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CICLO XX

$p27^{kip1}$ inhibits glioma growth, by impairing cell proliferation, motility and tumor-induced angiogenesis.

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SUMMARY

SUMMARY

The tumor suppressor gene *CDKN1B* encodes for a 27 kDa CDK inhibitory protein, $p27^{kip1}$, which together with its well established role in the inhibition of cell proliferation displays additional activities in the control of gene transcription and cell motility. $p27^{Kip1}$ thus represents a good candidate for a gene therapy approach, especially in those cancers refractory to the conventional therapies, like human glioblastoma.

Here, we show that overexpression of $p27^{Kip1}$ in glioblastoma cell lines induced cell cycle arrest and inhibition of cell motility through Extracellular Matrix (ECM) substrates. The use of adenoviral vectors in the treatment of glioblastoma *in vivo,* demonstrated that $p27^{Kip1}$ was able to block not only cancer cell growth but also local invasion and tumor induced neoangiogenesis. The latter effect was due to the ability of p27 to impair both endothelial cell growth and motility, thus preventing proper vessel formation in the tumor. The block of neoangiogenesis depended on cytoplasmic $p27^{Kip1}$ anti-migratory activity and was linked to its ability to bind to and inhibit the MT-destabilizing protein stathmin.

Our work provides the first evidence that a successful $p27^{Kip1}$-based gene therapy is linked to tumor microenvironment modification, thus opening new perspectives to the use of gene therapy approaches for the treatment of refractory cancers.

RIASSUNTO

Il "tumor suppressor gene" CDKN1B codifica per una proteina di 27 KDa, detta appunto $p27^{kip1}$. p27 è un inibitore delle chinasi-dipendenti dalle cicline (CKI) e oltre a svolgere un'azione inibitoria nei confronti della proliferazione cellulare, bloccando la progressione del ciclo cellulare, è coinvolto anche in ulteriori ruoli come nel controllo della motilità cellulare. p27 sembra così rappresentare un ottimo candidato per un possibile approccio di terapia genica, svolgendo uno sguardo particolare verso quei tumori insensibili alle convenzionali terapie come il glioblastoma multiforme, forma tumorale del sistema nervoso centrale.

In questo lavoro abbiamo dimostrato come la overespressione di p27 nelle linee di glioblastoma utilizzate sia in grado di inibire sia la progressione lungo il ciclo cellulare, sia la motilità cellulare attraverso diversi substrati della matrice extracellulare. Inoltre l'utilizzo dei vettori adenovirali, nel trattamento del glioblastoma *in vivo*, ha mostrato come la proteina p27 sia in grado di bloccare non solo la crescita tumorale ma anche di frenare la capacità delle cellule tumorali di invadere i tessuti circostanti sani e di inibire l'angiogenesi indotta dal tumore. Questo ultimo effetto è dato dalla capacità di p27 di ostacolare sia la crescita che la motilità delle cellule endoteliali, prevenendo così la formazione di vasi sanguigni all'interno del tumore stesso. p27, finché sia in grado di ridurre la neoangiogenesi e quindi di inibire la migrazione delle cellule endoteliali, deve localizzarsi a livello citoplasmatico, dove è in grado di legarsi e di inibire l'attività di stathmin, una proteina destabilizzante i microtubuli. I dati emersi da questo lavoro dimostrano come l'overespressione di p27, mediante l'utilizzo di vettori adenovirali, rappresenti una prospettiva futura per il trattamento di tumori refrattari, non soltanto perché in grado di ostacolare la crescita tumorale *in vivo* ma anche perché capace di apportare dei cambiamenti a livello del microambiente tumorale.

INTRODUCTION

1. CELL CYCLE

As “cell cycle” we define the process by which a cell correctly divides into two daughter cells and is central to the understanding of all life (Nurse, 2000). It is in fact largely accepted that is essential for their survival that the two daughter cells receive a full complement of all the organelles and a copy of the genome correctly duplicated. To ensure that DNA faithfully replicates and that the replicated chromosomes correctly segregates into the two newly divided cells, in all eukaryotic cells, cell cycle progression is stringently controlled (Heichman and Roberts, 1994; Wuarin and Nurse, 1996). In particular several mechanisms ensure that S phase is completed before mitosis begins and that M phase started only if the DNA has been faithfully replicated. This is possible since two Gap phases (the G1 separating the M and S phases, and the G2 between the S and M phases) are present in somatic cells and dictate the timing of cell division during which the control mechanisms principally act. However, in early embryonic cells, that need to fast replicate these two Gap phases seem to be absent. In living organisms the cells are usually in a state of quiescence called G0 and can re-enter into the cell cycle after stimuli derived from the local microenvironment such us growth factors stimulation, a process that can be reproduced in *in vitro* experiments. The beginning of the G1 phase is the only part of the cell cycle that seems to be dependent on growth factors stimulation. When cells are stimulated by growth factors to enter the cycle from G0, they generally require continuous mitogenic stimulation to be driven to the restriction point, after which mitogens can be withdrawn and cells will enter S phase and complete the cycle in their absence. This landmark study has been carried out about 30 years ago by Arthur Pardee (1974) that was able to demonstrate that, in a normal cell, growth factors are necessary to initiate and maintain the transition from early to late G1 phase since to the so called “restriction point” or G1 phase checkpoint. Once the cell had passed the restriction point it is irreversibly committed to complete cell division even if growth factor stimulation is removed. Once the restriction point has been passed, the cell enter S phase during which the DNA is duplicated. At this point, in the G2 phase, before the Mitosis (M phase) could start, the cell control that DNA has been faithful duplicated checks the internal signalling events necessary for a successful division. Progression of mitosis is controlled by signalling pathways that monitor the integrity of microtubule function, to ensure the fidelity of chromosome segregation.

Regulation by growth factors would be damaging for a cell, and consequently, mitosis is a growth factors-independent phase of the cell cycle. As cells exit mitosis, the cell cycle is reset, allowing the establishment of a new, competent replication state in GO or G1 phases.

1.1 CYCLINS AND CDKS

Progression through the cell cycle is orderly driven by Cyclin-Dependent Kinases (CDK) activity (Figure 1). CDKs are serine and threonine kinases, and their actions are dependent on associations with their activating subunits, cyclins (Hunt, 1991¸Sherr, 1994). Cyclin abundance is regulated by protein synthesis and degradation; the activity of CDKs is therefore regulated to a large degree by the presence of different cyclins. Also, cyclins possess overlapping, but distinct, functional activities, allowing further refinement of control and probably the timely and irreversible occurrence of cell-cycle transitions. Cyclin specificity can be achieved in various ways: cyclins are expressed or are present at stable levels at different times; they are differentially sensitive to cell-cycle-regulated inhibitors; they are differentially restricted to specific subcellular locations; or they bind specifically to only some phosphorylation targets. In some cases, intrinsic cyclin specificity has been traced to specific modular sequences in the cyclin protein (for example, nuclear localization sequences (NLS), destruction boxes that regulate proteolysis or hydrophobic patches that can mediate interactions with substrates). Cyclins are regulated at the level of protein degradation by ubiquitin-mediated proteolysis. Proteins that are tagged with a chain of ubiquitin molecules are degraded by the 26S proteasome. The sensitivity of different cyclins to different ubiquitin ligases constitutes an important mechanism for cyclin specificity in controlling the cell-cycle engine.

Restriction point control is mediated by two families of enzymes, the cyclin D- and E-dependent kinases. The D-type cyclins (D1, D2, and D3; Matsushime et al., 1991; Xiong et al.,1991; Motokura et al., 1991) interact combinatorially with two distinct catalytic partners: CDK4 and CDK6 (Matsushime et al., 1992; Meyerson et al., 1994). Whereas CDK4 and CDK6 are relatively long-lived proteins, the D-type cyclins are unstable, and their induction, synthesis, and assembly with their catalytic partners all depend upon persistent mitogenic signalling. In this sense, the D-type cyclins act as

growth factor sensors, forming active kinases in response to extracellular cues (Sherr, 1993). Indeed, the major function of the cyclin D pathway is to provide this link between mitogenic cues and the potentially autonomous cell cycle machinery which is composed primarily of CDK2 and CDK1 and their associated regulators. Thus D-type cyclins are usually absent from cell cycles that proceed independently of extrinsic mitogenic signals. Conversely, constitutive activation of the cyclin pathway can reduce or overcome certain requirements for cell proliferation and thereby contribute to oncogenic transformation. (Weinberg, 1995; Sherr, 1996; Bartkova et al., 1997). The activities of these kinases are regulated also by integrins. The expression of cyclin D1 and, thus, the activation of CDK4/6 are suppressed in cells that are not anchored to ECM (Zhu et al., 1996). Integrin signalling may be needed for the transcription of cyclin D1, because the cyclin D1 promoter is co-ordinately regulated by JNK and ERK (Albanese et al., 1996). Integrins also stimulate the p70 S6-kinase, which may promote cyclin D1 translation (Koyama et al., 1996). Finally, anchorage to the ECM is necessary for the down-regulation of the CDK2 inhibitors p21 and p27 and, thus, the activation of cyclin E-CDK2 (Zhu et al., 1996; Fang et al., 1996).

The mitogen-dependent accumulation of the cyclin D-dependent kinases triggers the phosphorylation of the Retinoblastoma protein (Rb), thereby helping to cancel its growth-repressive functions (Matsushime et al., 1992; Ewen et al., 1993; Kato et al., 1993). Rb represses the transcription of genes whose products are required for DNA synthesis. It does so by binding transcription factors such as the E2Fs (Nevins, 1998) and recruiting repressors such as histone deacetylases (Brehm et al., 1998; Luo et al., 1998; Magnaghi et al., 1998) and chromosomal remodelling SWI/SNF complexes (Zhang et al., 2000) to E2F-responsive promoters on DNA. Rb phosphorylation by the G1 CDKs disrupts these interactions (Harbour et al., 1999), enabling released E2Fs to function as transcriptional activators. Apart from a battery of genes that regulate DNA transcription, E2Fs induce the cyclin E and A genes. Cyclin E enters into a complex with its catalytic partner CDK2 (Koff et al., 1991; Lew et al., 1991; Dulic et al., 1992; Koff et al., 1992) and collaborates with the cyclin D-dependent kinases to complete Rb phosphorylation (Hinds et al., 1992; Kelly et al., 1998; Lundberg et al., 1998). This shift in Rb phosphorylation from mitogen-dependent cyclin D-CDK4/6 complexes to mitogen-independent cyclin E-CDK2 accounts in part for the loss of dependency on extracellular growth factors at the restriction point. Cyclin E-CDK2 also phosphorylates

substrates other than Rb, and its activity is somehow linked to replication origin firing (Fang et al., 1991; Krude et al., 1997). The activity of the cyclin E-CDK2 complex peaks at the G1-S transition, after which cyclin E is degraded and replaced by cyclin A. Cyclin A is particularly interesting among the cyclin family, because it can activate two different CDKs and function in both S and M phases. In mitosis, the precise role of cyclin A is obscure, but it may contribute to the control of cyclin B stability. Cyclin A starts to accumulate during S phase and is abruptly destroyed before previous to cyclin B which persists until metaphase plate is formed.

Other levels of CDKs regulation are represented by phosphorylation and de-phosphorylation of specific threonine and/or tyrosine residues that results either in the activation or in the inhibition of CDKs catalytic activity. In particular, CDKs have to be phosphorylated (on the threonine residue located in its 'T-loop') to fully open its catalytic cleft. In fact CDK7 together with cyclin H and the assembly factor MAT1 forms the CDK-activating kinase (CAK) complex, responsible for the activating phosphorylation of CDK1,CDK2, CDK4 and CDK6 (Fesquet et al.,1993; Fisher et al., 1994; Matsuoka et al., 1994; Kato et al., 1994). These phosphorylation is removed by the phosphatase KAP after the cyclin partner has been degraded, i.e. KAP binds to CDK2 and dephosphorylates Thr160 when the associated cyclin subunit is degraded or dissociates (Poon et al., 1995).

On the other hand, the cyclin−CDK complexes can be kept in an inactive state by phosphorylation on one, or sometimes two, residues in the ATP-binding site of the CDK. These residues are a conserved tyrosine in many CDKs that is phosphorylated by Wee1/Mik1, and an adjacent threonine residue that is phosphorylated in animal cells by Myt1 (reviewed in Nigg, 2001). Members of the Cdc25 family of phosphatases remove the phosphates from the ATP-binding site tyrosine and/or threonine of the CDK, and thus activate the cyclin-CDK complex (Morgan, 1995).

Finally, the cyclin-CDK complexes are further regulated by the binding of specific CDK inhibitor proteins (CKI) (Pines, 1999).

1.2 CKIs

The CKIs can be divided into 2 families on the basis of sequence; the INK4 family and the Kip/Cip family (reviewed in Hunter and Pines, 1994; Sherr and Roberts, 1995; Harper and Elledge, 1996). The INK4 proteins are almost entirely composed of

ankyrin repeats (Serrano et al., 1993), a putative protein-protein interaction motif and this family comprises four members: $p15^{INK4b}$ (Hannon and Beach, 1994), $p16^{INK4a}$ (Serrano et al., 1993) $p18^{INK4c}$ (Guan et al., 1994; Hirai et al., 1995), and $p19^{INK4d}$ (Chan et al.,1995; Hirai et al., 1995). In humans, INK4a and INK4b are closely linked on the short arm of chromosome 9 (Kamb et al.1994), whereas INK4c maps to chromosome 1 and INK4d maps to chromosome 19. In mice, the INK4c and INK4d genes are expressed in stereotypic patterns in different tissues during development in uterus, whereas INK4a and INK4b expression has not been detected prenatally (Zindy et al. 1997). These inhibitors are specific for the CDK4 and CDK6 kinases (Hannon et al., 1994; Hirai et al., 1995), that bind the D-type cyclins (Matsushime et al., 1992; Bates et al., 1994). These kinase complexes have been most closely linked to the control of G1 phase, especially with regard to the decision to proliferate or not according to the presence of growth factors, and to cell size control (reviewed in Sherr and Roberts, 1995; Sherr, 1994). The INK4 family inhibits the CDK4 subfamily by competing for binding with the D-type cyclins (Hirai et al., 1995; Guan et al., 1994). By preventing the D-type cyclins from binding CDK4 and 6, the INK4 proteins could be predicted to keep these kinases in an inactive state, because, in analogy with what has been demonstrated for the activation of CDK2 by cyclin A-CDK4 and 6, should neither be able to bind their substrates nor to hydrolyse ATP without the conformational change induced by binding with the cyclin Ds. However, to confirm this hypothesis it will require the resolution of the structures of the INK4 proteins and the CDK4/6 subfamily, that up to now has not been reported. The INK4 proteins are also able to inhibit CDK4/6 in a complex with cyclin D (Jeffrey et al., 2000), and it will be interesting to see whether this bears any relation to the inhibitory mechanisms employed by the Kip/Cip family.

The Kip/Cip family comprises three proteins; $p21^{Cip1/Waf1/Sdi1}$ (Xiong et al., 1993; el-Deiry et al., 1993; Harper et al., 1993; Noda et al., 1994), $p27^{Kip1}$(Polyak et al., 1994; Toyoshima and Hunter, 1994) and $p57^{Kip2}$ (Lee et al., 1995; Matsuoka et al., 1995). These inhibitors show tissue specific distribution patterns. For example in gastrointestinal tract, the best organ characterized, p21, p27 and p57 are not expressed in the basal cells, which are the proliferating stem cells of keratinocytes, whereas there are well expressed in the suprabasal nonproliferative compartment (el-Deiry et al., 1995; Fredersdorf et al., 1996). Intense costaining of p27 and p57 has been detected in some specialized tissue, such as neurons (postmitotic neurons in the intermediate zone),

lens, skeletal muscle, cartilage, lung (bronchial epithelium), kidney (podocytes) and skin (superbasal cells) during their development. However, some tissue express only p27, such as most lymphocytes, the retina, and the adrenal medulla (reviewed in Nakayama et al., 1998). Their expression regulation is also differently regulated following specific stimuli. Indeed p57 is imprinted (Matsuoka et al., 1996), and have been implicated in cell differentiation (Elledge et al., 1996), and in the response to stress. p21 transcription is upregulated in response to DNA damage by wild type but not mutant p53, and p27 was initially identified as the factor responsible for inhibiting proliferation in contact-inhibited and TGFβ-treated cells (Koff et al. 1993). Until now the Kip/Cip family had been thought specific for the cyclin-CDK complexes (for the G1 and S phase kinases) (Harper et al. 1995). However, there is a report showing that p21, a DNA-damage-inducible cell-cycle inhibitor, acts also as an inhibitor of the SAPK (kinases stress-activated protein), group of mammalian MAP, such as JNK, (Shim et al., 1996), suggested that p21 may participate in regulating signalling cascades that are activated by cellular stresses such as DNA damage.

The Kip/Cip family are 38–44% identical in the first 70 amino acid region of their amino terminus, and this region is sufficient to inhibit cyclin-CDK activity (Nakanishi et al. 1995; Chen et al. 1995; Chen et al. 1996). The kinase inhibitory domain, in fact, maps to the N-terminus (1-82) and contains the CDK binding site (28-82). A functional characterization of p21 mutants in the N-terminal domain reveals that cyclins bind to this domain independently of CDK2. Correlating with these results Fotedar et al. (1996) find that p21 can associate with cyclin-CDK kinases in two functionally distinct forms, one in which the kinase activity is inhibited and the other one in which the kinase is still active. The first one requires that p21 bind both the CDK and the cyclin. In the second type of interaction, p21 bind only the cyclin or the CDK.

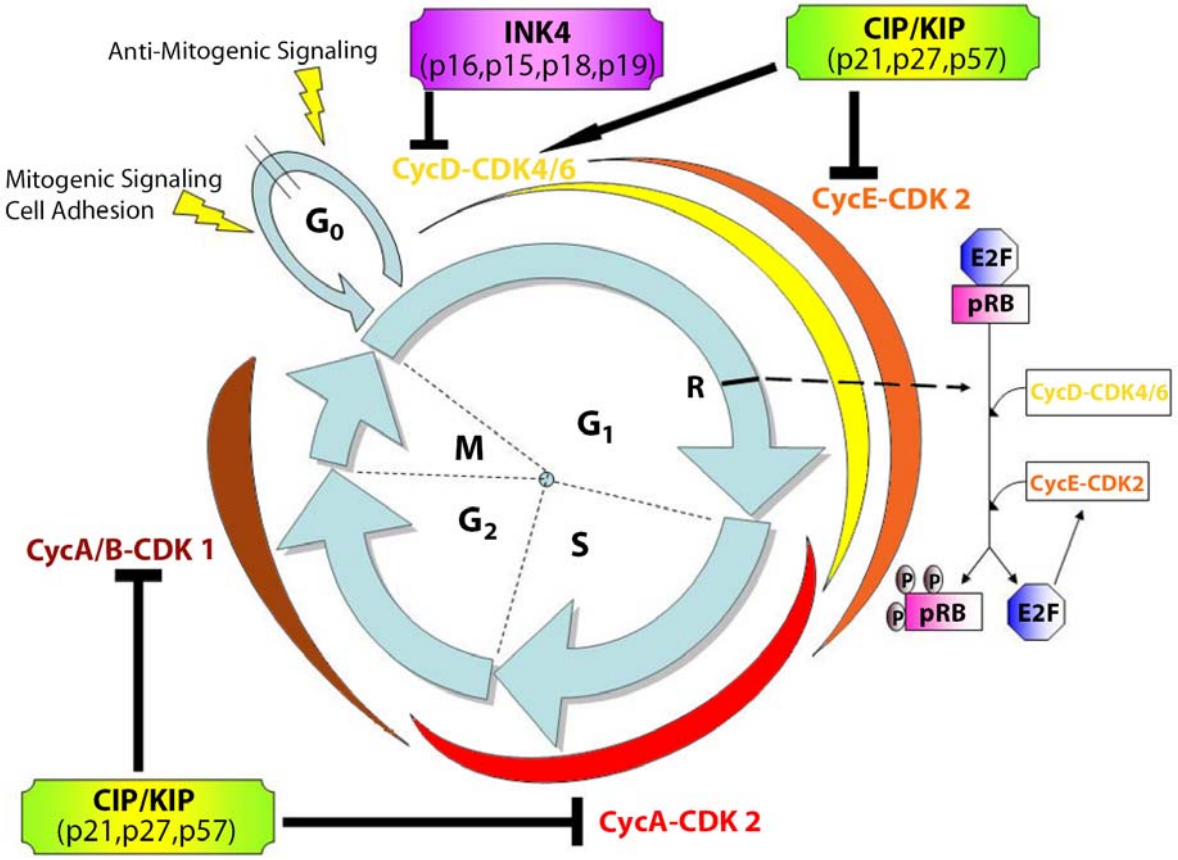


Figure 1. *Schematic representation of cell cycle regulation.* Mitogenic stimuli promote cell cycle progression from G0 to G1 inducing the expression of D type cyclins and lowering the expression of p27. Sequential activation of cyclin E-CDK2, cyclin A-CDK2, cyclin A-CDK1 and cyclin B-CDK1 allow the cells to pass through the restriction point (R) and to complete the mitotic division. The passage through the R point is due to the inactivation of the pRB protein by CDKs-dependent phosphorylation.

2. $p27^{Kip1}$

p27 was first identified as a CDK2-inhibitory activity detected in contact-inhibited or TGF-beta treated cells (Koff et al., 1993; Polyak et al., 1994; Polyak et al., 1994). At the same time, it was cloned in a tri-hybrid screen as a cyclin D-CDK4 interacting protein (Toyoshima and Hunter, 1994). In fact p27 has a crucial role in the G1-S transition, by interacting with and inhibiting cyclin E-CDK2 and cyclinA-CDK2 activity, thus blocking cell cycle progression. In early G1 p27 promotes cyclinD-CDK4/6 complex assembly and nuclear import, increasing cyclin D stability, all without inhibiting CDK4 kinase activity. In proliferating cells, p27 is primarily associated with

cyclin D-CDK4/6 complexes, but these complexes are catalytically active, whereas in G1 arrested cells p27 preferentially binds and inhibits CyclinE-CDK2. The sequestration of p27 by cyclin D-CDK4/6 complexes effectively frees CDK2 from inhibition and allows both CDK4/6 and CDK2 to remain active. In this way mitogen induction of cyclin D expression determines cell cycle progression both by activating CDK4 and by sequestering p27 thus favouring cyclin E-CDK2 activation. Activation of cyclin E by E2F, after hyper-phosphorylation of pRB, enables the formation of the active cyclin E-CDK2 complex. This is accelerated by the continued sequestration of Cip/Kip proteins into complexes with assembling cyclin D-CDK complexes. Cyclin E-CDK2 completes the phosphorylation of Rb, further enabling activation of E2F-responsive genes, including cyclin A. Once cyclin E-CDK2 is activated, it phosphorylates p27. This phosphorylation on Threonine 187 allows p27 to be recognized by the ubiquitin ligases and to be targeted for destruction by the 26S proteasomes (Pagano et al., 1995; Sheaff et al.,1997; Vlach et al., 1997; Montagnoli et al., 1999; Nguyen et al., 1999) . Therefore, cyclin E-CDK2 antagonizes the action of its own inhibitor. It follows that once cyclin E-CDK2 is activated, p27 is rapidly degraded, contributing to the irreversibility of passage through the restriction point. If cells are persistently stimulated with mitogens, cyclin D-dependent kinase activity remains high in the subsequent cell cycles, p27 levels stay low, and virtually all of the p27 can be found in complexes with the cyclin D-CDK4/6. However, when mitogens are withdrawn, cyclin D is rapidly degraded, and the pool of previously sequestered Cip/Kip proteins are mobilized to inhibit cyclin E-CDK2, thereby arresting progression usually within a single cycle.

Multiple extracellular stimuli regulate p27 abundance, which functions as a sensor of external signals to cell cycle regulation. In normal cells p27 is expressed at high levels in quiescence phase, whereas it decreases rapidly after mitogen triggering and cell cycle re-entry. A number of studies have shown that many anti-mitogenic signals induce the accumulation of p27, including cell–cell contact, growth factor deprivation, loss of adhesion to extracellular matrix, TGF-beta, cAMP, rapamycin or lovastatin treatment (Hengst and Reed, 1998) (Figure 2).

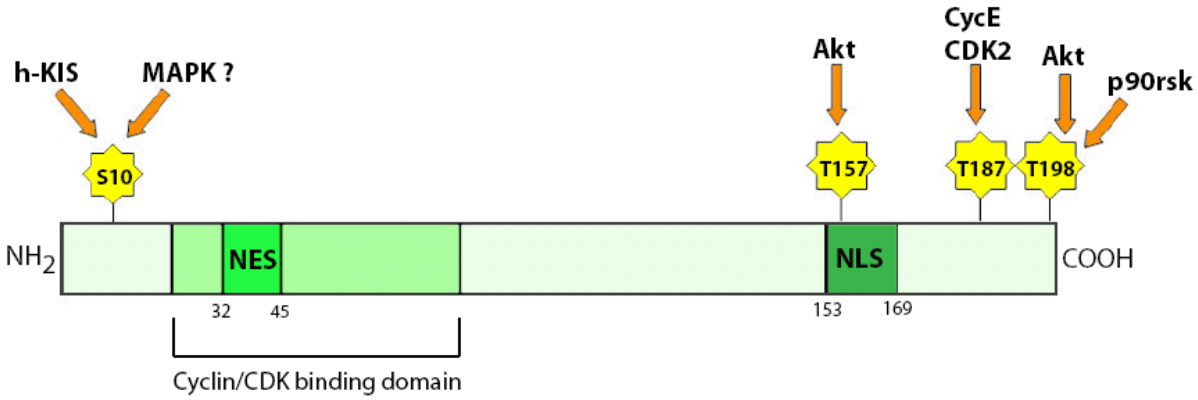


Figure 2. *Schematic representation of the principal p27 domains and of its major phosphorylation sites.*

2.1 Regulation of p27

In normal cells, the amount of p27 protein is high during G0 phase but decrease rapidly on re-entry of cells into G1 phase (Nourse et al., 1994; Reynisdottiret al., 1995). This rapid removal of p27 at the G0-G1 transition is required for effective progression of the cell cycle to S phase. The abundance of p27 is thought to be controlled by multiple mechanisms that operate at the level of its synthesis (transcription, translation), degradation and localization (Agrawal et al., 1996; Connor et al., 2003; Hengst et al., 1996; Ishida et al., 2002; Medema et al., 2000; Millard et al., 2000; Pagano et al., 1995; Rodier et al., 2001). In particular, a change in the subcellular localization of p27 induced by the exposure of quiescent cells to growth stimuli is crucial for the down-regulation of this protein at the G0-G1 transition. In G0 phase, p27 accumulates in the nucleus, where it inhibits the cyclin-CDK complexes. However, p27 undergoes rapid translocation from the nucleus into the cytoplasm by yet unspecified mechanism that seems to involve phosphotylation of Serine 10 by KIS (Kinase-interacting stathmin) (Boehm et al., 2002) or by MAPK (Rodier et al., 2005) and /or phosphorylation of Threonine 198 by AKT (Motti et al.,2004) or RSK (Fujita et al., 2003). The translocation of p27 from the nucleus to the cytoplasm is follow by its degradation by the ubiquitin-proteasome pathway involving the KPC1 and KPC2 proteins (Kamura et al., 2004). This ubiquitin dependent degradation of p27 is different from the one observed into the nucleus and that requires p27 phosphorylation onT187 by the cyclin

E-CDK2 complex and the binding to the SKP2 F-box protein (see also the next paragraph "p27 as a target of proteasome" (Figure 3).

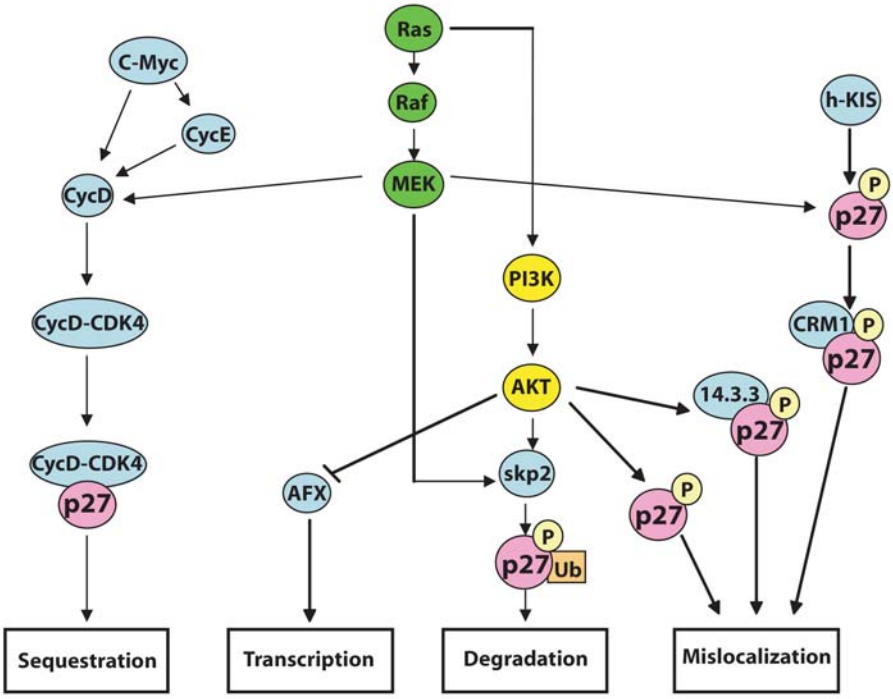


Figure 3. *Representation of the different intracellular pathways known to regulate p27 expression and function.*

2.2 Transcriptional regulation of p27

Although p27 mRNA levels are usually constant, there are some exceptions to this rule. p27 promoter activity has been shown to be positively regulated by Interleukin-6 signaling through STAT3 activation in melanoma cells (Kortylewski et al., 1999) and negatively regulated by PI3K and AKT through the inhibition of the AFX forkhead transcription factor (Medema RH et al., 2000). Recently Chassot et al. (2007), using a wounding model that induces cell-cycle entry of human dermal fibroblasts, demonstrated that p27mRNA is downregulated when cells progress into the G1 phase, and then it returns to its basal level when cells approach the S phase. By using a quantitative PCR screening they identified inhibitors of differentiation (Id3), a bHLH transcriptional repressor, as a candidate mediator accounting for p27 mRNA decrease.

Id3 silencing, using an small interfering RNA approach, reversed the injury mediated p27 downregulation demonstrating that Id3 is involved in the transcriptional repression of p27.

2.3 p27 as a target of proteasome

Proteolysis of many G1 regulatory proteins, including p27, is mediated by SCF ubiquitin ligases, each composed of four major subunits: Skp1, a cullin subunit called Cul1, Rbx1/Roc1 and one of the many F-box protein (Fbps). The substrate specificity of SCFs is due to distinct F-Box proteins, that recognize phosphorylated substrates and target them for ubiquitin-mediated degradation. The F-box protein involved in the ubiquitination and degradation of p27 is Skp2, first identified together with Skp1 as interactors of the Cyclin A-CDK2 complex, hence the name S phase kinase-associated proteins (Skps).

Degradation of p27 through the ubiquitin-proteasome pathway is a three-step process that requires: phosphorylation of p27 at threonine 187 residue by Cyclin E-CDK2 complex (Sheaff et al., 1997; Nguyen et al., 1999), recognition of T187-phosphorylated p27 by the ubiquitin ligase SCF-Skp2 (Montagnoli et al., 1999; Carrano et al., 1999) and SCF-Skp2-dependent ubiquitination and degradation of T187-phosphorylated p27 (Pagano et al., 1995; Carrano et al., 1999).

Skp2 also targets free cyclin E (not the one in complex with CDK2) for ubiquitylation (Nakayama et al., 2000). The importance of Skp2 in control of p27 abundance was confirmed by the observations that p27 accumulates at high levels in the cells of mice that lack Skp2 (Nakayama et al., 2004). It thus became widely accepted that Skp2 mediates p27 degradation at G0-G1 transition of the cell cycle. However, this apparently simple scenario turned out not to be quite so simple after all. Whereas mitogenic activation of resting cells induces rapid degradation of p27 at early to mid-G1 phase, Skp2 is not expressed until late G1 to early S phase, unequivocally later than the degradation of p27 start (Ishida et al., 2002; Hara et al., 2001). Moreover, this degradation of p27 occurs in the cytoplasm after export of the protein from the nucleus (Connor et al., 2003; Ishida et al., 2002; Rodier et al., 2001; Tomoda et al., 1999), whereas Skp2 is restricted to the nucleus (Miura et al., 1999; Maruyama et al., 2001). These discrepancies between the temporal and spatial patterns of p27 expression and

those of Skp2 expression suggested the existence of a Skp2-independent pathway for the degradation of p27. Indeed, the downregulation of p27 at the G0-G1 transition was found to occur normally in Skp2-/- cells and to be sensitive to proteasome inhibition, indicating that p27 is degraded at this time by a proteasome-dependent, but Skp2-indipendent mechanism (Hara et al., 2001). In contrast to the Skp2-dependent degradation occurring in the nucleus at S-G2 phases, this second pathway seems to occur in the cytosol and it is responsible for the G0-G1 degradation of p27. A recent study has described an ubiquitin ligase, named Kip1 ubiquitilation-promoting complex (KPC), that interacts with and ubiquitinates p27 in G1 phase into the cytoplasm of mammalian cells (Kamura et al., 2004; Hara et al., 2005; Kotoshiba et al., 2005). In accord with this point of view, the generation of mice double knock out for both p27 and Skp2 revealed that the Skp2-dependent p27 degradation participate principally in the S and G2 phases of the cell cycle rather than in G1 phase (Nakayama et al., 2004) (Figure 4).

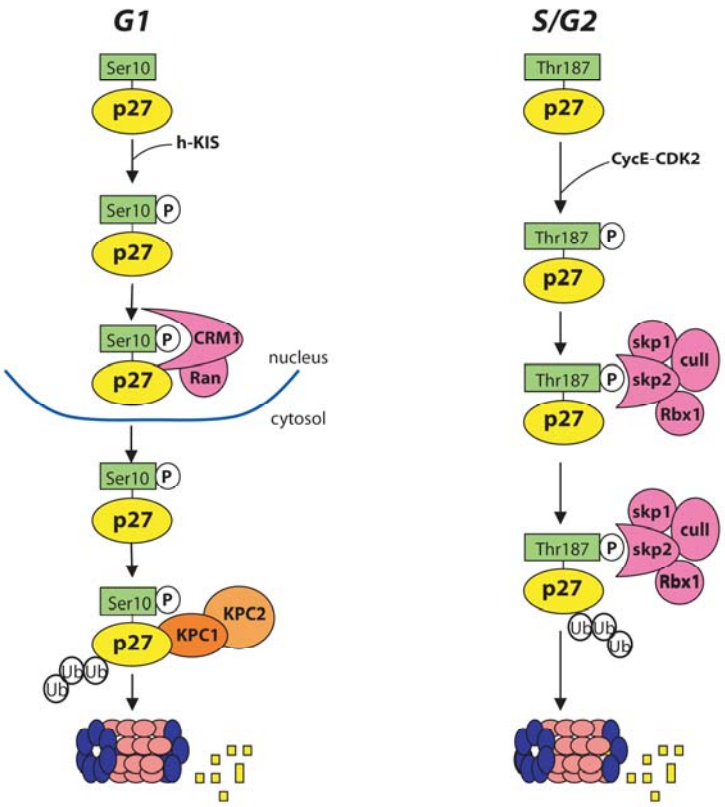


Figure 4. *Proposed models for p27 ubiquitin-dependent degradation*. On the left the proposed cytoplasmic degradation of p27 by the KPC complex, which has been reported to occur in early G1 phase. On the right, the Skp2 dependent p27 proteolysis that occurs in the early S and in G2 phases in the nucleus.

