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Regulation of malignant cell transformation by the stress-activated kinase p38α

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Regulation of malignant cell transformation by the stress-activated kinase p38α

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To my mother,

who demonstrated incredible bravery
in fighting cancer and always inspired
my life.

We miss you terribly.

“ Cuando nací, un agosto
ardiente calcinaba las casas.
Al romper en llanto, sintió
mi madre un nudo en la garganta.
Del beso que posó sobre mi cara,
aún noto la tibieza de sus labios.
De sus brazos, que acogieron mi cuerpo,
con ternura, todavía los siento [...]

Y me estremezco ante un beso,
y me quebranto ante un llanto.
Por eso quisiera que mis hijos
amaran como yo,
como mi madre lo hizo ayer
con sus besos y abrazos.
Con esos besos y abrazos
que aún llevo impresos en
los pliegues de mi piel.”

Maria del Carmen Llavador. *Palabras*

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I still recall when I had my first interview with Angel at the EMBL, in particular when he asked me the frightening question: "*what would you like to do for your PhD in my laboratory?"*. Truly, I did not know.
What I knew was that I wanted to shift from chemistry into biology, in particular into the cancer field, although I had no clue on how to undertake such step.
Luckily, just a few minutes earlier I had accidentally met Gustavo, a PhD student in Angel´s lab at that time, who casually gave me an overview of the projects in the lab and told me about an intriguing protein called p38 that, as he said: "*it looks like it might be a tumor suppressor*". This sounded fascinating.
So, I told Angel: "*well, I would like to work with this tumor suppressor, this...p38, isn´t it?*".
And that is how it all started.

So, first of all, I am particularly grateful to my supervisor, Angel Nebreda, who has given me the opportunity and the means during these years to learn how "big science" works and has guided me to become a better scientist. My most sincere "thank you" as well for being so available in the lab, for the "millions" of fruitful discussions that we have shared through these years, and for always depositing your trust on me.

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THESIS SUMMARY

Cancer is a dynamic process that requires the stepwise deregulation of mechanisms affecting various cellular traits. During my PhD, I have characterized how the stress-activated p38α MAPK signaling pathway regulates the processes of cellular migration, proliferation, and survival in the context of oncogene-induced malignant transformation, which recapitulates the mechanisms of cancer initiation at the cellular level.

My studies have been mostly based on the use of human and mouse cultured cells, which I have analyzed using both biochemical and cell biological approaches. In particular, the development of p38a-deficient cell lines and the application of retrovirally-based gene expression techniques have been very useful. The implementation of tools to measure the intracellular levels of reactive oxygen species (ROS) within living cells has also been key for my work.

I have found that p38α regulates the process of malignant transformation at various levels. First, p38α negatively regulates cell cycle progression induced by mitogenic signals in both exponentially proliferating and confluent cells. Oncogene-expressing cells proliferate faster in the absence of p38α, which may be accounted for by the negative effect of p38α on cyclin D1 expression. Similarly, p38α controls the process of cell-cell contact-inhibition, which requires $p27^{Kip1}$ accumulation and triggers G1-phase cell cycle arrest upon cell confluence. The process of contact inhibition is likely to involve uncharacterized membrane-associated signaling events. Accordingly, I have found that p38α regulates the membrane composition of oncogene-transformed cells. In addition to its negative role in cell proliferation, I have shown that p38α can interfere with the process of malignant transformation by sensing oxidative stress and inducing apoptosis. Thus, p38α becomes activated when oncogene-expressing cells accumulate high levels of carcinogenic ROS and, in turn, induces the elimination of the transformed cells by apoptosis. Interestingly, I have found that human cancer cell lines that contain high ROS levels have developed a mechanism to by-pass this p38α function. Finally, in contrast to its anti-proliferative and pro-apoptotic roles, I have found that p38α is an important mediator of cytokine-induce cell migration, a process that is thought to be important for cancer cell metastasis.

ZUSAMMENFASSUNG (THESIS SUMMARY IN GERMAN)

Regulierung der malignen Zelltransformation durch die stressaktivierte Kinase p38α

Krebsentstehung ist ein dynamischer Vorgang, der durch eine schrittweise Deregulierung zellulärer Prozesse gekennzeichnet ist. Das Ziel meiner Dissertationsarbeit war es, die Rolle der stressaktivierten p38α MAP Kinase bei der Regulierung von Zellmigration, Zellwachstum und Überleben im Hinblick auf onkogen-induzierte, maligne Zelltransformation aufzuklären. Die maligne Zelltransformation ist ein Prozess, der die Mechanismen des Beginns der Krebsentstehung auf zellulärer Ebene widerspiegelt.

Für die Forschungsarbeiten wurden hautsächlich humane und Maus Zellinien verwendet, welche biochemisch und zellbiologisch analysiert wurden.Von besonderer Bedeutung waren die Entwicklung von p38α MAPK-defizienten Zellinien und retrovirale Genexpressionstechniken. Außerdem war die Verwendung von Methoden zur Messung des intrazellulären Niveaus von reaktiven Sauerstoffradikalen (Englisch: “reactive oxygen species” = ROS) in lebenden Zellen essentiell für meine Arbeit.

Die im Rahmen dieser Dissertation erarbeiteten Forschungsergebnisse deuten darauf hin, dass die p38α MAP Kinase die maligne Zelltransformation auf verschiedenen Ebenen reguliert. Zum einen reguliert p38α in negativer Weise die durch mitogene Signale induzierte Progression des Zellzyklus sowohl in proliferierenden, als auch in konfluent wachsenden Zellen. Zellen, welche Onkogene exprimieren, wachsen schneller in Abwesenheit von p38α, was eventuell auf den negativen Effekt der p38α MAPK Kinase auf die Cyclin D1 Expression zurückzuführen ist. In ähnlicher Weise kontrolliert p38α MAPK den Vorgang der Kontaktinhibition in Zellkultur, für den eine Akkumulierung von $p27^{Kip1}$ notwendig ist, und der bei Zell-Zell Kontakt den Zellzyklus in der G1 Phase blockiert. An der Kontaktinhibition in Zellkultur sind wahrscheinlich andere noch unbekannte zellmembranassoziierte Signalübertragungswege beteiligt. In diesem Sinne habe ich zeigen können, dass p38α MAPK die molekulare Zusammensetzung der Zellmembranen von durch Onkogene transformierten Zellen steuert. Weiterhin konnte ich aufklären, dass p38α MAPK die maligne Zelltransformation dadurch beeinflusst, dass sie als Messmodul für oxidativen Stress fungiert und bei hohen Werten Apoptose induziert. Dabei wird p38α MAPK dann aktiviert, wenn Onkogen exprimierende Zellen hohe Levels an karzinogenem reaktivem Sauerstoff angesammelt haben. Die aktivierte p38α MAPK induziert daraufhin den programmierten Zelltod der transformierten Zellen. Zusätzlich konnte ich im Rahmen dieser Arbeit zeigen, dass Zellinien menschlicher Tumoren, die hohe intrazelluläre Niveaus von oxidativem Stress aufweisen, einen Kompensationsmechnismus entwickelt haben, diesen p38α gesteuerten Prozess zu umgehen. Schließlich konnte ich zeigen, dass p38α MAPK, im Gegensatz zu ihren anti-proliferativen und anti-apoptotischen Funktionen, ein wichtiges Regulationsglied der cytokininduzierten Zellwanderung ist. Dieser Vorgang scheint sehr wichtig für die Metastatisierung von Tumoren zu sein.

RESUMEN (THESIS SUMMARY IN SPANISH)

Regulación de la transformación celular maligna por la quinasa de estrés p38α

El cáncer es una enfermedad que surge tras la alteración progresiva de los mecanismos que regulan el correcto funcionamiento de la célula. Durante mi doctorado, he caracterizado como la ruta de señalización de la p38α MAP quinasa regula los procesos de migración, proliferación y supervivencia celulares en el contexto de la transformación celular maligna inducida por oncogenes. Dicho proceso recapitula a nivel celular los mecanismos asociados con la iniciación del cáncer.

Mis estudios se han centrado principalmente en el uso de células de mamífero en cultivo, tanto humanas como de ratón, que he analizado desde perspectivas bioquímicas y del campo de la biología celular. Es de destacar que el desarrollo de células deficientes en p38α me ha sido de especial utilidad, así como el uso de técnicas de expresión ectópica basadas en sistemas retrovirales. La puesta a punto de métodos para visualizar y cuantificar los niveles intracelulares de radicales libres de oxígeno (en inglés "reactive oxygen species = ROS") en células vivas ha sido también importante para mi trabajo.

Así, he encontrado que p38α regula el proceso de transformación celular maligna a varios niveles. Por una parte, p38α regula negativamente la progresión del ciclo celular inducida por mitógenos en células proliferando exponencialmente así como en condiciones de confluencia. De hecho, células transformadas por varios oncogenes proliferan más rápido en ausencia de p38α, lo que puede deberse al efecto negativo que p38α ejerce sobre la expresión de la ciclina D1. De forma similar, p38α regula el proceso de inibición por contacto inducido por contactos célula-célula en condiciones de confluencia celular, que requiere la acumulación de la proteína $p27^{Kip1}$ e induce una parada en la fase G1 del ciclo celular. Además, el proceso de inibición por contacto requiere probablemente la participación de eventos de señalización originados en la membrana plasmática y que aún no han sido caracterizados. A este respecto, p38α es capaz de regular directamente la composición proteíca de las membranas de células transformadas por oncogenes.

Aparte del efecto inibitorio de p38α en proliferación celular, también he demostrado que p38α puede interferir con el proceso de transformación celular maligna a través de su capacidad para detectar estrés oxidativo e inducir apoptosis. De hecho, p38α se activa en las células transformadas por oncogenes en respuesta a altos niveles de ROS intracelulares, de conocido carácter carcinogénico, e induce su eliminación por medio de una respuesta apoptótica. Es interesante destacar que algunas células cancerígenas humanas han desarrollado un mecanismo para desacoplar la activación de p38α de la acumulación de altos niveles de ROS, y esto les confiere una mayor capacidad tumorigénica.

Finalmente, en contraste con sus funciones anti-proliferativas y pro-apoptóticas, he demostrado la implicación de p38α en la migración celular inducida por citoquinas, un proceso que se sospecha es importante para la capacidad metastática de las células cancerígenas.

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- CUADRADO A., LAFARGA V., CHEUNG P., **DOLADO I.**, LLANOS S., COHEN P., NEBREDA A.R. (2007). A new p38 MAP kinase-regulated transcriptional coactivator that stimulates p53-dependent apoptosis. *EMBO J.* 26(8), 2115-2126.

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  Ignacio wrote the first draft of this review and actively contributed to the preparation of the final manuscript.

- **DOLADO I.**, SWAT A., AJENJO N., DE VITA G., CUADRADO A., NEBREDA A.R. (2007). p38α MAP kinase as a sensor of reactive oxygen species in tumorigenesis. *Cancer Cell* 11, 191-205.

  Ignacio performed the majority of the experiments and participated extensively in their design. He independently wrote the first manuscript draft and contributed importantly to its editing.

- ALFONSO P., **DOLADO I.**, SWAT A., NUNEZ A., CUADRADO A., NEBREDA A.R., CASAL J.I. (2006). Proteomic analysis of p38α mitogen-activated protein kinase-regulated changes in membrane fractions of RAS-transformed fibroblasts. *Proteomics* 6 Suppl 1, S262-271.

  Ignacio performed 30% of the experiments. He made substantial contributions to the experimental design as well as to the writing of the manuscript.

- FAUST D., **DOLADO I.**, CUADRADO A., OESCH F., WEISS C., NEBREDA A.R., DIETRICH C. (2005). p38α MAPK is required for contact inhibition. *Oncogene* 24, 7941-7945.

  Ignacio performed 30% of the experiments.

- ROUSSEAU S., **DOLADO I.**, BEARDMORE V., SHAPIRO N., MARQUEZ R., NEBREDA A.R., ARTHUR S.C., TESSIER-LAVIGNE M., GAESTEL M., CUENDA A., COHEN P. (2006). CXCL12 and C5a trigger cell migration via a PAK1/2-p38α MAPK-MAPKAP-K2-HSP27 pathway. *Cell Signal.* 18, 1897-1905.

  Ignacio performed 20-30% of the experiments.

- CUADRADO A., LAFARGA V., CHEUNG P., **DOLADO I.**, LLANOS S., COHEN P., NEBREDA A.R. (2007). A new p38 MAP kinase-regulated transcriptional coactivator that stimulates p53-dependent apoptosis. *EMBO J.* 26, 2115-2126.

  Ignacio performed less than 5% of the experiments. He identified $p18^{Hamlet}$ as a new p38 MAPK substrate and developed a $p18^{Hamlet}$ polyclonal antibody.

Dr. Ángel R. Nebreda

ABBREVIATIONS

AD	----	alcohol dehydrogenase
APC	----	adenomatosis polyposis coli
ARE	----	AU-riche element
AREBP	----	ARE-binding protein
BMK	----	Big MAPK
CAM	----	cell-cell adhesion molecule
CDK	----	cyclin-dependent kinase
CI	----	contact-inhibition
CSC	----	cancer stem cell
COX	----	cyclooxygenase
CYP	----	cytochrome P450 oxidase
ECM	----	extracellular matrix
eIF	----	eukaryotic initiation factor
EPHX	----	epoxide hydrolase
ERK	----	extracellular signal-regulated kinase
FBS	----	foetal bovine serum
FMO	----	flavin-containing monooxygenase
GADD	----	growth arrest and DNA damage–induced
GPx	----	glutathione peroxidase
GR	----	glutathione reductase
GSH	----	glutathione
GST	----	glutathione-S-transferase
HAT	----	histone acetyltransferase
HIF	----	hypoxia-inducible factor
HMG	----	high mobility group
IL	----	interleukin
KO	----	knock-out
JNK	----	Jun-N-terminal kinase
MAPK	----	mitogen-activated protein kinase
MAPKAPK	----	MAPK-activated protein kinase
MEF	----	mouse embryo fibroblast
MEK	----	MAPK kinase

MEKK	----	MAPK and ERK kinase kinase
MLK	----	mixed lineage kinase
MMR	----	mismatch repair
MK	----	MAPKAPK
MKK	----	MAPK kinase
MKKK	----	MAPK kinase kinase
MKKKK	----	MAPK kinase kinase kinase
MNK	----	MAPK-interacting kinase
NAT	----	N-acetyltransferase
NHEJ	----	non-homologous end-joining
NER	----	nucleotide-excision repair
NOS	----	nitric oxide synthase
NOX	----	NADPH oxidase
PAH	----	polyaromatic hydrocarbon
PAK	----	p21-activated kinase
pol	----	polymerase
pRb	----	retinoblastoma protein
Prx	----	peroxiredoxin
RNS	----	reactive nitrogen species
ROS	----	reactive oxygen species
RSK	----	p90 ribosomal S6 kinase
SAPK	----	stress-activated protein kinase
SC	----	stem cell
SOD	----	superoxide dismutase
STE	----	Sterile
STK	----	Ste20-like kinase
SULT	----	sulfotransferase
TAO	----	thousand and one-aminoacid kinase
TAK	----	TGFβ-activated kinase
TBP	----	TATA-bindig protein
TGF	----	transforming growth factor
TNF	----	tumor necrosis factor
TRAF	----	TNF receptor-associated factor
Trx	----	thioredoxin

TrxR	----	thioredoxin reductase
UDP	----	uracil diphosphate
UGT	----	UDP-glucuronosyltransferase
UV	----	ultraviolet
VEGF	----	vascular endothelial growth factor
WT	----	wild-type
XB	----	xenobiotic
Xox	----	xanthine oxidase

INTRODUCTION

INTRODUCTION

1. MALIGNANT TRANSFORMATION, TUMORIGENESIS AND CANCER

A) Concept definition

The adjectives transformed, tumorigenic and cancerous are normally used in the literature when referring to cellular capabilities typically associated with cancer, such as the abilities to proliferate without control or induce tumor growth. However, these terms should not be considered synonyms. Namely, a transformed cell is usually defined as one bearing relaxed or absent growth control mechanisms after having acquired at least one of the cancer hallmarks described below (see B and Figure 1). Noteworthy, a transformed cell does not need either to be tumorigenic or to display any obvious cancer-associated phenotype. This is the case for some immortalized cell lines that, despite proliferating limitless, do not show any other obvious abnormal phenotype when compared to primary cells (with the exception, perhaps, of being slightly more genomically unstable). Consequently, such cells are sometimes called "pre-malignant", although they have accomplished the first step in the course to cancer: the acquisition of a limitless lifespan. As an extension, tumorigenic refers to a transformed cell that has further progressed into the process of cellular transformation to the extent of being able to produce macroscopic tumors *in vivo*. Of note, a tumorigenic cell line will have normally accomplished most of the cancer-associated traits depicted in Figure 1, with the exception of the abilities to invade and metastasize. Finally, the term cancerous should be only used for tumorigenic cells that have also acquired the abilities to invade the surrounding tissues and metastasize to distant sites. These represent the ultimate achievements in cell transformation and underlay the true malignant nature of cancer (Chambers et al., 2002).

B) Traits of the cancer cell and how to analyze them technically

In a seminal review article in 2000, Hanahan and Weinberg (Hanahan and Weinberg, 2000) proposed that the complex array of phenotypes displayed by cancer cells may be organized in 6 cancer-associated traits (Figure 1) that, when properly quantified, will tell us the extent of malignancy of any given cell population. These are the cellular abilities to proliferate indefinitely (immortalization), to become independent of extracellular growth or anti-growth signals, to evade apoptosis, to induce a self-sustained supply of nutrients and oxygen (angiogenesis) and, ultimately, to invade and metastasize.

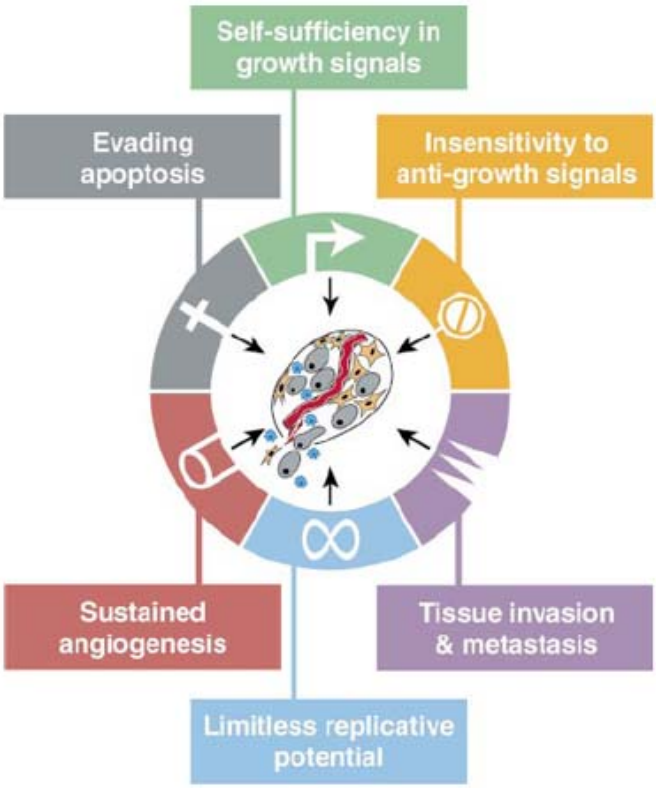

Figure 1. Cancer-associated cellular traits that are progressively adopted by normal cells when becoming cancerous.

Extracted from (Hanahan and Weinberg, 2000)

B.1) Immortalization

Cancer cells are immortal, meaning that they can continuously proliferate and grow. Indeed, the ability to proliferate limitless is both a characteristic of and a prerequisite for cell transformation, as it is the main mean to fix the successive genomic mutations that drive clonal expansion and progression in cancer (Hanahan and Weinberg, 2000; Nowell, 1976) (further discussed in C.1). For instance, a clear example of this capability is given by the widely used HeLa cells, which were originally derived from a human cervical cancer in 1951 and are still used nowadays as a common model cell line in cancer research (Masters, 2002).

However, the ability to proliferate limitless was not always considered an intrinsic characteristic of transformed cells, and during the first half of the 20th century it was believed that any cell population could be expanded *in vitro* indefinitely (Hayflick, 1998). Noteworthy, seminal work by Hayflick and Moorhead in the early 1960´s established that the replicative potential of any normal (non-transformed) cell type has a limit, the so-called “Hayflick limit”, after which the cells irreversibly exit the cell cycle (Hayflick, 1998). This process, which was afterwards named replicative “senescence” from the Latin word *senex* (meaning "old man" or "old age"), was shown to be ultimately due to the progressive telomere attrition that occurs within successive cell divisions (Bodnar et al., 1998; Serrano and Blasco, 2001), although its onset can be accelerated

by extracellular and oncogenic stresses in a process known as "premature senescence" (Serrano and Blasco, 2001). Thus, it is not surprising that the first selective pressure on normal cells to become cancerous likely consists in the acquisition of an impaired senescence response. Accordingly, virtually all human cancers express high levels of the telomere-maintaining enzyme telomerase (Shay and Bacchetti, 1997), whose expression is normally absent in most somatic cells except for stem and progenitor cells (Flores et al., 2006). Importantly, telomerase inhibition in human tumor cell lines attenuates their growth (Zumstein and Lundblad, 1999), further highlighting its causal role in human cancer cell immortalization. Furthermore, human cancers usually show defects as well in senescence-controlling signaling pathways downstream of telomere attrition and oncogenic/extracellular stresses (i.e. Arf-p53 and Ink4a-pRb pathways), which further cooperate with the abnormal telomerase expression for immortalization of human cancer cells (Serrano and Blasco, 2001). Of note, murine but not human cells constitutively express telomerase, thus the sole disruption of the Arf-p53 and Ink4a-pRb pathways is sufficient for their immortalization (Serrano and Blasco, 2001).

As for the technical means to evaluate cellular immortalization and senescence, there are both phenotypical and biochemical markers. Among the phenotypical markers for immortalization, the most classical approach is to evaluate whether the cells can proliferate beyond their Hayflick limit by continuously expanding them in culture (Bodnar et al., 1998). Conversely, several markers are used to validate the senescent state, such as the acquisition of a characteristic flat and enlarged morphology as well as the expression of the enzyme acidic beta-galactosidase (Collado and Serrano, 2006). Biochemically, the entry into the senescent state is characterized by the persistent mitogen-independent accumulation of cell cycle inhibitors (Figure 2) such as p53, $p21^{Cip1}$ and $p16^{INK4a}$, and by a sustained hypo-phosphorylated state of the retinoblastoma protein (pRb) (Collado and Serrano, 2006).

B.2) Independence of extracellular growth signals

The acquisition of a limitless replicative potential is a "gate-keeper" in cancer, meaning that a cell can only become a competent cancer cell once it can self-perpetuate indefinitely (Hanahan and Weinberg, 2000; Nowell, 1976). However, this capability only confers growth advantage to malignant cells if these are actually induced to enter the cell cycle (Figure 2) and proliferate. Noteworthy, normal cells highly rely on exogenous mitogenic stimulation at G1 for this decision, which is mostly coordinated by the Ink4a/pRb pathway (Hanahan and Weinberg, 2000) (Figure 2). In contrast, a virtually ubiquitous characteristic of cancer cells is their ability to proliferate in the absence of exogenous mitogenic stimulation, which makes them refractory to normal

homeostatic regulation (Hanahan and Weinberg, 2000). For instance, it has been estimated that at least half of human cancer cell lines bear distinct genetic or epigenetic alterations that impair proper cell cycle control at G1 (Kawabe, 2004; Reinhardt et al., 2007), including the overexpression of cyclin D1 and cyclin-dependent kinase (CDK) 4 as well as the silencing of $p16^{INK4a}$ and pRb (Vogelstein and Kinzler, 2004), which all impinge on the INK4a/pRb pathway (Figure 2). Furthermore, it is well-established that many oncogenes (i.e. Ras, Src, Raf) exert their transforming effects, in part, by providing the cell with a sustained endogenous supply of mitogenic signaling (Hanahan and Weinberg, 2000) that also unplugs cell proliferation from extracellular mitogenic stimulation. Similarly, it is increasingly recognized that cell types composing the tumor bed (i.e. fibroblasts, inflammatory cells, endothelial cells) can also contribute to the independence of tumor cells from extracellular mitogens by continuously providing certain proliferative cytokines such as interleukin (IL)-6 (Comoglio and Trusolino, 2005; Tlsty and Hein, 2001).

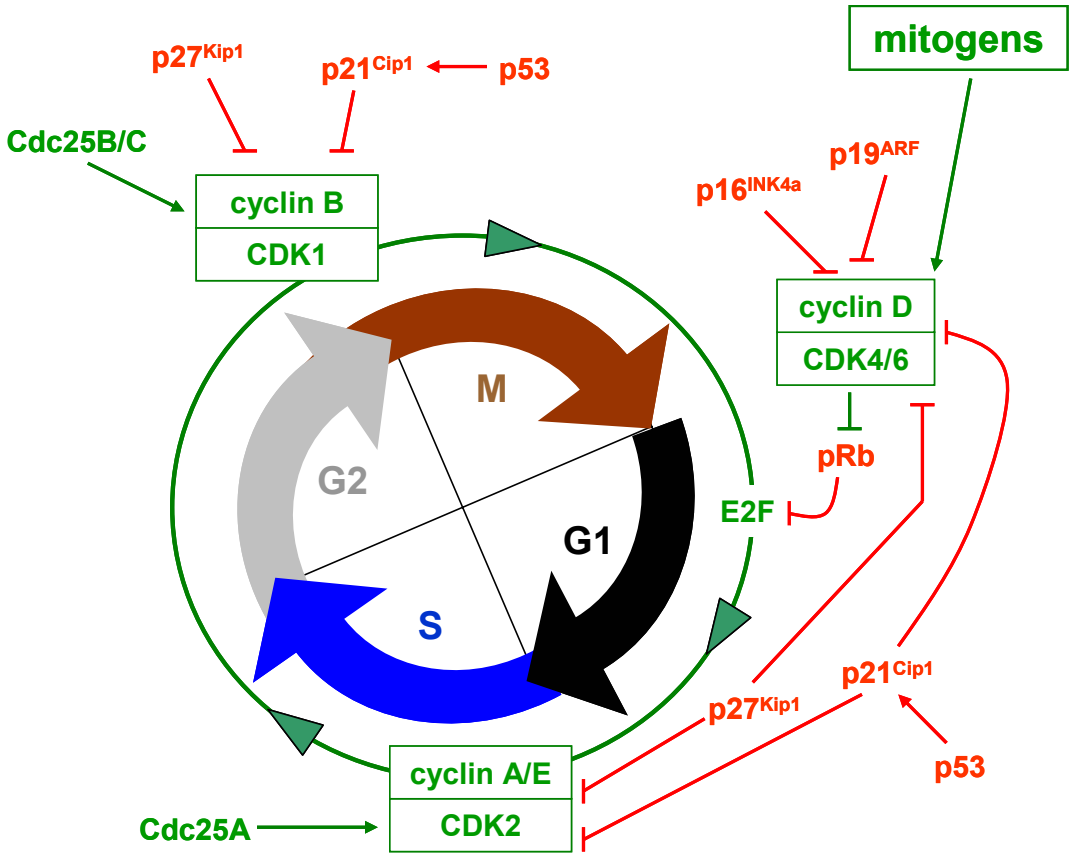

Figure 2. Simplified scheme of the mammalian cell cycle
The cell cycle is composed of four distinct phases that are driven by various phase-specific CDKs, namely the control stages of G1 and G2, as well as the DNA replicating S-phase and the M-phase, where a tetraploid cell divides into two diploid daughter cells. Proteins, protein complexes, and signaling events that drive cell cycle progression are shown in green. As shown, mitogenic stimulation is the entry point to the cell cycle through G1. The most significant cell cycle inhibitors are shown in red.

In practical terms, the extent of mitogen-independence acquired by a transformed cell line can be determined by comparing its growth rate in low-serum (0.5% foetal bovine serum -FBS-) *versus* high-serum conditions (10% FBS). Furthermore, it is also informative to compare the growth rate of the transformed cell line and its parental non-transformed counterpart in low-serum medium (Brancho et al., 2003; Dolado et al., 2007). Biochemically, mitogen-dependence is normally reflected by the enhanced accumulation of hypo-phosphorylated pRb following serum-deprivation (Dietrich et al., 1997).

B.3) Independence of anti-growth signals

In their course to malignancy, transformed cells must not only get provision of their own mitogenic signals but also overcome certain homeostatic anti-proliferative stimuli such as those triggered by an excess cell number or those that are concomitant with the process of cell differentiation (Hanahan and Weinberg, 2000). Noteworthy, such anti-proliferative signals are thought to be regulated in many cases at the membrane receptor level (Hanahan and Weinberg, 2000), as normal cells usually establish *in vivo* numerous protein-protein interactions with the surrounding cells (i.e. through cadherins and CAMs) and with the extracellular matrix (ECM) (i.e. through integrins) in order to evaluate if they are located in the right niche as well as whether cell division is required to compensate for cell loss. For instance, one of these cell-cell interactions, which is mediated by a yet poorly identified membrane receptor (Gradl et al., 1995), regulates the **contact-inhibition** (CI) response (Dietrich et al., 1997; Faust et al., 2005), which induces a rapid cell cycle exit when high cellular densities are achieved. Both the $p16^{Ink4a}$ and $p27^{Kip1}$ cell cycle inhibitors seem to be key regulators of the CI response (Polyak et al., 1994; Wieser et al., 1999). Consequently, the usual disruption of these pathways in cancer may explain, at least in part, why virtually all cancer cells are unresponsive to CI and have the potential to grow without temporal (see B.1) and spatial restrictions.

From the cell biology perspective, the loss of CI in transformed cells can be measured by a "focus formation assay" that evaluates the cell ability to grow in multi-layered cellular structures in culture (Dolado et al., 2007). Biochemically, the impaired accumulation of $p16^{Ink4a}$ and/or $p27^{Kip1}$ in confluent conditions of growth is a good marker to support the loss of the CI response.

Another powerful source of anti-proliferative signals is the process of cellular **differentiation**, in which pluripotent tissue stem/progenitor cells give rise to all the specialized cell types of a multicellular organism. Cell differentiation is indeed a progressive process in which cells steadily lose their proliferative capacity, to finally exit the cell cycle in an irreversible fashion at the stage of terminal differentiation. Of note, the vast majority of somatic cells in our organisms

are either terminally differentiated or on the way to this non-replicative state. In contrast, it is a well-known feature of cancer that human tumors show differentiation defects (Potter, 1978), meaning that virtually all human tumor cells are de-differentiated to some degree. However, it has been controversial for many years whether the undifferentiated nature of cancer arises from a differentiation back-tracking of already differentiated cells or from a block in differentiation of stem/progenitor cells (Gisselsson, 2007). Although no hypothesis has been discarded yet, the latter seems to be favoured by recent data (Bapat, 2007; Krivtsov et al., 2006; Widschwendter et al., 2007), to the extent that a new paradigm in cancer is arising that points to cancer as a stem/progenitor cell disease (Wicha et al., 2006) (see C.2). Accordingly, the ability of several oncogenes to block cell differentiation has been proposed to mediate their transforming activity, for example Notch in breast, pancreatic and lymphoid cancers (Sjolund et al., 2005) and Bcr-Abl in leukaemia (Klein et al., 2006).

Technically, the loss of differentiation is usually concomitant with a gain of proliferation. Thus, differentiation defects in transformed cells can be assessed *in vitro* by the lack of cell cycle arrest and organization into differentiated cellular structures (i.e. myotubules, myofibers) following treatment with differentiation-inducing agents (i.e. retinols, vitamin D, neurotrophins). Furthermore, differentiation defects can be also visualized immunologically and immunohistochemically, as the de-differentiated state is usually characterized by the absence of certain specialized cellular organelles (i.e. vacuoles, melanosomes) and proteins (i.e. hormone receptors, keratins) as well as by the presence of proteins whose expression is normally restricted to the foetal stage (i.e. carcinoembryonic antigen, alpha-fetoprotein) (McKinnell et al., 2006).

B.4) Apoptosis evasion

All the cancer-associated traits mentioned above are recognized as key contributors to the cell-autonomous transformed state (Hanahan and Weinberg, 2000), but those that impinge on the differentiation (see B.3) and apoptotic cell programs are considered of special relevance (Alberts et al., 2002). Indeed, whereas the acquisition of invasiveness is regarded as the deadliest characteristic of cancer, the development of drug resistance may account for the failure of treatment, and ultimately death, of more than 90% of metastatic cancer patients (Dean et al., 2005; Sporn, 1996). It is therefore not surprising that numerous proteins involved in the regulation of apoptosis have been found silenced or deregulated in most cellular and animal models for cancer, as well as in human tumors (Johnstone et al., 2002). Thus, although the acquisition of cell death resistance is not usually considered a “gate-keeper” in cancer (i.e. early tumors normally show an enhanced sensitivity to apoptosis than non-tumorigenic tissues), its

acquisition through the process of cell transformation not only amplifies the transforming effect of certain oncogenes (Brancho et al., 2003; Dolado et al., 2007) but also makes cancer cells refractory to treatment (Dean et al., 2005). At the molecular level, several mechanisms have been proposed to underlie the acquisition of such malignant phenotype, including the mutation or silencing of pro-apoptotic proteins (i.e. Fas, p53, Bax, Apaf-1) (Johnstone et al., 2002), the overexpression/overactivation of pro-survival factors (Akt, Ras, Bcl-2, Bcl_{xL}), the alteration of the protein composition of the plasma membrane (i.e. integrins) (Reddig and Juliano, 2005), and enhanced DNA-repair activity (Kohno et al., 2005). Of note, the ability of transformed cells to elude apoptosis induced by anchorage-independent growth conditions *in vitro* has been long recognized as the best predictor for their potential tumorigenicity *in vivo* (Shin et al., 1975).

From the cell biology point of view, there are numerous ways to measure cell alterations in the apoptotic program, both phenotypical and biochemically. At the phenotypic level, probably the most common assay is the "soft-agar assay" (Dolado et al., 2007), which measures the cell ability to grow in anchorage-independent conditions in which normal cells would undergo apoptosis. This assay can also be adapted to measure the acquisition of drug resistance (Fukazawa et al., 1996), although an alternative assay called "clonogenic assay" is normally used routinely to this end (Zips et al., 2005). Briefly, this consists in treating a limited number of sparsely-seeded cells and then measure which percentage of cells has survived and consequently self-expanded into macroscopic clones. At the cellular level, there are also several markers to asses the occurrence of apoptosis, such as the visualization of refringent and rounded-up poorly adherent cells in bright-field microscopy (Cuadrado et al., 2003), the appearance of apoptotic bodies in immunofluorescence (Garcia-Fernandez et al., 2002), the translocation of the plasma membrane phospholipid phosphatidylserine from the cytosol to the extracellular space (Cuadrado et al., 2003), the accumulation of a sub-G1 cell population by flow cytometric analysis (Cuadrado et al., 2003), and the fragmentation of the genomic DNA to nucleosome-sized particles (Dolado et al., 2007). Biochemically, apoptosis can be followed up as well by the accumulation or modification of several proteins, including the processed forms of poly-ADP-ribose polymerase (PARP) and pro-caspase-3 (Dolado et al., 2007).

B.5) Sustained angiogenesis

It is usually assumed that a cell has become tumorigenic once it has successfully acquired all the aforementioned cancer-associated traits: (1) limitless replication potential, (2) self-sufficiency in growth signaling, (3) insensitivity to anti-growth signals, and (4) unresponsiveness to apoptosis (Hanahan and Weinberg, 2000). This assumption is indeed usually validated in "soft-agar"

experiments by the cells ability to form microscopic tumor-like spheroids *in vitro* (Dolado et al., 2007; Shin et al., 1975).

However, it has been observed experimentally that many cells that are "tumorigenic" *in vitro* or that have been derived from human tumors or metastatic nodules are unable to produce macroscopic tumors in immuno-depleted mice (Naumov et al., 2006). This apparent contradiction is now starting to be understood. Indeed, whereas the above mentioned neoplastic properties are able to drive a cell-autonomous transformed state *in vitro*, further cell expansion *in vivo* into macroscopic tumors additionally requires a process called angiogenesis (Folkman, 1971; Folkman, 1990). In this, tumor cells of the incipient microscopic tumor stimulate the vascular system to provide them with new blood vessels, which then constitute a sustained supply of oxygen and nutrients to the tumor, as well as a way-out for its catabolites, and consequently boost tumor growth. The key relevance of angiogenesis for macroscopic tumor growth is such that it has been proposed that targeting tumor angiogenesis might be a way to turn cancer into a chronic and asymptomatic disease (Naumov et al., 2006; Pincock, 2005) (discussed in C.3 and I). Accordingly, although the process of angiogenesis was thought in the 1960´s to be dispensable for tumor growth (Birmingham, 2002), it is now well established that tumor growth beyond the size of 1-2 millimetres is always angiogenesis-dependent (Naumov et al., 2006). Furthermore, the acquisition of angiogenic capabilities critically influences tumor metastasis (Gupta et al., 2007). At the molecular level, angiogenesis is known to be stimulated by the incipient tumor in a process known as the "angiogenesis switch" (Hanahan and Folkman, 1996), in which tumor cells produce high local concentrations of endothelial cytokines and survival factors that attract new endothelial cells to the tumor bed. Of note, the transcription factor hypoxia-inducible factor 1 (HIF1), which is activated by the hypoxic conditions of the microscopic tumor, is known to play a key role in angiogenesis (Zhou et al., 2006).

In practical terms, the most widely used assay to asses the angiogenic potential of a transformed cell population is called the "cornea assay" (Jimenez et al., 2000; Phillips and Knighton, 1995), which consists in implanting the cells into the cornea of mice or rabbits and monitor new capillary formation with time, both in terms of capillary density and length.

B.6) Invasion and metastasis

The acquisition of an invasive and metastatic behavior is the ultimate achievement of the cancer cell and underlies the malignant nature of cancer. Indeed, the presence of metastases at cancer diagnosis readily precludes efficient cancer treatment, with chemotherapy constituting a mere palliative in such cases (McKinnell et al., 2006; McVie, 1999; Vogelstein and Kinzler, 2004). In

contrast with its key clinical importance, the metastatic process is poorly understood at the cellular level (Vogelstein and Kinzler, 2004), but it is thought to consist of 6 independent steps (McKinnell et al., 2006): (1) detachment from the primary tumor mass, (2) degradation of and migration through the stroma and the basement membrane layer, (3) intravasation into the blood/lymphatic systems, (4) translocation to other "fertile" body sites, (5) extravasation, and (6) settlement and re-growth.

Several alterations have been proposed to drive the metastatic phenotype at the molecular level (Hanahan and Weinberg, 2000), although it is not clear yet whether these normally result from the acquisition of a specific "metastatic" gene expression program (Gentile and Comoglio, 2004; Minn et al., 2005) or are just a consequence of the multiple mutations acquired through the cell transformation process (Nowell, 2002). Such alterations include misexpression of cell-cell adhesion proteins such as cadherins (i.e. E-cadherin) and other cohesive CAMs (i.e. N-CAM), which is observed in most invasive and metastatic tumor cells and is likely to facilitate their detachment from the primary tumor (Hanahan and Weinberg, 2000). In addition, not only the tumor cells of malignant tumors, but also the surrounding stromal (Comoglio and Trusolino, 2005; Tlsty and Hein, 2001) and inflammatory cells (Baniyash, 2006; Karin, 2006; Philip et al., 2004), are known to produce extracellular proteases (i.e. ECM metalloproteinases –MMPs-, lysil oxidases) that degrade the ECM and facilitate the invasion of the metastatic cells. Interestingly, metastatic cancer cells usually shift their expression pattern of integrins from those that bind the intact epithelium ECM proteins to others that show higher affinity for degraded stromal components (Hanahan and Weinberg, 2000), which likely directs their migration through the degraded epithelium towards the blood vessels. Of note, tumor cells do not metastasize stochastically, meaning that specific primary tumor types metastasize preferentially to certain secondary tissues, for example mammary tumors to bone, lung and liver; lung tumors to brain; or stomach tumors to ovary (McKinnell et al., 2006). This reproducible clinical phenomenon was first described in 1882 (Fuchs, 1882), and later proposed as the "seed and soil" theory in cancer (Paget, 1889). This states that primary tumor cells (the seed) from a particular tissue of origin are predisposed to settle and expand only in certain "fertile" secondary tissues (the soil), a process that we know today is largely influenced by the establishment of the appropriate biological interactions (i.e. chemokines/chemokine receptors) between the host tissue and the metastasizing tumor cells (McKinnell et al., 2006; Muller et al., 2001; Muller et al., 2006).

From the cell biology perspective, the metastatic phenotype is usually evaluated by several assays that independently measure different metastatic cell properties, such as the abilities to

invade and migrate through the ECM (Dolado et al., 2007; Rousseau et al., 2006) or to settle into secondary body sites (Kennedy and Davis, 2003).

C) Cancer at the molecular level

C.1) Cancer is an evolving and dynamic genetic disease

Early in the history of cancer research it was believed that cancer was a viral infectious disease (Nastac, 1967; Zil'ber, 1967), a view that was further fuelled in the 1960´s and 1970´s by the discovery of various tumorigenic rodent viruses (Harvey, 1964; Kirsten and Mayer, 1967; Peters et al., 1974; Rasheed et al., 1978). However, it was soon observed that DNA isolated from chemically- and virally-induced rodent tumors (Shih et al., 1979), as well as from human tumor cell lines (Der et al., 1982), was sufficient *per se* to transform virus-free cultured cells *in vitro*, which set the foundings for the current paradigm of cancer as a predominantly endogenous genetic disease (Bishop, 1991). Early in the 1980s, several genes were cloned and identified from such transforming DNA preparations, which were called oncogenes on behalf of "cancer-causing genes" (Malumbres and Barbacid, 2003). Closing the circle, it was soon acknowledged that the notorious cancer-causing viruses from the 1960´s were tumorigenic by virtue of encoding certain oncogenes in their genomes (Malumbres and Barbacid, 2003), hence establishing the basis for the viral origin of some human cancers. The ultimate recognition for the genetic origin of cancer was settled in 1989, when J.M. Bishop and H.E. Varmus were awarded the Nobel Prize in Medicine or Physiology for their contribution to the understanding of cancer as a genetic disease that, at that time, was thought to be mostly caused by the deregulation of endogenous proteins with potential oncogenic properties, namely proto-oncogenes.

It is well established today, almost 20 years later, that cancer is a genetic disease (Bishop, 1991; Vogelstein and Kinzler, 2004), which continuously progresses from benign to malignant stages through the steady acquisition of genomic mutations in key cell-regulatory genes, namely oncogenes, tumor-suppressors, and stability genes. Such mutations usually fall within four groups (Lengauer et al., 1998): point mutations, gene amplifications, chromosome translocations, and alterations in chromosome numbers, which usually occur concomitantly in cancer. The mutational burden of human cancer is such that around eleven thousand mutations per cell were found in a study of colorectal carcinoma (Stoler et al., 1999), although only about 1% of these is expected to affect coding regions (Lander et al., 2001). Accordingly, a recent study of various types of human colorectal- and breast cancer tumors have identified that individual tumors accumulate an average of 90 mutated genes, although only about 10% of these seem to play a causal role in tumorigenesis (Sjoblom et al., 2006). This continuous process of cancer evolution

through mutation, which we know proceeds through multiple rounds of clonal expansion (Nowell, 1976), is called malignant transformation at the cellular level (see 1.B) and tumor progression in the clinics (Nowell, 2002).

However, it has not been always accepted that subsequent rounds of growth-promoting mutations coupled to clonal expansion drive cancer progression. Indeed, in the 1950´s it was believed that neoplasms, once initiated, kept their characteristics "fixed" thereafter (Foulds, 1957). In contrast, we know today that the acquisition of progressive genomic mutations is indeed the driving force of cancer and one of its hallmarks (Cahill et al., 1999; Lengauer et al., 1998), as well as the main reason for its malignant behavior (Folkman et al., 2000). Nevertheless, it has been a subject of debate for a long time how, and at what stage, tumors acquire such a mutator phenotype (Loeb, 1991), as cells normally have multiple mechanisms to avoid and repair genomic mutations (Lengauer et al., 1998; Vogelstein and Kinzler, 2004). These include the intrinsic proof-reading nature of DNA replication; various cellular programs specifically aimed at repairing altered nucleosides (the nucleotide-excision repair –NER- system), mutated or lost nucleotides (the mismatch repair –MMR- system), and double-stranded DNA breaks (the non-homologous end-joining -NHEJ- system); as well as a plethora of checkpoints ensuring the proper execution of such programs. In this regard, it was found almost ten years ago that the sequence of mutational events affecting oncogenes, tumor-suppressors and stability genes during cancer progression is not totally random, meaning that there are certain cancer-associated gene groups that are under more mutational pressure than others in particular cancer stages (Kinzler and Vogelstein, 1996). For instance, it has been established that stability genes, which are involved in the detection and repair of DNA mutations through the above mentioned processes (i.e. MMR), are early targets in cancer (Kinzler and Vogelstein, 1996; Lengauer et al., 1998), and their inactivation provides the incipient cancer cells with the necessary plasticity to acquire more mutations and further progress towards malignant transformation. These stability genes include regulators of the NER system such as *MTH1*, MMR genes such as *MLH1* and *MSH2*, S-phase checkpoint genes such as those encoding BRCA1, BRCA2, ATM, ATR and p53, and M-phase checkpoint genes encoding proteins such as BUB1, Plk1 and Aurora. The importance of this group of genes in cancer is such that they are also called "gatekeepers", as their disruption and the subsequent acquisition of genomic instability represents a no-return point in cancer. In agreement with this, germinal mutations in stability genes such as *MLH1* and *MSH2* highly predispose humans to the occurrence of early colorectal carcinomas (Kinzler and Vogelstein, 1996).

In summary, the collection of multiple genomic mutations that drive cancer progression does not only have to occur within the same cell for cancer to develop, but also in some kind of orderly fashion, which might explain why cancer is a disease with such a long "incubation" period that sometimes comprises 30 to 40 years (Kinzler and Vogelstein, 1996).

C.2) Target cells in carcinogenesis

Cancer has been traditionally considered as a stochastic disease that does not distinguish among specific cell types or cell regulatory genes when inflicting its mutational burden. However, as discussed in C.1, recent evidence suggests that there must be indeed certain mutational order for cancer to progress.

On a related issue, evidence accumulated in recent years also suggests that not all cell types in our body are equally prone to cancer-causing mutations (Wicha et al., 2006), but only long-lived (i.e. 30-40 years) proliferation competent cells, with the ability to clonally expand, are likely relevant *in vivo* targets in cancer. However, the vast majority of cells in our body are neither proliferative nor long-lived. Namely, most of our cells are either in the non-proliferative, irreversible state of terminal differentiation or committed towards it (McKinnell et al., 2006). Furthermore, apart from not proliferating, our terminally differentiated cells are also being continuously substituted by newly-differentiated ones in the process of tissue-renewal, which makes them in some cases extremely short-lived. For instance, the average turn-over rate of hematopoietic and intestinal epithelial cells is 2-3 days (McKinnell et al., 2006). So, how can a 2-3 day long-lived, non-proliferative cell accumulate all the mutations required for cancer progression and subsequently drive a disease that takes 30 to 40 years to turn up clinically? The answer to this question is only now starting to be figured out and points towards a specific cellular subpopulation in our body, which is composed of tissue stem and progenitor cells, as the true target cells in carcinogenesis (Bapat, 2007; Houghton et al., 2006; Wicha et al., 2006). Indeed, stem cells (SC) constitutively express telomerase, thus they are long-lived (some would say immortal) and have an almost limitless replicative potential. Furthermore, SC show a variety of cellular characteristics that are not shared by the vast majority of somatic cells but which are also found in cancer cells (Li et al., 2007; Wicha et al., 2006). For instance, SC can survive and grow in the absence of anchorage to a substrate (i.e. "soft-agar" growth); are intrinsically drug-resistant by virtue of their asymmetric cell division, their low proliferative rates, their abundance on membrane protein pumps, and their high levels of anti-apoptotic signaling; and they have the innate abilities to migrate, invade, and metastasize. Thus, as useful as these SC properties may be for tissue renewal, it is scary to realize that normal SC in our body already display most of the

malignant characteristics associated with cancer cells, such as invasivity and drug resistance. Consequently, it has been argued that perhaps cancer does not arise through the complex mutational process described in B, but that it may conversely result from a deregulation in the normal process of tissue renewal (McKinnell et al., 2006; Wicha et al., 2006) (Figure 3), either by an abnormal SC behavior (i.e. unrestrained proliferation, migration to other tissues) (Houghton et al., 2006) or by a lack of commitment of undifferentiated stem/progenitors cells (Widschwendter et al., 2007). Interestingly, epigenetic more than genetic abnormalities might be the driving force of such stem/progenitor cell alterations (Krivtsov et al., 2006; Sparmann and van Lohuizen, 2006; Widschwendter et al., 2007). This new view summarizes what is called the "stem cell origin of cancer" hypothesis.

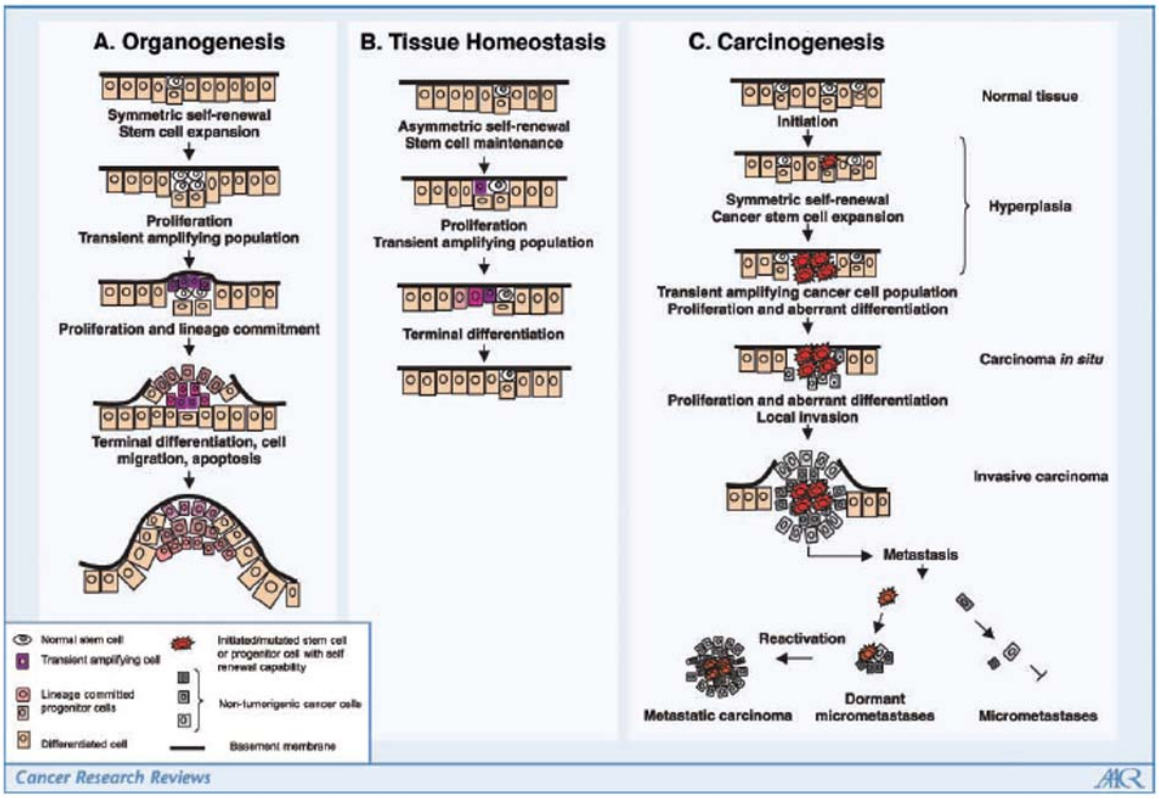

Figure 3. Cancer as a "caricature" of the normal tissue renewal process
Cancer may mostly arise by alterations in the normal process of stem/progenitor cell differentiation, as these cells already show most cancer-associated traits: they are immortalized, undifferentiated, migratory, invasive, and show high levels of anti-apoptotic signals. (Extracted from (Wicha et al., 2006))

Of note, cancer cells with SC-like properties have been already isolated from several human tumors and cancer cell lines (Dean et al., 2005) and named cancer stem cells (CSC). CSC have been shown to account for up to 1% of the total tumor cell population (Houghton et al., 2006)

and can be both initiators and sustainers of cancer, in agreement with the hypothesis of the stem cell origin of cancer. For instance, a study of human breast cancer (Al-Hajj et al., 2003) has shown that whereas as less as 200 tumor-derived CSC were able to resume tumor growth in mice, 20.000 cells isolated from the same tumor that did not displayed SC markers were unable to form tumors.

