. Intriguingly, RITA did not affect the mRNA level of α5 integrin in our conditions, suggesting that transcriptional and non transcriptional mechanisms may be involved. Additionally, a less efficient induction of p53 was observed with RITA compared to Nutlin-3a even at concentrations that inhibited cell clonogenic potential. Our data suggest that p53 activation below a threshold value, as obtained with RITA, is not sufficient to affect α5 integrin expression. Such a threshold mechanism has recently been proposed for p53-mediated growth arrest or apoptosis [36]. Nutlin-3a binds to mdm2 and RITA to p53 and both drugs increase p53 activity by inhibiting the mdm2–p53 axis. However, different outcomes on p53 target gene induction/repression and cell survival have been described for the two drugs. Nutlin-3a is more prone to induce cell cycle arrest whereas RITA preferentially induces apoptosis [25]. It remains to be determined whether differences in p53 induction and/or transcriptional target gene activation are implicated in the differential effects of Nutlin-3a and RITA on α5 integrin repression. In line with this, although both drugs are able to repress oncogene expression, only high doses of RITA achieve oncogene repression and efficient apoptosis [37]. In our experimental conditions, Nutlin-3a and RITA at high concentrations were shown to affect the survival of cells lacking p53. In fact, increasing amount of data point to a p53-independent role of these agents including the initiation of DNA damage response or activation of p73 and E2F1 instead of p53 by Nutlin [38,39] or enhancement of mdm2-dependent oncogene degradation by RITA [40]. In addition, it was recently shown that Nutlin-3a interacts with multiple anti-apoptotic Bcl2-family proteins which may be important for p53-independent effects of the drug [41].

5. Conclusions

In conclusions, our data add new evidences supporting that α5β1 integrins have a crucial role in colon cancer cell survival. Targeted therapies aiming to block their function or to repress their expression may be useful to sensitize these tumors to conventional chemotherapeutic drugs as suggested by our previous data [23]. The characterization of a negative crosstalk between α5β1 integrin and p53 in colon cancer cells might help to guide development of more efficient drug combinations for a selected subpopulation of patients bearing tumors with a p53wt and a high expression of α5β1 integrin.

Conflict of interest

None of the authors have any conflict of interest to be disclosed regarding this study.

Acknowledgements

The authors thank Pr. B. Vogelstein (Baltimore, USA) for the HCT116 cell lines, Dr. C. Blattner (Karlsruhe, Germany) for the p53wt-containing plasmid and Dr. F. Carreiras (Cergy-Pontoise, France) for human fibronectin. Work is supported by the University of Strasbourg, the Ligue Contre le Cancer (Comité du Grand Est), by Alsace contre le Cancer, by the Fondation ARC pour le Recherche sur le Cancer. H. Janouskova is a predoctoral fellow from the French Ministère des Affaires Etrangères and from the Fondation ARC pour la Recherche sur le Cancer. A.M. Ray is a predoctoral fellow from the French Ministère de l'Enseignement Supérieur et de la Recherche and from the Ligue contre le Cancer. F. Schaffner is a postdoctorant supported by the Ligue contre le Cancer.

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Please cite this article in press as: H. Janouskova et al., Activation of p53 pathway by Nutlin-3a inhibits the expression of the therapeutic target α5 integrin in colon cancer cells, Cancer Lett. (2013), http://dx.doi.org/10.1016/j.canlet.2013.03.018

APPENDIX 3:

Martin, S., **Janouskova, H**., & Dontenwill, M. 2013. *Integrins and p53 pathways in glioblastoma resistance to temozolomide*. Front Oncol, 2, 157.

frontiers in
ONCOLOGY

REVIEW ARTICLE
published: 31 October 2012
doi: 10.3389/fonc.2012.00157

Integrins and p53 pathways in glioblastoma resistance to temozolomide

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Glioblastoma is the most common malignant primary brain tumor. Surgical resection, post-operative radiotherapy plus concomitant and adjuvant chemotherapy with temozolomide (TMZ) is the standard of care for newly diagnosed glioblastoma. In the past decade, efforts have been made to decipher genomic and core pathway alterations to identify clinically relevant glioblastoma subtypes. Based on these studies and more academic explorations, new potential therapeutic targets were found and several targeting agents were developed. Such molecules should hopefully overcome the resistance of glioblastoma to the current therapy. One of the hallmarks of glioblastoma subtypes was the enrichment of extracellular matrix/invasion-related genes. Integrins, which are cell adhesion molecules important in glioma cell migration/invasion and angiogenesis were one of those genes. Integrins seem to be pertinent therapeutic targets and antagonists recently reached the clinic. Although the p53 pathway appears often altered in glioblastoma, conflicting results can be found in the literature about the clinically relevant impact of the p53 status in the resistance to TMZ. Here, we will summarize the current knowledge on (1) integrin expression, (2) p53 status, and (3) relationship between integrins and p53 to discuss their potential impact on the resistance of glioblastoma to temozolomide.