2.4 p27 functional inactivation

Beside modulation of its expression levels, subcellular localization also plays a pivotal role in governing p27 function, as mentioned above. It is widely accepted that to act as cell cycle inhibitor p27 must be located in the nucleus, whereas its cytoplasmic sequestration allows cell cycle progression (Belletti et al., 2005; Baldassarre et al., 2005). In fact, to inhibit Cyclin E-CDK2, p27 needs to be imported into the nucleus. Nuclear import depends on the presence of a nuclear localization signal (NLS) localized at the C-terminus of the protein (Zeng et al., 2000). Through its NLS sequence, p27 is recognize and targeted to the nucleus by Importin α3 and α5 in a complex with Importin β, and the small GTPase Ran (Sekimoto et al., 2004). As cells progress along the cell cycle, p27 shuttles between nucleus and cytoplasm. The cytoplasmic redistribution of p27 induced by mitogenic stimulation is dependent on the

phosphorylation of a specific serine residue (S10) (Rodier et al., 2004). The kinase responsible for S10 phosphorylation has been identified as the human kinase interacting stathmin (hKIS) (Boehm et al., 2002), a nuclear protein that, by phosphorylating p27 on S10 in response to mitogenic stimulation, promotes its nuclear export to the cytoplasm and overcomes p27-induced growth arrest. Also p27 nuclear export seems to be controlled and regulated, both by the presence of a leucine rich nuclear export signal (NES) within the CDK binding domain (amino acids 32-45) (Connor et al., 2003). p27 is solely nuclear in G0 and does not even bind to its exportin CRM1 until cells enter G1 following mitogen stimulation (Connor et al., 2003), suggesting that phosphorylation at Ser-10 may be implicated. However, recently the arginine-directed serine/threonine kinase Mirk/dyrk1B has been identified as a kinase active in G0 which phosphorylates p27 at Ser-10 without inducing its translocation from the nucleus (Deng et al., 2004). Mirk is transcriptionally up-regulated by the Rho family members (Deng et al., 2003) and itself functions as a transcriptional activator under control of the MKK3/p38 MAPK signalling system (Lim et al., 2002). Mirk and KIS exhibit markedly different transcriptional regulation so they do not occupy the same phase of the cell cycle. Cellular mitogens transcriptionally down-regulate Mirk/dyrk1B, whereas mitogens induce the transcription of KIS. Mirk levels and activity are highest in G0 and decline during G1 when KIS levels and activity are rising; KIS is not present during G0 to phosphorylate p27. Therefore, phosphorylation of p27 at Ser-10 has two outcomes which are cell cycle-dependent. Phosphorylation of p27 at Ser-10 during G0 by Mirk/dyrk1B stabilizes p27 and maintains p27 within the nucleus where it can bind to CDK2. In contrast, phosphorylation of p27 at Ser-10 by the KIS kinase in G1 enables p27 to bind to CRM1 and to be transported into the cytoplasm for destruction (Boehm et al., 2002; Deng. et al., 2003). Although further studies are required to give an explanation for that the same phosphorilation (S10) by two kinases results in different p27 fate.

Other phosphorylation sites of p27, such as Thr 157 and Thr 198 in the human protein (Thr 197 in mouse), have been implicated in the cytoplasmic translocation and localization of p27. The Thr 157 site of p27 is phosphorylated by the kinase AKT in cancer cells (Viglietto et al., 2002; Liang et al., 2002; Shin et al., 2002). This residue is located in the nuclear localization signal (NLS) of p27, and its phosphorylation prevents the nuclear localization of p27 by promoting the binding of its to 14-3-3 (Sekimoto et

al., 2004). However, the fact that Thr 157 is not conserved in mouse p27 shows that its phosphorylation is not a general mechanism for determining p27 localization. The Thr 198 site of human p27 (Thr 197 in mouse p27) is phosphorylated by AKT as well as ribosomal protein S6 kinase 1 (RSK1) and RSK2 (Fujita et al., 2002; Fujita et al., 2003). The 14-3-3 protein also bind to p27 via phosphorylated Thr 198, and this binding has been thought to be responsible for the cytoplasmic translocation of p27. Indeed, Thr 198-phosphorylated p27 is largely restricted to the cytoplasm in cell exposed to growth stimuli, and the nuclear export of p27 during G1 phase is delayed by mutation of this residue to Ala (Kossatz et al., 2006). Phosphorylation of p27 on Thr 198 thus appears to be required for the timely exit of p27 from the nucleus after mitogenic stimulation and seems to be determinant of p27 stability, preventing ubiquitylation and proteasomal turnover. Kossatz et al. (2006) demonstrated that the half-life of $p27^{T198A}$ (a non-phosphorylatable form of p27) is shorter than the wild-type p27 form. Then, they confirmed that the increased turnover of $p27^{T198A}$ at steady-state levels was due to increased ubiquitylation, in fact $p27^{T198A}$ was able to incorporate more His-ubiquitin when compared with wild-type p27. Furthermore, treatment with the proteasome inhibitor MG132 stabilized $p27^{T198A}$ leading to expression levels comparable to wild-type p27. This protein degradation is only partially due to the Skp2 protein patway.

The binding of p27 to Jab1 or CRM1 (directly through its NES) has also been implicated in p27 translocation (Tomoda et al., 1999; Connor et al., 2003).

As described for p27 cytoplasmic localization, also p27 sequestration into higher order complexes with cyclin D-CDK4 is important for regulating p27 functions. Various growth signalling pathways stimulate the assembly of these heterotrimeric complexes, containing p27 with cyclin Ds and CDK4/6. Indeed, the MEK/ERK pathway which induces cyclin D transcription, favours the assembly of the cycling D-CDK4 , and sequesters p27 in inactive cytoplasmic complexes (Cheng et al., 1998). Also the proto-oncogene c-Myc, by increasing the expression levels of cyclin D and cyclin E, is responsible for p27 sequestration (Vlach et al., 1996; Perez-Roger et al., 1999; Bouchard et al., 1999), and this molecular event appears essential for Myc-induced cell cycle progression. More importantly, p27 sequestration seems to be one of the relevant mechanisms to human cancerogenesis, at least for lymphomas (Sanchez-Beato et al., 1999) and thyroid carcinomas. Overexpression of cyclin D3 in thyroid cancers caused p27 cytoplasmic sequestration by cyclin D3-cdk complexes (Baldassarre et al., 1999).

Moreover, both the Ras/Raf/Mek1 and PI3K/Akt pathways may impinge on p27 localization indirectly by their direct effects on cyclin D levels since, as mentioned above. MAPK activation upregulates cyclin D1 transcripts while AKT-dependent phosphorylation of GSK3-β inhibits cyclin D1 degradation (Pruitt and Der, 2001). Both of these mechanisms would increase the subsequent sequestration of p27, and prevent its association with the CDK2 complexes. Additionally, many of the molecules important for the proliferation, differentiation, survival and also adhesion and migration commonly classified as oncogenes, including BCR-Abl, Ras, PI3K, Myc, STAT, Fak, Src, Erk, and Jun, have the ability to modulate and/or downregulate p27, so that its expression may be a powerful prognostic marker since it may represent the readout of multiple different signals transduction pathways known to be involved in the onset and/or the development of human tumours.

2.5 Another function of p27: relationship p27 and extracellular matrix (ECM)

The tight crosstalk that exists between cells and surrounding extracellular matrix (ECM) components influences both cell growth and survival. ECM also provides background information and architectural scaffold for cellular adhesion and migration, eventually affecting the behaviour of both normal and neoplastic cells (Bissell and Radisky, 2001). Signals from the ECM are transduced mainly from a family of glycoprotein called integrins (reviewed in Hood and Cheresh, 2002) that activate several intracellular pathways, resulting in the formation of focal adhesion contacts and cytoskeleton reorganization, finally leading to cell cycle progression (reviewed in Giancotti and Ruoslahti, 1999). It is well established that integrin stimulation is necessary for the correct proliferation of adhesion-dependent growing cells. The observation that adhesion to ECM directly influences the expression levels of cyclin D1 or of the p27 proteins (reviewed in Schwartz and Assoian, 2001) provides a molecular explanation for this phenomenon. More recently, a role for cell cycle-regulating proteins in cell adhesion or motility on ECM substrates has been proposed (Juliano, 2003). Among the cell cycle regulators an important role has been recently demonstrated for the CKI p27 in the regulation of cell motility. p27 seems to act as an inhibitor of cell motility directed by chemotactic or aptotactic stimuli in smooth muscle (Sun et al., 2001), mesangial (Daniel et al., 2004), and endothelial cells (Goukassian et al., 2001), although

the precise molecular mechanism is still unclear. On the other hand, cytoplasmic p27 was shown to positively regulate of cell migration in bi-dimensional assays (Denicourt and Dowdy, 2004). In fact, different research groups demonstrated that p27 stimulated the scatter motility of hepatocellular carcinoma cells and mouse embryonic fibroblasts in wound healing assay (McAllister et al., 2003; Besson et al., 2004), proposing that p27 can induce rearrangements of the actin cytoskeleton, either in a Rac-(McAllister et al., 2003) or RhoA-dependent manner (Besson et al., 2004). By contrast, other studies showed that p27 inhibits the migration of endothelial (Goukassian et al., 2001) and vascular smooth muscle cells (Sun et al., 2001) through or within ECM substrates and in vivo. Although all these studies suggest that p27 plays a role in cell migration, it is not clear whether the discrepancy between these reports (i.e. whether p27 inhibits or promotes cell migration) might be a reflection of differences in the activity of cytoplasmic p27 in different cell types and/or of differences in the migration assays that were used. Baldassarre et al. (2005) demonstrated that p27 expression inhibits the migration of HT-1080 fibrosarcoma cells and normal mouse fibroblasts. The authors were able to localize the migration-inhibitory activity of p27 to the C-terminal 28 amino acids of the protein. Based on these evidences, they hypothesized that the C-terminal domain might bind to interacting protein(s) that are important in the regulation of cell motility. Using a yeast two-hybrid assay, the authors identified stathmin as a partner protein that binds to p27 and demonstrated by co-immunoprecipitation in vivo interactions between p27 and stathmin in HT-1080 sarcoma cells, pork brain, mouse foetal brain and normal mouse fibroblasts adherent to fibronectin.

3. Stathmin

Stathmin is a ubiquitous cytosolic phosphoprotein, proposed to be a small regulatory molecule and a relay integrating diverse intracellular signalling pathways involved in the control of cell proliferation, differentiation and activities. It is also called by different names (e.g., p17, p18, p19, 19K, metablastin, oncoprotein 18, LAP18, and Op18/stathmin). Stathmin was first identified as a 17-kDa cytosolic protein that is rapidly phosphorylated when HL60 leukemic cells are induced to undergo terminal differentiation and cease to proliferate (Feurestein and Cooper, 1983). Studies in several other leukemic cell lines also showed that stathmin expression is drastically decreased

when the cells ceased to proliferate upon exposure to a variety of differentiation agents (Luo et al., 1991). A growing set of arguments indicates that stathmin participates to the control of cell proliferation. In first instance it has been demonstrated that stathmin is expressed at higher levels during development than in adult tissue, although its expression pattern in embryonic tissue is variable. Stathmin expression is high in undifferentiated multipotent cells of the early embryo (Doye et al., 1992) and then after a slightly decline in most tissue its expression peaks at the neonatal period (Doye et al., 1989; Koppel et al., 1990). In the adult it expression strongly decreases, remaining abundant mostly in the nervous system, in the testis and in the germinal centre of lymph nods (Koppel et al., 1990; Peschanski et al., 1993). An important regulation of stathmin expression was also noted during induction of differentiation in a variety of cell types, with a high expression level in undifferentiated cells followed by a dramatic reduction during induction of differentiation (Schubart et al., 1989). Also in solid tumours like breast and ovarian cancers, higher stathmin expression was found in poorly differentiated cancers with high proliferative potential respect to more differentiated and less proliferative tumours. Interestingly, it has been shown that Stathmin is overexpressed in several types of human cancers, suggesting that it could also play a role in tumorigenesis (Melhem et al., 1991; Curmi et al., 2000; Price et al., 2000). More recently, a point mutated Stathmin protein, which carries the Q18E substitution, was identified in esophageal cancer. It has been reported that this mutation impairs Stathmin phosphorylation on S 16, S 25 and possibly S 38, thus resulting oncogenic for mouse fibroblasts (Misek et al., 2002).

3.1 Stathmin as microtubule regulator during cell cycle progression

The initial clue that stathmin may play a role in mitotic progression came from genetic studies that involved either antisense RNA inhibition or forced overproduction of stathmin in leukemic cells. Antisense inhibition of stathmin expression results in a marked decrease in the rate of proliferation of K562 erythroleukemic cells and their accumulation in the G2-M phases of the cell cycle. Surprisingly, overproduction of stathmin in leukemic cells also resulted in growth inhibition and accumulation of cells in the G2-M phases. These apparently paradoxical findings of mitotic arrest with both antisense RNA inhibition and overproduction of stathmin were later explained by a

study by Belmont and Mitchison (1996) that identified stathmin as a cellular factor that promotes microtubule depolymerization by increasing their catastrophe rate. The microtubule depolymerising activity of stathmin is regulated by changes in its phosphorylation status that occur during cell cycle progression. The level of phosphorylation of stathmin is significantly increased in the mitotic phase of the cell cycle relative to interphase. Stathmin is phosphorylated in vivo on four distinct serine residues (Ser 16, Ser 25, Ser 38 and Ser 63) by different protein kinases. All four Ser residues are phosphorylated during mitosis (Figure 5). Cyclin-dependent kinases, CDK2 and CDK1, have been identified as the kinases that phosphorylates Ser 25 and ser 38 (Brattsand et al., 1994; Larsson et al., 1995). Besides cell cycle-regulated phosphorylation, stathmin is phosphorylated by members of mitogen-activated protein kinase (MAPK) on Ser 25 (Leighton et al., 1993; Marklund et al., 1993; Marklund et al., 1993), by the Ca^{2+}/calmodulin-dependent kinase IV/Gr on Ser 16 (Marklund et al., 1994) and by cyclin AMP-dependent protein kinase (PKA) on Ser 16 and Ser 63 (Beretta et al., 1993). Phosphorylation of all four serines in mitosis switches off stathmin microtubule-depolymerising activity and allows the assembly of the mitotic spindle at the onset of mitosis. Several studies indicates that at the mitosis onset the active cyclin B1-CDK1 complex phosphorylates stathmin on serine 25 and 38. This event probably induces a conformational switch allowing the subsequent phosphorylation of Ser 13 and 63 by still unknown kinases, that determines the complete silencing of stathmin activity during the M phase. At the end of mitosis, the separation between the new daughter cells is achieved by a process referred to as cytokinesis. Cross-talk between the mitotic spindle microtubules and the actin cytoskeleton is believed to be essential for the formation of the acto-myosin contractile ring that completes the process of cytokinesis. Studies by Johnson et al. (1999) suggested that stathmin may be involved in the microtubule-dependent events of cytokinesis. When cytokinesis was blocked by Bistratene A treatment of HL60 cells, the cells became polyploidy and multinucleated (Johnson et al., 1999). This was associated with an increase in the level of stathmin expression and its level of phosphorylation. In a different study, Daub et al. (2001) showed that stathmin may be a downstream target of Rac/Cdc42, which are Rho GTPase-related proteins that are involved in the regulation of the actyn cytoskeleton and the initiation of cytokinesis. Taken togheter, these studies suggest that the microtubule regulatory function of stathmin may also be

required for the morphological changes associated with cytokinesis and the entry into a new cell cycle. The activity of stathmin during the mitotic phase of the cell cycle is regulated by phosphorylation, at least in part, by $p34^{cdc2}$. Interestingly, new lines of evidence suggest that the activity of stathmin may also be regulated at the transcriptional level by p53 and E2F, two transcription factors that play critically important roles in the regulation of cell-cycle progression. It was first demonstrated that induction of p53 by DNA-damanging agents results in a drastic decrease in the level of stathmin expression (Ahn et al., 1999). It was later shown that the p53-induced downregulation of stathmin expression is associated with accumulation of glioblastoma cells in the the G2/M phase of the cell cycle (Johnsen et al., 2000). In a different study, the G2/M arrest that is induced by genotic stress in NIH3T3 was shown to be associated with E2F-mediated downregulation of stathmin expression (Polager and Ginsberg, 2003). These studies suggest that stathmin might be involved in checkpoint pathways that prevent cell-cycle progression following exposure to DNA-damaging agents.

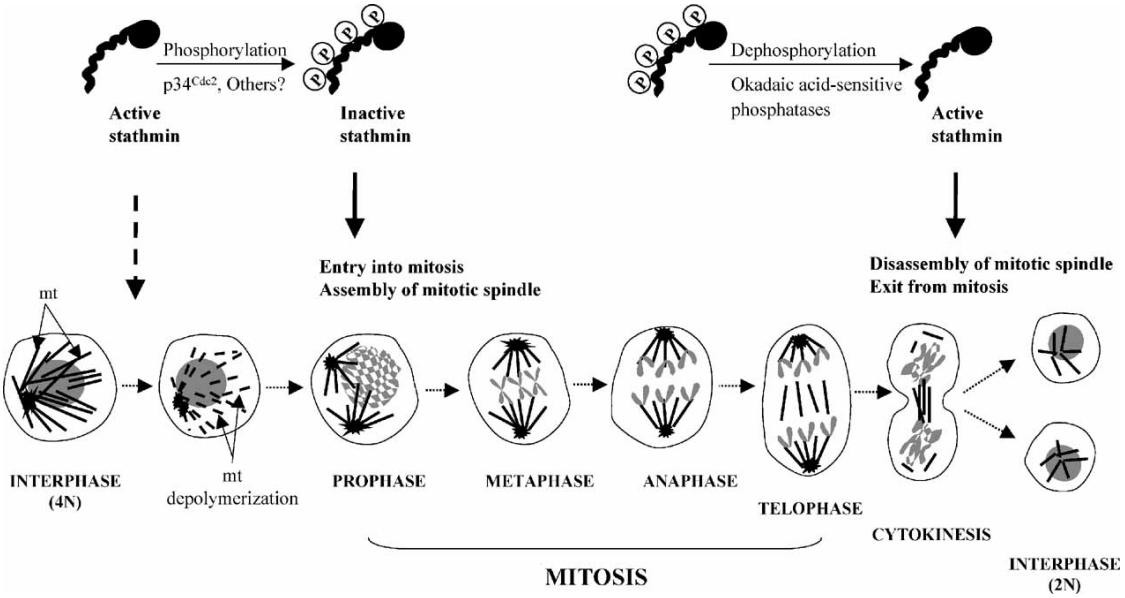


Figure 5. *Schematic model for the role of stathmin in the mitotic phase of the cell cycle.* The inactivation of stathmin by phosphorylation allows mitotic spindle assembly and entry into mitosis while its reactivation by dephosphorylation promotes mitotic spindle disassembly and exit from mitosis.

Microtubules consist of α/β tubulin heterodimers that exist in a state of continuous transition between polymerization and depolymerization events. This property, which is referred to as dynamic instability, is characterized by stochastic switching between phases of growth and phases of shrinkage. The transition from the phase of growth to the phase of shrinkage is known as "catastrophe" while the transition from the phase of shrinkage to the phase of growth is known as "rescue." In interphase, microtubules are long and relatively stable and their dynamics of growth and shrinkage are relatively slow. In contrast, at the onset of mitosis, when the interphase arrays of microtubules depolymerise and then repolymerise to assemble the mitotic spindle, microtubules are highly dynamic as a result of a marked increase in their rate of catastrophe (Belmont et al., 1990).

A least three major models have been proposed to explain the mechanisms by which stathmin destabilizes microtubules. In the first model, proposed by Belmont and Mitchison, stathmin destabilizes microtubules by increasing the rate of catastrophe during the dynamic transition between phases of growth and shrinkage (1996). A subsequent study by Curmi et al. suggested that stathmin slows elongation but does not act directly on microtubule ends to promote catastrophes (1997). The same group also showed that stathmin interacts with two molecules of dimeric α/βtubulin to form a tight ternary T2S complex (Jourdain et al., 1997). This group proposed an alternative model in which stathmin destabilizes microtubules by acting as a tubulin-sequestring protein, thus depleting the pool of tubulin available for polymerization (Figure 6). These discrepancies were later resolved by Howell et al., who showed that stathmin can have two different functional activities that are compatible with both models under different pH conditions. So they proposed a third model, in which stathmin mediates at least two distinct activities: a catastrophe-promoting activity, which requires the N-terminal region of stathmin, and tubulin-sequestering activity that requires the C-terminal region of stathmin (Howell et al., 1999; Larsson et al., 1999).

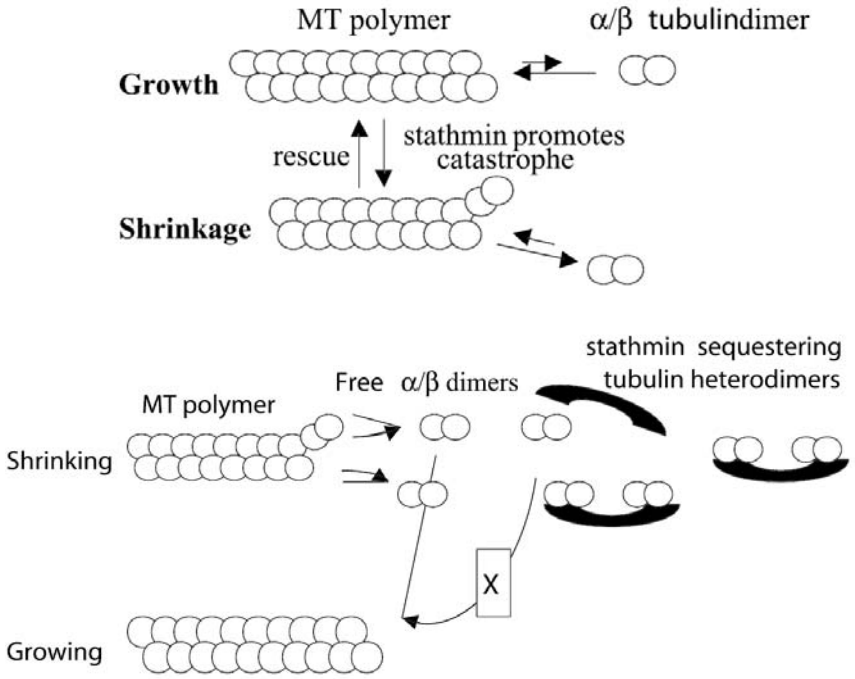


Figure 6. *Proposed models for stathmin induced destabilization of microtubules*. In the first one, stathmin promotes depolymerization of microtubules by increasing the rate of catastrophe during the dynamic transition between phases of growth and shrinkage. In the second one, stathmin binds to two molecules of tubulin heterodimer, therby depleting the pool of tubulin available for polymerization.

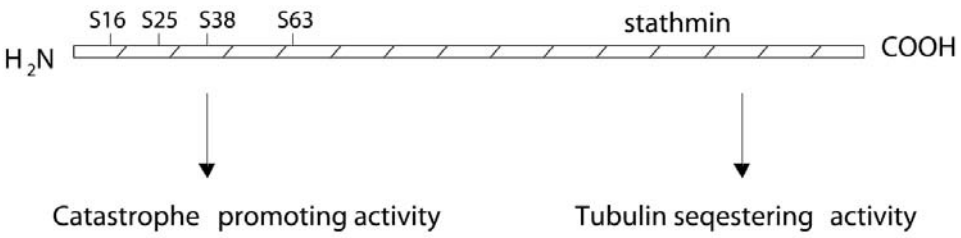


Figure 7. *Schematic representation of the dual activity of stathmin*. The N-terminal region promotes catastrophe-promoting activity and the C-terminal region promotes tubuli-sequestering activity.

3.2 Stathmin family

Stathmin is the most known members of a family of well conserved phosphoproteins which are the products of four distinct genes. They include SCG10, SCLIP and RB3 and its two splice variants RB3′ and RB3, all of which posses a common stathmin-like domain and additional specific NH_2-terminal extensions (Maucuer et al., 1993; Ozon et al., 1998; Ozon et al., 1997; Schubart et al., 1989; Stein et al., 1988). Stathmin is expressed ubiquitously, although more intensely in the nervous system, the other members of the stathmin family are mostly restricted to the nervous system. They are differentially regulated during neuronal differentiation and brain development (Ozon et al., 1998), and differentially expressed and regulated in the adult brain (Beilharz et al., 1998; Ozon et al., 1997; Ozon et al., 1999). Each protein has a MT-destabilizing activity (Cassimeris et al., 2002).

Beside regulation of cell cycle division, stathmin has been also implicated in many physiological processes like spermatogenesis (Marret et al.,1998) and neuritogenesis (Di Paolo et al., 1997), as also suggested by its expression pattern in adult tissue. In fact, the stathmin protein levels are dramatically increased during neurite outgrowth and synapse formation, suggesting a role for stathmin in neuronal differentiation (Di Paolo et al., 1997). The same group showed that inhibition of stathmin expression with antisense oligonucleotides prevents nerve growth factor (NGF)-induced differentiation of rat PC12 cells into sympathetic-like neurons. More recently, it has been demonstrated that stathmin phosphorylation on ser 16 is necessary for neuritis formation (Di Paolo et al., 1997), and, finally, stathmin was found to be expressed at high levels in the migrating cells of the rat olfactive system, suggesting its possible involvement in cell migration (Camoletto et al., 1997). Despite this plethora of functions that have been attributed to stathmin, the stathmin knockout mice generated by Schubart et al. (1996) seemed to develop normally and did not show any phenotype upon initial examination. However, more recently it has been reported that when stathmin-null mice age, they develop an axonopathy of the central and peripheral nervous system (Liedtke et al., 2002) and that have some alterations in their behaviour that resemble the human autism (Shumyatsky et al. 2005), further implicating stathmin in the physiology of nervous system. It is to note that Liedtke et al. showed increased expression of SCLIP, a stathmin-related protein, in the nervous system of stathmin null aging mice, showing

that SCLIP, like stathmin itself, is ubiquitously expressed suggesting that stathmin and SCLIP may be functionally redundant.

4. p27-stathmin interaction regulates cell migration

Baldassarre et al. (2005) showed that p27 interferes with the ability of stathmin to sequester tubulin, leading to increased microtubule polymerization. Although p27 and stathmin were originally discovered as important regulators of the eukaryotic cell cycle, both are now suggested to be involved in the process of cell migration. A dual role for stathmin in the regulation of cell proliferation and cell migration should not be surprising as microtubules are known to be important in both processes. The connection between the roles of p27 in cell proliferation and cell migration is not as obvious since these two functions of p27 are localized in different parts of the molecule and are exerted in different cellular compartments. The effects of p27 on cell motility were previously shown to be mediated through changes in the activities of Rho GTPases, which are important regulators of the actin cytoskeleton. The report of Baldassarre et al. provides the first indication that $p27^{Kip1}$ regulates migration by a direct effect on stathmin, a known regulator of the microtubule cytoskeleton.

Another question raised by this report is whether p27 and stathmin could also interact indirectly to regulate cell motility. Figure x illustrates four possible interactions between p27 and stathmin. The first is a direct interaction that represents the binding of p27 to stathmin, as suggested by Baldassarre and colleagues. The second is an indirect interaction between p27 and stathmin through the Rho GTPase pathway. Rac was previously shown to stabilize microtubules by phosphorylation-dependent inactivation of stathmin (Wittmann T et al., 2004). More recently, phosphorylation-dependent inactivation of stathmin, believed to be mediated by Rac, was shown to occur at the leading edge of motile A6 *Xenopus* cells (Niethammer et al., 2004). Interestingly, the stimulatory effects of p27 on cell migration also appear to require the activation of Rac (McAllister et al., 2003). Thus, p27 might regulate cell migration by activating Rac, which can promote phosphorylation of stathmin and lead to microtubule stabilization. In a third possible interaction, p27 might modulate stathmin activity indirectly through its inhibitory effects on cyclin–CDK complexes. As stathmin is a substrate for CDK1, inhibition of CDK1 by p27 might prevent the inactivation of stathmin and lead to

destabilization of microtubules. Alternatively, the inhibition of cyclin–CDK complexes by p27 might regulate stathmin function by a different mechanism. Because E2F transcription factors are known to activate the stathmin promoter (Polzin et al., 2004), inhibition of the CDK–Rb–E2F pathway by p27 might result in decreased stathmin expression and lead to microtubule stabilization. Finally, a fourth way in which p27 and stathmin might interact is through the human kinase interacting stathmin (hKIS). This kinase was first discovered as a stathmin partner protein and later shown to phosphorylate stathmin. Interestingly, KIS was recently shown to phosphorylate p27 and promote its translocation from the nucleus to the cytoplasm, where it has to be to regulate the activity of stathmin (Figure 8).

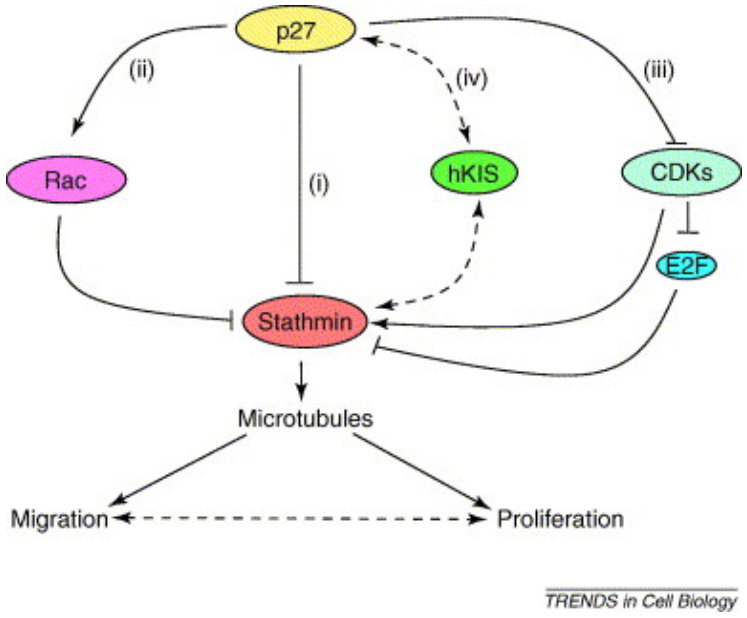


Figure 8. *Potential interactions between p27 and stathmin in the regulation of cell motility.* Four different mechanisms are described: (i) p27 might directly interact with stathmin and prevent its microtubule-destabilizing activity, (ii) p27 activates Rac, which can promote the phosphorylation of stathmin and inhibit its microtubule-destabilizing activity, iii) the inhibitory effect of p27 on cyclin-dependent kinases (CDKs) might result in either E2F-mediated downregulation of stathmin expressionor increased stathmin activity by means of CDK1 inactivation; (iv) p27 and stathmin might interact through the human kinase interacting stathmin (hKIS) that phosphorylates p27 and induces its translocation to the cytoplasm where stathmin is localized.

5. Effects of p27 loss in tumours

Over the last 15 years, many tumours suppressor gene have been identified. Their characterization has revealed prevalent involvement in human cancer, pinpointing the key mechanism that protect from tumour development. Because their cancer-preventive effects usually require the presence of only a single functional gene, prototypic tumour suppressor genes are recessive, requiring "two-hit" inactivation of both alleles (Knudson, 1971; Knudson et al, 1973; Comings, 1973). However, this strategy will fail under circumstances in which the second allele is epigenetically silenced or when the targeted gene is haploinsufficient for tumour suppression, a situation in which functional loss of only one allele confers a selective advantage for tumour growth (Cook and McCaw, 2000; Quon and Berns, 2001).

One well-defined instance of haploinsufficiency in the mouse involves the Cdk inhibitor, p27 (Fero et al., 1998). Animals lacking one copy of *Kip1* develop tumours spontaneously late in life and are highly sensitive to tumour induction by chemical carcinogens; however, the tumours that arise retain the normal *Kip1* allele, which encodes a fully functional protein. Although tumours arise faster in *Kip1* null animals, these data argue that a reduced dosage of p27, rather than its absolute absence, can contribute to cancer susceptibility. Yet, nullizygous animals for p27 display a further increase in tumour rate, and this observation lead many to a new definition for p27 that is a "dosage-dependent" tumour suppressor gene. The use of mouse models proved extremely useful to address the question of whether loss of p27 is casual or a secondary effect of tumour development. Experiments in p27-deficient mice established that p27 acts as a dosage-dependent tumour suppressor. $p27^{-/-}$ mice display increased body size of about 20-30% respect to wild-type littermates (Fero et al., 1996; Nakayama et al., 1996; Kiyokawa et al., 1996), due to increased cellularity of tissue, demonstrating a key role of p27 in the control of tissue growth also in vivo. In line with the dosage-dependent of tumour suppressive activity of p27, heterozygous mice showed about 50% of p27 protein expression and an intermediate phenotype in term of weight, indicating that, even in vivo, control of proliferation and body size is extremely sensitive to the levels of p27 protein. $p27^{-/-}$ mice are predisposed to spontaneous pituitary adenomas and develop multiple organ hyperplasia. Testing with radiation or chemical carcinogens revealed that p27 null mice were extremely susceptible to developing tumours in

multiple tissue, including adenomas, adenocarcinomas of the intestine and the lung, granulosa cell tumours of the ovary and uterine tumours of several histological subtype (Fero et al., 1998). In addition to a reduced tumour free survival, p27-heterozygous and deficient mice showed increased tumour multiplicity in the diverse sites. The analysis of biochemical pathways that contribute to neoplasia is facilitated by the comparison of specific mouse mutations, or even better, by combination of mutations. While p27-null mice are relatively tumour-free, loss of p27 in mouse models carrying other specific oncogenic lesions cooperates in enhancing tumour frequency and malignancy, decreasing tumour latency and increasing the organ spectrum of tumour formation.
A similar dosage effect might be functioning in human tumours, where point mutations or deletions in the coding region of the CDKN1B gene, are extremely rare even in the presence of loss of heterozygosity at chromosome 12p12-13 (Ponce-Castaneda et al., 1995), where the human CDKN1B gene is localized (Kawamata et al., 1995). Yet, loss or decrease of p27 protein expression is frequently observed in human cancer and this correlates with poor patient survival; in fact a plethora of studies shows involvement of p27 protein reduction or loss in wide variety of human tumours, including carcinomas of the colon, breast, lymphomas and soft tissue sarcomas. Multivariate analysis showed that reduced levels of p27 are of independent prognostic significance for many of these tumours. A number of recent studies have demonstrated the prognostic significance of p27 in human cancer. Decreased levels of total p27 protein are associated with high tumour grade and stage in human breast (Catzavelos et al., 1997), colorectal (Ciaparrone et al., 1998; Sgambato et al., 1999; Fredersdorf et al., 1997), and gastric cancer (Yasui et al., 1997), among others. Reduction of p27 correlates significantly with decreased survival in breast (Catzavelos et al., 1997; Porter et al., 1997; Tan et al., 1997), colorectal (Loda et al., 1997), gastric (Ciaparrone et al. 1998; Mori et al., 1997), ovary (Masciullo et al., 1999; Newcomb et al., 1999), prostate (Cote et al., 1998), bladder transitional cells (Del Pizzo et al., 1999; Korkolopoulou et al., 2000), and oesophageal squamous cell carcinoma patients (Shamma et al., 2000), among others.

5.1 p27 in brain tumors

In non-astrocytic and non oligodendrocytic tumors of the nervous system, such as meningiomas and malignant medulloblastomas, neuroblastomas and malignant

lymphomas, p27 is often poorly expressed perhaps reflecting lack of p27 expression in the normal counterpart of tumour cells. p27 is expressed in normal oligodendrocytes and in differentiated glial tumours, decreasing with anaplasia and malignancy. Cavalla et al. (1999) proposed p27 as an independent prognostic factor for human gliomas and found that p27 decreased with anaplasia and almost disappeared in glioblastoma (GBM), whereas Skp2 was absent or poorly expressed in well differentiated astrocytomas and it was diffusely or focally expressed in most GBM (Schiffer et al., 2002). However, controversial data came from other smaller studies, reporting wide and high p27 expression in a panel of 20 human malignant gliomas (Naumann et al., 1999) and no apparent correlation between p27 expression and any other parameters tested, pointing to the heterogenecity of gliomas.

The classification of neurological tumours is based on the predominant cell type(s), which is generally determined by morphological and immunohistochemical criteria. After development ceases, neurons become post-mitotic and only a small compartment of stem cells remain, whereas glial cells retain the ability to proliferate throughout life. In this context, it is perhaps not surprising that most adult neurological tumours are of glial origin. These tumours are termed gliomas, and include tumours that are composed predominantly of astrocytes (astrocytomas), oligodendrocytes (oligodendrogliomas), mixtures of various glial cells (for example, oligoastrocytomas) and ependymal cells (ependymomas) (Kleihues et al., 2000; Maher et al., 2001; Holland, 2001).

In the central nervous system (CNS), multipotent neural stem cells in the ventricular/subventricular zones of the embryonic neural tube give rise to three main cell types in the mature CNS — neurons, and oligodendrocytes and astrocytes. The classification of neurological tumours is based on their predominant cell type(s). For example, astrocytoma is composed primarily of astrocytes, oligodendroglioma is composed primarily of oligodendrocytes, and oligoastrocytoma contains both astrocytic and oligodendroglial components. A few pediatric brain tumours, such as medulloblastoma, are derived from neuronal precursor cells.

The World Health Organization (WHO) grading system classifies gliomas into grades I–IV based on the degree of malignancy, as determined by histopathological criteria. In the CNS, grade I gliomas generally behave in a benign fashion and, in many cases, might even be circumscribed, whereas grade II–IV gliomas are malignant and diffusely infiltrate throughout the brain. Astrocytomas are the most common CNS neoplasms,

accounting for more than 60% of all primary brain tumours. The most malignant form of infiltrating astrocytic neoplasm — glioblastoma multiforme (GBM) (WHO grade IV astrocytoma) — is one of the most aggressive human cancers, with a median survival of less than 1 year. Grade IV astrocytoma can be divided into two subtypes based on clinical characteristics: primary and secondary GBM. Primary GBM arises as a *de novo* process, in the absence of a pre-existing low-grade lesion, whereas secondary GBM develops progressively from low-grade astrocytoma, generally over a period of 5–10 years.

GBM is defined by the hallmark features of uncontrolled cellular proliferation, diffuse infiltration, propensity for necrosis, robust angiogenesis, intense resistance to apoptosis and rampant genomic instability. As reflected in the old name "multiforme," GBM presents with significant intratumoral heterogeneity on the cytopathological, transcriptional, and genomic levels.