C.3) Cancer as a systemic disease: beyond oncogenes and tumor suppressors

Cancer has been long studied as a cell autonomous disease and treated in consequence, namely by just focusing on the killing of the cancer cells. Indeed, the current "gene-centric" paradigm of cancer assumes that if one day we understood all the genetic alterations occurring in a cancer cell, then we might reverse or target them individually and perhaps cure cancer (Folkman et al., 2000). However, this view has at least two major potential pitfalls: on the one hand, the number of genetic alterations in human tumors may be in some cases overwhelming, perhaps in the number of thousands (Stoler et al., 1999), and thus impossible to target in practice. On the other hand, due to the dynamic nature of cancer, both the number and type of genetic alterations, as well as their causal roles in cancer, may change with time, specially under the selective pressure of cytotoxic drugs targeting specific alterations.

In contrast, the window of therapeutic interventions against cancer might be significantly extended if we consider cancer not as a cell-autonomous disease, but as the systemic disease that it is. Indeed, it is increasingly recognized that the tumor environment plays a key role in tumor progression as important as the tumor cells themselves (Comoglio and Trusolino, 2005; Folkman et al., 2000). For instance, the innate immune system, which normally plays a key role in the initiation of the immune response against pathogens, has been shown to facilitate cancer progression and metastasis by triggering chronic inflammation at tumor sites (Hussain et al., 2003), which in turn induces local production of a variety of angiogenic and pro-survival cytokines. Consequently, the presence of tumor-infiltrating macrophages and other leukocytes in cancer patients is usually considered a bad prognostic factor in the clinics. Accordingly, it has been estimated that chronic inflammation contributes to one in four of all cancer cases worldwide (Hussain et al., 2003). Conversely, whereas tumors usually capitalize on the pro-carcinogenic effects of the innate immune system (i.e. inflammation, angiogenesis), it is well established that one of the obligate steps in cancer progression is the evasion of the adaptative immune system, a process traditionally referred to as "tumor escape" that is also considered one of the hallmarks of cancer (Shu et al., 1997). On a related issue, the vascular system has been also proposed to be a key extra-tumoral resource *in vivo* for cancer to survive and progress

(Folkman et al., 2000; Folkman and Kalluri, 2004) (see B.5). How tumor cells gain such a valuable resource is a topic of intense research, but it is thought that tumors usually induce a high local concentration of angiogenic factors in their surroundings, which normally occurs through a reduction in the expression of anti-angiogenic factors (i.e. endostatin, thrombospondin) and/or by an enhanced production of pro-angiogenic ones (i.e. vascular endothelial growth factor –VEGF-, interleukin (IL)-8). Even the tumor matrix fibroblasts, which were once thought to play only structural roles, are now known to also cooperate in tumor progression by expressing certain matrix degrading proteases (i.e. MMPs), specially when senescent (Comoglio and Trusolino, 2005).

C.4) The double-edged role of free radicals in cancer

C.4.1) The chemical nature of free radicals

The terms free radicals, oxidative stress and antioxidants are nowadays relatively common in our everyday life, as there is an increasing tendency in the media to advertise new foods with claimed medicinal effects on human health (Mandel et al., 2005). However, this was not always the case, and in the 1950´s free radicals and antioxidants were almost unheard of in the clinical and biological sciences, not to say among the general public, despite chemists had known about them for years in the contexts of radiation, polymer and combustion technologies (Gutteridge and Halliwell, 2000). So, what are free radicals? Free radicals are low molecular weight organic or inorganic molecules that show a high and unspecific chemical reactivity by virtue of having unpaired electrons in their most outer electron shell. To distinguish them from non-radical species, free radicals are usually depicted with a single dot on the atom sustaining an unpaired electron, which is followed by the net charge of the molecule when applicable (Figure 4).

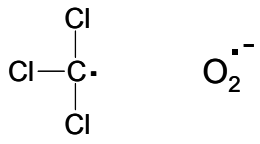

Figure 4. Examples of biologically significant free radicals
Left. Free radical produced from the organic compound Cl_4C (carbon tetrachloride), which is a recognized human toxicant known to act through a radical-mediated mechanism (Slater, 1984).
Right. Radical superoxide resulting from the incomplete reduction of oxygen in the mitochondria. It usually accounts for up to 2% of the total oxygen consumed by normal cells (Pelicano et al., 2004).

In biology, free radicals are mostly classified depending on whether the unpaired electrons lay on oxygen or nitrogen atoms. Hence, oxygen-derived free radicals are called reactive oxygen species (ROS) and are the main cause of oxidative stress. The most notorious ROS representatives are the hydroxyl and superoxide radicals (Figures 4 and 5). Of note, although

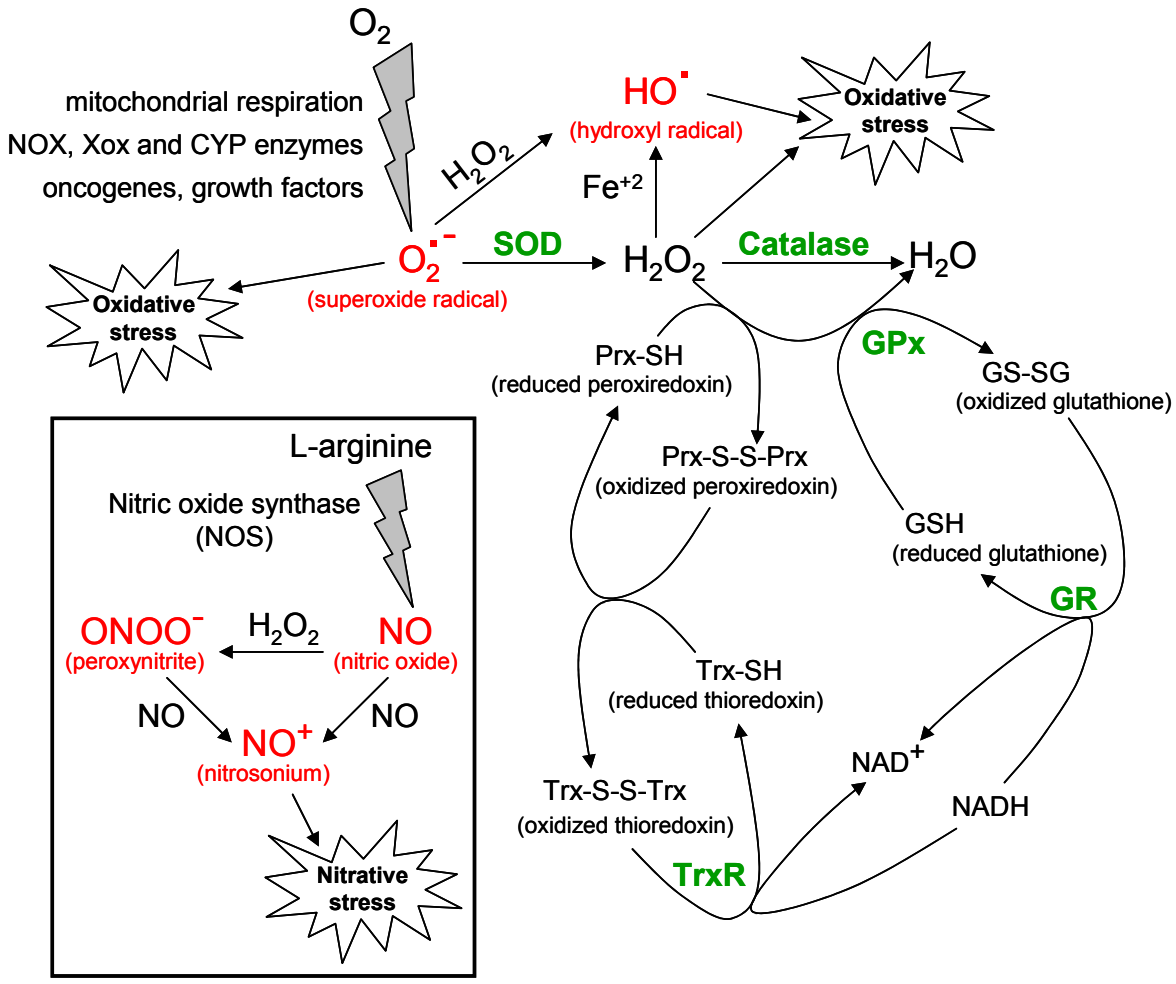

Figure 5. Pathways leading to the production and scavenging of ROS and RNS *in vivo*
Top left. Intracellular superoxide can be produced by several mechanisms. These include electron leakage from the mitochondrial electron transport chain, signaling by several oncogenes (i.e. Ras, Myc) and growth factors (i.e. epidermal growth factor), as well as a result of the enzymatic activity of several proteins, such as the NADPH oxidases (NOXs) and the metabolic enzymes xanthine oxidase (Xox) and cytochrome P450 oxidase (CYP). The term oxidative stress globally means oxidative damage to DNA, proteins, lipids, and sugars.
Top middle and right. The radical superoxide is in turn converted to various species, both reactive and innocuous, through chemical as well as enzymatic activities. Species that are specially reactive, or which have been mentioned in the text, are shown in red. The most relevant antioxidant enzymes in this context are shown in green, which are superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), and thioredoxin reductase (TrxR).
Bottom left. The inset shows an scheme of how nitric oxide and peroxynitrite are normally produced physiologically, as well as why an excess of nitric oxide usually leads to nitrative stress, namely through the production of the alkylating nitrosonium specie. Nitrative stress refers to any damage produced by RNS on DNA, proteins, lipids, or sugars, such as their nitrosation by the nitrosonium specie.

hydrogen peroxide (H_2O_2) is not a free radical *per se*, it is also considered a ROS member as it is normally converted into hydroxyl radicals *in vivo* in the presence of copper and iron ions (Gutteridge and Halliwell, 2000). Similarly, nitrogen-derived free radicals are called reactive

nitrogen species (RNS) and their outmost representative precursors are nitric oxide and peroxynitrite (Espey et al., 2002) (Figure 5). Noteworthy, low levels of both ROS and RNS are continuously produced in our cells and play important physiological roles (Gutteridge and Halliwell, 2000), in contrast to the widespread believe that free radicals are just deleterious metabolic cellular by-products. These include processes as diverse as gene expression (Allen and Tresini, 2000), cell proliferation and survival (Hancock et al., 2001; Kamata and Hirata, 1999), pathogen clearance by the immune system, and blood vessel permeability. Indeed, R.F. Furchgott, L.J. Ignarro and F. Murad shared the 1998 Nobel Prize in Medicine or Physiology by their discoveries of the key physiological roles of nitric oxide on the vascular system.

C.4.2) Cancer as a disease of free radicals overload

As mentioned above, free radicals are normally produced at low levels within our cells and play important physiological roles. Furthermore, their levels and subcellular localization are normally regulated within the cell by a plethora of antioxidant systems (Figure 5) that continuously sustain the intracellular reducing environment (Gutteridge and Halliwell, 2000). Nevertheless, it is thought that 1 in 2 males and 1 in 3 females that develop cancer by the age of 85 owe this burden to a lifetime exposure to low levels of free radicals (DePinho, 2000). In agreement with this, the fact that cancer is not anymore a disease of the elder seems to be accounted for, at least in part, by the numerous insults of modern life that result in increased ROS levels within our body (Halliwell, 2002). These include repeated exposure to ultraviolet (UV) light (Thomas-Ahner et al., 2007) as well as to polyaromatic hydrocarbons (PAHs) contained in cigarette- and car exhaust smoke (Halliwell, 2002), eating of benzpyrene present in overcooked food (Halliwell, 2002), breathing of traces of asbestos and nickel spread in the environment (Halliwell, 2002), and also the development of chronic inflammatory conditions derived from wrong alimentary habits (i.e. colitis) or continuous exposure to chemicals (i.e. pancreatitis), allergens, and stress (i.e. dermatitis) (Hussain et al., 2003). Indeed, chronic inflammatory conditions coursing through the continuous production of high levels of free radicals not only contribute to about 25% of cancer cases worldwide, but also increase the risk of cancer in healthy individuals in more than two hundred-fold in certain cases (Table 1) (Hussain et al., 2003). Accordingly, it is estimated that about 80% of cancers would be avoided if we change our lifestyle (McKinnell et al., 2006).

However, in contrast with the aforementioned correlative evidence, free radicals have been traditionally discredited as a “serious” cause of cancer, as they have been usually perceived more as a consequence than a cause of the transformed state. Conversely, free radicals have been known to damage DNA for at least 50 years (Phillips, 1956), were also shown 30 years ago to

Inflammatory condition	Cancer to which it predisposes	Fold-risk ratio
Haemochromatosis	Liver	219
Barrett´s oesophagus	Oesophageal	50-100
Viral hepatitis B	Liver	88
Viral hepatitis C	Liver	30
Urinary bladder catheterization	Bladder	5-28
Human papillomavirus	Cervix	16
Schistosoma hematobium	Bladder	2-14
Helicobacter pylori	Gastric	10
Ulcerative colitis	Colon	6
Schistosoma japonicum	Colon	1.2-6.0
Crohn´s disease	Colon	3
Pancreatitis	Pancreatic	2-3
Prostatitis	Prostate	2

Table 1. Chronic inflammation in humans highly predisposes to cancer
Various types of inflammatory conditions are shown (left column) that course through the production of high levels of free radicals. The middle column shows the types of cancer to which they predispose, together with the fold-increase risk (right column) compared to inflammation-free individuals.

Adapted from (Hussain et al., 2003)

accumulate to high levels in cancerous but not in normal cells (Swartz and Gutierrez, 1977), were proposed to play a causal role in cancer already in 1983 (Ames, 1983), and were then shown to transform cell cultures *in vitro* one year later (Zimmerman and Cerutti, 1984). Furthermore, a recent reborn interest in the free radical field is shedding new light on the causal role of these species in cancer. For instance, the NCBI publication record on "oxidative stress and cancer" for the period 2001-2006 more than doubles that one comprising the years 1950-2000. Thus, apart from the clear-cut example of inflammation, whose carcinogenic effects are mostly driven by the production of free radicals and certain inflammatory cytokines (Baniyash, 2006; Hussain et al., 2003; Karin, 2006; Philip et al., 2004), free radicals are currently emerging as potent carcinogens at all cancer stages, namely initiation, promotion and progression. For instance, by increasing DNA mutational rates to comparable levels as other well-known carcinogens (i.e. PAHs, aflatoxins) (Halliwell, 2002) free radicals are considered powerful cancer initiators (Toyokuni, 2006). Similarly, ROS have been shown to promote proliferation of various cell types *in vitro* (Irani et al., 1997; Polytarchou et al., 2005), which highlights their potential relevance in cancer promotion (i.e. clonal expansion). Furthermore, exposure of several cancer cell lines to inflammation- or chemically-induced ROS boosts their migratory and invasive behaviors (Okada et al., 2006; Payne et al., 2005; Polytarchou et al., 2005), indicating a likely role of free radicals in promoting cancer cells to the autonomous and invasive phenotype. Of note, *in vivo* experiments with animal models also support the causal role of free radicals in cancer. Namely, knock-out (KO) mice for distinct antioxidant enzymes that regulate ROS levels *in vivo* (i.e. superoxide dismutase –SOD-, glutathione peroxidase –GPx-) (Figure 5) not only have higher levels of ROS in their tissues, but also suffer from higher rates of spontaneous tumors (Halliwell, 2007). Similarly, mice deficient in Mth1, a key NER enzyme involved in the

repair of DNA oxidative lesions, also show higher rates of spontaneous lung, liver, and stomach tumours (Tsuzuki et al., 2001).

C.4.3) Free radicals in the clinics: a word of caution

According to the evidence provided above, it can be concluded that free radicals are potent DNA mutagenic species (Halliwell, 2002), which likely exert their carcinogen effects by providing transformed cells with greater plasticity for malignant progression through enhanced DNA-mutational rates (Toyokuni, 2006). In agreement with this, free radicals have been shown to predispose to the onset of cancer *in vivo* (Halliwell, 2007; Thomas-Ahner et al., 2007; Tsuzuki et al., 2001). Thus, should it be concluded that free radicals are to be always avoided in cancer both with preventive and therapeutic objectives? The straight answer seems to be "yes", as extracted from the mounting evidence in support of their numerous carcinogenic roles. For instance, mouse models in which tumorigenesis is known to be driven by oncogene- or UV light-induced free radicals (Calvisi et al., 2004; Factor et al., 2000; Wilgus et al., 2003) show reduced tumor burden when treated with antioxidants. Similarly, cultured cancer cell lines bearing high levels of intracellular ROS show attenuated malignant phenotypes after ectopic expression of antioxidant enzymes (Church et al., 1993; Fernandez-Pol et al., 1982). On a related issue, medical trials testing the potential of anti-inflammatory drugs as human cancer chemopreventive agents are now ongoing (Hussain et al., 2003).

A less studied effect of free radicals, specially within the context of cancer treatment, is their pro-apoptotic activity. For instance, it is known that cellular exposure to free radicals above a certain threshold irreversibly leads to cell death (Pelicano et al., 2004; Trachootham et al., 2006). Furthermore, several cancer chemotherapeutic agents (i.e. cisplatin, arsenic trioxide), as well as radiotherapy, are known to exert their cytotoxic effects through ROS-mediated mechanisms (Pelicano et al., 2004). Consequently, it has been recently proposed that artificially increasing the intracellular free radical burden in cancer patients may be useful for therapy (Pelicano et al., 2004; Trachootham et al., 2006), as well as that antioxidant uptake by cancer patients may interfere with chemotherapeutic treatments (D´Andrea, 2005).

Although this is a topic of intense debate (Block et al., 2007; Moss, 2006), the available evidence suggests that whereas free radicals scavenging might be beneficial as a cancer preventive measure, their overproduction might be conversely useful for cancer therapy, although it is still to be determined whether this might apply to all chemotherapeutic schedules or only to particular subsets.

C.5) Detoxification enzymes in cancer: cleaner is not always better

When entering first time into the biochemistry of free radicals and antioxidants, it is easy to bump into enzymes with intriguingly similar names. Such is the case for NADPH oxidase (Nox) and cytochrome P450 oxidase (CYP) (Figure 5), as well as for the glutathione-related enzymes glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST). Indeed, with names so similar, it is normal to think that these enzymes all belong to the same or similar protein families and are involved in the regulation of intracellular free radical levels (Figure 5). However, this is a misconception. Whereas Nox enzymes are committed to the production of superoxide radicals and modulate through them various cellular processes (Lambeth et al., 2000), CYP proteins are detoxification enzymes that only produce superoxide accidentally (Hines and McCarver, 2002). Similarly, GPx and GR are key antioxidant enzymes involved in the reduction of cellular superoxides (Figure 5), whereas GST is a metabolic enzyme that uses GSH to inactivate electrophiles (Figure 6) and that cannot directly modulate free radical levels. In this respect, the most relevant cellular antioxidant enzymes are shown in Figure 5.

C.5.1) Antioxidant *versus* detoxification enzymes

Living organisms are continuously exposed to exogenous substances that are not normally found within their cells, called xenobiotics (XBs) (meaning “alien to life”). These include substances such as hormones, PAHs, aromatic amines, dioxins, and halogenated aromatic hydrocarbons, which normally produce a toxic effect when not disposed of properly (i.e. cancer). Consequently, organisms have developed a sequential two-step detoxification system to efficiently get rid of such substances, which is composed of two classes of enzymes: class I and class II detoxification enzymes (Figure 6). Not surprisingly, xenobiotic detoxification enzymes are found in all living organisms from prokaryotes to humans (Nebert and Vasiliou, 2004), in agreement with their key role for properly adapting to the environment.

Class I detoxification enzymes execute the first step of xenobiotic metabolism and consist of three protein families, namely the cytochrome P450 oxidases (CYPs), the flavin-containing monooxygenases (FMOs), and the alcohol dehydrogenases (ADs) (Hines and McCarver, 2002); with CYPs being by far the most widely studied family due to their putative dominant role (Ding and Kaminsky, 2003; Hines and McCarver, 2002). Enzymes within this class have the major roles of both making xenobiotics more bioavailable (i.e. more soluble) and also transforming them in appropriate substrates for the following class II detoxification enzymes. Interestingly, both tasks are normally achieved through a single reaction type, namely by sequentially oxidizing the xenobiotic at appropriate motifs (i.e. double bonds, hydroxyl groups) (Figure 6,

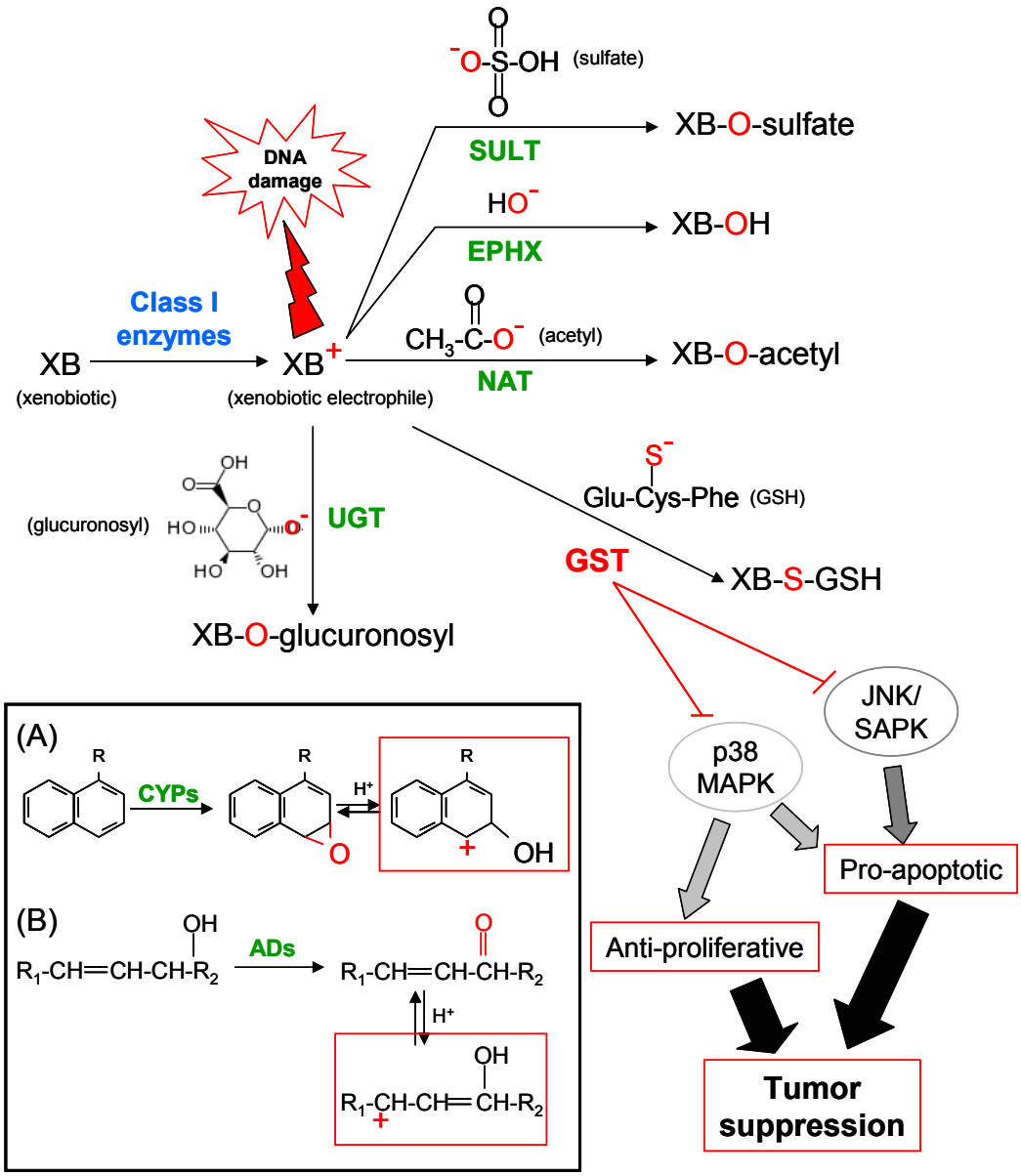

Figure 6. Xenobiotic metabolism by class I and class II detoxification enzymes
Top left. The first step in the metabolism of XBs consists in their chemical activation, which is normally achieved through sequential oxidative reactions performed by class I detoxification enzymes (see **Inset**). As a result, highly reactive electrophile intermediates are generated (XB^+) that will be subsequently processed by class II enzymes (see **Top right**). Importantly, as such reactive electrophiles are highly alkylating molecules, they can potentially induce widespread cellular damage if not rapidly inactivated by class II enzymes.
Top right. Several families of class II enzymes are shown in green, except for GSTs that are shown in red. They usually inactivate the XB electrophile by means of conjugating it with various negatively-charged hydrophilic molecules, whose reactive atoms are shown in red.
Bottom right. GST proteins have been recently shown to play cell signaling roles in addition to their detoxification function. Namely, Gstp1 has been shown to inhibit the JNK/SAPK pathway, whereas Gstm1 and Gstm2 can inhibit the p38 MAPK pathway. Of note, these GST signaling roles are considered oncogenic as they disrupt tumor-suppressor mechanisms.
Inset. The inset shows two examples of XB electrophiles (squared in red) generated from different substrates by phase I detoxification enzymes. **(A)** Single oxidation of a double bond within a general PAH structure by CYPs. **(B)** Example of oxidation of a lineal unsaturated hydrocarbon by alcohol dehydrogenase (AD) enzymes.

inset) (McKinnell et al., 2006). In turn, this normally results in enhanced xenobiotic solubility, as it increases the oxygen content of its structure (Figure 6, inset A), and also usually creates unstable electrophilic (positively charged) intermediates that serve as perfect substrates for the class II detoxification enzymes. Once activated, xenobiotics are conjugated by class II enzymes with various negatively-charged hydrophilic compounds (Figure 6) that do not only increase xenobiotic solubility further, but also facilitate xenobiotic excretion (Jedlitschky et al., 1994)). Of note, class II enzymes are composed of five major families: glutathione-S-transferases (GSTs), N-acetyltransferases (NATs), UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and epoxide hydrolases (EPHX), which differ in the type of molecule that they conjugate with the XB electrophile (Figure 6, top right) (McCarver and Hines, 2002). As they are specially significant for the present thesis work (Dolado et al., 2007), it is worth noting that human GSTs are composed of 16 cytosolic proteins distributed in 6 families (Nebert and Vasiliou, 2004), namely GST *alpha* (Gsta1-5), *mu* (Gstm1-5), *pi* (Gstp1), *omega* (Gsto1-2), *theta* (Gstt1-2) and *zeta* (Gstz1). Interestingly, five among them (Gsta1, Gstt1, Gstm1, Gstm2, and Gstp1) have been widely studied as prognostic or risk-modulating factors in human cancer (Beeghly et al., 2006; Dalhoff et al., 2005; Dolado et al., 2007; Henderson and Wolf, 2005; McIlwain et al., 2006; Parl, 2005) (see also C.5.2).

Concerning their tissue-expression pattern, both class I and II detoxification enzymes are highly expressed in tissues with an intense exposure to xenobiotics, such as the liver and the respiratory and gastrointestinal tracts (Ding and Kaminsky, 2003; Hines and McCarver, 2002; McCarver and Hines, 2002). Interestingly, the fact that they are mostly expressed in xenobiotic-enriched tissues may be accounted for by their substrate-dependent inducible expression. Furthermore, class I and II detoxification enzymes have been also recently found to be expressed in numerous extra-hepatic tissues in addition to those mentioned above (i.e. breast, ovary, prostate), where they are thought to influence cancer development by incompletely understood mechanisms (see C.5.2).

C.5.2) Detoxification enzymes and cancer: a matter of equilibrium

As mentioned above, the cooperative functions of class I and II detoxification enzymes are critical for the cellular disposal of compounds that frequently have carcinogenic effects (i.e. dioxins, PAHs). Accordingly, reduced activity of some of these enzymes has been found to predispose to some cancer types, which is believed to be accounted for by a general lack of xenobiotic detoxification (Dalhoff et al., 2005). For instance, the homozygous deletion of the GSTM1 gene has been shown to predispose to lung, bladder and colorectal carcinomas. Similarly, reduced Gstp1 activity has been correlated with higher prostate cancer risk, and

reduced activity of SULT and NAT enzymes has been correlated with enhanced risk of colorectal and bladder cancers, respectively. Of note, class II enzyme alterations as those just mentioned here would not be only expected to affect xenobiotic clearance (Dalhoff et al., 2005), but also to lead to enhanced accumulation of alkylating xenobiotic electrophiles due to insufficient conjugation (Figure 6), which may likely account in the first place for the carcinogenic effect of such loss-of-function alterations.

However, in contrast with the assumed anti-cancerous activity of these detoxification enzymes, it has been recently found as well that the overexpression of wild-type (WT) or gain-of-function alleles of some of these enzymes can both predispose to some cancer types and also interfere with cancer treatment. For instance, high CYP expression has been correlated with increased azoximethane-induced bladder cancer risk, which is probably due to the accumulation of higher levels of azoximethane electrophiles (McKinnell et al., 2006), and has also been proposed to predispose to breast cancer (Tsuchiya et al., 2005). Similarly, gain-of-function NAT alleles have been shown to predispose to bladder cancer (Dalhoff et al., 2005). However, the most notorious examples are the GST isoforms Gstm1 and Gstp1. Hence, whereas Gstm1 expression reduces the risk of cancer in tissues normally exposed to a high xenobiotic burden (i.e. lung, colon; see above), in which high expression of CYPs is also expected, Gstm1 has been conversely shown to predispose to breast cancer (Parl, 2005), as well as to be associated with ovary cancer progression and worse survival (Beeghly et al., 2006). Furthermore, Gstm1 expression in several cancer types precludes efficient therapy (McIlwain et al., 2006), and Gstm1-overexpressing human cancer cells are resistant to apoptosis (see Appendix A2). Similarly, Gstp1 has been shown to be a prevalent protein in many solid tumors and is normally found overexpressed in drug-resistant cancers (Townsend et al., 2005). Accordingly, GSTs are currently emerging as new promising targets in cancer therapy (Guengerich, 2005; Townsend et al., 2005; Turella et al., 2005), with two such drugs targeting GSTs being already in phase II clinical trials (McIlwain et al., 2006).

However, how can we reconcile that some of these detoxification enzymes (i.e. CYPs, Gstm1, Gstp1) apparently have simultaneous anti-cancerous and tumorigenic roles? Although there is not a straight answer to this question, some rational guessing can be done in the light of the available evidence (Figure 6). For instance, it has been recently shown that GSTs can play cell signaling roles independently of their detoxification activity. Namely, Gstp1 can inhibit the JNK/SAPK pathway by directly sequestering JNK (Adler et al., 1999), whereas Gstm1 and Gstm2 can inhibit the p38 pathway by interfering with the activation of the p38 upstream kinase Ask1 (Cho et al., 2001). Thus, whereas concomitant expression of class I and GST enzymes in

normal tissues would be expected to have an anti-cancerous role due to efficient xenobiotic disposal, the sole expression of GST proteins in some tissues might conversely predispose to cell transformation through inhibition of two pathways with known tumor-suppressive roles (Bulavin and Fornace, 2004; Kennedy et al., 2003). Similarly, the required equilibrated expression among class I and II detoxification enzymes may also explain the apparent oncogenic role displayed by some class I enzymes (i.e. CYPs in bladder cancer, see above). Whereas the concomitant expression of both class enzymes would normally result in low steady-state levels of reactive xenobiotic electrophiles, enhanced expression of class I enzymes without a parallel increase in phase II enzymes might result in the accumulation of such carcinogenic species and predispose to cancer.

Thus, situations far from the equilibrium between class I and class II detoxification enzymes may likely predispose to cancer, either due to the accumulation of highly reactive electrophilic species (class I levels >> class II) or to the inactivation of tumor suppressor pathways (class II levels >> class I).

D) Human cancer in the clinics: current status and future perspectives

Only a few months ago it was the 35th anniversary of the National Cancer Act, the opening salvo of the US government´s battle to eradicate cancer that was signed in the US Congress in 1971. Noteworthy, President Nixon himself declared at the signing of such document that cancer would be cured by 1976. Today, bearing a 30-year delay, the current US National Cancer Institute prediction has been expanded to 2015 (Leaf, 2004), perhaps too optimistically, again.

In these almost 40 years of cancer research little advance has been achieved in actually improving clinical cancer therapy (Leaf, 2004), although basic cancer research has generated an impressive amount of knowledge. For example, in a recent review of more than 200 ongoing clinical trials of new anti-cancer drugs (Nygren and Larsson, 2003), only 5% of them showed statistically significant therapeutic benefits, but even in these few cases the patient survival rate was just extended in a few months at best. Accordingly, it is no secret that the only cancer types that have significantly benefited from therapeutic improvements in these four decades are a small group of rare children tumors (i.e. Wilms´ tumor, Erwing´s sarcomas) and some low-incidence adult cancers (i.e. testicular cancer, Hodgkin´s disease, leukaemia, osteosarcoma) (Leaf, 2004; McVie, 1999). In contrast, the most common cancer types in adults, namely melanoma and those of the breast, lung, colon, prostate, and ovary, have only improved their famous “5-year survival rate” by virtue of better diagnostic tools, but not because of improved therapeutics (Leaf, 2004;

McVie, 1999). So, what has been done on behalf of the patients during these last 40 years and where is the problem?

Apparently, there are various problems that are usually ignored. On the one hand, little attention is usually given to the deadliest characteristic of cancer, namely the ability to metastasize (see B.6 for details). Indeed, it is estimated that less than 0.5% of cancer project grants sent to the US National Cancer Institute propose to focus primarily on the understanding of the metastatic process, whereas more than 92% of them do not even mention the word metastasis (Leaf, 2004). Accordingly, our knowledge of the metastatic process is scarce (Gupta and Massague, 2006) and no cancer drug is still designed to directly target the metastatic process. Not surprisingly, we do not know how to manage metastatic cancer in the clinics, which is nowadays virtually lethal in 100% of the cases (McKinnell et al., 2006). Consequently, much effort will have to be placed on the understanding of metastasis, if we pretend to target this deadly cancer trait clinically in the near future. In this respect, some advances have been recently accomplished in the understanding of the genetic basis of the metastatic process (Gupta et al., 2007).

Another side of the problem, although more difficult to solve, concerns the dominant role that mice models have achieved in cancer research. Indeed, although they have been (and actually are) an invaluable tool in basic cancer research (Van Dyke and Jacks, 2002), their applicability as models to predict human cancer response in the clinics has been recently questioned (Cespedes et al., 2006; Corpet and Pierre, 2005). Indeed, mice and humans differ in multiple aspects when it comes to cancer. The most obvious one is that they have a totally different pharmacokynetic/pharmacodynamic profile, which is a key determinant of the dose-response ratio as well as of the toxicity of any given drug. Furthermore, mice do not only require a lower number of genetic lesions than humans to develop cancer (Hamad et al., 2002), but also the same type of genetic lesions often leads to different cancer types in mouse and humans and to lower metastatic rates in mice (Cespedes et al., 2006). Accordingly, established mouse models for human cancer (i.e. colorectal cancer) rarely recapitulate all the genetic lesions observed in humans (Kinzler and Vogelstein, 1996). Thus, should it be proposed to stop using mice as a model in clinical cancer research? As this would be clearly deleterious for cancer research, part of the problem could be solved if we try to “humanize” cancer mouse models to make them recapitulate human disease with greatest similarity (Cespedes et al., 2006). Furthermore, more studies should be launched in order to establish pharmacokynetic/pharmacodynamic correlations between mice and humans.

Another source of criticism is the dual “gene-centric” and “tumor-centric” view of current cancer research (Folkman et al., 2000). It is sometimes assumed that cancer is a disease that has solely

to do with the tumor cells ("tumor-centric" view), meaning that targeting the tumor is the major goal. In contrast, there is evidence that such approach often results in enhanced cancer progression and worse prognosis (Camphausen et al., 2001; Leaf, 2004), which only reflects the systemic nature of cancer (see C.3). Similarly, it is widely believed that cancer cure will result from first identifying and then targeting all the genetic lesions present in cancer cells ("gene-centric" view). However, as previously discussed (see C.3), such approach might prove extremely difficult in practical terms, given the intrinsic instabilility of cancer cells. Consequently, some have argued that improved cancer therapies might result from targeting the genomically stable tumor matrix cells instead of the tumor cells themselves, on the assumption that as a systemic disease, cancer needs the system (organism) resources to progress (Folkman et al., 2000). Accordingly, new anti-angiogenic therapies are currently emerging that target the tumor-associated endothelial cells instead of the tumor itself (Gasparini, 2001; Kerbel and Folkman, 2002), with promising preclinical results. On a related issue, the increasing interest in the link between stem cells and cancer (see C.2) may as well lead to the development of new therapies, namely CSC-targeted approaches. These, in contrast to the current treatments that target the numerous (partially) differentiated cells within a tumor, would be conversely directed against the true sustainers of tumor growth (C.2).

Finally, a word on the obscure concept of cancer prevention. When mentioning "cancer prevention", it is usually assumed that we should immediately stop sunbathing, smoking, getting stressed, eating processed foods, etc. if we want to avoid cancer. Indeed, this could be a good preventive measure, as it is estimated that up to 80% of cancer cases are directly related to our lifestyle (McKinnell et al., 2006). However, it is totally unrealistic to rely on people changing their lifestyles in order to prevent cancer, despite it is increasingly recognized that cancer prevention, more than therapy, will probably be the key to make of cancer a chronic and asymptomatic disease in the future (Leaf, 2004; Pincock, 2005; Vogelstein and Kinzler, 2004). To solve this contradiction, a new branch of cancer research has been recently founded to try to prevent the clinical manifestation of cancer without disturbing people lifestyles, namely the field of cancer chemoprevention. Broadly, this new field pretends to detect cancer somewhere along the line of the 30 to 40 years that it normally takes to turn up clinically (Kinzler and Vogelstein, 1996) and stop or slow its progression with low-toxicity drugs. Although there have already been the first clinical trials on this regard (i.e. anti-inflammatory drugs for colon cancer) (Castellone et al., 2006; Morrow and Jordan, 2007; Ulrich et al., 2006) much needs to be done in developing less toxic drugs, identifying new targets, and, specially, in finding and validating *in vivo* prognostic clinical markers for such a "pre-cancerous" physiological state.

2. MITOGEN-ACTIVATED PROTEIN KINASES

E) The mitogen-activated protein kinase (MAPK) family. A brief overview

MAPKs consist of a highly-conserved family of proline-directed Ser/Thr protein kinases that play key signal transduction roles in eukaryotic cells. MAPKs are required for the coordination of the cell responses to virtually all types of extracellular stimuli (Cobb et al., 1994; Kyriakis and Avruch, 2001), which probably accounts for their high extent of conservation through eukaryotes. For instance, human MAPKs can normally compensate for the absence of their orthologs in yeast (Atienza et al., 2000; Galcheva-Gargova et al., 1994). Nevertheless, as the first MAPKs to be identified, the ERKs (Boulton et al., 1991) (see below), were discovered as mitogen-activated proteins, subsequent MAPK family members have been also traditionally referred to as mitogen-activated protein kinases (MAPKs). However, this is misleading in some cases, as some MAPKs are not activated by mitogens at all, and also does not properly reflects the wide range of stimuli to which MAPKs respond. Accordingly, at least eight different MAPK signaling cascades have been identified up to date in mammals (Bogoyevitch and Court, 2004), with each one showing some stimulus selectivity. These are the extracellular signal-regulated kinases (ERKs) ERK1 and ERK2; the Jun-N-terminal kinases (JNKs) JNK1, JNK2 and JNK3; the p38 MAP kinases p38α, p38β, p38γ, p38δ; the Big MAPK 1 (BMK1)/ERK5 cascade; and the less studied, atypical MAPKs ERK3, ERK4, ERK7, and ERK8 (Figure 7). Hence, whereas the ERK pathway has been long known to be preferentially activated by mitogens (Cobb et al., 1994), both the JNK and p38 MAPK cascades are mostly responsive to stress and inflammatory cytokines (Kyriakis and Avruch, 2001), which has more recently gained them both the more appropriate name of stress-activated protein kinases (SAPKs) (Kyriakis and Avruch, 2001). In contrast, little is known about the stimulus selectivity of the atypical ERK members ERK3 to ERK8 (Coulombe and Meloche, 2006), meanwhile the BMK1/ERK5 pathway is known to be activated by both mitogenic and stress stimuli (Coulombe and Meloche, 2006; Kyriakis and Avruch, 2001). In turn, once activated, MAPKs lead to the coordination of numerous cellular responses, which range from proliferation to apoptosis and inflammation, although it is suspected that the relative activity of different isoforms within each MAPK subfamily can modulate the cell response towards different outcomes. Accordingly, MAPK isoforms often show different functional capabilities and tissue expression patterns. For instance, JNK1 and JNK2 are expressed ubiquitously in mice and humans, whereas JNK3 expression is mostly restricted to brain, heart and testis (Derijard et al., 1994; Kyriakis et al., 1994). Furthermore, JNK2 has been proposed to have an oncogenic activity, while JNK1 and JNK3 are tumor suppressor candidates

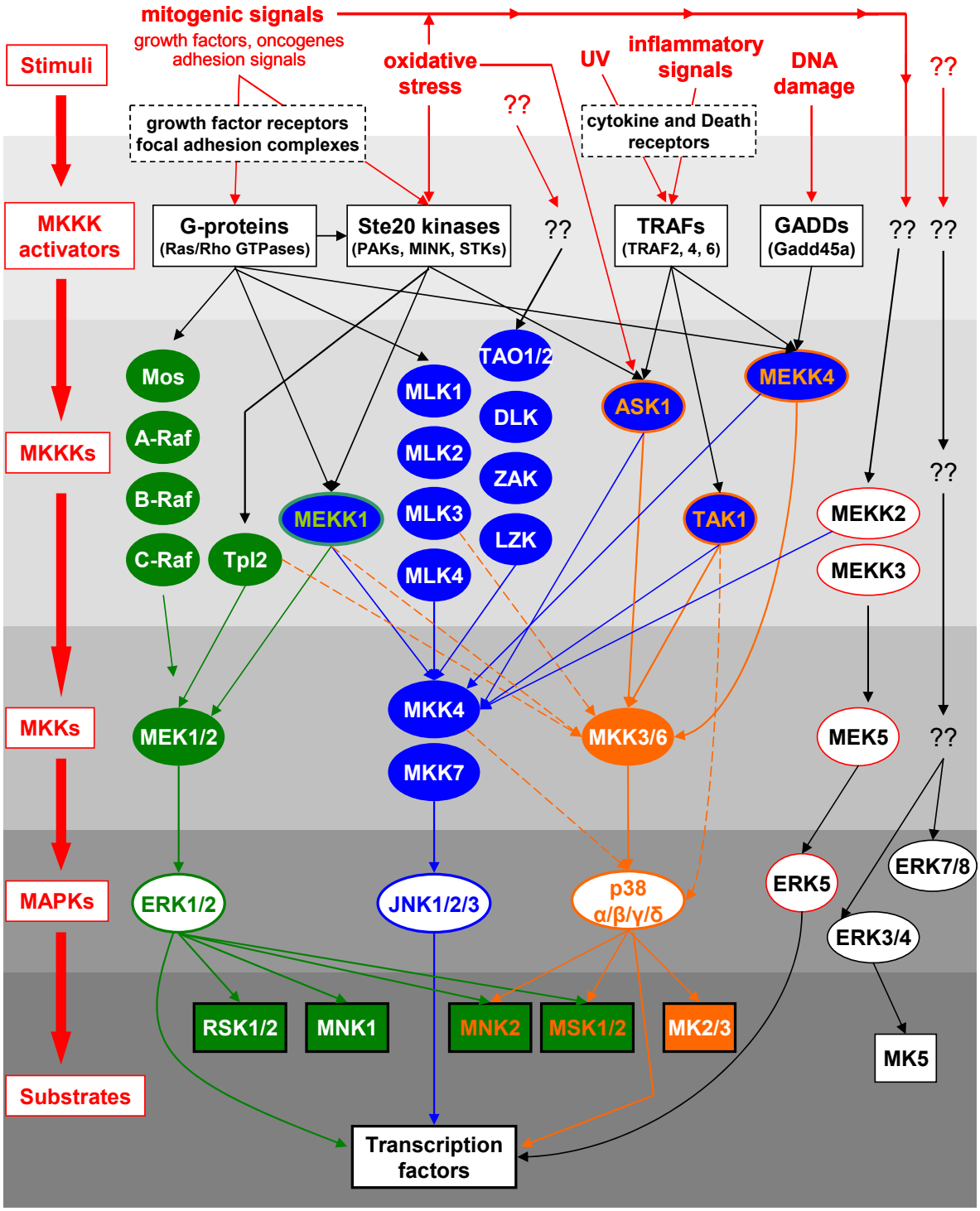

Figure 7. Molecular wiring of the MAPK pathway

The MAPK module. The MAPK signaling pathway can be divided into 2 subfamilies, the canonical and the atypical MAPKs. The canonical MAPKs are activated in a three-tiered fashion and are composed of the ERKs (ERK1/2) (green), the JNKs (blue), the p38 MAPKs (orange), and the ERK5/BMK1 pathway (red circle). The atypical MAPKs (black) do not have recognized MKKs, MKKKs or MKKK activators, and consist of ERK3, ERK4, ERK7 and ERK8.

The MKK module. The canonical MAPKs are activated by upstream MKKs, which are shown in different colours in accordance to the MAPK subfamily that they activate. MKK4 can also cooperate in the activation of p38 MAPKs (orange dashed line).

The MKKK module. MKKs are activated by numerous MKKKs, which are shown in green for the activators of the ERK pathway and in blue for those activating the JNKs. MKKKs shared by the ERKs and the JNKs combine blue and green, and those shared by JNKs and p38 MAPKs are depicted in blue and orange. Note that the p38 MAPK subfamily is the only one that does not have specific MKKKs. TAK1 has been shown to activate p38 MAPKs as well in a MKK-independent manner (Ge et al., 2002) (orange dashed line). MEKK1, Tpl2 and MLK3 are connected with p38 MAPKs through dashed lines as their *in vivo* relevance for p38 MAPK activation has to be confirmed yet.
MKKK activators are the last step between MKKKs and cellular receptors (examples are shown in dashed squares). Some MKKK activators are shown that activate specific downstream MKKKs with certain specificity. For instance, RAFs (A-, B-, C-) and MLKs (MLK1-4) are mostly activated by G-proteins, whereas TAK1 is preferentially activated by TRAFs. Conversely, MKKK activators upstream of TAO1/2, DLK, ZAK and LZK have not been properly characterized yet. It is mostly at the level of the crosstalk between MKKK activators and MKKKs where most extracellular cues are funnelled into the activation of specific MAPKs. Questions marks are placed where signaling components have not been identified. **The substrates**. MAPK downstream kinases are shown in the appropriate colour code.

(Rennefahrt et al., 2005). Similarly, p38α and p38β, but not p38γ and p38δ, are ubiquitously expressed proteins (Nebreda and Porras, 2000), although p38α is normally expressed at higher levels than p38β and is usually regarded as the dominant isoform within this MAPK subfamily (Nebreda and Porras, 2000; Perdiguero et al., 2007). Accordingly, p38α deficiency is embryonic lethal in mice (Adams et al., 2000; Allen et al., 2000; Mudgett et al., 2000; Tamura et al., 2000) and adult p38α conditional knock-out mice show impaired lung differentiation (Ventura et al., 2007). In contrast, mice defective in any of the other p38 MAPK isoforms do not show any significant alteration (Beardmore et al., 2005; Sabio et al., 2005). Furthermore, whereas p38α and p38β are usually thought as functionally overlapping kinases, p38α activity has been widely associated with apoptosis induction (Porras et al., 2004), whereas p38β has been shown to be anti-apoptotic in some cases (Kaiser et al., 2004; Nemoto et al., 1998; Silva et al., 2006).

Interestingly, whereas individual stimulus selectivities usually serve to classify MAPKs in different subfamilies (in combination with the extent of homology within their kinase domains), the highly conserved mechanism of activation represents an unifying characteristic of the MAPK family (Figure 7). Indeed, unlike other signal transduction pathways, MAPKs are activated in a characteristic multi-tiered module that provides them with a great degree of signal robustness and sensitivity (Klipp and Schaber, 2007) and that also serves to amplify extracellular stimuli (i.e. through switch-like all-or-none mechanisms) as well as to integrate many signals into defined cellular responses. MAPKs require dual Thr and Tyr phosphorylation on their activation loops for full activation (Thr-Xaa-Tyr motif), which is catalyzed by a family of upstream MAPK-specific kinases called MAPK kinases (MKKs or MAP2Ks). There are seven MKKs in mammals (Bogoyevitch and Court, 2004; Ichijo, 1999) (Figure 7), including MEK1 and MEK2

in the ERK pathway, MEK5 in the BMK1/ERK5 cascade, MKK4 and MKK7 in the JNK pathway, and MKK3, MKK6 and MKK4 in the p38 MAPK pathway. In turn, MKKs are known to be activated by upstream kinases (MKKKs or MAP3Ks) that also show some MKK specificity, although not as much as MKKs for MAPKs, as well as certain stimulus selectivity (see F.1 and Figure 7) (Ichijo, 1999; Stalheim and Johnson, 2007). Of note, although there are only seven MKKs, the number of MKKKs in mammals adds up to twenty members (Stalheim and Johnson, 2007), which has led some to suggest that it is mainly at the level of MKKKs where it is decided which MAPKs will be activated in response to specific stimuli (Figure 7) and, consequently, which type of cellular response will be triggered (Ichijo, 1999; Stalheim and Johnson, 2007). Noteworthy, MKKKs rarely act as sensors themselves but need to be coupled to cell receptors through upstream proteins in order to efficiently rely signaling cues (Figure 7). These proteins upstream of MKKKs are usually referred to as MKKKKs or MAP4Ks and include Sterile 20 (STE20) family kinases such as the MINKs (Nicke et al., 2005), the p21-activated kinases (PAKs) (Ichijo, 1999), and the STE20-like kinases (STKs) (Figure 7). Other proteins that are known to function upstream of MAP3Ks and are sometimes integrated with MAP4Ks are small G-proteins of the Ras and Rho GTPase families, scaffold proteins such those belonging to the tumor necrosis factor (TNF) receptor-associated factors (TRAFs) (Ichijo, 1999) and the growth arrest and DNA damage–induced (GADD) proteins (Takekawa and Saito, 1998).
In turn, MAPK activation leads to the phosphorylation of downstream targets. These include numerous transcription factors (Crump et al., 2007), although some MAPKs can also activate other kinases (see F.2 and Figure 7) (Kotlyarov and Gaestel, 2007; Ono and Han, 2000) and many other proteins (Ono and Han, 2000).
Of note, neither MKKs nor MKKKs have been identified so far for the MAPK cascades of ERK3, ERK4, ERK7, and ERK8 (Bogoyevitch and Court, 2004). This has led to the proposal that these MAPKs might not be activated in the canonical and well-conserved multi-tiered fashion described above.