Keywords: integrin, p53, temozolomide, glioblastoma, chemoresistance

INTRODUCTION

Glioblastoma is characterized by rapidly dividing cells, high degree of vascularity, invasion into the normal brain tissue, and an intense resistance to death-inducing stimuli. Significant advances have been made in understanding the molecular genetics underlying the heterogeneity of glioblastoma and their resistance to therapies. However, standard therapy including surgical resection and radiotherapy with concomitant and adjuvant chemotherapy using temozolomide (TMZ) remains poorly efficient (Stupp et al., 2005). Prognostic and predictive markers are continuously proposed based on large scale genomic data. In the recent years, emphasis has been given to the predictive impact of O^6-methylguanine-DNA methyltransferase (MGMT) expression/activity, a DNA repair enzyme that protects cells against alkylating drugs such as TMZ (Stupp et al., 2009). The promoter of the MGMT gene is methylated in 40–45% of glioblastoma and the enzyme is not expressed in the majority of these cases (Hegi et al., 2005; Silber et al., 2012). While the contribution of MGMT to TMZ resistance is highly documented, tumors in which MGMT is not the primary determinant of treatment outcome also exist (Carlson et al., 2009; Combs et al., 2011). Integrins have been proposed to play a role in the aggressiveness of gliomas and have been implicated in radio/chemoresistance in different types of tumors (Aoudjit and Vuori, 2012). The p53 protein has been largely studied in gliomas but its prognostic value has not been consistently established. In line with our recent data proposing an $\alpha_5\beta_1$ integrin-p53 axis with potential implication in TMZ resistance (Janouskova et al., 2012), we will summarize here the current knowledge on integrins and p53 status in glioblastoma.

INTEGRINS IN GLIOMA

BIOLOGY OF INTEGRINS

Integrins are heterodimeric cell surface receptors that mediate cell adhesion to the extracellular matrix (ECM) and support cell–cell interactions in a multitude of physiological and pathological situations. They are at least 24 known αβ heterodimers formed by a combination of 18 α and 8 β subunits bound non-covalently. Natural ligands of integrins are component of the ECM such as vitronectin, collagen, or fibronectin. Each αβ integrin pair has a defined set of ECM protein (Hynes, 2002). The repertoire of integrins present at the membrane dictates therefore the extent to which a cell will behave on a specific matrix and respond to its environment. Once engaged with the ECM, integrins cluster and recruit various signaling and adaptor proteins to form focal adhesion complexes (Geiger et al., 2001). These complexes activate intracellular downstream signaling pathways including NF-κB, PI3K, Src, or Ras-MAP kinases (Hynes, 2002; Legate et al., 2009). Such pathways regulate functions involved in motility, cytoskeleton organization, adhesion, proliferation, survival, and gene transcription. Integrins link ECM to the actin cytoskeleton through FAK/ILK/SFK/Rho proteins pathway providing the traction necessary for cell motility (Geiger et al., 2001). Integrins regulate the localization and the activity of urokinase-type plasminogen activator (uPA)/uPA receptor (Ghosh et al., 2000; Wei et al., 2007; Bass and Ellis, 2009) and matrix metalloproteinases (MMPs; Lamar et al., 2008; Morozevich et al., 2009) therefore controlling ECM remodeling and the invasive process.

Beside their mechanical functions and despite the lack of intrinsic kinase activity, integrins are true signaling molecules.