Genetic studies of GBMs, particularly secondary GBMs, indicate that there are distinct genetic pathways involved in the initiation and progression of these neoplasms. Several genetic pathways are involved in the initiation versus progression of secondary glioblastoma multiforme (GBM). For example, loss of *TP53* and activation of the growth-factor–RTK (receptor tyrosine kinase)–RAS pathway (such as through overexpression of *PDGF*/*PDGFR* (platelet-derived growth factor/PDGF receptor) or loss of neurofibromatosis type 1 (*NF1*)) are involved in the initiation of pilocytic (grade I) or low-grade (grade II) astrocytoma. These can progress to anaplastic astrocytoma (grade IV) or secondary GBM (grade IV), which has been associated with disruption of the retinoblastoma (RB) pathway (through loss of *RB* or amplification/overexpression of *CDK4*). In primary GBM, the same genetic pathways are dismantled, although through different mechanisms. For example, disruption of the p53 pathway often occurs through loss of the gene that encodes ARF, or less frequently through amplification of *MDM2*. Disruption of the RB pathway occurs through loss of the gene that encodes INK4A. Amplification and/or mutation of the gene that encodes epidermal growth factor receptor (EGFR) is the most frequently detected genetic defect that is associated with primary GBM, also activity of the phosphatase PTEN is also frequently disrupted in this type of tumour. PTEN, at 10q23.3, is a tumour suppressor gene that was altered in 30-44% of high-grade gliomas (Wang et al.,1997). It has been suggested that PTEN may correlate with p27 activity, and loss of PTEN has been linked inversely to the

duration of survival (Li and Sun, 1998). PTEN expression may cause the accumulation of p27, raising the possibility that the phosphatidylinositol-3 kinase signalling pathway may regulate the level of p27 and block cell cycle progression in the G1-phase. The expression of p27 in malignant astrocytomas appears to reflect the proliferation activity of tumour cells.

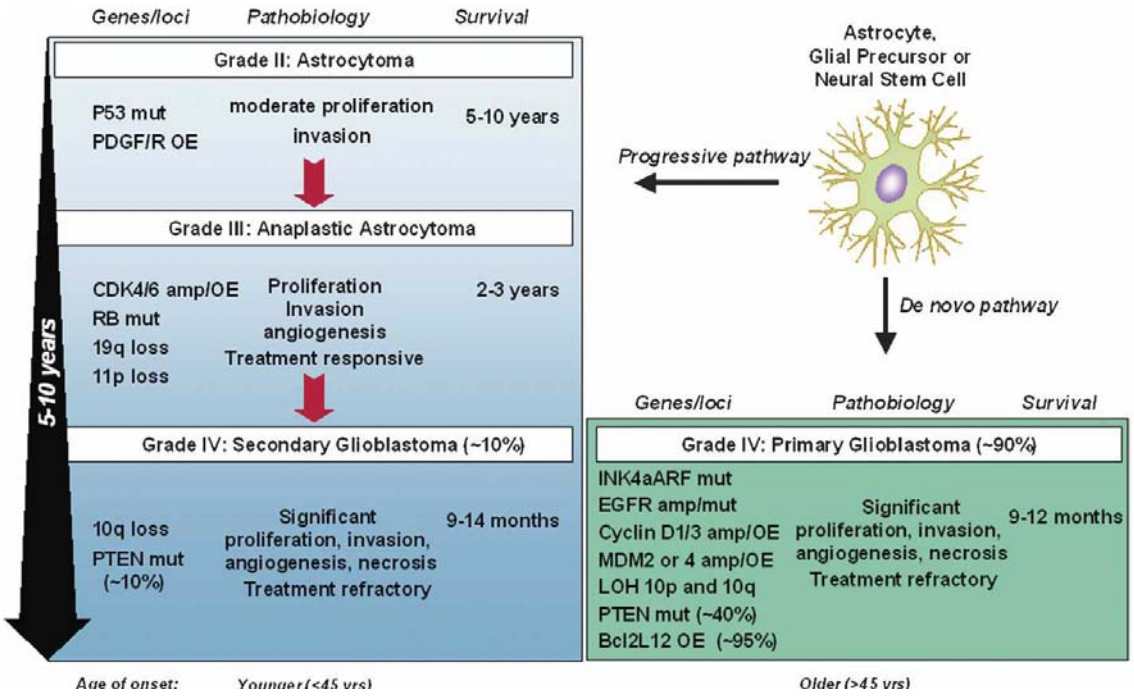


Figure 9. *Chromosomal and genetic aberrations involved in the genesis of glioblastoma.* Shown are the relationships between survival, pathobiology, and the molecular lesions that lead to the formation of primary (de novo) and secondary (progressive) glioblastomas. Although histologically indistinguishable, these grade IV gliomas occur in different age groups and present distinct genetic alterations affecting similar molecular pathways.(Furnari et al., 2007).

6. Gene therapy approach in GBM

Established treatments such as surgery, radiation and chemotherapy have not altered the median survival of glioblastoma and since these failures reflect the highly invasive nature of glioblastoma, as well as the fact that few cells are actively replicating at any given point in time, therapies need to act in areas of the brain distant from the site of tumour origin and for long after their introduction. GBMs are good targets for gene

therapy as they are generally well localised and rarely metastasise outside the central nervous system.

Over the past decade, laboratory studies and early clinical trials have raised hope that these therapeutic requirements may be fulfilled by gene therapy using non-replicating transgene-bearing viruses, oncolytic viruses or migratory stem cells to deliver tumoricidal transgenes (reviwed in Aghi and Rabkin, 2005). To date, vectors based on retroviruses or adenoviruses have been used most frequently in cancer gene (Dyer and Herrling, 2000). Studies comparing either retrovirus producing cells and replication deficient adenoviral vectors' efficiency in transducing human glioma tumours found higher gene transfer efficiency and greater survival times with replication deficient adenoviral vectors (Puumalainen et al., 1998; Sandmair et al., 2000).

Under natural conditions, adenoviruses are usually associated with minor human diseases such as upper respiratory tract infections, keratoconjunctivitis, and gastroenteritis. Under experimental conditions, early clinical vaccination with wild-type live adenoviruses showed no significant side effects, demonstrating the relative safety of adenoviruses as vector for in vivo gene therapy. They efficiently infect and transfer genes in a wide variety of cell type including dividing and non-dividing cells. Unlike retroviruses, adenoviruses do not integrate into the host genome. This gives adenoviral vectors an important safety feature for in vivo gene therapy. In addition, given the fact that main goal of cancer gene therapy is to eliminate rather than rectify malignant cells, there is generally little need for integration. Viewed from one perspective, a disadvantage of using adenoviral vectors for long-term gene expression is their capacity to elicit strong inflammatory and immunological responses. However, when viewed from another perspective, this immunogenicity may enhance tumour-killing in cancer gene therapy. Furthermore, adenoviral vectors are easy to manipulate by classical recombinant techniques and express transgenes at high levels, are producible at high titers.

6.1 Adenoviruses

To date, 49 different human adenoviruses serotypes have been isolated and classified into six distinct subgroups (A through F) (Wadell, 1984). The serotypes 5 and 2 of subgroup C viruses have been used most widely for construction of gene transfer

vectors. Adenoviruses are nonenveloped viruses. The icosahedral capsid of adenoviruses consists of predominantly three proteins: hexon (in red Figure 10), penton base (in yellow Figure 10) and fiber (in green Figure 10) along with several minor proteins including VI, VIII, IX, IIIa and IVa2.

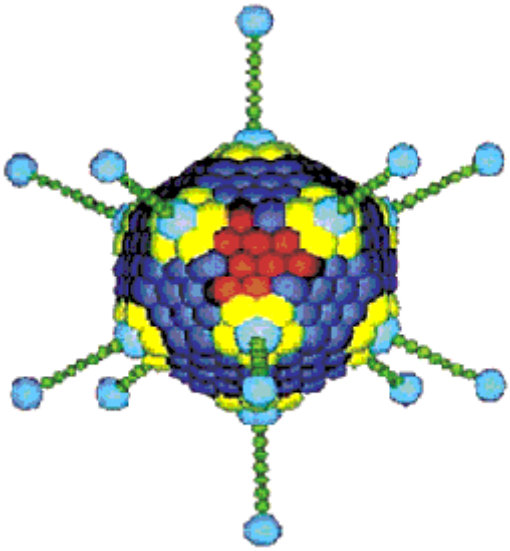


Fig 10. *Schematic representation of adenoviral capsid.*

The adenoviral genome contains a linear double-stranded DNA of approximately 36 kb, with relatively short terminal repeats (ITRs) whose inverted structures plays a role in replication of the DNA. (Rekosh et al., 1977). The adenoviral genome encodes for at least 30 different mRNA transcripts (Mathews and Shenk, 1991), which can be divided into early and late phases based upon the onset of viral DNA replication. The viral proteins encoded by early genes are involved in modulation of the cell cycle, viral DNA replication, and invasion of the host immune system. The late gene products are mainly viral structural proteins necessary for virus assembly, such as hexon, penton base and fiber. The E1A and E1B proteins are the first viral early proteins to be produced from early region 1 (E1) after the viral DNA enters the nucleus. These proteins are essential for viral replication. They engage in a number of regulatory functions, including activation of gene transcription within the other early and late regions. The replication cycle of adenovirus type 5starts with the attachment of the viral fiber protein to a primary cellular receptor (Nemerow, 2000). Three types of cell surface molecule have been identified as a primary cellular receptor for adenovirus: the coxsackievirus group

B and adenovirus receptor (CAR) (Bergelson et al., 1997), the MHC-I α2 subunit (Hong et al., 1997) and sialic acid saccharides on glycoproteins (Arnberg et al., 2000). The interaction between fiber and a primary receptor allows the penton base protein to bind to a second cellular receptor, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins, which promotes internalization of the adenoviral particle via receptor mediated endocytosis (Wickham et al., 1993). Once inside a cell, a sequential disassembly of the outer capsid proteins allows the virus to escape from endosome and enter the cytoplasm. Virus particles are subsequently transported to the nuclear pore complex and release the viral DNA into the nucleus to initiate viral gene expression (Greber et al., 1993). The E1A protein is first produced, which induces expression of the other early proteins encoded by the E1B, E2, E3 and E4 regions. Viral DNA replication begins approximately 7 hours after infection, after which late proteins expressed from the late regions are synthetized. Viral assembly begins in cell nucleus at 20 to 24 hours after infection, and after 2 to 3 days the cells dead and release virus particles (Figure 11).

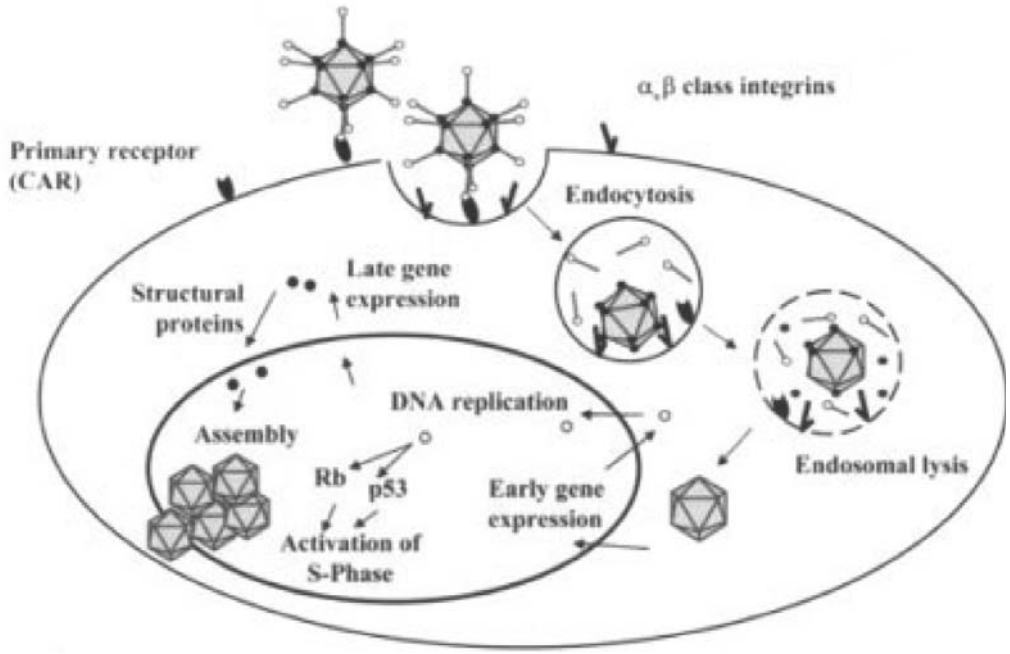


Figure 11. *The adenovirus infection pathway*. Cell entry is initiated by high-affinity binding of the fiber to its primary receptor, CAR. CAR binding is followed by endocytosis mediated by penton-base interaction with cellular $\alpha_v\beta_.$ integrins. After endosomal lysis, viral DNA is transported to the nucleus through a microtubule-mediate process, and viral genes or transgenes are expressed.

Adenoviruses can accommodate up to 7.5 kb of foreign DNA without significant effects on their stability and infectivity. However, if a larger size of foreign DNA has to be inserted, this would necessitate deletion of part of the viral genome. The latter manipulation has led to the development of three types of adenoviral vectors: the first generation viral vectors are usually recombinant adenoviruses that contain transgenes in replacing E1A and E1B genes (Bett et al., 1995). The purpose of the E1 deletion is to abolish the potential oncogenicity of adenovirus vectors, and to make vector replication deficient as well as to expand the cloning capacity of adenoviral vectors. Since E1 proteins are essential for viral replication, the propagation of E1-deleted adenoviral vectors requires complement cell lines to provide in trans the E1 protein, such as HEK 293 cell lines. The major drawback of first generation adenoviral vectors is their tendency to elicit strong immune responses against the viral vector capsid proteins and infected cells expressing viral antigens. Second generation adenoviral vectors have been constructed by further deletion of additional early genes necessary for viral DNA replication, in fact these vectors with double deletions (E1 and E3) or triple deletions (E1, E3 and E4) can accommodate up to 10 kb of foreign DNA (Gao et al., 1996; He et al., 1998). Recently a third generation of adenovirus vectors has been reported; it is being developed by removing all viral genes except the cis-acting sequences necessary for viral DNA replication and packaging (Kochanek, 1999; Russell, 2000). These gutless or mini adenoviral vectors can accommodate large inserts up to 37 kb. In addition, they appear to give less immune responses and much prolonged expression of transgenes in vivo.

The first generation vectors are the ones that are used most frequently for delivering immunogenes, tumor suppressor gene and chemogenes in vivo in cancer gene therapy. Their cloning capacity is compatible with most applications and they are easy to growth to large quantities. Immune responses, especially characteristic of the early generation adenoviruses, may have provided additional anti-tumor effects in the context of most cancer treatment protocols (Kay et al., 2001; Sandmair et al., 2000; Danthinne and Imperiale, 2000).

MATERIALS and METHODS

1. Cell culture

Human embryonic kidney cells (HEK 293, ATCC CRL-1573) were used to generate recombinant Adenovirus (Ad) and were grown in Dulbecco's Modified Eagle's Medium (DMEM, Cambrex Bio Science) with 10% FBS and Penicillin-Streptomycin 1%.

Human malignant glioma cell lines U87MG, U138MG, T98G, U251MG, SF268, SBN75, U373, SF539, SF295, SBN19 were kindly provided by Prof. A. Fusco, Università Federico II di Napoli (Italy) and maintained in RPMI-1640 medium (Cambrex Bio Science) supplemented with 10% heat inactivated FBS and Penicillin-Streptomycin 1%.

HUVEC (Human Umbelical Vein Endothelial Cells) were isolated from human umbelical cord vein as described previously (Jaffe et al., 1973). Cells were cultured in Medium 199 (GIBCO) supplemented with 20% FBS, Penicillin-streptomycin 1%, 50 µg/ml heparin and bovine brain extract (0.5%) and plated in dishes previously coated with 1% porcine skin gelatin.

2. Tissue samples

A total of 8 brain tumors were collected and diagnosed at University La Sapienza Rome (Italy) according to the World Health Organization (WHO) criteria. For immunohistochemical staining, 4 glioblastoma multiformes (GBM, Grade IV) and 4 anaplastic astrocytomas (AAs, Grade III) were evaluated using an anti-human p27 antibody from Dako.

3. Recombinant adenovirus

Tetracycline-inducible recombinant Ads were constructed according provider's instructions (Adeno X Tet ON Expression System 2, Clontech). Briefly, the Donor Vector (pDNR-CMV) containing p27 gene was combined with Adeno-X TRE Acceptor Vector using a Adeno-X LP Reaction Mix, pre-aliquoted solution composed of Cre Reaction Buffer, BSA and pLP-Adeno-X Viral DNA. Cre Recombinase was added and incubated at room for 15 min. Cre bound to the loxP sites on both the Donor Vector and acceptor Vector, cleaved the DNA and then catalyzed strand exchange and ligation of

the DNA. As a result, p27 gene and chloramphenicol resistance gene were transferred in unison from the Donor Vector to the Acceptor Vector (Fig 1). Recombinants was isolated by transforming ElectroMAX^{TM} DH10β cells (Invitrogen) with electroporation and selecting on medium containing both chloramphenicol and sucrose: chloramphenicol to select for cells containing recombinant vectors, sucrose to select against those with Donor Vectors. Following selection, recombinant clones were positively identified by PCR and restriction analysis using PI-Sce I, I-Ceu I (Clontech) and XhoI (Promega) enzymes and then the chosen positive clones were amplified and purified using QIAGEN Plasmid Purification Maxi Kit. Adenoviruses were produced by transfecting with FuGENE (Roche) recombinant adenoviral vectors into HEK 293 cells. Before transfection, the vectors were digested with Pac I to linearize the DNA and to expose the inverted terminal repeats (ITRs) that flank the Adeno-X genome. Then HEK 293 cells were checked every day for cytopathic effect (CPE), and once detached from the plate were harvested and centrifuged at 3000 rpm for 5 min. Because adenovirus remains associated with cells until late in the infection cycle, high titer virus was obtained by lysing cells with a series of freeze-thaw cycles. After the third cycle, the pellet debris was centrifuged to recover the viral suspensions, which stored at -20°C. Then recombinant adenoviruses were amplified to prepare high-titer stocks. For animals experiments recombinant Ads were purified (BD Adeno-X Virus Purification kit) and eventually concentrated using Centricon Centrifugal Filter Devices YM-50.

The Knockout RNAi System (Clontech) was used following the manufacturer's procedures and inserting the appropriate sequence:

Stathmin Si RNA 1: Target sequence: 5'-tcccatgaagctgaggtc-3' (from base 268 Genbank X53305)

Stathmin Si RNA 2: Target sequence: 5'-cgtttgcgagagaaggata-3' (from base 445 Genbank X53305)

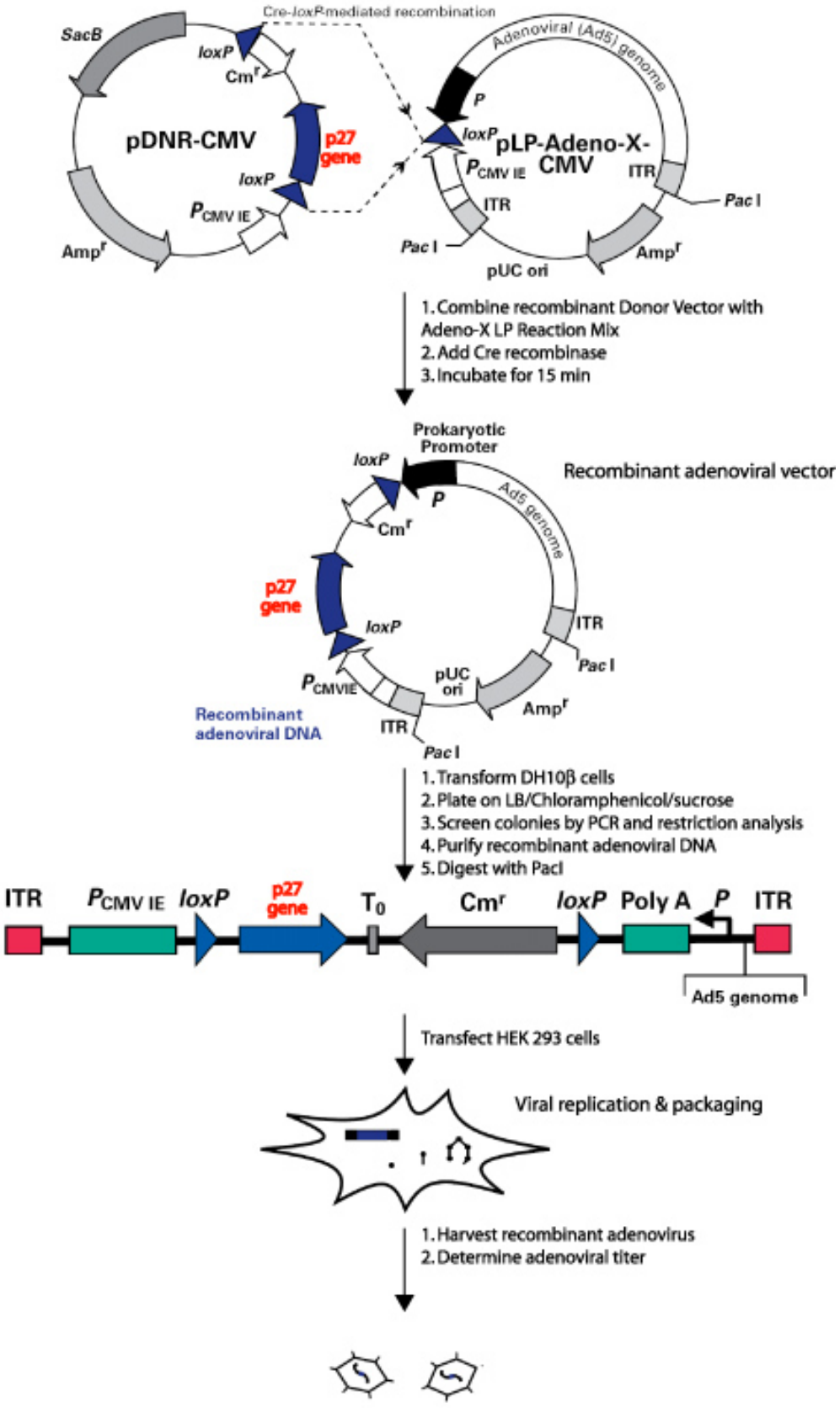


Figure 1. Constructing recombinant adenovirus.

4. X-gal staining

GBM cells were transduced (or not) as indicated with AdTRE-βGal/AdTet-ON in presence or absence of doxycycline and 48 hrs post transduction were fixed 2% formaldehyde and 0,2% glutaraldehyde for 5 min at 4°C. Fixed cells then were washed and incubated with x-gal solution overnight at 37°C; the positive cells, expressing the β-galactosidase protein, turned blue.

To assay β-galactosidase activity, lysates prepared from transduced cells with AdTRE-βGal/AdTet-ON as indicated were performed using β-galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega) according to the manufacture's instructions.

5. Titration of adenoviral stocks

Viral stock titers were determined using HEK 293 cells. These cells were seeded in 12-well plate at density $2x10^5$/well and infected with 100 µl of viral sample 10-fold serial dilutions from 10^{-2} to 10^{-6}. After 48 hrs at 37°C, cells were fixed with ice-cold 100% methanol, washed three times with PBS + 1% BSA and incubated with goat anti-hexon primary antibody (1:400) (Chemicon) for 1 hr at 37°C. Incubation with anti-goat secondary antibodies HRP conjugate was performed for 1 hrs at 37°C in PBS 1% BSA, then DAB solution was added for 10 min at room temperature. Once removed DAB, brown positive cells were counted with 20X objective, and the mean number of positive cells in each well was calculated. The infectious units (ifu/ml) for each well were calculated as follows: [(infected cells/field)x(fields/well)]/[volume virus (ml)x(dilution factor)].

6. Preparation of Cell lysates, Immunoblotting and Immunoprecipitation

Cells were harvested and lysed in cold NP-40 buffer containing protease inhibitors (Roche), sodium orthovanadate 1mM and DTT 1mM. After incubation on ice for 20 min, the lysates were centrifuged at max speed for 15 min at 4°C to recover the supernatant.

For nuclear/cytoplasmatic fractions, cell monolayers were washed and harvested in ice-cold PBS and a ice-cold hypotonic buffer (buffer A), containing protease inhibitors, were added to a cell pellet (250-800 µl for 100mm dish). After incubation on ice for 15 min, 10% of NP-40 were added and after vortex treatment the samples were centrifuged at 6000 rpm for 1 min at 4°C to recover a cytoplasmic fraction (supernatant). The pellet was washed three times with sucrose buffer and resuspended in 1/5 of the starting volume in ice-cold hypertonic buffer (buffer C) containing protease inhibitors. After incubation on ice for 20 min, the samples were centrifuged at max speed for 15 min at 4°C to recover a nuclear fraction (supernatant).

Immunoprecipitation experiments were performed using 0.5/1.0 mg of total lysate resuspended in HNTG buffer and protein A/G-sepharose (Amersham). True Blot™ reagent (e-Bioscience) was used as secondary anti-mouse or anti-rabbit antibody.

For immunoblotting proteins were separated in 4-20% SDS-PAGE (Criterion Precast Gel, Biorad) and transferred to nitrocellulose membranes (Amersham). Blots were blocked with dried milk in TBS-T and incubated at 4°C overnight with primary antibodies. Primary antibodies were from: Transduction Laboratories (p27, CDK1, CDK2 and Beclin-1), Santa Cruz (p27 C19, p27 N20, p27, CDK1, Cyclin B1, Cyclin A, vinculin, Akt, c-Abl, caspase3 and caspase7), Sigma (Stathmin and α-tubulin), Cell Signaling (pS473Akt) and Upstate Biotechnology (Histone-H1). Then the membranes were washed, incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies and further processed for chemiluminescent detection (ECL Kit, Amersham).

7. Migration experiments

GBM cell lines in response to ECM substrates was assessed by fluorescence-assisted transmigration invasion and motility assay (FATIMA) (Spessotto et al., 2000). The procedure is based on the use of Transwell-like inserts carrying fluorescence shielding porous polyethylene terephthalate (PET) membranes (polycarbonate-like material with 8 µm pores), HTS FluoroBlokTM inserts (Becton-Dickinson, Falcon, Milan, Italy).

For migration, bottoms of HTS Fluoroblok (Becton Dickinson) were coated on the upper side with 20µg/ml FN, 10µg/ml Vitronectin, 10µg/ml Collagen I or 10µg/ml

Collagen VI in bicarbonate buffer at 4°C overnight and then saturated with PBS 1% BSA for 2 hrs at room temperature. Cells were labeled with DiI (Molecular Probes) for 20 minutes at 37°C before being seeded in the Fluoroblok™ upper chamber and then incubated at 37°C for the indicated times. Migratory behavior of the cells was then monitored at different time-intervals by independent fluorescence detection from the top (corresponding to non-transmigrated cells) and bottom (corresponding to transmigrated cells) side of the membrane using the computer-interfaced *GENios Plus* (TECAN Italia S.r.L.). Each experiment was performed at least 3 times, in duplicate. In some experiments migration was blocked at the indicated time point, by fixing the Fluoroblok membranes in 4% PFA and the Fluoroblok™ membrane mounted on a slide, to allow the count of migrated cells.

For 3D Matrigel™ invasion assay, Fluoroblok™ upper chamber were coated with 80μg/ml Matrigel™ overnight at 4°C, then allowed to dry at room temperature and re-hydrated with PBS+1% BSA. Transduced GBM cells were seeded in the Fluoroblok™ upper chamber coated and then incubated at 37°C for the two days. Fluoroblok™ membranes were then fixed in 4% PFA, cells stained with Propidium Iodide as indicate above and the Fluoroblok™ membranes mounted on a slide, to allow the count of migrated cells. At least 10 randomly selected field/membrane were counted in 2 different experiments performed in duplicate.

8. Matrigel™ evasion assay

For 3D Matrigel™ evasion assay, cells ($3x10^5$/ml) were transduced as indicated and 48 hours later were included in Matrigel™ (6 mg/ml, Becton Dickinson) or in Collagen I (1 mg/ml, Becton Dickinson) drops and allowed up side down to polymerized at 37°C for 1 hr. Then the drops were incubated for the indicated times in complete medium. Cell motility were observed by transmission microscopy using a Nikon TS100/F microscope. Images were collected using a digital camera (Nikon).

9. Growth curve

Cell proliferation was evaluated using growth curve. GBM cells were transduced (or not) as indicated and 48 hrs later were seeded in 6 cell well plate at density $1x\ 10^5$

cell/well. Cells were trypsinized, and counted after trypan blue (Sigma) staining as the indicated time.

10. MTT assay

Cell proliferation was evaluated also using MTT assay. GBM cells were transduced (or not) as indicated and 48 hrs later were seeded in 96 well plates at density 1000 or 5000 cells/well for MTT assay (Sigma). For this assay at the indicated times, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) yellow reagent was added to the cells and incubated for 4 hours at 37°C in complete medium. MTT is reduced to purple formazan in the mitochondria of living cells. Medium was then discarded and after solubilization with dimethyl sulfoxide (DMSO), the reduced form of this colorimetric substrate was quantified in an Elisa plate reader at 560 nm.

11. Cell cycle analysis

U87MG, U251MG and HUVEC were trypsinized 48 hours post-transduction and fixed with 70% ice-cold ethanol. Fixed cells were twice washed with PBS and labeled with propidium iodide staining. The stained cells were subjected to flow cytometry analysis (FACS) with a FACScan and a FACSCalibur instrument (BD Biosciences). Data were analyzed with a cell cycle analysis software program to calculate the distribution of cells in G1, S and G2/M phases of the cell cycle.

12. Colony assay

For colony assay, 48 hrs post-transduction cells were trypsinized, counted and seeded at density 1000 and 5000 cell/100 mm dish and incubated in complete growth medium with or without doxycycline as indicated. Two weeks later plates were stained with crystal violet solution and colonies counted.

13. Immunofluorescence Analysis

For immunofluorescence staining, HUVEC plated on Matrigel™ drops or GBM cells adhered to FN coated coverslips for the indicated time were fixed in PBS 4% paraformaldehyde (PFA) at room temperature (RT), permeabilized in PBS 0.2% Triton X-100 and blocked in PBS 1% BSA, 10% normal goat serum. Incubation with primary antibodies was performed for 3 hrs at RT (or overnight at 4°C) in PBS 1% BSA and 1% normal goat serum, then samples were washed in PBS and incubated with secondary antibodies for 1 hour at RT. Nuclear staining was performed with 5 μg/ml Propidium Iodide in PBS supplemented with 100μg/ml of RNase A for 30 minutes at RT or with 1 μg/ml Hoechst 33258 in PBS for 10 minutes at RT. Coverslips were mounted in Mowiol 4–88 (Calbiochem-Novabiochem) containing 2.5% (w/v) DABCO (Sigma). Images were acquired with a Leica TCS SP2 confocal system (Leica Microsystems Heidelberg, Mannheim, Germany), using the Leica Confocal Software (LCS) or using a Nikon Diaphot 200 epifluorescent microscope.

14. Animal Experiments

Human glioblastoma xenografts were established by subcutaneous injection of 10^7 U87MG cells into female athymic nude mice (Harlan, 8 weeks of age). When tumors were ca 40-60 mm^3 (about 15 days from injection) the animals (10 mice/group of treatment) were randomly divided into groups according to experimental design and intratumoral injections were performed with $2x10^9$ total ifu (AdTRE/AdTet-ON, ratio 80:40) and repeated five times every two days. Tumor size was measured with a caliper three times a week and volume was calculated (0.5x length x $width^2$). Unless differently indicated, animals were sacrificed after 15 days of treatment and tumor analysis was performed.

In the tumor prevention models, 7 x 10^6 U87MG cells were transduced with ratio 80:40 AdTRE/AdTet-ON and then harvested 48 hrs later. Cells were then washed, resuspended in DMEM without red phenol and injected subcutaneously into nude mice (8 mice/group of treatment). Animals were sacrificed after 25 days for tumor analysis. For *in vivo* induction of p27 expression, two days before cell injection and for all the

course of the experiments, drinking water was supplemented with 1 mg/ml doxycycline and 2.5% w/v sucrose, and changed every 2 days.

15. Histological evaluation and determination of microvessel density on xenograft tumor sections

Explanted tumors were formalin-fixed and included in paraffin. At least 10 sections/tumor stained with H&E were analyzed in blind by an expert pathologist in order to evaluate the presence of local invasion.

For microvessel density analysis, explanted tumors were included in OCT and frozen in liquid N2. Sections were incubated with anti-mouse CD31 (PECAM-1) Monoclonal antibody 1:20 (BD Pharmigen, Cat. 550274) and visualized via three-step staining procedure in combination of biotinylated polyclonal anti-rat Igs mouse-adsorbed (Vector Laboratories, BA-4001, 5 ug/ml) and Streptavidin-HRP (Sigma, 1:400) using DAB as substrate. The slides were then counterstained with hematoxylin. The number and the length of CD31-positive vessel were calculated in blind using a Leica microscope coupled with the Leica IM software. Ten randomly selected fields/section and at least 4 sections/tumor were analyzed. For each treatment group, 4 different tumors were analyzed. Only vessels >10μm in length were considered.

16. Cytokines/ growth factors expression pattern using an antibody array.

Parental and Ad/p27^{wt} transduced U87MG cells were plated at low density ($5x10^5$ cells) in 100mm dishes. After 48 hours medium was changed to serum free RPMI medium supplemented with 1% BSA and 1μg/ml of doxycycline (6 ml/dish) and cells cultured at 37°C for additional 4 days before collection. The conditioned mediums were centrifuged to eliminate cells debris and then used fresh on HUVE cells tube-formation experiments or analyzed for their cytokines contents using the RayBio® Human Cytokine Antibody Array C series 2000, (Cat H0108020) from RayBiotech. Membranes were scanned using the Gel Analyzer scanner coupled with the QuantiONE software (BioRad). Each cytokine expression was then normalized against the positive and negative controls and results were expressed in arbitrary units.

17. TUNEL assay

GBM cells apoptosis detection was performed using Apotag Fluorescein Direct In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA) on GBM cells adhered to coverslips according to the manufacture's instructions. The apoptosis rate was calculated as the ratio of positive cells on the total number.

18. Tube-formation assay

HUVEC were transduced using a ratio 60:30 of recombinant AdTRE p27s/AdTet-ON, in the presence of 1.0 μg/ml doxycycline 48 hrs before seeding on Matrigel^{TM}. Matrigel (18 mg/ml, BD Bioscience) was thawed at 4°C overnight, diluited to 12 mg/ml with Dulbecco Modified Eagle Medium (DMEM; Cambrex Bio Science) and 80 μl were quickly added to each well of a LABTEK plate and allowed to solidify for 30 min at 37°C. Once solid, $5{,}5x10^4$ transduced HUVE cells were seeded in each well resuspended in 400 μl of U87 conditioned medium and incubated at 37°C for 2 hrs. In some experiments, HUVEC were also transduced with Ad/stathmin siRNA at MOI 500 and 24 hrs later with AdTRE $p27^{wt}$, as described above. Non-transduced HUVEC were used as control. Results are expressed as number of tube-like structures/field (magnification 200 x). Tube formation was visualized with a camera equipped inverted Nikon ECLIPSE TS100 microscope.

19. Detection of autophagy

To detect acidic vesicular organelles (AVOs) characteristic of autophagy, we performed vital staining with acridine orange. Cells were transduced with AdTRE p27s, and after 5 days were stained with 1 μg/ml acridine orange (Sigma) for a period of 20 min at 37°C. Microphotographs were obtained using the Leica Confocal Software (LCS) or using a Nikon Diaphot 200 epifluorescent microscope. In acridine orange-stained cells, the cytoplasm and nucleus fluoresce bright green, whereas acidic compartments such as AVOs fluoresce bright red. To inhibit the acidification of AVOs, cells were pre-treated with 200 nM Bafilomycin A (Sigma) for 30 min at 37°C before addition of acridine orange. For FACS analysis, cells were stained with acridine orange

for 20 min, removed from the plate with trypsin-EDTA, and collected in phenol red-free growth medium. Green (510-530 nm) and red (>650 nm) fluorescence emission from 10^4 cells was measured with a FACSCalibur from Becton Dickinson.

20. Statistical analysis

Statistical significance of the results was determined by using the paired and unpaired Student's t-test. A value of $p < 0.05$ was considered significant.