F) Signal transduction by p38 MAPK

F.1) Upstream activators

As mentioned above, up to twenty MKKKs have been identified so far that activate the four different canonical MAPK subfamilies with certain selectivity (Figure 7). These can be divided into six groups (Stalheim and Johnson, 2007): (1) the MAPK and ERK kinase kinases (MEKKs) MEKK1-5; (2) the mixed lineage kinases (MLKs) MLK1-4, DLK, LZK and ZAK; (3) the thousand and one-amino acid kinases (TAOs) TAO1-2 and Tpl2; (4) the Raf kinases A-Raf, B-

Raf and C-Raf; and the single-membered groups of (5) Mos, and (6) transforming growth factor (TGF) β-activated kinase 1 (TAK1). Interestingly, some among these MKKKs have been shown to be preferentially activated by certain stimuli, hence coupling particular signaling cues with specific MAPK pathways (Figure 7). For instance, MEKK4 has been shown to couple the DNA-damage response with the activation of the p38 and JNK MAPK pathways through the GADD family member Gadd45a (Takekawa and Saito, 1998). Similarly, TAK1 has been shown to be preferentially activated by pro-inflammatory cytokines (Stalheim and Johnson, 2007), whereas MEKK5/Ask1 is a key transducer of oxidative stress signals (Matsukawa et al., 2004). On a related issue, both MEKK1 and MEKK4 have been shown to activate downstream MKKs in response to UV light (Rosette and Karin, 1996; Takekawa et al., 1997; Zhuang et al., 2006), meanwhile Raf kinases (Downward, 2003), as well as Tpl2 and MEKK5/Ask1 (Dolado et al., 2007; Nicke et al., 2005), are key targets of oncogenic signals. Noteworthy, whereas there are specific MKKKs for the ERK and JNK pathways (Mos and Raf kinases for ERKs and LZK and MLK1-4 for JNKs), no specific MKKKs have been found yet for the p38 MAPK pathway. Furthermore, among the twenty MKKKs, six can activate the ERK pathway and fourteen lay upstream of JNKs (Figure 7), meanwhile only three of them have been shown to be relevant for the activation of p38 MAPKs *in vivo*, namely TAK1, MEKK5/Ask1, and MEKK4 (Ichijo, 1999). Accordingly, although some other MKKKs have been shown to activate p38 MAPKs *in vitro* (i.e. TAOs, MLK3, MEKK1), mouse models lacking these proteins do not show significant alterations in the activation of the p38 MAPK pathway (Stalheim and Johnson, 2007).

On a related issue, mouse models deficient for p38 MKKs have confirmed that MKK3 and MKK6 are the dominant MKKs in the p38 pathway (Brancho et al., 2003), although MKK4 can also cooperate for p38 MAPK activation in some cases (Nebreda and Porras, 2000; Zarubin and Han, 2005).

F.2) Downstream targets

Activation of the p38 MAPK pathway leads to the phosphorylation and activation of a wide range of transcription factors (i.e. p53, CREB, Elk1, ATF2) and transcriptional repressors (i.e. HBP1), which are also shared with other MAPK subfamilies in some cases (i.e. ATF2 with JNKs, Elk1 with JNKs and ERKs). Indeed, p38 MAPKs can regulate in a phosphorylation-dependent manner the activity of more than twenty transcription-associated factors (see F.2.1 and Figure 8), in agreement with the fact that MAPKs drive a significant proportion of their effects through changes in gene expression (Crump et al., 2007). This effect is not exclusively driven by direct p38 phosphorylation, as p38 and other MAPK subfamilies can also activate downstream

Ser/Thr kinases that mediate some of their cellular effects (Hauge and Frodin, 2006; Kotlyarov and Gaestel, 2007). These include the p90 ribosomal S6 kinases (RSKs) RSK1 and RSK2, the MAPK-activated protein kinases (MAPKAPKs or MKs) MK2, MK3 and MK5, the MAPK-interacting kinases (MNKs) MNK1 and MNK2, and the mitogen- and stress-activated kinases (MSKs) MSK1 and MSK2. Interestingly, these subgroups of MAPK-downstream kinases show some differences among them as well as distinct MAPK specificities (Kotlyarov and Gaestel, 2007) (Figure 7). For instance, whereas MKs and MNKs have one catalytic domain, RSKs and MSKs have two, which implies complex regulation of their catalytic activities (Kotlyarov and Gaestel, 2007). Furthermore, whereas RSKs are activated only by ERKs, MKs are preferentially activated by p38 MAPKs. Conversely, MSKs and MNKs can be phosphorylated by both ERKs and p38 MAPKs (Kotlyarov and Gaestel, 2007), with the exception of MNK2, which is only activated by ERKs but not by p38 MAPKs *in vivo* (Parra et al., 2005; Scheper et al., 2003). Similarly, although MK5 was long thought to be exclusively activated by p38 MAPKs, it is now clear that MK5 represents a target of the atypical MAPKs ERK3 and ERK4 (Kotlyarov and Gaestel, 2007). Of note, the JNK pathway has no known downstream kinases up to date (Kotlyarov and Gaestel, 2007).

F.3) Regulation of gene expression by p38 MAPK

One of the cellular processes that p38 MAPK is more frequently associated with is the apoptotic response (Dolado et al., 2007; Porras et al., 2004; Wada and Penninger, 2004), which is known to be regulated by p38 through several gene expression-independent mechanisms (see G.1 and Figure 1 from Appendix 1). Other cellular processes, such as proliferation and cell migration (see G.1 and G.2), are also known to be regulated by p38 MAPK, at least in part, through post-translational mechanisms (Frey et al., 2006; Rousseau et al., 2006). However, despite the aforementioned examples, one of the most important roles of p38 when coordinating the cell response lays at the level of gene expression, namely by regulating both DNA transcription and mRNA translation. Various cellular processes as diverse as inflammation (Saklatvala, 2004), differentiation (Lluis et al., 2006), angiogenesis (Carter et al., 1999; Gauthier et al., 2005), and even apoptosis (Bulavin et al., 1999; Cuadrado et al., 2007; Porras et al., 2004), have been shown to be regulated by p38 MAPK through gene-expression dependent mechanisms.

F.3.1) Transcriptional regulation

The regulation of gene expression by p38 MAPK depends to a large extent on its effect on DNA transcription, which is coordinated through several mechanisms. Probably the one that has been most intensively studied consists in the phosphorylation-dependent regulation of transcription

factors (Figure 8), including both activators and repressors (Perdiguero and Munoz-Canoves, 2007). Transcription factors activated by p38 MAPK include p53, which induces the expression of proteins involved in cell cycle arrest (i.e. $p21^{Cip1}$, 14-3-3) and apoptosis (i.e. Apaf-1, Noxa) (Bensaad and Vousden, 2005); MEF2, which regulates the expression of muscle-specific genes required for muscle differentiation (Lluis et al., 2006); and NF-*k*B, which induces the expression of a wide range of pro-inflammatory and pro-survival proteins (Karin, 2006). Conversely, p38 can also repress gene transcription. For instance, p38 can phosphorylate and activate the transcriptional repressor HBP1, which in turn shuts down the expression of various genes, including cyclin D1 (Yee et al., 2004). In addition, p38 can phosphorylate certain transcription activators, such as NFATc4 (Yang et al., 2002), and induce their export from the nucleus, which also results in the downregulation of gene transcription. Of note, transcription factors can be either directly phosphorylated by p38 MAPK or indirectly through downstream kinases (Figure 8).

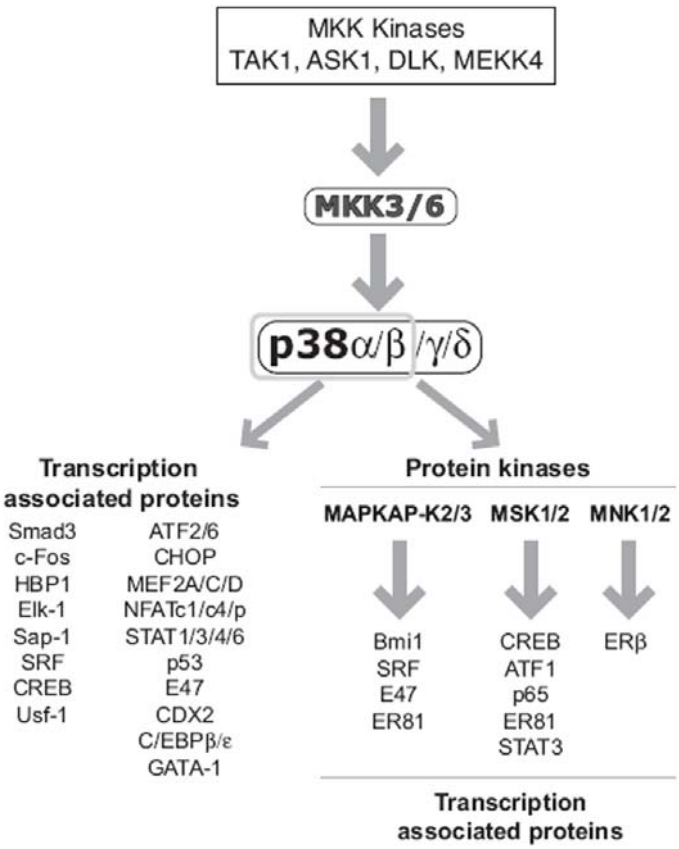

Figure 8. Regulation of gene expression by p38 MAPKs.
p38 MAPKs can regulate gene expression by modulating the activity of multiple transcription factors; either by direct phosphorylation or through their downstream kinases MKs, MSKs and MNKs. p38α and p38β have been more intensively studied in this context.

Adapted from (Perdiguero and Munoz-Canoves, 2007)

In addition to their key role as regulators of many transcription factors, p38 MAPKs can also play other fundamental roles in gene transcription (Crump et al., 2007) (Figure 9). For instance, p38 has been shown to phosphorylate and activate the TATA-binding protein (TBP) that, when activated, binds to the TATA element on gene promoters and nucleates the assembly of transcription complexes, thus inducing gene expression. Accordingly, p38 MAPK has been shown to prime the expression of NF-*k*B and AP-1 target promoters through this mechanism (Carter et al., 1999; Carter et al., 2001), hence cooperating in the transcriptional activity of these two important mammalian transcription factors. Furthermore, a recently discovered mechanism of transcriptional regulation by p38 MAPK, which has been shown to be conserved from yeast to humans (Alepuz et al., 2003; Perdiguero et al., 2007; Proft et al., 2006), consists in the recruitment of RNA polymerase II to target gene promoters. Interestingly, p38 is also currently emerging as a key regulator of gene transcription at another level, namely by inducing the remodelling of the chromatin structure in target genes (Crump et al., 2007). For instance, the direct, as well as MSK-mediated, p38-induced phosphorylation of certain transcription factors, such as Elk1 and CREB, has been shown to recruit certain histone acetyltransferases (HATs) (i.e. CBP/p300, P/CAF) to p38 target gene promoters (Crump et al., 2007). Of note, histone acetylation is known to lead to loosened histone-chromatin interactions and thus facilitate promoter accessibility to and subsequent transcriptional initiation by transcription factors (Narlikar et al., 2002). Besides recruiting HATs to the chromatin, p38 has also been shown to phosphorylate, in a MSK-dependent manner, the nucleosomal proteins histone H3 and high-mobility group (HMG)-14 (Clayton and Mahadevan, 2003), which in turn leads to a more accessible nucleosomal structure for HATs. Interestingly, HATs are not the only kind of chromatin-remodelling enzymes that p38 targets to the chromatin. A family of chromatin-remodelling complexes, of which the SWI/SNF complex is the best characterized, have been shown to be recruited by p38 to its target genes in a phosphorylation-dependent manner (Simone et al., 2004). These complexes, in contrast to HATs, are much bigger, depend on ATP for their activity, and do not loose histone-DNA contacts by indirectly modifying histones, but directly impair histone-DNA interactions through their helicase catalytical activity (Kadam and Emerson, 2003; Narlikar et al., 2002).

p38 MAPK has also been recently found to act as a transcriptional elongation factor in yeast (Pascual-Ahuir et al., 2006; Pokholok et al., 2006; Proft et al., 2006), although it remains to be determined whether this transcriptional role of p38 is conserved as well in higher eukaryotes.

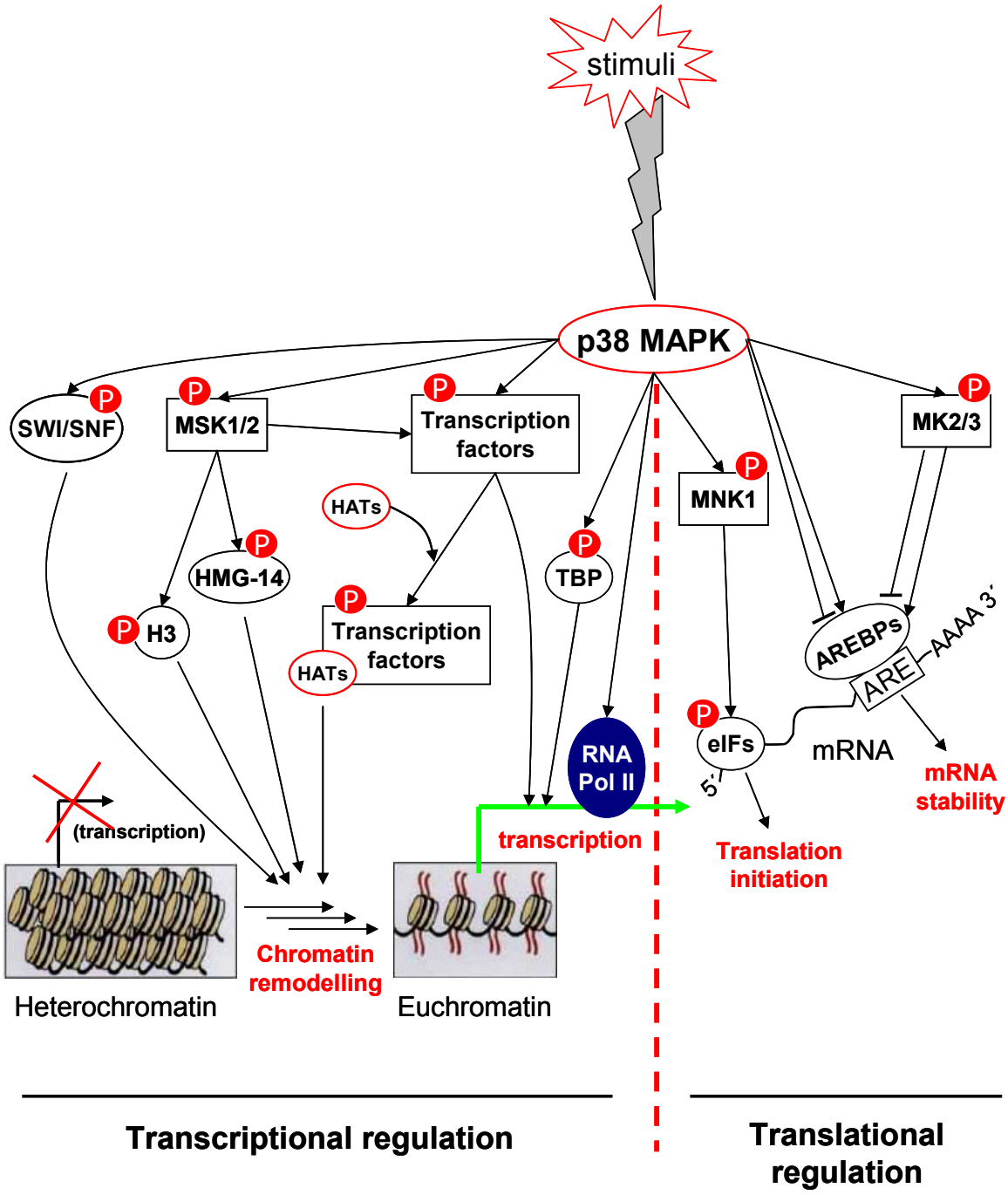

Figure 9. p38 MAPK can regulate gene expression by transcriptional and translational mechanisms
Left. p38 MAPK can regulate gene transcription by impinging on both **chromatin remodelling and transcription** initiation. Mechanisms involved in the regulation of chromatin remodelling include the phosphorylation-dependent (indicated with a white "P" inside of a red circle) recruitment of chromatin remodelling enzymes to p38-target promoters (i.e. SWI/SNF, HATs), as well as the MSK-mediated phosphorylation of the nucleosomal proteins histone H3 and HMG-14. In addition, p38 can also regulate transcription initiation by priming target promoters through recruitment of TBP and RNA polymerase II (RNA pol II), as well as by activating a wide range of transcription factors (see Figure 8).
Right. p38 can also regulate gene expression at the **translational** level by regulating translation initiation through the MSK-mediated phosphorylation of eukaryotic initiation factors (eIFs). Furthermore, p38 can modulate mRNA stability by regulating, either directly or through its downstream MKs, the binding as well as protein levels of certain AU-rich element (ARE)-binding proteins (AREBPs).

F.3.2) Translational regulation

As mentioned above, the interplay between p38 and downstream MSKs is important for the regulation of gene transcription (Figure 9). Conversely, MKs and MNKs have been reported as central coordinators of the regulation of mRNA translation by p38 MAPK (Kotlyarov and Gaestel, 2007). MKs and MNKs have been proposed to act at different levels within the mRNA translation machinery (Figure 9). Namely, whereas MNKs mainly act at the level of translation initiation (Kotlyarov and Gaestel, 2007), such as by phosphorylating and activating the translation eukaryotic initiator factor 4E (eIF4E) (Waskiewicz et al., 1999), MKs act in concert with p38 MAPK in the stabilization of mRNA transcripts (Kotlyarov and Gaestel, 2007). The latter is normally coordinated by modulating the binding to RNA as well as the expression levels of certain proteins, such as Tristetrapolin (TTP) and HuR, which in turn regulate mRNA stability in an AU-rich element (ARE)-dependent manner (Rodriguez-Gabriel and Russell, 2007). Noteworthy, whereas the activation of p38 MAPK is normally associated with the stabilization of mRNA transcripts (Saklatvala, 2004), it has also been shown that certain mRNAs can be destabilized by p38 or its downstream kinases (Ambrosino et al., 2003). Of note, whereas the regulation of gene transcription is important for the functions of p38 MAPK in cell differentiation (Lluis et al., 2006; Perdiguero and Munoz-Canoves, 2007) (see G.1), its involvement in translational regulation seems to be critical for its pro-inflammatory roles (Saklatvala, 2004) (see G.2).

G) p38 MAPK roles in cancer

p38 MAPK was originally identified more than ten years ago as a protein mediating the inflammatory and stress responses in eukaryotic cells (Freshney et al., 1994; Han et al., 1994; Lee et al., 1994; Rouse et al., 1994). Consequently, it has been intensively studied since then mostly as a potential therapeutic target in human inflammatory diseases (Kumar et al., 2003) as well as a key coordinator of the cell responses to all kind of extracellular stresses (i.e. radiation, oxidative stress, heat shock) (Kyriakis and Avruch, 2001). In addition, it has been recently recognized that p38 MAPKs, specially the p38α isoform, are also involved in the regulation of a wide range of cellular processes (Nebreda and Porras, 2000), including the cell response to numerous endogenous stress types such as those originated from death receptors (i.e. Fas, TNF receptor) (Porras et al., 2004; Wada and Penninger, 2004) and the mitochondria (Emerling et al., 2005) to the stress triggered by oncogenic signals. Consequently, the p38 MAPK field has experienced in the last five years an increasing interest in unveiling the functions of p38 in the context of oncogene-induced cellular transformation. Interestingly, whereas much has been

learnt on the functions of p38α in cancer (see G.1 and G.2), very little is known on the other p38 isoforms. Namely, p38α was found to counteract oncogenic signals by inducing cell cycle arrest (Chen et al., 2000) and senescence (Haq et al., 2002; Wang et al., 2002), which led to the proposal of p38α as a tumor-suppressor protein (Bulavin and Fornace, 2004). Accordingly, the negative regulation of proliferation by p38α was subsequently shown to mediate its inhibitory role on Ras-induced breast tumorigenesis *in vivo* (Bulavin et al., 2004). Furthermore, p38α has been found more recently to coordinate other tumor-suppressor mechanisms, ranging from the regulation of cell differentiation to the induction of apoptosis (see G.1 and Appendix A1), which has consolidated the early association between p38α and tumor suppression.

In contrast, there has also been a steadily growing body of evidence in support of p38α having a pro-oncogenic role in cancer (Rennefahrt et al., 2005) (see G.2 and Appendix A1), although this has generally attracted less attention than its tumor-suppressor roles.

Thus, the intense research carried out in recent years on p38α and cancer has provided a complex, counter-intuitive scenario where p38α can play either anti- or pro-tumorigenic roles (Figure 10) depending on the tumor type and cancer stage (Appendix A1).

G.1) Tumor-suppressive roles: proliferation, differentiation and survival

p38α was first recognized as a tumor suppressor based on the observation that it could inhibit the **proliferation** of oncogene-transformed cells *in vitro* (Chen et al., 2000). Consequently, the mechanisms by which p38α induces cell cycle arrest or cell cycle exit (i.e. senescence) in oncogene-transformed cells have been the subject of intense research (discussed in detail in Appendix A1). For the sake of clarity, it is worth mentioning that p38α has been shown to negatively regulate cell cycle progression at both the G1 and G2 phases of the cell cycle trough transcriptional as well as post-translational mechanisms (Bulavin and Fornace, 2004). For instance, p38α has been shown to induce G1 cell cycle arrest by negatively regulating the expression of cyclin D1 (Lavoie et al., 1996) as well as by up-regulating the CDK inhibitor $p16^{INK4a}$ (Bulavin et al., 2004; Wang et al., 2002). Similarly, p38α can also induce, in a phosphorylation-dependent manner, the degradation of D-type cyclins (Casanovas et al., 2004; Casanovas et al., 2000) and Cdc25A (Goloudina et al., 2003) as well as the stabilization of the CDK inhibitor $p21^{Cip1}$ (Kim et al., 2002) and the cyclin D1 transcriptional repressor HBP1 (Yee et al., 2004). Furthermore, the transcription factor p53 is also an important target of p38α in the regulation of G1 cell cycle arrest (Bulavin and Fornace, 2004), as the activation of p53 by p38α can result in the expression of the cell cycle inhibitor $p21^{Cip1}$. Noteworthy, the induction of G1 cell cycle arrest under conditions of high cellular density, a process called cell-cell contact

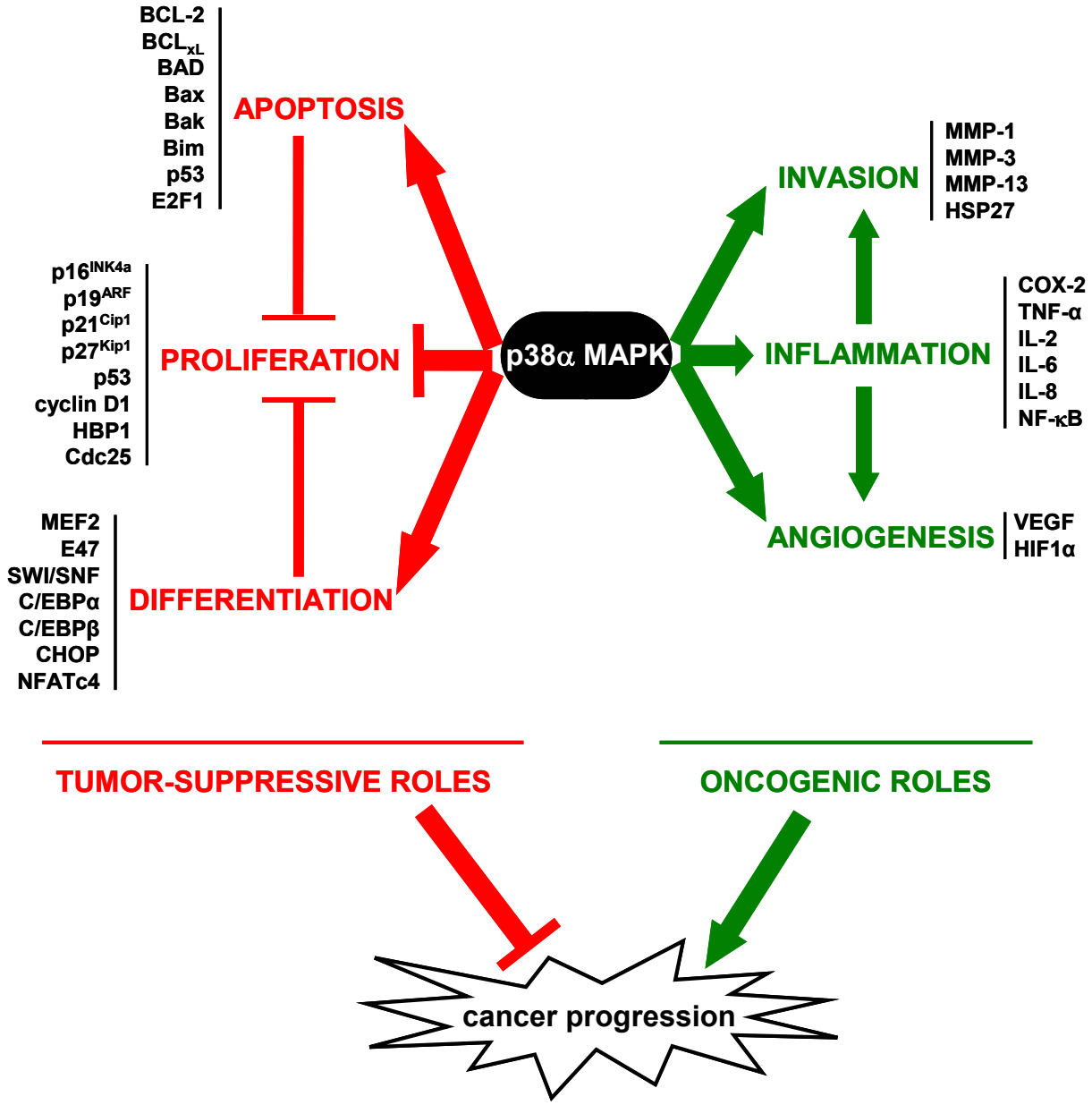

Figure 10. The two-sided effect of p38α MAPK in cancer
p38α modulates cellular transformation and cancer progression by impinging on virtually all cancer-associated traits (see 1.B), which might explain its sometimes opposite roles in tumorigenesis (for details see Appendix A1).
Right. The pro-oncogenic functions of p38α (see G.2) are depicted in green. These include its pro-inflammatory effect as well as its role inducing the expression of various pro-angiogenic (i.e. COX-2, VEGF, IL-8) and pro-metastatic factors (i.e. MMPs). Furthermore, p38α can also indirectly induce angiogenesis by activating the catalytical α-subunit of the transcription factor HIF1. p38α is also a key mediator of cancer cell migration through its downstream target hsp27.
Left. p38α can also coordinate various tumor-suppressor functions (red) through numerous downstream targets (black). These affect the induction of apoptosis, differentiation and cell cycle arrest. Interestingly, whereas p38α modulates apoptosis and proliferation through both transcriptional (i.e. p16^{INK4a}, cyclin D1) and post-translational mechanisms (i.e. BCL-2, Cdc25), the regulation of cell differentiation by p38α is mostly mediated by transcription factors (black).

inhibition (see B.3), is also regulated by p38α and probably involves the CDK inhibitor $p27^{Kip1}$ (Faust et al., 2005). On a related issue, p38α can induce a G2/M cell cycle arrest or delay through several mechanisms (Mikhailov et al., 2005), among which it is noteworthy to mention the one consisting in the MK2-mediated phosphorylation of Cdc25B and Cdc25C, which in turn results in the inhibition of Cdk2 (Manke et al., 2005; Rennefahrt et al., 2005). Of note, the anti-proliferative role of p38α has been recently shown to be of relevance as well *in vivo*, namely in determining the proliferative lifespan of hematopoietic SCs (Ito et al., 2006). It will be interesting to know whether this mechanism also applies to CSCs.

In addition to the multiple, direct anti-proliferative mechanisms described above, p38α is also known as a key regulator of the process of **differentiation**, which indirectly leads to cell cycle exit (Lluis et al., 2006; Perdiguero and Munoz-Canoves, 2007). Of note, whereas the regulation of cell proliferation by p38α involves both transcriptional and post-translational mechanisms (see above), p38α is thought to regulate cell differentiation predominantly by transcriptional means (Lluis et al., 2006; Simone et al., 2004) (Figure 10). As for its relevance in tumor suppression, it has been shown that forced activation of p38α in several human cancer cell lines triggers a more differentiated, less-transformed phenotype (Finn et al., 2004; Puri et al., 2000). Similarly, p38α has been shown to protect the lungs of oncogenic K-Ras-bearing mice from tumor burden *in vivo*, which is likely accounted for by the differentiation-inducing activity of p38α in lung epithelial stem and progenitor cells (Ventura et al., 2007). Accordingly, p38α has been shown to induce the *in vitro* differentiation of SCs from various origins (Aouadi et al., 2006; Forte et al., 2006; Schmelter et al., 2006), although whether p38α might also induce the differentiation of CSCs (see C.2) into less malignant cells needs further investigation.

The third tumor suppressor role of p38α consists in the induction of **apoptosis**, which has been shown to mediate its tumor-suppressor activity in many cases (Appendix A1 and A2). For instance, sustained activation of p38α has been shown to mediate apoptosis induction in numerous tumorigenic cell lines following various stimuli, including chemotherapeutic drugs (Olson and Hallahan, 2004), death receptor signals (Farley et al., 2006; Grethe and Porn-Ares, 2006; Hou et al., 2002), UV irradiation (Bulavin et al., 1999), oxidative stress (Dolado et al., 2007), and conditions that mimic the tumor environment such as serum withdrawal and substrate detachment (Fassetta et al., 2006; Porras et al., 2004). Accordingly, p38α is a well-known regulator of both the intrinsic (via cell death receptors) and extrinsic (via mitochondria) apoptotic pathways in eukaryotes, which normally modulates through both transcriptional as well as post-translational means (see Figure 1 from Appendix 1). These include phosphorylation-dependent mechanisms, such as the inactivation of the pro-survival proteins Bcl-2 and Bcl_{xL} (De Chiara et

al., 2006; Farley et al., 2006; Grethe et al., 2004) and the activation of the mitochondrial pro-apoptotic proteins BAD, Bim, Bax and Bak (Cai et al., 2006; Grethe et al., 2006; Grethe and Porn-Ares, 2006; Kim et al., 2006). Furthermore, p38α can also transcriptionally upregulate the pro-apoptotic proteins Fas, Bax, Apaf-1 and Noxa through the transcription factors p53, E2F1 and STAT3 (Bulavin et al., 1999; Cuadrado et al., 2007; Hou et al., 2002; Porras et al., 2004; Sanchez-Prieto et al., 2000).

G.2 Oncogenic activities: inflammation, invasion, angiogenesis and migration

In spite of the tumor-suppressive mechanisms described above, there is growing evidence indicating an oncogenic role for p38α in cancer (Appendix A1). For instance, increased levels of active p38α have been correlated with enhanced malignancy of follicular lymphoma (Elenitoba-Johnson et al., 2003), as well as of non-small-cell lung, thyroid and lymph node-positive breast carcinomas (Esteva et al., 2004; Greenberg et al., 2002; Pomerance et al., 2006). Furthermore, p38α has been shown to contribute to the invasiveness of breast, prostate and pancreatic cancers (Chen et al., 2004; Dreissigacker et al., 2006; Kim et al., 2003), as well as to the maintenance of the neoplastic phenotype in Ras-transformed human fibroblasts and epithelial cells (Weijzen et al., 2002). The use of chemical inhibitors has also shown the requirement of p38α (and perhaps p38β as well) for the proliferation of cancer cell lines, including chondrosarcoma, prostate carcinoma and melanoma cells (Halawani et al., 2004; Recio and Merlino, 2002; Ricote et al., 2006).

How is it possible that the originally considered tumor suppressor p38α (Bulavin and Fornace, 2004) coordinates all the aforementioned oncogenic functions? As discussed in Appendix A1, this is likely reflecting the involvement of p38α in the cellular processes of inflammation, migration and invasion (Figure 10). Indeed, since its dicovery in 1994, p38α has been intensively linked to the process of **inflammation** (Freshney et al., 1994; Lee et al., 1994), namely by inducing the production of a wide range of pro-inflammatory cytokines as well as by mediating the signal rely from inflammatory cytokine receptors. Accordingly, p38α has been long pursued as a drugable target in the treatment of chronic inflammatory diseases, with several clinical trials currently ongoing (Kumar et al., 2003; O'Neill, 2006). Consequently, given the previously discussed carcinogenic nature of chronic inflammation (see C.3 and C.4.2), it is not surprising that p38α can actually contribute to cancer progression in certain scenarios. For instance, p38α can induce the expression of the pro-inflammatory protein cyclooxygenase (COX)-2 (Carter et al., 1999; Gauthier et al., 2005), which has been correlated with bad prognosis in breast cancer (Gauthier et al., 2005; Gupta et al., 2007; Minn et al., 2005) as well as with the development of

drug resistance in bladder (T24) and cervix (HeLa) carcinoma cell lines. Indeed, COX-2 may promote cancer progression by enhancing both cell survival (Surh et al., 2001) and invasivity (Gupta et al., 2007; Timoshenko et al., 2006). In the same line, the pro-inflammatory cytokines TNF-α and IL-1, the pro-survival cytokines IL-2 and IL-6 and the angiogenic cytokine IL-8 have all been shown to be induced post-transcriptionally by p38α, sometimes indirectly through MK2 (Kumar et al., 2003; Saklatvala, 2004) (see F.2.2 and Figure 9). In addition, as mentioned before (see F.2.1), p38α can also prime the transcriptional activity of the pro-inflammatory transcription factor NF-*k*B (Carter et al., 1999). Thus, p38α can cooperate in cancer progression by inducing angiogenesis, survival, and invasion both via direct mRNA stabilization and indirectly by NF-*k*B-mediated transcription of pro-inflammatory proteins (Karin, 2006).

p38α can also have a more direct role in tumor **invasion** and **angiogenesis** than merely through its role in inflammation. For instance, p38α has been shown to induce the expression of various ECM-degradating metalloproteinases such as MMP-1, MMP-3, and MMP-13 (Ono and Han, 2000; Saklatvala, 2004), which are key proteins for matrix remodeling and degradation by metastatic cells (see B.6). Furthermore, the pro-angiogenic factor VEGF (see B.5) has been also shown to be expressed in a p38α-dependent manner in various cytokine-stimulated cellular systems (Wang et al., 2004; Yamamoto et al., 2001; Yoshino et al., 2006), which may be accounted for, at least in part, by the positive effect of p38α on the activation of the angiogenesis-inducing transcription factor HIF-1 (see B.5) (Emerling et al., 2005; Nakayama et al., 2007; Shemirani and Crowe, 2002). Of note, recent experiments using mouse xenografts and cultured cancer cells support a role for p38α in lung metastasis *in vivo* (Hiratsuka et al., 2006; Matsuo et al., 2006).

Furthermore, in addition to help creating the right conditions for metastasis to occur, such as by enhancing tumor angiogenesis (i.e. VEGF expression) and matrix degradation (i.e. MMPs expression), p38α may also regulate cancer cell **migration**. Accordingly, p38α has been shown to mediate the migration of IL-12-stimulated HeLa cells and Ras-transformed breast (MCF10A) and pancreatic (PANC-1) cell lines (Dreissigacker et al., 2006; Kim et al., 2003), as well as of other cell types stimulated by several chemotactic stimuli (McMullen et al., 2005; Rousseau et al., 2006). Interestingly, the role of p38α in cell migration seems to rely mostly on its ability to induce actin polymerization and cytoskeleton remodeling through its downstream kinase MK2, which phosphorylates the protein Hsp27 and induces its release from F-actin caps (Rousseau et al., 2006).

H) p38 MAPK as a putative target in cancer therapy?

p38α has been proposed to have a tumor suppressor role based on its ability to negatively regulate cell proliferation and to induce cell death (see G.1). Accordingly, a variety of anti-cancer drugs require a functional p38 MAPK pathway for efficient action (Olson and Hallahan, 2004). However, most of these studies have been limited to the investigation of p38α functions in the context of the self-autonomus cancer cell and during tumor initiation. On the other hand, the potential implication of p38α in the interplay between tumor cells and the extracellular matrix or the immune system are largely unknown, despite the key roles of these interactions for cancer progression (see B.6 and C.3). Consequently, we do not have much information on the roles of p38α in advanced cancer stages, as well as in the regulation of tumor environment-induced paracrine signaling. This might underlie the apparent controversy on the role of p38α in cancer (Appendix A1): whereas p38α may suppress tumor initiation through its involvement in cell cycle arrest as well as in the induction of differentiation and apoptosis, it seems to conversely serve oncogenic functions in cancer progression (i.e. metastasis) (Figure 10). This may explain why p38α has not been found mutated or down-regulated in human cancers to date, in contrast to other well-established tumor suppressor proteins (Johnstone et al., 2002).

Thus, given the complex network of tumorigenesis-related functions coordinated by p38α (see G), it is likely that both the type and stage of the tumor will have to be carefully taken into account in any rational attempt to modulate p38α activity for cancer therapy. For instance, whereas p38α activation by chemotherapeutic agents may prove beneficial for the first-line treatment of early-stage non-recurrent solid tumors (which are naturally sensitized to apoptosis), the same approach might have little effect or even result hazardous in apoptosis-resistant metastatic cancers, which might in turn capitalize on the oncogenic functions of p38α to further migrate and invade. Further work, including the use of specific mouse tumor models, should help to better define the roles of p38α in *in vivo* tumorigenesis and the potential value of this signaling pathway as a target for cancer therapy.

OUTLOOK

I have characterized several mechanisms by which p38α can regulate the process of malignant transformation, namely by inducing cell cycle arrest or apoptosis as well as by mediating cell migration.

According to these results, p38α emerges as an important negative regulator of cancer initiation due to its function as a free radical sensor, which induces apoptosis of tumorigenic cells in response to high levels of reactive oxygen species (ROS). It would be interesting to study whether apoptosis induction is the only mechanism coordinated by p38α to counteract ROS accumulation or if additional p38α-mediated mechanisms exist that impinge on ROS-producing pathways or cellular antioxidant enzymes. Furthermore, the ROS-mediated tumor suppressor role of p38α and its interplay with GST proteins should be evaluated *in vivo*. To this end, it would be informative to analyze whether levels of p38α activity and GST protein expression inversely correlate in human tumor samples. In addition, wild-type and p38α knock-out mice could be used to study whether p38α interferes with the carcinogenic effect of ROS-producing chemicals or ROS-inducing oncogenes, and if treatment with GST inhibitors cooperates with p38α at inhibiting tumorigenesis. However, several technical considerations will have to be addressed in order to perform these experiments. For example, reliable methods for monitoring ROS levels *in vivo* need to be optimized. Moreover, it has to be determined whether chemicals that act through ROS-mediated mechanisms are sufficiently tumorigenic in mice as single agents or if they require the synergistic effect of additional oncogenic lesions.

Conversely, it would be also interesting to study whether p38α may play pro-tumorigenic functions *in vivo* in certain cancer stages, for instance through its key roles in the inflammatory response or in cytokine-mediated cell migration. In this regard, wild-type and p38α knock-out mice could be treated with established carcinogenic protocols and the appearance of metastases in target organs could be later monitored and characterized. Subsequent use of organ-specific p38α knock-out mice might help to characterize whether p38α expression is more important in the cancer cell (“the seed”) or in the niche (“the soil”) for metastasis to occur. Importantly, targeting of p38α kinase activity in these experiments, either by the use of chemical inhibitors or through the generation of mice expressing kinase-dead p38α, would be informative with the aim of evaluating p38α as a potential target in cancer therapy.

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APPENDIX

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Regulation of tumorigenesis by p38α MAP kinase

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Abstract

p38α is a stress-activated protein kinase that can suppress tumor formation by negatively regulating cell cycle progression or by inducing apoptosis. More recently, the ability of p38α to induce cell differentiation has also been connected to tumor suppression. Accordingly, several proteins that can potentially down-regulate the activity of p38α have been found over-expressed in human tumors and cancer cell lines. However, p38α can impinge on cancer progression by modulating other cellular responses, in addition to proliferation and differentiation, such as cell migration as well as the processes of invasion and inflammation. This could explain why, in some cancer types, p38α activation has been correlated with malignancy and poor prognosis rather than with tumor suppression. Here, we will review the evidence connecting p38α to distinct cancer traits and will discuss the mechanisms that may account for the oncogenic and tumor suppressor roles of p38α.

Abbreviations: CDK, cyclin-dependent kinase; CREB, cAMP-responsive element binding protein; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; ERK, extracellular-signal regulated kinase; GCSF, granulocyte colony stimulating factor; HDAC, histone deacetylase; HIF, hypoxia-inducible factor; IL, interleukin; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; MDA-7, melanoma differentiation-associated 7; MEFs, mouse embryo fibroblasts; MK, MAPK-activated protein kinase; MMP, matrix metalloproteinase; MSK, mitogen- and stress-activated kinase; NF-*k*B, nuclear factor *k*B; PAK, p21-activated kinase; ROS, reactive oxygen species; TNF, tumor necrosis factor; UV, ultraviolet; VEGF, vascular endothelial growth factor.

1 Introduction

p38α mitogen-activated protein kinase (MAPK), also known as RK, CSBP and SAPK2a, was originally identified as a 38-kDa protein that mediated the inflammatory effect of several cytokines, as well as the target of anti-inflammatory drugs and an important regulator of the cellular responses to stress (Freshney et al. 1994; Han et al. 1994; Lee et al. 1994; Rouse et al. 1994). Three other p38 MAPK family members were subsequently identified (p38β, p38γ and p38δ), with p38β showing the highest similarity to p38α in terms of substrate specificity and sensitivity to inhibitors (Nebreda and Porras 2000; Ono and Han 2000).

In addition to the p38 MAPK family, three other major MAPK pathways have been characterized in mammals: the mitogenic extracellular-signal regulated kinases (ERK1 and ERK2), the stress-activated c-jun-N-terminal kinases (JNK1, JNK2 and JNK3) and the ERK5/BMK1 cascade. These four MAPK pathways are structurally related and share some regulatory mechanisms, but have been shown to play different roles in tumorigenesis. For instance, constitutive activation of the ERK1/ERK2 pathway is necessary and sufficient for cell transformation (Cowley et al. 1994; Mansour et al. 1994) and has been detected in many human tumors (Gollob et al. 2006). Consequently, inhibitors of the ERK1/ERK2 pathway have been developed for cancer therapeutics (Sebolt-Leopold and Herrera 2004). The role of the JNK pathway in cancer is less straight forward, as reviewed elsewhere (Engelberg 2004; Manning and Davis 2003; Rennefahrt et al. 2005). Essentially, JNKs can have both oncogenic effects, based on their ability to activate the proto-oncogene c-Jun, and tumor suppressor roles, due to their pro-apoptotic activity (Fan and Chambers 2001; Kennedy et al. 2003; Tront et al. 2006). It is therefore likely that the overall effect of JNKs in tumor development will depend on the balance between oncogenic and tumor-suppressive functions, which in turn may be affected by factors such as signal intensity and JNK isoform cross-talk. For example, apoptosis induction has been associated only with sustained JNK activation (Brozovic et al. 2004) (Ventura et al. 2006). Furthermore, whereas JNK2 has been proposed as an oncogene, JNK1 and JNK3 are tumor suppressor candidates (Rennefahrt et al. 2005).

Evidence accumulated over the last years support the function of p38α as a tumor suppressor (Bulavin and Fornace 2004), which seems to be mostly based on the ability of p38α to negative regulate cell cycle progression as well as to mediate apoptosis induction. For instance, mouse embryo fibroblasts (MEFs) deficient in either p38α (Bulavin et al. 2002) or the p38 MAPK activators MKK3 and MKK6 (Brancho et al. 2003) are able to produce higher oncogene-induced tumor burden in nude mice than their wild-type counterparts. In addition, genetic inactivation in mice of the PPM1D/Wip1 phosphatase, which can de-phosphorylate p38 MAPKs, results in reduced mammary tumorigenesis that correlates with increased p38 MAPK activity levels (Bulavin et al. 2004). p38α can also suppress tumor formation by inducing terminal differentiation (Puri et al. 2000). In agreement with these observations, several proteins that can potentially down-regulate p38α signaling have been found over-expressed in human tumors and cancer cell lines, including the phosphatases PPM1D/Wip1 and DUSP26/Mkp8 (Bulavin et al. 2002; Li et al. 2002; Yu et al. 2006) and the ASK1 inhibitors Gstm1 and Gstm2 (Dolado et al. 2007). Furthermore, some human tumors, such as hepatocellular carcinomas (Iyoda et al. 2003), have lower p38 MAPK activity levels than the corresponding non-tumorigenic tissues. While these results are all consistent with a tumor suppressor role of p38α, the picture is more complex if one takes into account the function of p38α in cell migration as well as in key processes for cancer progression such as invasion and inflammation. In this review we will discuss the evidence linking p38α to distinct cancer features.

2 Cell cycle regulation

Cell cycle deregulation is considered one of the hallmarks of cancer and is normally associated with uncontrolled cell proliferation and checkpoint failure. Indeed, more than half of human cancer cells are thought to have impaired G1 checkpoints (Kawabe 2004). Interestingly, p38α can negatively regulate cell cycle progression both at the G1/S and the G2/M transitions, and several mechanisms have been proposed to be involved in each case.

2.1 Inhibition of the G1/S transition

p38α can induce a G1/S delay in response to stress either at the transcriptional level, by down-regulating cyclin D1 (Lavoie et al. 1996) or by up-regulating the cyclin-dependent kinase (CDK) inhibitor $p16^{INK4a}$ (Bulavin et al. 2004; Wang et al. 2002), or by phosphorylation-mediated mechanisms that trigger the degradation of D-type cyclins (Casanovas et al. 2004; Casanovas et al. 2000) and Cdc25A (Goloudina et al. 2003) or the stabilization of the CDK inhibitor $p21^{Cip1}$ (Kim et al. 2002a) and the cyclin D transcriptional repressor HBP1 (Yee et al. 2004). In addition, p38α can modulate the expression levels and activity of the tumor suppressor p53 both at the transcriptional and post-translational levels (Bulavin and Fornace 2004), which also contributes to the p38α-mediated G1 cell cycle arrest through the induction of $p21^{Cip1}$. Of note, the up-regulation of $p21^{Cip1}$ expression mediated by p38α, but in a p53-independent manner, is also essential for $H\text{-}Ras^{G12V}$-induced cell cycle arrest (Nicke et al. 2005). Furthermore, the induction of G1 cell cycle arrest under conditions of high cellular density, a process called cell-cell contact inhibition, is also regulated by p38α and probably involves the CDK inhibitor $p27^{Kip1}$ (Faust et al. 2005). Noteworthy, the ability of p38α to regulate progression through the G1 phase of the cell cycle has been recently shown to be relevant for both myogenesis and hematopoiesis in vivo. Thus, in response to the accumulation of reactive oxygen species (ROS), p38α induces the up-regulation of the G1 cell cycle inhibitors $p16^{INK4a}$ and $p19^{ARF}$, which in turn limit the lifespan of hematopoietic stem cells (Ito et al. 2006). Conversely, p38α-deficient myoblasts are impaired in cell cycle exit and continuously proliferate in differentiation-promoting conditions, which may be accounted for by the enhanced activation of JNK/c-Jun in the absence of p38α (Perdiguero et al. 2007).

2.2 Inhibition of the G2/M transition

p38α has also been associated with G2/M cell cycle arrest or delay induced by a variety of stresses, including ultraviolet (UV) light, methylating agents, osmotic shock, and inhibitors of topoisomerase II or histone deacetylases (Mikhailov et al. 2005). At the mechanistic level, MAPK-activated protein kinase 2 (MAPKAP-K2, lately referred to as MK-2) has been proposed as a key mediator of the p38α-induced G2/M arrest (Manke et al. 2005; Reinhardt et al. 2007). MK-2 is activated by p38α phosphorylation and in turn can phosphorylate Cdc25B and Cdc25C, which induces their binding to 14-3-3 proteins. This prevents Cdc25 from activating the Cdc2/Cyclin B complex and inhibits mitosis entry. Interestingly, Cdc25B can be also directly phosphorylated by p38α, independently of MK-2 (Lemaire et al. 2006), but the contribution of this phosphorylation to the p38α-induced G2/M arrest needs further investigation. In addition, down-regulation of cyclins A and B expression may also contribute to the G2 cell cycle arrest induced by p38α in vitro (Garner et al. 2002) as well as in vivo (Engel et al. 2005). Moreover, recent studies in mouse thymocytes support an in vivo role for p38α in the p53-mediated G2/M arrest in response to DNA breaks (Pedraza-Alva et al. 2006).

2.3 Stimulation of cell cycle progression

As mentioned above, the ability of p38α to phosphorylate specific proteins is important for the induction of cell cycle arrest. Intriguingly, it has been recently proposed that p38α positively regulates mitotic progression in HeLa cells in a kinase-independent fashion (Fan et al. 2005). The relevance of this observation and the putative mechanism involved remain to be elucidated.

However, it should be noted that the *S. cerevisiae* p38 MAPK homologue Hog1 might regulate transcription by a mechanism that does not require its kinase activity (De Nadal et al. 2004; Proft et al. 2006).

It is conceivable that the function of p38α in cell cycle progression could depend on both the type and the malignant stage of the cell. Accordingly, p38α stimulates proliferation in transformed chondrosarcoma cells, but not in primary non-tumorigenic chondrocytes (Halawani et al. 2004; Yosimichi et al. 2001). On the other hand, p38α activation has no effect in the proliferation of hepatocarcinoma cells (Aguirre-Ghiso et al. 2004) or E1A/H-Ras^{G12V}-transformed MEFs, but inhibits cell cycle progression in primary MEFs (Bulavin et al. 2004).

An extensive review of the literature (Table 1) argues that p38α activation in fibroblasts, hematopoietic and epithelial cells, either by UV light, osmotic shock, oncogenic H-Ras or active MKK3 and MKK6, leads in all cases to cell cycle arrest or delay, which is in agreement with the above mentioned p38α anti-proliferative roles. Noteworthy, this effect appears to be independent of the transformed phenotype of the cells (Table 1). In contrast, p38α has a positive role in the proliferation of cytokine-stimulated hematopoietic cells and human breast cancer cells (Table 2).