Integrins regulate proliferation by controlling the expression of cyclin D1 which permits cells to enter the S-phase of the cell cycle (Fournier et al., 2008). Integrins relay survival or apoptotic signals depending on the surrounding environment. Integrin ligation promote survival through various mechanisms including increased anti-apoptotic proteins (bcl-2, FLIP; Aoudjit and Vuori, 2001b; Matter and Ruoslahti, 2001; Uhm et al., 1999), activation of PI3K–Akt (Aoudjit and Vuori, 2001a) or NF-κB pathway (Scatena and Giachelli, 2002; Courter et al., 2005). Unligated integrins were reported to promote apoptosis through the so-called integrin-mediated death (IMD) a mechanism dependent, or not, on caspases activation in anchorage-dependent cells (Stupack et al., 2001; Jan et al., 2004). However, tumor cells are often IMD-resistant and unligated integrins rather promote anchorage-independent growth, survival, and metastasis than apoptosis (Desgrosellier et al., 2009). Additionally, cross-talks occur between integrins, cytokines, and growth factor receptors. Optimal growth factor stimulation relies on integrin-mediated adhesion to an appropriate ECM protein. $\alpha_v\beta_{3/5}$ and $\alpha_5\beta_1$ interact with growth factor receptors (VEGFR2, c-Met, FGFR1, PDGFR, EGFR, TIE-2, and IGF-1R) to promote full activation of each receptor and maximal signal transduction (increased MAPK and Akt activity) resulting in enhanced cell migration, proliferation, survival, and angiogenesis (Friedlander et al., 1995; Eliceiri, 2001; Alam et al., 2007; Soung et al., 2010). Integrins were also reported to bind directly growth factor (such as angiopoietins or VEGF) allowing the transduction of information in the absence of the receptor (Carlson et al., 2001; Hutchings et al., 2003). In short, integrins sense, interpret, and distribute information so that cancer cells adjust and respond to their microenvironment.

INTEGRIN EXPRESSION AND FUNCTION IN GLIOMA

Clustering of transcriptomic data from high grade glioma predicted poor survival in subclasses of tumors overexpressing ECM components such as fibronectin which is the preferred ligand of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (Geiger et al., 2001; Freije et al., 2004; Bredel et al., 2005; Colin et al., 2006; Tso et al., 2006). Functional analysis revealed gliomagenesis and glioblastoma networks composed of genes that play a role in integrin signaling including fibronectin, α_3 and α_5 integrins (Bredel et al., 2005). Gingras et al. (1995) investigated glioblastoma for the expression of cell adhesion molecules including integrins that might distinguish tumor from normal adjacent brain tissue. Results showed that glioblastoma expressed α_2, α_3, α_5, $\alpha_6\beta_1$, and $\alpha_v\beta_3$ integrins at significantly higher level than normal brain tissue suggesting that these integrins might play a role in the development or the progression of glioma (Gingras et al., 1995). β_8 and $\alpha_5\beta_1$ integrins were commonly expressed in a perinecrotic or perivascular pattern in glioblastoma (Riemenschneider et al., 2005). Higher levels of α_5 and β_3 integrin mRNA were measured in glioblastoma as compared to normal brain or low grade astrocytoma (Kita et al., 2001). Average $\alpha_v\beta_3$ integrin expression in glioblastoma seemed to exceed those in low grade glioma at the protein level although mRNA levels of both subunits were not discriminative between glioblastoma and low grade glioma (Schnell et al., 2008). In another study, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ integrins were shown to be expressed at consistently higher levels than $\alpha_v\beta_3$ integrins in human glioma cell explants (Mattern et al., 2005). We and others showed recently that $\alpha_5\beta_1$ integrin expression in biopsies from patient with glioma correlated with poor prognosis and tumor aggressiveness (Cosset et al., 2012; Holmes et al., 2012; Janouskova et al., 2012).