21. Technical Appendix

DAB solution: 25 µl imidazole 1M, 40 µl H_2O_2 3%, in 2,5ml of 0,05M Tris-HCl pH 7.6 and 25 mg DAB (3,3'-Diaminobenzidine)

x-gal solution: 250 µl x-gal (40 mg/ml), 33mg potassium ferricyanide, 42,5mg potassium ferrocyanide, 20 µl $MgCl_2$ 1 M, in 10 ml PBS

NP40 lysis buffer: 0.5% NP40, 50mM HEPES pH 7, 250mM NaCl, 5mM EDTA, 0.5mM EGTA pH 8

TBS-T: 20mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Tween 20

HNTG: 20mM HEPES, 150mM NaCl, 10% glycerol, 0,1% Triton-100

Mowiol 4-88: 4,8g Mowiol 4-88, 12g glycerol, 12ml H_2O plus 24ml TRIS 2M pH 8.5, 2,5% DABCO (Sigma)

buffer A: 10mM HEPES pH 7.9, 0,1mM EGTA pH 7.8, 0,1mM EGTA pH 8, 10mM KCl

sucrose buffer: 0,32M sucrose, 3mM $CaCl_2$, 2mM Mg-Acetate, 0,1mM EDTA pH 8, 10mM Tris pH 8

buffer C: 20mM HEPES pH 7.9, 1mM EDTA pH 8, 1mM EGTA pH 8, 400mM NaCl

bicarbonate buffer: 0,2 M Na_2CO_3, 0,2 M $NaHCO_3$

RESULTS

1. Cytoplasmic p27 expression correlates with glioblastoma cell motility

In malignant glioblastoma (GBM), low p27 expression levels have been often associated with poor prognosis and increased proliferation (Kirla et al., 2003; Mizumatsu et al., 1999; Piva et al., 1997; Tamiya et al., 2001). Smaller studies did not confirm this correlation, (Nakasu et al., 1999; Naumann et al., 1999) a discrepancy that could be due to the heterogeneity of gliomas (Zagzag et al., 2003) or to the different cutoff used to evaluate p27 positive cells within the tumors. In order to give our contribution on this topic anaplastic astrocytoma (AA) and GBM sections were evaluated by immunohistochemistry for p27 expression and localization. In all cases GBM (n=4) samples showed a low expression pattern of p27 compared to AA (n=4) (Figure 1), suggesting a correlation between low expression of p27 and tumor grade. In both types of tumors p27 staining was predominantly nuclear.

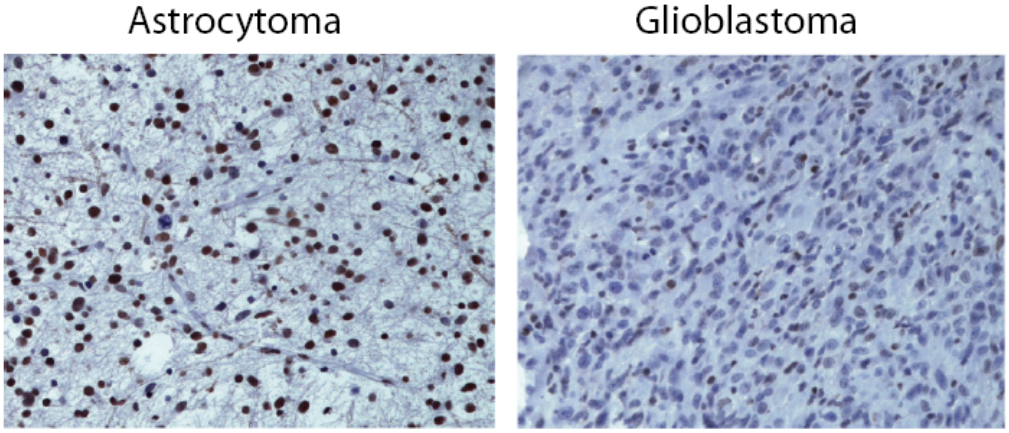


Figure 1. p27 expression correlates with tumor grade. Representative microphotographs of immunohistochemical analysis of p27 expression on different human glioblastoma and anaplastic astrocytoma specimens. Low levels of p27 (in brown) were observed in glioblastoma samples compare to astrocytoma.

Few studies have addressed systematically the impact of p27 cytoplasmic localization in tumor progression. The largest one performed in breast carcinomas demonstrated that patients with high cytoplasmic expression of p27 had a better survival respect to patients with low expression, although the best prognosis was found in patients with high nuclear p27 staining (Liang et al., 2002). We evaluated p27 expression and localization in 10 different GBM cell lines by Western blot. Except for T98G cells, p27

is generally expressed at low levels in the analyzed GBM cell lines (Figure 2A) in line with the expression in primary tumors. Differential extraction of cytoplasmic and nuclear proteins demonstrated that in the majority of the cell lines p27 presented an enriched nuclear localization although some protein was also present in the cytoplasm (Figure 2B). A comparison of only the cytoplasmic fractions revealed that in U251 and SBN75 cell lines p27 was almost completely absent, while two cell lines, T98G and U138MG, expressed high levels of cytoplasmic p27 (Figure 2C).

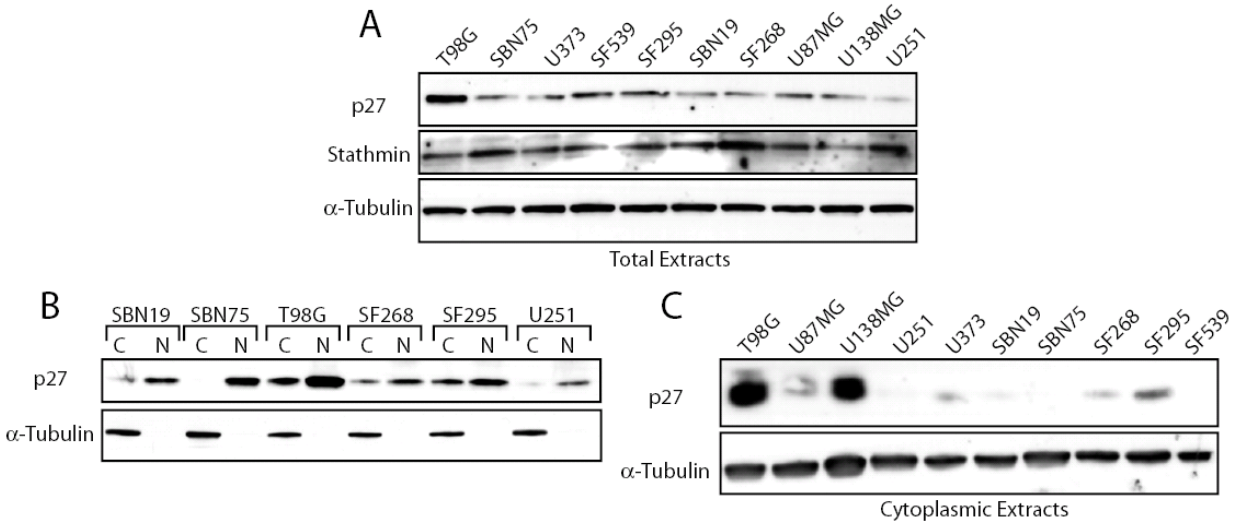


Figure 2. p27 expression and localization in 10 different GBM cell lines A. Western Blot analysis of p27 and stathmin expression in total extracts of different GBM cell lines. α-tubulin was used as loading control. **B**. Western Blot analysis of p27 expression in cytoplasmic (C)/nuclear(N) extracts of the indicated GBM cell lines. α-tubulin was used as a loading control of the cytoplasmic fractions and as purity control of nuclear extracts. **C**. Western Blot analysis of p27 in GBM cell lines cytoplasmic extracts. α-tubulin was used as loading control.

To assess if the different cellular localization of p27 could determine differences in the growth rate, MTT proliferation assay was performed on some representative cell lines: T98G and U138 MG cells displaying strong cytoplasmic p27 expression, and U87MG and SF268 displaying low cytoplasmic levels. Results showed that no direct correlation could be established between cytoplasmic levels of p27 and proliferation rates (Figure 3).

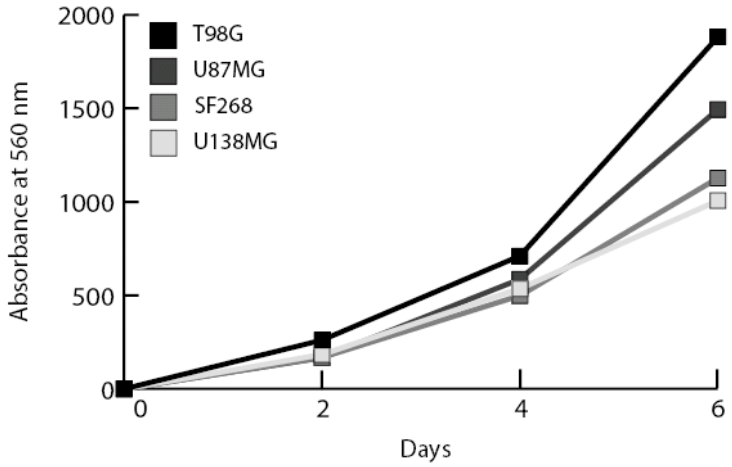


Figure 3. Proliferation assay on indicated cell lines. MTT assay used to evaluate cell proliferation of the indicated cell lines.

To determine if this differential cellular localization could be related to ECM-driven cell motility, as previously proposed (Baldassarre et al., 2005), migration assays on different ECM substrates were performed. Interestingly, T98G and U138 MG cells, which had strong p27 cytoplasmic expression, exhibited a decreased migration rate through vitronectin, collagen I, collagen IV (Figure 4) and fibronectin (FN) (data not shown), compared to SF268 and U87MG cells which expressed low cytoplasmic p27 levels. These results support the concept that p27 inhibits cell growth when located into the cell nucleus, whereas the control of cell motility is to ascribe to its cytoplasmic pool, and this is also for GBM cell lines.

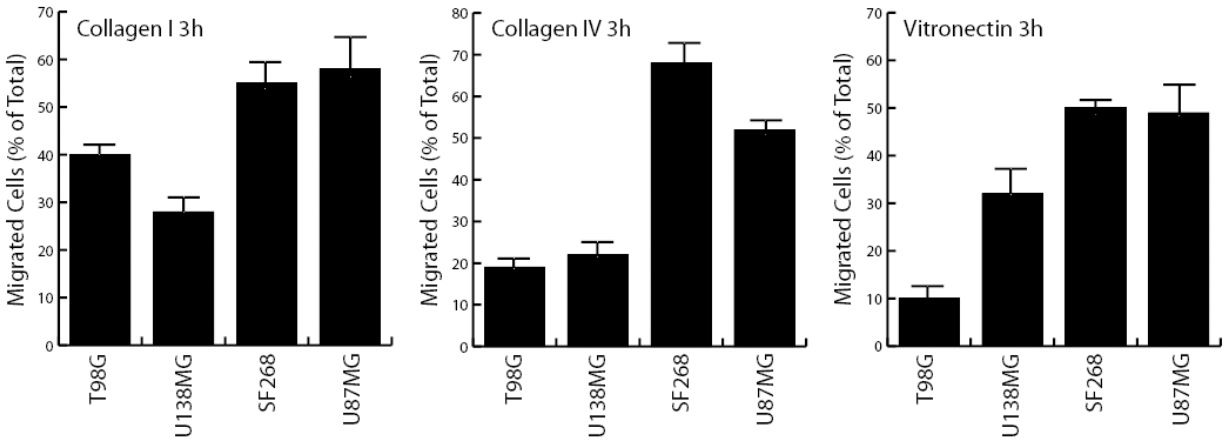


Figure 4. **Cytoplasmic p27 expression correlates with glioblastoma cell motility**. Migration assay of the indicated cell lines through collagen I, IV and vitronectin coated membranes. Results are expressed as percentage of migrated cells and represents the mean value ± SD of two independent experiments each performed in duplicate.

2. Preparation of different recombinant adenoviruses (AdTRE) expressing $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$

In this work we analyzed the effects of human p27 protein on glioblastoma cells growth and motility using either its wild type form ($p27^{wt}$) and two mutants: one a truncated mutant lacking the last 28 aminoacids ($p27^{1-170}$) and a second one carries a point mutation resulting in the substitution of threonine 187 with alanine ($p27^{T187A}$), which impairs p27 degradation via the ubiquitin-dependent proteasome pathway. This mutant represents a better control of the $p27^{1-170}$ mutant than the wt protein, since it also lacks the T187. Based on this notion and to determine the effects of p27 overexpression on growth and motility of GBM cells *in vitro* and *in vivo*, different recombinant adenoviruses (AdTRE) were prepared expressing $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$.

The $p27^{wt}$ and two mutants $p27^{1-170}$ and $p27^{T187A}$ were amplified by a PCR reaction and then cloned into a pDNR-CMV vector (Figure 1 Material and Methods). This vector is both a donor vector and an expression vector. It contains the immediate early promoter of human cytomegalovirus located just upstream of the multiple cloning site, and an SV40 polyadenilation signal just downstream. In Figure 5 we tested and verified the expression of $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$ after transfection in HEK 293 cells by Western blot analysis. p27 is highly expressed in cells transfected with the pDNR-CMV

p27s expression vectors while its levels are very low in cells untransfected or transfected with the empty vector (Figure 5).

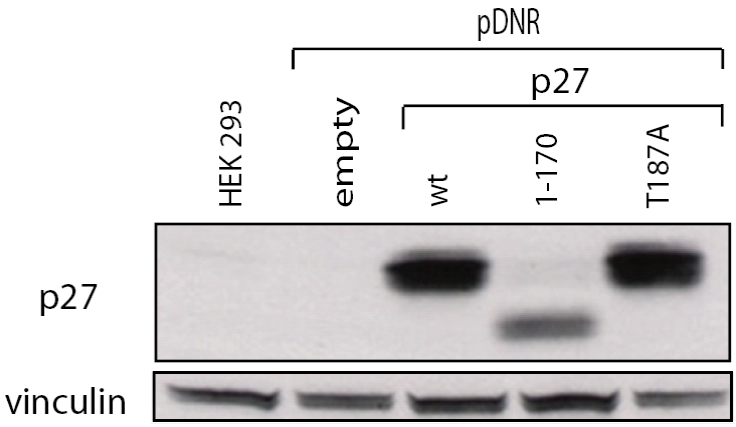


Figure 5. Expression of $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$ after transfection in HEK 293 cells. Western Blot analysis of p27 expression in total protein lysates extracts from transfected HEK293 with pDNR-CMV, pDNR-CMV$p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$. Vinculin was used as loading control.

To generate from the Donor Vectors the respective adenoviral vectors, the Adeno-XTRE Acceptor vector from Clontech was used. As reported in the methods (Figure 1 Material and Methods), it is based on the ability of the Cre Recombinase to bind to the loxP sites on both the pDNR vector and Adeno acceptor vector, cleaves the DNA and then catalyzes strand exchange and ligation of the two plasmids. The correct recombination were verified by double digestion with PI-Sce I and I-Ceu I enzymes or by restriction with Xho I. The digested were resolved on a 0,8% agarose/EtBr gel. In Figure 6 a typical digestion analysis is reported showing the presence of expected fragments after the digestion of the recombinant pLP-Adeno –XTRE with both the PI-Sce I/I-Ceu I and Xho I enzymes (arrows), whose size changes if the p27 gene and the chloramphenicol resistance gene have been inserted into the Ad Acceptor vector.

For example the digestion of pLP-Adeno –XTRE with Xho I should give a fragment of 3,8 kb in absence of recombination that, after recombination, should shifted to a higher molecular weight (5,3 kb). Similarly, the PI-Sce I/I-Ceu I digestion should give a fragment size of 1,7 kb that should shift to 3,2 kb after recombination (Figure 6).

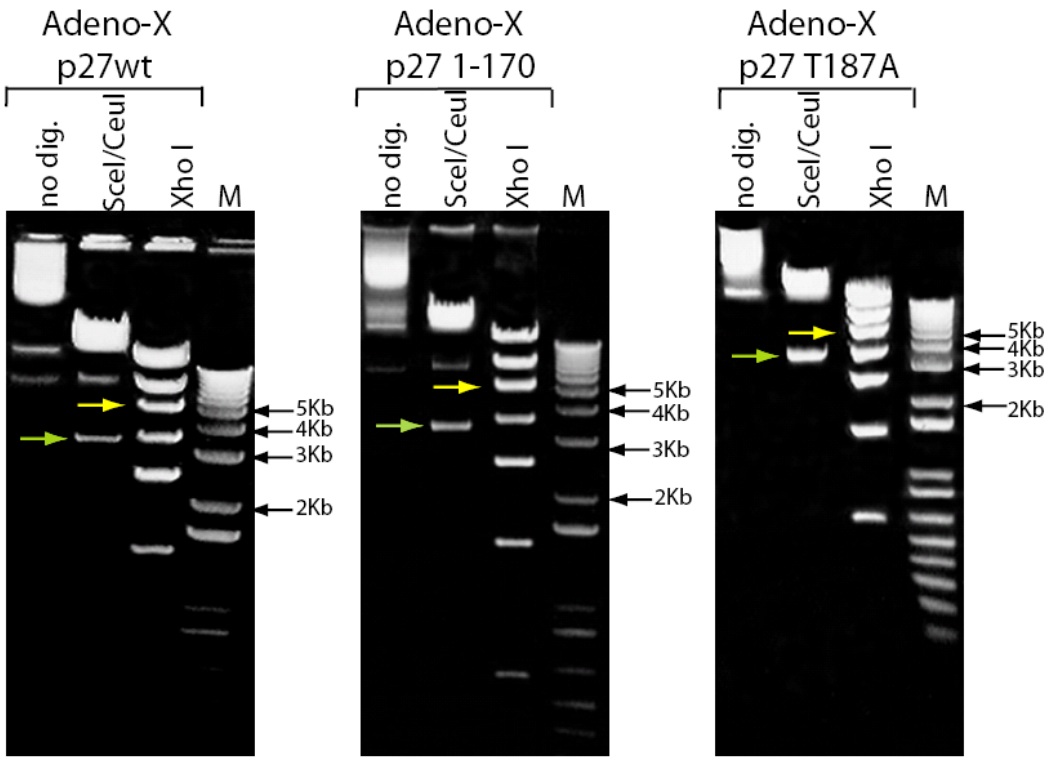


Figure 6. Digestion of pLP Adeno-XTRE $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$. Electrophoretic analysis of pLP Adeno-XTRE $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$, digested with PI-Sce and I-Ceu I or with Xho I restriction enzymes and resolved on a 0,8% agarose gel. The arrows indicated expected size after a correct recombination of the p27 gene on viral genome.

To accommodate DNA insert and to produce a replication-incompetent adenoviral vector, extensive portions of the Early regions 1 (E1) and 3 (E3) of wild type adenovirus have been deleted from the Ad5 genome in pLP-Adeno-XTRE. Because the E1 elements have been eliminated, an early passage of HEK 293 cells, that express the E1-encoded trans-complementing factors, are required to propagate and titrate recombinant adenoviruses. HEK 293 infected cells typically round up and may detach from the plate in "grape-like" clusters after infections with adenoviruses; these morphological changes are collectively referred to as the cytopathic effect (Figure 7A) and represent clear signs that the cells are producing the viruses. To determine the title of each virus production/stock we take advantage of the fact that the viruses produce the viral hexon proteins against which specific antibodies have been generated. Briefly, infected HEK 293 cells were fixed and stained with the anti-hexon protein antibody,

incubated with a horseradish peroxidase-conjugated secondary antibody and finally stained with DAB-substrate. In this manner only the infected HEK 293 cells will appear as dark brown colored (Figure 7B). Then the titer can be determined by counting the number of brown cells since and each stained cell corresponds to a single infectious unit (ifu).

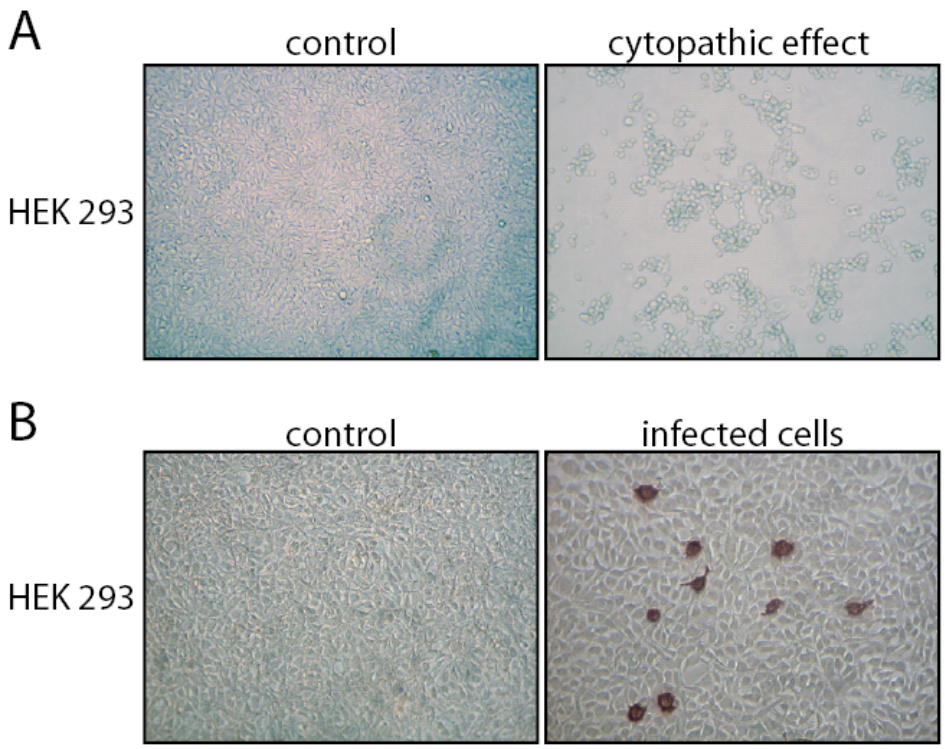


Figure 7. Amplification and titration of the AdTRE. Typical images of infected HEK293 are shown, demonstrating the presence of cytopathic effect (**A**) and the immunocytochemical with the anti-hexon protein antibody. Infected cells are colored in dark brown cells by DAB as explained in the text (**B**). (magnification 100X).

3. Standardization of doxycycline-regulated adenoviral expression system in GBM cell lines

Once the viruses have been correctly cloned, produced and titrated, we started then standardization their expression using four different GBM cell lines U87MG, U251MG, U138MG and SF268. Target cells were co-transduced with two viruses: the AdTet-ON (Clontech), that provides the constitutive expression of the reverse

tetracycline-controlled transactivator (rtTA), and the AdTRE βGal, able to express the β-galactosidase protein, after the induction with doxycycline. The standardization of this adenoviral system consisted in determining the most suitable co-transduction ratio and doxycycline concentration in order to achieve a good inducible β-galactosidase protein expression in the vast majority of glioblastoma cells. To this aim, cells transduced with the indicated ratios of AdTRE β-gal and AdTet-ON were harvested 48 hours later the transduction and tested in β-galactosidase assay (Promega) (Figures 8A and 8B) as reported in the methods section. For example the best co-transduction ratio and doxycycline concentration for U87MG cell line were 80:40 (AdTRE βGal:AdTet-ON) and 1μg/ml respectively, able to achieve a good inducible β-galactosidase protein expression.

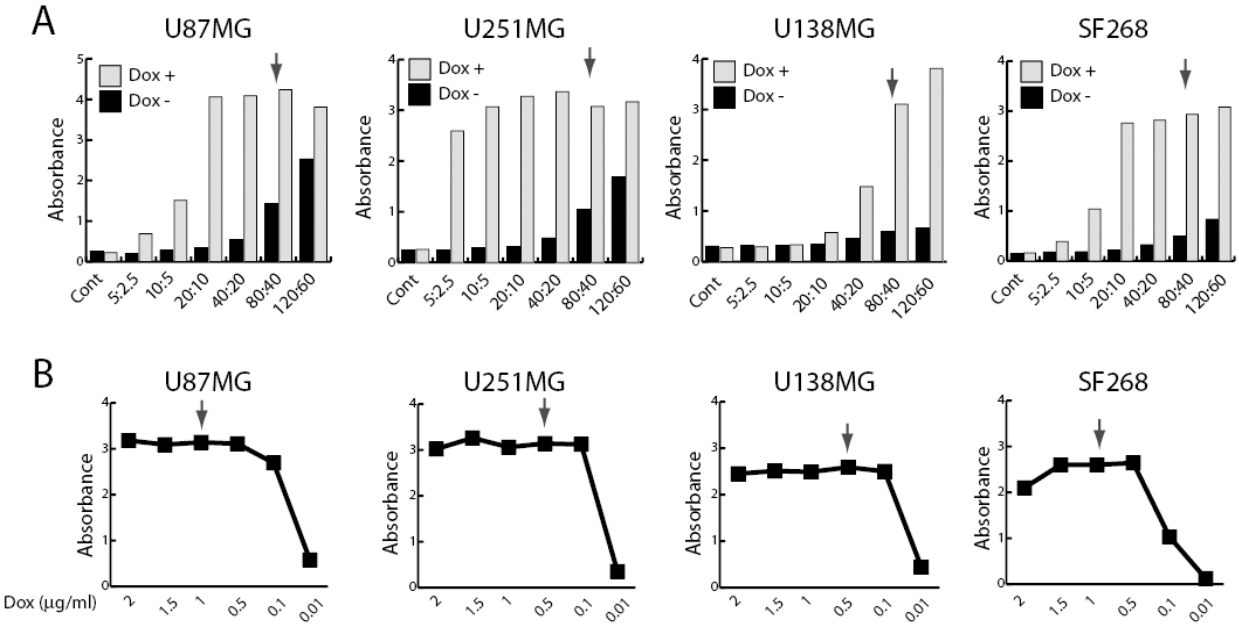


Figure 8. Standardization of doxycycline-regulated adenoviral expression system in GBM cell lines. A. Determination of the suitable co-transduction ratio for the different glioblastoma cell lines using AdTRE βgal and AdTet-ON. Cells were transduced with the indicated ratios in the presence of 2μg/ml doxycycline and 48 hours later total cell extracts were tested in β-galactosidase assay. Arrows indicate the chosen co-transduction ratio. **B**. Cells were transduced with the most suitable ratio AdTRE β-gal/AdTet-ON as determined and incubated in presence of indicated doxycycline concentrations for 48 hours before the assessment of β-galactosidase activity. Data in A and B are representative of 3 independent experiments.

Transduction efficiency for the different cell lines was also determined by X-gal staining to reveal *in situ* the β-gal activity in transduced cells. Briefly, GBM cells transduced with AdTRE β-gal and AdTet-ON at different ratio and exposed or not to doxycycline for 48 hrs were fixed in glutaraldehyde/formaldehyde solution and then incubated with the substrate X-Gal at 37°C for 14-16 hours (Figure 9). The photos of a typical field of all tested cell lines demonstrated that by increasing the MOI of AdTRE βgal and AdTet-ON maintaining the same ratio (i.e. 2:1), it was possible to transduce a higher percentage of cells without losing the inducible expression.

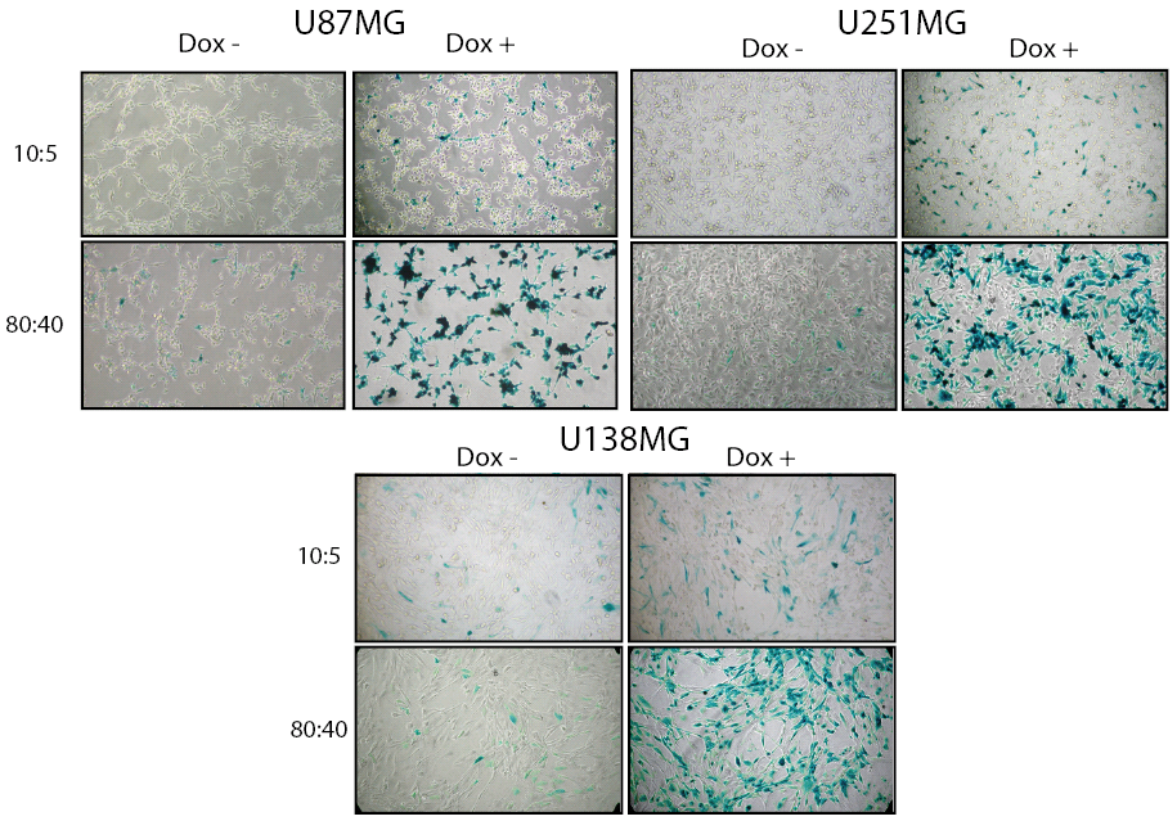


Figure 9. Transduction efficiency for U87MG, U251MG and U138MG cells by X-gal staining. U87MG, U251MG and U138MG cells were transduced with the indicated ratios of AdTRE βgal/AdTet-ON in presence/absence of doxycycline and 48 hours later stained for β-Gal activity.

Then the recombinant adenoviruses expressing $p27^{wt}$ and two mutants $p27^{1-170}$ and $p27^{T187A}$ were tested on the same four GBM cell lines. To obtain regulated p27

expression, target cells were co-transduced with two viruses: the AdTet-ON and the AdTRE that encodes for the $p27^{wt}$ or mutant proteins in presence of doxycycline. Time course analysis of p27 overexpression in different glioma cell lines showed that the maximal p27 expression in all the cell lines tested was achieved at 48-72 hours after viral transduction (Figure 10A). Moreover, U87MG, U251MG and U138MG cells were also co-transducted using increasing adenoviral ratios and after 72 hours post-transduction were analyzed by Western Blot (Figure 10B). Maximal expression of the three exogenous p27 proteins was obtained using 80 adenoviral particles of AdTRE $p27^{wt}$, $p27^{1-170}$ or $p27^{T187A}$ and 40 particles of AdTet-ON per cell.

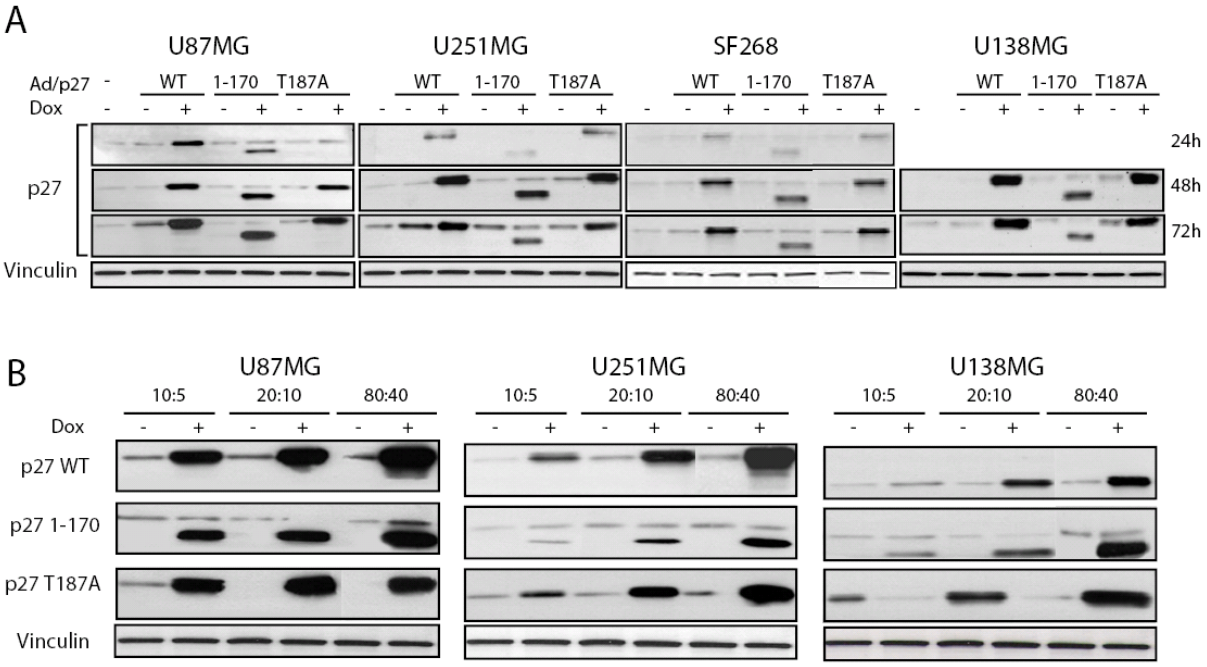


Figure 10. Overexpression of $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$ on GBM cell lines. A. Time course analysis of p27 overexpression in different glioma cell lines. Co-transduction ratios AdTRE p27s/Ad Tet ON 10:5 were used. Total cell extracts were prepared at the indicated times post-transduction and different p27s expression was analyzed in Western Blot. Vinculin was used for loading control. **B**. p27 overexpression in U87MG, U251MG and U138MG cells using increasing co-transduction ratios and analyzed in Western Blot 72 hours post-transduction. Vinculin was used for loading control.

4. p27 inhibits GBM cells growth

Biological characterization of p27 overexpression on GBM cell growth was performed using as a model the U87MG and U251MG cells. GBM cells previously transduced with the different AdTRE p27 in the presence of doxycycline except otherwise indicated were plated in six well plates and counted daily using the Trypan blue staining (Figure 11). Overexpression of $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$ was able to reduce the cell growth respect to untreated cells, or cells transduced with the AdTRE p27 but not induced to express the protein with doxycycline.

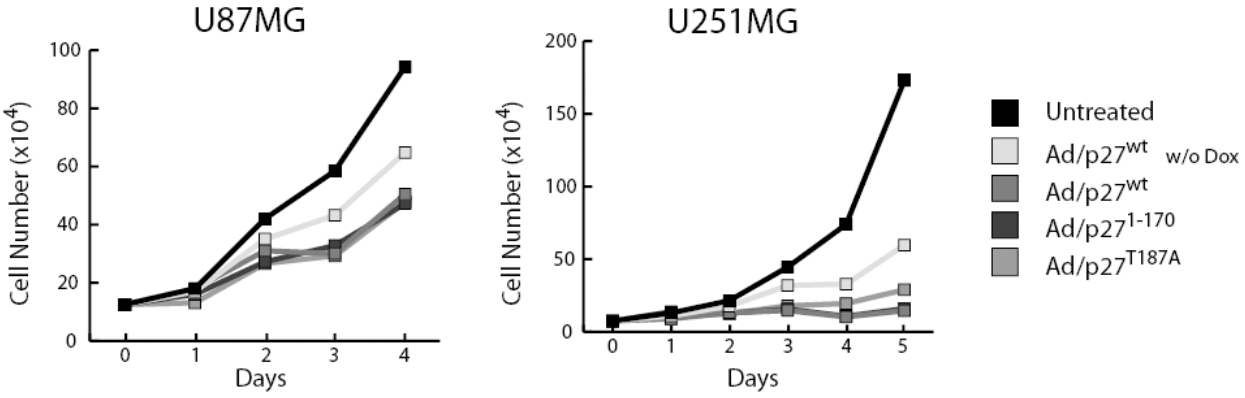


Figure 11. p27 inhibits GBM cells growth. Cell co-transduced with the different AdTRE p27s/AdTet-On (80:40) were plated and counted using the Trypan blue staining. The number of viable cells at each time point is reported in the graph and represents the mean of two independent experiments performed in triplicate.

To confirm this result, U87MG and U251MG cells were transduced with the different AdTRE p27s and 72 hours later fixed and analyzed by flow cytometry for their DNA content (Figure 12A). All three adenoviruses were able to arrest the cell cycle progression in G1 phase, reducing the percentage of cell present in S and G2/M phases. The amount of untreated U87MG cells in G1 and S phases was approximately 68% and 16% respectively while the percentage of transduced cells with all three different AdTRE p27 was around 80% in G1 phase and 8% in S phase. Moreover, the expression levels of cyclin A (S phase marker) and cyclin B1 (marker of G2/M phases) were strongly decreased in U87 MG and U251MG cells transduced with the different AdTRE p27s as revealed by Western Blot analysis (Figures 12B). The same proitein extracts were immunoprecipitated with cyclin B1 (Figure 12C), CDK1 (Figure 12D) and p27

(Figure 12E) antibodies to evaluate the p27 ability of the different p27 proteins to bind to cyclin-CDK complexes. Immunoprecipitation analysis confirmed that $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$ were able to bind to cyclin B1, CDK1 and CDK2 with similar affinity, thus supporting the functional evidences that overexpression of p27 and its mutants induces cell cycle arrest.