Table 1. p38α as a mediator of cell cycle arrest

Cell type[a]	Cell line	Malignancy[b]	Inducer	Phenotype (mechanism)[c]	Reference
FI	BJ	-	Oncogenic H-Ras, Active MKK3 and MKK6	Irreversible cell cycle arrest (↑ $p16^{INK4a}$)	(Wang et al. 2002)
	MEFs	-	*Ppm1d*/Wip1 deletion	Irreversible cell cycle arrest (↑ $p16^{INK4a}$ and $p19^{ARF}$)	(Bulavin et al. 2004)
	CCL-39	-	Active MKK3	G1 delay (↓ cyclin D1)	(Lavoie et al. 1996)
	CCL-39	-	Active MEKK3	G1/S arrest (↑ $p21^{Cip1}$) G2/M arrest (↓ cyclins A and B1)	(Todd et al. 2004)
	CCL-39	-	Active MEKK3		(Garner et al. 2002)
	NIH 3T3	-	MKK3, MKK6, Cdc42	G1/S arrest	(Molnar et al. 1997)
	NIH 3T3	-	Sodium arsenite	Inhibition of proliferation (↑ $p21^{Cip1}$)	(Kim et al. 2002b)
	NIH 3T3	-	Active MKK6	Inhibition of proliferation	(Chen et al. 2000)
	DFs	-	UV irradiation	G2/M arrest (Cdc25B phosphorylation)	(Bulavin et al. 2001)
EN	Cardio-myocytes	-	Active MKK3	Inhibition of proliferation (↓ cyclins A2 and B)	(Engel et al. 2005)
	Myoblasts	-	Serum withdrawal	Cell cycle exit (↓JNK/c-Jun pathway)	(Perdiguero et al. 2007)
HE	T cells	-	Active MKK6	Mitotic arrest (↑ p53)	(Pedraza-Alva et al. 2006)
	Granta519	+	Osmotic shock	G1 delay (↓ cyclin D1)	(Casanovas et al. 2000)
	Daudi	+	Etoposide	G2/M arrest (Cdc25 phosphorylation)	(Kurosu et al. 2005)
EP	Ptk1	-	Topoisomerase II and HDACs inhibitors	G2/M arrest	(Mikhailov et al. 2004)
	mIMCD3	-	Osmotic shock	G2/M arrest	(Dmitrieva et al. 2002)
	HOSE	-	Oncogenic H-Ras	Inhibition of proliferation (↑ $p21^{Cip1}$)	(Nicke et al. 2005)
	RIE-1	+	Active MKK6	Inhibition of proliferation (↓ cyclin D1)	(Pruitt et al. 2002)
	T24	+	Sodium arsenite Active MKK6	Inhibition of proliferation	(Chen et al. 2000)
	U2OS	+	UV irradiation	G2/M arrest (Cdc25 phosphorylation)	(Manke et al. 2005)
	HeLa	+	UV irradiation	G2/M arrest (Cdc25B phosphorylation)	(Bulavin et al. 2001)
	HeLa	+	Osmotic shock	S phase arrest (Cdc25A degradation)	(Goloudina et al. 2003)
	U87MG	+	Temozolomide	G2/M arrest (Cdc25C phosphorylation)	(Hirose et al. 2003)

[a] FI, fibroblast; EN, endothelial; HE, hematopoietic; EP, epithelial
[b] -, non-transformed; +, transformed/tumorigenic
[c] ↑, up-regulation; ↓, down-regulation

Thus, p38α implication in both the production of inflammatory cytokines and the signal rely from inflammatory cytokine receptors (see Section 5.1) may determine its pro-oncogenic activity in cytokine-sensitive neoplasias, such as hematological malignancies (Platanias 2003), prostate cancer (Ricote et al. 2006), and melanoma (Recio and Merlino 2002). Perhaps, low levels of active p38α (i.e. after cytokine stimulation) can stimulate proliferation, in contrast to the strong, stress-induced p38α activation that mediates cell cycle arrest. On the other hand, the pro-tumorigenic effect of p38α in human breast cancer (Lee et al. 1999; Neve et al. 2002) might be accounted for by the participation of p38α-activated transcription factors, such as the cAMP-responsive element binding protein (CREB) (Sabbah et al. 1999), in the proliferative response triggered by the oestrogen receptor, which is usually overexpressed in breast cancer cells. Consequently, p38α has been found over-activated and associated with poor outcome in 20% of primary human breast carcinomas (Esteva et al. 2004) and has been suggested as an early marker for mammoplasty screening (Gauthier et al. 2005).

Table 2. p38α as an inducer of cell proliferation

Cell type[a]	Cell line	Malignancy[b]	Inducer	Mechanism[c]	Reference
HE	CT6	-	IL-2, IL-7	N.D.	(Crawley et al. 1997)
	BaF3	-	GCSF	N.D.	(Rausch and Marshall 1999)
	OCI-AML5	+	GCSF	N.D.	(Srinivasa and Doshi 2002)
	Mo7e	+	TNF-α	N.D.	(Liu et al. 2000)
	MM.1S	+	-	↑ paracrine IL-6 expression (by bone marrow stromal cells)	(Hideshima et al. 2003)
EP	22Rv1	+	Oncostatin-M (OSM)	N.D.	(Godoy-Tundidor et al. 2005)
	T47D	+	Heregulinβ1 (HRG)	↑ cyclins D1 and D2	(Neve et al. 2002)
	MCF-7	+	Estradiol Spermine	↑ cyclin D1	(Lewis et al. 2005)
	MCF-7	+	$pp60^{v\text{-}src}$	↑ cyclin D1	(Lee et al. 1999)

[a] HE, hematopoietic; EP, epithelial
[b] -, non-transformed; +, transformed/tumorigenic
[c] N.D., no determined; ↑, up-regulation

3 Regulation of cell survival and apoptosis

Alterations in cell survival programs are thought to play important roles in cancer. In fact, whereas the acquisition of invasiveness is regarded as the deadliest characteristic of cancer, the development of drug resistance may account for the failure of treatment, and ultimately death, of more than 90% of metastatic cancer patients (Dean et al. 2005). It is therefore not surprising that numerous proteins involved in the regulation of apoptosis have been found silenced or deregulated in animal cancer models and human tumors (Johnstone et al. 2002), hence providing a molecular basis for the common clinical occurrence of cancer drug resistance and recurrence. In this context, the modulation of p38α activity might be a strategy worth exploring for sensitizing cancer cells to apoptotic death, which might prove useful for cancer therapy.

3.1 Apoptosis induction

p38α mediates apoptosis induction in normal and tumorigenic cell lines following various stimuli (Table 3), including chemotherapeutic drugs (Olson and Hallahan 2004), death receptor signals (Farley et al. 2006; Grethe and Porn-Ares 2006; Hou et al. 2002; Porras et al. 2004), UV irradiation (Bulavin et al. 1999) and conditions that mimic the tumor environment such as serum withdrawal and substrate detachment (Fassetta et al. 2006; Porras et al. 2004). In some cases, apoptotic stimuli trigger p38α activation via secondary effects such as ROS production or the

induction of DNA damage (Figure 1). In particular, the ability of p38α to sense oncogene-induced ROS and to induce apoptosis is likely to play a key role in the suppression of tumor initiation (Dolado et al. 2007).

Table 3. p38α as a mediator of the apoptotic response

Cell type[a]	Cell line	Inducer	Mechanism[b]	Reference
FI	NIH3T3	Cisplatin	↑ p53	(Sanchez-Prieto et al. 2000)
	MEFs	Serum withdrawal	ERK inhibition, ↑ Fas and Bax	(Porras et al. 2004)
	MEFs	Serum withdrawal	Akt inhibition	(Zuluaga et al. 2007)
	MEFs	Serum withdrawal	Bcl-2 phosphorylation	(De Chiara et al. 2006)
EN	Cardiomyocytes	Serum withdrawal, UV irradiation, Anti-Fas antibody	ERK inhibition, ↑ Fas and Bax	(Porras et al. 2004)
	Cardiomyocytes	H_2O_2	ERK inhibition	(Liu and Hofmann 2004)
	EA.hy926	Doxorubicin TNF-α	↓ BCL_{XL} ↓BAD phosphorylation	(Grethe et al. 2004; Grethe et al. 2006)
	HDMECs	γ-irradiation	N.D.	(Kumar et al. 2004)
HE	Thymocytes	FasL	Cytosol translocation of Bcl-2 and BCL_{XL}	(Farley et al. 2006)
	Thymocytes	Active MKK6	↓ Bcl-2	(Merritt et al. 2000)
	S49.1	Glucocorticoids	N.D.	(Miller et al. 2005)
	CEM	Glucocorticoids	↑ Bim	(Lu et al. 2006)
	Jurkat	Cannabinoids (THC)	N.D.	(Herrera et al. 2005)
	Jurkat	Oxidative stress	N.D.	(Chen et al. 2006)
	ML-1	Vinblastine	N.D.	(Stadheim et al. 2001)
	U937	Paclitaxel	N.D.	(Yu et al. 2001)
EP	MCF7	UV irradiation	↑ p53	(Bulavin et al. 1999)
	HepG2	Methyl-cholanthrene	↑ p53	(Kwon et al. 2002)
		Staurosporine, H_2O_2	Bax phosphorylation	(Kim et al. 2006)
	PC12	Arsenite	Bim phosphorylation	(Cai et al. 2006)
	H1299, H460	γ-irradiation	Bak and Bax conformational activation	(Choi et al. 2006)
	HeLa	Taxol, Nocodazole	Bak and Bax conformational activation	(Deacon et al. 2003)
	A2780S	Cisplatin	Bak and Bax conformational activation	(Yuan et al. 2003)
	2008	Cisplatin	↑ FasL	(Mansouri et al. 2003)
	SW480	Cisplatin	↓EGFR pro-survival signaling	(Zwang and Yarden 2006)
	WM35, MeWo	mda-7/IL-24	↑ GADD proteins	(Sarkar et al. 2002)
	CGNs	Anti-Fas antibody	Rb inhibition	(Hou et al. 2002)
	CRC cells	Serum withdrawal, substrate detachment	N.D.	(Fassetta et al. 2006)
	HN4	Deferoxamine	N.D.	(Lee et al. 2006)
	Eca109, HaCaT, HeLa, TOV21G, OV-90, SK-OV-3	Cisplatin	N.D.	(Brozovic et al. 2004; Coltella et al. 2006; Losa et al. 2003; Zhang et al. 2005)
	MCF7, TOV21G, OV-90, SK-OV-3	Paclitaxel	N.D.	(Bacus et al. 2001; Coltella et al. 2006)

[a] FI, fibroblast; EN, endothelial; HE, hematopoietic; EP, epithelial
[b] N.D., not determined;↑, up-regulation; ↓, down-regulation

Several mechanisms have been proposed to underlie the induction of apoptosis by p38α (Figure 1), including the phosphorylation-dependent inactivation of the pro-survival proteins Bcl-2 and Bcl_{xL}, as well as the activation of the pro-apoptotic proteins BAD, Bim, Bax and Bak. BAD activation may be triggered by the PP2A-mediated inhibition of ERK1/ERK2 and Akt, three

kinases that can phosphorylate and directly inhibit BAD function (Table 3). In addition, de novo transcription of pro-apoptotic genes such as Fas, Bax and Apaf-1 by the transcription factors p53, E2F1 or STAT3, can mediate the regulation of both the extrinsic and intrinsic apoptotic pathways by p38α (Bulavin et al. 1999; Hou et al. 2002; Porras et al. 2004; Sanchez-Prieto et al. 2000). Furthermore, a recent report has found that p38α may sensitize cells to apoptosis by phosphorylating the epidermal growth factor receptor (EGFR) and inducing its internalization (Zwang and Yarden 2006). Studies in mice also support a pro-apoptotic role for p38α in vivo (Wada and Penninger 2004; Yang et al. 2006).

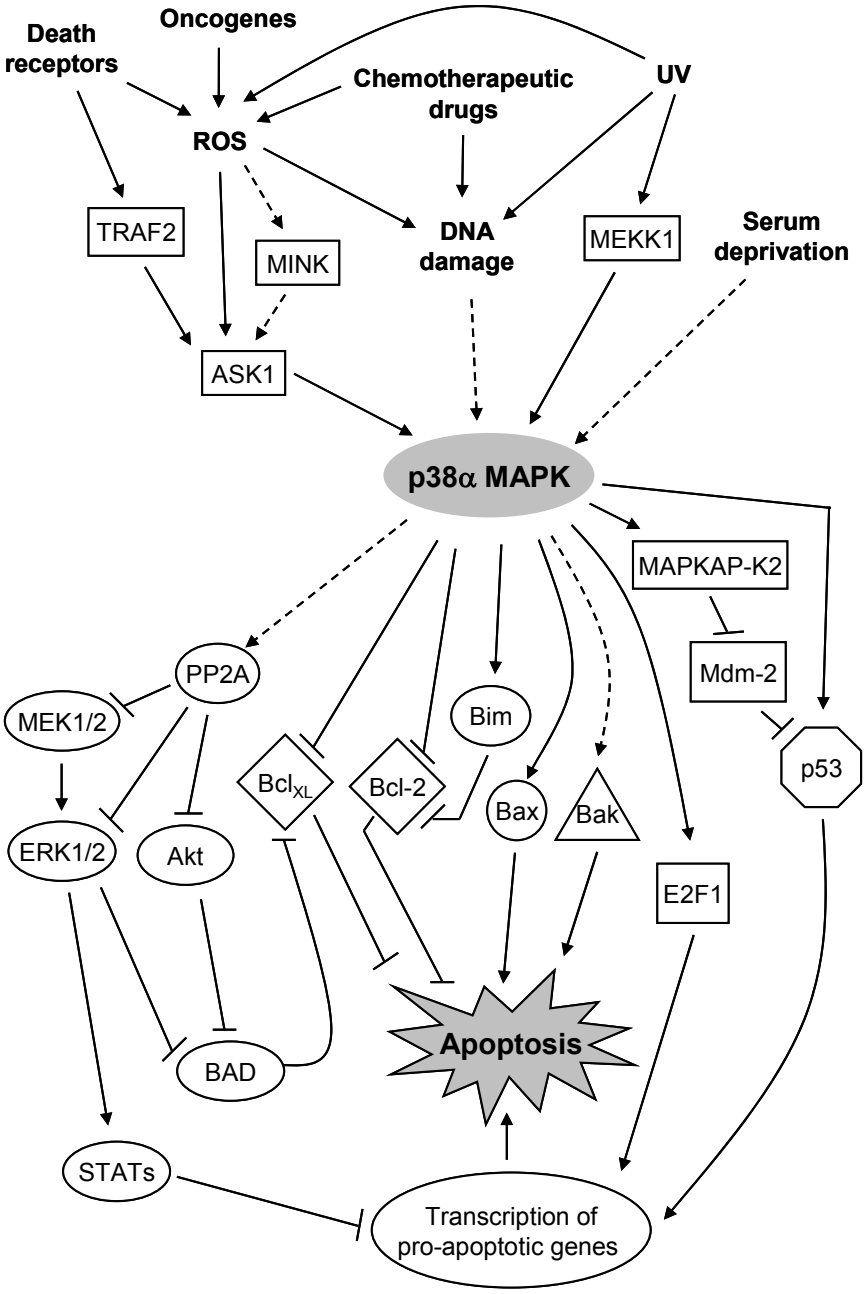

Figure 1. Pathways potentially involved in the pro-apoptotic effect of p38α
A variety of apoptotic stimuli can activate p38α and some of the upstream kinases involved in particular pathways have been identified, such as MEKK1 for UV irradiation (Zhuang et al. 2006) or MINK (Nicke et al. 2005) and ASK1 (Matsukawa et al. 2004) for oxidative stress. In turn, p38α activation can induce apoptosis by several mechanisms; see text for details. The dashed lines indicate mechanisms that are not well characterized.

Whether these different mechanisms operate simultaneously in p38α-induced apoptosis or their individual contribution is regulated in a context-dependent manner are still open questions. It is noteworthy, however, that whereas p38α has been shown to promote apoptosis in fibroblasts and endothelial cells through the p38α-induced and PP2A-dependent inactivation of the ERK1/ERK2 and Akt survival pathways (Grethe and Porn-Ares 2006; Porras et al. 2004; Zuluaga et al. 2007), this mechanism might not be operative in tumorigenic cell lines (Li et al. 2003). Indeed, substrate attachment has been shown to be necessary for the PP2A-mediated dephosphorylation of Akt in non-transformed cells (Zuluaga et al. 2007), suggesting that alterations in cell adhesion proteins usually found in cancer cells (i.e. down-regulation of E-cadherin or miss-expression of integrins) may contribute to impair, at least in part, p38α-mediated pro-apoptotic signaling in tumor cells.

In addition to the malignant state of the cell, the stimulus type may also determine how p38α induces apoptosis. For instance, the p38α-mediated inhibition of the ERK1/ERK2 pathway seems to be consistently used for membrane receptor-induced apoptosis (Grethe and Porn-Ares 2006; Porras et al. 2004), whereas p38α and p53 have been linked in several cases of DNA damage-induced apoptosis (Bulavin et al. 2001; Kwon et al. 2002; Sanchez-Prieto et al. 2000). As mentioned above, p38α can contribute to p53 stabilization and activation by several mechanisms, including direct phosphorylation by p38α (Bulavin and Fornace 2004) as well as MK-2 mediated inhibition of Mdm-2 (Weber et al. 2005). Furthermore, we have recently identified a new p38α substrate named ZnF-HIT1 or p18^{Hamlet} that contributes to DNA damage-induced apoptosis by stimulating the expression of p53-regulated pro-apoptotic genes (Cuadrado et al. 2007).

Table 4. p38α as an inhibitor of apoptosis

Cell line	Inducer	Mechanism[a]	Reference
Myocytes	TNF-α	↑ NF-κB/IL-6	(Craig et al. 2000)
Jurkat T cells	-	N.D.	(Nemoto et al. 1998)
Leukocytes	-	Caspases-3/8 inactivation by phosphorylation	(Alvarado-Kristensson et al. 2004)
CLL B cells	-	↑ MMP-9	(Ringshausen et al. 2004)
CLL B cells	mda-7/IL-24	↑ IL-2	(Sainz-Perez et al. 2006)
Thymocytes, Jurkat	Photodynamic therapy	N.D.	(Cappellini et al. 2005)
Macrophages	Lipopolysaccharide	↑ NF-κB-regulated genes	(Park et al. 2002b)
HeLa, T24	Photodynamic therapy	↑ COX-2	(Hendrickx et al. 2003)
Daudi	Etoposide	Induction of G2/M arrest	(Kurosu et al. 2005)
U1810 NSCLC	γ-irradiation	↑ Ku86, ↑ Ku-DNA-binding (increased DNA repair)	(Cosaceanu et al. 2006)
PC-12	TNF-α	Induction of differentiation	(Park et al. 2002a)
MCF-7	TNF-α	↑ NF-κB	(Weldon et al. 2004)

[a] N.D., not determined;↑, up-regulation

3.2 Anti-apoptotic roles

In contrast to the well-established pro-apoptotic effects of p38α, several studies have also described previously unexpected pro-survival roles (Table 4). These anti-apoptotic effects seem to be mainly related to the inflammatory response (Kumar et al. 2003; Saklatvala 2004) (see Section 5), as p38α mediates cell survival in several cases by inducing anti-apoptotic inflammatory signals, such as the cytokine interleukin (IL)-6 or the transcriptional regulator nuclear factor *k*B (NF-*k*B) (Table 4). However, independently of its role in inflammation, p38α

can also protect differentiated PC12 tumor cells from apoptosis induced by tumor necrosis factor (TNF)-α (Park et al. 2002a). Since differentiated, non-proliferative cells are usually refractory to apoptosis, it is plausible that p38α may sometimes mediate cell survival by inducing differentiation, for example in neuronal cells (Mao et al. 1999), and cell cycle arrest. Accordingly, p38α can induce a quiescent cancer state, known as cancer dormancy, which has been proposed to be important for the acquisition of drug resistance in cancer (Ranganathan et al. 2006).

p38α has also been involved in the establishment of the G2/M checkpoint in response to various cytotoxic agents (see Section 2.2). This cellular response is aimed at repairing damaged DNA and it is therefore necessary for normal cell homeostasis, but it may also potentially lead to apoptosis resistance in cancer cells by antagonizing chemotherapy-induced DNA damage (Kawabe 2004; Reinhardt et al. 2007). Indeed, one of the hallmarks of cancer cell resistance to apoptosis is enhanced DNA-repair activity (Kohno et al. 2005). The involvement of p38α in DNA-repair signaling suggests that after certain stimuli p38α might, instead of triggering apoptosis, induce cell cycle arrest and DNA repair, thus protecting cancer cells from apoptosis. In agreement with this idea, p38α activation has been associated with apoptosis-protective effects in hematopoietic cancer cells exposed to DNA-damaging agents such as etoposide (Kurosu et al. 2005) and photodynamic therapy-induced ROS (Cappellini et al. 2005). Furthermore, p38α mediates cell survival in U1810 non-small-cell lung cancer cells exposed to γ-irradiation by increasing the expression and activity of the DNA-repair proteins Ku86 and Ku70 (Cosaceanu et al. 2006).

In summary, the implication of p38α in DNA repair and cell differentiation (see Section 4) illustrates how tumor-suppressive mechanisms of normal cells can sometimes be switched to promote survival in cancer cells.

3.3 Reconciling pro- and anti-apoptotic functions

In the light of the above considerations, the bottom-line question is why the activation of p38α leads to apoptosis induction in some cases whereas it enhances survival in others. A popular explanation is that cell type-specific differences may account in most cases for the variability observed. However, other hypothesis may be proposed, such as the influence of the signal intensity/duration or the cross-talk with other signaling pathways.

The biological output of p38α activation might be determined by the intensity/duration of the signal. Hence, high activation of p38α following chemotherapeutic drugs would normally lead to apoptosis, whereas milder stimuli such as cytokines could result in the expression of anti-apoptotic proteins (i.e. IL-6) and the subsequent enhancement of cell survival. Indeed, cisplatin is known to require high and sustained activation of p38α for apoptosis induction, whereas apoptotic resistance correlates with transient activation of p38α (Brozovic et al. 2004; Losa et al. 2003; Mansouri et al. 2003). In agreement with this, whereas acute exposure to cisplatin leads to p38α-mediated apoptosis in a wide range of tumor cell lines (Table 3), chronic exposure of HeLa or MCF7 cells to increasing doses of cisplatin or TNF-α, respectively, leads to the development of p38α-dependent drug resistance (Brozovic et al. 2004; Weldon et al. 2004). These two antagonistic effects of p38α on cell survival could be explained by the differential regulation of p38α target recruitment depending on p38α activity levels, as previously proposed for other proteins such as p53 (Bensaad and Vousden 2005), MKK6 (Alonso et al. 2000) and ERK (Murphy and Blenis 2006). Thus, low levels of p38α activity may trigger the expression of pro-survival or differentiation-inducing proteins (Lluis et al. 2006; Saklatvala 2004), whereas higher p38α levels might additionally induce pro-apoptotic signaling that, due to its higher intensity or faster induction, could override the concomitant pro-survival signals. Accordingly, several p38α-mediated apoptotic mechanisms have been shown to function at the post-translational level (i.e. Bcl-2/Bcl_{xL} inactivation by phosphorylation), arguing in favor of a faster kinetic of induction

than, for example, the translational-dependent regulation of survival cytokines by p38α (Saklatvala 2004).

Another factor that may regulate the biological output of p38α activation could be the cross-talk with other signaling pathways. For instance, apart from inducing p38α activation, cisplatin activates the pro-survival PI3K/Akt pathway in breast cancer cells, which compensates for the p38α-mediated apoptotic signal (Winograd-Katz and Levitzki 2006). Akt has also been reported to inhibit the activation of p38α by MEKK3 and ASK1 in epithelial and endothelial cells (Gratton et al. 2001; Yuan et al. 2003), hence providing protection against the pro-apoptotic activity of p38α. Consequently, the interplay between the p38α and PI3K/Akt pathways may determine the extent of p38α-mediated apoptosis in response to chemotherapy treatments. In agreement with this, the chemotherapeutic agent taxol can activate pro-survival proteins such as NF-*k*B and p21-activated kinase (PAK) in parallel to p38α, which also results in a reduced apoptotic effect (Olson and Hallahan 2004). The JNK pathway, which is activated by most p38α-activating stimuli due to the sharing of several upstream regulators by both MAPK pathways (Ichijo 1999), can also sometimes have opposite effects to p38α on apoptosis-inducing proteins, such as BAD (Tourian et al. 2004). In conclusion, the activation of p38α may not necessarily always lead to apoptosis induction, due to the opposing effects of pro-survival pathways that are concomitantly activated by the same stimuli.

Finally, the relative activity levels of different p38 MAPK family members may also explain the controversial effect of p38α activation on cell survival. In particular, p38β has been proposed to have anti-apoptotic effects in various cell lines (Kaiser et al. 2004; Nemoto et al. 1998; Silva et al. 2006).

4 Regulation of cell differentiation

The first link between cancer and deregulated cell differentiation was the identification of embryonic biochemical markers in hepatomas, which led to the suggestion that lack of differentiation might contribute to tumor formation (Potter 1978). Subsequently, numerous reports have confirmed the link between cancer and cellular de-differentiation. For instance, the ability of several oncogenes to block cell differentiation has been proposed to mediate their transforming activity, for example Notch in breast, pancreatic and lymphoid cancers (Sjolund et al. 2005) and Bcr-Abl in lymphoblastic leukaemia (Klein et al. 2006). Furthermore, the early observation that most cells within a tumor are differentiated and weakly tumorigenic, has lead to the formulation of a recent theory that postulates that a few undifferentiated cancer stem cells within a tumor (i.e. < 1%) are the true initiators and sustainers of cancer (Houghton et al. 2006). This has been supported by the isolation of cancer cells with stem cell-like properties from several human tumors and cancer cell lines (Dean et al. 2005). Consequently, treatments that specifically induce stem cell differentiation might represent an attractive approach for cancer therapy (Edsjo et al. 2006).

Along these lines, p38α is emerging as an important regulator of differentiation in several cell types, including adipocytes, neurons, myocytes and hematopoietic cells (Lluis et al. 2006; Nebreda and Porras 2000; Uddin et al. 2004). Indeed, p38α can orchestrate the cellular differentiation process by multiple mechanisms, such as by activating differentiation-inducing transcription factors (i.e. MyoD and MEF2), by promoting cell cycle exit prior to the onset of differentiation (Perdiguero et al. 2007) or by targeting chromatin-remodeling enzymes to specific loci, thereby inducing the transcription of differentiation-specific genes (for further details see the chapters by Perdiguero and Muñoz-Cánoves and by Crump et al.). In vivo studies with mice have also recently shown the importance of p38α for myoblast differentiation (Perdiguero et al. 2007) as well as for maintenance of the differentiated state in adult cardiomyocytes (Engel et al. 2005), although in both cases the primary effect of p38α seems to be at the level of the cell cycle arrest required for differentiation.

Interestingly, forced activation of p38α in human cancer cell lines such as muscle rhabdomyosarcoma (Puri et al. 2000) and renal carcinoma A-498 cells (Finn et al. 2004) triggers a more differentiated and less transformed phenotype. We have also recently found that p38α-deficient mice are highly sensitized to K-Ras^{G12V}-induced lung tumorigenesis, which is mostly due to the inability of lung epithelial cells to undergo accurate differentiation in the absence of p38α (Ventura *et al*., submitted). These results provide in vivo evidence for the relevance of the differentiation-inducing activity of p38α for tumor suppression. p38α has also been shown to induce the in vitro differentiation of embryonic stem cells into cardiomyocytes (Aouadi et al. 2006; Schmelter et al. 2006), as well as to mediate a proliferation arrest, which was concomitant with the onset of differentiation, in hepatocyte growth factor-treated mesenchymal stem cells (Forte et al. 2006). Whether p38α might also induce the differentiation of cancer stem cells needs further investigation.

5 Inflammation

The roles of p38α in the production of pro-inflammatory cytokines and in the signal rely from cytokine receptors have been intensively studied. This work has led to the development of several p38α inhibitors currently undergoing clinical trials for inflammatory diseases (Kumar et al. 2003; O'Neill 2006; Saklatvala 2004). However, there is some concern that inhibition of p38α to ameliorate chronic inflammation might result in a higher predisposition to cancer, given the evidence in support of p38α as a tumor suppressor (Bulavin and Fornace 2004; Dolado et al. 2007). On the other hand, chronic inflammation is also a potent cancer promoter (Baniyash 2006; Karin 2006; Philip et al. 2004), which has been linked to enhanced cancer cell survival as well as to the induction of DNA damage, angiogenesis and invasion. Thus, the pro-inflammatory role of p38α may not only contribute to cancer progression but also compromise cancer treatment by increasing the sensitivity of normal tissues to chemotherapeutic drugs (Li et al. 2006; Ramesh and Reeves 2005).

p38α can induce the expression of the pro-inflammatory protein cyclooxygenase (COX)-2 (Park et al. 2002b), which has been correlated with bad prognosis in breast cancer as well as with the development of drug resistance in bladder (T24) and cervix (HeLa) carcinoma cell lines. Indeed, COX-2 may promote cancer progression by enhancing both cell survival (Surh et al. 2001) and invasivity (Timoshenko et al. 2006). Furthermore, the pro-inflammatory cytokines TNF-α and IL-1, the pro-survival cytokines IL-2 and IL-6 and the angiogenic cytokine IL-8 have all been shown to be induced post-transcriptionally by p38α, some times indirectly through p38α-regulated kinases such as MK-2 and mitogen- and stress-activated kinase (MSK)-1 (Arthur and Darragh 2006; Kumar et al. 2003; Saklatvala 2004). In addition, p38α may induce the transcription of some of these cytokines by enhancing the activity of transcription factors such as NF-*k*B (Carter et al. 1999; Karin 2006). Thus, p38α can cooperate in cancer progression by inducing angiogenesis and invasion both via direct mRNA stabilization and indirectly by NF-*k*B-mediated transcription of pro-inflammatory proteins.

6 Cell migration and invasion

p38α may also have a direct role in tumor invasion and angiogenesis, independently of its role in inflammation. For instance, p38α has been shown to induce the expression of metalloproteinases (MMPs) such as MMP-1, MMP-3, and MMP-13 (Ono and Han 2000; Saklatvala 2004), which are key proteins for matrix remodeling and degradation by metastatic cells (Coussens et al. 2002). Vascular endothelial growth factor (VEGF), a potent inducer of tumor survival and angiogenesis (Carmeliet 2005), has been also shown to be expressed in a p38α-dependent manner in various cytokine-stimulated cellular systems (Wang et al. 2004a; Yamamoto et al. 2001; Yoshino et al. 2006). In addition, p38α may cooperate as well in the overexpression of

VEGF and other angiogenic factors in hypoxic tumors in a process known as the "angiogenic switch", which correlates with enhanced cancer aggressiveness and bad prognosis (Hanahan and Folkman 1996; Zhou et al. 2006). Indeed, p38α can activate hypoxia-inducible factor (HIF)-1, a transcription factor that plays a key role in the hypoxia-driven expression of angiogenic factors, at least in part through the stabilization of its α-subunit (Emerling et al. 2005; Nakayama et al. 2007; Shemirani and Crowe 2002). In contrast, p38α has been attributed a metastasis suppressor role in human ovarian cancer (Hickson et al. 2006), although this has not been confirmed to date in other tumor types, including prostate, breast and pancreatic cancers (Vander Griend et al. 2005; Wang et al. 2004b). Furthermore, recent experiments using mouse xenografts and cultured cancer cells support a role for p38α in lung metastasis (Hiratsuka et al. 2006; Matsuo et al. 2006).

In addition to help creating the right conditions for metastasis, by enhancing tumor angiogenesis (i.e. VEGF expression) and matrix degradation (i.e. MMPs expression), p38α may also regulate cancer cell migration. Thus, p38α mediates the migration of IL-12-stimulated HeLa cells and Ras-transformed breast (MCF10A) and pancreatic (PANC-1) cell lines (Dreissigacker et al. 2006; Kim et al. 2003), as well as of other cell types stimulated by several chemotactic stimuli (McMullen et al. 2005; Rousseau et al. 2006). At the mechanistic level, the role of p38α in cell migration seems to rely mostly on its ability to induce actin polymerization and cytoskeleton remodeling through its downstream kinase MK-2, which phosphorylates the protein Hsp27 and induces its release from F-actin caps (Rousseau et al. 2006). Additionally, MK-2 may also activate the protein kinase LIMK1, which in turn phosphorylates and inactivates the actin-depolymerazing protein cofilin (Kobayashi et al. 2006). A recent report has also implicated p38α in epithelial cell migration, although via a more indirect mechanism. Namely, p38α can induce the phosphorylation of ligand-bound EGFR at tyrosine-1045, which triggers EGFR degradation. This p38α effect prevents recycling of the active EGFR receptor from the early endosomes back to the plasma membrane and seems to be associated with a proliferation-to-migration shift in the cellular response (Frey et al. 2006).

7 Concluding remarks

p38α has been proposed to have a tumor suppressor role based on its ability to negatively regulate cell proliferation and to induce cell death (Bulavin and Fornace 2004). Accordingly, a variety of anti-cancer drugs require a functional p38α pathway for efficient action (Olson and Hallahan 2004). However, most of these studies have been limited to the investigation of p38α functions in the context of the cancer cell and during tumor initiation. On the other hand, the potential implication of p38α in the interplay between tumor cells and the extracellular matrix or the immune system are largely unknown, despite the key roles of these interactions for cancer progression (Baniyash 2006; Comoglio and Trusolino 2005).

Consequently, we do not have much information on the roles of p38α in advanced cancer stages, as well as in the regulation of tumor environment-induced paracrine signaling. This might underlie the apparent controversy on the role of p38α in cancer. Thus, in spite of the many tumor-suppressive functions of p38α (see Sections 2.1, 2.2, 3.1 and 4), increased levels of phosphorylated p38α have been correlated with malignancy in follicular lymphoma (Elenitoba-Johnson et al. 2003), as well as in non-small-cell lung, thyroid and lymph node-positive breast carcinomas (Esteva et al. 2004; Greenberg et al. 2002; Pomerance et al. 2006). Furthermore, p38α has been shown to contribute to the invasiveness of breast, prostate and pancreatic cancers (Chen et al. 2004; Dreissigacker et al. 2006; Kim et al. 2003), as well as to the maintenance of the neoplastic phenotype in Ras-transformed human fibroblasts and epithelial cells (Weijzen et al. 2002). In addition, the use of chemical inhibitors such as SB203580 has shown the requirement of p38α (and maybe also p38β) for the proliferation of cancer cell lines, including chondrosarcoma, prostate carcinoma and melanoma cells (Halawani et al. 2004; Recio and

Merlino 2002; Ricote et al. 2006). Thus, whereas p38α may suppress tumor initiation, it seems to serve oncogenic functions in cancer progression. Perhaps, this explains why p38α has not been found mutated or down-regulated in human cancers to date, in contrast to other well-established tumor suppressor proteins (Johnstone et al. 2002).

Given the complex network of tumorigenesis-related functions coordinated by p38α, it is likely that both the type and stage of the tumor will have to be carefully taken into account in any rationale attempt to modulate p38α activity for cancer therapy. Hence, whereas p38α activation by chemotherapeutic agents may prove beneficial for first-line treatment of early-stage non-recurrent solid tumors (which are naturally sensitized to apoptosis), it might have little effect or even result hazardous in apoptosis-resistant metastatic cancers, which might in turn take advantage of p38α activity to further migrate and invade or to enter an apoptosis-resistant but reversible dormant state (Ranganathan et al. 2006). Similarly, chemotherapy-induced activation of p38α might result in therapeutic benefit in well-irrigated solid tumors, especially in combination with anti-angiogenic therapy to target for example VEGF. However, the same approach might be questionable in the case of hypoxic tumors that, apart from being less sensitive to chemotherapy due to poor blood irrigation, may instead capitalize on p38α activation by the treatment-induced inflammatory response to enhance angiogenesis and further invasion. In contrast, p38α inhibition might be of therapeutic benefit in these types of aggressive and resistant tumors (i.e. metastatic and hypoxic), if combined with simultaneous activation of p38α-independent apoptotic signaling. Another potential use of the p38α inhibitors could be in combination with drugs that induce DNA damage, because cancer cell death could be stimulated by impairing p38α-mediated cell cycle arrest and repair mechanisms (Reinhardt et al. 2007). Further work, including the use of specific mouse tumor models, should help to better define the roles of p38α in tumorigenesis and the potential value of this signaling pathway as a target for cancer therapy.

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p38α MAP kinase as a sensor of reactive oxygen species in tumorigenesis

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Abstract

p38α is a stress-activated protein kinase that negatively regulates malignant transformation induced by oncogenic H-Ras, although the mechanisms involved are not fully understood. Here, we show that p38α is not a general inhibitor of oncogenic signaling, but that it specifically modulates transformation induced by oncogenes that produce reactive oxygen species (ROS). This inhibitory effect is due to the ROS-induced activation of p38α early in the process of transformation, which induces apoptosis and prevents the accumulation of ROS and their carcinogenic effects. Accordingly, highly tumorigenic cancer cell lines have developed a mechanism to uncouple p38α activation from ROS production. Our results indicate that oxidative stress sensing plays a key role in the inhibition of tumor initiation by p38α.

Significance

The characterization of tumor suppressors whose activity could be stimulated for cancer therapy is an area of intense research. We show that the ability of p38 MAPK to induce apoptosis in response to the detection of reactive oxygen species (ROS) plays an important inhibitory role in tumor initiation. This activity is likely to be relevant for human cancer, as the tumorigenicity of cancer cell lines correlates with increased levels of glutathione S-transferase (GST) proteins that specifically desensitize p38α activation from ROS accumulation. Our results illustrate a mechanism used by cancer cells for the inactivation of tumor-suppressor pathways and suggest that restoring the ROS-induced activation of p38 MAPK, for example by targeting GST proteins, may be of potential therapeutic interest.

Introduction

Cancer is a complex disease that involves the disruption of cell and tissue homeostasis via a series of successive genetic changes (Hanahan and Weinberg, 2000). These include activating mutations in the *H-*, *N-*, and *K-ras* proto-oncogene family members, which have been found to be mutated or overexpressed in more than 30% of human tumors (Bos, 1989).

Ras-induced tumorigenesis is accompanied by a number of biochemical changes, including the activation of the ERK MAP kinase (MAPK)-, PI3K-, and RalGDS-signaling pathways (Downward, 2003). Furthermore, increased intracellular levels of reactive oxygen species (ROS) have also been reported to mediate some biological effects of oncogenic H-Ras, such as mitogenesis in fibroblasts (Irani et al., 1997), the onset of premature senescence in primary cells (Lee et al., 1999; Nicke et al., 2005), the generation of genomic instability (Woo and Poon, 2004), and malignant transformation (Mitsushita et al., 2004). In contrast, N-Ras has not been linked to oxidative stress yet, whereas K-Ras has been reported to either increase or decrease intracellular ROS levels depending on the cellular context (Maciag and Anderson, 2005; Santillo et al., 2001). The ability of other oncogenes, apart from Ras, to induce ROS production has not been described; however, BCR/ABL (Sattler et al., 2000) and several growth factor receptors that signal through Ras, such as the transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) receptors, have all been reported to raise intracellular ROS levels in hematopoietic cells (Sattler et al., 1999).

Oxidative stress has been traditionally considered as a toxic by-product of cellular metabolism, but it has been recently appreciated that ROS are actively involved in the regulation of signal-transduction pathways (Hancock et al., 2001), and that they can also cooperate with oncogenic signaling in cellular transformation and cancer (Suh et al., 1999; Woo and Poon, 2004). The carcinogenic effects of ROS accumulation have been proposed to operate at various levels, including changes in gene expression (Allen and Tresini, 2000), increased proliferation and DNA-mutational rates (Irani et al., 1997; Toyokuni, 2006), and genomic instability (Woo and Poon, 2004). Furthermore, high levels of ROS have been detected in several human cancer cell lines (Szatrowski and Nathan, 1991), as well as in human tumors from different tissues (Toyokuni et al., 1995). ROS have also been implicated in the proliferation of melanoma, breast carcinoma, and fibrosarcoma human tumor cell lines (Church et al., 1993; Fernandez-Pol et al., 1982). Taken together, these reports support the causal link between oxidative stress and cancer, which was proposed 20 years ago (Ames, 1983).

p38α MAPK plays an important role in the coordination of the cellular responses to many stress stimuli. The signaling pathways leading to the activation of p38α involve several upstream MAP3Ks, with apoptosis signal-regulating kinase 1 (ASK1) (MAP3K5) playing a major role in p38α activation by oxidative stress (Tobiume et al., 2001). ASK1 activation is thought to involve both oligomerization and autophosphorylation, which is prevented in non-stressed cells by the binding of stress-sensitive proteins. Two of these ASK1-binding proteins are thioredoxin (Trx) and glutathione S-transferase Mu-1 (Gstm1), which dissociate from ASK1 after oxidative stress and heat shock, respectively (Dorion et al., 2002; Matsukawa et al., 2004). Interestingly, overexpression of Gstm1 inhibits p38α activation by oxidative stress, which might be accounted for by the binding of both Gstm1 and Trx to the same N-terminal region of ASK1 (Cho et al., 2001).

In addition to its key role as a coordinator of the cellular responses to stress, p38α has also been shown to regulate other cellular processes in a cell-type-specific manner (Nebreda and Porras, 2000). Of note, p38α negatively regulates the malignant transformation induced by oncogenic H-Ras, and several mechanisms have been proposed to explain this putative tumor-suppressor role, including inhibition of the ERK pathway (Li et al., 2003), induction of premature senescence (Wang et al., 2002) or of a p53-dependent cell cycle arrest (Bulavin et al., 2002), and upregulation of cell cycle inhibitors, such as $p16^{INK4a}$ (Bulavin et al., 2004) and $p21^{Cip1}$ (Nicke et

al., 2005). Other reports indicate that p38α may also antagonize malignant transformation induced by N-Ras in fibroblasts (Wolfman et al., 2002) and by K-Ras in colon cancer cells (Qi et al., 2004), although the mechanisms involved are poorly understood.
Here, we show that p38α is not a general inhibitor of oncogenic signaling, but that it specifically modulates malignant transformation induced by oncogenes that produce ROS. Interestingly, some human cancer cells can bypass the inhibitory role of p38α on ROS accumulation, and this leads to enhanced tumorigenicity. Thus, oxidative stress sensing by p38α MAPK is an important mechanism by which to negatively regulate the onset of cancer.

Results

p38α-deficient MEFs are sensitized to H-RasV12-induced transformation

To investigate the effect of p38α on H-Ras-induced transformation, fibroblasts derived from wild-type (WT) and p38α$^{-/-}$ mouse embryos were immortalized by the 3T3 protocol. Consistent with previous reports (Brancho et al., 2003; Chen et al., 2000; Faust et al., 2005), we found that immortalized WT and p38α$^{-/-}$ mouse embryo fibroblasts (MEFs) proliferated with comparable rates, although upon H-RasV12 transduction (Figure 1A) or under low-serum conditions (Figure S1; see the Supplemental Data available with this article online), p38α$^{-/-}$ MEFs proliferated faster than WT MEFs. This was consistent with higher levels of cyclin D1 in exponentially proliferating p38α$^{-/-}$ MEFs expressing H-RasV12 (Figure 1B), as expected from the known ability of p38α to downregulate cyclin D1 expression (Lavoie et al., 1996). However, in contrast to the described role of p38α as a modulator of the p16^{Ink4a}/p19^{Arf} pathways in primary stem cells and breast tumorigenesis (Bulavin et al., 2004; Ito et al., 2006), we observed no differences in the levels of p16^{Ink4a} between H-RasV12-expressing WT and p38α$^{-/-}$ MEFs (Figure 1C), whereas p19^{Arf} was not detected by immunoblotting (data not shown).
We also analyzed the ability of p38α-deficient cells to grow in soft agar, which is considered a better marker for in vivo tumorigenesis than the rates of proliferation. We found that H-RasV12-expressing p38α$^{-/-}$ MEFs were 40% less adherent (data not shown) and showed a more refringent morphology than H-RasV12-transduced WT MEFs (Figure 1D). Moreover, H-RasV12-transformed p38α$^{-/-}$ MEFs formed bigger foci and were able to produce about 9-fold more colonies in soft agar than H-RasV12-transduced WT MEFs (Figures 1E and 1F). Importantly, the differences between WT and p38α$^{-/-}$ MEFs could be rescued by reintroduction of p38α in H-RasV12-expressing p38α$^{-/-}$ cells (Figures 1G and 1H), arguing that the observed differences are directly due to the absence of p38α, and not to secondary genetic alterations. Of note, we could not detect p53 protein expression in immortalized WT and p38α$^{-/-}$ MEFs, either when proliferating or after stress treatments (Figure 1B and data not shown), suggesting that p38α inhibits H-RasV12-induced transformation of fibroblasts by p53-independent mechanisms, in agreement with previous work (Bulavin et al., 2004). The in vivo relevance of these observations was confirmed by injecting nude mice subcutaneously with H-RasV12-transformed p38α$^{-/-}$ MEFs, which gave rise to tumors significantly faster than H-RasV12-transformed WT MEFs (Figure 1I).

Sustained activation of p38α inhibits H-RasV12-induced transformation, but not ERK activation

NIH3T3 fibroblasts are immortalized, highly contact-inhibited cells that carry a homozygous deletion in the entire *INK4a/ARF* locus. To confirm whether p38α could negatively regulate H-RasV12-induced transformation independently of p16^{Ink4a} and p19^{Arf}, NIH3T3 fibroblasts were transfected with H-RasV12, either alone or together with the specific p38 MAPK activator MKK6DD. Expression of MKK6DD resulted in efficient activation of endogenous p38α (Figure

2A), which correlated with the inhibition of H-RasV12-induced transformation, as determined by the less refringent morphology and the reduced number of both foci formation and anchorage-independent growth (Figure 2B).

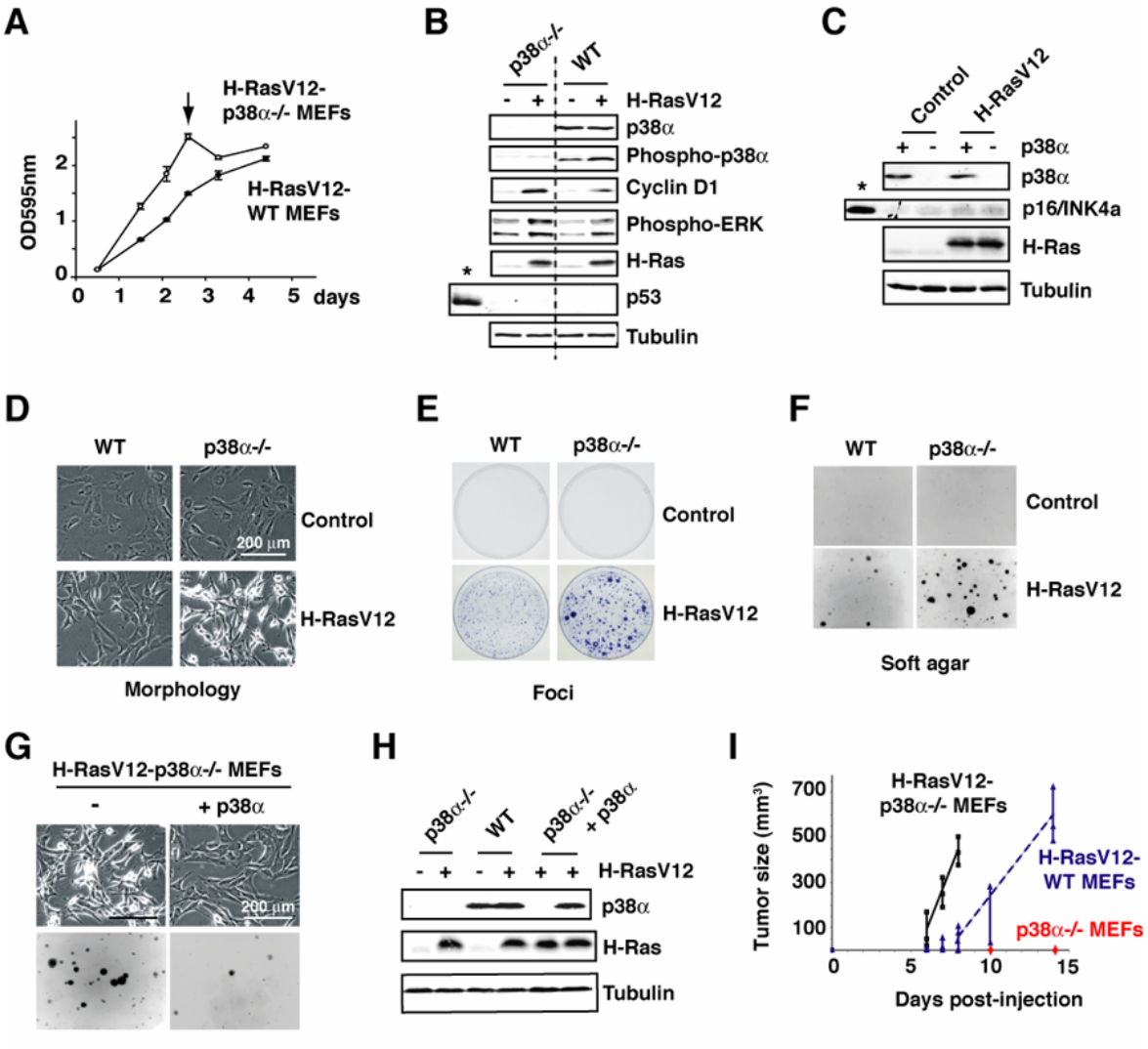

Figure 1. p38α negatively regulates H-RasV12-induced malignant transformation
(A) Proliferation rates of H-RasV12-expressing WT and p38α$^{-/-}$ MEFs. The arrow indicates when H-RasV12-expressing p38α$^{-/-}$ cells achieved confluence. The error bars show SD.
(B and **C)** Total cell lysates from exponentially proliferating WT and p38α$^{-/-}$ MEFs (50 μg total protein) were analyzed by immublotting with the indicated antibodies. Primary and SV40 LT-Ag-immortalized MEFs were used as controls for p53 and p16^{INK4a} immunoblotting, respectively (indicated by asterisks).
(D–F) H-RasV12-expressing WT and p38α$^{-/-}$ MEFs as well as control cells transduced with the empty vector were selected with puromycin (1.5 μg/ml) for 1 week and then compared in terms of **(D)** morphology, **(E)** ability to form foci, and **(F)** anchorage-independent growth in soft agar.
(G and **H)** p38α$^{-/-}$ MEFs were rescued by forced expression of p38α and then analyzed for **(G)** anchorage-independent growth and morphology, as well as by **(H)** immublotting with the indicated antibodies.
(I) Immunodeficient nude mice were injected subcutaneously with control p38α$^{-/-}$ (rhombus) and H-RasV12-expressing WT (triangles) or p38α$^{-/-}$ (squares) MEFs, and tumor size was measured periodically. Error bars show SD.

The ability of p38 MAPK to inhibit ERK activation has been previously documented (Li et al., 2003). We therefore investigated whether the negative effect of p38α on HRasV12-induced transformation could be accounted for by interfering with the activation of the ERK pathway,

since this has been shown to be important for the H-Ras-induced transformation of mouse fibroblasts (Cowley et al., 1994; Mansour et al., 1994). We found that exponentially proliferating H-RasV12-WT MEFs contained similar phospho-ERK levels as H-RasV12-p38α$^{-/-}$ cells (Figure 1B). Moreover, kinetic analysis of ERK activation in response to serum stimulation showed no differences between p38α$^{-/-}$ and WT MEFs, either in the presence or absence of H-RasV12 expression (Figure 2C). We also confirmed that MKK6DD expression affected neither the basal nor the oncogene-induced levels of active ERK in NIH3T3 fibroblasts (Figure 2D). These results indicated that p38α was not inhibiting H-RasV12-induced transformation of MEFs and NIH3T3 cells by interfering with ERK activation.