In glioma, integrins were often studied because of their crucial role in tumor cell invasion (Gritsenko et al., 2012). Both β_1 subunit-containing integrins (Paulus et al., 1996) and $\alpha_v\beta_3$ integrins control glioma cell invasion (D'Abaco and Kaye, 2007). $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ integrins are overexpressed in multidrug-resistant glioma cells and are responsible for their increased adhesive and invasive capacities (Hikawa et al., 2000). The laminin-5 receptor $\alpha_3\beta_1$ integrin is mainly expressed in areas of tumor cell invasion and support glioma cell migration and invasion (Tysnes et al., 1996; Mahesparan et al., 2003; Kawataki et al., 2007). $\alpha_v\beta_3$ integrins promote migration and adhesion in various glioma cells (Gladson and Cheresh, 1991; Friedlander et al., 1996) and inhibition of $\alpha_v\beta_3$ integrin with neutralizing antibody inhibited migration and invasion selectively in cell lines that contained a high level of integrin expression (Wild-Bode et al., 2001). Our laboratory extensively investigated $\alpha_5\beta_1$ integrins in glioma. We showed that $\alpha_5\beta_1$ integrins increased proliferation, clonogenic survival, adhesion, migration, and invasion of various glioma cell lines (Maglott et al., 2006; Bartik et al., 2008; Martin et al., 2009; Cosset et al., 2012). We also reported that the expression of $\alpha_5\beta_1$ integrins in glioma is controlled by caveolin-1 (Martin et al., 2009; Cosset et al., 2012). Interestingly, it was shown recently that invasive recurrent glioblastoma, resistant to antiangiogenic therapy, overexpress $\alpha_5\beta_1$ integrin and its ligand fibronectin (DeLay et al., 2012). $\alpha_v\beta_3$ integrin/ILK/RhoB pathway (Monferran et al., 2008) and β_1 integrin/AKT/p130Cas/paxillin (Cordes et al., 2006) controlled the radiosensitivity of glioma cells by regulating radiation-induced cell death. Recruitment of $\alpha_v\beta_{3/5}$ integrin in glioblastoma cells is induced by hypoxia. It follows the activation of FAK/RhoB/GSK3β pathway leading to HIF-1α induction and the transcription of proangiogenic factors (Skuli et al., 2009). Finally, surface expression of $\alpha_6\beta_1$ integrin in U87MG cells enhanced cell spreading and attachment on laminin-111, increased proliferation, decreased apoptosis due to serum starvation and increased migration and invasion of U87MG cells both *in vitro* and *in vivo* (Delamarre et al., 2009).

INTEGRINS IN GLIOMA ANGIOGENESIS

Induction of angiogenesis is essential for a tumor to grow beyond 1–2 mm and glioblastoma exhibit prolific angiogenesis. Poorly expressed in resting endothelial cells, $\alpha_5\beta_1$ and $\alpha_v\beta_{3/5}$ integrins are highly upregulated on endothelium cells during tumor angiogenesis (Bussolati et al., 2003; Avraamides et al., 2008; Desgrosellier and Cheresh, 2010) and rapidly accessible in tumor blood vessels (Magnussen et al., 2005). They stimulate endothelial cell proliferation, promote migration, and lumen formation (Mettouchi and Meneguzzi, 2006). Although $\alpha_5\beta_1$ integrin is undoubtedly recognized as a proangiogenic factor, controversial results for αvβ3 integrin questioned its role (Hodivala-Dilke, 2008; Robinson and Hodivala-Dilke, 2011). Overexpression of $\alpha_v\beta_3$ integrin in glioma exerted growth-suppressive effects *in vivo* that are linked

to vascular defects (Reynolds et al., 2002; Kanamori et al., 2004, 2006). However, antagonists to both $\alpha_5\beta_1$ and $\alpha_v\beta_{3/5}$ integrins are able to inhibit tumor angiogenesis (Brooks et al., 1995; Friedlander et al., 1995; Kim et al., 2000).

INTEGRINS IN GLIOMA STEM CELLS

Brain tumors also contain highly tumorigenic and therapeutically resistant pluripotent stem cells referred as glioma stem or initiating cells. The glioma stem cell hypothesis incorporates a model in which only a small subset of cells, the glioma stem cells, can initiate tumor. This hypothesis was confirmed very recently *in vivo* (Lathia et al., 2011). Elevated levels of $\alpha_6\beta_1$ integrins were found in glioma stem cells and seem to be a reliable new marker to enrich for glioma stem cells (Lathia et al., 2010).

Integrins are implicated at various levels of glioma development and progression. Blocking their functions may affect both tumoral cells and endothelial cells and these characteristics made them attractive therapeutic targets for glioblastoma (Chamberlain et al., 2012; Goodman and Picard, 2012). Emphasis on $\alpha_v\beta_3$ integrins has been given recently as cilengitide, their prototypical small peptide antagonist, is currently evaluated in phase III clinical trials in glioblastoma (Tabatabai et al., 2010). Interestingly, the outcome in a phase II trial was particularly good in patients with a methylated MGMT gene promoter (Stupp et al., 2010). Emerging data showing the role of $\alpha_5\beta_1$ integrin in glioblastoma give some hope for new therapeutic propositions in the near future.