A

	Untreated	Dox (1µg/ml)	Ad/p27^{wt} w/o Dox	Ad/p27^{wt}	Ad/p27$^{1-170}$	Ad/p27^{T187A}
U87MG	G1 64% S 18% G2 18%	G1 68% S 16% G2 16%	G1 68% S 13% G2 19%	G1 83% S 7% G2 10%	G1 78% S 9% G2 13%	G1 80% S 9% G2 11%
U251MG	G1 70% S 18% G2 12%	G1 67% S 18% G2 15%	G1 75% S 15% G2 10%	G1 88% S 6% G2 6%	G1 82% S 8% G2 10%	G1 86% S 7% G2 7%

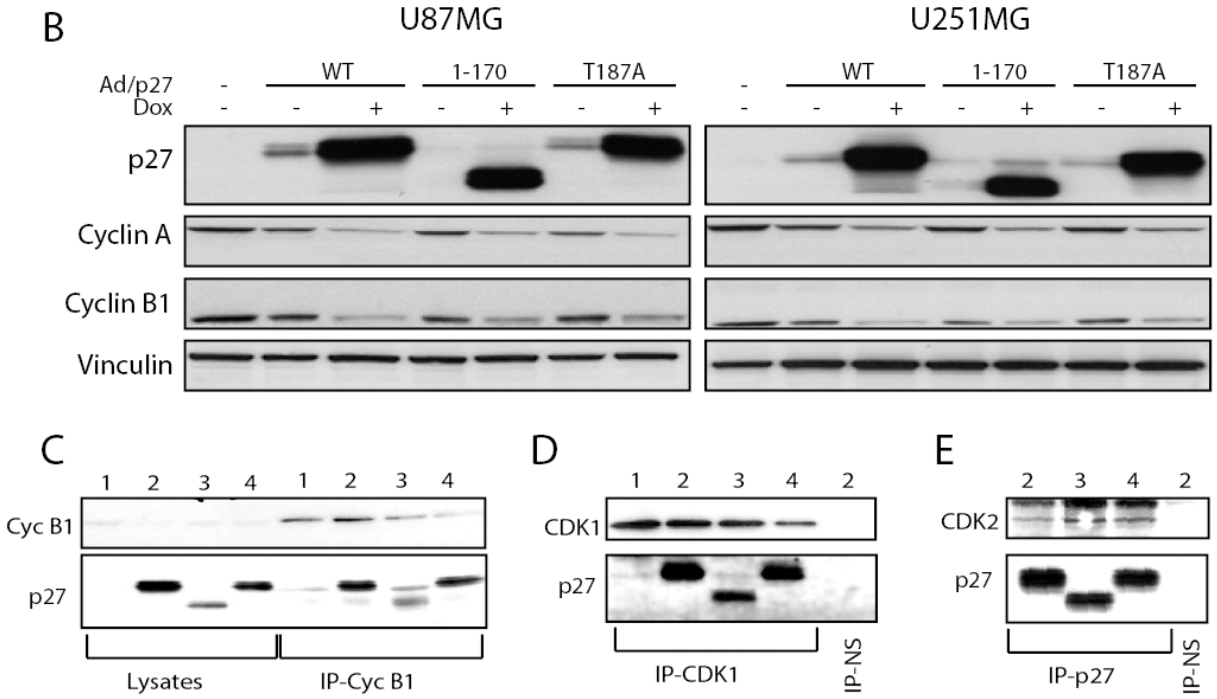


Figure 12. p27 inhibits GBM cells growth by binding to different cyclin-CDK complexes . **A**. U87MG and U251MG cells were transduced with the different AdTRE p27s and 72 hours later fixed and analyzed by flow cytometry for their DNA content. The percentage of cells in G1, S and G2/M phases of the cell cycle is reported and represents the mean of two independent experiments. **B**. U87MG and U251MG cells were transduced with the different AdTRE p27s and 72 hours later the protein extracts were analyzed for p27, cyclin A and cyclin B1 expression. Vinculin was used for loading control. **C, D and E**. Immunoprecipitation analysis of p27/cyclin/Cdk complexes in U87MG cells untreated (1) or transduced for 72 hrs with AdTRE/$p27^{wt}$ (2), $p27^{1-170}$ (3) and $p27^{T187A}$ (4). Total Cell lysates (500 µg) were immunoprecipitated with anti Cyclin B1 (**C**), anti-CDK1 (**D**) or anti-p27 (**E**) antibodies and analyzed for p27, Cyclin B1, CDK1 and CDK2 expression as indicated. IP-NS indicates the immunoprecipitation with a non relevant antibody.

Then U87MG and U251MG cells transduced with the increasing MOI of different AdTRE p27s in the presence or not of doxycycline, were plated in 100 mm dishes at the diluition of 1000 or 5000 cells/dish, allowed to grown for 15 days and then stained with crystal violet (Figure 13 photos). The number of colonies in each dish was counted and the obtained results were expressed as percentage of growth inhibition comparing the colony numbers of each experimental condition to the colony numbers formed by untreated cells (Figure 13 histograms). Also in long term growth assay, such is the colony assay, all the adenoviruses were able to significantly impair cell growth with AdTRE $p27^{1-170}$ resulting the most effective one. Importantly, the effect of p27 on growth inhibition seems to be dose-dependent since by increasing the MOI of recombinant adenoviruses used it was possible to enhance the capability of the three adenoviruses to block the cell cycle progression.

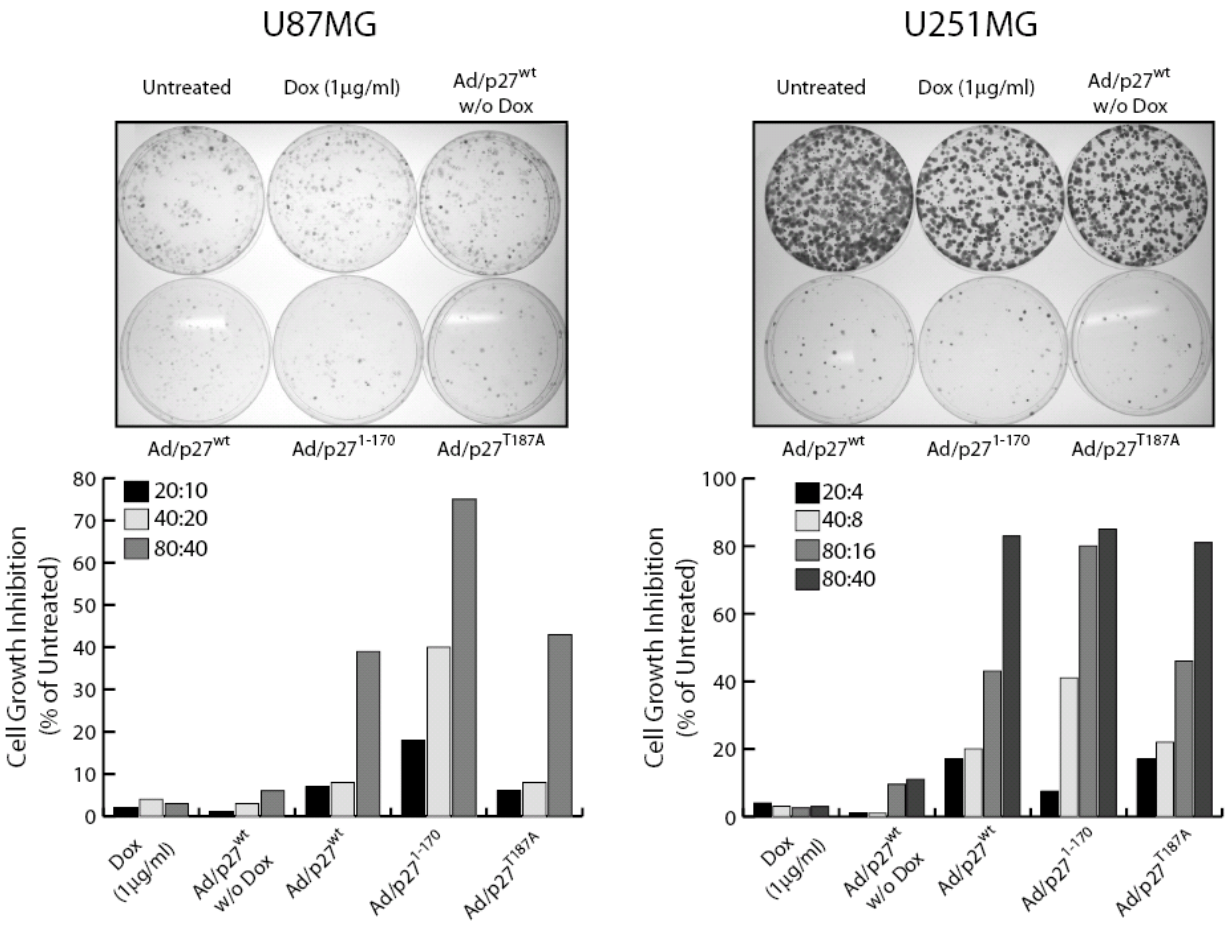


Figure 13. p27 inhibits GBM cells growth. Colony formation assay. U87MG and U251MG cells transduced with the increasing MOI of the indicated AdTRE in the presence or not of doxycycline as indicated, were plated in 100 mm dishes (1000 and 5000 cells/dish) allowed to grown for 15 days and then stained with crystal violet (upper photos show a typical effect observed using the co-transduction ratio 80:40). The histograms showed the percentage of growth inhibition comparing the colony numbers of each experimental condition to the colony numbers formed by untreated cells. Data represents the mean value of three independent experiments performed in duplicate.

5. Effects of p27 on autophagy

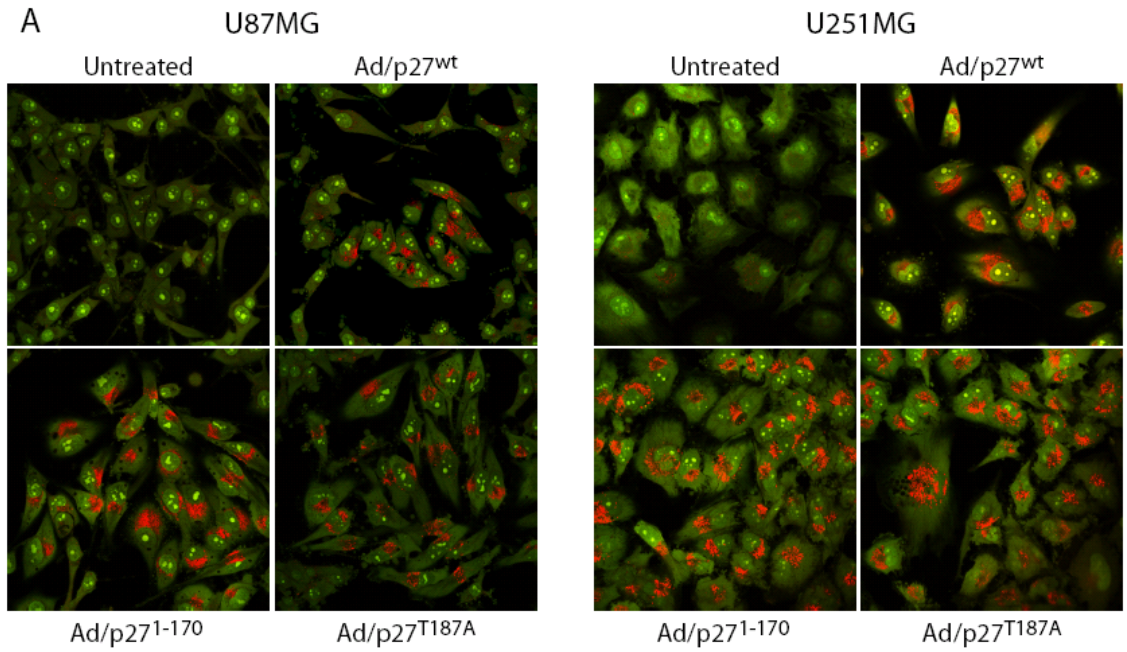
It has been controversial whether p27 expression leads tumour cell death beside to induce growth arrest. In fact while the induction of growth arrest by p27 is largely accepted (Sherr and Roberts, 1995; Craig et al., 1997), the regulation of apoptosis by this CKI is more controversial. p27 overexpression has been shown to induces apoptosis in several cell lines (Katayose et al., 1997; Schreiber et al., 1999), but also to protect cells from apoptosis (Hiromura et al., 1999). Recently, Komata et al. (2003) proposed a

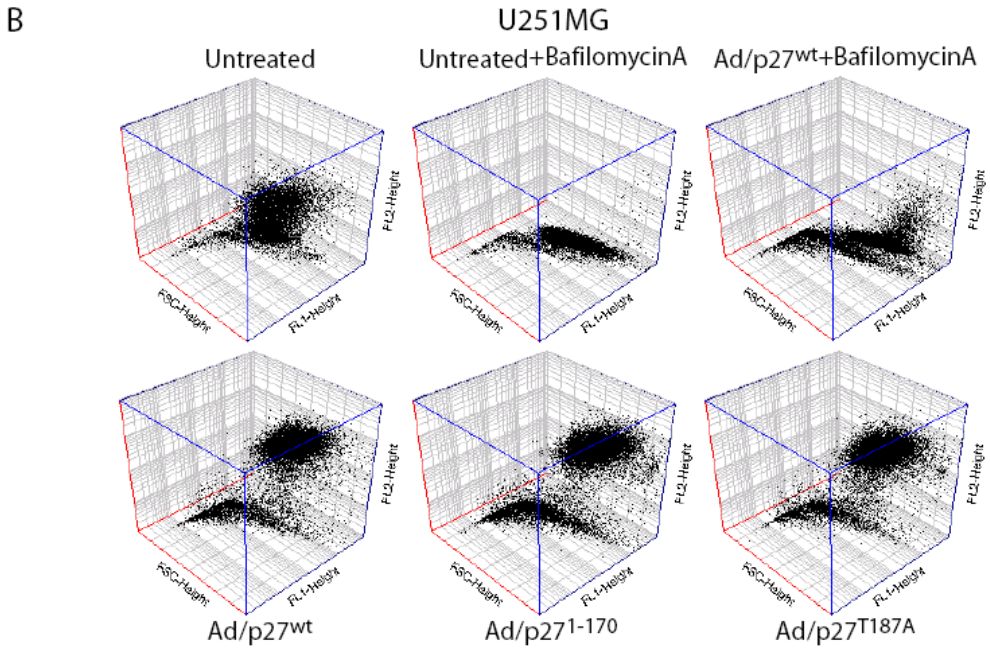
new role for p27, showing that in malignant glioma cells p27 overexpression induced growth arrest and autophagic cell death, but not apoptosis. This finding has been then supported by Liang and co-workers (2007), who demonstrated that regulation of p27 expression by AMPK could represent one of the mechanisms mediating the decision of the cell to enter autophagy or apoptosis in response to growth factor deprivation.

In order to clarify the possible pro-apoptotic and/or pro-autophagic role of p27 in our model system, we initially analyzed if p27 overexpression leaded to cell death the U87MG and/or U251MG cell lines using the TUNEL assay. The incidence of TUNEL-positive cells in U87MG and U251MG cells treated with the different AdTRE p27s for 3 or 5 days after transduction was less than 1% (data not shown), thus we speculated that it is unlikely that apoptosis is involved in the antitumor effect of p27 on malignant glioma cells. We next evaluated the presence of autophagy in p27 transduced cells using the lysosomotropic agent acridine orange. In fact, Paglin et al (2001) demonstrated that formation of acidic vesicular organelles (AVO) was detected in radiation-induced autophagy. The acidification of AVO, which results from the fusion of autophagosomes with lysosomes, was mediated by the vacuolar H^+-ATPase and detectable with acridine orange, permitting to quantify AVO accumulation. In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red. The intensity of the red fluorescence is proportional to the degree of acidity. Thus, U87MG and U251 cell lines were transduced with AdTRE $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$ and 5 days post transduction were treated for 20 min with 1 μg/ml of acridine orange to detect AVOs (Figure 14A). Induction of p27 expression correlated with a clear increase of AVOs level (red vesicles), in contrast, no change in red/green fluorescence ratio was observed in the controls. Then we evaluated the effect of Bafilomycin A1, a specific inhibitor of vacuolar type H+-ATPase, which prevented AVO formation on U251 transduced with AdTRE p27s. Five days post transduction U251MG cells were treated or not with 200 nM of Bafilomycin A for 30 min and then incubated for 20 min with 1 μg/ml of acridine orange. These cells were analyzed by flow cytometry to quantify the amount of AVO acidification in the different colture conditions. This is possible by calculating the amount of green and red fluorescence ratio in the population examined. Data in Figure 14B show that the majority of untreated and/or Bafilomycin A treated cells exhibited mainly green fluorescence with minimal red fluorescence, in contrast p27 overexpression increased

the strength of the bright red fluorescence. When the FACS data were reported as red:green fluorescence ratio (Figure 14B table), it was evident that AdTRE p27s increased of about 10 fold the amount of re fluorescence. Bafilomycin A prevented this increasing.

When cell death involves autophagy, it is designated as type II programmed cell death, in contrast to apoptosis, which is referred to as type I (Bursch et al., 2000). The morphological and biochemical features of autophagic cell death and apoptosis are generally distinct (Bursch et al., 2000; Bursch, 2001). In autophagic cell death, unlike apoptotic cell death, caspases are not activated and an increased number of acid autophagic vesicles are present. To confirm that p27 proteins overexpression may effectively induce on autophagy, Western blot analysis for apoptosis and autophagic markers have been performed on U87MG and U251MG cells transduced with the different AdTRE p27s and analyzed 72 hrs post-transduction (Figure 15). Overexpression of p27 did not lead to caspase 3 and 7 activation (apoptotic markers) but it did increased Beclin-1 expression in U87MG cells (the essential autophagy regulator) and Cathepsin D expression in U251MG cells (a lysosomal protease). Therefore, we confirmed that in glioblastoma cells p27 overexpression induced autophagy although further studies are necessary to investigate the molecular mechanisms (Beclin and/or Cathepsin) underlying this p27-effect. Similarly, whether p27-induced autophagy is a survival or a cell death mechanisms for glioblastomas *in vivo*, it will also require future work.





	Untreated	Untreated BafilomycinA	Ad/p27wt BafilomycinA	Ad/p27wt	Ad/p271-170	Ad/p27T187A
ratio red:green	0,35	0,01	0,02	3,9	3	3,6

Figure 14. **p27 induces autophagy**. **A**. Representative microphotographs of transduced U87MG (left) and U251MG (right) cells with AdTRE p27s, as indicated, and treated with 1 µg/ml of orange acridine for 15 min. (magnification 400X). **B.** Flow cytmetry analysis of red and green fuorescence in acridine orange-stained cells, treated as indicated. FSC as forward scatter (cell size), FL-1 as green fluorescence and FL-2 as red fluorescence. In the table the red:green fluorescence ratio is reported and represents the mean value of three independent experiments.

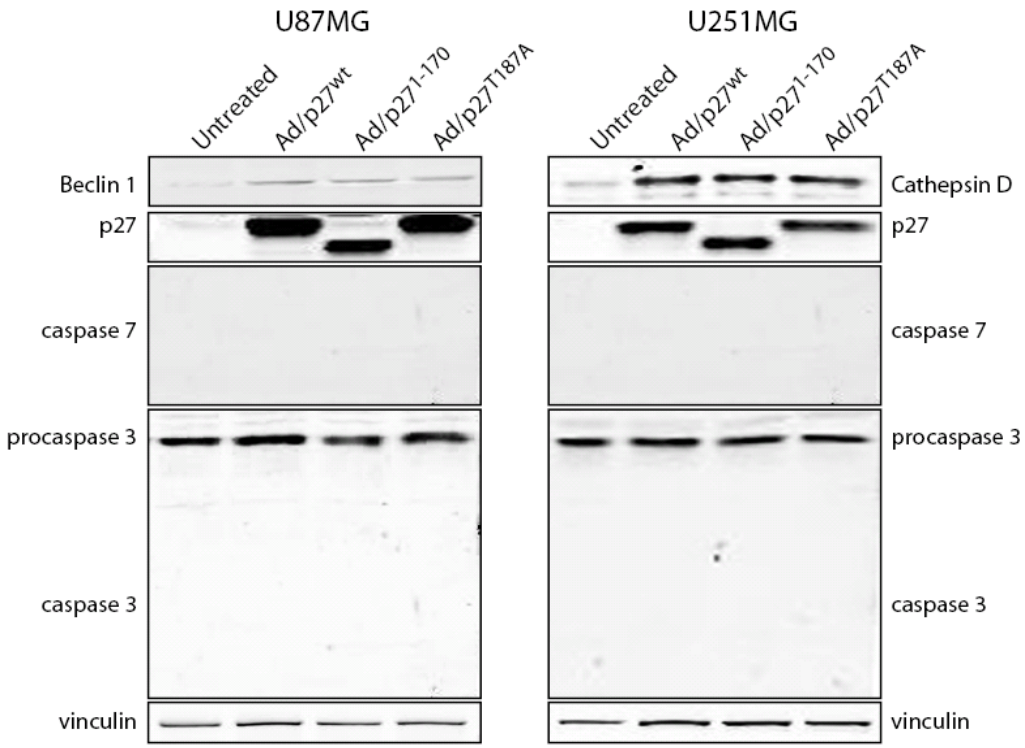


Figure 15. p27 induces, caspase-indipendent, autophagy. Western Blot analysis of p27, caspase 3 and 7, Beclin-1 and Cathepsin D of the indicated GBM cell lines. α-vinculin was used as loading control.

6. Effects of p27 on GBM cells motility

At this point we demonstrated that p27 induced cell growth inhibition by inducing cell cycle arrest and possibly autophagic cell death. We next asked whether in this model system p27 could also induce cell motility inhibition as evidenced in sarcomas (Baldassarre et al., 2005).

To determine the effects of the different p27 proteins on glioblastoma cell motility, U87MG and U251MG cells were transduced with the AdTRE p27/AdTet-ON (80:40) and 48 hrs post-transduction colored with the lipophilic dye DiI and seeded on FN-coated Fluorobloks and allow to migrate for 6-8 hrs (Figure 16). The photograph showed representative fields of migrated (in the bottom of the transwell membrane) U87MG and U251MG cells transduced or not, while the graphs showed the quantification of migrated cells and represent the mean of at least three independent experiments performed in duplicate. Overexpression of $p27^{wt}$ and $p27^{T187A}$ induced a significant impairment of U87MG cell migration (65 and 67.5% of inhibition,

respectively), while $p27^{1-170}$ was much less effective (30% of migration inhibition; $p \leq 0.01$ vs $p27^{wt}$ and $p27^{T187A}$ t-student test) (Figure 16 left). Conversely, overexpression of p27 did not alter the migration pattern of U251MG cells (Figure 16 right).

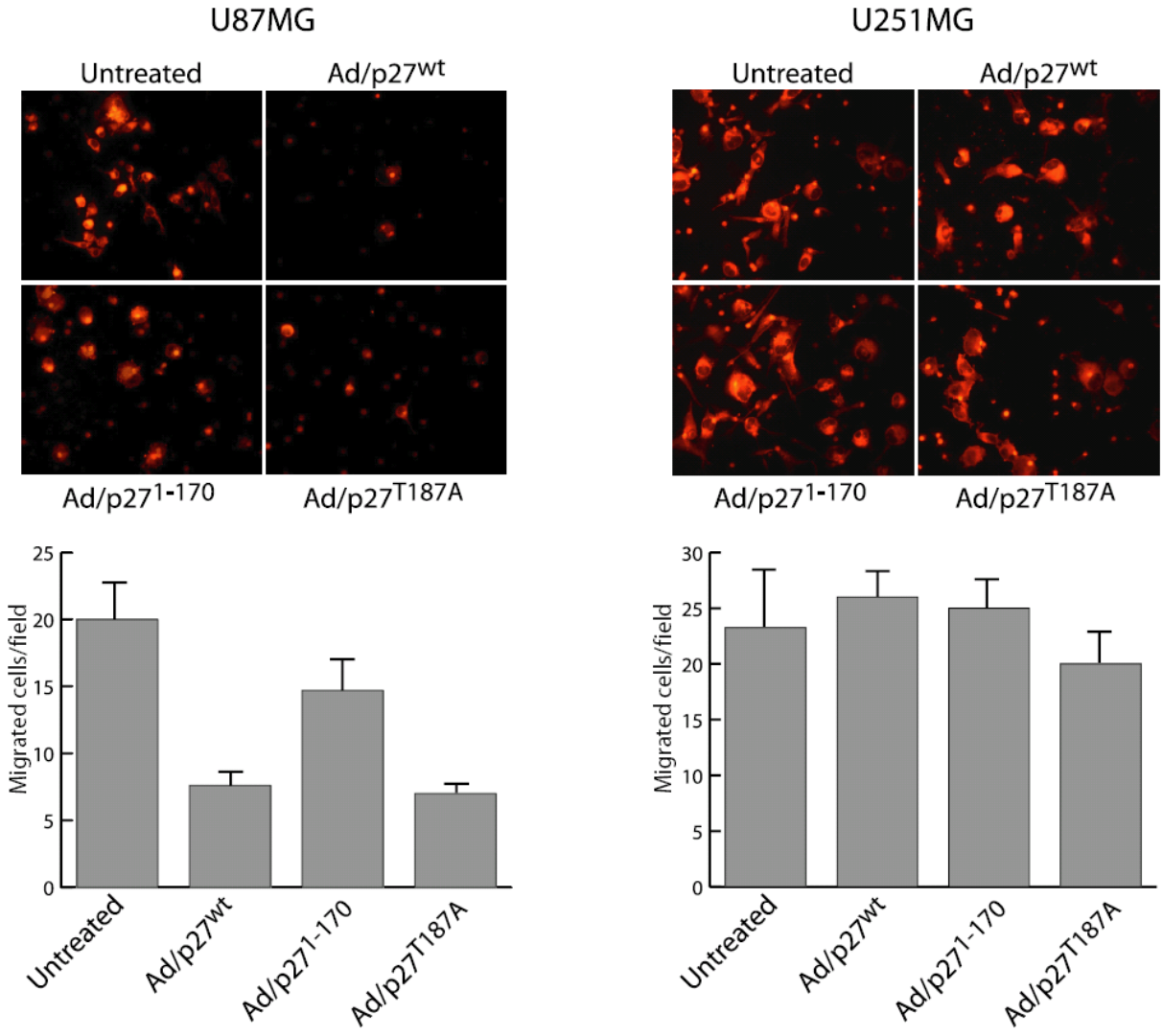


Figure 16. Effects of p27 on GBM cells motility. U87MG and U251MG ells were transduced with the indicated AdTREs and 48 hrs post-transduction seeded on FN-coated fluoroblocks and allow to migrate for 6-8 hrs. The upper photograph show representative fields of migrated U87MG (left) and U251MG (right) cells treated as indicated. The lower graphs show the quantification of migrated U87MG (left) and U251MG (right) and represent the mean of at least three independent experiments performed in duplicate.

In agreement with the above results, $p27^{wt}$ and $p27^{T187A}$ inhibited U87MG cells motility also in other migration assays, such as invasion and evasion assay, evaluating cell movements under three-dimensional (3D) conditions (Figures 17A and 17B). U87MG cells transduced with the different AdTRE p27s, after 48 hrs post-transduction were plated on Matrigel™ coated Fluorobloks and allowed to invade for 48 hrs (Figure 17A) or included in Matrigel™ drops and incubated at 37°C for 5 days (Figure 17B). Again, $p27^{1-170}$ failed to properly inhibit cell migration in both types of assays, confirming that the C-terminal portion of p27 is required to fully inhibit ECM-driven cell motility (Baldassarre et al. 2005). In fact cells treated with AdTRE $p27^{wt}$ and $p27^{T187A}$ were unable to invade the three-dimensional matrix (Figure 17A) and to move out from Matrigel™ drops (Figure 17B) respect to untreated and AdTRE $p27^{1-170}$ transduced cells.

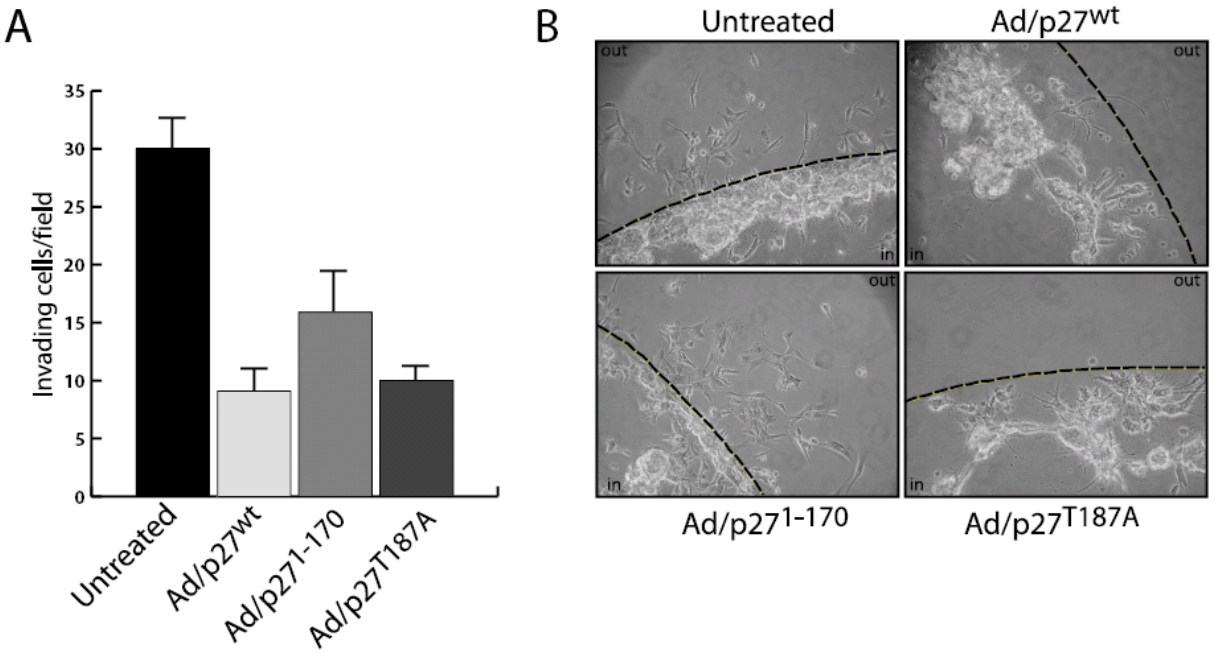


Figure 17. $p27^{wt}$ and $p27^{T187A}$ inhibit U87MG cell motility. **A.** Quantification of invading U87MG cells transduced as indicated, plated on Matrigel™ coated fluoroblocks and allow to invade for 48 hrs. Data expressed as invading cells/field represent the mean (±SD) of three independent experiment performed in duplicate. **B.** Matrigel evasion assay of U87MG cells included in Matrigel™ drops and incubated at 37°C for 5 days. Representative microphotographs are shown. (magnification 100X).

To understand the molecular basis of the different motility behavior observed in U87MG versus U251MG cells, we first investigated the localization of endogenous and overexpressed p27 proteins in these cells adhered to FN for 1 hour by Western Blot and immunofluorescence analysis. U87MG cells showed a predominant p27 cytoplasmic localization either in non-transduced or transduced cells (Figures 18A and B); in contrast, in U251MG cells p27 mainly localized in the nuclear compartment (Figure 18A and B). Western blot analysis revealed that, even if very small, a fraction of overexpressed protein is also present in the cytoplasm of U251MG cells, but it is mainly cleaved at its C-terminus, as revealed using antibodies recognizing the C-Terminus (C19) or the N-Terminus of p27 protein (N20) (Figure 18B).

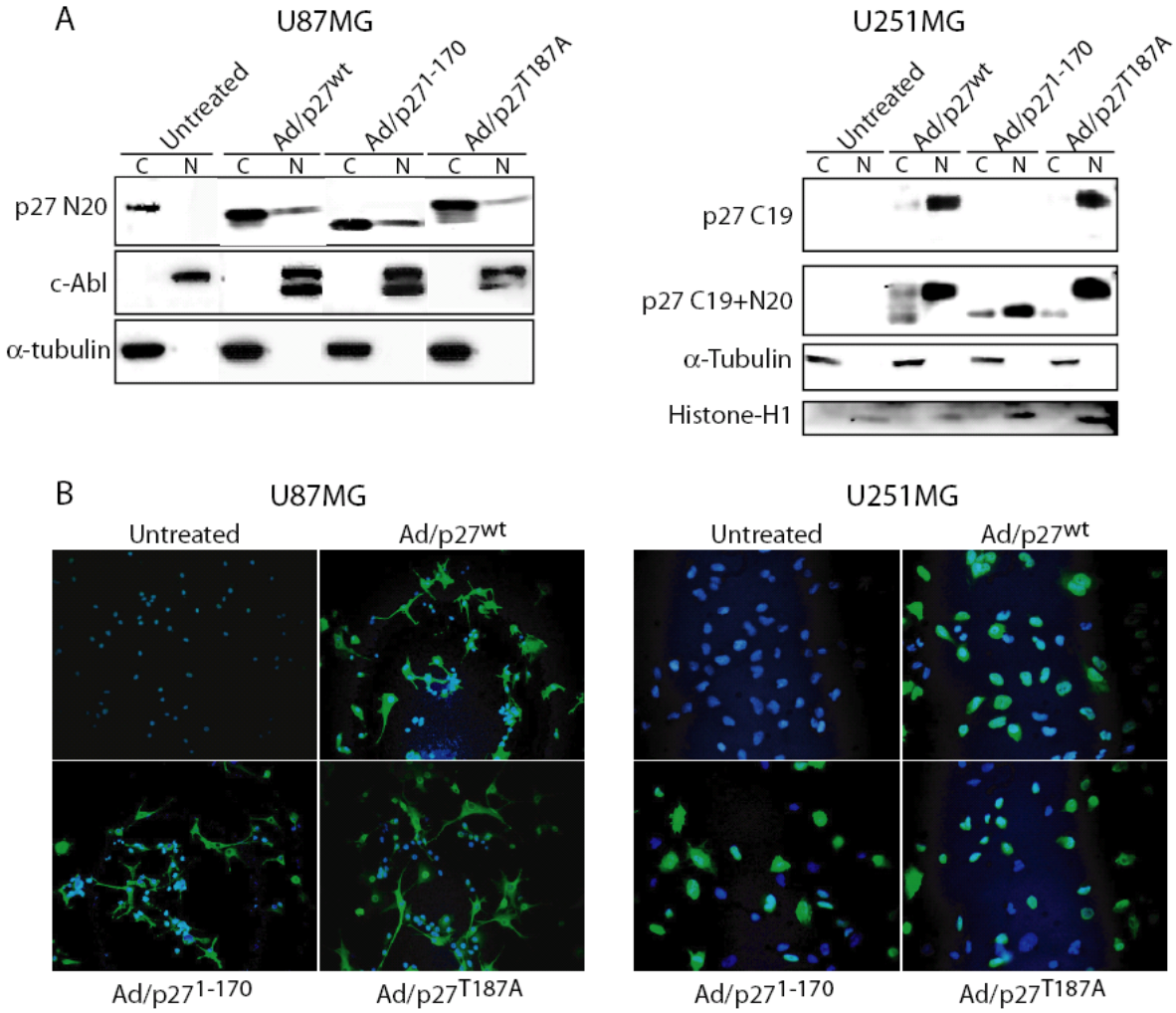


Figure 18. p27 localization in GBM cells. A. Western blot analysis of p27 expression in cytoplasmic/nuclear extracts of U87MG and U251MG cells transduced as indicated and plated on FN for 1 hour. α-tubulin was used as control for cytoplasmic fractions and c-Abl or Histone H1 as controls for nuclear fractions. The p27 N20 antibody is directed against an epitope present at the N-terminal part of the protein while the C19 antibody that recognized an epitope present at the C-terminus of the protein. Accordingly, the N20 but not the C19 antibody recognized the $p27^{1-170}$ mutant. **B.** Immunofluorescence analysis of GBM adhered to FN for 1 hour confirmed that p27 (green) had a predominant cytoplasmatic localization in U87MG cells and an enriched nuclear localization in U251MG cells. Hoechst nuclear staining is shown in blue.

Interestingly, the caspase inhibitor Z-VAD –FMK almost completely prevented p27 cytoplasmic degradation (Figure 19) suggesting that the cytoplasmic cleavage of p27 in U251MG cells is dependent on caspase activity. It is thus possible that the different p27 subcellular localization coupled with a cytoplasmic specific caspase-dependent C-

terminus deletion is the cause of the lack of inhibition of migration observed in U251MG cells.