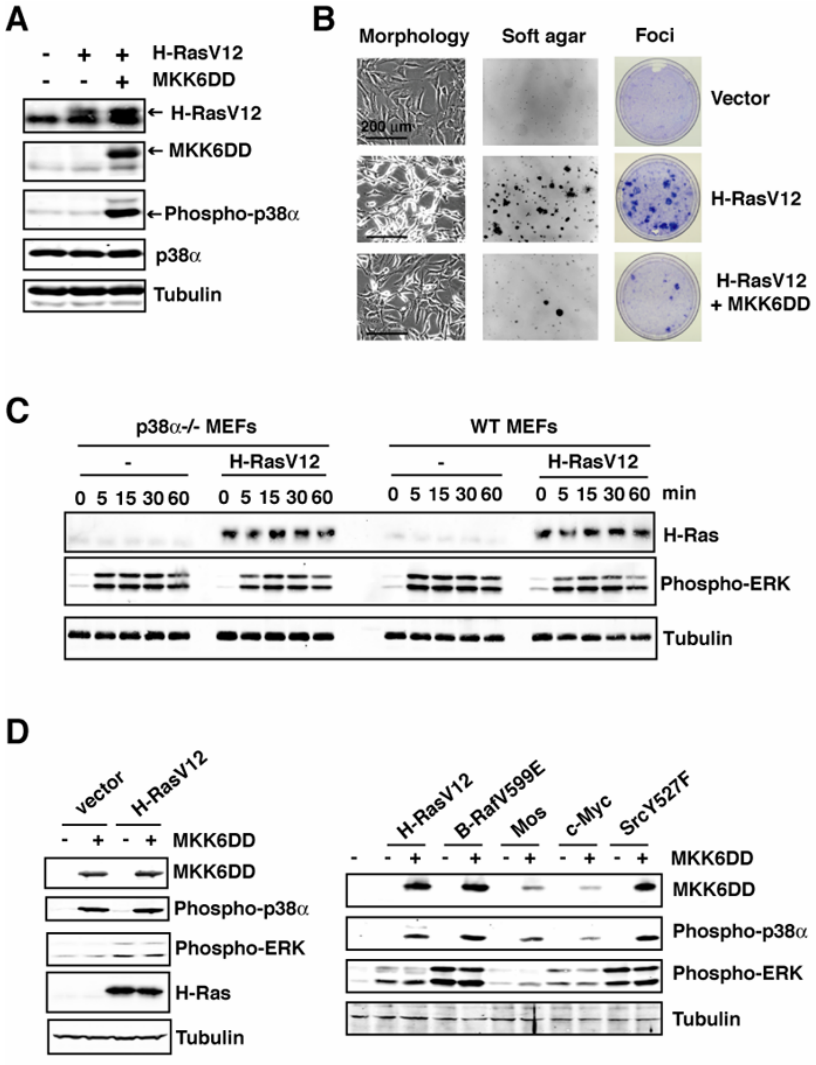

Figure 2. p38α regulates H-RasV12-induced transformation independently of the *INK4a/ARF* locus and the ERK pathway

(A) NIH3T3 fibroblasts were transfected with H-RasV12 in combination with MKK6DD or an empty vector and 48 h later were analyzed by immunoblotting with the indicated antibodies.

(B) NIH3T3 cells stably expressing H-RasV12 alone or in combination with MKK6DD were analyzed for transformation-associated morphological changes (left panels) and anchorage-independent growth in soft agar (middle panels). NIH3T3 cells were also transiently transfected with HRasV12 or H-RasV12 plus MKK6DD and were analyzed for foci formation during the course of 3 weeks (right panels).

(C) Kinetics of ERK activation in the indicated cell lines after incubation in 0.5% serum for 60 h, followed by stimulation with 10% FBS.

(D) NIH3T3 cells were transiently transfected with the indicated oncogenes, together with MKK6DD or an empty vector, and 48 h later were analyzed by immunoblotting.

p38α inhibits H-RasV12-induced ROS accumulation by triggering apoptosis

Our results indicated that p38α was able to inhibit H-RasV12-induced transformation of pre-malignant fibroblasts independently of both p53 and the *INK4a/ARF* locus, and without interfering with ERK activation. Next, we investigated the effect of p38α on the production of ROS, a well-known biological consequence of oncogenic H-Ras expression (Irani et al., 1997).
We found that H-RasV12-p38α$^{-/-}$ MEFs accumulated significantly higher levels of intracellular ROS than HRasV12-WT MEFs (Figure 3A). Quantitative analysis showed that H-RasV12-WT MEFs contained 2- to 3-fold higher ROS levels than non-transformed WT or p38α$^{-/-}$ cells, whereas ROS levels detected in p38α$^{-/-}$ MEFs expressing H-RasV12 were ~25-fold higher (Figure S2). Interestingly, the higher levels of ROS in H-RasV12-p38α$^{-/-}$ MEFs not only correlated with their enhanced transformed phenotype, but also with two known outcomes of oxidative stress, which are reduced intracellular phosphatase activity and high levels of genomic instability (Figures S3A and S3B). In support of the idea that high intracellular ROS levels could play a causal role in the malignant phenotype, we were able to inhibit the more dramatic transformed morphology of H-RasV12-expressing p38α$^{-/-}$ MEFs by incubation with the antioxidant glutathione (Figure 3B).
To further analyze the interplay between p38α, ROS accumulation, and H-RasV12-induced transformation, we used a 4-hydroxytamoxifen (OHT)-inducible ER-HRasV12 system (De Vita et al., 2005). Surprisingly, we found that, early in H-RasV12 induction with OHT, both p38α$^{-/-}$ and WT MEFs contained comparable ROS levels (Figure 3C, upper panel). However, long-term accumulation of ROS was only observed in p38α$^{-/-}$ cells. In order to elucidate why WT cells were not able to accumulate ROS, we monitored the time-dependent appearance of the ERHRasV12-induced transformed morphology in WT and p38α$^{-/-}$ MEFs (Figure 3C, lower panel). We observed that both WT and p38α$^{-/-}$ MEFs acquired a similar transformed morphology shortly after OHT treatment. However, WT cells undergo up to 10-fold more apoptosis than p38α$^{-/-}$ cells when treated with OHT for 1 week (Figure 3C, arrows, and Figure 3D), which occurs in parallel with a drop in their ROS levels between 7 and 10 days after OHT treatment, depending on the experiment. The occurrence of apoptosis was confirmed biochemically by the accumulation of processed p85 PARP (Figure 3E), and its key role for ROS downregulation in WT MEFs was further supported by incubation with the pan-caspase inhibitor ZVAD-fmk. Indeed, the inhibition of apoptosis by ZVAD-fmk (Figure 3F) interfered with ROS downregulation in WT MEFs at late times after ER-HRasV12-induction, without affecting ROS levels in p38α$^{-/-}$ cells (Figure S4). Extended treatment with OHT for up to 3 weeks did not have any further effect on the surviving cells, which showed sustained low levels of ROS. Moreover, no apoptotic crisis was observed in p38α$^{-/-}$ MEFs expressing ER-HRasV12 or in control WT and p38α$^{-/-}$ MEFs treated with OHT, arguing in favor of an early p38α-mediated inhibitory mechanism in response to oncogenic H-RasV12-induced ROS. In agreement with this, phospho-p38α levels transiently increased after OHT treatment, in parallel with p85 PARP accumulation (Figure 3E), and decreased after 2 weeks of treatment.
Next, we studied whether the observed increase in p38α activity was necessary for H-RasV12-induced apoptosis. WT MEFs expressing ER-HRasV12 were incubated with the p38 MAPK inhibitor SB203580, and apoptosis was quantified 8 days after OHT treatment. As expected, SB203580 strongly impaired the H-RasV12-induced activation of the p38 MAPK pathway and the subsequent apoptotic response (Figure 3F), without affecting the H-RasV12-induced accumulation of ROS (data not shown), which indicates that p38α activation lays downstream of ROS and is required for apoptosis induction by H-RasV12.

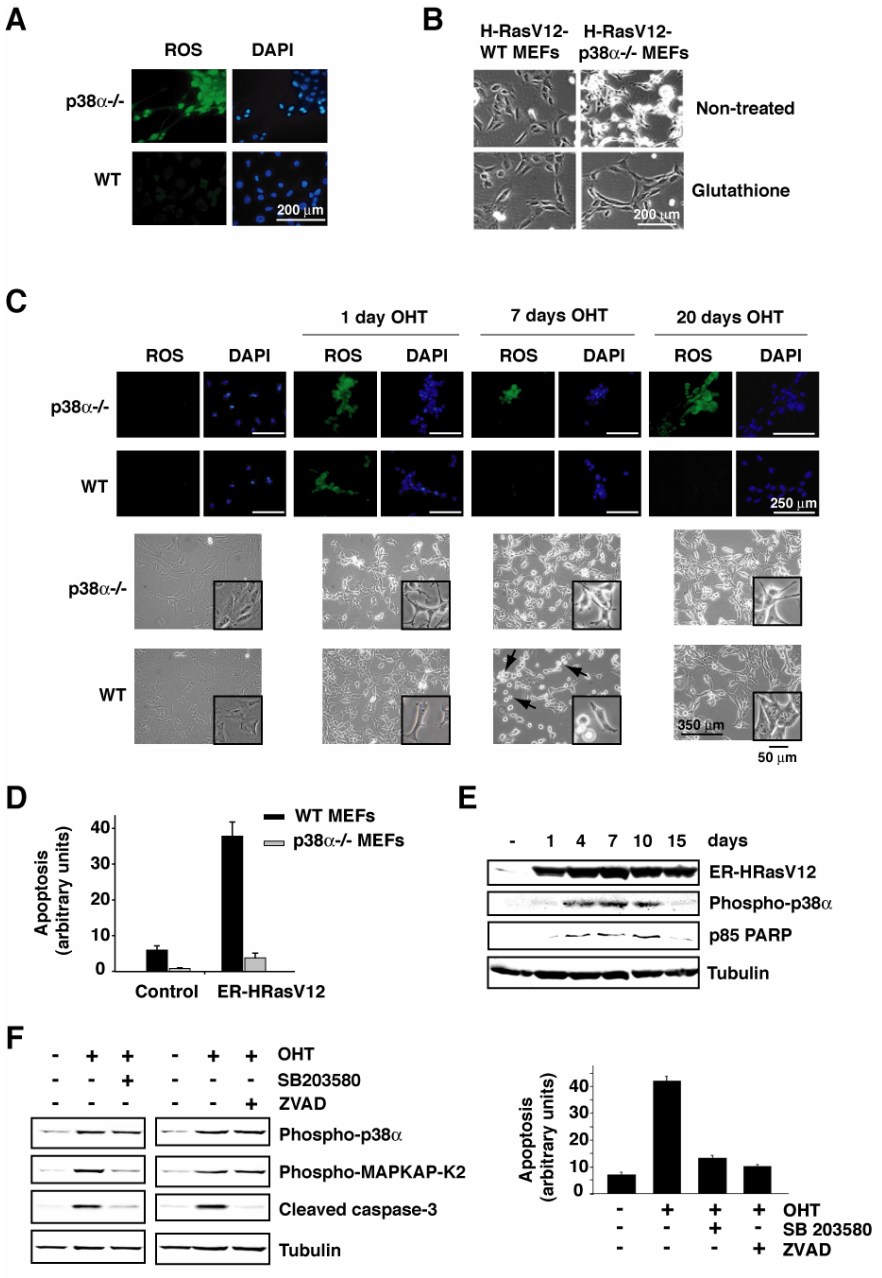

Figure 3. p38α prevents the long-term accumulation of H-RasV12-induced high levels of ROS through an apoptotic response

(A) WT and p38α$^{-/-}$ MEFs stably expressing H-RasV12 were analyzed for intracellular ROS levels by immunofluorescence.

(B) WT and p38α$^{-/-}$ MEFs were transduced with H-RasV12 and then selected for 1 week with either 1 μg/ml puromycin alone (top panels) or in combination with reduced glutathione (10 mM for 3 days, followed by 5 mM for 4 days; bottom panels) before the pictures were taken.

(C) WT and p38α$^{-/-}$ MEFs expressing an OHT-inducible H-RasV12 construct (ER-HRasV12) were treated for different times with 1 μM OHT, and ROS accumulation was visualized by immunofluorescence (top panel). Transformation-associated morphological alterations were also monitored in parallel (bottom panel). The insets show magnifications of representative fields.

(D) Apoptosis was determined in empty vector- and ER-HRasV12-transduced WT and p38α$^{-/-}$ MEFs after 8 days of treatment with 1 μM OHT. Error bars show SD.

(E) ER-HRasV12-expressing WT MEFS were treated with 1 μM OHT and analyzed by immunoblotting with the indicated antibodies.

(F) ER-HRasV12-expressing WT MEFs were treated with 1 μM OHT for 4 days and then incubated for another 4 days with OHT together with SB203580 (10 μM) or ZVAD-fmk (20 μM). Cell lysates were analyzed by immunoblotting with the indicated antibodies. Apoptosis was quantified by an ELISA assay (right panel). Error bars show SD.

H-RasV12-induced ROS accumulation is mediated by the ERK and Rac1 pathways and involves NADPH oxidases

To elucidate which pathways mediate the long-term accumulation of ROS in H-RasV12-transformed p38α$^{-/-}$ MEFs, we used rotenone, an inhibitor of the mitochondrial electron transport chain, and diphenylene iodonium chloride (DPI), an inhibitor of NADPH oxidase (NOX) enzymes that are major mediators of the non-mitochondrial ROS production (Kamata and Hirata, 1999). Incubation with rotenone did not affect H-RasV12-induced ROS accumulation in p38α$^{-/-}$ MEFs, whereas DPI basically abolished it (Figure S5A). *Nox* genes have been previously associated with cellular transformation and cancer (Suh et al., 1999). In agreement with this, we found that the *Nox1* and *Nox4* mRNAs were upregulated in H-RasV12-transformed p38α$^{-/-}$ MEFs, suggesting that these two NOX family members may be involved in H-RasV12-induced ROS production in fibroblasts (Figure S5B).
We also used chemical inhibitors to investigate the contribution of different Ras-activated signaling pathways to ROS production. Our results indicated that H-RasV12-induced ROS accumulation in p38α$^{-/-}$ MEFs was mediated by cooperative action of the ERK and Rac1 pathways, but did not require PI3K activity (Figures S5C and S5D).

Uncoupling p38α activation from oxidative stress enhances H-RasV12-induced transformation

We have recently identified *gstm2* as one of the genes that may be regulated by p38α in H-RasV12-transformed MEFs (unpublished data). Gstm2 was more than 90% homologous to Gstm1 (Figure 4A), another member of the same GST family whose overexpression has been reported to inhibit the oxidative stress-induced activation of p38 MAPK by binding to and inhibiting ASK1 (Cho et al., 2001; Dorion et al., 2002). *gstm1* has been associated with elevated breast cancer risk (Parl, 2005) and is also overexpressed in tumors from brain, skin, and kidney, according to the Cancer Genome Anatomy Project database (http://cgap.nci.nih.gov/Tissues). To address the putative role of Gstm2 as a modulator of p38α function in HRasV12-induced tumorigenesis, we investigated whether Gstm2 overexpression could affect the activation of p38α by H-RasV12. In agreement with a recent report detailing the use of ovarian epithelial cells (Nicke et al., 2005), we found that the H-RasV12-induced activation of p38α was impaired in the presence of the antioxidant N-acetyl-L-cysteine (NAC), suggesting that ROS are also key mediators of the activation of p38α by H-RasV12 in fibroblasts. Interestingly, Gstm2 overexpression inhibited the activation of p38α (and its activator MKK6) by HRasV12, but interfered neither with the activation of other H-Ras-regulated signaling pathways, such as the PI3K/Akt pathway, nor with the activation of p38α by other stimuli such as UV (Figure 4B). This result argues that Gstm2 specifically uncouples p38 MAPK activation from HRasV12-induced ROS accumulation. We confirmed that Gstm2 can also interact with ASK1 (Figure 4C), which probably accounts for its ability to inhibit ROS-induced p38 MAPK activation, as it has been proposed for Gstm1 (Cho et al., 2001). Consistent with the ability of Gstm2 to interfere with p38α activation, we detected long-term ROS accumulation in H-RasV12-WT MEFs expressing Gstm2 (Figure 4D). Most importantly, accumulation of ROS in H-RasV12-WT MEFs after Gstm2 overexpression correlated with a stronger transformed phenotype, similar to that observed in H-RasV12-p38α$^{-/-}$ MEFs, both at the morphological level and by the enhanced ability of the cells to grow in soft agar (Figure 4E). Of note, the growth of H-RasV12-p38α$^{-/-}$ MEFs in soft agar was not affected by Gstm2 overexpression (data not shown), suggesting that p38α was an important target for the Gstm2 effect observed in WT cells. These results strongly support the hypothesis that p38α functions as a key oxidative stress sensor in oncogenic transformation by H-RasV12.

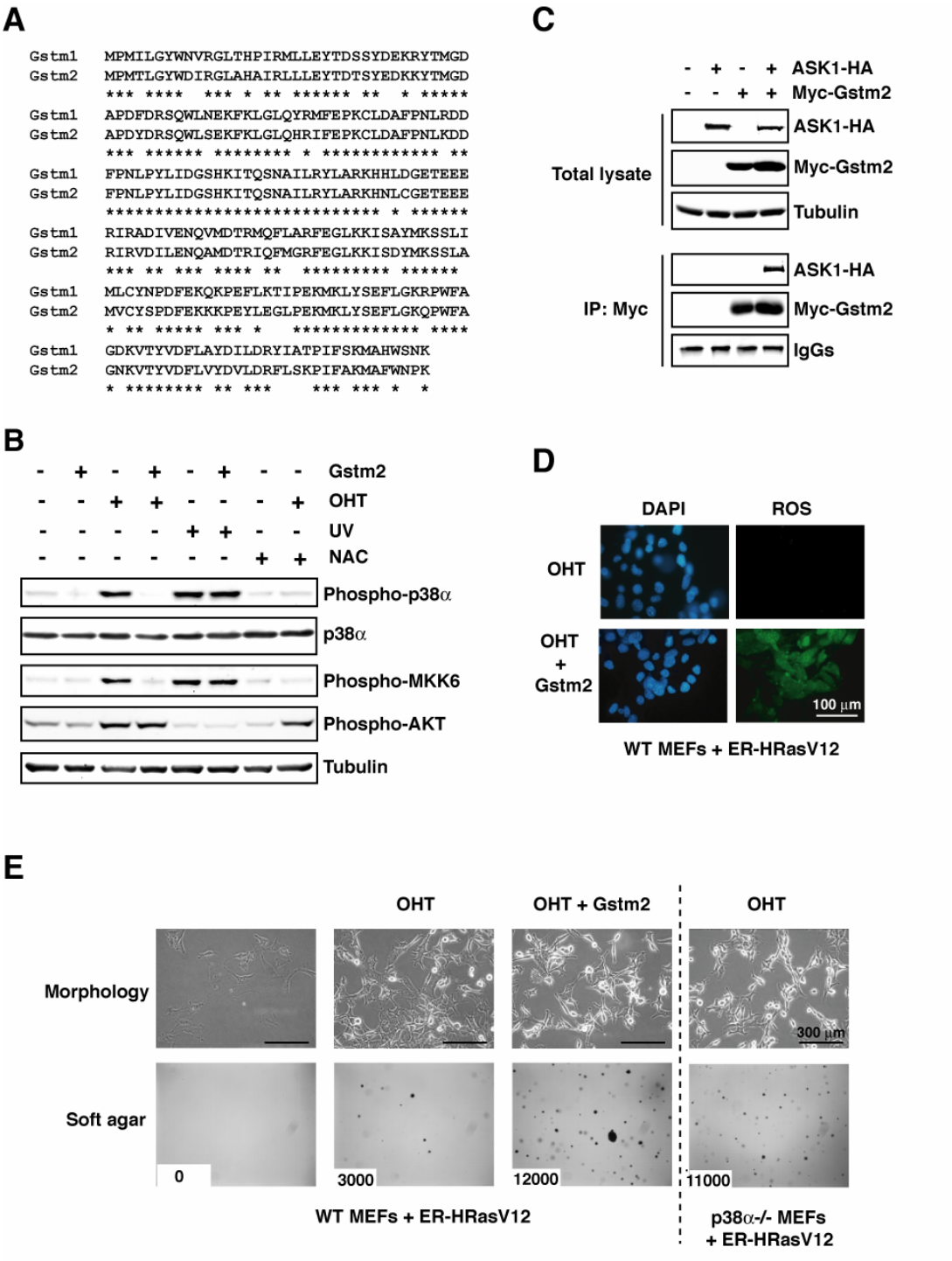

Figure 4. Uncoupling ROS accumulation from p38α activation results in enhanced tumorigenicity

(A) Amino acid sequence alignment of murine Gstm1 (NP_034488) and Gstm2 (NP_032209). Asterisks indicate identical amino acids.

(B) ER-HRasV12-expressing WT MEFs were transduced with murine Gstm2 or an empty vector and then either stimulated with UV irradiation or treated with 1 μM OHT for 5 days. One sample was co-incubated with 5 mM NAC during the last 16 h of OHT treatment. Total cell lysates were analyzed by immunoblotting with the indicated antibodies.

(C) Lysates from 293T cells transfected with plasmids expressing ASK1-HA and Myc-Gstm2 were subjected to immunoprecipitation with Myc antibody. The total lysates and the Myc immunoprecipitates were analyzed by immunoblotting with the indicated antibodies.

(D and **E)** ER-HRasV12-expressing WT MEFs were transduced with murine Gstm2 or an empty vector, treated with 1 μM OHT for 3 weeks, and then analyzed for **(D)** intracellular ROS levels and **(E)** transformation-associated morphological alterations and anchorage-independent growth in soft agar.

p38α specifically regulates malignant transformation by ROS-inducing oncogenes

We next investigated whether the role of p38α as a ROS sensor in H-RasV12- induced transformation could be extended to other oncogenes. Thus, WT and p38α$^{-/-}$ MEFs were transduced with a panel of oncogenes, covering different pathways and subcellular localizations, and were analyzed in terms of anchorage-independent growth, focus formation, and intracellular ROS levels (Table 1). As with H-RasV12, none of the oncogenes tested were able to induce long-term accumulation of ROS in WT MEFs. However, the oncogenes NeuV664E and N-RasV12 did induce high ROS levels in p38α$^{-/-}$ MEFs that were comparable to those observed for H-RasV12 (Table 1). Interestingly, ROS accumulation in p38α$^{-/-}$ MEFs expressing N-RasV12 or NeuV664E correlated with a more dramatic transformed phenotype in these cells than in WT MEFs expressing the same oncogenes. No differences in soft agar growth were observed between WT and p38α$^{-/-}$ MEFs with the other oncogenes, including K-RasV12, which was consistent with their inability to induce long-term accumulation of high ROS levels in MEFs (Table 1). These results suggest that p38α functions as an oxidative stress sensor in tumorigenesis, with the capacity to downregulate malignant transformation by oncogenes that induce ROS production. Of note, p38α negatively regulated the induction of focus formation by oncogenic forms of Raf-1, B-Raf, and K-Ras, which do not produce high ROS levels (Table 1), suggesting that p38α may also have ROS-independent anti-oncogenic functions.

Table 1. Effect of p38α on anchorage-independent growth and focus formation induced by different oncogenes in mouse fibroblasts

Oncogene	ROS[1] (p38α$^{-/-}$ MEFs)	Soft agar[2] (p38α$^{-/-}$ MEFs)	Focus formation	
			p38α$^{-/-}$ MEFs[3]	NIH3T3 + MKK6DD[4]
Neu V664E	+	Enhanced	Enhanced	Reduced
H-RasV12	+	Enhanced	Enhanced	Reduced
N-RasV12	+	Enhanced	Enhanced	Reduced
K-RasV12	-	As WT	Enhanced	ND
B-Raf V599E	-	As WT	Enhanced	Reduced
Raf-1 22W	-	As WT	Enhanced	Reduced
RalGDS-CAAX	-	As WT	As WT	ND
Rac1 N115I	-	As WT	As WT	ND
MEK1 ΔN	-	As WT	As WT	As NIH3T3
v-Mos	-	As WT	As WT	ND
c-Src Y527F	-	As WT	As WT	As NIH3T3
SV40 LT-Ag	-	As WT	As WT	As NIH3T3
v-Jun	-	As WT	As WT	As NIH3T3
c-Myc	-	As WT	As WT	As NIH3T3

[1]ROS levels were visualized by immunofluorescence; + indicates ROS accumulation to high levels.
[2]Soft agar was used to measure anchorage-independent growth in p38α$^{-/-}$ MEFs and compared to WT MEFs.
[3]Focus formation in p38α$^{-/-}$ MEFs as compared to WT MEFs.
[4]Focus formation in NIH3T3 fibroblasts expressing the p38 MAPK activator MKK6DD *versus* NIH3T3 fibroblasts.
ND, not determined.

Gstm2 impairs p38α activation by oxidative stress in human epithelial cells

Gstm2 was able to specifically inhibit p38α activation after oncogene-induced oxidative stress, without affecting other H-Ras-activated pathways in murine fibroblasts (Figure 4B). To complement these observations, we overexpressed Myc-tagged Gstm2 in HEK293 human epithelial cells, which then were stimulated with H_2O_2, sorbitol, UV irradiation, and cisplatin, or co-transfected with HRasV12. We confirmed that Gstm2 efficiently inhibited p38α activation induced by H-RasV12 or H_2O_2 (Figures S6A and S6B). However, the Gstm2 inhibitory effect was more modest in the cases of osmotic shock, UV irradiation, and cisplatin treatment (Figure S6C). On the other hand, Gstm2 overexpression did not affect ERK activation by any of these stimuli and either only partially inhibited or had no effect on JNK activation induced by H_2O_2 and HRasV12, respectively (data not shown). These results indicate that Gstm2 targets a key regulator of the oxidative stress-induced activation of p38 MAPK (i.e. ASK1), whereas additional pathways may contribute to JNK activation by oxidative stress.

ROS accumulation in human cancer cell lines correlates with enhanced tumorigenicity

Our results indicated that oncogene-induced ROS accumulation correlated with enhanced tumorigenicity in fibroblasts. However, many human neoplasms originate from epithelial cells, in which little is known about ROS levels. Thus, we compared intracellular ROS levels and tumorigenic potential in a panel of human epithelial cell lines derived from colon, prostate, breast, and lung tumors. We observed a strong correlation in all tissues between high levels of ROS and efficient anchorage-independent growth (Figure 5). These results suggest that ROS accumulation may enhance the malignant phenotype of cancer cells, in agreement with the pro-carcinogenic effects mediated by oxidative stress. In contrast, we observed no correlation between oxidative stress accumulation and the invasivity of these cancer cell lines (Figure 5). This was confirmed by the lack of effect of antioxidant treatment on the invasivity of MDA-MB-231 cells (data not shown).
Thus, intracellular ROS levels seem to correlate with the tumorigenic potential of human cancer cells, but not with their invasive capacity.

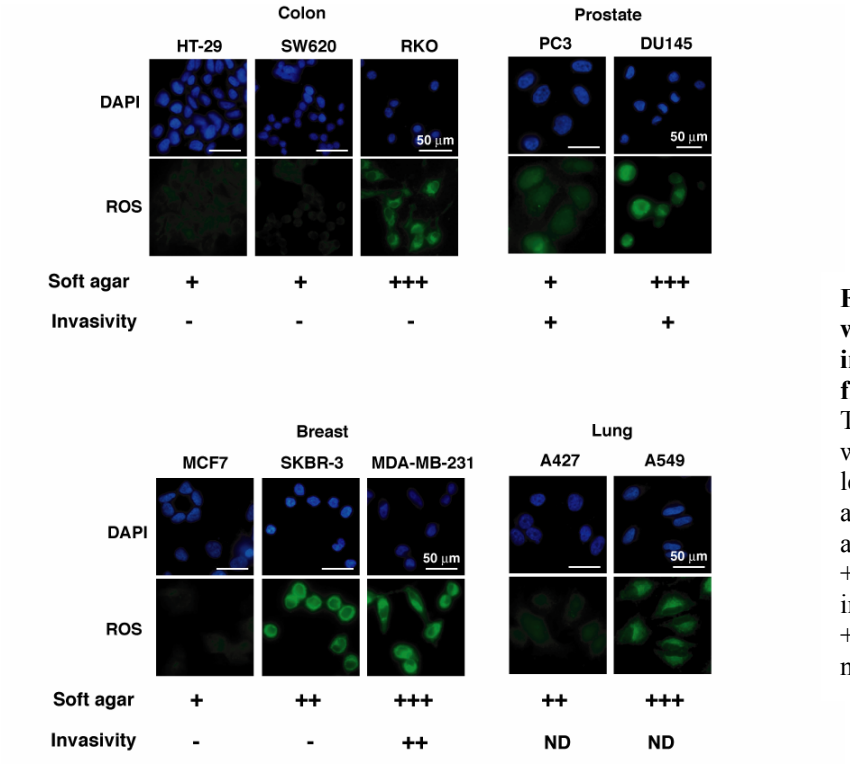

Figure 5. High levels of ROS correlate with enhanced tumorigenicity, but not invasivity, in human cancer cell lines from different tissues
The indicated human cancer cell lines were analyzed for intracellular ROS levels by immunofluorescence, for anchorage-independent growth in soft agar (+, 2,000–4,000; ++, 5,000–7,000; +++, 10,000–17,000 colonies), and for invasivity in matrigel chambers (-, <15; +, 25–50; ++, >90 arbitrary units). ND, not determined.

Cancer cell lines with high ROS levels are partially impaired in p38α activation

The identification of cancer cell lines that contained high levels of ROS despite expressing normal levels of p38α (Figure 6A) was intriguing, given the ability of p38α to sense oxidative stress and negatively regulate ROS accumulation. Thus, we investigated the pattern of p38α activation in response to H_2O_2-induced oxidative stress in colon and breast cancer cell lines, which contained various levels of ROS. As shown in Figures 6A and 6B, H_2O_2 treatment activated p38α about 2-fold more efficiently in ROS-negative than in ROS-positive cancer cells. Interestingly, no differences in the activation of p38α were observed when cells were exposed to other stresses such as UV irradiation and osmotic shock (Figure 6C) or cisplatin treatment (data not shown). Our results therefore indicate that cancer cell lines with high ROS levels have developed specific mechanisms by which to desensitize p38α activation from oxidative stress, most likely in order to tolerate the high levels of ROS. Of note, whereas p38α activation was partially uncoupled from oxidative stress in ROS-producing cancer cells, JNKs, particularly the p54 JNK isoform, appeared to be more efficiently activated (Figure 6B). On the other hand, the ERK pathway was similarly activated in ROS-positive and ROS-negative cancer cell lines (Figure 6B).

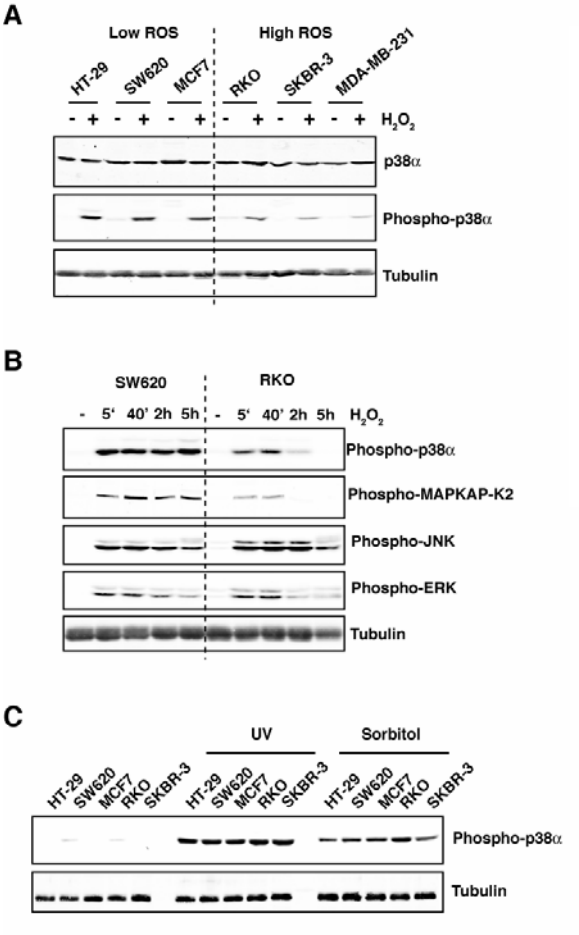

Figure 6. Human cancer cell lines with high ROS levels are partially impaired in p38α activation

(A) Cell lines derived from human colon and breast tumors, which contained either low or high ROS levels, were exposed to 1 mM H_2O_2 for 30 min and analyzed by immunoblotting with the indicated antibodies.

(B) ROS-negative SW620 and ROS-positive RKO colon cancer cells were treated with 5 mM H_2O_2 for different times and analyzed by immunoblotting with the indicated antibodies.

(C) ROS-negative (HT-29, SW620, MCF7) and ROS-positive (RKO, SKBR-3) cancer cell lines were exposed either to UV irradiation or osmotic shock, and phospho-p38 levels were analyzed by immunoblotting.

As mentioned above, Gstm1 has been reported to inhibit the activation of p38 MAPK by oxidative stress, a function that we have shown is also shared by Gstm2. Interestingly, Gstm1 mRNA and protein levels were very high in most ROS-positive cancer cell lines, while they were absent or expressed at very low levels in ROS-negative cells (Figure 7A).

Next, we analyzed whether higher expression levels of Gstm proteins could account for the differences in ROS accumulation and p38α activation observed in cancer cell lines. First, we

found that p38α activation was enhanced by siRNA-mediated knock-down of Gstm1 in the ROS-producing cancer cell lines MDA-MB-231 and A549 (Figure 7B) as well as in DU145 (data not shown). Similar results were obtained upon knock-down of Gstm2 in the cancer cell lines MCF7 (Figure 7B) and SW620 (data not shown), which express Gstm2, but not Gstm1 (Figure 7A and Figure S7). Interestingly, the activation of p38α observed upon knock-down of Gstm1 and Gstm2 correlated in all cases with enhanced apoptosis (Figure 7B). Conversely, overexpression of Gstm2 in MCF7 and SW620 cells resulted in reduced basal levels of activated p38α, as well as in the desensitization of p38α to oxidative stress (Figure 7C). Finally, overexpression of Gstm2 led to the accumulation of higher levels of ROS and the acquisition of a more malignant phenotype in MCF7 cells (Figure 7D), as well as in SW620 cells (data not shown). Of note, we did not observe changes in ROS levels after Gstm downregulation in the cancer cell lines mentioned above (data not shown), suggesting that Gstm proteins function downstream of ROS.
Taken together, these results argue that upregulation of Gstm proteins may be responsible for the partially impaired activation of p38α in ROS-producing cancer cells. Thus, Gstm1 and Gstm2 may inhibit the ROS-sensing and tumor-suppressor function of p38α in human epithelial cells, which is in agreement with their association with increased malignancy of several types of cancer.

Discussion

p38α MAPK was identified as a protein kinase that coordinates the cellular responses to many types of stresses, including those that trigger oxidative stress production. In addition, p38α has been recently shown to mediate physiological processes in response to endogenous ROS, such as the regulation of the lifespan of murine hematopoietic stem cells (Ito et al., 2006). Here, we show that the ability of p38α to trigger apoptosis in response to oncogene-induced ROS accumulation plays a key role in the regulation of malignant transformation. Interestingly, highly tumorigenic human cancer cells can override this p38α function.

p38α as a negative regulator of malignant transformation

Previous studies have established p38α as a negative regulator of H-RasV12-induced cellular transformation, an effect that can be mediated by p53 and the $p16^{Ink4a}$ and $p19^{Arf}$ pathways (Bulavin et al., 2002, 2004). Our results indicate that p38α can also inhibit H-RasV12-induced tumorigenesis in the absence of a functional p53 response and independently of $p16^{Ink4a}$/$p19^{Arf}$. It therefore appears that the mechanisms by which p38α can impinge on malignant transformation may vary depending on the cell type and, probably, also between primary and immortalized cells (Ito et al., 2006; Li et al., 2003).
We show here that the ability of p38α to detect oxidative stress production early in the process of oncogenic H-Ras-induced transformation is important for its inhibitory effect on tumorigenesis. We have also extended this p38α-mediated inhibitory mechanism to other oncogenes, providing a molecular basis for the specificity of p38α as a tumor suppressor. Namely, we found that p38α functions as a tumor surveillance system activated by ROS, which, in turn, inhibits tumor initiation, at least in part, by inducing apoptosis. In agreement with this, ROS-induced sustained activation of p38α has been implicated in apoptosis induction (Tobiume et al., 2001), which can be mediated by both transcriptional and post-transcriptional mechanisms (Porras et al., 2004; Wada and Penninger, 2004), although low levels of oxidative stress can also induce a p38 MAPK-dependent cell cycle arrest (Kurata, 2000). Oxidative stress sensing, therefore, represents a major mechanism for the inhibitory effect of p38α on oncogene-induced transformation.

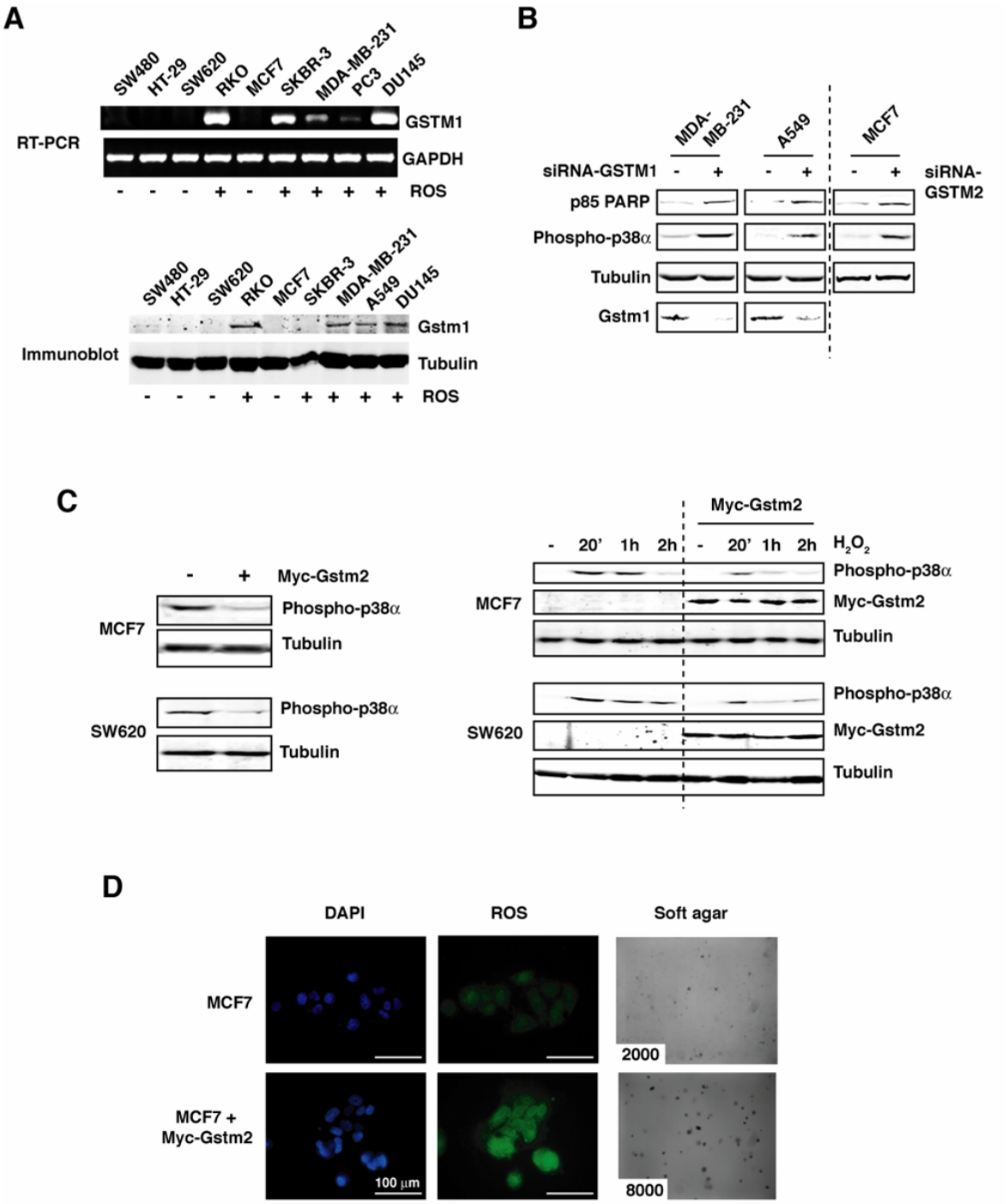

Figure 7. Gstm1 and Gstm2 modulate the activation of p38α by oxidative stress in several human cancer cell lines

(A) *gstm1* (NM_000561) expression was analyzed by both RT-PCR (top panel) and western blot (bottom panel) in several human cancer cell lines.

(B) Downregulation of Gstm1 and Gstm2 by siRNA enhances both p38α activation and the basal apoptotic levels (as indicated by the accumulation in p85 PARP) of human cancer cell lines. Analysis by qRT-PCR confirmed that treatment with *gstm2* siRNA downregulated the *gstm2* mRNA levels to about 40% of those observed in untreated MCF7 cells.

(C) Myc-Gstm2 overexpression in the ROS-negative cancer cell lines MCF7 and SW620 reduces the basal levels of phospho-p38α (left panels) and impairs the activation of p38α by oxidative stress (right panels).

(D) MCF7 cells stably infected with Myc-Gstm2 or empty vector (EV) were analyzed for intracellular ROS levels (middle panels) and anchorage-independent growth in soft agar (right panels).

Mechanisms of p38α activation by oncogene-induced ROS

Several oncogenes have been reported to induce ROS accumulation and to rely on high ROS levels for efficient transformation (Mitsushita et al., 2004), but the molecular links between oncogene activation and ROS production are not completely understood. We found that oncogenic H-Ras-induced accumulation of ROS in p38α$^{-/-}$ cells requires NOX activity and is blocked by the combined use of ERK and Rac1 inhibitors. This is consistent with previous work showing that ERK can induce transcriptional upregulation of Nox1 (Mitsushita et al., 2004), whereas Rac1 cooperates in the assembly of the fully active NOX complex at the plasma membrane (Hancock et al., 2001).

Our results are in agreement with recent work showing that activation of p38α in response to oncogenic H-Ras requires ROS production (Nicke et al., 2005). Furthermore, the slow kinetics of p38α activation, which takes 3–4 days from the onset of oncogenic H-Ras signaling, suggests that p38α is probably activated as a consequence of the high ROS levels accumulated in the cells, rather than as a direct target of H-Ras signaling. Then, how do oncogene-induced ROS lead to p38α activation? One of the key mediators of ROS-induced p38α activation is ASK1 (Matsukawa et al., 2004). Thus, it is foreseen that oncogene-induced ROS would oxidize certain cysteine residues of Trx and induce its dissociation from ASK1, hence inducing the activation of the JNK and p38 pathways. Of note, whereas ASK1 is the major mediator of p38α activation by ROS (Tobiume et al., 2001), MEKK1 may collaborate with ASK1 for the activation of JNK by oxidative stress (Xia et al., 2000; Yujiri et al., 2000).

In addition to the well-characterized role of ASK1 in the activation of p38α by oxidative stress, a recent report has also identified the Ste20 family kinase MINK (MAP4K6) as a novel mediator of the H-RasV12-induced activation of p38 MAPK (Nicke et al., 2005). The mechanism by which H-RasV12 activates MINK is unknown but requires both ERK activation and ROS production. In turn, MINK may lead to the activation of the p38 MAPK pathway through both ASK1 and Tpl2. The interplay between the direct activation of ASK1 by ROS and the participation of ASK1 in ROS-induced MINK signaling is unclear, but it might reflect different ROS-induced cellular responses dependent on signal duration or intensity. Thus, whereas ROS-induced, Trx-dependent activation of ASK1 has normally been associated with the induction of apoptosis (Tobiume et al., 2001), the MINK-mediated ASK1 activation by ROS results in cell cycle arrest (Nicke et al., 2005).

The ROS-p38α connection in human cancer

Suppression of apoptosis is thought to be an important aspect of tumor development (Evan and Vousden, 2001), and multiple mechanisms for inhibition of apoptosis have been identified in human tumors. Furthermore, inactivation of apoptotic proteins potentiates malignant transformation in vitro (Kennedy and Davis, 2003). In agreement with these observations, we show that p38α can inhibit cell tumorigenicity by triggering oncogene-induced apoptosis mediated by ROS. The relevance of this finding was confirmed by the observation that human cancer cell lines have developed a mechanism by which to uncouple p38α activation from oxidative stress production, which results in enhanced tumorigenicity. This mechanism relies on the ability of the GST family members Gstm1 and Gstm2 to impair p38α activation in response to ROS accumulation (Cho et al., 2001; Dorion et al., 2002). Indeed, the ability of highly tumorigenic cancer cell lines to accumulate very high levels of ROS correlates with the upregulation of Gstm1 and Gstm2. Taken together, these results suggest that the p38 MAPK-signaling pathway may suppress tumor formation in vivo by inducing apoptosis. Furthermore, certain members of the GST family may function as potential oncogenes in human cancer, by impairing the normal inhibitory responses, such as apoptosis, triggered by p38α in response to ROS accumulation. Consistent with this idea, a positive correlation between the expression of

several GST family members and cancer progression has been recently proposed (Parl, 2005; Townsend and Tew, 2003).

Collectively, our data indicate that cancer cells may undergo positive selection for high intracellular ROS levels in their course for proliferative advantages. Thus, the carcinogenic effects associated with increased ROS levels might provide cancer cells with greater plasticity for malignant progression. Interestingly, the associated overexpression of Gstm1 and Gstm2 could be a way to specifically suppress the apoptotic effects of p38α in response to ROS, without affecting other cellular processes mediated by p38α that might be important for the viability of the cancer cell. This may explain the lack of evidence for the loss of p38α expression or activity in human cancer, and it suggests a new category of tumor-suppressor proteins in which a partial loss of function specifically impairs their negative contribution to cancer cell survival, while allowing other functions that might be important for malignant progression. This idea is in agreement with evidence indicating that p38 MAPK might, in some cases, contribute to cancer progression, for example by mediating cancer cell migration (Kim et al., 2003), by activating the transcription factor HIF-1 (Emerling et al., 2005; Nakayama et al., 2007) or by other mechanisms (Elenitoba-Johnson et al., 2003; Weijzen et al., 2002). Consistent with this idea, we have found that inhibition of p38 MAPK impairs the proliferation and anchorage-independent growth of some cancer cell lines (data not shown). Thus, whereas p38α can negatively regulate tumor initiation by triggering apoptosis in response to oncogene-induced ROS, the overall effect of p38 MAPK inhibition for human cancer is likely to be highly dependent on the tumor type and cancer stage.

Experimental Procedures

Cell culture

WT and p38α$^{-/-}$ primary MEFs were derived from E11.5 and E12.5 embryos (Ambrosino et al., 2003). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin (all from GIBCO-Invitrogen) and were immortalized by following the 3T3 protocol. The established cell lines represent pools of at least 100 independent clones. NIH3T3 mouse fibroblasts were maintained under subconfluent conditions in DMEM, as described above; however, in this case, DMEM was supplemented with 10% heat-inactivated newborn calf serum (NBCS). When used for focus formation assays, NBCS was reduced to 5%. The HEK293-derived, virus-packaging cell lines Ampho-Pak and 293T were obtained from Clontech and M. Serrano (CNIO), respectively. The human cancer cell lines DU145 and HT-29 were kindly provided by M. Robledo and J. Bravo (CNIO), respectively. The remaining cancer cell lines were a kind gift from A. Muñoz (Biomedical Research Institute Alberto Sols, Madrid). All human cancer cell lines were cultured in DMEM with 10% FBS.

Retroviral infections

Expression constructs are described in Supplemental Data. Retroviruses were produced in 293T cells by transient transfection. Culture supernatants were collected 48 h (first supernatant) and 72 h (second supernatant) post-transfection, filtered (0.45 μm filter, PVDF, Millipore), and supplemented with 4 μg/ml polybrene (Sigma). MEFs at ~$5x10^5$ cells per 10-cm dish were infected with 6 ml of the first supernatant, supplemented 24 h later with 3 ml of the second supernatant, and purified 48 h post-infection with either 1-2 μg/ml puromycin for 1 week or 150-200 μg/ml hygromycin for 2 weeks. Pools of at least 10^5 independent clones were normally used. The data for H-RasV12, B-RafV599E, c-SrcY527F, SV40 LT-Ag, and v-Jun are representative of studies in which at least three different cell populations that were independently isolated were used. For other oncogenes, pools were isolated independently twice.

For the rescue experiments, two rounds of retroviral infection were performed as previously described (Ambrosino et al., 2003). MSCV or MSCV-p38α was first expressed in p38α$^{-/-}$ MEFs, followed by transduction with H-RasV12.
Retroviral transduction of MCF7 and SW620 human cancer cells with Myc-tagged Gstm2 was performed as described above, except that 293 Ampho-Pak packaging cells were used instead of 293T cells. Antibiotic selection was carried out with 100-150 μg/ml hygromycin for MCF7 and 250-300 μg/ml hygromycin for SW620.

Transformation and tumorigenicity assays
NIH3T3-based focus formation assays and anchorage-independent growth in soft agar were performed by following standard procedures.
Tumorigenicity assays in nude mice were performed in accordance with institutional guidelines (EMBL Animal Care and Use Committee). Details are provided in Supplemental Data.
For the focus formation assays with immortalized MEFs, 9x10^5 WT or p38α$^{-/-}$ cells were seeded per 10-cm plate and were infected with the virus-containing supernatants from 293 Ampho-Pak cells transfected with oncogene-encoding retroviral vectors. Cells were maintained in DMEM with 10% FBS, and the medium was changed every 2-3 days. Foci were counted 10-15 days after transduction.
The in vitro invasion assays were carried out in BD BioCoat Matrigel chambers (Becton Dickinson) as described in Supplemental Data.

Determination of intracellular ROS levels
To visualize intracellular ROS levels, proliferating cells were grown on coverslips, washed once with warm PBS, and incubated with 10 μM 2′-7′-dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes D399) in warm PBS supplemented with 5.5 mM glucose. After 10 min at 37ºC, PBS was replaced with complete culture medium, and cells were incubated for an additional 10-15 min, washed once again with warm PBS, and fixed in 4% formalin (Sigma). Coverslips were incubated with 1 μM 4,6-diamidino-2-phenylindole (DAPI) for nuclei staining (Sigma) and were mounted in Mowiol (Calbiochem), and intracellular ROS levels were visualized by using an inverted fluorescence microscope, Leica DM5000B, coupled to a Leica DC500 camera. Pictures were taken at 63x magnification with the Leica IM50 software. Where indicated, cells were pretreated for 12-16 h with rotenone (R8875, Sigma), DPI (D2926, Sigma), LY294002 (Calbiochem), NSC23766 (Calbiochem), or PD98059 (Calbiochem) before ROS visualization.
For ROS quantification, cells were treated as described above with 10 μM DCF-DA, trypsinized, and analyzed by FACS as described (Nicke et al., 2005).

Immunoblot analysis
Cell lysates were prepared as described (Alonso et al., 2000), separated by SDS-PAGE, and analyzed by immunoblotting by using the Odyssey Infrared Imaging System (Li-Cor, Biosciences). Details on the procedure and antibodies used are described in Supplemental Data.

Cell treatments and assays for survival and proliferation
To induce p38 MAPK activation, cells were treated with 1-5 mM H_2O_2 (Sigma) for 5 min to 5 h, 0.4 M sorbitol (Sigma) for 6 h, and 25 μM cisplatin (Sigma) for 8-10 h or UV stimulated by using a Stratalinker apparatus, followed by 30 min in the 37ºC incubator.
Cell proliferation assays were performed by using the MTT cell proliferation Kit I (Roche Diagnostics, Mannheim, Germany). For proliferation assays with MEFs, 1000 cells/well were seeded in triplicate, and cell numbers were monitored during the course of 5 days. Experiments were repeated at least three times.

For apoptosis assays with MEFs expressing the OHT-inducible ERHRasV12 construct, 8000 cells/well were seeded in triplicate, and apoptosis was measured with the Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics GmbH, Germany). Experiments were performed twice.

Knock-down of Gstm1 and Gstm2 by siRNA

Human *gstm1* and *gstm2* as well as control (siGLO) siRNA oligos were obtained from Dharmacon. MDA-MB-231 and A549 cells were transfected with 200 nM and 150 nM *gstm1* siRNA, respectively. MCF7 cells were transfected with 100 nM *gstm2* siRNA. In all cases, DharmaFECT1 buffer was used (Dharmacon). After 3 days, cells were scraped, and the lysates were analyzed by immunoblot.

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Supplemental Data

Supplementary Figures

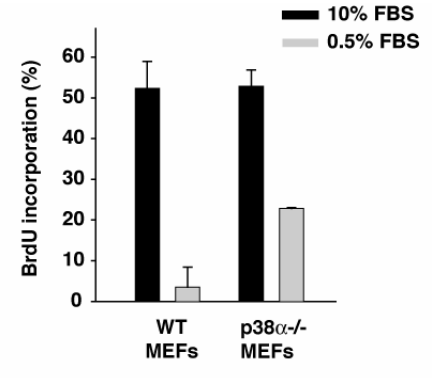

Figure S1. p38α negatively regulates MEF proliferation in low-serum conditions
BrdU incorporation was quantified in serum starved (0.5% FBS) WT and p38α$^{-/-}$ MEFs, as well as following stimulation with 10% FBS. Error bars = SD.