p53 PROTEIN IN GLIOMA

p53, the "guardian of the genome," is certainly one of the most widely studied protein in human glioma. Activation of the tumor suppressor p53 by stress signals triggers different cellular programs such as cell cycle arrest, apoptosis, differentiation, DNA repair, autophagy, and senescence through complex network and signaling pathways (Levine and Oren, 2009; Vousden and Prives, 2009; Sullivan et al., 2012). Gaining a better understanding of how transcriptional and non-transcriptional functions of p53 integrate will be of great importance for the proposal of new therapeutic options (Dai and Gu, 2010; Speidel, 2010). Somatic p53 missense mutations are found in approximately 50% of all human cancers. Intensive research on p53 status as a classical molecular marker led to controversial results and non-significant clinical impact, particularly in the glioma field.

p53 STATUS IN GLIOMA

As most mutations in p53 gene led to the accumulation of p53 in the nucleus, nuclear overexpression of p53 was usually considered as a marker of mutation. Several studies showed that the expression of p53 is correlated at 90% with its mutation (Figarella-Branger et al., 2011). Detection of p53 mutation by the yeast functional assay that measures quantitatively mutant p53 alleles and qualitatively the loss of p53 competence was also employed and compared to conventional techniques including DNA sequencing (Tada et al., 1997; Fulci et al., 2000). Overall results indicate that p53 mutations often occurred in low grade gliomas (WHO grade II astrocytoma; Bourne and Schiff, 2010) and thus is a frequent event in the pathological progression of secondary glioblastoma (WHO Grade IV; Gladson et al., 2010). Secondary glioblastoma arise from a preexisting grade II or III astrocytoma in contrast with primary glioblastoma that form *de novo*. Primary glioblastoma represent about 90% of glioblastoma. p53 gene mutations are present in about 30% of primary glioblastoma, and occur more frequently in secondary glioblastoma (65%; Ohgaki et al., 2004; Zheng et al., 2008). A recent integrated genomic analysis identified four relevant subclasses of glioblastoma (proneural, mesenchymal, neural, and classical glioblastoma). p53 mutation was observed in 54, 32, 21, and 0% of tumors from the proneural, mesenchymal, neural, and classical glioblastoma subtype, respectively (Verhaak et al., 2010). Interestingly, classical glioblastoma benefit from more aggressive therapy regimen than the others (Verhaak et al., 2010). In fact, the prognostic value of p53 status may be reconsidered according to these data.

p53 STATUS AND GLIOBLASTOMA PROGNOSIS

No clear consensus has been reached about the prognostic value of p53 status despite numerous studies (**Table 1**). A clear picture remains difficult to draw due to the different techniques used to evaluate p53 (including immunostaining on tumor tissues, direct sequencing of p53 gene, and functional assays) and the complexity of patient cohort composition. Data illustrating an association of p53 with survival always point to a longer survival when p53 is mutated (Tada et al., 1998; Schiebe et al., 2000; Birner et al., 2002; Burton et al., 2002). However, the majority of studies do not validate p53 as an independent prognostic marker for glioblastoma (Kraus et al., 2001; Simmons et al., 2001; Shiraishi et al., 2002; Rich et al., 2005; Ruano et al., 2009; Weller et al., 2009; Levidou et al., 2010; Rossi et al., 2011). Overall it means that the prognostic impact of p53 aberrations is only marginal when considered in a global glioblastoma patient population. Reevaluation of this impact in clinically relevant glioblastoma subpopulations (see above) and association with specific molecular signatures will certainly be of interest in the future.

p53 AND GLIOMA-INITIATING STEM CELLS

Recent studies begin to shed light onto the role of p53 in the regulation of neural stem cells (NSCs). NSCs are self-renewing cells in the central nervous system that can generate both neurons and glia. An elegant study showed that dual inactivation of p53 and PTEN in murine NSC promotes an undifferentiated state with high renewal potential and generates tumors with a high grade glioma phenotype (Zheng et al., 2008). Although the role of p53 in brain tumor stem cells has not been well established, data suggest that loss of differentiation and increase in neurosphere renewal may be linked to the disruption of the p53 pathway in glioma (Molchadsky et al., 2010; Mendrysa et al., 2011; Spike and Wahl, 2011). To achieve a permanent eradication of brain tumors, it is noteworthy that glioma-initiating stem cells have to be considered and in this way their p53 status and functions need to be further explored.

p53 AND TMZ

Despite expressing mainly a wild-type p53 and thus being expected to be sensitive to DNA-damaging agents, primary glioblastoma resist standard therapies including chemotherapy with TMZ. This intriguing observation is in debate and the role of p53 status

Table 1 | Evaluation of p53 status in glioblastoma.