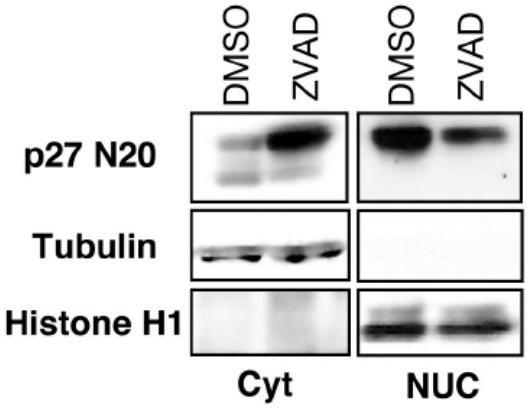


Figure 19. p27 cytoplasmic cleavage, caspase-dependent, in U251MG cells. Western blot analysis of p27 expression in the nucleus and in the cytoplasm of U251MG cells transduced with AdTRE $p27^{WT}$ and then treated for 48 hours with vehicle (DMSO) or with the caspase inhibitor Z-VAD–FMK (35μM, R&D Systems).

7. Doxycycline-regulated p27 expression impairs glioblastoma growth and invasion *in vivo*

In order to verify whether *in vivo* the various AdTRE p27s were able to inhibit glioblastoma cell growth, U87MG cells known to be able to grow when implanted subcutaneously in nude mice were used. On the contrary U251MG cells could not be used since they are unable to form tumors in the same type of assay (Ke et al., 2002). U87MG xenografts were established using pre-transduced cells and their growth was followed in animals fed with doxycycline. As controls, untreated and AdTRE βGal transduced cells were used. In this experiment, AdTRE $p27^{1-170}$ transduction resulted the most effective in growth inhibition (60%), whereas $p27^{wt}$ and $p27^{T187A}$ overexpression gave 43% and 54% of tumor growth inhibition, respectively (Figure 20), in line with the *in vitro* experiments (Figure 13). These data demonstrated that p27 overexpression is able to reduce GBM cell growth *in vivo*.

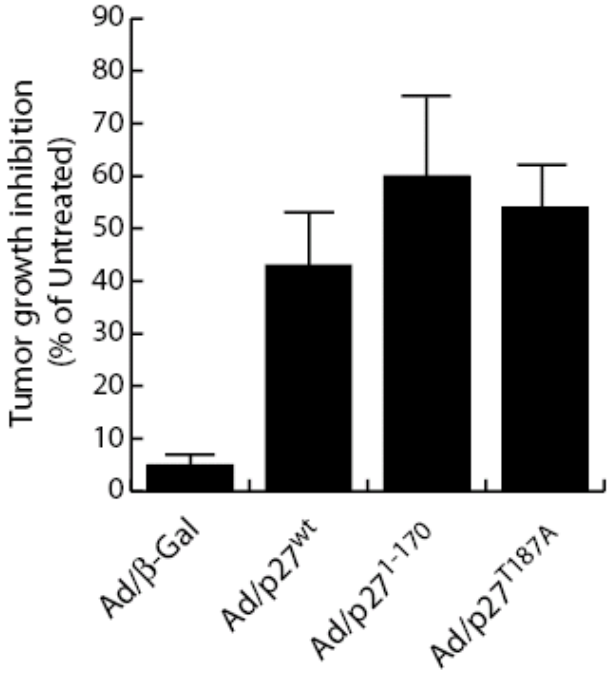


Figure 20. p27 impairs glioblastoma cells growth *in vivo*. U87MG cells transduced with the indicated AdTREs or left untreated were injected sudcutaneously in nude mice fed with doxycycline and allow to growth for 15 days. Mice were then sacrificed and tumor mass evaluated. Data are expressed as percent of growth inhibition (i.e. 50% represent the reduction to the half of the tumor mass expressed in grams) exerted by each AdTRE respect to untreated tumors. In each group of treatment 6/8 animals were used.

Next, we asked whether the various AdTRE p27s could be used to treat glioblastoma *in vivo*. To this aim, U87MG xenografts tumors were established in nude mice by subcutaneous injection. Tumor treatments, consisting of 5 intratumoral injections of the various AdTRE p27s, started as the tumors reached the volume of about 50 mm^3 (Figure 21). In a pilot experiment (n= 3/group of treatment) mice transduced with AdTRE $p27^{wt}$ and AdTRE $p27^{1-170}$ were fed or not with doxycycline and the tumor growth was evaluated every 3 days up to 24 days after injection unless noted. Western blot analysis demonstrated that also *in vivo* the transgene expression was regulated by doxycycline (Figure 21B). Induction of p27 expression by doxycycline treatment (Figure 21B) reduced tumor growth and increased the survival of mice of about 10 days (Figure 21A), demonstrating that growth inhibition was specifically due to p27 expression, and not to adenoviral transduction.

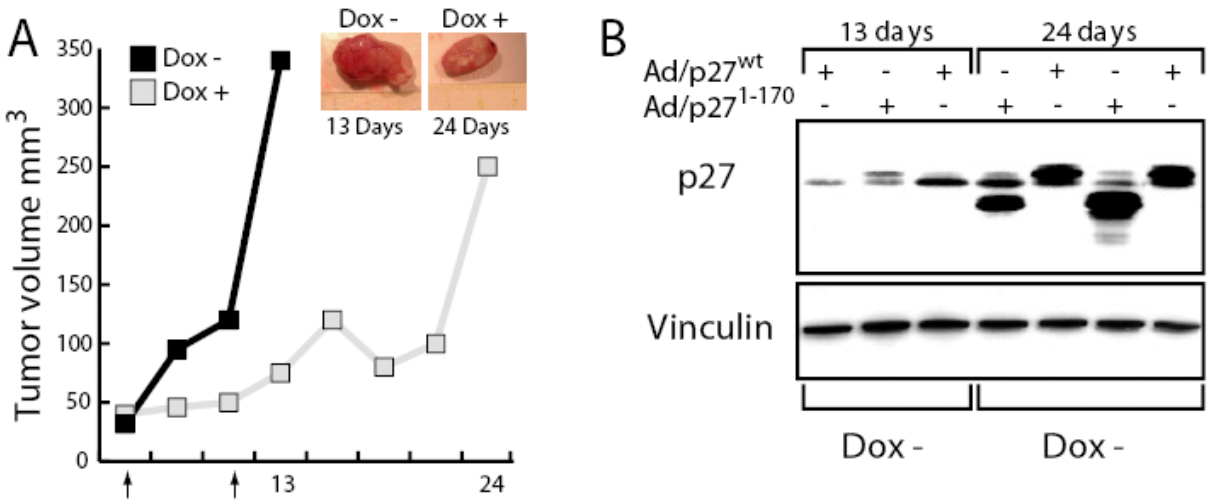


Figure 21. p27 impairs glioblastoma growth *in vivo*. A. Representative *in vivo* tumor growth of xenografts tumors treated with 5 intratumoral injection of AdTRE $p27^{wt}$ in mice fed with (Dox+) or without doxycycline (Dox-) in drinking water. In the inset typical images of the explanted tumors is reported. Arrows indicated the start and the end of the treatment. Mice fed without doxycycline have been sacrificed earlier (13 days after the first intratumoral injection of the adenovirus) for humanitarian reasons. **B**. Western Blot analysis of p27 expression in total protein lysates extracts from animals treated as in A and fed with (Dox+) or without doxycycline (Dox-) in drinking water. Vinculin was used as loading control.

In a larger experiment (n= 8-10 mice/group of treatment) all AdTRE p27s tested significantly inhibited U87MG growth (Figure 22), compared to untreated and AdTRE βGal transduced tumors. When tumor growth inhibition was evaluated using the tumor weight as a parameter, a significantly stronger activity was observed for AdTRE $p27^{wt}$ and $p27^{T187A}$ compared to AdTRE $p27^{1-170}$ (60% and 70% vs. 45% of tumor growth inhibition, respectively) (Figure 22).

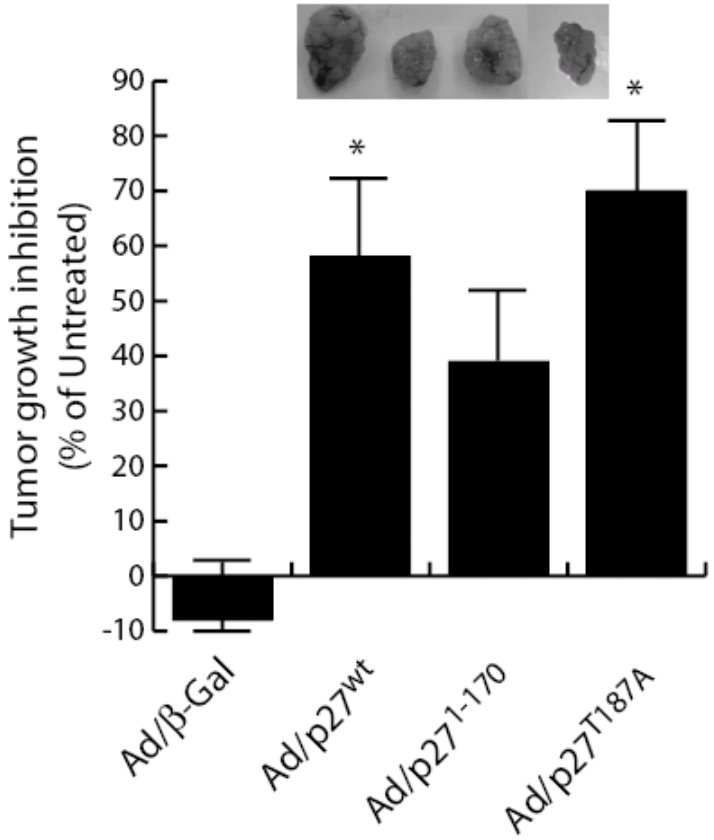


Figure 22. $p27^{wt}$ and $p27^{T187A}$ impair GBM growth *in vivo*. Tumor growth inhibition exerted by AdTRE β-Gal, $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$ intratumoral injection performed as in Figure 21 in animals fed with doxycycline. Mice were then sacrificed 15 days after the first adenovirus intratumoral injection. Percent of tumor growth inhibition was calculated on tumor mass expressed in grams as in Figure 21 and and represent mean ± SD of 8-10 animals/group of treatment. * = $p<0.05$ respect to the AdTRE $p27^{1-170}$ treatment.

Histological analysis of explanted tumors was carried out to search for signs of local invasion. Samples were judged as positive when at least 2 foci/section of invasive growth were found. Interestingly, we found that only 1 out of 7 different AdTRE $p27^{wt}$ treated-tumors exhibited signs of local invasion (12.5%) (Figures 23A and B), whereas untreated and AdTRE $p27^{1-170}$ treated tumors showed a local invasion in 57% and 60% of cases, respectively.

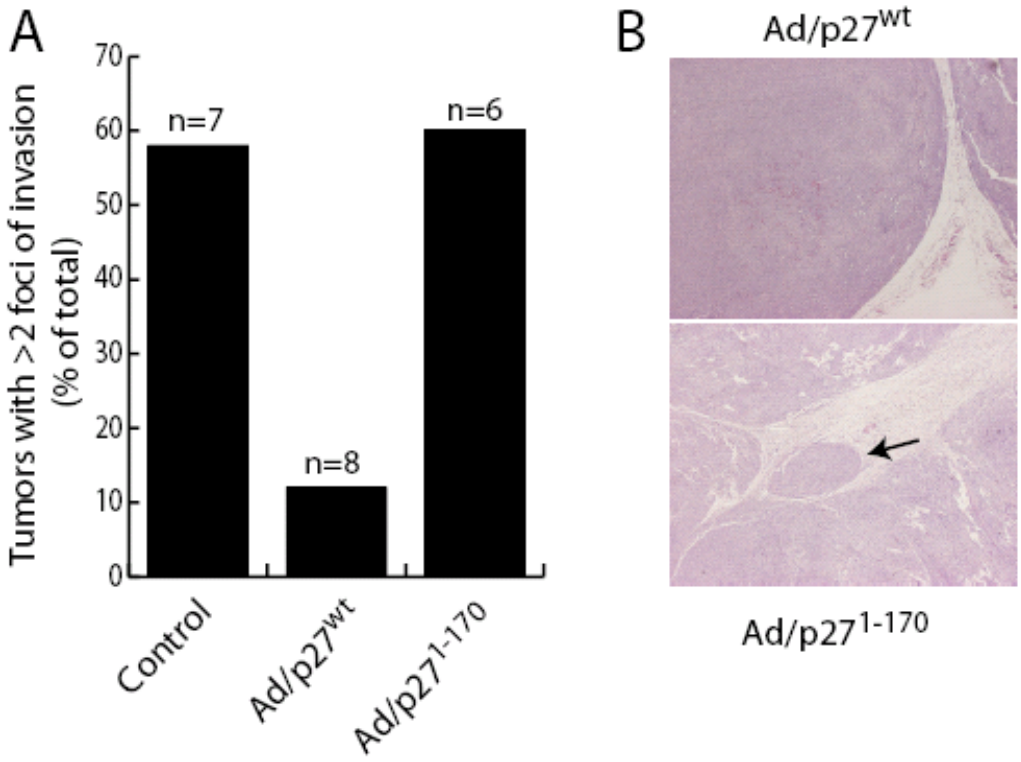


Figure 23. p27^{wt}, but not p27$^{1-170}$, inhibits local invasion. A. Histological evaluation of local invasion in explanted tumors treated as in Figure 22. The percentage of tumors with more than 2 zone of local invasion is reported. The number of analyzed tumors/group of treatment is reported in the graph. **B.** Typical images of GBM local invasion in AdTRE p27^{wt} and p27$^{1-170}$ treated tumors. The black arrow indicates the zone of invasion. (Magnification 40X).

p27 inhibition of cell invasion was linked to its ability to bind the MT-destabilizing protein stathmin (Baldassarre et al., 2005). Accordingly, in tumors treated with AdTRE p27^{wt}, but not with AdTRE p27$^{1-170}$, stathmin co-precipitated with the transduced protein (Figure 24), whereas the two proteins similarly bound to cyclin-CDK complexes (Figures 12C, D and E). Overall, these data demonstrated that intratumoral gene therapy approach based on p27 overexpression is able to significantly inhibit glioblastoma cell growth and invasion and that the C-terminal portion of p27 is necessary for the proper p27 activity *in vivo*.

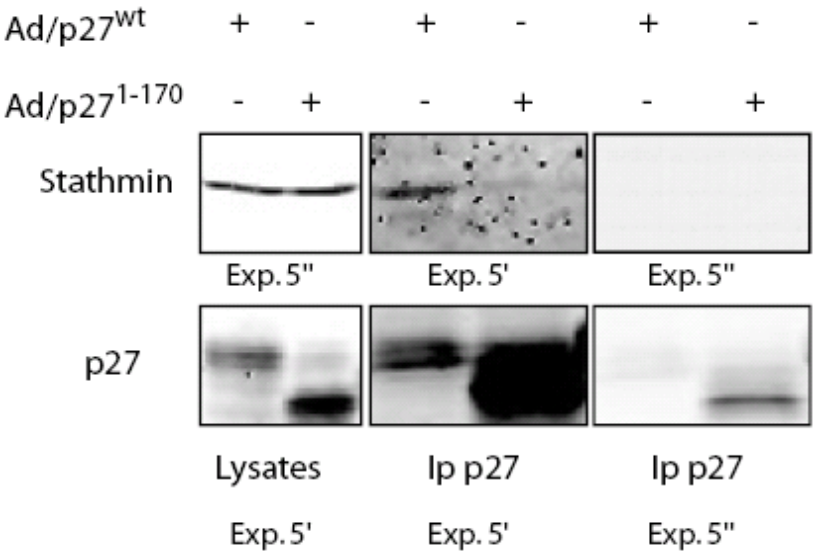
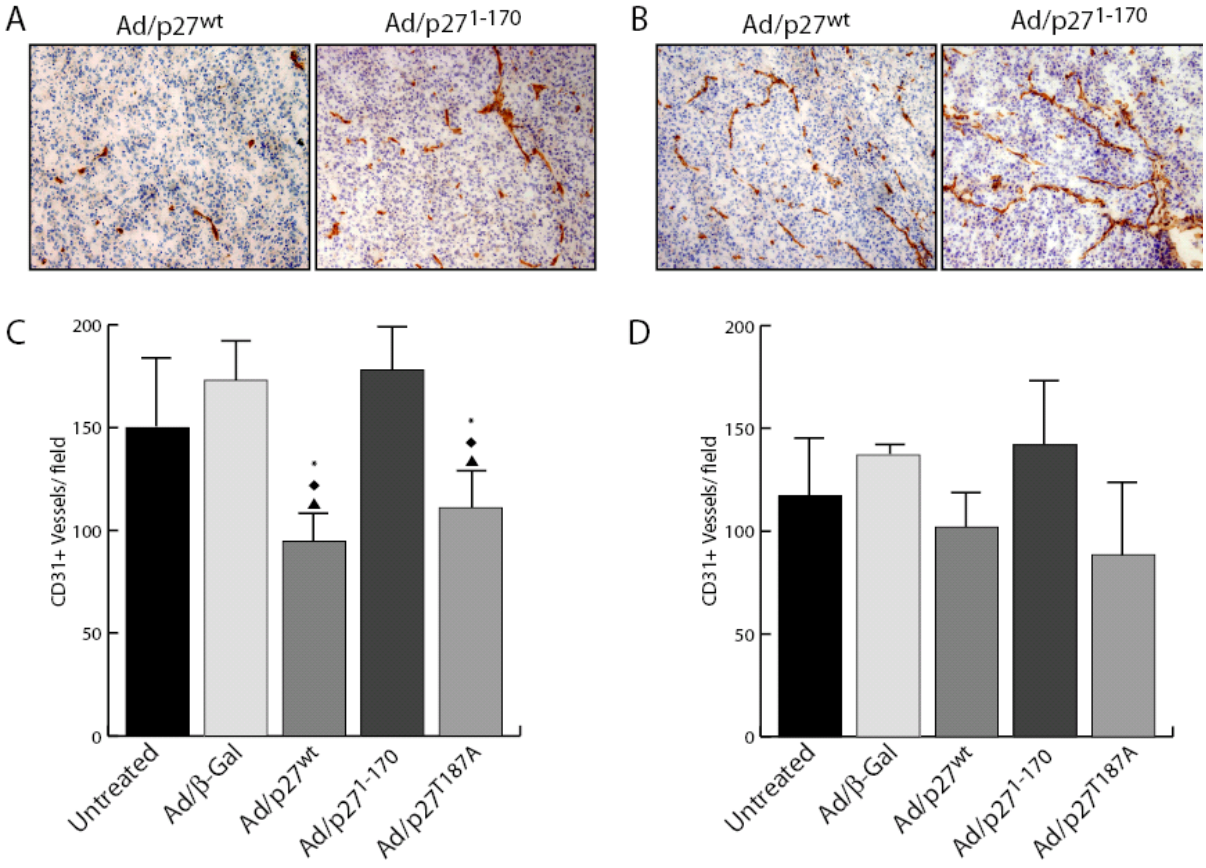


Figure 24. $p27^{wt}$, but not $p27^{1-170}$, co-immunoprecipitates with stathmin *in vivo*. Expression of p27 and stathmin in whole cell lysates (left panel) and in p27-immunoprecipitated proteins (middle and right panels), from AdTRE $p27^{wt}$ and $p27^{1-170}$ treated tumors. Stathmin co-precipitated with overexpressed $p27^{wt}$ but not with $p27^{1-170}$. The exposure time is indicated under each panel.

8. p27 expression affects tumor vascularization

The data collected using the intratumoral treatment were in apparent contrast with the tumor growth inhibition observed when U87MG cells were transduced with the various AdTRE p27s before tumor cells injection in nude mice. In this type of experiment, in fact, AdTRE $p27^{1-170}$ showed the strongest tumor growth inhibition, in accord with *in vitro* experiments. Thus, we speculated that intratumoral injections of AdTRE p27s could function not only on cancer cells but also by altering the tumor microenvironment. This hypothesis was also supported by the fact that AdTRE $p27^{wt}$ and $p27^{T187A}$ exerted a higher growth inhibitory activity when injected in preformed tumors respect to when pre-transduced in U87MG cells (compare Figures 20 and 22). Previous works demonstrated that inducible overexpression of $p27^{wt}$ and $p27^{T187A}$ in endothelial cells blocked DNA replication, inhibited cellular migration and tubulogenesis *in vitro*, and impaired angiogenesis in a mouse model of hind limb ischemia (Goukassian et al., 2001). Taking into account these results and our *in vivo* observations (Figure 20-24), we investigated whether p27 overexpression had any effect on U87MG tumor neoangiogenesis. To this aim we analyzed microvessel density in

explanted tumors derived from the intratumoral or from the pre-transduced treatments, using the anti CD31 (PECAM) antibody. When AdTRE $p27^{wt}$ and $p27^{T187A}$ were administered intratumorally, they significantly reduced the number (Figures 25A and B) and the length (Figure 25E) of CD31 positive vessels respect to control, AdTRE βGal or AdTRE $p27^{1-170}$ treated tumors. Conversely, when the same analysis was carried on tumors formed by pre-transduced cells, no significant difference was observed among the treatments, and tumors formed by untreated or pre-transduced U87MG cells presented similar vascularization (Figures 25C-E).



E

CD31 + Vessels Median length (µm)

Treatments	In vitro transduced	In vivo transduced	In vitro/In vivo ratio
Untreated	49,295	55,84	0,88
Ad/β-Gal	46,59	53,295	0,87
Ad/p27^{wt}	57,275	26,98 *	2,12
Ad/p27$^{1-170}$	37,75	40,94 **	0,92
Ad/p27^{T187A}	55,38	26,62 *	2,08

* $p < 0.0001$ vs Untreated, Ad/β-Gal and Ad/p27$^{1-170}$

** $p = 0.01$ vs Untreated or Ad/β-Gal

Figure 25. **p27 expression affects tumor vascularization. A/C.** Typical images of tumors treated with intratumoral injections of the indicated adenoviruses (**A**) or formed from pre-transduced cells (**C**) and stained with the anti-CD31 (PECAM) ab (in brown). (Magnification 250X). **B/D**. Quantification of vessel densities/field in tumors treated as indicated by intratumoral injection (**B**) or formed from pre-transduced cells (**D**). Microvessel density in AdTRE p27^{wt} and p27^{T187A} treated tumors was reduced by at least 37% compared with untreated tumors. *, $p< 0.05$ vs. untreated, ♦, $p< 0.01$ vs p27$^{1-170}$-treated and vs. AdTRE β-gal treated tumors, as calculated by t-student test. **E**. Comparative analysis of vessel median length in tumors formed from pre-transduced cells, treated with Ads intratumoral injections. Median vessel length ratio obtained comparing both treatments. Statistical significance was obtained using the t-student test.

To understand whether tumor cells affected the microenvironment and surrounding cell niches, a semiquantitative proteomic analysis of U87MG conditioned medium derived from non-transduced and AdTRE $p27^{wt}$ transduced cells on 174 known cytokine demonstrated no significant differences in the amount of pro-angiogenic factors produced by U87MG cells (Table 1). In fact, for example, U87MG conditioned medium derived from non-transduced and AdTRE $p27^{wt}$ transduced cells produced the same amount of IL-8 (Brat et al., 2005), VEGF (Cantarella et al., 2006), PDGF-AA, TIMP-2, TGF-β2, angiogenic factors in gliomas (Kargiotis et al., 2006; Fischer et al., 2005). These data suggested that the different tumor vascularization was due to a direct effect of p27 on mouse endothelial cells rather than to a modification of tumor cells growth factor production.

Cytokines, Chemokines & Growth Factors	*U87MG Conditioned Medium*	*Ad/p27^{wt}-transduced U87MG Conditioned Medium*
Activin-A[b]	+	+
Angiogenin[b]	+	++
Angiopoietin-2[a]	+	+
BDNF[c]	+	+
BMP-4	+	+
BMP-5	+	+
BMP-6[b]	+	+
BMP-7	+	+
b-FGF[a,b,c,d]	+	+
FGF-9[d]	+	+
GCSF[c,d]	+	+
GDNF[d]	++	++
GROα[b]	+	+
HGF[a,c]	+	+
IGF-2[a,c]	+	+
IL-1α[c]	+	+
IL-6[*a,b,c*]	+	+
IL-8[*a,b,c,d*]	+++	+++
MMP-1[b]	++	++
MMP-9[b]	+	+
NAP-2[a]	++	++
b-NGF[b,c]	+	+
Oncostatin M[b]	+	+
PDGF-AA[a]	++	+++
PDGF-AB[a]	+	+
RANTES	++	++
TIMP-1	+++	+++
TIMP-2[a,d]	+++	+++
TIMP-4	+++	+++
TGF-β2[a]	++	++
TGF-β3[a]	+	+
TNF-α[b]	+	+
VEGF[a,b]	++	++
VEGF-D[a,b]	+	+

Table 1. Expression of Cytokines, Chemokines and growth factors in U87MG conditioned medium. *a: angiogenic factors in gliomas; b: involved in angiogenesis*; *c: soluble factors involved in glioma invasion; d: detected in GBM cell lines CMs*
Semiquantitative analysis of proteins expression was evaluated respect to positive control furnished by manufacturers expressed in arbitrary units (AU) and categorized as follow: Neg= <0.1 AU, + = >0.1<0.4 AU, ++ = ≥ 0.4 < 0.8 AU,+++ = ≥ 0.8 AU.

9. Standardization of doxycycline-regulated adenoviral expression system in HUVEC

To analyze the role of p27 on tumor induced angiogenesis, we used HUVEC (Human Umbelical Vein Endothelial Cells) as a model system. We determined the optimal co-transduction ratio for the HUVEC using Ad TREβGal/AdTet-ON. Cells were transduced with the different Ad ratios and 48 hours later total extracts were tested in β-galactosidase assay. The best co-transduction ratio for these cells was 60 particles of AdTRE βGal and 30 particles of AdTet-ON (arrow indicated in Figure 26A). After HUVEC were transduced with AdTRE βGal/AdTet-ON 60 :30 and incubated for 72 hours in presence of different doxycycline concentrations, and then β-galactosidase activity was determined on total extracts. The doxycycline concentration chosen for further experiments in these cells was 1 μg/ml (arrow indicated in Figure 26B).

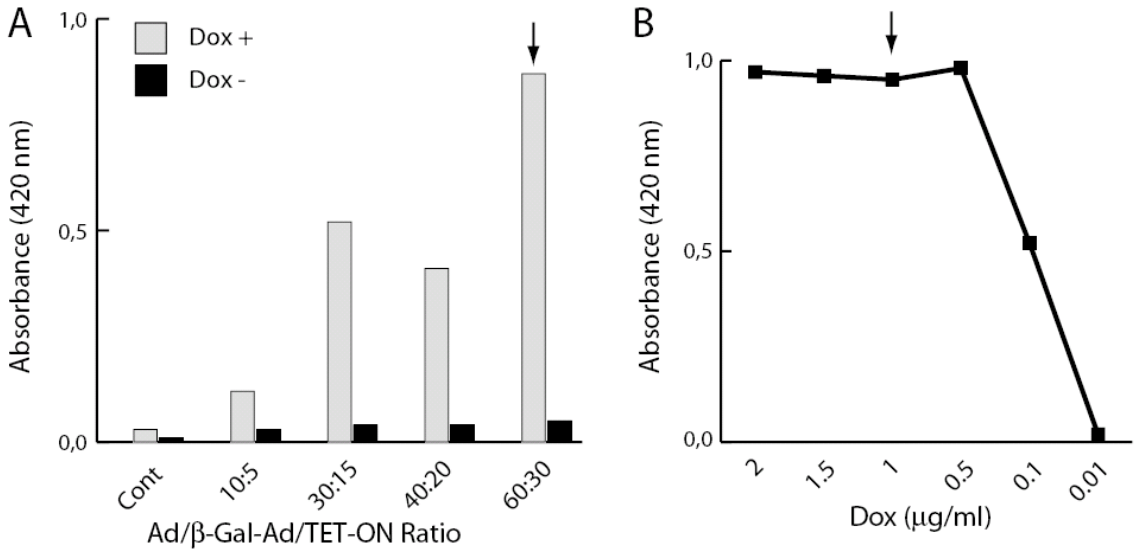


Figure 26. **Standardization of doxycycline-regulated adenoviral expression system in HUVEC**. **A.** Determination of the suitable co-transduction ratio for HUVE Cells using AdTRE βGal and AdTet-ON. Cells were transduced with the indicated ratios in the presence of 2μg/ml doxycycline and 48 hours later total cell extracts were tested in β-galactosidase assay. Arrows indicate the chosen co-transduction ratio. **B.** HUVE Cells were transduced with the most suitable ratio AdTRE βGal/AdTet-ON as determined (60:30) and incubated in presence of indicated doxycycline concentrations for 48 hours before the assessment of β-galactosidase activity. Data in A and B are representative of 3 independent experiments.

Then the recombinant adenoviruses expressing $p27^{wt}$ and two mutants $p27^{1-170}$ and $p27^{T187A}$ were tested on HUVEC. To obtain regulated p27 expression, target cells were co-transduced with two viruses: the AdTet-ON (moi 30) and the AdTRE that encodes for the p27 wt or mutant proteins (moi 60) in presence or absence of doxycycline. Time course analysis of p27 over-expression in HUVEC showed that the maximal p27 expression in this cell line was between 48-72 hours after viral transduction (Figure 27) by Western Blot.

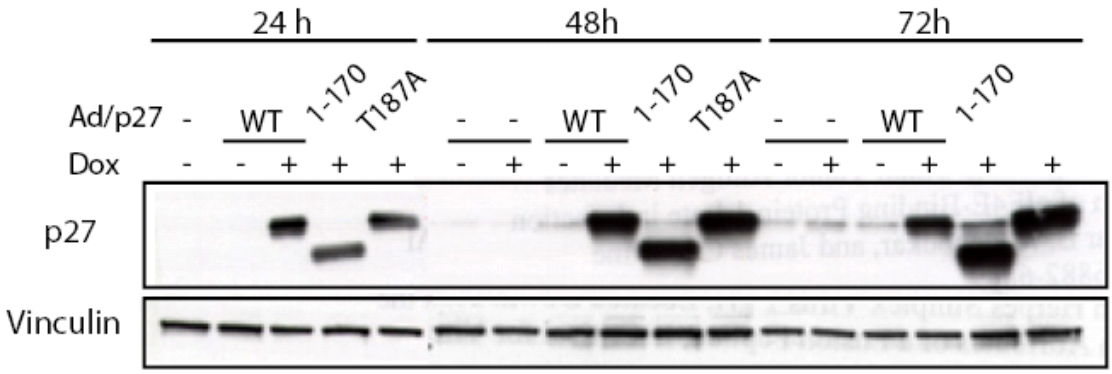


Figure 27. Time course analysis of p27 overexpression in HUVEC. Co-transduction ratios AdTRE p27s/AdTet-ON 60:30 were used. Total cell extracts were prepared at the indicated times post-transduction and different p27s expression was analyzed in Western Blot. Vinculin was used for loading control.

Biological characterization of p27 overexpression on cell growth was performed on HUVE cells. HUVEC were transduced with the different AdTRE p27s and 72 hours later fixed and analyzed by flow cytometry for their DNA content (Fig 28). Over-expression of $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$ resulted in a similar proliferation inhibition, as expected, in fact all three AdTRE p27s were able to arrest the cell cycle progression in G1 phase, reducing the cell percentage in S and G2 phases. The amount of untreated HUVEC in G1 and S phases was approximately 31% and 36% respectively while the percentage of transduced cells with all three different AdTRE p27s shifted to 60% in G1 phase and 15% in S phase.

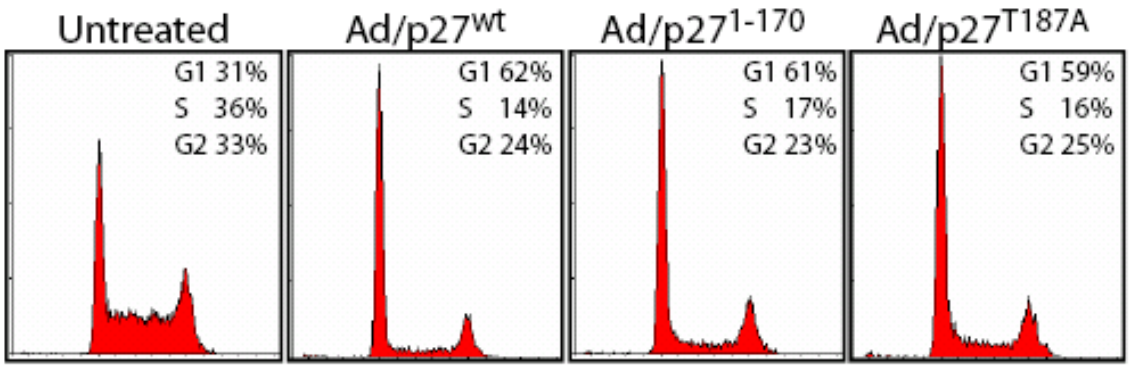


Figure 28. p27 inhibits HUVEC proliferation. FACS analysis of DNA content distribution in HUVEC untreated or transduced for 72 hours with the indicated AdTRE p27 in the presence of doxycycline. A typical experiment (out of 3) is shown. The percentage of cells in the different phases of the cell cycle is shown in each graph.

10. p27 impairs endothelial cells motility through Stathmin

It is well known that endothelial cells are capable of aggregating in vitro to form capillary-like structures (Folkman and Haudenschild, 1980; Maciag et al., 1982). This process, which is thought to mimic the process by which endothelial cells form capillaries *in vivo*, requires specializes culture conditions. When endothelial cells were grown on basement membrane collagens (such as Matrigel, a substrate composed of basement membrane proteins), they did not proliferate but did aggregate and form tube-like structures at early culture times (Madri and Williams, 1983).

We first evaluated the effects of U87MG conditioned medium (U87-CM) on HUVEC motility, comparing it to the HUVEC standard growth medium. U87 conditioned medium was obtained growing U87MG cells in RPMI serum free medium plus 1% of BSA. HUVEC were seeded on Matrigel gel and incubated with M199 growth medium or with U87-conditioned medium (Figure 29). Interestingly, U87-CM profoundly increased HUVEC ability to form tube-like structures respect to normal growth medium.

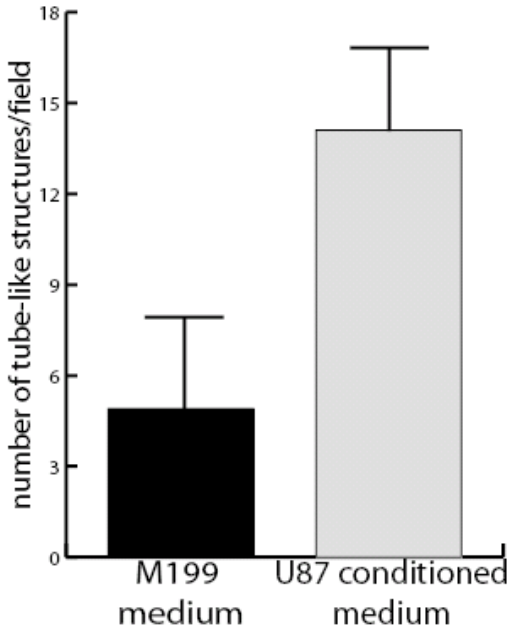


Figure 29. U87-CM increases HUVEC ability to form tube-like structures. Number of tube-like structures/field (magnification 200x) formed by HUVEC plated on Matrigel™ and incubated for 2 hrs in M199 enriched medium (M199) or in U87MG conditioned medium (U87 CM). Results represent the mean (± SD) of 3 independent experiments in which 10 different randomly selected fields were counted.

Then, we evaluated the effect of $p27^{wt}$, $p27^{1-170}$ and $p27^{T187A}$ on HUVEC motility stimulated by U87-CM. HUVEC were transduced with the different AdTRE p27s and 48 hrs post-transduction were seeded on Matrigel and incubated with U87 conditioned medium (Figure 30). As shown in Figure 30, $p27^{wt}$ and $p27^{T187A}$ significantly impaired tube-like structures formation by HUVEC (4.8±2.1 and 5.5±3 structures/field, respectively, respect to 18±8 of non transduced cells; $p \leq 0.01$), whereas no significant difference was induced by $p27^{1-170}$ respect to control cells (11±4 vs. 18±8, respectively; $p=0.2$) (Figure 30).