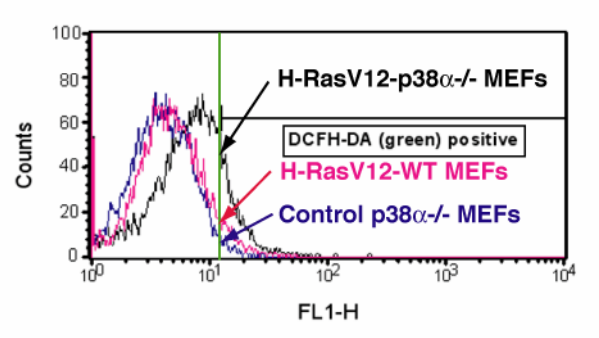

Figure S2. WT and p38α$^{-/-}$ MEFs stably expressing H-RasV12 were analyzed for intracellular ROS levels by FACS analysis.

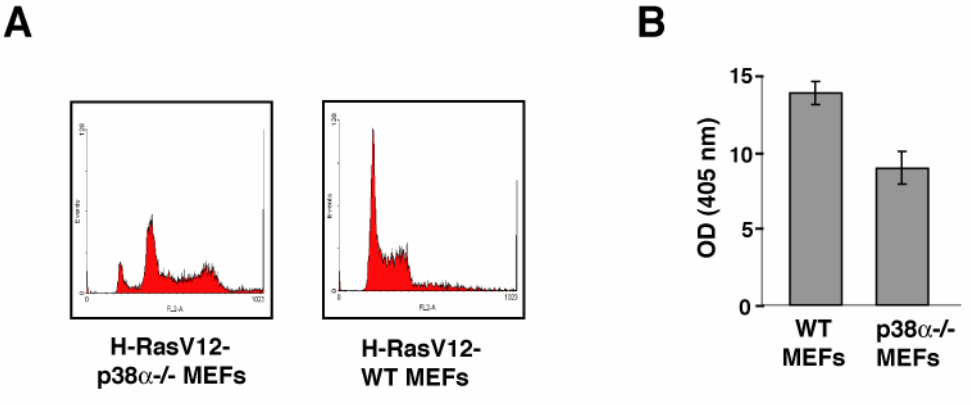

Figure S3. WT and p38α$^{-/-}$ MEFs stably expressing H-RasV12 were analyzed by flow cytometry analysis **(A)**. Intracellular protein phosphatase activity levels were measured on pNPP **(B)**. Error bars = SD.

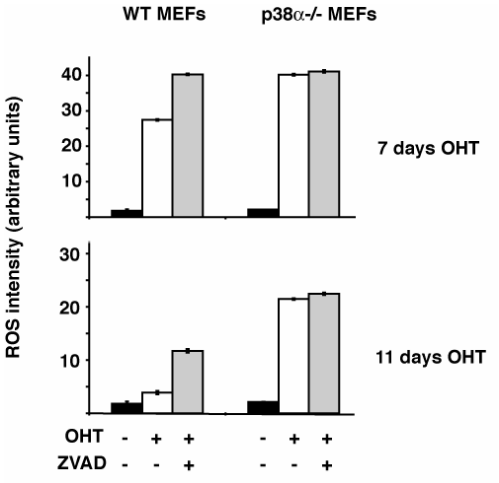

Figure S4. WT and p38α$^{-/-}$ MEFs were induced with 1 μM OHT for the indicated times, with simultaneous incubation in the presence of 25 μM ZVAD-fmk during the last 3 days of OHT treatment.
ROS levels were quantified by FACS immediately after cell collection as described in Methods. Error bars = SD.

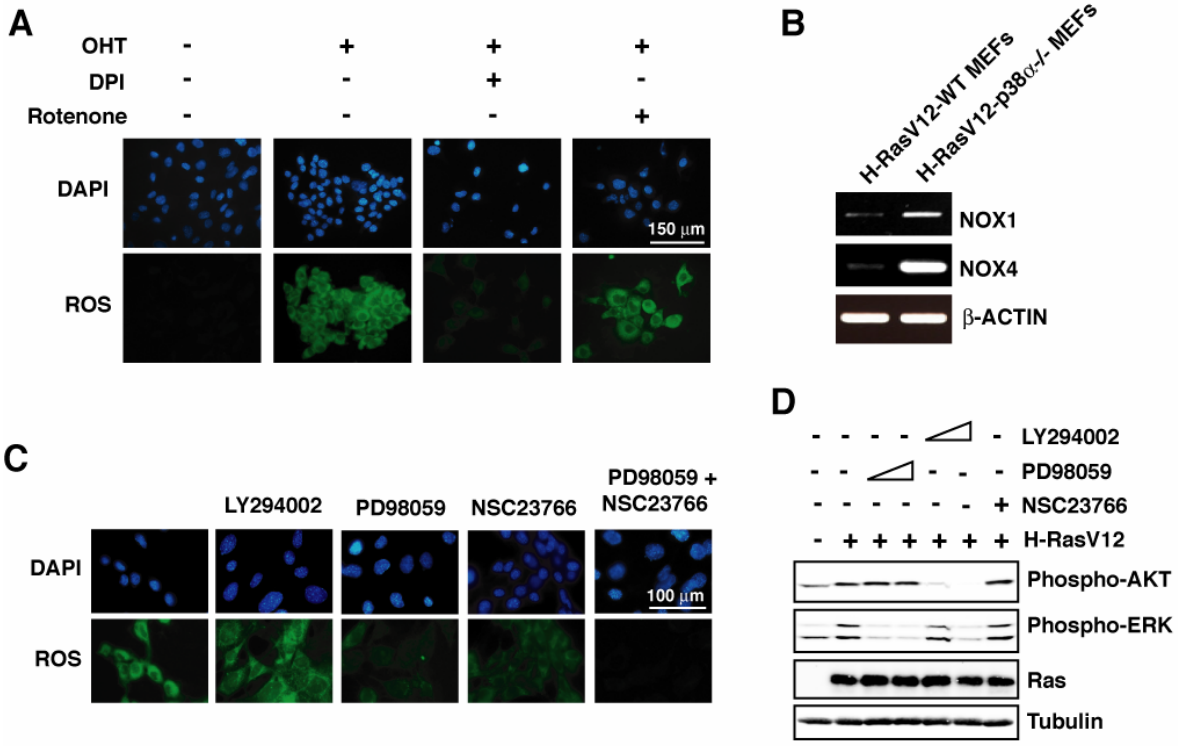

Figure S5. H-RasV12-induced ROS production involves the Rac1 and ERK pathways and is mediated by NADPH oxidases
(A, C) ER-HRasV12-expressing p38α$^{-/-}$ MEFs were treated with 1 μM OHT for 48 h and intracellular ROS levels were visualized by immunofluorescence. Where indicated, samples were also treated for the last 12-16 h with rotenone (1 μg/ml), DPI (2.5 μM), the PI3K inhibitor LY294002 (LY, 20 μM), the MEK inhibitor PD98059 (PD, 20 μM), the Rac1 inhibitor NSC23766 (NSC, 50 μM) or a combination of NSC23766 and PD98059.
(B) *Nox1* and *Nox4* mRNA levels were analyzed by RT-PCR in established H-RasV12-expressing WT and p38α$^{-/-}$ MEFs.
(D) H-RasV12-expressing p38α$^{-/-}$ MEFs were incubated for 2 h with LY294002 (20 and 30 μM), PD98059 (15 and 20 μM), and NSC23766 (50 μM), and were then analyzed by immunoblotting with the indicated antibodies.

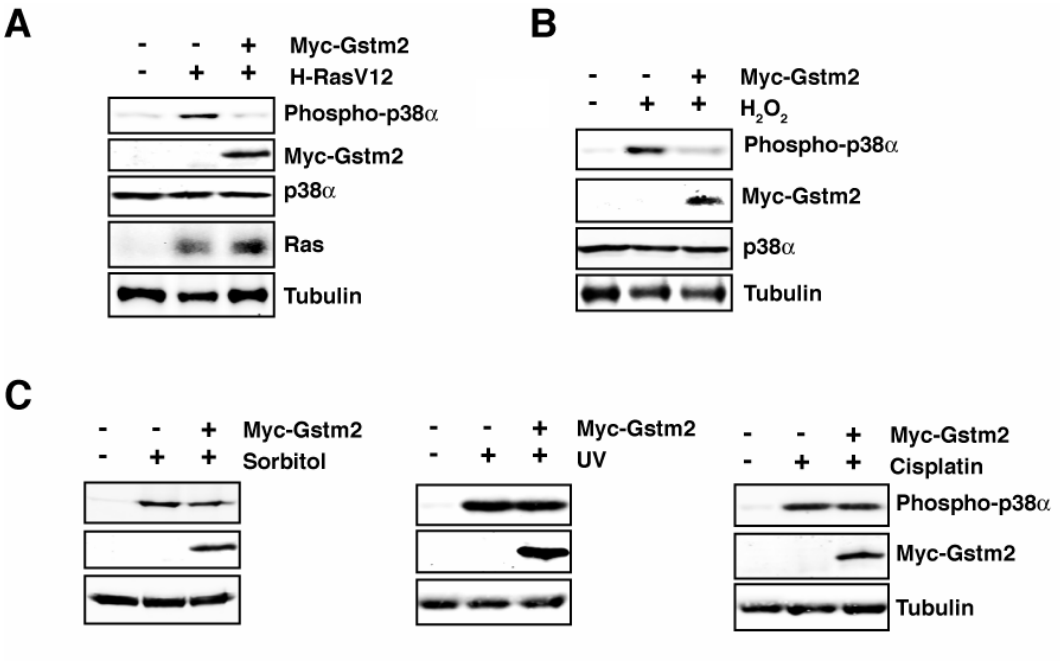

Figure S6. Gstm2 overexpression selectively impairs the activation of p38α by oxidative stress
(A) HEK293 cells were transfected with H-RasV12, either alone or together with Myc-Gstm2, and 48 h later were analyzed by immunoblotting with the indicated antibodies.
(B-C) HEK293 cells were transfected with either Myc-Gstm2 or an empty vector and 48 h later were treated with 5 mM H_2O_2 for 30 min **(B)**, 0.4 M sorbitol for 6 h **(C, left)**, UV irradiation followed by 30 min incubation at 37ºC **(C**, middle**)**, and 25 μM cisplatin for 8 h **(C**, right**)**, and then analyzed by immunoblot with the indicated antibodies.

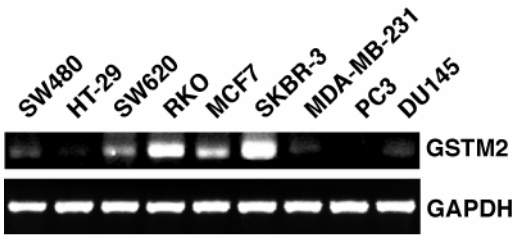

Figure S7. *gstm2* (NM_000848) expression was analyzed by RT-PCR in several human cancer cell lines.

Supplemental Experimental Procedures

Expression constructs

The following plasmids were used for the generation of retroviruses: pBabe (Morgenstern and Land, 1990), pLPC (the gene of interest is expressed from the CMV promoter and the gene for puromycin resistance is driven by an LTR), pWZL (co-expresses the gene of interest with a selectable marker translated from an internal ribosomal entry site) and pZIPneo (Jat et al., 1986). pBabe-Puro-based vectors encoding H-RasV12, N-RasV12 and K-RasV12 4A and 4B, a pZIPneo derivative containing the SV40 LT-Ag, as well as pLPC-Hygro-based vectors encoding Raf-1 22W (a Raf-1 mutant lacking 305 N-terminal residues) (Stanton et al., 1989), Rac1 N115I (Khosravi-Far et al., 1995), and RalGDS-CAAX (Matsubara et al., 1999), were kindly provided by M. Serrano (CNIO, Madrid). RSV-vJun and pcDNA3-cMyc, were kind gifts from P. Angel (DKFZ, Heidelberg) and M. Eilers (University of Marburg, Germany), respectively, and were subsequently cloned as BamHI-EcoRI fragments into pBabe-Puro. pSGT-Src527 expressing an activated form of chicken c-Src with the mutation Y527F was kindly provided by G. Superti-

Furga (CeMM Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna) and cloned as a SalI-SalI fragment into pBabe-Puro. R. Marais (The Institute of Cancer Research, London) kindly provided us with the B-Raf-encoding cDNA containing the activating mutation V599E, which we cloned as a BamHI-SalI fragment into pBabe-Puro after removing an internal BamHI site by site-directed mutagenesis (QuikChange, Stratagene). An additional step of mutagenesis was required after cloning to introduce a start codon. The pBabe-Puro-NeuT vector, expressing the rat Erbb2 cDNA with the activating mutation V664E, was kindly provided by P. Sicinski (Dana Farber Cancer Institute, Boston). Retroviral vectors expressing v-Mos and activated MEK1 were kind gifts from G. Vande Woude (Van Andel Research Institute) and N. Ahn (University of Colorado), respectively. A pCDNA3.1-based construct encoding HA-tagged human ASK1 (Morita et al., 2001) was kindly provided by H. Ichijo (The University of Tokyo). To re-express p38α MAPK in p38α-deficient mouse fibroblasts, we used the retroviral construct MSCV-p38α (Ambrosino et al., 2003). pBabe-Puro-ER-HRasV12 was used to develop the OHT-inducible H-RasV12 system (De Vita et al., 2005). pEFmlink-MKK6DD (Alonso et al., 2000) has been previously described. A mouse Gstm2 cDNA was obtained from the RIKEN cDNA collection (AK002845), PCR amplified with the primers 5´-cgGGATCCatgcctatgacactaggttac-3´ and 5´-cgGAATTCctactttgggttccaaaaggc-3´ and cloned as a BamHI-EcoRI fragment into pWZLHygro. A Myc-tagged Gstm2 derivative (pWZL-Myc-Gstm2) was prepared by site-directed mutagenesis (QuikChange, Stratagene) and used to express Myc-Gstm2 in human cancer cell lines. Myc-Gstm2 was also subcloned BamHI-EcoRI into pCDNA3.1 to generate the construct pCDNA-Myc-Gstm2, which was used for transient transfection experiments in HEK293 and 293-T cells.

Transformation and tumorigenicity assays

For NIH3T3-based focus formation assays, approximately $8x10^5$ cells were plated per 10-cm dish, and the medium was changed the following day. Four hours later, cells were transfected by calcium phosphate co-precipitation with 2-3 μg of oncogene-encoding plasmid in combination with 7-8 μg of either pEFmlink alone or pEFmlink-MKK6DD. Medium was changed after overnight incubation with the precipitates, and 48 h later plates were fed with 5% NBCS-containing medium. Medium was subsequently changed every 3-4 days. Foci were counted after 3 weeks following fixation with 4% formalin (F5554, Sigma) and Giemsa staining (Merck). Experiments were performed in duplicates and repeated at least twice.

To measure anchorage-independent growth in soft agar, cells were cultured in 6-cm plates previously covered in soft agar bottom layer (DMEM with 0.5% agar, 1% pen/strep, 1% L-glutamine and 10% fetal bovine serum). The top layer contained $5x10^4$ cells in DMEM with 0.35% agar, 1% pen/strep, 1% L-glutamine and 10% fetal bovine serum. Medium was added to the top layer to prevent drying of the agarose gel. After 14 days, colonies were stained with 0.1% crystal violet (Sigma), and colonies bigger than 0.2 mm were counted. Pictures of representative fields were taken at 40x magnification with a Leica MZ6 microscope coupled to a RS Photometrics camera. Colony formation assays were always measured in duplicates.

For the tumorigenicity assays in nude mice, $2x10^5$ empty vector- or H-RasV12- expressing $p38\alpha^{-/-}$ MEFs were injected subcutaneously in triplicates into the right flank of six 10-week-old immunodepleted female mice. H-RasV12-WT MEFs were injected on the left side of all 6 animals. Tumor growth was monitored for two weeks, but mice were killed when tumor size reached 500 mm^3.

Matrigel invasion assay

The in vitro invasion assays were carried out in BD BioCoat Matrigel chambers (Becton Dickinson). After re-hydrating the chambers for 2 h in the tissue culture incubator, $4x10^5$ cells in 2 ml of DMEM with 10% FBS were added into each of the upper chambers in duplicates. After 20 h of incubation, the non-invading cells on the upper side of the chamber membranes were

removed. The invading cells to the opposite side of the chamber membranes were fixed with 4% formalin (Sigma) for 15 min at RT and stained with 0.1% crystal violet (Sigma) for 10 min. Then, chambers were washed several times by immersion in a large beaker filled with distilled water and let to dry at room temperature. For ROS modulation in MDA-MB-231 cells, $2x10^5$ cells were incubated during the time of the experiment (20 h) with either reduced L-glutathione (G6013, Sigma), N-acetyl-L-cysteine (NAC) (A9165, Sigma) or diphenylene iodonium chloride (DPI) (D2926, Sigma). To quantify cell invasion, crystal violet-positive invading cells were extracted with 10% acetic acid and the resulting solutions were spectrophotometrically measured at 595 nm. Experiments were performed in duplicates.

Analysis of nuclear DNA content and intracellular phosphatase activity

Flow cytometry analysis was used to determine ploidy of the nuclei. Cells were washed in PBS, fixed by drop-wise addition of 1 ml of cold 70% ethanol and allowed to stand on ice for 10 min. Cell pellets were resuspended in 200 µl of PBS containing 10 µg/ml RNase A and incubated at 37°C for 30 min. Propidium iodide (200 µg/ml) was added and cell cycle profiles were obtained in a FACScan flow cytometer (Becton-Dickinson). For analysis, only signals from single nuclei were considered (10^4 nuclei/assay).

pNPP assays to measure the intracellular phosphatase activity levels were performed with 100 µg of cell lysates following the manufacturer's instructions (Enzolyte™ pNPP protein phosphatase assay kit, Anaspec). Reactions were incubated for 30 min and pNPP hydrolysis was measured at 405 nm. Data are representative of two independent replica experiments performed in duplicates.

RT-PCR analysis

Total RNAs were extracted from exponentially proliferating cell cultures using the RNeasy Kit (Qiagen) according to manufacturer´s instructions. cDNA was prepared using M-MLV reverse transcriptase (Invitrogen). Primers for PCR amplification of mouse *Nox1* (NM_172203) and *Nox4* (NM_015760), as well as of human *gstm2* (NM_000848) have been already published (Ebert et al., 2003; Wingler et al., 2001). For human *gstm1* (NM_000561), the primers 5´-ccatgatactggggtactgg-3´ and 5´-aaatatacggtggaggtcaagg-3´ were used. PCR primers were always used at a final concentration of 160 nM and RT-PCR conditions were as follows: reverse transcription at 37ºC for 90 min, denaturation at 95ºC for 2 min, followed by 30-35 cycles of denaturation at 95ºC for 45 s, annealing at 55ºC for 45 s and elongation at 72ºC for 60 s. A final step of elongation at 72ºC for 10 min was performed. The products of the RT-PCR reaction were analyzed by 1% agarose gel electrophoresis.

Quantitative Real-Time PCR (qRT-PCR) analysis was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems) to confirm the downregulation of *gstm2* in MCF7 cells. Total RNA from control or *gstm2* siRNA-transfected MCF7 cells (1 µg) was reverse transcribed, diluted 1:10 and amplified in triplicates by PCR (40 cycles) with the primers 5´-atgggggacgctcctgact-3´ and 5´-aagtcagggctgtagcaaac-3´ using 2X SYBR GreenER qPCR SuperMix (Invitrogen) in a final volume of 20 µl. Relative quantities (Δ cycle threshold values) were obtained by normalizing against *gapdh*.

Immunoblot Analysis

For preparation of cell lysates, subconfluent cell cultures were washed twice with ice-cold PBS, scraped on ice and harvested by centrifugation at 400x*g* and 4ºC for 10 min in a refrigerated table-top centrifuge (Eppendorf). The resulting pellets were stored at -80ºC until required or immediately resuspended in 100-600 µl of IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2.5 mM benzamidine, 2 µM microcystin and 10 µg/ml of leupeptin and aprotinin). The suspension was vortexed, incubated on ice for 10 min, and centrifuged for 10 min at 16000×*g* and 4ºC to remove insoluble material. Protein lysates were quantified by the Bradford assay

using BSA as standard (Protein Assay Kit, Bio-Rad), and 40-70 μg of total protein were resolved by SDS-PAGE. Proteins were electro-transferred to NC membranes (Protran, Schleicher & Schuell) using semi-dry (Hoefer TE 77, GE Healthcare) or wet transfer (Trans-well Blot®, Bio-Rad) systems. Membranes were blocked with 5% non-fat dry milk in PBS for 1 h at RT and incubated with the following antibodies: c-Myc (9E10, Santa Cruz Biotechnology), p38 MAPK (C20-G, Santa Cruz Biotechnology), ERK1 (C16, Santa Cruz Biotechnology), JNK1 (FL, Santa Cruz Biotechnology), Ras (clone 18, BD Transduction Laboratories), ER-α (MC20, Santa Cruz Biotechnology), cyclin D1 (A-12, Santa Cruz Biotechnology), murine p53 (Ab-3, Calbiochem), p16Ink4a (M-156, Santa Cruz Biotechnology), murine p19ARF (ab80, Abcam), Gstm1 (C15, Santa Cruz Biotechnology), PARP p85 fragment (G7341, Promega), tubulin (clone DM1A, Sigma), MKK6 (home-made polyclonal), phospho-MKK6 (9231, Cell Signaling Technology), phospho-p38 (9211, Cell Signaling Technology), phosphorylated JNK (9251, Cell Signaling Technology), phospho-ERK (9101, Cell Signaling Technology), phospho-MAPKAP-K2 (3041, Cell Signaling Technology) and phospho-AKT (9271, Cell Signaling Technology).
After washing out the primary antibodies, membranes were incubated with Alexa Fluor 680-conjugated secondary antibodies (Molecular Probes) for 1h at RT and visualized and quantified using the Odyssey Infrared Imaging System (Li-Cor, Biosciences).
For immunoblotting with cleaved Caspase-3 antibodies (9661, Cell Signaling Technology) a different lysis buffer was used as previously described (Porras et al., 2004).

Coimmunoprecipitation of ASK1 and Gstm2

To test the physical interaction between ASK1 and Gstm2, HA-tagged human ASK1 and Myc-tagged mouse Gstm2 were ectopically expressed in 293-T cells in a 3:7 ratio. 48 h later, cells were collected and lysed as previously described (Cho et al., 2001). Protein lysates (4 mg) were adjusted to a final volume of 500 μl with lysis buffer and incubated with 2 μg of a Myc polyclonal antibody (ab9106, ABCAM) for 1 h at 4ºC. The immunocomplexes were recovered by incubation with protein G-sepharose 4B beads (Amershan) for 15 min at 4ºC in a rotating wheel, washed 3 times with lysis buffer and analyzed by SDS-PAGE. The HA immunoblot was performed using a rat polyclonal antibody (Roche).

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Proteomic analysis of p38α MAP kinase-regulated changes in membrane fractions of Ras-transformed fibroblasts

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Abstract

Oncogenic Ras signalling has been long known to play an important role in tumorigenesis and human cancer. In this report, we have used the sensitive 2D differential gel electrophoresis (DIGE) coupled to mass spectrometry for the identification of proteins differentially expressed at the cell membrane level between oncogenic H-RasV12-transformed wild type and p38α-deficient mouse embryo fibroblasts (MEFs). Following trifluoroethanol solubilization, a total of 76 proteins were differentially regulated. After peptide mass fingerprinting, 63 spots containing 42 different proteins were unequivocally identified by MALDI-TOF mass spectrometry coupled with data base interrogation. As expected, many of them were membrane proteins. Six proteins were selected for further validation studies based on their potential functional link with malignant transformation and signal transduction. These were prohibitin (PHB), protein disulfide isomerase 3 (PDIA3), focal adhesion kinase 2 (FAK2), c-GMP dependent protein kinase 2 (KGP2), NADH-ubiquinone oxidoreductase 30 kDa subunit (NUGM) and translationally controlled tumor protein (TCTP). All these proteins were up-regulated in the membranes of H-RasV12-transformed p38α-/-cells, except for prohibitin, which was down-regulated. An excellent correlation was found between DIGE results and western blot studies, indicating the reliability of the 2D-DIGE analysis. The available evidence about the putative function of the identified proteins supports the emerging role of p38α as a negative regulator of tumorigenesis. Further studies are in progress to elucidate the implications of these findings in the regulation of H-Ras-induced transformation by p38α signalling.

Key words: p38 MAPK, Ras, transformation, membrane fractions, 2D-DIGE, MALDI-TOF

Introduction

Oncogene-induced malignant transformation is a complex process, which profoundly affects several cellular traits, including proliferation, cell morphology and anchorage-dependent growth [1]. *ras* genes were the first oncogenes to be implicated in human cancer and have been found to be deregulated in up to 30% of human tumors [2] as well as in many cellular and animal models for malignant transformation and cancer [3].

p38α is the most abundant p38 mitogen-activated protein kinase (MAPK) family member in mammals. This family consists of four serine/threonine protein kinases, which play key roles in many cellular processes, most notably in the stress response but also in the regulation of proliferation, differentiation and survival of various cell types [4]. In addition, emerging evidence indicates that reduced p38 MAPK activity may facilitate H-Ras-induced malignant transformation [5]. Several mechanisms have been proposed to explain the putative tumor suppressor role of p38 MAPK, including an inhibitory effect on the ERK pathway [6], the induction of a p53-dependent cell cycle arrest [7], an irreversible process of premature senescence [8] and the modulation of the apoptotic response [9].

We were interested in the role of p38 MAP kinase signalling in oncogenic H-Ras-induced malignant transformation. We have found that p38α-/- mouse embryonic fibroblasts (MEFs) have normal fibroblast morphology and similar exponential proliferation rates to wild type (WT) cells. Moreover, p38α-/- MEFs do not form foci in over-confluent conditions and are unable to grow in soft agar. However, upon expression of oncogenic H-RasV12, p38α-deficient fibroblasts show a more dramatic transformed phenotype, as determined by several assays such as loss of contact inhibition [10] and growth in soft agar (Dolado et al., manuscript in preparation). As these processes are likely to be modulated by membrane proteins [11], our observations suggested that p38α may influence either the signal relay or the protein composition of cell membranes.

Fluorescent two-dimensional differential gel electrophoresis (2D-DIGE) is becoming a major tool to assess changes in protein expression profiles of cells or whole tissues. Even minor differences in protein expression can be detected and quantified across multiple samples with statistical confidence. The comparison of spot intensities using fluorescent labelling, in combination with the DeCyder software, is more objective than the conventional approach, which is based on the comparison of spot-brightness between different gel images obtained by silver or Coomassie staining [12].

Here, we report the application of 2D-DIGE analysis for the proteomic characterization of membrane fractions from H-RasV12-transformed WT and p38α-deficient MEFs. Several differentially expressed membrane and membrane-associated proteins were identified that may play a role in the regulation by p38α of H-RasV12-induced malignant transformation.

Materials and Methods

Cell culture and membrane protein extraction

H-RasV12-expressing WT and p38α-/- MEFs were generated by retroviral transduction (Dolado *et al*., manuscript in preparation) following spontaneous immortalization of MEFs according to the 3T3 protocol [13]. $1.5x10^6$ cells were seeded on 15-cm Falcon tissue culture dishes (BD Biosciences) and grown for 2 days in DMEM supplemented with 10% foetal bovine serum (Invitrogen), 1% L-glutamine (Invitrogen) and 1% penicillin-streptomycin (GIBCO). H-RasV12-expressing MEFs were exponentially proliferating, with a confluence of about 80% at the time of subcellular fractionation. Cells were washed twice with ice-cold PBS, scraped on ice and harvested by centrifugation at 400xg and 4ºC for 10 min. The resulting pellets were resuspended in 2 ml of isotonic lysis buffer (20 mM Tris-HCl pH 7.5, 125 mM NaCl, 1 mM $MgCl_2$, 25 mM

β-glycerolphosphate, 20 mM NaF, 0.1 mM sodium vanadate, 1 mM PMSF, 2.5 mM benzamidine, 2 μM microcystin, 10 μg/ml leupeptin and 10 μg/ml aprotinin) and membranes were disrupted by sequential instant-freezing in liquid nitrogen followed by sonication with a Branson Sonifier 250 (Branson Ultrasonics Corporation). After initial centrifugation at 400xg and 4ºC for 10 min to discard unbroken cells and cell debris, cell membrane fractions were centrifuged at 16100xg and 4ºC for 15 min. The resulting pellets, composed of plasma membranes and other subcellular membranes, were washed twice with ice-cold isotonic lysis buffer and extracted in solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS and 25 mM Tris-HCl pH 8.2). Typically, about 600 μg of proteins were obtained from a 15-cm tissue culture plate of MEFs. For total extracts, parallel MEF plates grown under the same culture and confluence conditions were washed twice with ice-cold PBS, scraped on ice, harvested by centrifugation at 400xg and 4ºC for 10 min and lysed with IP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EGTA, 5 mM EDTA, 20 mM NaF, 0.1 mM sodium vanadate, 1 mM PMSF, 2.5 mM benzamidine, 2 μM microcystin, 10 μg/ml leupeptin and 10 μg/ml aprotinin). All protein extracts were stored at -80ºC until required. Three different replica experiments were carried out. Two of them were used for the identification of membrane proteins differentially expressed between H-RasV12-transduced WT and p38α-/- MEFs. The third experiment was used for western blot validation of six selected candidates (see 3.5).

D-DIGE

Protein extracts were labelled according to the manufacturer´s instructions (GE Healthcare). Briefly, 50 μg of membrane fractions from H-RasV12-transduced WT and p38α-/- MEFs (from now on WT and p38α-/- for simplicity) were minimally labelled with 400 pmol of Cy3 or Cy5 fluorescent dye on ice for 30 min in the dark. The labelling reaction was quenched with 0.2 mM lysine. An internal pool was generated by combining equal amounts of WT and p38α-/- extracts. This pool was labelled with Cy2 fluorescent dye and was included in all gel runs. Following the labelling reaction, WT and p38α-/- extracts, together with a pool aliquot, were mixed and run in a single gel. 50% trifluoroethanol (TFE) was added to the rehydration buffer to increase the solubility of the membrane proteins and to improve the IEF [14]. Samples were focused using broad-range (pH 3-11, 24 cm) IPG strips (GE, Healthcare). DeStreak™ reagent was used, instead of conventional reducing agents, to avoid unspecific oxidations of basic proteins. IPG strips were equilibrated for 15 min with gentle shaking in 50 mM Tris-HCl, pH 8.8 containing 6 M urea, 4% w/v SDS, 65 mM DTT, 30% glycerol and a trace of bromophenol blue. Iodoacetamide (53 mM) was added to the second equilibration solution instead of DTT, and strips were then incubated for 15 min in this solution. Standard continuous SDS-PAGE for the second dimension (12%) was carried out at 15 mA per gel for 16 h.

Proteins were visualized by using a fluorescence scanner at appropriate wavelengths for the Cy2, Cy3 and Cy5 dyes (Typhoon 9400™, GE Healthcare). Total protein was detected by post-staining of the gels with Sypro Ruby (Molecular Probes). Briefly, gels were first fixed in 30% methanol and 7.5% acetic acid overnight and then incubated with Sypro Ruby for 4 h. After washing three times with water, proteins were visualized by fluorescence scanning.

Image analysis was carried out with the DeCyder 5.01 software (GE Healthcare). The differential in-gel analysis (DIA) module was used for pair-wise comparisons of each WT and p38α-/- membrane extracts to the mixed standard present in each gel and for the calculation of normalized spot volume/protein abundance. Replicate gels were used to calculate average abundance changes and paired Student's t-test p-values for each protein across the four replica gels. This was done by using the DeCyder biological variation analysis (BVA) module and the Cy3:Cy2 and Cy5:Cy2 ratios for each individual protein.

Peptide mass fingerprinting by MALDI-TOF MS

Changes detected by 2D-DIGE analysis were matched with Sypro Ruby protein patterns and spots were selected for picking according to the post-stained image. Spots of interest were excised from the gel automatically using an Ettan-Picker™ robot and subjected to tryptic digestion according to a previous protocol [15] with minor variations [16]. Proteins were first reduced (10 mM DTT) and then alkylated (50 mM iodoacetic acid). Following vacuum-drying, the gel pieces were incubated with modified porcine trypsin (Promega) at a final concentration of 10 ng/μl in 50 mM ammonium bicarbonate for 16 h at 37ºC. Supernatants were collected, vacuum-dried, re-dissolved in 10 μl 0.1% TFA and 0.5 μl added onto a matrix consisting of 0.5 μl of 5 mg/ml 2-5-dihydroxybenzoic acid in water:acetonitrile (2:1) with 0.1% TFA. MALDI-TOF MS analysis of the samples was carried on a mass spectrometer Autoflex (Bruker Daltonics) in positive ion reflector mode. The ion acceleration voltage was 20 kV. Each spectrum was internally calibrated with the masses of two trypsin autolysis products. For PMF identification, the tryptic peptide mass maps were transferred through MS BioTools™ program (Bruker Daltonics) as inputs to search Swiss-Prot using Mascot software (Matrix Science). Up to one missed tryptic cleavage was considered and a mass accuracy of 50 ppm was used for all tryptic-mass searches.

Western blot analysis

Protein concentrations were determined by the Bradford assay using BSA as standard (Protein Assay Kit, BioRad). Total, cytoplasmic and membrane protein extracts (50 μg) were mixed with 4x SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue) and resolved by SDS-PAGE in 12% acrylamide gels. Proteins were detected immunologically following semi-dry electrotransfer (Hoefer TE 77, GE Healthcare) onto nitrocellulose membranes (PROTRAN, Schleicher & Schuell). The membranes were blocked with 5% non-fat dry milk in PBS for 1 h at room temperature and incubated overnight at 4ºC with the following primary antibodies: anti-cGKII (1:100, Santa Cruz, polyclonal) for KGP2, anti-HRF (1:250, BD Transduction Laboratories, polyclonal) for TCTP, anti-PHB (1:200, Neomarkers, polyclonal), anti-ERp57 (1:200, Stressgen, polyclonal) for PDIA3, anti-PYK2/CAKβ (1:500, BD Transduction Laboratories, monoclonal) for FAK2, anti-OxPhos (1:200, Molecular Probes, monoclonal) for NUGM, anti-p38 (1:1000, Santa Cruz, polyclonal), anti-Ras (1:500, BD Transduction Laboratories, monoclonal) and anti-GAPDH (1:1000, Santa Cruz, polyclonal). After washing out the primary antibodies, membranes were incubated with Alexa Fluor 680 conjugated secondary antibodies (Molecular Probes) for 1 h at room temperature and blots were visualized and quantified using the Odyssey Infrared Imaging System (Li-Cor, Biosciences).

Results and Discussion

Morphological alterations in H-RasV12-transformed WT and p38α-/- MEFs

Control WT and p38α-/- MEFs showed the typical flat morphology of fibroblasts (Fig.1A, top panels). However, upon retroviral delivery of H-RasV12, p38α-/- MEFs acquired a more spindle-shaped refringent morphology than WT MEFs (Fig.1A, bottom panels). Fig. 1B shows the expression levels of H-RasV12 and p38α in the two cell lines analyzed. The morphological changes also correlated with reduced adhesion properties and more efficient anchorage-independent growth of the H-RasV12-transformed p38α-/- MEFs (Dolado et al., in preparation). Thus, the absence of p38α correlates with more profound H-RasV12-induced alterations in morphology and cell adhesion properties, which might be important for malignant transformation [17].

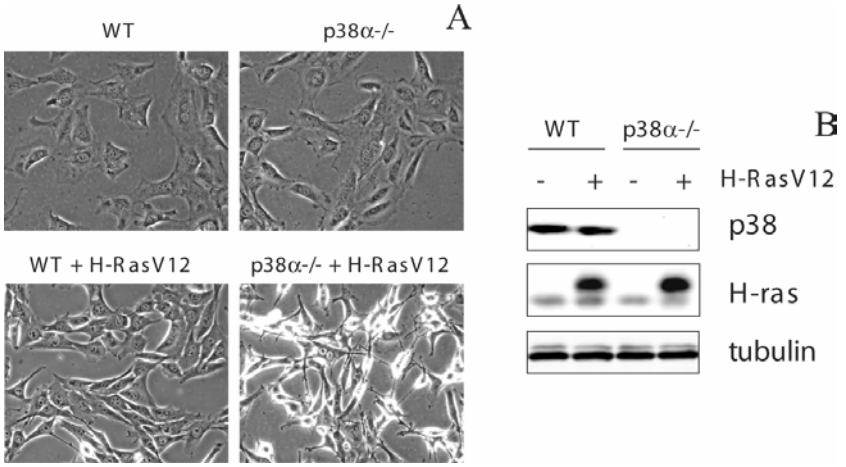

Figure 1. Enhanced transformed phenotype of p38α-deficient fibroblasts upon H-RasV12 transduction.
(A) Oncogenic H-RasV12 expressing p38α-/- cells show a more refringent morphology than wild-type fibroblast. **(B)** Expression of p38α and H-RasV12 was validated by western blotting using total lysates prepared from the cell lines in **(A)**.

Isolation of membrane fractions from H-RasV12-transformed WT and p38α-/- MEFs.

To identify potential p38α-regulated membrane proteins in the context of H-RasV12 malignant transformation, oncogenic H-RasV12-expressing WT and p38α-/- MEFs were cultured under standard conditions and membrane fractions were prepared from exponentially proliferating MEFs. Total, membrane and cytosolic fractions were separated by SDS-PAGE and analyzed by immunoblotting The results of the fractionation are shown in Fig. 2. We used EGFR, pan-Ras and caveolin-1 antibodies to confirm that membrane fractions were enriched in membrane proteins and cytosolic fractions mostly depleted of them. We also used p38 and GAPDH antibodies to show that cytosolic proteins were quantitatively recovered in the cytosolic fractions and were not detected in membrane fractions. Three completely independent replica experiments of membrane protein isolation were performed with good reproducibility. We confirmed in all cases that GAPDH was only detected in the cytosolic fractions, whereas caveolin-1 and EGFR levels were highly enriched in the membrane fractions.

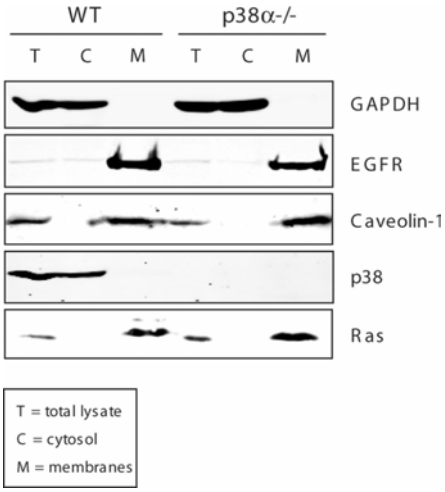

Figure 2. Isolation of membrane fractions from H-RasV12-transformed WT and p38α-/- MEFs.
Total, cytosolic and membrane fractions of exponentially proliferating H-RasV12-expressing WT and p38α-/- MEFs were analyzed by western blotting with the indicated antibodies. To assess the quality of the fractionation, GAPDH was used as a cytosolic marker and EGFR and caveolin-1 as membrane markers.

Differential protein composition between membrane fractions of H-RasV12-transformed p38α-/- and WT MEFs

Membrane fractions from two independent replicas of H-RasV12-expressing WT and p38α-/- MEFs, containing both integral membrane proteins and membrane-associated proteins, together with an internal standard control, were differentially labelled and analyzed by 2D-DIGE. Four replica gels were considered for the quantitative and statistical analysis using the DeCyder™ software. This analysis revealed changes in the abundance of 76 proteins, with a statistical variance of the p38α-/- *vs* WT spot volume ratios within the 99th confidence level (Student's T-test; $p < 0.01$). Fig. 3 shows a representative 2D gel image indicating those proteins whose expression levels changed consistently between the two biological samples. 63 out of the 76 proteins analysed were unambiguously identified by peptide mass fingerprinting (83% identification rate). These 63 spots correspond to 42 different proteins. In some cases, the same protein was identified in different spots over the gel, suggesting the occurrence of post-translational modifications. In a few other cases, two or three different proteins were identified in the same spot, probably due to the broad pH range used for the IEF. In these particular cases, the ratio obtained resulted from the combination of all the proteins present in the spot, and further experiments are necessary to really confirm changes in individual proteins. Proteins that were analysed but not identified are also indicated in Fig 3 with a circle. They correspond mainly to low molecular weight proteins.

Fig. 4 illustrates the quantitative analysis of six proteins that were chosen for further characterization. The graph view shows the average ratio of each protein (referred as the standardized Log abundance, according to the internal standard) in H-RasV12 p38α-/- and WT MEFs. The p value is also indicated. As Student's T-test was used for the statistical analysis, protein abundance differences being considered statistically significant when the T-test $p<0.05$. Due to the low number of biological replicates included, a more stringent criteria ($p<0.01$) has been used in this report for selecting significant changes in protein abundance. In all cases the T-test p values are well below this threshold. The insert shows a representative 3-D view of the abundance of each protein in the membranes of H-RasV12 p38α-/- and WT MEFs, as revealed by DIGE analysis of one of the 2-D gel replicas used for the statistical analysis.

All the spots indicated in Fig. 3 were further analysed by MALDI-TOF MS. Table 1 summarizes the 42 unique distinctly deregulated proteins that were unambiguously identified by PMF, taking into account the Mascot score, the sequence coverage and the number of matching peptides over all the searched ones. Proteins were grouped according to the biological processes in which they are likely to participate, i.e. signal transduction and cell communication, cytoskeleton, energy and metabolism, chaperone activity and transport. As expected, most of the proteins showed membrane localization, either in the inner/outer mitochondrial membranes, in the nuclear and plasma membranes or in the endoplasmic reticulum. Some are integral proteins involved in the transport of molecules across membranes, i.e. metaxin 2 (MTX2) in the mitochondria or nucleoporin N107 in the nuclear membrane. In other cases, we identified proteins associated to cell surface receptors, such as alpha-2-macroglobulin receptor-associated protein (LRPAP1) and focal adhesion kinase (FAK2). These findings exemplify how the inclusion of TFE in the rehydration buffer makes possible to analyze hydrophobic samples, such as those consisting of membrane proteins, by 2D-DIGE.

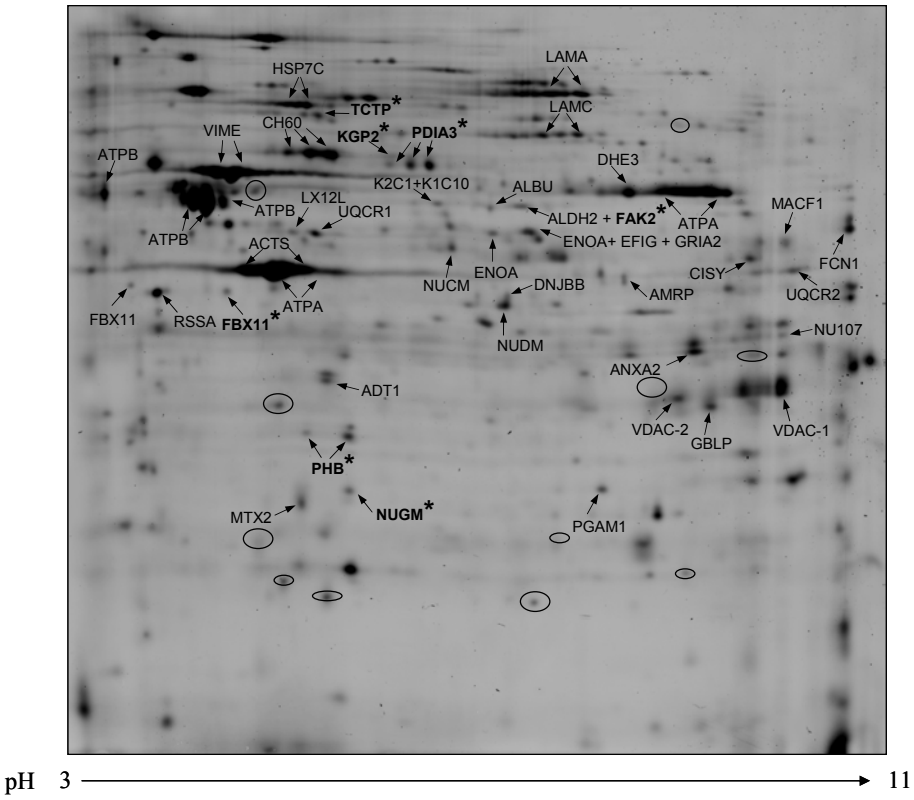

Figure 3: Image of a representative 2D-DIGE gel corresponding to the membrane proteome of proliferating H-RasV12 MEFs. Protein extracts from the membrane fractions of H-RasV12-expressing WT and p38α-/- cells were differentially labelled with Cy3 and Cy5, respectively. A mixed internal standard combining all the proteins from both extracts, labelled with an additional Cy2 dye, was included in all gels. IPG strips (pH: 3-11) were used for the IEF and standard SDS-PAGE for the second dimension. The image shows the spot map corresponding to the mixed internal standard, which is common to all gels analysed. Spots marked with a code-name indicates all those proteins identified whose abundance changes when comparing the p38α-/- *vs* the WT extracts was within a 99th confidence level. Proteins marked with an asterisk correspond to the selected proteins chosen for Western blot analysis. Proteins that were not identified by PMF are indicated with a circle.

Functional selection of membrane proteins differentially expressed in H-RasV12-transformed WT and p38α-/- MEFs

To validate the 2D-DIGE results, seven proteins were selected based on their potential connection with oncogenic transformation as well as with Ras or p38 MAPK signalling. These proteins were prohibitin (PHB), protein disulfide isomerase 3 (PDIA3), focal adhesion kinase 2 (FAK2), c-GMP dependent protein kinase 2 (KGP2), NADH-ubiquinone oxidoreductase 30 kDa subunit (NUGM), translationally-controlled tumor protein (TCTP) and F-box only protein 11 (FBX11), which probably binds to phosphorylated proteins promoting their ubiquitination and proteasome-mediated degradation.

PHB is a potential tumor suppressor protein, which was down-regulated in the membranes of H-RasV12-expressing p38α-/- cells. PHB has been reported to have antiproliferative activity and can block the G1/S cell cycle transition in several human cancer cell lines such as MCF7 [18]. A possible mechanism involves PHB binding to the retinoblastoma protein, functioning as a repressor of E2F-mediated transcription and proliferation [19]. The down-regulation of PHB in H-RasV12 transformed p38α-/- cells is consistent with the higher proliferative rate of these cells when compared with H-RasV12 WT MEFs (data not shown), suggesting that PHB and p38α MAP kinase might have a synergistic effect in the negative regulation of proliferation and tumorigenesis.

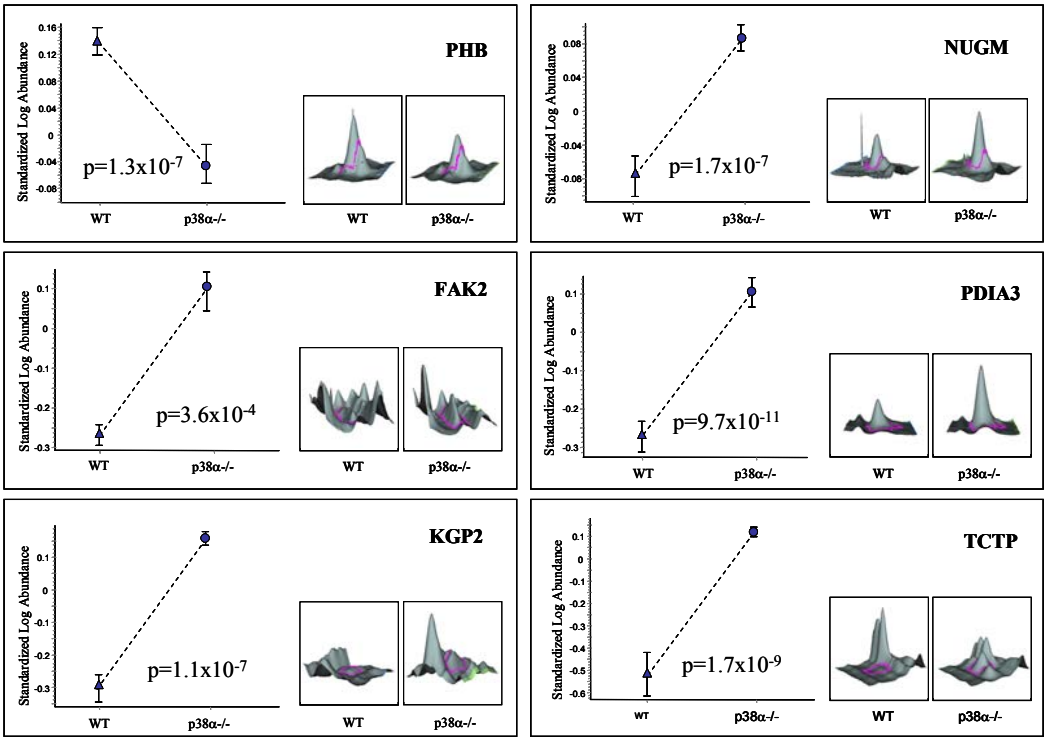

Figure 4: Differential expression of six selected proteins in membrane fractions of H-RasV12 transformed p38α-/- and WT MEFs. The graph view represents the average ratio of expression for each selected protein, as obtained by computational analysis with DeCyder™ 5.0. Statistical analysis allowed for the detection of significant abundance changes (99th confidence level) based on the variance of the mean change within the replicates included, p-values are indicated. The insert shows a representative 3-D view for each protein in a particular replica gel.

The rest of the selected proteins were all up-regulated in the membranes of p38α-/- MEFs upon H-RasV12 expression. Consistent with the stronger transformed phenotype of these cells, several of these proteins have been previously described to be up-regulated in some tumours and may be associated to tumour progression. PDIA3, for example, is also known as a 58 kDa glucose-regulatory protein, which becomes up-regulated in NIH 3T3 mouse fibroblasts transformed by Ras or Src oncogenes. PDIA3 is activated by stress and has been also found to be up-regulated in breast, uterus, lung and stomach tumors [20]. FAK2 is a cytoplasmic tyrosine kinase related to focal adhesion kinase. It can associate with tyrosine kinase receptors such as Her2 and Her3 and plays an important role in regulating the invasiveness of several cell lines derived from breast and glia cancers. FAK2 is also found over-expressed in gastrointestinal tumors [21,22]. NUGM is an NADH dehydrogenase of the mitochondrial oxidative respiratory chain complex I [23]. Its potential link with mitochondrial oxidative stress production makes NUGM an interesting putative p38α-regulated protein due to the known co-operation between oxidative stress and oncogenic signalling in malignant transformation and cancer [24-26]. KGP2, also called PRKG2, is a c-GMP dependent protein kinase type II [27], which has been shown to be involved in brain tumor progression driven by retroviral insertional mutagenesis in mice [28]. Finally, TCTP, also known as histamine-releasing factor or HRF, is a microtubule-stabilizing cytoplasmic protein, which associates to membranes. TCTP has been implicated in many important cellular processes, such as cell growth, cell cycle control, malignant transformation and the modulation of the stress and apoptotic responses [29]. In particular, TCTP has been reported to be stabilized by the anti-apoptotic protein MCL1 [30]. The down-regulation of TCTP in H-RasV12 WT cells might indicate some degree of antagonism between its anti-apoptotic activity and the well-established

pro-apoptotic activity of p38α under many stressful stimuli [31]. The accumulating evidence for a negative role of p38α in tumorigenesis [5], suggest that the genetic inactivation of this signalling pathway in p38α-/- MEFs might be responsible for the increased membrane localization of all these tumour progression-related proteins, thus facilitating oncogenic transformation.