Evaluation of the p53 status	Number of patients	% of p53 mutant	p53: prognostic marker?	Reference
Sequencing/yeast functional assay	42	43	YES (longer survival for patients with p53mut tumors)	Tada et al. (1998)
Sequencing	75	32	YES (longer survival for patients with p53mut tumors)	Schiebe et al. (2000)
Sequencing/immunostaining	110	19	NO	Simmons et al. (2001)
Sequencing/immunostaining	93	22	NO	Kraus et al. (2001)
Sequencing/yeast functional assay	123	31	NO	Shiraishi et al. (2002)
Sequencing/immunostaining	41 long-term survivors	25	YES (longer survival for patients with p53 positive tumors)	Burton et al. (2002)
	48 short-term survivors	31		
Immunostaining	114	/	YES (longer survival for patients with p53 positive tumors)	Birner et al. (2002)
Sequencing	41	27	NO	Rich et al. (2005)
Sequencing/immunostaining	194	/	NO	Ruano et al. (2009)
Sequencing/immunostaining	291	15	NO	Weller et al. (2009)
Immunostaining	77 Meta analysis	/	NO	Levidou et al. (2010)
Immunostaining	106	/	NO	Rossi et al. (2011)

in response to TMZ has been largely addressed in preclinical studies. Conflicting results have been obtained (**Table 2**) and show either an improved capability of TMZ to inhibit cell viability when p53wt is functionally inhibited (Hirose et al., 2001; Xu et al., 2001, 2005a,b; Dinca et al., 2008; Blough et al., 2011) or a sensitization of cells to drugs when p53wt is functional (Hermisson et al., 2006; Roos et al., 2007). The former studies suggested that glioma cells with an intact p53 gene are selectively impaired in the proapoptotic functions of p53wt while retaining the potential to mediate relevant DNA repair and cell cycle arrest. Treatment with TMZ induced a persistent cell cycle arrest and an increase in p21 (a cell cycle regulator) in functional p53-expressing cells which showed morphological and biochemical features of senescent cells (Hirose et al., 2001; Martinkova et al., 2010). In cells impaired for p53 function or with a mutant p53, TMZ induced a transient cell cycle arrest and cell death via apoptosis or mitotic catastrophe (Hirose et al., 2001; Martinkova et al., 2010) as well as attenuation of DNA repair (Xu et al., 2005b). When TMZ-triggered apoptosis

Table 2 | Role of p53 in TMZ outcome.

Material	p53 inhibition	Effect of p53 modulation on TMZ sensibility	Reference
Glioblastoma cell lines (U87MG, LNZ308)	By oncoprotein E6	Increased sensibility	Hirose et al. (2001)
Glioblastoma cell lines (SWB95, SWB77, SWB33, SWB40, SWB39, SWB61, D54) xenografts		p53-independent cell cycle arrest	Bocangel et al. (2002)
Glioblastoma cell lines (U87MG, LNZ308)	By pifithrin-α	Increased sensibility	Xu et al. (2005a)
Glioblastoma cell lines (D54, A172)	By oncoprotein E6	Increased sensibility	Xu et al. (2005b)
Glioblastoma cell lines (U87MG, U373MG, U251MG, U138MG, LN18, LN428, LN319, LNT229, LN308, D247MG, T98G)	By siRNA	Decreased sensibility	Hermisson et al. (2006)
Glioblastoma cell lines (U87MG, U138MG)	By pifithrin-α	Decreased sensibility	Roos et al. (2007)
Glioblastoma cell lines (U87MG) xenografts of biopsies	By pifithrin-α	Increased sensibility	Dinca et al. (2008)
Glioblastoma cell lines (U87MG, LNZ308, LN443, SF767, U251N, U373)	By siRNA	In cell lines : increased sensibility	Blough et al. (2011)
Cancer stem cells		In stem cells : decreased sensibility	

was reported for both p53wt and p53mutant cells, pathways involved differed with activation of the FAS apoptotic pathway or the mitochondrial apoptotic pathway, respectively (Roos et al., 2007). Thus adverse effects of p53wt activities are increasingly recognized and may participate in chemoresistance of diverse cancers including glioma (Kim et al., 2009; Martinez-Rivera and Siddik, 2012). In one recent report, the effect of p53 status on response to TMZ was explored in glioma-initiating stem cells. It was shown that tumor stem cells are resistant to TMZ when p53 is mutated and sensitive to TMZ when intact (Blough et al., 2011). These data add a new level of complexity in the relationship between p53 status and TMZ sensitivity in glioma.