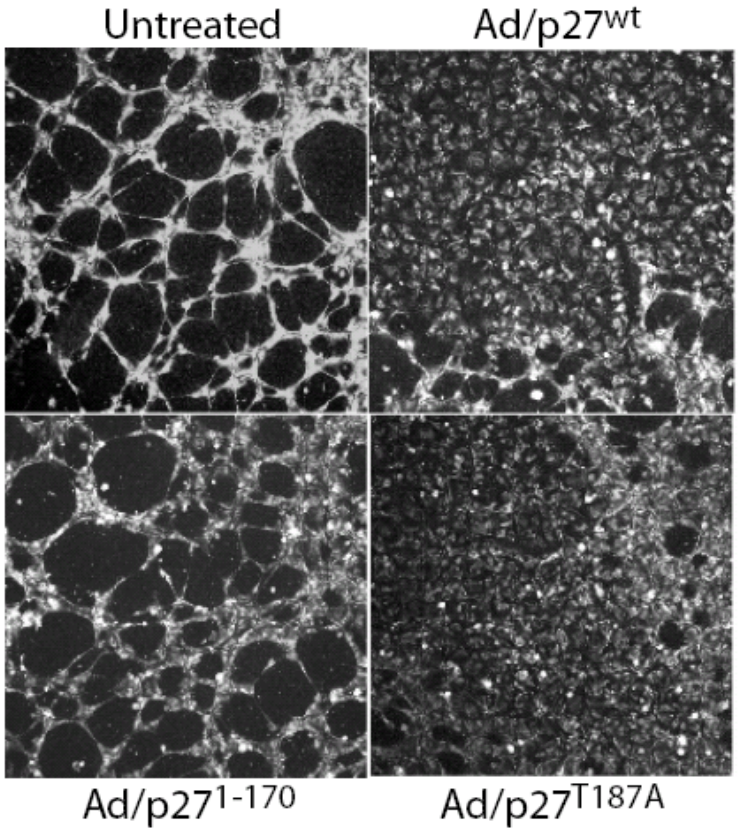


Figure 30. p27^{wt} and p27^{T187A} impair tube-like formation by HUVEC. Typical images formed by HUVEC transduced as indicated and two days later plated on Matrigel^{TM} and incubated for 2 hrs in U87CM. (magnification 200X).

We previously described that the negative effects of p27 on sarcoma cell migration requires two fundamental modifications: the cytoplasmic localization of p27 in response to cell–ECM interaction, and the consequent p27 binding to the microtubule-destabilizing protein stathmin (Baldassarre et al., 2005).

We thus verified whether in endothelial cells the same mechanism was functioning. We first investigated the localization of endogenous p27 proteins in HUVE cells adhered to FN for 1 hour by nucleo-cytoplasmic proteins extraction and by Western Blot analysis. p27 was clearly shuttled from nucleus to cytoplasm in an adhesion-dependent manner, in fact cytoplasmic shift of p27 is clearly observed after 30 and 60 minutes of adhesion to FN (Figure 31A). To confirm the activation of intracellular pathways due to cell-ECM contact, we analyzed the adhesion dependent activation of AKT using a phospho-specific antibody directed against its phosphorylated ser 473. Then we analyzed if, after cytoplasmic localization in response to cell–ECM interaction, p27 was able to bind to

stathmin. By co-immunoprecipitation analysis, we observed an increased adhesion-dependent association between p27 and stathmin (Figure 31B).

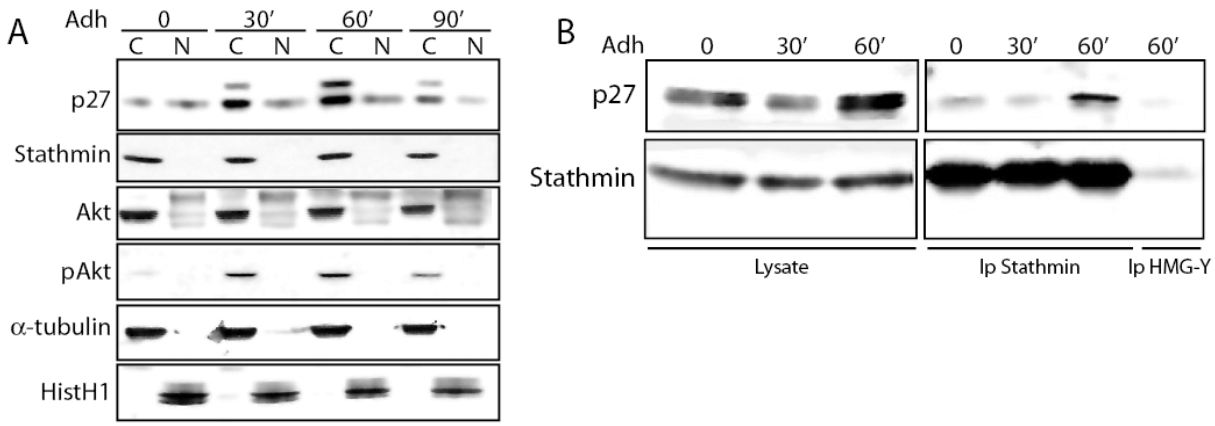


Figure 31. Cytoplasmic localization of p27 and consequent binding to stathmin. A. HUVEC were adhered to FN coated plates and at the indicated times cytoplasmic/nuclear extracts were prepared (C/N) and analyzed in Western Blot analysis for p27 and stathmin expression. Tubulin and histone H1 expression were used as controls for the cytoplasmic fractions and nuclear purity and loading fractions, respectively. **B**. Expression of endogenous p27 and stathmin in cytoplasmic extracts of HUVEC serum starved (0) and then adhered to FN for 30 and 60 minutes and in the same extracts immunoprecipitated with an anti-stathmin ab. IP-NS indicates the immunoprecipitation performed with a non specific rabbit IgG.

Then we analyzed p27 and stathmin endogenous expression in HUVEC plated on Matrigel and allowed to form tube-like structures for 2 hours at 37°C by immunofluorescence analysis. As shown in Figure 32 we noticed an enriched stathmin (red) expression in cells forming the tubes on Matrigel. Importantly, p27 (green) seemed to colocalize with stathmin (yellow) in HUVEC moving on Matrigel.

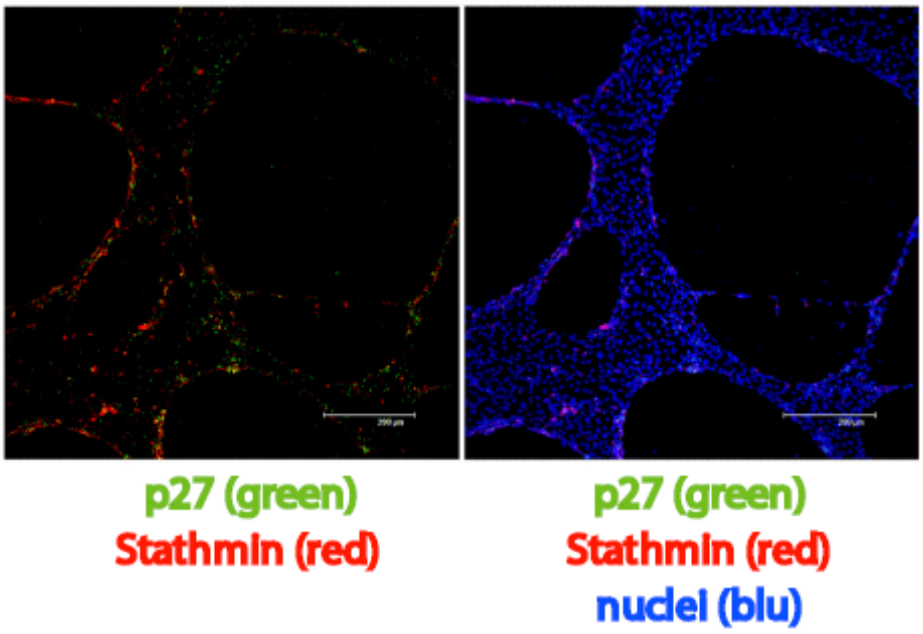


Figure 32. Immunofluorescence analysis of p27 (green) and stathmin (red) expression in HUVEC plated on Matrigel and allowed to form tube-like structures for 2 hours at 37°C. An enriched stathmin expression is observed in cells forming the tubes. A scatter colocalization (yellow) of endogenous p27 and stathmin is also observed. In the lower panel the nuclear staining (blu) of the same field is shown.

Our data support the hypothesis that p27 regulates HUVEC cell motility through the binding and the inhibition of stathmin. To prove this hypothesis, we used an adenoviral system encoded for a specific small-interference to silence stathmin. HUVEC were co-transduced with AdTRE $p27^{wt}$ and 24 hrs later with AdSTM-siRNA (or not) and seed on Matrigel basement (Figure 33). The overexpression of p27 and the silencing of stathmin were confirmed by Western Blot analysis (Figure 33 the inner panel). The downregulation of stathmin in HUVEC resulted in a strong inhibition of tube-like structures formation that was not affected by co-overexpression of $p27^{wt}$ protein. This results confirm that stathmin expression is necessary for proper endothelial cell motility and that its association with p27 could be an important event in the regulation of endothelial cell motility, both *in vitro* and *in vivo*.

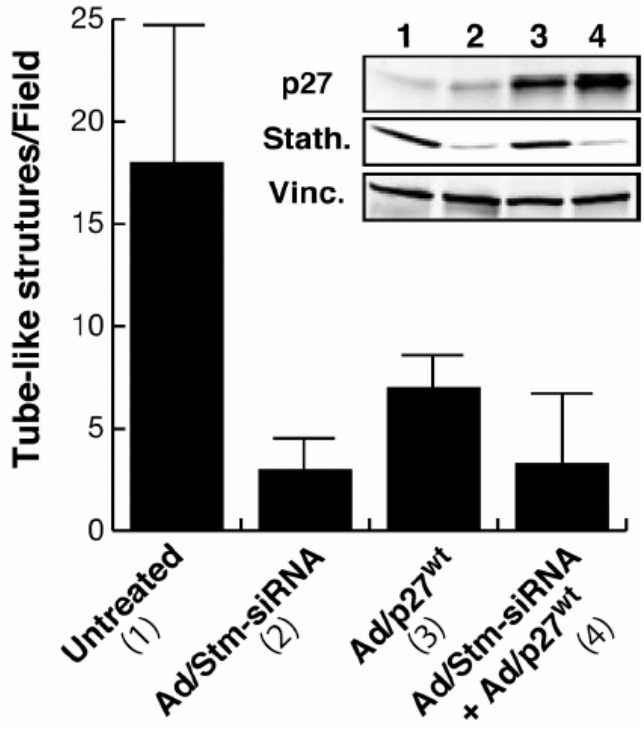


Figure 33. p27 regulates HUVEC cells motility through stathmin. Number of tube-like structures/field (magnification 200x) formed by HUVEC transduced with the indicated adenovirus and 3 days later plated on Matrigel™ and incubated for 2 hrs in U87-CM. Results represent the mean (± SD) of 2 independent experiments in which 10 different randomly selected fields were counted. In the inset the expression of stathmin and p27 in HUVEC used in a typical experiment is reported. Lane 1 Untreated cells; Lane 2-4 cells were transduced with the indicated adenovirus: 2 = Ad/Stathmin siRNA; 3 = Ad/TRE $p27^{wt}$; 4 =Ad/TRE $p27^{wt}$ + Ad/Stathmin siRNA.

DISCUSSION

Uncontrolled cell proliferation is one of the hallmarks of cancer (Hanahan and Weinberg, 2000). This could be caused by mutation or misregulation of cell-cycle regulatory genes and proteins (Sherr and McCormick, 2002). Among the various cell cycle regulatory proteins, particular interest has received the role of the CKI p27 in tumor progression. In fact, many human cancers express decreased amounts of p27 compared to normal tissues, and reduced expression correlates with increased tumor aggressiveness and a poor clinical outcome (Bloom and Pagano, 2003; Slingerland and Pagano, 2000; Philipp-Staheli et al., 2001). Although, conflicting data on the expression of p27 in human gliomas and its role as an independent prognostic factor have been reported (Kirla et al., 2003; Zagzag et al., 2003), it seems that grade IV astrocytomas (GBM) usually display decreased expression of p27 protein respect to grade I-III tumors (Tamiya et al., 2001). Moreover, some studies of astrocytomas have reported an inverse relationship between p27 expression and proliferative activity within the same tumors assessed by Ki67 immunostaining (Fuse et al., 2000; Nakasu et al., 1999). In our small case record, by comparing anaplastic astrocytomas versus glioblastomas, we supported the correlation between low expression pattern of p27 and tumor grade. Primary GBM show a low nuclear p27 expression respect to astrocytomas and this finding has been then confirmed in a panel of GBM cell lines.

GBM are usually refractory to conventional therapies, including aggressive surgical resection, radiation, and chemotherapy. Their diffuse, highly infiltrative nature precludes complete resection, leading to almost inevitable recurrence. Gene therapy is a promising alternative because GBM consist of localized rapidly dividing cells in a background of nondividing cells, and rarely form distant metastasis. This enables direct inoculation of the tumor, or postoperative tumor cavity, with the gene therapy agent. These notions suggest that the introduction of p27 gene into GBM cells is expected to be a promising strategy to inhibit their malignant cellular proliferation. Considering that, we generate a doxycycline-regulated adenoviral system to obtain a modulated p27 expression to be used in a gene therapy program on different human glioblastoma cell lines. Using this experimental approach we obtained several promising results. First of all, we demonstrated that three different AdTRE p27s are able to transduced all tested GBM cell lines and that by increasing the MOI of adenoviruses used, it is possible to transduce a significant percentage (more than 80%) of GBM cells obtaining a good expression of the transgene. Our *in vitro* work, demonstrated that p27 is able to inhibit

glioblastoma cell growth and in long term growth assay, such is the colony assay, AdTRE $p27^{1-170}$ results the most effective one. This effect may due to the different regulation of p27 mutants, in fact $p27^{1-170}$ lacks both T187 and T198, two key residues involved in p27 protein stability regulation. p27 is phosphorylated at T198 in a PI3K/Akt dependent manner in breast (Motti et al., 2004) and in thyroid cancer cells (Motti et al., 2005), and this phosphorylation contributes to p27 cytoplasmic mislocalization. In astrocytomas, mutations of PTEN, a central negative regulator of the PI3K/AKT signaling cascade, are rarely observed in low-grade tumors and occur frequently in glioblastoma multiforme (Kitange et al., 2003), suggesting that the use of p27 mutant in the tumor treatment, lacking T187 and the T198, may synergize to prevent the cell cycle progression along G1 phase (Kossatz et al., 2006).

Furthermore, we confirmed that in glioblastoma cells p27 proteins overexpression effectively induces on autophagy, as previously proposed (Komata et al., 2003). Autophagy can promote cell adaptation and survival, but under some conditions it could lead to cell death (Maiuri et al., 2007). A number of studies have reported that autophagy, or autophagic cell death, is activated in cancer cells that are derived from tissues such as breast, colon, prostate and brain, in response to various anticancer therapies. For example, Temozolomide (TMZ), a DNA alkylating agent, induces autophagy, but not apoptosis, in malignant glioma cells (Kanzawa et al., 2004). Other anticancer treatments that induce autophagy or autophagic cell death include γ-irradiation, which induces autophagy in cancer cells of epithelial origin, such as breast cancer, prostate cancer and colon cancer (Paglin et al., 2001), and also in malignant glioma cells (Ito et al., 2005; Yao et al., 2003). Additionally, Liang et al., (2007) demonstrated that LKB1-AMPK pathway-dependent phosphorylation of p27 at Thr 198 stabilizes p27 and permits cells to survive after growth factor withdrawal and metabolic stress through autophagy. The authors suggest that autophagy may contribute to tumor-cell survival under conditions of growth factor deprivation, disrupted nutrient and energy metabolism, or during stress of chemotherapy, although no prove exists demonstrating whether p27-induced autophagy in cancer represents a survival or a death factor. In fact, the role of autophagy in cancer is still largely unknown, and even the most fundamental question, whether autophagy kills cancer cells or protects them from unfavorable destiny, has not been clearly answered. Thus further studies are necessary

to better understand the significance and the molecular mechanisms underlying this p27 and mutants-effect.

Moreover, our *in vitro* work demonstrated that p27 is able to inhibit also cell motility in glioblastoma cells. This effect depends on the C-terminal portion of the protein and on its cytoplasmic displacement. Accordingly, in U251MG p27 overexpression did not interfere with cell motility due to the fact that in these cells p27 is retained in the nucleus and is cleaved at its C-terminus by caspase activity. Previous work demonstrated that caspase are able to cleave p27 (Loubat et al., 1999) and this cleavage leads to the cytosolic sequestration of p23, results from the proteolysis of p27 by a 'caspase-3-like' protease, but does not alter its binding properties to CDK2 and CDK4 kinases. Our data demonstrated that in U251MG cells cleavage of p27 occurs at high rate in the cytoplasm suggesting that several mechanisms control p27 expression, localization and activity in GBM cells.

Importantly, the use of different mutants allowed us to demonstrate that p27 may also play important roles in the control of tumor microenvironment modification, specifically inhibiting tumor-induced neoangiogenesis. This behavior is not due to a modification of tumor cells growth factor production, but it is a direct effect of p27 on mouse endothelial cells. In fact the same amount of angiogenic factors in gliomas, or involved in angiogenesis is detected, analyzing the conditioned medium derived from tumor overexpressing p27 or not.

Here, we provide the first formal demonstration that p27/stathmin interaction plays a pivotal role in the control of tumor cell invasion in *in vivo* models, confirming our previous results using *in vitro* experiments (Baldassarre et al., 2005). Recent data suggest that p27 could also play a pro-tumorigenic role in mice (Besson et al., 2007), however whether the “oncogenic” activity of p27 depend on its cytoplasmic (Denicourt et al., 2007) or nuclear part is still to be determined and whether it is dependent on its role in the control of cell motility is similarly unexplored (Sicinski et al., 2007). Interestingly, recent data suggest that the knock-out of p27 in human mammary carcinoma cells results in increased cell growth and motility (Yuan et al., 2007), supporting again its tumor suppressive role, at least in human cancer cells. Our data suggest that, at least in part, the motility inhibition exerted by p27 is mediated by its interaction with stathmin in the cytoplasm of tumor and/or endothelial cells through the last 28 amino acids at p27 C-terminal. In fact $p27^{1-170}$ is unable to bind to and inhibit

stathmin activity both *in vitro* and *in vivo*. Using a siRNA approach on HUVEC we confirm that stathmin expression is necessary for proper endothelial cell motility and that the effects of stathmin downregulation on HUVEC motility are not affected by co-concomitant overexpression of p27wt protein. It is increasingly clear that modulation of microtubules dynamics plays an important role in the regulation of several aspects of cell physiology, including cell motility (Etienne-Manneville, 2004). Thus, it is not surprising that interfering with stathmin activity can result in the alteration of several aspects of the cell physiology. Yuan et al. (2006) demonstrated that stathmin overexpression is associated with tumour progression, early recurrence, and poor prognosis in hepatocellular carcinoma. Moreover, in accord with our findings, recent data demonstrate that high stathmin expression in glioblastoma represents a marker of poor prognosis (Ngo et al., 2007). Additionally, Atweh and colleagues (2007) recently confirmed a prominent role for stathmin in the control of HUVEC motility, showing that the exposure of endothelial cells to anti-stathmin adenovirus resulted in a dose-dependent inhibition of proliferation, migration, and differentiation into capillary-like structures, thus independently confirming our finding.

In summary, we report here that, at least in GBM, p27-based gene therapy could represent a promising approach, and provide evidence that its tumor suppressive activity is due not only to block of cell proliferation but also to tumor microenvironment modification, opening new way to look at gene therapy approach in human cancer.

REFERENCES

Aghi M, Rabkin S. (2005). Viral vectors as therapeutic agents for glioblastoma. *Curr Opin Mol Ther*. Oct;7(5):419-30. Review.

Agrawal D, Hauser P, McPherson F, Dong F, Garcia A, Pledger WJ. (1996). Repression of p27kip1 synthesis by platelet-derived growth factor in BALB/c 3T3 cells. *Mol Cell Biol*. Aug;16(8):4327-36.

Ahn J, Murphy M, Kratowicz S, Wang A, Levine AJ, George DL. (1999). Down-regulation of the stathmin/Op18 and FKBP25 genes following p53 induction. *Oncogene*. Oct 21;18(43):5954-8.

Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A, Pestell RG. (1995). Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J Biol Chem*. Oct 6;270(40):23589-97.

Arnberg N, Edlund K, Kidd AH, Wadell G. (2000). Adenovirus type 37 uses sialic acid as a cellular receptor. *J Virol*. Jan;74(1):42-8.

Baldassarre G, Belletti B, Bruni P, Boccia A, Trapasso F, Pentimalli F, Barone MV, Chiappetta G, Vento MT, Spiezia S, Fusco A, Viglietto G. (1999). Overexpressed cyclin D3 contributes to retaining the growth inhibitor p27 in the cytoplasm of thyroid tumor cells. *J Clin Invest.* Oct;104(7):865-74.

Baldassarre G, Belletti B, Nicoloso MS, Schiappacassi M, Vecchione A, Spessotto P, Morrione A, Canzonieri V, Colombatti A. (2005). p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion. *Cancer Cell*. Jan;7(1):51-63.

Bartkova J, Lukas J, Bartek J. (1997). Aberrations of the G1- and G1/S-regulating genes in human cancer. *Prog Cell Cycle Res*.;3:211-20. Review.

Bates S, Bonetta L, MacAllan D, Parry D, Holder A, Dickson C, Peters G. (1994). CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of the cyclin-dependent kinases that associate with cyclin D1. *Oncogene*. Jan;9(1):71-9.

Beilharz EJ, Zhukovsky E, Lanahan AA, Worley PF, Nikolich K, Goodman LJ. (1998). Neuronal activity induction of the stathmin-like gene RB3 in the rat hippocampus: possible role in neuronal plasticity. *J Neurosci*. Dec 1;18(23):9780-9.

Belletti B, Nicoloso MS, Schiappacassi M, Chimienti E, Berton S, Lovat F, Colombatti A, Baldassarre G. (2005). p27(kip1) functional regulation in human cancer: a potential target for therapeutic designs. *Curr Med Chem*.;12(14):1589-605. Review.

Belmont LD, Hyman AA, Sawin KE, Mitchison TJ. (1990). Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell.* Aug 10;62(3):579-89.

Belmont LD, Mitchison TJ. (1996). Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. *Cell*. Feb 23;84(4):623-31.

Beretta L, Dobránsky T, Sobel A. (1993). Multiple phosphorylation of stathmin. Identification of four sites phosphorylated in intact cells and in vitro by cyclic AMP-dependent protein kinase and p34cdc2. *J Biol Chem.* Sep 25;268(27):20076-84.

Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*. Feb 28;275(5304):1320-3.

Besson A, Gurian-West M, Schmidt A, Hall A, Roberts JM. (2004). p27Kip1 modulates cell migration through the regulation of RhoA activation. *Genes Dev*. Apr 15;18(8):862-76.

Besson A, Hwang HC, Cicero S, Donovan SL, Gurian-West M, Johnson D, Clurman BE, Dyer MA, Roberts JM. (2007). Discovery of an oncogenic activity in p27Kip1 that causes stem cell expansion and a multiple tumor phenotype. *Genes Dev*. Jul 15;21(14):1731-46.

Bett AJ, Krougliak V, Graham FL. (1995). DNA sequence of the deletion/insertion in early region 3 of Ad5 dl309. *Virus Res.* Nov;39(1):75-82.

Bloom J, Pagano M. (2003). Deregulated degradation of the cdk inhibitor p27 and malignant transformation. *Semin Cancer Biol.* Feb;13(1):41-7. Review.

Boehm M, Yoshimoto T, Crook MF, Nallamshetty S, True A, Nabel GJ, Nabel EG. (2002). A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression. *EMBO J*. Jul 1;21(13):3390-401.

Bouchard C, Thieke K, Maier A, Saffrich R, Hanley-Hyde J, Ansorge W, Reed S, Sicinski P, Bartek J, Eilers M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. *EMBO J*. Oct 1;18(19):5321-33.

Brat DJ, Bellail AC, Van Meir EG. (2005). The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro Oncol.* Apr;7(2):122-33. Review.

Brattsand G, Marklund U, Nylander K, Roos G, Gullberg M. (1994). Cell-cycle-regulated phosphorylation of oncoprotein 18 on Ser16, Ser25 and Ser38. *Eur J Biochem*. Mar 1;220(2):359-68.

Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*. Feb 5;391(6667):597-601.

Bursch W, Ellinger A, Gerner C, Fröhwein U, Schulte-Hermann R. Programmed cell death (PCD). (2000). Apoptosis, autophagic PCD, or others? *Ann N Y Acad Sci*.;926:1-12. Review.

Bursch W. (2001). The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ*. Jun;8(6):569-81. Review.

Camoletto P, Peretto P, Bonfanti L, Manceau V, Sobel A, Fasolo A. (1997). The cytosolic phosphoprotein stathmin is expressed in the olfactory system of the adult rat. *Neuroreport.* Sep 8;8(13):2825-9.

Cantarella G, Risuglia N, Dell'eva R, Lempereur L, Albini A, Pennisi G, Scoto GM, Noonan DN, Bernardini R. (2006). TRAIL inhibits angiogenesis stimulated by VEGF expression in human glioblastoma cells. *Br J Cancer*. May 22;94(10):1428-35.

Carrano AC, Eytan E, Hershko A, Pagano M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol*. Aug;1(4):193-9.

Cassimeris L. (2002). The oncoprotein 18/stathmin family of microtubule destabilizers. *Curr Opin Cell Biol.* Feb;14(1):18-24. Review.

Catzavelos C, Bhattacharya N, Ung YC, Wilson JA, Roncari L, Sandhu C, Shaw P, Yeger H, Morava-Protzner I, Kapusta L, Franssen E, Pritchard KI, Slingerland JM. (1997). Decreased levels of the cell-cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. *Nat Med*. Feb;3(2):227-30.

Cavalla P, Piva R, Bortolotto S, Grosso R, Cancelli I, Chiò A, Schiffer D. (1999). p27/kip1 expression in oligodendrogliomas and its possible prognostic role. *Acta Neuropathol*. Dec;98(6):629-34.

Chan FK, Zhang J., Cheng L., Shapiro DN and Winoto A. (1995). Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. *Mol. Cell. Biol.* 15, pp. 2682–2688.

Chassot AA, Turchi L, Virolle T, Fitsialos G, Batoz M, Deckert M, Dulic V, Meneguzzi G, Buscà R, Ponzio G. (2007). Id3 is a novel regulator of p27kip1 mRNA in

early G1 phase and is required for cell-cycle progression. *Oncogene*. Aug 23;26(39):5772-83.

Chen J, Jackson PK, Kirschner MW, Dutta A. (1995). Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature*. Mar 23;374(6520):386-8.

Chen J, Saha P, Kornbluth S, Dynlacht BD, Dutta A. (1996). Cyclin-binding motifs are essential for the function of p21CIP1. *Mol Cell Biol*. Sep;16(9):4673-82.

Cheng M, Sexl V, Sherr CJ, Roussel MF. (1998). Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). *Proc Natl Acad Sci U S A*. Feb 3;95(3):1091-6.

Ciaparrone M, Yamamoto H, Yao Y, Sgambato A, Cattoretti G, Tomita N, Monden T, Rotterdam H, Weinstein IB. (1998). Localization and expression of p27KIP1 in multistage colorectal carcinogenesis. *Cancer Res*. Jan 1;58(1):114-22.

Comings DE. A general theory of carcinogenesis. (1973). *Proc Natl Acad Sci U S A*. Dec;70(12):3324-8.

Connor MK, Kotchetkov R, Cariou S, Resch A, Lupetti R, Beniston RG, Melchior F, Hengst L, Slingerland JM. (2003). CRM1/Ran-mediated nuclear export of p27(Kip1) involves a nuclear export signal and links p27 export and proteolysis. *Mol Biol Cell*. Jan;14(1):201-13.

Cook WD, McCaw BJ. (2000). Accommodating haploinsufficient tumor suppressor genes in Knudson's model. *Oncogene*. Jul 13;19(30):3434-8.

Cote RJ, Shi Y, Groshen S, Feng AC, Cordon-Cardo C, Skinner D, Lieskovosky G. (1998). Association of p27Kip1 levels with recurrence and survival in patients with stage C prostate carcinoma. *J Natl Cancer Inst*. Jun 17;90(12):916-20.

Craig C, Wersto R, Kim M, Ohri E, Li Z, Katayose D, Lee SJ, Trepel J, Cowan K, Seth P. (1997). A recombinant adenovirus expressing p27Kip1 induces cell cycle arrest and loss of cyclin-Cdk activity in human breast cancer cells. *Oncogene.* May 15;14(19):2283-9.

Curmi PA, Andersen SS, Lachkar S, Gavet O, Karsenti E, Knossow M, Sobel A. (1997). The stathmin/tubulin interaction in vitro. *J Biol Chem*. Oct 3;272(40):25029-36.

Curmi PA, Noguès C, Lachkar S, Carelle N, Gonthier MP, Sobel A, Lidereau R, Bièche I. (2000). Overexpression of stathmin in breast carcinomas points out to highly proliferative tumours. *Br J Cancer*. Jan;82(1):142-50.

Daniel C, Pippin J, Shankland SJ, Hugo C. (2004). The rapamycin derivative RAD inhibits mesangial cell migration through the CDK-inhibitor p27KIP1. *Lab Invest*. May;84(5):588-96.

Danthinne X, Imperiale MJ. (2000). Production of first generation adenovirus vectors: a review. *Gene Ther*. Oct;7(20):1707-14.

Daub H, Gevaert K, Vandekerckhove J, Sobel A, Hall A. (2001). Rac/Cdc42 and p65PAK regulate the microtubule-destabilizing protein stathmin through phosphorylation at serine 16. *J Biol Chem*. Jan 19;276(3):1677-80.

Del Pizzo JJ, Borkowski A, Jacobs SC, Kyprianou N. (1999). Loss of cell cycle regulators p27(Kip1) and cyclin E in transitional cell carcinoma of the bladder correlates with tumor grade and patient survival. *Am J Pathol*. Oct;155(4):1129-36.

Deng X, Ewton DZ, Pawlikowski B, Maimone M, Friedman E. (2003). Mirk/dyrk1B is a Rho-induced kinase active in skeletal muscle differentiation. *J Biol Chem*. Oct 17;278(42):41347-54.

Deng X, Mercer SE, Shah S, Ewton DZ, Friedman E. (2004). The cyclin-dependent kinase inhibitor p27Kip1 is stabilized in G(0) by Mirk/dyrk1B kinase. *J Biol Chem*. May 21;279(21):22498-504.

Denicourt C, Dowdy SF. (2004). Cip/Kip proteins: more than just CDKs inhibitors. *Genes Dev*. Apr 15;18(8):851-5.

Denicourt C, Saenz CC, Datnow B, Cui XS, Dowdy SF. (2007). Relocalized p27Kip1 tumor suppressor functions as a cytoplasmic metastatic oncogene in melanoma. *Cancer Res*. Oct 1;67(19):9238-43.

Di Paolo G, Lutjens R, Osen-Sand A, Sobel A, Catsicas S, Grenningloh G. (1997). Differential distribution of stathmin and SCG10 in developing neurons in culture. *J Neurosci Res*. Dec 15;50(6):1000-9.

Doye V, Kellermann O, Buc-Caron MH, Sobel A. (1992). High expression of stathmin in multipotential teratocarcinoma and normal embryonic cells versus their early differentiated derivatives. *Differentiation*. Jun;50(2):89-96.

Doye V, Soubrier F, Bauw G, Boutterin MC, Beretta L, Koppel J, Vandekerckhove J, Sobel A. (1989). A single cDNA encodes two isoforms of stathmin, a developmentally regulated neuron-enriched phosphoprotein. *J Biol Chem*. Jul 25;264(21):12134-7.

Dulić V, Lees E, Reed SI. (1992). Association of human cyclin E with a periodic G1-S phase protein kinase. *Science*. Sep 25;257(5078):1958-61.

Dyer MR, Herrling PL. (2000). Progress and potential for gene-based medicines. *Mol Ther*. Mar;1(3):213-24. Review.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell*. Nov 19;75(4):817-25.

el-Deiry WS, Tokino T, Waldman T, Oliner JD, Velculescu VE, Burrell M, Hill DE, Healy E, Rees JL, Hamilton SR, et al. (1995). Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. *Cancer Res*. Jul 1;55(13):2910-9.

Elledge SJ, Winston J, Harper JW. (1996). A question of balance: the role of cyclin-kinase inhibitors in development and tumorigenesis. *Trends Cell Biol*. Oct;6(10):388-92.

Etienne-Manneville S. (2004). Actin and microtubules in cell motility: which one is in control? *Traffic*. Jul;5(7):470-7. Review.

Ewen ME, Sluss HK, Sherr CJ, Matsushime H, Kato J, Livingston DM. (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell*. May 7;73(3):487-97.

Fang F, Newport JW. (1991). Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell*. Aug 23;66(4):731-42.

Fang F, Orend G, Watanabe N, Hunter T, Ruoslahti E. (1996). Dependence of cyclin E-CDK2 kinase activity on cell anchorage. *Science*. Jan 26;271(5248):499-502.

Fero ML, Randel E, Gurley KE, Roberts JM, Kemp CJ. (1998). The murine gene p27Kip1 is haplo-insufficient for tumour suppression. *Nature*. Nov 12;396(6707):177-80.

Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai LH, Broudy V, Perlmutter RM, Kaushansky K, Roberts JM. (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell.* May 31;85(5):733-44.

Fesquet D, Labbé JC, Derancourt J, Capony JP, Galas S, Girard F, Lorca T, Shuttleworth J, Dorée M, Cavadore JC. (1993). The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. *EMBO J*. Aug;12(8):3111-21.

Feuerstein N, Cooper HL. (1983). Rapid protein phosphorylation induced by phorbol ester in HL-60 cells. Unique alkali-stable phosphorylation of a 17,000-dalton protein detected by two-dimensional gel electrophoresis. *J Biol Chem.* Sep 10;258(17):10786-93.

Fischer I, Gagner JP, Law M, Newcomb EW, Zagzag D. (2005). Angiogenesis in gliomas: biology and molecular pathophysiology. *Brain Pathol.* Oct;15(4):297-310. Review.

Fisher RP, Morgan DO. (1994). A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell*. Aug 26;78(4):713-24.

Folkman J, Haudenschild C. (1980). Angiogenesis in vitro. *Nature*. Dec 11;288(5791):551-6.

Fotedar R, Fitzgerald P, Rousselle T, Cannella D, Dorée M, Messier H, Fotedar A. (1996). p21 contains independent binding sites for cyclin and cdk2: both sites are required to inhibit cdk2 kinase activity. *Oncogene*. May 16;12(10):2155-64.

Fredersdorf S, Burns J, Milne AM, Packham G, Fallis L, Gillett CE, Royds JA, Peston D, Hall PA, Hanby AM, Barnes DM, Shousha S, O'Hare MJ, Lu X. (1997). High level expression of p27(kip1) and cyclin D1 in some human breast cancer cells: inverse correlation between the expression of p27(kip1) and degree of malignancy in human breast and colorectal cancers. *Proc Natl Acad Sci U S A*. Jun 10;94(12):6380-5.

Fredersdorf S, Milne AW, Hall PA, Lu X. (1996). Characterization of a panel of novel anti-p21Waf1/Cip1 monoclonal antibodies and immunochemical analysis of p21Waf1/Cip1 expression in normal human tissues. *Am J Pathol*. Mar;148(3):825-35.

Fujita N, Sato S, Katayama K, Tsuruo T. (2002). Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. *J Biol Chem*. Aug 9;277(32):28706-13.

Fujita N, Sato S, Tsuruo T. (2003). Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization. *J Biol Chem.* Dec 5;278(49):49254-60.

Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, Hahn WC, Ligon KL, Louis DN, Brennan C, Chin L, DePinho RA, Cavenee WK. (2007). Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev.* Nov 1;21(21):2683-710. Review.

Fuse T, Tanikawa M, Nakanishi M, Ikeda K, Tada T, Inagaki H, Asai K, Kato T, Yamada K. (2000). p27Kip1 expression by contact inhibition as a prognostic index of human glioma. *J Neurochem*. Apr;74(4):1393-9.

Gao GP, Yang Y, Wilson JM. (1996). Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. *J Virol*. Dec;70(12):8934-43.

Giancotti FG, Ruoslahti E. (1999). Integrin signaling. *Science*. Aug 13;285(5430):1028-32. Review.

Goukassian D, Díez-Juan A, Asahara T, Schratzberger P, Silver M, Murayama T, Isner JM, Andrés V. (2001). Overexpression of p27(Kip1) by doxycycline-regulated adenoviral vectors inhibits endothelial cell proliferation and migration and impairs angiogenesis. *FASEB J*. Sep;15(11):1877-85.

Greber UF, Willetts M, Webster P, Helenius A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. *Cell.* Nov 5;75(3):477-86.

Guan KL, Jenkins CW, Li Y, Nichols MA, Wu X, O'Keefe CL, Matera AG, Xiong Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev*. Dec 15;8(24):2939-52.

Hanahan D, Weinberg RA. (2000). The hallmarks of cancer. *Cell*. Jan 7;100(1):57-70. Review.

Hannon GJ, Beach D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature*. Sep 15;371(6494):257-61.

Hara T, Kamura T, Kotoshiba S, Takahashi H, Fujiwara K, Onoyama I, Shirakawa M, Mizushima N, Nakayama KI. (2005). Role of the UBL-UBA protein KPC2 in degradation of p27 at G1 phase of the cell cycle. *Mol Cell Biol*. Nov;25(21):9292-303.

Hara T, Kamura T, Nakayama K, Oshikawa K, Hatakeyama S, Nakayama K. (2001). Degradation of p27(Kip1) at the G(0)-G(1) transition mediated by a Skp2-independent ubiquitination pathway. *J Biol Chem*. Dec 28;276(52):48937-43.

Harbour JW, Luo RX, Dei Santi A, Postigo AA, Dean DC. (1999). Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell*. Sep 17;98(6):859-69.

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell.* Nov 19;75(4):805-16.

Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai LH, Zhang P, Dobrowolski S, Bai C, Connell-Crowley L, Swindell E, et al. (1995). Inhibition of cyclin-dependent kinases by p21. *Mol Biol Cell*. Apr;6(4):387-400.

Harper JW, Elledge SJ. (1996). Cdk inhibitors in development and cancer. *Curr Opin Genet Dev.* Feb;6(1):56-64. Review.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. (1998). A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A.* Mar 3;95(5):2509-14.

Heichman KA and Roberts JM. (1994). Rules to replicate by. *Cell*. Nov 18;79(4):557-62.

Hengst L, Reed SI. (1996). Translational control of p27Kip1 accumulation during the cell cycle.Hengst L, Reed SI. *Science*. Mar 29;271(5257):1861-4.

Hengst L, Reed SI. (1998). Inhibitors of the Cip/Kip family. *Curr Top Microbiol Immunol*.;227:25-41.

Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI, Weinberg RA. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell*. Sep 18;70(6):993-1006.

Hirai H, Roussel MF, Kato JY, Ashmun RA and Sherr CJ. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol. Cell. Biol.* 15, pp. 2672–2681.

Hiromura K, Pippin JW, Fero ML, Roberts JM, Shankland SJ. (1999). Modulation of apoptosis by the cyclin-dependent kinase inhibitor p27(Kip1). *J Clin Invest*. Mar;103(5):597-604.

Holland EC. (2001). Progenitor cells and glioma formation. *Curr Opin Neurol*. Dec;14(6):683-8. Review.

Hong SS, Karayan L, Tournier J, Curiel DT, Boulanger PA. (1997). Adenovirus type 5 fiber knob binds to MHC class I alpha2 domain at the surface of human epithelial and B lymphoblastoid cells. *EMBO J*. May 1;16(9):2294-306.

Hood JD, Cheresh DA. (2002). Role of integrins in cell invasion and migration. *Nat Rev Cancer*. Feb;2(2):91-100. Review.

Howell B, Larsson N, Gullberg M, Cassimeris L. (1999). Dissociation of the tubulin-sequestering and microtubule catastrophe-promoting activities of oncoprotein 18/stathmin. *Mol Biol Cell*. Jan;10(1):105-18.

Hunt T. (1991). Cell biology. Cell cycle gets more cyclins. *Nature*. Apr 11;350(6318):462-3.

Hunter T, Pines J. (1994). Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. *Cell*. Nov 18;79(4):573-82. Review.

Ishida N, Hara T, Kamura T, Yoshida M, Nakayama K, Nakayama KI. (2002). Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. *J Biol Chem*. Apr 26;277(17):14355-8.

Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y. (2005). Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. *Int J Oncol.* May;26(5):1401-10.

Jaffe EA, Nachman RL, Becker CG, Minick CR. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest.* Nov;52(11):2745-56.

Jeffrey PD, Tong L, Pavletich NP. (2000). Structural basis of inhibition of CDK-cyclin complexes by INK4 inhibitors. *Genes Dev*. Dec 15;14(24):3115-25.

Johnsen JI, Aurelio ON, Kwaja Z, Jörgensen GE, Pellegata NS, Plattner R, Stanbridge EJ, Cajot JF. (2000). p53-mediated negative regulation of stathmin/Op18 expression is associated with G(2)/M cell-cycle arrest. *Int J Cancer*. Dec 1;88(5):685-91.

Johnson WE, Watters DJ, Suniara RK, Brown G, Bunce CM. (1999). Bistratene A induces a microtubule-dependent block in cytokinesis and altered stathmin expression in HL60 cells. *Biochem Biophys Res Commun*. Jun 24;260(1):80-8.

Jourdain L, Curmi P, Sobel A, Pantaloni D, Carlier MF. (1997). Stathmin: a tubulin-sequestering protein which forms a ternary T2S complex with two tubulin molecules. *Biochemistry.* Sep 9;36(36):10817-21.

Juliano R. (2003). Movin' on through with Cdc2. *Nat Cell Biol*. Jul;5(7):589-90. Review.

Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS 3rd, Johnson BE, Skolnick MH. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. *Science*. Apr 15;264(5157):436-40.

Kamura T, Hara T, Matsumoto M, Ishida N, Okumura F, Hatakeyama S, Yoshida M, Nakayama K, Nakayama KI. (2004). Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase. *Nat Cell Biol*. Dec;6(12):1229-35.

Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. (2004). Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ.* Apr;11(4):448-57.

Kargiotis O, Rao JS, Kyritsis AP. (2006). Mechanisms of angiogenesis in gliomas. *J Neurooncol*. Jul;78(3):281-93.

Katayose Y, Kim M, Rakkar AN, Li Z, Cowan KH, Seth P. (1997). Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. *Cancer Res.* Dec 15;57(24):5441-5.

Kato J, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev*. Mar;7(3):331-42.

Kato JY, Matsuoka M, Strom DK, Sherr CJ. (1994). Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. *Mol Cell Biol*. Apr;14(4):2713-21.

Kawamata N, Morosetti R, Miller CW, Park D, Spirin KS, Nakamaki T, Takeuchi S, Hatta Y, Simpson J, Wilcyznski S, et al. (1995). Molecular analysis of the cyclin-dependent kinase inhibitor gene p27/Kip1 in human malignancies. *Cancer Res.* Jun 1;55(11):2266-9.

Kay MA, Glorioso JC, Naldini L. (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med.* Jan;7(1):33-40. Review.

Ke LD, Shi YX, Yung WK. (2002). VEGF(121), VEGF(165) overexpression enhances tumorigenicity in U251 MG but not in NG-1 glioma cells. *Cancer Res*. Mar 15;62(6):1854-61.

Kelly BL, Wolfe KG, Roberts JM. (1998). Identification of a substrate-targeting domain in cyclin E necessary for phosphorylation of the retinoblastoma protein. *Proc Natl Acad Sci U S A*. Mar 3;95(5):2535-40.

Kirla RM, Haapasalo HK, Kalimo H, Salminen EK. (2003). Low expression of p27 indicates a poor prognosis in patients with high-grade astrocytomas. *Cancer.* Feb 1;97(3):644-8.

Kitange GJ, Templeton KL, Jenkins RB. (2003). Recent advances in the molecular genetics of primary gliomas. *Curr Opin Oncol*. May;15(3):197-203. Review.

Kiyokawa H, Kineman RD, Manova-Todorova KO, Soares VC, Hoffman ES, Ono M, Khanam D, Hayday AC, Frohman LA, Koff A. (1996). Enhanced growth of mice

lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell.* May 31;85(5):721-32.

Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, Cavenee WK. (2002). The WHO classification of tumors of the nervous system. *J Neuropathol Exp Neurol.* Mar;61(3):215-25; discussion 226-9. Review.

Knudson AG Jr, Strong LC, Anderson DE. (1973). Heredity and cancer in man. *Prog Med Genet*.;9:113-58. Review.

Knudson AG Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A*. Apr;68(4):820-3.

Kochanek S. (1999). High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum Gene Ther*. Oct 10;10(15):2451-9. Review.

Koff A, Cross F, Fisher A, Schumacher J, Leguellec K, Philippe M, Roberts JM. (1991). Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. *Cell*. Sep 20;66(6):1217-28.

Koff A, Giordano A, Desai D, Yamashita K, Harper JW, Elledge S, Nishimoto T, Morgan DO, Franza BR, Roberts JM. (1992). Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science*. Sep 18;257(5077):1689-94.

Koff A, Ohtsuki M, Polyak K, Roberts JM, Massagué J. (1993). Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-beta. *Science*. Apr 23;260(5107):536-9.

Komata T, Kanzawa T, Takeuchi H, Germano IM, Schreiber M, Kondo Y, Kondo S. (2003). Antitumour effect of cyclin-dependent kinase inhibitors (p16(INK4A), p18(INK4C), p19(INK4D), p21(WAF1/CIP1) and p27(KIP1)) on malignant glioma cells. *Br J Cancer.* Apr 22;88(8):1277-80.

Koppel J, Boutterin MC, Doye V, Peyro-Saint-Paul H, Sobel A. (1990). Developmental tissue expression and phylogenetic conservation of stathmin, a phosphoprotein associated with cell regulations. *J Biol Chem*. Mar 5;265(7):3703-7.

Korkolopoulou P, Christodoulou P, Konstantinidou AE, Thomas-Tsagli E, Kapralos P, Davaris P. (2000). Cell cycle regulators in bladder cancer: a multivariate survival study with emphasis on p27Kip1. *Hum Pathol*. Jun;31(6):751-60.

Kortylewski M, Heinrich PC, Mackiewicz A, Schniertshauer U, Klingmüller U, Nakajima K, Hirano T, Horn F, Behrmann I. (1999). Interleukin-6 and oncostatin M-

induced growth inhibition of human A375 melanoma cells is STAT-dependent and involves upregulation of the cyclin-dependent kinase inhibitor p27/Kip1. *Oncogene*. Jun 24;18(25):3742-53.

Kossatz U, Vervoorts J, Nickeleit I, Sundberg HA, Arthur JS, Manns MP, Malek NP. (2006). C-terminal phosphorylation controls the stability and function of p27kip1. *EMBO J*. Nov 1;25(21):5159-70.

Kotoshiba S, Kamura T, Hara T, Ishida N, Nakayama KI. (2005). Molecular dissection of the interaction between p27 and Kip1 ubiquitylation-promoting complex, the ubiquitin ligase that regulates proteolysis of p27 in G1 phase. *J Biol Chem*. May 6;280(18):17694-700.

Koyama H, Raines EW, Bornfeldt KE, Roberts JM, Ross R. (1996). Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell*. Dec 13;87(6):1069-78.

Krude T, Jackman M, Pines J, Laskey RA. (1997). Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell.* Jan 10;88(1):109-19.

Larsson N, Melander H, Marklund U, Osterman O, Gullberg M. (1995). G2/M transition requires multisite phosphorylation of oncoprotein 18 by two distinct protein kinase systems. J *Biol Chem.* Jun 9;270(23):14175-83.

Larsson N, Segerman B, Howell B, Fridell K, Cassimeris L, Gullberg M. (1999). Op18/stathmin mediates multiple region-specific tubulin and microtubule-regulating activities. *J Cell Biol*. Sep 20;146(6):1289-302.

Lee MH, Reynisdóttir I, Massagué J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev.* Mar 15;9(6):639-49.

Leighton IA, Curmi P, Campbell DG, Cohen P, Sobel A. (1993). The phosphorylation of stathmin by MAP kinase. *Mol Cell Biochem*. Nov;127-128:151-6.

Lew DJ, Dulić V, Reed SI. (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell*. Sep 20;66(6):1197-206.

Li DM, Sun H. (1998). PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. *Proc Natl Acad Sci U S A*. Dec 22;95(26):15406-11.

Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB. (2007). The energy sensing

LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol*. Feb;9(2):218-24.

Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. (2002). PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med.* Oct;8(10):1153-60.

Liedtke W, Leman EE, Fyffe RE, Raine CS, Schubart UK. (2002). Stathmin-deficient mice develop an age-dependent axonopathy of the central and peripheral nervous systems. *Am J Pathol.* Feb;160(2):469-80.

Lim S, Zou Y, Friedman E. (2002). The transcriptional activator Mirk/Dyrk1B is sequestered by p38alpha/beta MAP kinase. *J Biol Chem.* Dec 20;277(51):49438-45.

Loda M, Cukor B, Tam SW, Lavin P, Fiorentino M, Draetta GF, Jessup JM, Pagano M. (1997). Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med.* Feb;3(2):231-4.

Loubat A, Rochet N, Turchi L, Rezzonico R, Far DF, Auberger P, Rossi B, Ponzio G. (1999). Evidence for a p23 caspase-cleaved form of p27[KIP1] involved in G1 growth arrest. *Oncogene.* Jun 3;18(22):3324-33.

Lundberg AS, Weinberg RA. (1998). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol Cell Biol*. Feb;18(2):753-61.

Luo RX, Postigo AA, Dean DC. (1998). Rb interacts with histone deacetylase to repress transcription. *Cell*. Feb 20;92(4):463-73.

Luo XN, Arcasoy MO, Brickner HE, Mistry S, Schechter AD, Atweh GF. (1991). Regulated expression of p18, a major phosphoprotein of leukemic cells. *J Biol Chem*. Nov 5;266(31):21004-10.

Maciag T, Kadish J, Wilkins L, Stemerman MB, Weinstein R. (1982). Organizational behavior of human umbilical vein endothelial cells. *J Cell Biol.* Sep;94(3):511-20.

Madri JA, Williams SK. (1983). Capillary endothelial cell cultures: phenotypic modulation by matrix components. *J Cell Biol*. Jul;97(1):153-65.

Magnaghi P, Roberts C, Lorain S, Lipinski M, Scambler PJ. (1998). HIRA, a mammalian homologue of Saccharomyces cerevisiae transcriptional co-repressors, interacts with Pax3. *Nat Genet*. Sep;20(1):74-7.

Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK, DePinho RA. (2001). Malignant glioma: genetics and biology of a grave matter. *Genes Dev*. Jun 1;15(11):1311-33. Review.

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol.* Sep;8(9):741-52. Review.

Marklund U, Brattsand G, Osterman O, Ohlsson PI, Gullberg M. (1993). Multiple signal transduction pathways induce phosphorylation of serines 16, 25, and 38 of oncoprotein 18 in T lymphocytes. *J Biol Chem*. Dec 5;268(34):25671-80.

Marklund U, Brattsand G, Shingler V, Gullberg M. (1993). Serine 25 of oncoprotein 18 is a major cytosolic target for the mitogen-activated protein kinase. *J Biol Chem*. Jul 15;268(20):15039-47.

Marklund U, Larsson N, Brattsand G, Osterman O, Chatila TA, Gullberg M. (1994). Serine 16 of oncoprotein 18 is a major cytosolic target for the Ca2+/calmodulin-dependent kinase-Gr. *Eur J Biochem*. Oct 1;225(1):53-60.

Marret C, Avallet O, Perrard-Sapori MH, Durand P. (1998). Localization and quantitative expression of mRNAs encoding the testis-specific histone TH2B, the phosphoprotein p19, the transition proteins 1 and 2 during pubertal development and throughout the spermatogenic cycle of the rat. *Mol Reprod Dev*. Sep;51(1):22-35.

Maruyama S, Hatakeyama S, Nakayama K, Ishida N, Kawakami K, Nakayama K. (2001). Characterization of a mouse gene (Fbxw6) that encodes a homologue of Caenorhabditis elegans SEL-10. *Genomics*. Dec;78(3):214-22.

Masciullo V, Sgambato A, Pacilio C, Pucci B, Ferrandina G, Palazzo J, Carbone A, Cittadini A, Mancuso S, Scambia G, Giordano A. (1999). Frequent loss of expression of the cyclin-dependent kinase inhibitor p27 in epithelial ovarian cancer. *Cancer Res*. Aug 1;59(15):3790-4.

Mathews MB, Shenk T. (1991). Adenovirus virus-associated RNA and translation control. *J Virol.* Nov;65(11):5657-62. Review.

Matsuoka M, Kato JY, Fisher RP, Morgan DO, Sherr CJ. (1994). Activation of cyclin-dependent kinase 4 (cdk4) by mouse MO15-associated kinase. *Mol Cell Biol*. Nov;14(11):7265-75.

Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, Harper JW, Elledge SJ. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev*. Mar 15;9(6):650-62.

Matsuoka S, Thompson JS, Edwards MC, Bartletta JM, Grundy P, Kalikin LM, Harper JW, Elledge SJ, Feinberg AP. (1996). Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor, p57KIP2, on chromosome 11p15. *Proc Natl Acad Sci U S A*. Apr 2;93(7):3026-30.

Matsushime H, Ewen ME, Strom DK, Kato JY, Hanks SK, Roussel MF, Sherr CJ. (1992). Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. *Cell*. Oct 16;71(2):323-34.

Matsushime H, Roussel MF, Ashmun RA, Sherr CJ. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell.* May 17;65(4):701-13.

Maucuer A, Moreau J, Méchali M, Sobel A. (1993). Stathmin gene family: phylogenetic conservation and developmental regulation in Xenopus. *J Biol Chem*. Aug 5;268(22):16420-9.

McAllister SS, Becker-Hapak M, Pintucci G, Pagano M, Dowdy SF. (2003). Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions. *Mol Cell Biol*. Jan;23(1):216-28.

Medema RH, Kops GJ, Bos JL, Burgering BM. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature*. Apr 13;404(6779):782-7.

Melhem RF, Zhu XX, Hailat N, Strahler JR, Hanash SM. (1991). Characterization of the gene for a proliferation-related phosphoprotein (oncoprotein 18) expressed in high amounts in acute leukemia. *J Biol Chem*. Sep 25;266(27):17747-53.

Meyerson M, Harlow E. (1994). Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol Cell Biol*. Mar;14(3):2077-86.

Millard SS, Vidal A, Markus M, Koff A. (2000). A U-rich element in the 5' untranslated region is necessary for the translation of p27 mRNA. *Mol Cell Biol*. Aug;20(16):5947-59.

Misek DE, Chang CL, Kuick R, Hinderer R, Giordano TJ, Beer DG, Hanash SM. (2002). Transforming properties of a Q18-->E mutation of the microtubule regulator Op18. *Cancer Cell.* Sep;2(3):217-28.

Mistry SJ, Bank A, Atweh GF. (2007). Synergistic antiangiogenic effects of stathmin inhibition and taxol exposure. *Mol Cancer Res*. Aug;5(8):773-82.

Miura M, Hatakeyama S, Hattori K, Nakayama K. (1999). Structure and expression of the gene encoding mouse F-box protein, Fwd2. *Genomics*. Nov 15;62(1):50-8.

Mizumatsu S, Tamiya T, Ono Y, Abe T, Matsumoto K, Furuta T, Ohmoto T. (1999). Expression of cell cycle regulator p27Kip1 is correlated with survival of patients with astrocytoma. *Clin Cancer Res*. Mar;5(3):551-7.

Montagnoli A, Fiore F, Eytan E, Carrano AC, Draetta GF, Hershko A, Pagano M. (1999). Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. *Genes Dev*. May 1;13(9):1181-9.

Morgan DO. (1995). Principles of CDK regulation. *Nature*. Mar 9;374(6518):131-4. Review.

Mori M, Mimori K, Shiraishi T, Tanaka S, Ueo H, Sugimachi K, Akiyoshi T. (1997). p27 expression and gastric carcinoma. *Nat Med*. Jun;3(6):593.

Motokura T, Bloom T, Kim HG, Jüppner H, Ruderman JV, Kronenberg HM, Arnold A. (1991). A novel cyclin encoded by a bcl1-linked candidate oncogene. *Nature*. Apr 11;350(6318):512-5.

Motti ML, Califano D, Troncone G, De Marco C, Migliaccio I, Palmieri E, Pezzullo L, Palombini L, Fusco A, Viglietto G. (2005). Complex regulation of the cyclin-dependent kinase inhibitor p27kip1 in thyroid cancer cells by the PI3K/AKT pathway: regulation of p27kip1 expression and localization. *Am J Pathol.* Mar;166(3):737-49.

Motti ML, De Marco C, Califano D, Fusco A, Viglietto G. (2004). Akt-dependent T198 phosphorylation of cyclin-dependent kinase inhibitor p27kip1 in breast cancer. *Cell Cycle*. Aug;3(8):1074-80.

Nakanishi M, Robetorye RS, Adami GR, Pereira-Smith OM, Smith JR. (1995). Identification of the active region of the DNA synthesis inhibitory gene p21Sdi1/CIP1/WAF1. *EMBO J*. Feb 1;14(3):555-63.

Nakasu S, Nakajima M, Handa J. (1999). Anomalous p27kip1 expression in a subset of malignant gliomas. *Brain Tumor Pathol.*;16(1):17-21.

Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY, Nakayama K. (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell.* May 31;85(5):707-20.

Nakayama K, Nagahama H, Minamishima YA, Matsumoto M, Nakamichi I, Kitagawa K, Shirane M, Tsunematsu R, Tsukiyama T, Ishida N, Kitagawa M, Nakayama K, Hatakeyama S. (2000). Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. *EMBO J*. May 2;19(9):2069-81.

Nakayama K, Nagahama H, Minamishima YA, Miyake S, Ishida N, Hatakeyama S, Kitagawa M, Iemura S, Natsume T, Nakayama KI. (2004). Skp2-mediated degradation of p27 regulates progression into mitosis. *Dev Cell*. May;6(5):661-72.

Nakayama K, Nakayama K. (1998). Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. *Bioessays*. Dec;20(12):1020-9.

Naumann U, Weit S, Rieger L, Meyermann R, Weller M. (1999). p27 modulates cell cycle progression and chemosensitivity in human malignant glioma. *Biochem Biophys Res Commun*. Aug 11;261(3):890-6.

Nemerow GR. (2000). Cell receptors involved in adenovirus entry. *Virology*. Aug 15;274(1):1-4. Review.

Nevins JR. (1998). Toward an understanding of the functional complexity of the E2F and retinoblastoma families. *Cell Growth Differ*. Aug;9(8):585-93. Review.

Newcomb EW, Sosnow M, Demopoulos RI, Zeleniuch-Jacquotte A, Sorich J, Speyer JL. (1999). Expression of the cell cycle inhibitor p27KIP1 is a new prognostic marker associated with survival in epithelial ovarian tumors. *Am J Pathol.* Jan;154(1):119-25.

Ngo TT, Peng T, Liang XJ, Akeju O, Pastorino S, Zhang W, Kotliarov Y, Zenklusen JC, Fine HA, Maric D, Wen PY, De Girolami U, Black PM, Wu WW, Shen RF, Jeffries NO, Kang DW, Park JK. (2007). The 1p-encoded protein stathmin and resistance of malignant gliomas to nitrosoureas. *J Natl Cancer Inst*. Apr 18;99(8):639-52.

Nguyen H, Gitig DM, Koff A. (1999). Cell-free degradation of p27(kip1), a G1 cyclin-dependent kinase inhibitor, is dependent on CDK2 activity and the proteasome. *Mol Cell Biol*. Feb;19(2):1190-201.

Niethammer P, Bastiaens P, Karsenti E. (2004). Stathmin-tubulin interaction gradients in motile and mitotic cells. *Science*. Mar 19;303(5665):1862-6.

Nigg EA. (2001). Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol*. Jan;2(1):21-32. Review.

Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR. (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res*. Mar;211(1):90-8.

Nourse J, Firpo E, Flanagan WM, Coats S, Polyak K, Lee MH, Massague J, Crabtree GR, Roberts JM. (1994). Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature*. Dec 8;372(6506):570-3.

Nurse P. (2000). A long twentieth century of the cell cycle and beyond. *Cell*. Jan 7;100(1):71-8. Review.

Ozon S, Byk T, Sobel A. (1998). SCLIP: a novel SCG10-like protein of the stathmin family expressed in the nervous system. *J Neurochem*. Jun;70(6):2386-96.

Ozon S, El Mestikawy S, Sobel A. (1999). Differential, regional, and cellular expression of the stathmin family transcripts in the adult rat brain. *J Neurosci Res*. Jun 1;56(5):553-64.

Ozon S, Maucuer A, Sobel A. (1997). The stathmin family -- molecular and biological characterization of novel mammalian proteins expressed in the nervous system. *Eur J Biochem*. Sep 15;248(3):794-806.

Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF, Rolfe M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science.* Aug 4;269(5224):682-5.

Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J. (2001). A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res*. Jan 15;61(2):439-44.

Pardee AB. (1974). A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci U S A*. Apr;71(4):1286-90.

Perez-Roger I, Kim SH, Griffiths B, Sewing A, Land H. (1999). Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). EMBO J. Oct 1;18(19):5310-20.

Peschanski M, Hirsch E, Dusart I, Doye V, Marty S, Manceau V, Sobel A. (1993). Stathmin: cellular localization of a major phosphoprotein in the adult rat and human CNS. J *Comp Neurol.* Nov 22;337(4):655-68.

Philipp-Staheli J, Payne SR, Kemp CJ. (2001). p27(Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. Exp Cell Res. Mar 10;264(1):148-68.

Pines J. (1999). Four-dimensional control of the cell cycle. *Nat Cell Biol*. Jul;1(3):E73-9. Review.

Piva R, Cavalla P, Bortolotto S, Cordera S, Richiardi P, Schiffer D. (1997). p27/kip1 expression in human astrocytic gliomas. *Neurosci Lett*. Oct 3;234(2-3):127-30.

Polager S, Ginsberg D. (2003). E2F mediates sustained G2 arrest and down-regulation of Stathmin and AIM-1 expression in response to genotoxic stress. *J Biol Chem*. Jan 17;278(3):1443-9.

Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev*. Jan;8(1):9-22.

Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, Massagué J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*. Jul 15;78(1):59-66.

Polzin RG, Benlhabib H, Trepel J, Herrera JE. (2004). E2F sites in the Op18 promoter are required for high level of expression in the human prostate carcinoma cell line PC-3-M. *Gene*. Oct 27;341:209-18.

Ponce-Castañeda MV, Lee MH, Latres E, Polyak K, Lacombe L, Montgomery K, Mathew S, Krauter K, Sheinfeld J, Massague J, et al. (1995). p27Kip1: chromosomal mapping to 12p12-12p13.1 and absence of mutations in human tumors. *Cancer Res*. Mar 15;55(6):1211-4.

Poon RY, Hunter T. (1995). Dephosphorylation of Cdk2 Thr160 by the cyclin-dependent kinase-interacting phosphatase KAP in the absence of cyclin. *Science.* Oct 6;270(5233):90-3.

Porter PL, Malone KE, Heagerty PJ, Alexander GM, Gatti LA, Firpo EJ, Daling JR, Roberts JM. (1997). Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med*. Feb;3(2):222-5.

Price DK, Ball JR, Bahrani-Mostafavi Z, Vachris JC, Kaufman JS, Naumann RW, Higgins RV, Hall JB. (2000). The phosphoprotein Op18/stathmin is differentially expressed in ovarian cancer. *Cancer Invest*.;18(8):722-30.

Pruitt K, Der CJ. (2001). Ras and Rho regulation of the cell cycle and oncogenesis. *Cancer Lett*. Sep 28;171(1):1-10. Review.

Puumalainen AM, Vapalahti M, Agrawal RS, Kossila M, Laukkanen J, Lehtolainen P, Viita H, Paljärvi L, Vanninen R, Ylä-Herttuala S. (1998). Beta-galactosidase gene transfer to human malignant glioma in vivo using replication-deficient retroviruses and adenoviruses. *Hum Gene Ther*. Aug 10;9(12):1769-74.

Quon KC, Berns A. (2001). Haplo-insufficiency? Let me count the ways. *Genes Dev*. Nov 15;15(22):2917-21.

Rekosh DM, Russell WC, Bellet AJ, Robinson AJ. (1977). Identification of a protein linked to the ends of adenovirus DNA. *Cell*. Jun;11(2):283-95.

Reynisdóttir I, Polyak K, Iavarone A, Massagué J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev*. Aug 1;9(15):1831-45.

Rodier G, Makris C, Coulombe P, Scime A, Nakayama K, Nakayama KI, Meloche S. (2005). p107 inhibits G1 to S phase progression by down-regulating expression of the F-box protein Skp2. *J Cell Biol*. Jan 3;168(1):55-66.

Rodier G, Montagnoli A, Di Marcotullio L, Coulombe P, Draetta GF, Pagano M, Meloche S. (2001). p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis. *EMBO J*. Dec 3;20(23):6672-82.

Russell WC. (2000). Update on adenovirus and its vectors. *J Gen Virol*. Nov;81(Pt 11):2573-604. Review.

Sánchez-Beato M, Camacho FI, Martínez-Montero JC, Sáez AI, Villuendas R, Sánchez-Verde L, García JF, Piris MA. (1999). Anomalous high p27/KIP1 expression in a subset of aggressive B-cell lymphomas is associated with cyclin D3 overexpression. p27/KIP1-cyclin D3 colocalization in tumor cells. *Blood*. Jul 15;94(2):765-72.

Sandmair AM, Vapalahti M, Ylä-Herttuała S. (2000). Adenoviruses as gene delivery vectors. *Adv Exp Med Biol*. 465:423-9. Review.

Schiffer D, Cavalla P, Fiano V, Ghimenti C, Piva R. (2002). Inverse relationship between p27/Kip.1 and the F-box protein Skp2 in human astrocytic gliomas by immunohistochemistry and Western blot. *Neurosci Lett*. Aug 9;328(2):125-8.

Schreiber M, Muller WJ, Singh G, Graham FL. (1999). Comparison of the effectiveness of adenovirus vectors expressing cyclin kinase inhibitors p16INK4A,

p18INK4C, p19INK4D, p21(WAF1/CIP1) and p27KIP1 in inducing cell cycle arrest, apoptosis and inhibition of tumorigenicity. *Oncogene*. Mar 4;18(9):1663-76.

Schubart UK, Banerjee MD, Eng J. (1989). Homology between the cDNAs encoding phosphoprotein p19 and SCG10 reveals a novel mammalian gene family preferentially expressed in developing brain. *DNA*. Jul-Aug;8(6):389-98.

Schubart UK, Yu J, Amat JA, Wang Z, Hoffmann MK, Edelmann W. (1996). Normal development of mice lacking metablastin (P19), a phosphoprotein implicated in cell cycle regulation. *J Biol Chem*. Jun 14;271(24):14062-6.

Schwartz MA, Assoian RK. (2001). Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. *J Cell Sci*. Jul;114(Pt 14):2553-60. Review.

Sekimoto T, Fukumoto M, Yoneda Y. (2004). 14-3-3 suppresses the nuclear localization of threonine 157-phosphorylated p27(Kip1). *EMBO J.* May 5;23(9):1934-42.

Serrano M, Hannon GJ, Beach D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*. Dec 16;366(6456):704-7.

Sgambato A, Ratto C, Faraglia B, Merico M, Ardito R, Schinzari G, Romano G, Cittadini AR. (1999). Reduced expression and altered subcellular localization of the cyclin-dependent kinase inhibitor p27(Kip1) in human colon cancer. *Mol Carcinog*. Nov;26(3):172-9.

Shamma A, Doki Y, Tsujinaka T, Shiozaki H, Inoue M, Yano M, Kawanishi K, Monden M. (2000). Loss of p27(KIP1) expression predicts poor prognosis in patients with esophageal squamous cell carcinoma. *Oncology*. Feb;58(2):152-8.

Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. (1997). Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev*. Jun 1;11(11):1464-78.

Sherr CJ, McCormick F. (2002). The RB and p53 pathways in cancer. *Cancer Cell* Aug;2(2):103-12.

Sherr CJ, Roberts JM. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* May 15;9(10):1149-63. Review.

Sherr CJ. (1996). Cancer cell cycles. *Science*. Dec 6;274(5293):1672-7. Review.

Sherr CJ. (1994). G1 phase progression: cycling on cue. *Cell*. Nov 18;79(4):551-5. Review.

Sherr CJ. (1994). Growth factor-regulated G1 cyclins. *Stem Cells*.12 Suppl 1:47-55; discussion 55-7. Review.

Sherr CJ. (1993). Mammalian G1 cyclins. *Cell*. Jun 18;73(6):1059-65. Review.

Shim J, Lee H, Park J, Kim H, Choi EJ. (1996). A non-enzymatic p21 protein inhibitor of stress-activated protein kinases. *Nature*. Jun 27;381(6585):804-6.

Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J, Arteaga CL. (2002). PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nat Med*. Oct;8(10):1145-52.

Shumyatsky GP, Malleret G, Shin RM, Takizawa S, Tully K, Tsvetkov E, Zakharenko SS, Joseph J, Vronskaya S, Yin D, Schubart UK, Kandel ER, Bolshakov VY. (2005). stathmin, a gene enriched in the amygdala, controls both learned and innate fear. *Cell*. Nov 18;123(4):697-709.

Sicinski P, Zacharek S, Kim C. (2007). Duality of p27Kip1 function in tumorigenesis. *Genes Dev*. Jul 15;21(14):1703-6. Review.

Slingerland J, Pagano M. (2000). Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol.* Apr;183(1):10-7.

Spessotto P, Giacomello E, Perris R. (2000). Fluorescence assays to study cell adhesion and migration in vitro. *Methods Mol Biol*. 139:321-43. Review.

Stein R, Mori N, Matthews K, Lo LC, Anderson DJ. (1988). The NGF-inducible SCG10 mRNA encodes a novel membrane-bound protein present in growth cones and abundant in developing neurons. *Neuron.* Aug;1(6):463-76.

Sun J, Marx SO, Chen HJ, Poon M, Marks AR, Rabbani LE. (2001). Role for p27(Kip1) in Vascular Smooth Muscle Cell Migration. *Circulation.* Jun 19;103(24):2967-72.

Tamiya T, Mizumatsu S, Ono Y, Abe T, Matsumoto K, Furuta T, Ohmoto T. (2001). High cyclin E/low p27Kip1 expression is associated with poor prognosis in astrocytomas. *Acta Neuropathol*. Apr;101(4):334-40.

Tan P, Cady B, Wanner M, Worland P, Cukor B, Magi-Galluzzi C, Lavin P, Draetta G, Pagano M, Loda M. (1997). The cell cycle inhibitor p27 is an independent prognostic marker in small (T1a,b) invasive breast carcinomas. *Cancer Res*. Apr 1;57(7):1259-63.

Tomoda K, Kubota Y, Kato J. (1999). Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1. *Nature*. Mar 11;398(6723):160-5.

Toyoshima H, Hunter T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell*. Jul 15;78(1):67-74.

Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, Fusco A, Santoro M. (2002). Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nat Med*. Oct;8(10):1136-44.

Vlach J, Hennecke S, Alevizopoulos K, Conti D, Amati B. (1996). Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. *EMBO J.* Dec 2;15(23):6595-604.

Vlach J, Hennecke S, Amati B. (1997). Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. *EMBO J*. Sep 1;16(17):5334-44.

Wadell G. (1984). Molecular epidemiology of human adenoviruses. *Curr Top Microbiol Immunol.* 110:191-220.

Wang SI, Puc J, Li J, Bruce JN, Cairns P, Sidransky D, Parsons R. (1997). Somatic mutations of PTEN in glioblastoma multiforme. *Cancer Res*. Oct 1;57(19):4183-6.

Weinberg RA. (1995). The molecular basis of oncogenes and tumor suppressor genes. *Ann N Y Acad Sci.* Jun 30;758:331-8. Review.

Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell*. Apr 23;73(2):309-19.

Wittmann T, Bokoch GM, Waterman-Storer CM. (2004). Regulation of microtubule destabilizing activity of Op18/stathmin downstream of Rac1. *J Biol Chem*. Feb 13;279(7):6196-203.

Wuarin J and Nurse P. (1996). Regulating S phase: CDKs, licensing and proteolysis. *Cell*. Jun 14;85(6):785-7. Review.

Xiong Y, Connolly T, Futcher B, Beach D. (1991). Human D-type cyclin. *Cell*. May 17;65(4):691-9.

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature*. Dec 16;366(6456):701-4.

Yao KC, Komata T, Kondo Y, Kanzawa T, Kondo S, Germano IM. (2003). Molecular response of human glioblastoma multiforme cells to ionizing radiation: cell cycle arrest, modulation of the expression of cyclin-dependent kinase inhibitors, and autophagy. *J Neurosurg*. Feb;98(2):378-84.

Yasui W, Kudo Y, Semba S, Yokozaki H, Tahara E. (1997). Reduced expression of cyclin-dependent kinase inhibitor p27Kip1 is associated with advanced stage and invasiveness of gastric carcinomas. *Jpn J Cancer Res*. Jul;88(7):625-9.

Yuan RH, Jeng YM, Chen HL, Lai PL, Pan HW, Hsieh FJ, Lin CY, Lee PH, Hsu HC. (2006). Stathmin overexpression cooperates with p53 mutation and osteopontin overexpression, and is associated with tumour progression, early recurrence, and poor prognosis in hepatocellular carcinoma. *J Pathol*. Aug;209(4):549-58.

Yuan Y, Qin L, Liu D, Wu RC, Mussi P, Zhou S, Songyang Z, Xu J. (2007). Genetic screening reveals an essential role of p27kip1 in restriction of breast cancer progression. *Cancer Res*. Sep 1;67(17):8032-42.

Zagzag D, Blanco C, Friedlander DR, Miller DC, Newcomb EW. (2003). Expression of p27KIP1 in human gliomas: relationship between tumor grade, proliferation index, and patient survival. *Hum Pathol*. Jan;34(1):48-53.

Zeng Y, Hirano K, Hirano M, Nishimura J, Kanaide H. (2000). Minimal requirements for the nuclear localization of p27(Kip1), a cyclin-dependent kinase inhibitor. *Biochem Biophys Res Commun*. Jul 21;274(1):37-42.

Zhang HS, Gavin M, Dahiya A, Postigo AA, Ma D, Luo RX, Harbour JW, Dean DC. (2000). Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell*. Mar 31;101(1):79-89.

Zhu X, Ohtsubo M, Böhmer RM, Roberts JM, Assoian RK. (1996). Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. *J Cell Biol*. Apr;133(2):391-403.

Zindy F, Quelle DE, Roussel MF, Sherr CJ. (1997). Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene*. Jul 10;15(2):203-11.