Validation by immunoblotting of the differential protein expression data obtained by 2D-DIGE in the membrane fractions of H-RasV12-transformed MEFs

All DIGE results for the selected proteins were validated by immunoblotting using specific commercial antibodies. The same subcellular fractions previously described (see Figure 2) were used to test membrane expression levels of PHB, NUGM, FAK2, PDA3, KGP2 and TCTP. The only protein that could not be confirmed by immunoblotting due to the lack of available commercial antibodies was FBX11. Fig. 5 illustrates the results together with the numerical values for the variation ratio, obtained by densitometric analysis of the membrane fractions immunoblot. Interestingly, the expression changes were mainly observed in the membrane protein fraction, except for PDIA3 and TCTP, which is in good agreement with their main cytoplasmic location. Membrane-located PHB displayed two different bands, which might correspond to two different phosphorylation states. This finding and its functional implications would require further studies. In all the proteins analysed there was an excellent quantitative correlation between DIGE and western blot ratios in the membrane fraction. However, little differences in expression were observed in the total extracts, which stress the importance of sub-cellular fractionation for the analysis of proteome profiles. This is consistent with recent publications suggesting the cumuli of proteins in particular cellular location as a mechanism to control protein function [32,33].

Concluding Remarks

The majority of the described mechanisms by which oncogenic Ras drives cellular transformation occur at the cytosolic level, a context where p38 MAPK has already been shown to have inhibitory roles [5-9]. However, it is also known that oncogenic Ras contributes to cellular transformation in many other ways. There is evidence, for example, that oncogenic Ras can down-regulate the expression levels of several membrane proteins, such as caveolin-1 and $\alpha_5\beta_1$-integrin, which positively correlates with enhanced transformation and strong alterations in cell growth, morphology, adhesion and cytoskeleton organization [34-36]. Consistent with this, we have observed that H-RasV12-transformed p38α-deficient MEFs have lower β_1-integrin levels than H-RasV12-transformed WT MEFs (unpublished observations), suggesting that p38α may modulate some of these H-RasV12-induced alterations in membrane proteins expression levels that could be important for transformation. With this hypothesis in mind, we have used 2D-DIGE coupled with MALDI-TOF and data base interrogation to identify integral-to-membrane or membrane-associated proteins potentially connected with Ras transformation and whose membrane abundance appears to be regulated by p38α.

The identified proteins include both integral-membrane proteins located in different membrane compartments and cytoplasmic membrane-associated proteins. Some of these proteins were found in mitochondrial membranes or endoplasmic reticulum. Interestingly, mitochondria carry out multiple cellular functions, including energy production, cell proliferation and apoptosis. Moreover, a role for mitochondria in maintaining genomic stability, an important element in cancer progression [25], has been established [37]. It will be interesting to further investigate the role of these mitochondrial proteins in the interplay between p38α MAPK signalling and H-RasV12-induced cellular transformation and tumour growth. In general, the proteins identified were involved in energy or metabolism processes, structure maintenance (i.e. nuclear envelope lamin), signal transduction (i.e. tyrosine kinases and known kinase substrates) or the ubiquitin-

Table 1. Differentially expressed proteins between p38α-/- and WT cells after oncogenic H-RasV12 transformation

AC[1]	Protein name	Average ratio[2]	T-*test*	Common name and function	Expected subcellular location
Signal transduction					
Q61410	KGP2	2,82	1,10E-07	c-GMP-dependent Ser/Thr protein kinase activity	Plasma membrane
P07356	ANXA2	1,27	6,20E-05	Annexin A2, Calcium-regulated membrane-binding protein	Lamina beneath plasma membrane
P68040	GBLP	-1,79	2,60E-07	Receptor of activated protein kinase C 1	Cytoplasm/Nucleous/Plasma membrane
P14206	RSSA	-1,52	3,10E-10	40S ribosomal protein	Cytoplasm/nucleolus/ribosome
Q9QVP9	FAK2[3]	2,38	0,00036	Tyr protein kinase family. FAK subfamily	Cytoplasm/membrane-associated
P67778	PHB	-1,61	1,30E-07	Prohibitin, Inhibits DNA synthesis; role in regulating proliferation	Cytoplasm/inner mitochondrial membrane
P63028	TCTP	4,32	1,70E-09	Translationally controlled tumor protein/antiapoptotic function	Cytoplasm
Q9QXZ0	MACF1	2,28	1,10E-10	Microtubule-actin crosslinking factor 1	Cytoplasm
O70165	FCN1	-1,54	5,10E-07	Ficolin 1, Defense-immunity protein activity/lectin activity	Secreted/Plasma membrane
Structural proteins					
P04104	K2C1[3]	1.52	1,50E-07	Cytokeratin 1, Structural protein, kininogen binding protein	Plasma membrane
P48678	LAMA	2.52	2,50E-09	Lamin A, Structural nuclear component; may interact with chromatin	Nuclear membrane
P11516	LAMC	1,88	8,70E-09	Lamin C, Structural nuclear component; may interact with chromatin	Nuclear membrane
P20152	VIME	-1,61	1,90E-12	Vimentin, Structural constituent of cytoskeleton	Cytoplasm/nucleolus
P68134	ACTS	-1.64	9,50E-06	Actin, Structural constituent of cytoskeleton	Cytoplasm
P02535	K1C10[3]	1.52	1,50E-07	Cytokeratin 10, Structural protein, generally associated with keratin 1	Cytoplasm
Energy and Metabolism					
P56480	ATPB	-1,5	0,00011	ATP synthase beta chain (catalytic subunit)	Mitochondrial membrane
P26443	DHE3	1,49	1,40E-06	Glutamate dehydrogenase	Mitochondrial matrix
P47738	ALDH2[3]	2,38	0,00036	Aldehide dehydrogenase	Mitochondrial matrix
Q99LC3	NUDM	1,41	3,60E-10	NADH-ubiquinone oxidoreductase	Mitochondrial matrix

Table 1. Continued

Q9CZU6	CISY	2,64	4,50E-09	Citrate synthase	Mitochondrial matrix
Q9DCT2	NUGM	1,45	1,70E-07	Transfer of electrons from NADH to the respiratory chain	Mitochondrial inner membrane
Q03265	ATPA	-1,72	2,30E-08	ATP synthase alpha chain (regulatory subunit)	Mitochondrial inner membrane
Q91WD5	NUCM	1,28	2,30E-06	NADH-ubiquinone oxidoreductase	Mitochondrial inner membrane
Q9CZ13	UQCR1	-1,32	5,10E-07	Component of ubiquinol-cytochrome c reductase complex	Mitochondrial inner membrane
Q9DB77	UQCR2	-1,48	0,00062	Component of ubiquinol-cytochrome c reductase complex	Mitochondrial inner membrane
P27773	PDIA3	2,56	9,70E-11	Protein disulfide-isomerase, rearrangement of -S-S- protein bonds	Endoplasmic reticulum lumen
P17182	ENOA[3]	2,11	1,50E-10	Alpha enolase, Multifunctional: glycolysis, growth control, etc	Cytoplasm/Plasma membrane
Q9D8N0	EFIG[3]	2,11	1,50E-10	Elongation factor 1-gamma, Translation regulatory protein	Cytoplasm
P39654	LX12L	-1,26	3,60E-05	Arachidonate 12-lipoxygenase, Leukotrienes biosynthesis	Cytoplasm
Q9DBJ1	PGAM1	2,99	1,00E-13	Phosphoglycerate mutase 1	Cytoplasm
Q7TPD1	FBX11	1,49	2,50E-08	F-box only protein 11, ubiquitination of phosphorylated proteins	Unknown
Chaperones					
Q99KV1	DNJBB	1.31	6,50E-6	DnaJ homolog, Chaperone activity	Endoplasmic reticulum
P55302	AMRP	-1,46	7,50E-10	Alpha-2-macroglobulin receptor-associated protein	Intracellular /Cell surface receptors/ ER
P63017	HSP7C	-1,36	3,50E-08	Heat-shock protein 70 kDa, Chaperone activity	Cytoplasm
P63038	CH60	1.72	5,10E-09	Heat shock protein 60 kDa, mitochondrial protein import	Mitochondrial matrix
Transport					
P07724	ALBU	2,05	1,60E-08	Albumin precursor, Transporter activity	Secreted
Q8BH74	NU107	1,62	0,0033	Nucleoporin, Assembly of peripheral proteins into the nuclear pore	Nuclear membrane
O88441	MTX2	-2,49	4,70E-13	Metaxin 2, Transport of proteins into the mitochondrion	Mitochondrial outer membrane
Q60930	VDAC-2	-1,18	0,0002	Voltage-dependent anion-selective channel protein 2	Mitochondrial outer membrane
Q60932	VDAC-1	-1,18	4,40E-06	Voltage-gated ion channel protein 1	Outer mitochondrial/Plasma membrane
P48962	ADT1	-1.27	1,50E-05	ATP carrier protein	Inner mitochondrial/Integral membrane
P23819	GRIA2[3]	2,11	1,50E-10	Glutamate receptor 2	Integral membrane protein

[1] Primary accession number in SwissProt
[2] Average ratio between p38α-/- and WT spot volumes for each protein in all the replica gels analysed
[3] More than one protein were identified in this spot, thus the abundance ratio corresponds to the combination of all these proteins

-proteasome system. We could not identify integrins or other adhesion receptors probably because the large size of these proteins, which makes its separation and identification by 2D gels very difficult. Six of the identified proteins were selected based on their putative involvement in oncogenic transformation and p38 MAPK signalling for western blot analysis. An excellent correlation was observed between the p38α-/- to WT membrane protein ratios of DIGE and western blot analysis, confirming the sensitivity and reliability of DIGE for detection and quantification of even minor changes in protein expression. In contrast, gene expression analysis by RT-PCR showed only partial correspondence with the 2D results (data not shown).
Down-regulation of PHB and up-regulation of NUGM, FAK2, PDA3, KGP2 and TCTP in p38α-/- cells following H-RasV12 transformation are all in good agreement with the emerging role of p38α as a tumour suppressor. Further studies are in progress to elucidate the potential implication of these proteins in the regulation of H-Ras-induced transformation by p38α. This report demonstrates the feasibility of using 2D gels to analyze membrane proteomes and to elucidate proteins involved in signalling pathways and malignant transformation.

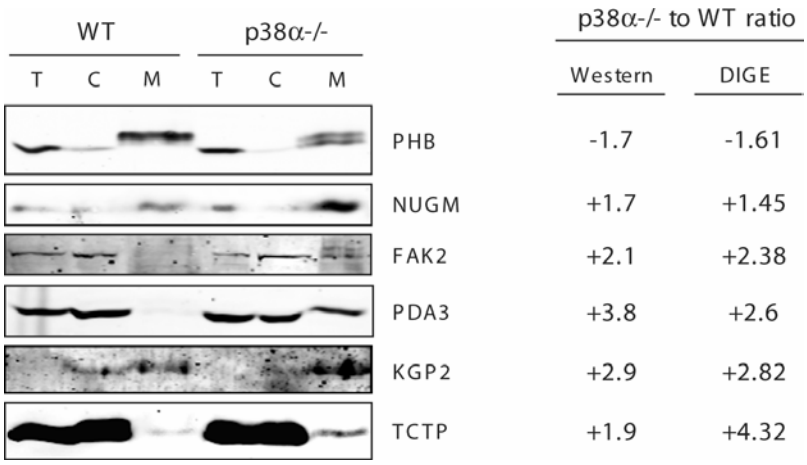

Figure 5: **Confirmation of DIGE results by western blot analysis**.
Total, cytosolic and membrane fractions of exponentially proliferating H-RasV12-expressing WT and p38α-/- MEFs were analyzed by western blotting with the indicated antibodies. The expression of several putative p38α-regulated proteins identified by DIGE with a potential role in oncogenic transformation was analyzed. All these proteins showed different expression levels between WT and p38α-/- cells, as quantified by densitometry with the Odyssey Infrared Imaging System.

Acknowledgements

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p38α MAPK is required for contact inhibition

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Abstract

Proliferation of non-transformed cells is regulated by cell-cell contacts, which are referred to as contact-inhibition. Despite its generally accepted importance for cell cycle control, knowledge about the intracellular signalling pathways involved in contact-inhibition is scarce. In the present work we show that p38α MAPK is involved in the growth-inhibitory signalling cascade of contact-inhibition in fibroblasts. p38α activity is increased in confluent cultures of human fibroblasts compared to proliferating cultures. Time course studies show a sustained activation of p38α in response to cell-cell contacts in contrast to a transient activation after serum-stimulation. The induction of contact-inhibition by addition of glutaraldehyde-fixed cells is impaired by pharmacological inhibition of p38 as well as in p38α-/- fibroblasts. Further evidence for a central role of p38α in contact-inhibition comes from the observation that p38α-/- fibroblasts show a higher saturation density compared to wild-type fibroblasts, which is reversed by reconstituted expression of p38α. In agreement with a defect in contact-inhibition, $p27^{Kip1}$ accumulation is impaired in p38α-/- fibroblasts compared to wild-type fibroblasts. Hence, our work shows a new role for p38α in contact-inhibition and provides a mechanistic basis for the recently proposed tumour-suppressive function of this MAPK pathway.

Key words: contact-inhibition, p38 MAPK, fibroblasts

Supplementary Information is available at Oncogene website (http://www.nature.com/onc)

Introduction

Proliferation of non-transformed cells is controlled by mitogenic and anti-mitogenic signals. The receptors and downstream effectors of proliferative stimuli exerted by growth factors or growth hormones have extensively been studied during the last decades. It is generally accepted that important anti-proliferative signals are mediated by cell-cell contacts, a cellular mechanism which is referred to as contact-inhibition (Eagle and Levine, 1967). Contact-inhibition plays a fundamental role in regulating homeostasis *in vitro* and *in vivo*. For example, non-transformed cells are arrested in G1-phase *in vitro* at a critical cell density forming a confluent monolayer. In contrast, transformed cells are characterized by the loss of contact-inhibition (Abercrombie, 1979) manifested by a higher saturation density and the emergence of multi-layered foci. Despite its importance for cell cycle control, knowledge about the intracellular signaling cascade mediating contact-inhibition is still scarce.

In FH109 human embryonal fibroblasts (Wieser et al., 1985), contact-inhibition is exclusively mediated by the interaction of two cell membrane proteins on adjacent cells, i.e. by the glycoprotein contactinhibin (Wieser et al., 1990) which binds to its receptor referred to as contactinhibin-receptor (Gradl et al., 1995). Anti-proliferative signals from the engaged receptor then lead to G1 arrest by increased association of the tumor suppressor $p16^{Ink4}$ with Cdk4 (Wieser et al., 1999) and by increased association of $p27^{kip1}$ with the Cdk2/cyclin E complex (Dietrich et al., 1997). As a consequence, the retinoblastoma gene product (pRB) remains in the hypophosphorylated state thus inhibiting progression into S-phase (Dietrich et al., 1997; Polyak et al., 1994; Wieser et al., 1999). Furthermore, the activation and nuclear translocation of PKCδ is involved in contact-inhibition in human and murine fibroblasts (Heit et al., 2001).

Several reports have described a correlation between p38 MAPK (mitogen-activated protein kinase) activation and cell cycle arrest either in response to environmental stress, during senescence or by ectopic expression of p38 MAPK upstream activators (Wang and Ron, 1996; Molnar et al., 1997; Ellinger-Ziegelbauer et al., 1999; Wang et al., 2002; Haq et al., 2002). However, there is little direct evidence for a physiological role of p38 MAPK in cell cycle control in mammalian cells (for review see Ambrosino and Nebreda, 2001). Like all MAPKs, p38 MAPK is activated by dual phosphorylation at conserved threonine and tyrosine residues mediated by MKK3 or MKK6, which themselves are activated by MKK kinases. Four different p38 family members have been identified so far, with p38α being the major isoform in fibroblasts (for reviews see Ono and Han, 2000; Nebreda and Porras, 2000; Shi and Gaestel, 2002).

Results

To gain more insight into the intracellular signalling cascade mediating contact-inhibition we investigated if p38 MAPK is involved in contact-inhibition in fibroblasts. We first analyzed if p38 activity was enhanced in confluent, G1-arrested FH109 fibroblasts compared to exponentially proliferating cultures. Cells were seeded either sparsely or to confluence, which induces G1 arrest, and cultured for 24 h (Dietrich et al., 1997). As a positive control for p38 phosphorylation, serum-starved cells were stimulated for 30 min with 10% fetal calf serum (FCS) or anisomycin (10 μg/ml), a translation inhibitor widely used as a potent inducer of p38 activity. Total cell extracts were prepared and subjected to Western blot analysis to monitor p38 phosphorylation. The blots were stripped and reprobed with pan-anti-p38-antibody to control equal loading. Figure 1A clearly shows that p38 phosphorylation is increased in confluent, G1-arrested cultures compared to proliferating cultures. The single band detected by the pan-anti-p38-antibody was shown to represent p38α since it was only detected by an anti-p38α antibody, but not p38β, p38δ, or p38γ specific antibodies (data not shown). RT-PCR to analyze mRNA expression of different p38 isoforms revealed a strong expression of p38α, almost undetectable levels of p38β and weak expression of p38δ and p38γ (data not shown). In addition to

phosphorylation, we measured kinase activity and found that p38α activity was also enhanced about two-fold in confluent cultures compared to proliferating cultures (Figure 1B). Serum-stimulated and anisomycin-treated cultures served as positive controls.

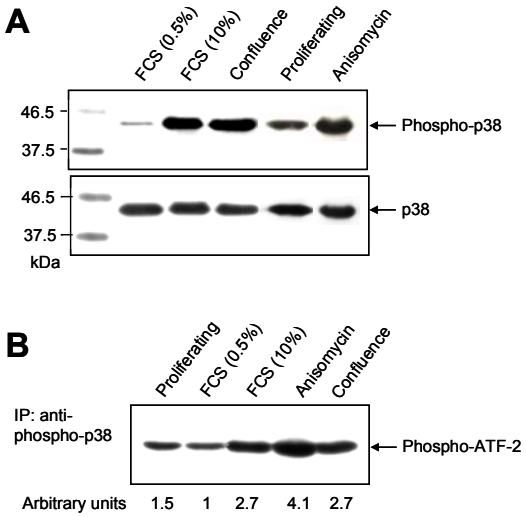

Figure 1. Increased p38α MAPK activity in confluent G1-arrested cells.
(A) Enhanced phosphorylation of p38α in confluent cells. FH109 human embryonal lung fibroblasts (Wieser et al, 1985; Dietrich et al., 1997) were routinely cultured in DMEM, supplemented with 10% FCS, penicillin and streptomycin (each 100 U/ml). Cells were either sparsely seeded (proliferating) or to confluence in the presence of 10% FCS and cultured for 24 h. For positive controls, serum-deprived (0.5% FCS) cells were stimulated for 30 min with either 10% FCS or anisomycin (10 µg/ml). Total cell extracts were prepared by lysing the cells in hot Laemmli sample buffer (Laemmli, 1970) and protein concentration was determined according to *Smith et al*. (1985). 50 µg protein/lane were subjected to SDS-PAGE (10%) followed by Western blot analysis with a phospho-specific (T180/Y182)-anti-p38 antibody (1:1000, Cell Signaling) according to the manufacturer´s instructions. The blots were stripped and reprobed with anti-pan-p38 antibody (1:1000, Cell Signaling) to control equal loading.
(B) Enhanced kinase activity of p38α in confluent, G1-arrested cells. Cells were seeded and treated as described in **(A)**. Activity of p38α was measured after immunoprecipitation with a monoclonal anti-phospho-p38 antibody using an ATF-2 fusion protein as substrate according to the manufacturer´s instructions (Cell Signaling). Phosphorylation was visualized after SDS-PAGE and Western blotting by an anti-phospho-ATF-2 antibody according to the manufacturer´s instructions (Cell Signaling). Quantification was performed with NIH Image (version 1.61). One representative experiment is shown out of three each leading to similar results.

Our data showed that p38α phosphorylation and activity was elevated in confluent, G1-arrested FH109 cells. Time course studies revealed that the increase in p38α phosphorylation was already maximal at 2 h after adherence to the culture dish and lasted for at least 24 h (Figure 2A), indicating a sustained activation of p38α in confluent cultures. The activation of p38α correlated with induction of G1-arrest, as demonstrated by the appearance of the hypophosphorylated species of pRB, which inhibit entry into S-phase (Figure 2B).
To show that p38α is indeed persistently activated in response to cell-cell contacts, we made use of an established cell culture model to study contact-inhibition (Wieser et al., 1985; Wieser and Oesch, 1986; Wieser et al., 1999; Heit et al., 2001; Nakatsuji and Miller, 1998). A contact-inhibition-like state was initiated by the addition of glutaraldehyde-fixed FH109 cells derived from confluent cultures to sparsely seeded fibroblasts. In confluent but not proliferating cultures, contactinhibin is active (Wieser et al., 1990; Wieser et al., 1995). Since the growth inhibitory activity of contactinhibin is exclusively mediated by N-glycans, it is not affected by the fixation process. In previous studies we have demonstrated that this system mimics cell cycle arrest and differentiation, which occur in confluent cultures (Wieser et al., 1985; Wieser and Oesch, 1986;

Wieser et al., 1999; Heit et al., 2001). This system enables us to specifically investigate signalling events induced by cell-cell contacts which are different from those induced after serum-starvation (Dietrich et al., 1997) and, furthermore, to perform time course studies of cell-

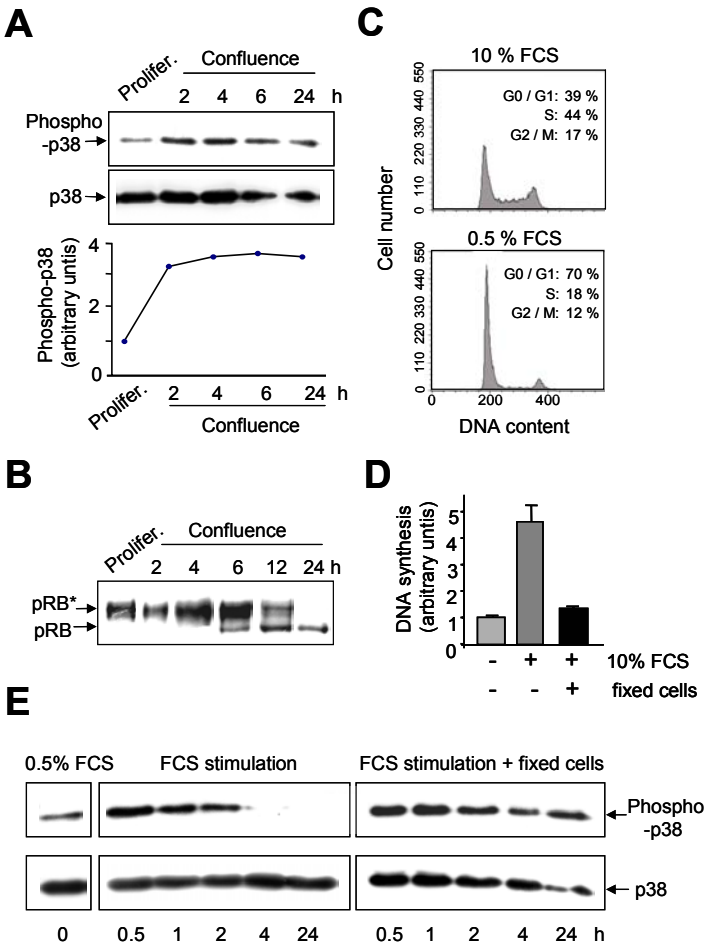

Figure 2. Persistent p38α MAPK phosphorylation in response to cell-cell contacts correlates with inhibition of DNA-synthesis.

(A) Proliferating cells (prolifer.) were seeded to confluence and harvested 2, 4, 6, and 24 h after adherence to the culture dish. Western blot analysis was performed as described in Figure 1. Phospho-p38α signal quantification after normalization to the loading control is shown in the lower panel.

(B) Activation of p38α correlates with dephosphorylation of pRB. Proliferating cells (prolifer.) were seeded to confluence and harvested 2, 4, 6, 12, and 24 h after adherence to the culture dish. Western blot analysis was performed with anti-pRB-antibody (1:1000, Cell Signaling) according to the manufacturer´s instructions.

(C) FH109 cells were sparsely seeded in the presence of 10% or 0.5% FCS. After 24 h, propidium iodide staining was performed and the cells analysed by a FACS Calibur.

(D) Imitating contact-inhibition by the addition of glutaraldehyde-fixed cells blocks serum-induced DNA-synthesis. FH109 cells were sparsely seeded in 96-well plates in DMEM with 0.5% FCS and cultured for 24 h to achieve partial synchronization. Cells were stimulated either with 10% FCS alone or together with glutaraldehyde-fixed cells ($1.5x10^5/cm^2$) to imitate contact-inhibition. For the fixation process, confluent FH109 cultures were trypsinized, washed and fixed by dropwise addition of glutaraldehyde according to *Oesch et al.* (1987). 20 h after addition of 10% FCS or glutaraldehyde-fixed cells plus 10% FCS, DNA-synthesis was determined by measurement of [^{3}H]thymidine incorporation into DNA after labeling with 9.25 kBq/well of [^{3}H]thymidine for another 4 h. Incorporated radioactivity was determined by liquid scintillation spectometry as described (Dietrich et al., 1996). Results are expressed as x-fold induction compared to serum-starved control values and given as means±SEM, (n=4-5).

(E) Sustained p38α phosphorylation after imitating contact-inhibition by the addition of glutaraldehyde-fixed cells. FH109 cells were seeded and treated as described in **(D)**. Cells were lysed in hot Laemmli sample buffer at the indicated time points. Western blot analysis was performed as described in Figure 1.

cell contact-dependent signalling. FH109 cells were sparsely seeded and serum-starved to achieve partial synchronization in the G1-phase of the cell cycle (Figure 2C). Cells were either stimulated with FCS or exposed to glutaraldehyde-fixed cells plus FCS to imitate contact-inhibition. DNA-synthesis was measured and total cell extracts prepared at various time-points. While FCS-stimulation led to a 4-5-fold induction of DNA synthesis, this was reduced to 1.3-fold by simultaneous addition of glutaraldehyde-fixed cells and FCS (Figure 2D), which correlated with the reduction of DNA-synthesis achieved by seeding the cells to confluence (Heit et al., 2001). Western blot analysis revealed that serum-stimulation resulted in transient phosphorylation of p38α whereas imitating contact-inhibition by the addition of glutaraldehyde-fixed cells (from confluent cultures) led to the sustained activation of p38α for at least 24 h (Figure 2E). No increase in p38α phosphorylation was detected after addition of glutaraldehyde-fixed cells derived from proliferating cultures (data not shown).

To investigate whether p38α was required for contact-inhibition, we used the p38α and p38β inhibitor SB203580 (Cuenda et al., 1995; Cohen, 1997). In the presence of SB203580 (10 μM), the inhibition of DNA-synthesis induced by glutaraldehyde-fixed cells was reduced from 70 % to 33 % indicating that p38α activity is required for contact-inhibition (Figure 3A). These data were confirmed by using immortalized mouse embryonic fibroblasts (MEFs) derived from p38α-/- mice and their wild-type (wt) littermates. As shown in Figure 3B, addition of glutaraldehyde-fixed cells to wt fibroblasts induced a similar decrease in DNA-synthesis (50 ± 8.8 %) as described for FH109 cells. Interestingly and in line with the results obtained with SB203580, fixed cells from confluent cultures were much less efficient at inhibiting DNA synthesis in p38α-/- fibroblasts (Figure 3B), supporting the conclusion that p38α is required for contact-inhibition.

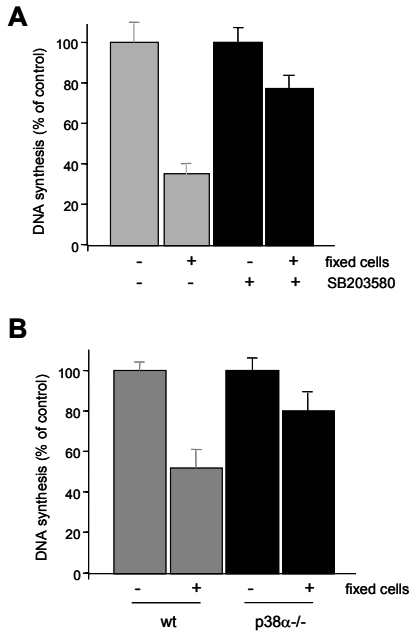

Figure 3. Inhibition of p38α MAPK activity impairs contact-inhibition.
(A) FH109 cells were seeded and treated with 10% FCS or glutaraldehyde-fixed cells plus 10% FCS as described in Figure 2D either in the absence or in the presence of SB203580 (10 μM).
(B) Wt and p38α-/- MEFs were seeded and treated as described in **(A)**. Results are expressed as % of control values in the absence of fixed cells and given as means±SEM, (n = 4-6).

To provide direct evidence for a pivotal role of p38α in contact-inhibition, we compared the growth curves of immortalized wt and p38α-/- MEFs. Both cell lines exhibited a similar growth rate, but p38α-/- fibroblasts showed an almost two-fold higher saturation density compared to wild type (wt) fibroblasts (Figures 4A and 4B). This effect was reversed by reconstituted expression of p38α (Figure 4C), arguing against a possible non-specific effect derived from the immortalization of p38α-/- fibroblasts. Furthermore, primary p38α-/- MEFs also showed higher saturation densities than wt MEFs (Figure 4D). Interestingly, although p38α-/- MEFs displayed a two-fold higher saturation-density they did not form spontaneous foci, indicating that contact-

inhibition was impaired but not totally absent in p38α-/- fibroblasts. In line with this idea, pRB dephosphorylation was also delayed in p38α-/- fibroblasts (Figure 4E).
pRB can be phosphorylated by Cdk4/cyclin D and Cdk2/cyclin E and several authors have demonstrated the p38 MAPK-mediated down-regulation of cyclin D1 (Lavoie et al., 1996; Conrad et al., 1999; Ellinger-Ziegelbauer et al., 1999; Casanovas et al., 2000). The fact that cyclin D1 is not decreased in contact-inhibited FH109 cells (Dietrich et al., 1997) argues against cyclin D1 as a main downstream target of p38α in contact-inhibition. Since $p27^{Kip1}$ is known to be upregulated in confluent cells (Figure 4E; Dietrich et al., 1997; Polyak et al., 1994) we analysed a potential link between p38α activation and $p27^{Kip1}$ accumulation. Indeed, $p27^{Kip1}$ accumulation was delayed and partially impaired in p38α-/- fibroblasts (Figure 4E). A similar effect on $p27^{Kip1}$ accumulation was detected in confluent FH109 cells treated with SB203580 (Supplementary figure). Binding of $p27^{Kip1}$ is known to inhibit Cdk2 phosphorylation at Thr160, which results in reduced Cdk2 kinase activity. Active Cdk2, which is phosphorylated at Thr160 and dephosphorylated at Thr14 and Tyr15, is identified as a faster-migrating 33 kDa form (Dietrich et al., 1997; Dulic et al., 1992). As expected, $p27^{Kip1}$ accumulation at increasing cell-densities correlated with the disappearance of the 33kDa active form of Cdk2 from wt cells (Figure 4E). Interestingly, the loss of active Cdk2 was slower in p38α-/- than in wt fibroblasts, in good correlation with the reduced accumulation of $p27^{Kip1}$ in p38α-/- cells (Figure 4E). Taken together, the results support a role for $p27^{Kip1}$ as a likely new target of p38α signalling in contact-inhibition.

Discussion

Our results provide several lines of evidence indicating that a switch from transient to a prolonged activation of p38α induces G1-arrest in contact-inhibited cultures: (i) p38α activity is enhanced in confluent, G1-arrested cultures compared to proliferating cultures, (ii) p38α phosphorylation is sustained in confluent cultures correlating with dephosphorylation of pRB, (iii) p38α phosphorylation is transient after serum-stimulation which leads to an induction of DNA-synthesis, whereas simultaneous addition of glutaraldehyde-fixed cells and serum results in a sustained p38α phosphorylation and strong inhibition of serum-induced DNA-synthesis.
The sustained activation of p38α may be the result of increased activity of upstream kinases or decreased activity of downstream phosphatases. Interestingly, we detected down-regulation of a dual-specificity phosphatase with substrate preference for the MAPK family in confluent mouse fibroblasts using high-density microarray analysis (manuscript in preparation), which would give a plausible explanation for the sustained activation of p38α in contact-inhibited cultures. The precise role of phosphatases in p38α activation during contact-inhibition is currently investigated.
Constitutive activation of p38 MAPKs due to the depletion of the WIP1 phosphatase results in resistance to malignant transformation *in vivo*, indicating that p38 MAPKs may inhibit tumour formation (Bulavin et al., 2002, 2004). In the present work, we describe a hitherto unknown role of p38α MAPK in contact-inhibition. This novel physiological function of p38α in cell cycle control provides further mechanistic support for the idea that p38α may act as a suppressor of tumorigenesis.

Acknowledgements

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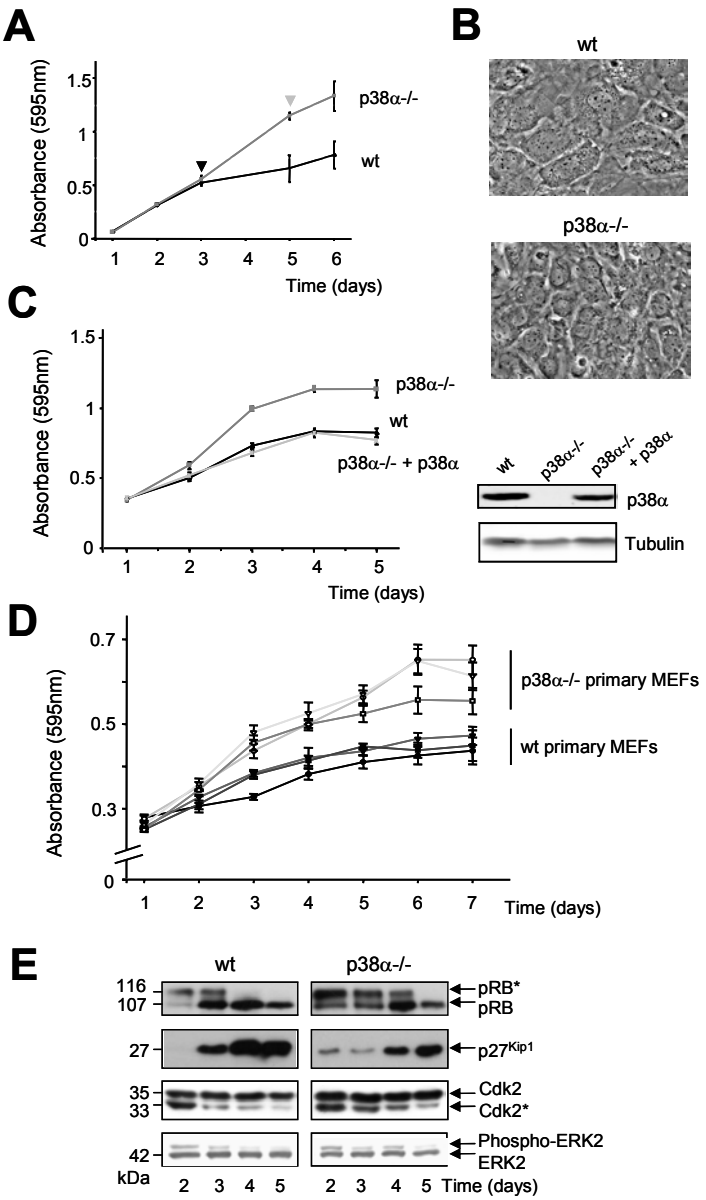


Figure 4. Impaired contact-inhibition in p38α-/- fibroblasts.

(A) Spontaneously immortalized wt and p38α-/- MEFs were seeded in triplicates at 1000 cells/well (96-well plates) and their proliferation monitored with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Roche Diagnostics GmbH). No significant proliferative differences were observed between both cell lines in subconfluent conditions (up to day 3, black arrowhead). However, p38α-/- MEFs achieved an almost two-fold higher saturation density than their wt counterparts (grey arrowhead).

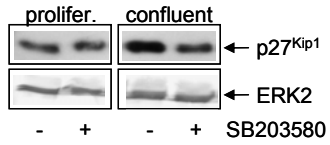
(B) Representative fields showing the saturation density of wt and p38α-/- cells two days after confluence.

(C) Reconstitution of p38α in the p38α-/- background reverses the contact-inhibition defect observed in the immortalized p38α-/- MEFs. wt and p38α-/- MEFs, either infected with a p38α-encoding retrovirus or with the retroviral vector, were seeded in triplicates at 1500 cells/well ($5x10^4$ cells/cm^2) and allowed to proliferate until absorbance stabilization.

(D) Three different sets of wt and p38α-/- primary MEFs (P4 or less) were seeded in quadruplicates at 1500 cells/well in 96-well plates and proliferation determined as in **(A)**. The confluence cell densities achieved by all three p38α-/- MEF lines was significantly higher than those of the wt MEFs.

(E) Wt and p38α-/- MEFs were seeded as described in **(C)** and total cell extracts were prepared at the indicated times. Cell extracts derived from wt and p38α-/- MEFs were loaded onto the same gel, blotted onto the same membrane and exposed to the same film during subsequent ECL-detection to guarantee for identical exposure times. Western blot analysis was performed with anti-pRB (1:1000, Cell Signaling), anti-$p27^{Kip1}$ or anti-Cdk2 antibodies (each 1:1000, Santa Cruz) according to the manufacturer´s instructions. The blot was stripped and re-hybridized with anti-ERK2-antibody (1:2000, Santa Cruz) to control equal loading. pRB* indicates hyperphosphorylated pRB; Cdk2* indicates active Cdk2 phosphorylated at Thr160.

Supplementary Figures



Supplemental Figure 1. **Pharmacological inhibition of p38α MAPK impairs $p27^{Kip1}$ accumulation in confluent FH109 cells.**
FH109 cells were either sparsely seeded (proliferating = prolifer.) or to confluence either in the absence or presence of SB203580 (10 μM) and harvested after 24 h. Western blot analysis was performed as described in figure 4D.

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CXCL12 and C5a trigger cell migration via a PAK1/2 - p38α MAPK - MAPKAP-K2 - HSP27 pathway.

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Abstract

Cell migration is critical for many processes, such as angiogenesis, inflammation, development and wound healing, and is also involved in tumour progression and metastasis. Here we show that CXCL12, complement factor 5a (C5a), hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF)-BB, which stimulate cell migration, also activate p38α MAPK. Pharmacological inhibition of this protein kinase with SB 203580 or BIRB 0796, or the genetic ablation of p38α MAPK, blocked cell migration induced by the aforementioned chemo-attractants. Macrophages from mice lacking one or more of the other p38 MAPK isoforms showed normal cell migration in response to C5a. We also show that the activation of p38α MAPK in response to CXCL12 requires the p21-activated protein kinases (PAK)-1 and PAK-2. MAPKAP-K2 is a protein kinase that is activated by p38α MAPK. Reducing its expression using RNA interference blocked CXCL12-induced HeLa cell migration, while macrophages from mice that do not express MAPKAP-K2 failed to migrate in response to C5a. Moreover, RNA interference against the small heat shock protein 27 (HSP27), a physiological substrate of MAPKAP-K2, blocked the CXCL12-induced cell migration. These results demonstrate a general and essential role of the PAK-p38α MAPK-MAPKAP-K2-HSP27 signalling pathway in mediating the effects of chemotactic stimuli on cell migration.

Key words: p38 MAPK, MAPKAP-K2, HSP27, PAK, cell motility, metastasis.

Introduction

Cell migration is not only required for many normal physiological processes, but also for the movement of tumour cells from primary foci to secondary sites of implantation (metastasis), a phase associated with decreased rates of cancer survival. In breast cancer, for example, there is a distinct metastatic pattern where tumour cells migrate to the regional lymph nodes, bone marrow, liver and lung. Cells at these sites express high levels of the G-protein coupled receptors CXCR4 and CCR7 [1], which are normally involved in leukocyte trafficking. It is thought that tumour cells hijack this system to migrate to secondary sites of implantation [2]. The respective ligands for CXCR4 and CCR7, namely the chemokines CXCL12/SDF-1α and CCL21/6Ckine, are expressed at high levels in organs representing the first destinations of metastasising breast cancer cells [1]. Thus signalling pathways triggered by activation of these receptors, such as phosphatidylinositol (PtdIns) 3-kinase and mitogen-activated protein kinase (MAPK) pathways [3], may play key roles in establishing the primary sites of metastasis.

Another signal important for metastasis is Hepatocyte Growth Factor (HGF), also known as Scatter Factor [4]. Like CXCL12, the binding of HGF to its tyrosine kinase receptor c-Met, triggers activation of both the phosphatidylinositol (PtdIns) 3-kinase and MAPK pathways [5]. Similarly, Platelet-Derived Growth Factor (PDGF)-BB, a well known mitogen and motogen [6] that promotes wound healing [7], activates these signalling pathways when it engages the PDGF receptor.

The anaphylatoxin complement factor 5a (C5a) has also been shown to be a strong chemo-attractant stimulating monocytes / macrophages to migrate to sites of wound healing and inflammation. C5a acts via its Gi-coupled receptor [8] to activate the PtdIns 3-kinase and MAPK signalling pathways [9].

The p38α MAPK is known to relay chemotactic signals that are relevant to tumour progression and subsequent metastasis. These include angiogenic signals from VEGF to the actin cytoskeleton in endothelial cells [10, 11], invasion of human breast epithelial cells by H-Ras [12], N-formyl-L-leucyl-L-phenylalanine (fMLP)-induced neutrophil migration [13] and migration of vascular smooth muscle cells [14]. The roles proposed for this pathway in cell motility have so far relied on the use of SB 203580, a relatively specific inhibitor of p38α and p38β MAPKs [15, 16].

In this study we have investigated the role of the different isoforms of the p38 MAPK family in mediating cell motility using a combination of pharmacological inhibitors, RNA interference (RNAi) and 'knockout" cells that do not express particular protein kinases. This led us to identify key target signalling molecules involved in mediating chemotactic cell migration via this pathway.

Materials and methods

Materials

CXCL12 was from R&D systems (Abingdon, UK), C5a from Calbiochem (Nottingham, UK), PDGF-BB and HGF from Sigma-Aldrich (Poole, UK) and Silencer siRNA construction kit from Ambion (Abingdon, UK). BIRB 0796 was synthesized as described [17]. The sources of all other materials are detailed elsewhere [18].

Antibodies and immunoblotting

Total and phosphorylated PAK1/2, total and phosphorylated p38 MAPK, and phosphorylated (Thr308) PKB were from Cell Signalling Technologies (Hitchin, UK). The monoclonal antibody that recognises vinculin, clone hVIN-1, was purchased from Sigma V9131. Rabbit anti-sheep IgG and goat anti-rabbit IgG, both conjugated to peroxidase, were obtained from Perbio Science Ltd. (Tattenhall, UK). Alexa Fluor 594 anti-mouse antibody was from Molecular Probes (Leiden, The Netherlands). YL 1/2 antibody (for microtubule staining) was from Sera Lab (Crawley Down, UK). Fluorescein conjugated anti-rat IgG was from Lorne Diagnostics

(Reading, UK). Immunoblotting was also carried out using the ECL detection system (Amersham Pharmacia Biotech). A quantification of blots was performed using the Li-Cor Odyssey® infrared imaging system.

Cell culture and cell lysis

Bone-marrow-derived macrophages (BMDM), transformed embryonic fibroblasts (MEFs) from wild type and p38α MAPK-deficient mice [19] and HeLa cells were prepared, maintained and lysed as described [18].

Cell migration assays

Cell migration was assayed in a 5 μm transwell (macrophages) or 8 μm transwell (HeLa and MEF) co-culture chamber following a modified Boyden chamber assay [10]. Briefly, cells were left to adhere for 1h on the upper membrane of the chamber, which was pre-coated with human fibronectin (5 μg/ml) for HeLa and MEF experiments only. The cells were stimulated with various agonists, added to the lower chamber, and left to migrate for 4 h. Cells on the upper membrane were then scraped using a cotton swab, fixed in 3.7% (v/v) formaldehyde and stained with Mayer's hematoxylin. Five fields were counted under 100-fold magnification, each data point being carried out in triplicate.

Wound Migration Assay

Cells were plated in culture dishes at confluence and pre-treated with mitomycin C (30 μg/ml) for 60 min before the injury line was made with a tip of 1.5 mm diameter. After rinsing with PBS, cells were allowed to migrate in complete medium, and photographs were taken (x 100) at the indicated time points.

siRNA construction and transfection

The following siRNAs were synthesized and used for the RNA interference studies. Human PAK1, TAACGGCCTAGACATTCAA (siRNA-1) or GGATGATGATGATGATGAT (siRNA-2); human MAPKAP-K2: ACCACCAGCCACAACUCUU [20]; human hnRNP A0 CAGUGGACCGUACAGAGGC; human PAK2, CTACAGACCTCCAATATCA (siRNA-1) or ACACACGGTCTGTAATTGA (siRNA-2); human HSP27 AATGCTCCTCCAGTCGGGTAT (siRNA 1) or ATGCTCCTCCAGTCGGGTA (siRNA-2) were prepared using the Silencer siRNA construction kit (Ambion) according to the manufacturer's instructions. Experiments were performed using a strain of easily transfectable HeLa cells, generously provided by Professor Jacques Pouyssegur (CNRS**,** Nice, France). These cells were transfected twice at 24 h intervals with 60 nM of the specified siRNA using Oligofectamine (Invitrogen) and used 24 h following the second transfection [21].

Immunofluorescence

MEFs were grown on coverslips, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS. Saturation was performed using 1% BSA in PBS for 1 h at room temperature. Actin fibres were visualized by using rhodamine-conjugated phalloidin (Molecular Probes) and focal adhesion-associated vinculin was stained with a commercially available monoclonal antibody which specifically recognizes vinculin at cell-cell and cell-substrate contacts, followed by incubation with an anti-mouse secondary antibody coupled to Alexa-594 (Molecular probes). Coverslips were mounted in Vectashield (Vector Laboratories) and pictures were taken using an inverted fluorescence microscope Zeiss Axiophot.

Results

SB 203580 inhibits cell migration induced by various agonists.

It was reported previously that inhibiting the activity of p38α and p38β MAPKs with SB 203580 led to inhibition of cell migration in different systems (see Introduction). We confirmed that this was also the case for HeLa cells stimulated with PDFG-BB, HGF or CXCL12, three chemo-attractants acting via distinct types of receptor to activate p38 MAPKs (Fig1A). The presence of SB 203580 did not affect basal cell migration (data not shown). Moreover, another unrelated chemical inhibitor, BIRB 0796, at concentrations (0.1 μM) that inhibit p38α and p38β MAPKs specifically [17], also led to complete inhibition of HeLa-cell migration (Fig. 1A). Similar results were obtained in primary bone-marrow-derived macrophages (BMDM) stimulated with C5a (Fig 1B).

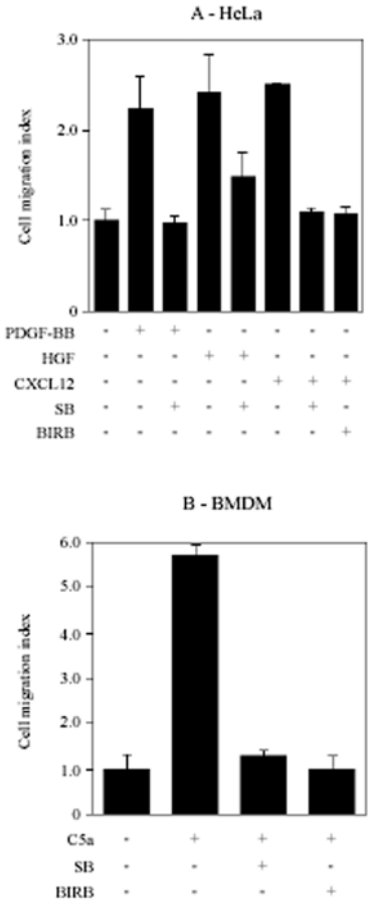

Figure 1. SB 203580 or BIRB 0796 inhibits PDGF-BB, HGF, CXCL12 and C5a-induced cell migration.
A. HeLa cells were incubated for 30 min without or with 5 μM SB 203580 (SB) or 0.1 μM BIRB 0796 (BIRB) added to both chambers of the vessel used to measure cell migration. They were then stimulated for 4 h with 15 ng/ml PDGF-BB or 20 ng/ml HGF or 10 nM CXCL12 as indicated. The migration of the cells was measured as a Migration Index, defined as the number of cells migrating in response to the agonist relative to the basal level of cell migration. Similar results were obtained in three independent experiments. Further details are given under Methods.
B. Bone-marrow-derived macrophages (BMDM) were incubated for 30 min without or with 5 μM SB 203580 or 0.1 μM BIRB 0796 added to both chambers of the vessel used to measure cell migration. They were then stimulated for 4 h with 1 μg/ml C5a. Cell migration was measured as in **A**. Similar results were obtained in two independent experiments.

p38α MAPK is the only p38 isoform involved in transducing C5a, HGF and PDGF-BB-driven cell motility.

The p38 MAPK family comprises four members, namely p38α, p38β, p38γ and p38δ [22]. Mice lacking one or more of these protein kinases have been generated [11, 23, 24] and all are viable with the exception of the p38α MAPK knockout, which is embryonic lethal. The p38α MAPK knockout has a defect in angiogenesis [11], consistent with a putative role for this protein kinase in cell migration. However, this does not preclude the possibility that other protein kinases of this family also play a role in regulating cell motility. More importantly, SB 203580 does not distinguish between effects mediated by p38α and p38β MAPKs, as both are potently inhibited

by this compound. In contrast, p38γ and p38δ MAPKs are not affected by SB 203580 [25]. We therefore studied the migration of primary macrophages from mice lacking different p38 isoforms. Mice lacking p38β MAPK, both p38β and p38δ MAPKs or both p38γ and p38δ MAPKs showed normal cell migration in response to C5a that was still sensitive to the effect of SB 203580 (Fig.2). These results point to p38α MAPK as the sole isoform responsible for transducing the C5a signal leading to cell migration.

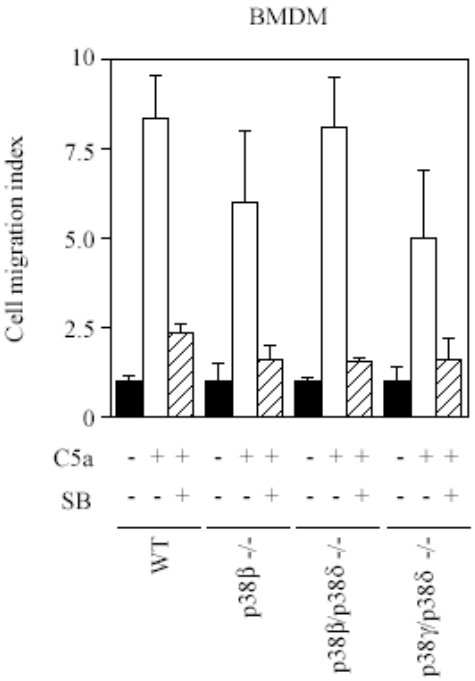

Figure 2. Macrophages deficient in p38β, p38γ and p38δ MAPKs show normal migration in response to C5a.
Bone-marrow-derived macrophages (BMDM) were isolated from wild type (WT) mice, p38β MAPK-deficient mice, mice deficient in both p38β MAPK and p38δ MAPK and mice deficient in both p38γ and p38δ MAPKs, as indicated, and incubated for 30 min without or with 5μM SB 203580 (SB) added to both chambers of the vessel used to measure cell migration. They were then stimulated for 4 h with 1 μg/ml C5a. Cell migration was measured as in Fig.1. Similar results were obtained in two independent experiments.