INTEGRINS AND p53

Although p53 itself is functional in a great majority of primary glioblastoma, inactivation of the p53 signaling pathway occurred in the form of ARF deletions, amplifications of mdm2 or mdm4 leading to p53 signaling alterations in 87% of glioblastoma (Network, 2008). Additionally several oncogenes such as the glioma oncoprotein Bcl2L12 (Stegh et al., 2010) were reported to be overexpressed in p53wt tumors and to impair p53 signaling pathway. Few reports investigated the relationship between integrins and p53 signaling pathways. Both integrin-dependent activation (Lewis et al., 2002) and inhibition (Bao and Stromblad, 2004) of p53 signaling have been suggested in different tumoral settings except gliomas. For example, Stromblad and colleagues demonstrated that $\alpha_v\beta_3$ integrin impacts negatively on p53wt activity in melanoma cells (Bao and Stromblad, 2004; Smith et al., 2012). We proposed recently that $\alpha_5\beta_1$ integrin plays a similar role in high grade glioma (Janouskova et al., 2012). We demonstrated that overexpression of the α_5 integrin subunit in p53wt U87MG cells impaired the activation of p53 and its transcriptional activity in response to TMZ. Under such conditions, cells became resistant to this alkylating agent. No such effects were found in p53 mutant glioma cell lines. Interestingly, higher levels of α_5 integrin were found in p53wt tumor biopsies than in p53 mutant tumor biopsies suggesting a link between this specific integrin and p53 status *in vivo*. Our *in vitro* studies also demonstrated that SJ749 and K34c, two specific non-peptidic antagonists of $\alpha_5\beta_1$ integrin, improved the therapeutic action of TMZ in a p53-dependent way (Martinkova et al., 2010). Molecular pathways involved in the integrin-dependent chemoresistance in p53wt tumors are currently unknown and deserve further studies. Proteins implicated in integrin signaling have been shown to shuttle between the membrane and the nucleus providing a potential mechanism for communication between integrins and p53. In particular FAK, the main kinase activated by integrins, is known to interfere with p53 activity in the nucleus (Lim et al., 2008; Golubovskaya and Cance, 2011). It will also be interesting to discriminate the potential effect of $\alpha_5\beta_1$ integrin on transcriptional and non-transcriptional functions of p53. Our data are the first to demonstrate relationships between an integrin and the TMZ-dependent activation of p53 in glioma possibly explaining the resistance to TMZ of a subgroup of patients. It is tempting to suggest that inhibition of $\alpha_5\beta_1$ integrin by specific antagonists might be an adjuvant treatment to standard therapies in patients expressing high level of $\alpha_5\beta_1$ integrin and p53wt.

CONCLUSION

Despite few and sometimes conflicting data available both on integrin expression and p53 status as prognostic and/or predictive markers for high grade glioma, a reevaluation of their roles is warranted. Due to the growing knowledge on glioblastoma heterogeneity and subclassification, it becomes reasonable to address these questions more accurately in well defined subpopulations of patients. Key issues need still to be addressed before proposing $\alpha_5\beta_1$ integrin expression level and p53 status as relevant biomarkers to stratify group of patients which may be more responsive to TMZ.

ACKNOWLEDGMENTS

We sincerely apologize to those whose work we were unable to discuss due to space limitations. Work in the author's laboratory is supported by the Ligue Contre le Cancer (Comité du Grand Est), by Alsace contre le Cancer, by the Fondation ARC pour le Recherche sur le Cancer. Hana Janouskova is a predoctoral fellow from the French Ministère des Affaires Etrangères and from the Fondation ARC pour la Recherche sur le Cancer.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 September 2012; paper pending published: 18 September 2012; accepted: 16 October 2012; published online: 31 October 2012.

Citation: Martin S, Janouskova H and Dontenwill M (2012) Integrins and p53 pathways in glioblastoma resistance to temozolomide. Front. Oncol. ***2****:157. doi: 10.3389/fonc.2012.00157*

This article was submitted to Frontiers in Radiation Oncology, a specialty of Frontiers in Oncology.