Embryonic fibroblasts (MEF) derived from mice lacking p38α MAPK showed impaired wound healing in a scratch assay (Fig. 3A). Moreover, the migration of these MEF in response to both PDGF-BB and HGF was greatly reduced compared to their wild-type counterpart (Fig. 3C). Taken together, these results demonstrate that p38α MAPK is a key component of the signalling mechanism transducing chemo-attractant signals.

siRNAs directed against PAK1 and PAK2 block the activation of p38α MAPK, as well as cell migration induced by CXCL12.

To further investigate the signal transduction pathway leading to increased cell motility, we used siRNA against components of the p38α MAPK signalling pathway to determine their role in transducing signals from the membrane to the cytoskeleton.
It has been reported that the protein kinases PAK1 and PAK2 can transmit the signal from chemoattractant molecules to the actin cytoskeleton, making them putative candidates to transmit the signals from CXCR4, the receptor for CXCL12, to p38α MAPK (reviewed in [26]). HeLa cells were incubated with siRNAs against PAK1 and PAK2 alone or in combination, and stimulated with CXCL12. These experiments demonstrated that PAK1 and PAK2 both become activated following treatment with CXCL12 (Fig. 4C), and that the siRNAs against each PAK kinase reduced their expression (Fig. 4A and C). Interestingly, the siRNA against either PAK1 or PAK2 caused a partial reduction in CXCL12-induced activation of p38α MAPK, while activation was largely suppressed after transfection of siRNA against both isoforms (Fig. 4B).

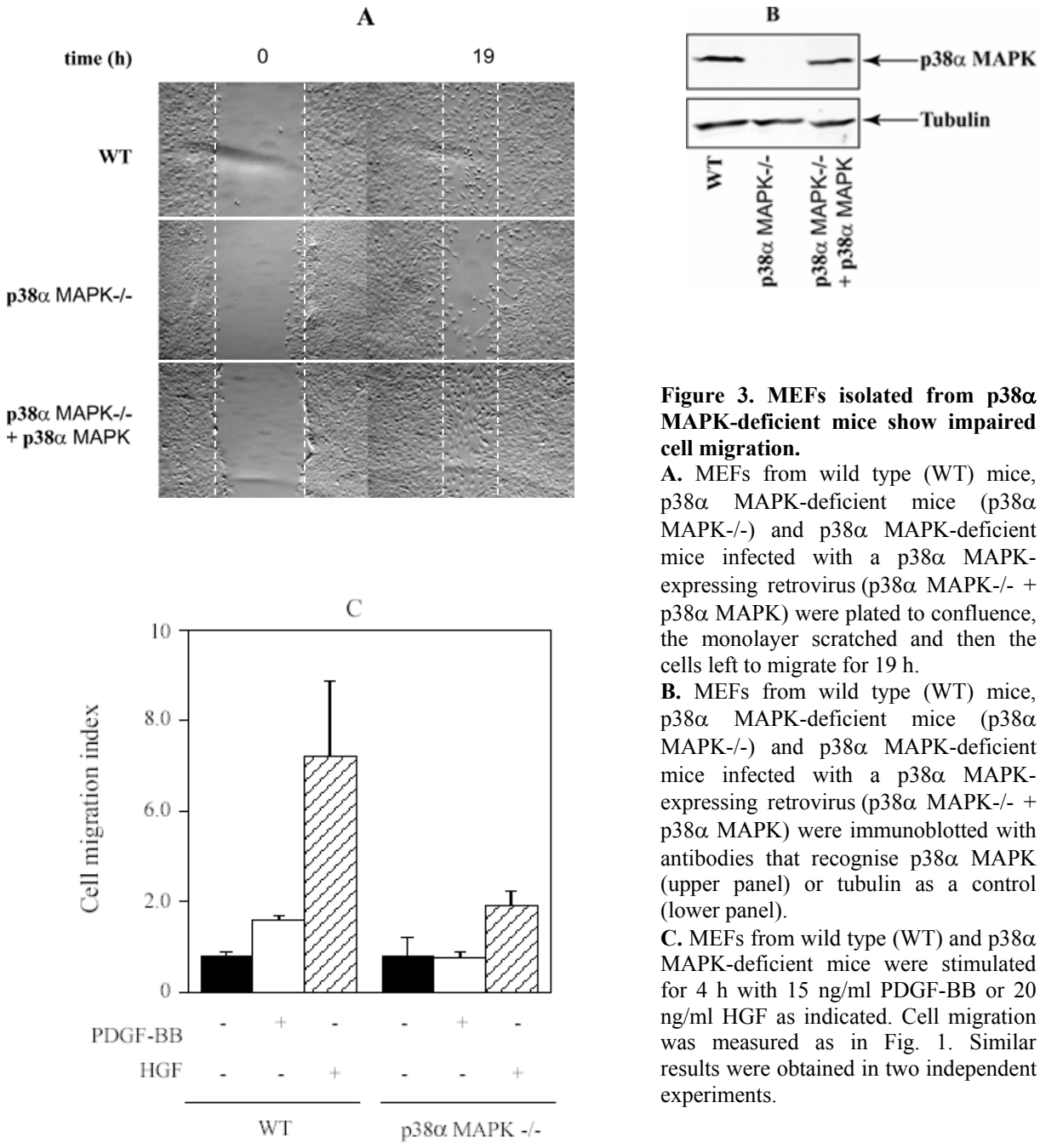

Figure 3. MEFs isolated from p38α MAPK-deficient mice show impaired cell migration.

A. MEFs from wild type (WT) mice, p38α MAPK-deficient mice (p38α MAPK-/-) and p38α MAPK-deficient mice infected with a p38α MAPK-expressing retrovirus (p38α MAPK-/- + p38α MAPK) were plated to confluence, the monolayer scratched and then the cells left to migrate for 19 h.

B. MEFs from wild type (WT) mice, p38α MAPK-deficient mice (p38α MAPK-/-) and p38α MAPK-deficient mice infected with a p38α MAPK-expressing retrovirus (p38α MAPK-/- + p38α MAPK) were immunoblotted with antibodies that recognise p38α MAPK (upper panel) or tubulin as a control (lower panel).

C. MEFs from wild type (WT) and p38α MAPK-deficient mice were stimulated for 4 h with 15 ng/ml PDGF-BB or 20 ng/ml HGF as indicated. Cell migration was measured as in Fig. 1. Similar results were obtained in two independent experiments.

Similar results were obtained with either siRNA-1 (Fig 4A and B) or siRNA-2 (data not shown) for each PAK isoform. Other signalling pathways activated by this chemokine, such as the PtdIns 3-kinase pathway, were unaffected, as indicated by the phosphorylation of PKB at Thr308 (Fig. 4C). Moreover, when cells were stimulated with anisomycin or IL-1, no reduction in p38α MAPK phosphorylation was observed in the presence of both PAK1 and PAK2 siRNA (Fig. 4D). This indicates that these agonists couple to the activation of p38α MAPK by a distinct mechanism that is independent of PAK1/PAK2. Taken together, our results demonstrate that signalling from CXCR4 to p38α MAPK is mediated by both PAK1 and PAK2. Knockdown of PAK1 and PAK2 completely inhibited CXCL12-induced cell migration (Fig. 4E), consistent with a key role for p38α MAPK in this process.

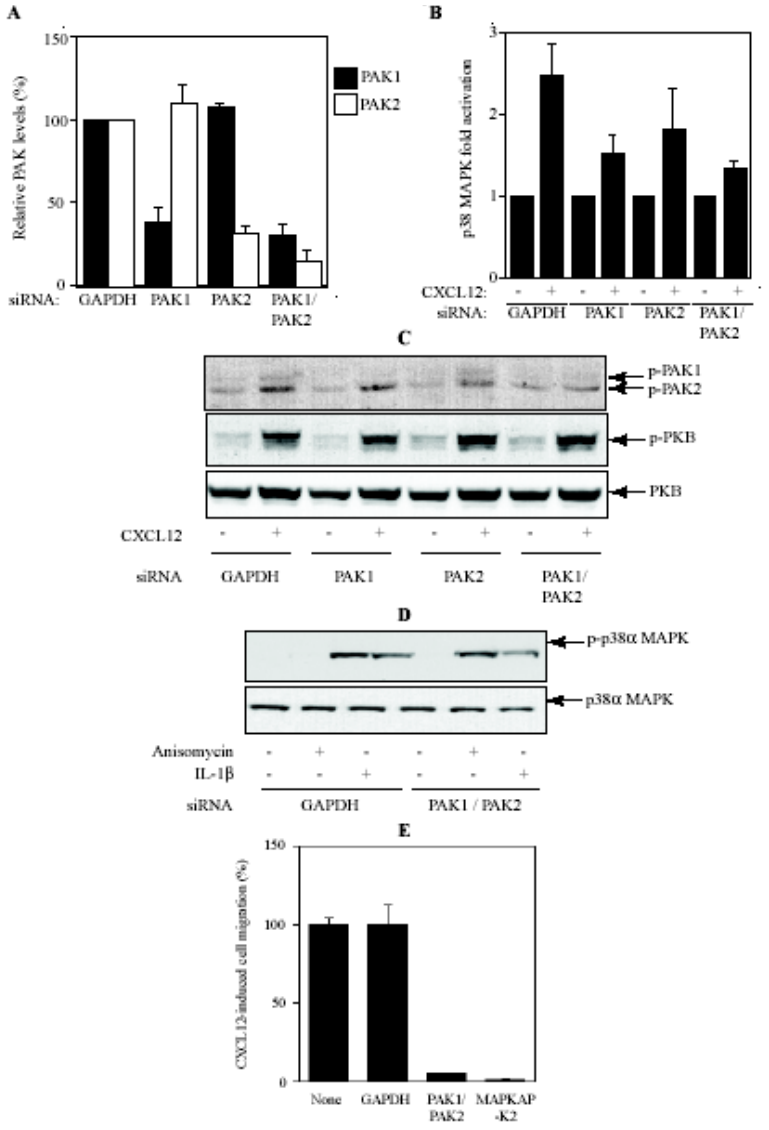

Figure 4. PAK1 and PAK2 are required for the activation of p38α MAPK.

A. HeLa cells were transfected with siRNA against GAPDH or PAK1 (siRNA-1-see Methods) or PAK2 (siRNA-1-see Methods) or both PAK1 and PAK2, as indicated. After cell lysis, the extracts were immunoblotted with antibodies that recognise PAK1 and PAK2. The levels of PAK1 (black bars) or PAK2 (white bars) protein were quantified and expressed as the % of protein present compared to the control levels and expressed as the average of two distinct experiments +/- SEM.

B. HeLa cells were transfected as in **A**. The cells were then either left untreated or exposed for 5 min to 10 nM CXCL12. After cell lysis, the extracts were immunoblotted with antibodies that recognise p38α MAPK phosphorylated at the Thr-Gly-Tyr motif (p-p38α MAPK) or all forms of p38α MAPK. The levels of phosphorylated p38α MAPK were quantified and expressed as fold activation over untreated cells. The results are presented as the average of three distinct experiments +/- SEM.

C. Hela cells were transfected as in **A**. After cell lysis, the extracts were immunoblotted with antibodies that recognise PAK1 and PAK2 (p-PAK1, p-PAK2) phosphorylated at Ser199 and Ser204 (PAK1) or Ser192 and Ser197 (PAK2) respectively, and PKB phosphorylated at Thr308 (p-PKB) or all forms of PKB.

D. HeLa cells were transfected with siRNA against GAPDH or against both PAK1 and PAK2 (siRNA-1), as indicated. The cells were then either left untreated or exposed for 15 min to 10 μg/ml anisomycin or for 15 min to 10 ng/ml IL-1. HeLa cell extracts were immunoblotted with antibodies that recognises phosphorylated p38α MAPK (p-p38α MAPK) or that recognise phosphorylated and unphosphorylated protein equally well (p38α MAPK).

E. HeLa cells were transfected with siRNA against GAPDH, against both PAK1 and PAK2 (siRNA-1) or against MAPKAP-K2, as indicated. The cells were then left to adhere for 1 h to the upper membrane of the assay vessel, then stimulated for 4 h with 10 nM CXCL12. Cell migration was expressed as a percentage of CXCL12-induced cell migration in the absence of siRNA. Similar results were obtained in three independent experiments.

MAPKAP-K2 lies "downstream" of p38α MAPK in the pathway leading to cell motility.

p38α MAPK activates several other protein kinases in cells, including MAPK-activated protein kinase-2 (MAPKAP-K2) and mitogen and stress-activated protein kinase-1 (MSK1) and MSK2 (reviewed in [22, 27]. Primary macrophages derived from mice lacking MAPKAP-K2, showed impaired cell motility in response to C5a, whereas mice lacking both MSK1 and MSK2 did not (Fig. 5A). These results were supported by the finding that siRNA knockdown of MAPKAP-K2 also prevented CXCL12 induced cell migration (Fig. 4E), demonstrating that MAPKAP-K2 is an essential link in the p38α MAPK-induced cell motility.
MAPKAP-K2 phosphorylates numerous proteins in cells, including HSP27 [28], hnRNP A0 [18] and NOGO-B [20]. We therefore examined which of these substrates could be involved in mediating MAPKAP-K2-dependent cell migration. Macrophages from mice lacking both NOGO-A and NOGO-B [29] showed a normal response to C5a (Fig. 5A), indicating that this substrate is involved in regulating a different process. In HeLa cells stimulated with CXCL12, knockdown of HSP27 impaired cell migration, but no effect was observed if hnRNP A0 or GAPDH were down-regulated using RNA interference (Fig. 5B). These results suggest that HSP27 is involved in mediating MAPKAP-K2-dependent cell migration**.** The phosphorylation of hnRNP A0 by MAPKAP-K2 induces its binding to AU-rich elements in the 3'-untranslated region of mRNAs encoding some pro-inflammatory cytokines and may be involved in regulating the stability and/or translation of these messages [18].

p38α-MAPK deficient MEF show impaired cytoskeletal organization

HSP27 is known to play a role in mediating re-organization of the actin cytoskeleton in response to stress [30]. Moreover, inhibition of the p38α MAPK pathway with SB 203580 can prevent actin re-organization into stress fibres induced by VEGF [10]. Therefore we investigated whether MEF from p38α MAPK-deficient mice showed a particular cytoskeletal phenotype. Organization of focal adhesions and stress fibers is sometimes induced by using plates covered with collagen or fibronectin or by adding extracellular matrix proteins to the culture medium, especially in the case of poorly adherent cellular systems such as Ras-transformed mouse fibroblasts. However, fully adherent cells such as non-transformed MEFs, already show robust F-actin stress fibers and focal adhesion complexes under normal culture conditions, in the absence of any further treatment. To support the idea that p38α may play a role in cell migration by mediating actin polymerization and cytoskeleton remodeling, we analyzed how the absence of p38α affected F-actin stress fiber formation in exponentially proliferating MEFs. Under these conditions, there were more and longer F-actin stress fibers in WT than in p38α-/- MEFs. Moreover, stress fibers were better distributed and parallel in the case of WT MEFs (Fig. 6A, panel a), but show an abnormal radial distribution in p38α-/- MEFs (Fig. 6A, panel b). Additionally, a marked reduction in the number of focal adhesions can be observed in the MEF from p38α MAPK-deficient animals as illustrated by staining for vinculin, a protein found at focal adhesion sites (Fig. 6B). These results suggest that p38α MAPK is a key enzyme involved in the organization of the cytoskeleton.

Discussion

Here we have studied the role of p38α MAPK in transducing the chemotactic signals of various agonists. All chemo-attractants tested (PDGF-BB, HGF, CXCL12 and C5a) induced the activation of p38α MAP kinase and blocking its activity suppressed cell migration in response to these stimuli. Moreover, MEF that do not express p38α MAP kinase failed to migrate properly, whereas MEF lacking one or more of the other p38 MAPK isoforms migrated normally. These results clearly demonstrated that p38α MAP kinase is the enzyme in this sub-family that is responsible for mediating cell migration in response to chemotactic stimuli.

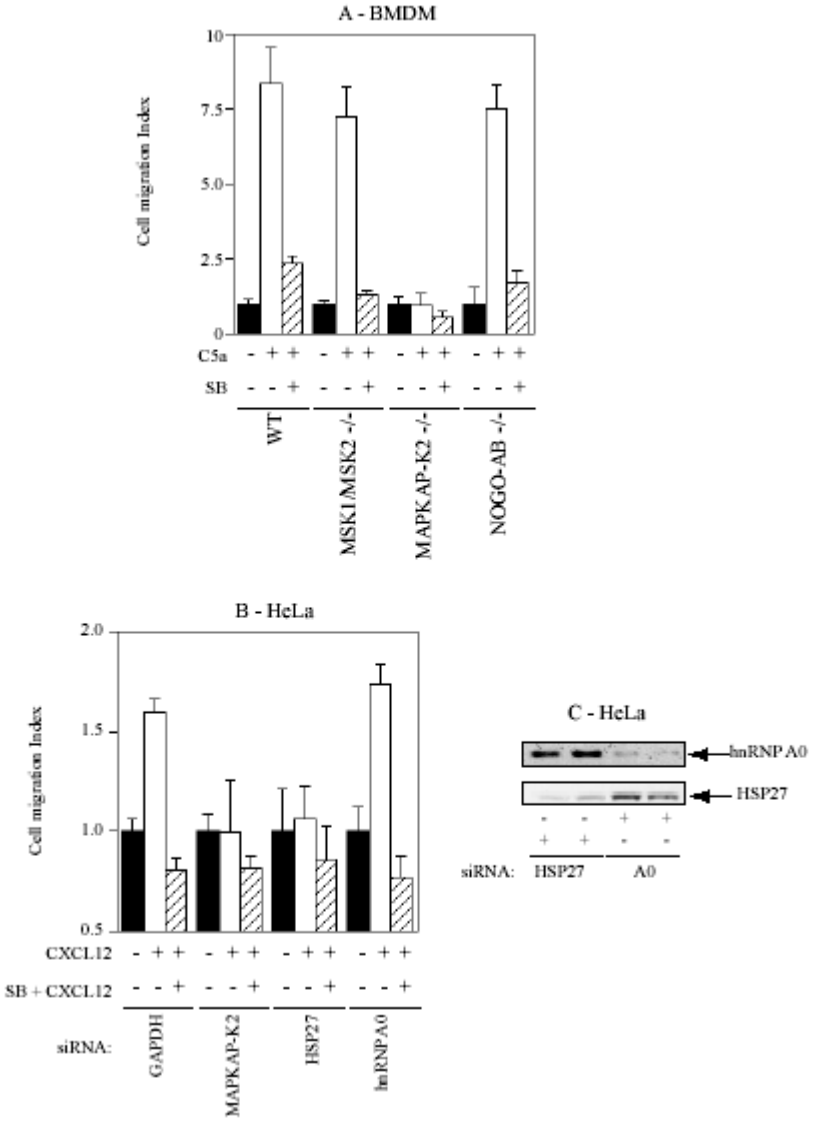

Figure 5. MAPKAP-K2 and HSP27 are involved in transducing the chemotactic signals from p38α MAPK.
A. BMDM were isolated from wild type (WT) mice or mice deficient in the proteins indicated, and incubated for 30 min without or with 5 μM SB 203580 (SB) added to both chambers of the vessel used to measure cell migration. They were then stimulated for 4 h with 1 μg/ml C5a. Cell migration was measured as in Fig 1. Similar results were obtained in two independent experiments.
B. HeLa cells were transfected with siRNA against GAPDH, MAPKAP-K2, HSP27 (siRNA-1 plus siRNA-2 – see Methods) or hnRNP A0, incubated for 30 min without or with 5 μM SB 203580 (SB) added to both chambers of the vessel used to measure cell migration and then stimulated for 4 h with 10 nM CXCL12. Migration was measured as in Fig. 1.
C. The extracts from cells treated with siRNA for HSP27 (siRNA-1 plus siRNA-2) or siRNA for hnRNP-A0 (30 μg protein) were immunoblotted as in Fig 4 using antibodies that recognise all forms of HSP27 (upper panel) or hnRNP A0 (lower panel) equally well.

We then investigated which other signalling components were involved in mediating the activation of p38α MAPK by chemotactic stimuli and how p38α MAPK triggers cell migration. PAK1/PAK2 are known to bind to, and be activated by, small GTPases of the Rho family, like RAC and CDC42, and thus are involved in transducing the effects of these small GTP binding proteins on the cytoskeleton (reviewed in[26]). We found that greatly reducing the expression of PAK1 and PAK2 using RNA interference blocked both the activation of p38α MAPK and the migration of HeLa cells in response to CXCL12. Other laboratories have shown that the

overexpression of catalytically inactive PAK1 inhibits smooth muscle cell migration in response to PDGF and greatly reduces PDGF-induced phosphorylation of p38 MAPK [31]. Importantly, we found that the CXCL12-stimulated phosphorylation of p38α MAPK was not blocked by wortmannin (an inhibitor of PtdIns 3-kinase) or PD 184352 (an inhibitor of the activation of ERK1/ERK2) (data not shown) demonstrating that these pathways, which are also known to be required for cell migration [32], operate through signalling components distinct from the PAK-p38α MAPK pathway.

Following its activation, p38α MAPK phosphorylates many proteins, including the protein kinases MAPKAP-K2 and the closely related MAPKAP-K3, MSK1 and the closely related MSK2 as well as MNK1 and the closely related MNK2. Eliminating the expression of MAPKAP-K2 by RNA interference inhibited the CXCL12-induced migration of HeLa cells, while BMDM from MAPKAP-K2-deficient mice did not migrate in response to C5a. In contrast, migration was unimpaired in BMDM that do not express MSK1 and MSK2. These results are consistent with studies in other cells, which showed that MAPKAP-K2-deficient neutrophils lost directionality when migrating in response to fMLP [33] and that cell migration was greatly impaired in MAPKAP-K2-deficient fibroblasts and vascular smooth muscle cells [34].

A number of substrates have been described for MAPKAP-K2 that could potentially be involved in mediating this signal, such as NOGO-B [20]. NOGO-B has been reported to co-localise with caveolin at cell membranes and to stimulate the migration of endothelial cells. However, NOGO-B was reported to inhibit the migration of vascular smooth muscle cells [35]. We found that macrophages from mice lacking NOGO-A and NOGO-B did not show any impairment of cell motility in response to C5a, making it unlikely that the phosphorylation of this protein mediates the effect of MAPKAP-K2 on cell migration.

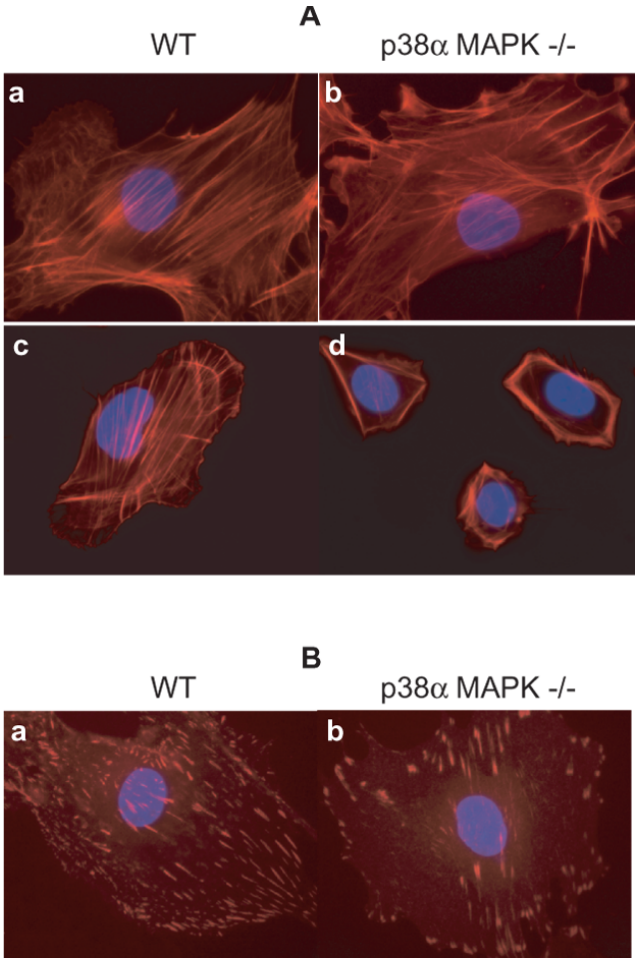

Figure 6. p38α MAPK plays an important role in the organization of the cytoskeleton.
A. MEF spontaneously immortalised (a and b) or immortalised with SV40 large T antigen (c and d) from wild type (a and c) or p38α MAPK-deficient mice (b and d) were stained for F-actin with rhodamine-conjugated Phalloidin.
B. MEFs from WT (a) or p38α-MAPK deficient mice (b) were stained with an antibody raised against vinculin coupled to Alexa-594 anti-mouse-IgG antibody.

Three other physiological substrates of MAPKAP-K2, HSP27 [28], LSP1 [36] and CapZIP [37], have been proposed to play a role in regulating actin dynamics and are therefore candidates to mediate the effects of MAPKAP-K2 on migration. Unphosphorylated HSP27 is an actin cap-binding protein *in vitro* [30, 38] and its phosphorylation by MAPKAP-K2 is thought to release it from actin in cells. This seems to induce new sites of actin nucleation, leading to the enhanced actin polymerisation [39, 40] needed to extend the leading edge of migrating cells. We found that siRNA against HSP27 blocked C5a-induced cell migration. Consistent with this observation, another laboratory has shown that infection of smooth muscle cells with adenoviruses expressing a catalytically inactive mutant of p38α MAPK, or an HSP27 mutant which cannot be phosphorylated by MAPKAP-K2, inhibits their migration in response to PDGF [14]. It could be envisaged that a set of MAPKAP-K2 substrates (HSP27, LSP1, CAPZIP) are all involved in co-ordinating an increase in actin dynamics to adapt the cytoskeleton of cells to particular environmental stresses and chemotactic stimuli.

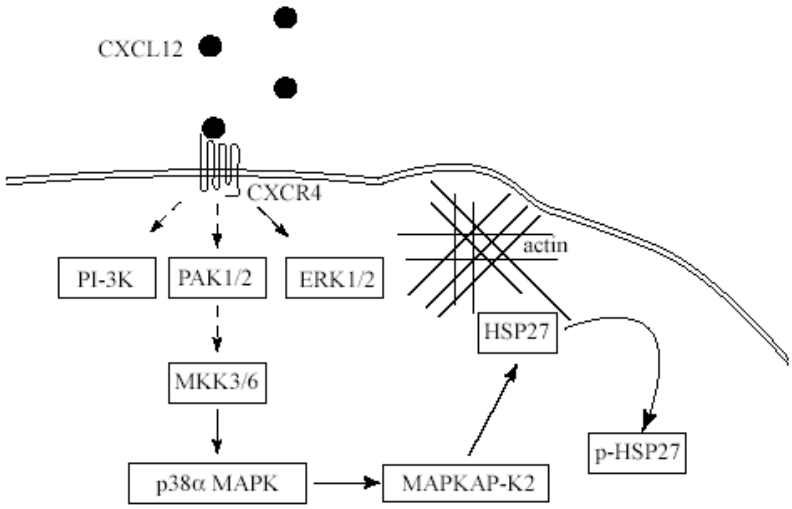

Figure 7. Proposed p38α MAPK-dependent pathway by which chemotactic agents stimulate cell migration. The binding of chemoattractants, such as CXCL12, to their receptors activates numerous intracellular signalling pathways, including the p38α MAPK pathway. In the present study, we have delineated a pathway that leads from such receptors to the sequential activation of PAK1/PAK2, p38α MAPK and MAPKAP-K2, which then phosphorylates HSP27. Phosphorylated HSP27 has been linked with increase actin dynamics that could contribute to the extension of the leading edge of the cell membrane, where it is enriched [28]. In conjunction with other signals, such as the PI3-kinase and classical MAPK cascade, the activation of this pathway may lead to the formation and stabilisation of the necessary actin structures for cell motility.

Conclusions

In summary, the experiments reported in this paper indicate that CXCL12 and C5a stimulate cell migration through the signalling pathway illustrated in Fig 7. Finally, our work points to MAPKAP-K2 as an interesting target for the development of anti-cancer/metastatic drugs, as well as anti-inflammatory drugs.

Acknowledgements

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A new p38 MAP kinase-regulated transcriptional co-activator that stimulates p53-dependent apoptosis

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Abstract

The p38 MAPK signaling pathway plays an important role in stress-induced cell-fate decisions by orchestrating responses that go from cell cycle arrest to apoptosis. We have identified a new p38 MAPK-regulated protein that we named p18^{Hamlet}, which becomes stabilized and accumulates in response to certain genotoxic stresses such as UV or cisplatin treatment. Overexpression of p18^{Hamlet} is sufficient to induce apoptosis, whereas its downregulation reduces the apoptotic response to these DNA damage-inducing agents. We show that p18^{Hamlet} interacts with p53 and stimulates the transcription of several pro-apoptotic p53 target genes such as PUMA and NOXA. This correlates with enhanced p18^{Hamlet}-induced recruitment of p53 to the promoters. In proliferating cells, low steady-state levels of p18^{Hamlet} are probably maintained by a p53-dependent negative feed-back loop. Therefore, p18^{Hamlet} is a new cell-fate regulator that links the p38 MAPK and p53 pathways and contributes to the establishment of p53-regulated stress responses.

Key words: p38 MAP kinase, p53, transcriptional co-activator, apoptosis, stress response

Introduction

Cells are continuously exposed to a variety of environmental stresses and, as a consequence, sometimes have to take the important decision whether to live or not to live. Several signaling pathways are involved in the stress-induced cell fate decisions. One of these pathways leads to the activation of the p38 mitogen-activated protein kinase (MAPK) cascade that coordinates cell responses to many types of stresses including UV, chemotherapeutic agents and oncogenes.

Four p38 MAPKs have been identified in higher eukaryotes. The most widely expressed and studied family member is p38α, which can be activated by the MAPK kinases MKK6, MKK3 and MKK4. Many proteins can be phosphorylated by p38 MAPKs including protein kinases and a growing list of transcription factors. The set of substrates targeted by p38 MAPKs in each particular case is thought to be an important determinant for the specificity of the cellular responses, which can be as diverse as cytokine production, cell differentiation, cell cycle arrest or apoptosis (Bulavin and Fornace, 2004; Nebreda and Porras, 2000; Ono and Han, 2000).

Activation of p38α in response to several anti-cancer agents is necessary and, in some cases, sufficient, to induce apoptosis in a variety of cancer cell lines (Coltella et al., 2006; Deacon et al., 2003; Poizat et al., 2005; Sanchez-Prieto et al., 2000). These results, together with the ability of p38α to positively regulate several tumor suppressor pathways and to attenuate oncogenic signals, have led to the proposal that this protein may function as a tumor suppressor (Bulavin and Fornace, 2004).

There is good evidence supporting a role for p38α in the regulation of the tumor suppressor protein p53, mainly through the phosphorylation of p53 induced by several types of stress (Bulavin et al., 1999; She et al., 2000). p53 is one of the most commonly mutated genes in human cancers and its loss of function is believed to result in increased genomic instability, with the subsequent acquisition of additional oncogenic mutations (Vousden and Prives, 2005). The protein level and transcriptional activity of p53 are upregulated in response to many stresses, including DNA damage. Upon activation, p53 coordinates a complex cellular response, which can lead to reversible cell-cycle arrest, an irreversible senescence-like state or apoptosis (Vousden and Lu, 2002). The role of p53 in maintenance of genome integrity involves multiple control mechanisms, including various post-translational modifications, such as phosphorylation, acetylation, ubiquitination and sumoylation (Bode and Dong, 2004). These modifications may increase the half-life of the p53 protein, which results in a rapid rise in intracellular p53 levels, and also enhances its ability to bind to specific promoter DNA sequences.

An additional and attractive mechanism of p53 regulation has emerged in the last years as a collection of transcriptional co-activators that influence p53 activity, usually without modifying the p53 protein (Coutts and La Thangue, 2005). These co-activators confer specificity to the p53 response since they are upregulated in response to certain types of stresses and, in some cases, enhance the ability of p53 to activate the transcription of genes involved in a particular response. This is the case of the ASPP (apoptotic-stimulating proteins of p53) family of proteins, which can interact with p53 and specifically stimulate the expression of the pro-apoptotic genes BAX and PIG3 (Samuels-Lev et al., 2001). Another example is hnRNP K (heterogeneous nuclear ribonucleoprotein K), recently identified as a transcriptional co-factor for p53 that has a crucial role in DNA-damage-induced cell-cycle arrest (Moumen et al., 2005).

We report here a new p38α-regulated protein, which we named $p18^{Hamlet}$ based on its ability to control life-or-death cell fate decisions. $p18^{Hamlet}$ accumulates in response to genotoxic stresses and induces the transcriptional activation of several p53 target genes such as NOXA and PUMA.

Results

Identification of a new p38 MAPK substrate

We performed yeast two-hybrid screenings to identify new proteins that could mediate the biological responses of p38α (Cheung et al., 2003). In these experiments, we found that human p38α specifically interacted with a poorly characterized protein of 18 kDa (NP_006340). We named this protein p18^{Hamlet}, based on its function, which is related to the regulation of cell-fate decisions, as described below. Two interesting features of p18^{Hamlet} were a C-terminal zinc finger-HIT1 type domain, which has been described as a protein-protein interaction domain in the Trip3 co-activator of hepatocyte nuclear factor-4a (Iwahashi et al., 2002) and a bipartite nuclear localization signal (Fig. 1A). p18^{Hamlet} was conserved along the evolutionary scale from yeast to human (Fig. 1B).

We first investigated whether p18^{Hamlet} was able to bind to different members of the p38 MAPK family. For this analysis, we performed *in vitro* pull-down assays with ^{35}S-labeled p38 MAPKs and recombinant GST-fused p18^{Hamlet} protein. As expected from the yeast two-hybrid results, p18^{Hamlet} was able to interact with p38α and also with p38β but not with p38γ and p38δ or with the p38 activator MKK6 (Fig. 1C and Supplementary Fig. 1). The interaction between p18^{Hamlet} and p38α was also observed in transfected HEK-293T cells, and was independent of the activation loop phosphorylation of p38α (Fig. 1D). We also detected interaction of the endogenous p18^{Hamlet} with Myc-p38α transfected in HEK-293T cells (Fig. 1E), but we failed to detect interaction of both endogenous proteins. Of note, we could neither detect interaction between the transfected proteins using p18^{Hamlet} or p38α antibodies for the immunoprecipitation (not shown), suggesting that the antibodies might sterically interfere with or somehow affect complex formation. We also confirmed that both p38α and p38β phosphorylated p18^{Hamlet} in vitro with similar efficiencies (Fig. 1F), whereas p38γ or p38δ did not phosphorylate p18^{Hamlet} (Supplementary Fig. 1).

The sequence of p18^{Hamlet} contains only one consensus MAPK phosphorylation site (Ser/Thr-Pro) at Ser124. However, mutation of this Ser to Ala did not affect in vitro phosphorylation of p18^{Hamlet} by p38α (not shown). Using generic phospho antibodies, we found that p18^{Hamlet} was phosphorylated by p38α *in vitro* on Thr residues (Fig. 2A). Based on this result, we individually mutated the nine Thr residues present in human p18^{Hamlet} and found four (Thr6, Thr64, Thr71, and Thr103) that could be potentially phosphorylated by p38α. However, the quadruple mutant T6A, T64A, T71A and T103A (4xT/A) was still partially phosphorylated by p38α (Supplementary Fig. 2), suggesting that additional residues might be involved.

We found that p38 MAPK activation by UV treatment also correlated with Thr phosphorylation of p18^{Hamlet} , and this phosphorylation was significantly reduced when the cells were pre-treated with the p38 MAPK inhibitor SB203580 (Fig. 2B). This suggests that p38 is involved in UV-induced p18^{Hamlet} phosphorylation. The high conservation of Thr103 as a phosphorylation site in p18^{Hamlet} proteins from different species (Fig. 1A), together with the *in vitro* phosphorylation experiments, suggested that this residue could be an important target for p38 MAPK. Indeed, mutation of Thr103 impaired the p38 MAPK-mediated phosphorylation of p18^{Hamlet} in cells, as determined by the reduced signal observed with the phospho-Thr antibody, when both wt and the T103A p18^{Hamlet} proteins were co-expressed with the p38 activator MKK6-DD (Fig. 2C). We developed an antibody that specifically recognized phospho-Thr103 (Fig. 2D) and confirmed that p18^{Hamlet} was phosphorylated on this residue in UV-treated cells (Fig. 2E). It is important to note that both the generic phospho-Thr and the specific phospho-Thr103 antibodies recognize p18^{Hamlet} phosphorylated on Thr103. However, in vitro kinase assays indicate that this was not the only p38α-dependent phosphorylation residue present in p18^{Hamlet} (Supplementary Fig. 2).

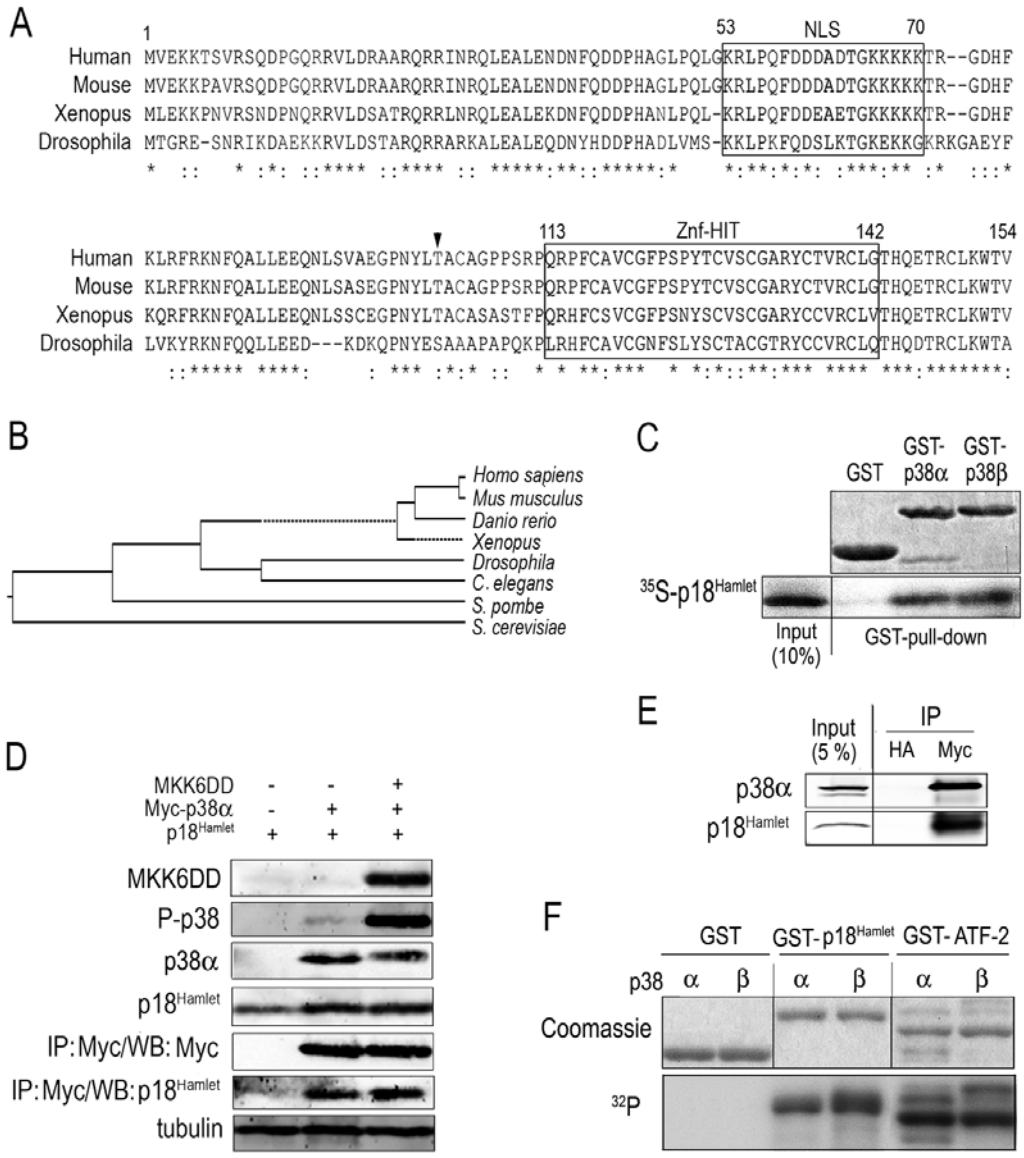

Fig. 1 p18^{Hamlet} is an evolutionary conserved substrate of the p38α and p38β MAPKs

(A) Amino acid sequence alignment of p18^{Hamlet} proteins. Identical and similar residues are indicated by asterisks and two dots, respectively. The domains corresponding to the Nuclear Localization Signal (NLS) and the Zinc-finger HIT type I domain (Znf-HIT) are boxed. The arrowhead indicates a Thr residue that was selected to generate specific phospho-antibodies. Sequence alignment was performed using the ClustalW program.

(B) Phylogenetic tree of p18^{Hamlet} proteins from *Homo sapiens* (NM_006349), *Mus musculus* (BC026751), *Xenopus* (NM_001017056), *Drosophila* (NP_608895), *Danio rerio* (AAH67648), *C.elegans* (NP_504477), *S.pombe* (CAB60106) and *S.cerevisiae* (NP_013671).

(C) GST pull-down assay were performed with the indicated GST-fused proteins and ^{35}S-labelled p18^{Hamlet}.

(D) HEK-293 cells were transfected with 5 μg of Myc-p38α, MKK6DD and p18^{Hamlet}, as indicated. 48 h after transfection, total cell lysates were prepared and analyzed by Western blotting together with Myc immunoprecipitates (IP).

(E) HEK-293 cells were transfected with Myc-p38α, and 48 h after transfection, p38α was immunoprecipitated with Myc and HA (as a negative control) antibodies.

(F) Kinase assays were performed using activated p38α and p38β MAPKs (200 ng) and GST, GST-p18^{Hamlet} and GST-ATF-2 (1 μg) in the presence of ^{32}P-γ-ATP. Coomassie staining shows the proteins used.

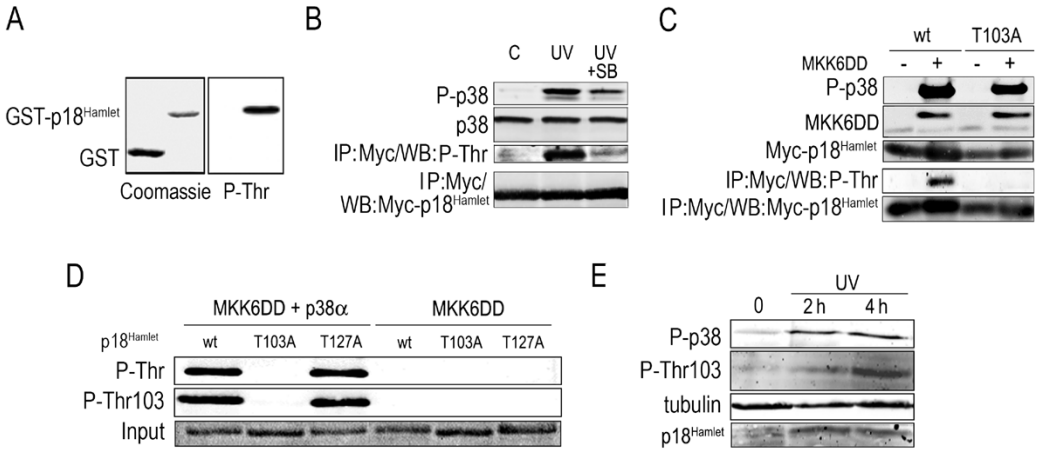

Fig. 2 p38α phosphorylates several Thr residues in p18^{Hamlet}
(A) GST-p18^{Hamlet} or GST proteins (500 ng) were phosphorylated with p38α *in vitro* and then analyzed by Western blotting with phospho-Thr antibodies.
(B) HEK-293 cells were transfected with Myc-p18^{Hamlet} and 48 h later were treated with UV alone or in the presence of 10 μM SB203580. 3 h after irradiation, total cell lysates were prepared and analyzed by Western blotting together with Myc immunoprecipitates.
(C) HEK-293 cells were transfected with Myc-p18^{Hamlet} wt and T103A either alone or together with MKK6DD, as indicated, and p18^{Hamlet} phosphorylation was analyzed by Myc immunoprecipitation followed by Western blotting with phospho-Thr antibody. Total cell lysates were also analyzed by Western.
(D) GST-p18^{Hamlet} wt, T103A and T127A proteins were incubated with p38α and MKK6DD or with MKK6DD alone and then analyzed by Western blotting with both phospho-Thr103-p18^{Hamlet} and generic phospho-Thr antibodies.
(E) HeLa cells overexpressing p18^{Hamlet} were UV irradiated, lysed at the indicated times after irradiation and analyzed by Western blotting with the phospho-Thr103-p18^{Hamlet} antibody.

p18^{Hamlet} protein levels are regulated by p38 MAPK

The mRNA levels of p18^{Hamlet} varied significantly among different human tissues and cell lines and appeared to be significantly high in some cases, in contrast with the p18^{Hamlet} protein, which was usually hard to detect (Supplementary Fig. 3 and data not shown). We therefore investigated the possibility that p18^{Hamlet} protein stability could be regulated. As shown in Fig. 3A, incubation with the proteasome inhibitor MG132 resulted in the accumulation of endogenous p18^{Hamlet} protein in both mouse embryonic fibroblasts (MEFs) and human osteosarcoma U2OS cells. To demonstrate that p18^{Hamlet} was a target of the ubiquitin-proteasome system, we transfected U2OS cells with Myc-p18^{Hamlet} alone or in combination with HA-Ubiquitin and then analyzed the Myc-p18^{Hamlet} immunoprecipitates by immunoblotting with anti-p18^{Hamlet} antibody. In this experiment, we detected a smear of slowly migrating p18^{Hamlet} forms that were not observed in extracts form cells transfected with either HA-Ubiquitin or p18^{Hamlet} alone, suggesting that they probably correspond to p18^{Hamlet}-ubiquitin conjugates. This was further supported by the recognition of the slowly migrating forms of p18^{Hamlet} with an anti-HA antibody (Fig. 3B). MG132 concentrations that inhibit the proteasome have been also reported to activate p38 MAPK (Wu et al., 2004). We confirmed that MG132-induced p18^{Hamlet} accumulation correlated with the phosphorylation of p38 MAPK in MEFs, but this effect was abolished when the MG132 treatment was performed in the presence of the p38 MAPK inhibitor SB203580 (Supplementary Fig. 4). These results strongly suggest that p18^{Hamlet} accumulation requires the activation of p38 MAPK.
Next, we investigated the effect of genotoxic stresses that activate the p38 MAPK pathway, such as UV (Kyriakis and Avruch, 1996), on the endogenous p18^{Hamlet} protein levels. In agreement with the above results, UV-induced p38 MAPK phosphorylation correlated with a small (about

two-fold) but reproducible increase in endogenous p18^{Hamlet} protein levels in different cell lines (Fig. 3C). The amount of p18^{Hamlet} protein typically peaked between 1 and 6 h after the treatment and later on decreased to levels lower than untreated cells. Importantly, the UV-induced accumulation of p18^{Hamlet} was prevented by pre-treatment with SB203580 (Fig. 3D).
We then investigated whether p18^{Hamlet} accumulation was specific for UV-induced p38 MAPK activation or if it was a more general response to stress. Treatment of cells, with the chemical DNA damage-inducing agent cisplatin induced p38 MAPK activation, which was accompanied by a significant increase in endogenous p18^{Hamlet} levels (Fig. 3E).

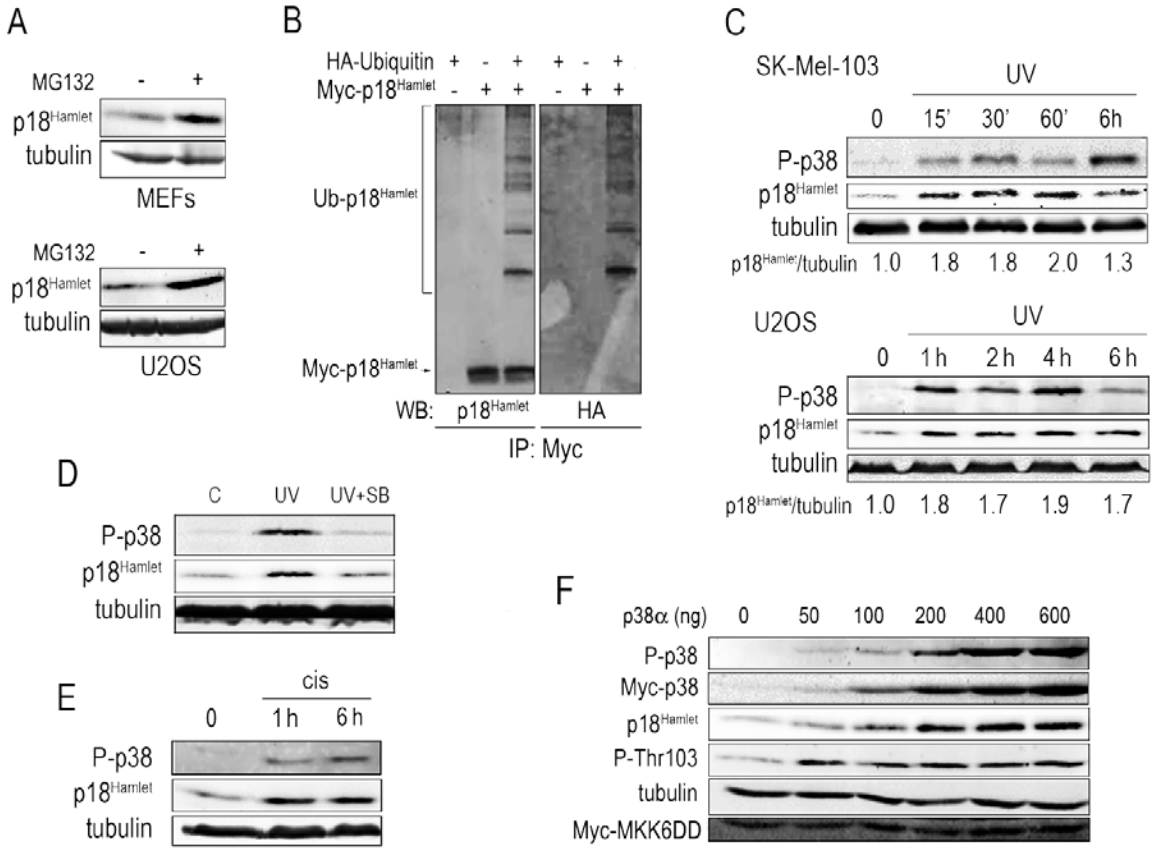

Fig. 3 Accumulation of p18^{Hamlet} protein in response to DNA damage-inducing agents
(A) MEFs and U2OS cells were treated with the proteasome inhibitor MG132 (25 μM) for 2 h and then lysed. Expression of endogenous p18^{Hamlet} was analyzed by Western blotting.
(B) U2OS cells were transfected with HA-Ubiquitin and Myc-p18^{Hamlet}, as indicated, and 16 h after transfection were treated with MG132 (25 μM) for 5 h. Myc immunoprecipitates were analyzed by Western blotting using p18^{Hamlet} and HA antibodies.
(C) SK-Mel-103 and U2OS cells were treated with UV and cell lysates were analyzed by Western blotting using the indicated antibodies.
(D) SK-Mel-103 cell lysates were prepared 3 h after UV treatment, either in the presence or absence of SB203580 (SB, 10 μM), and analyzed by Western blotting.
(E) SK-Mel-103 cells were treated with cisplatin for the indicated times and p18^{Hamlet} accumulation was analyzed by Western blotting.
(F) U2OS cells were co-transfected with MKK6DD (600 ng), p18^{Hamlet} (1 μg) and increasing amounts of p38α as indicated. 24 h after transfection, lysates were prepared from both attached and floating cells (that express higher levels of p18^{Hamlet}) and analyzed by Western blotting.

To further strengthen the connection between p38 MAPK activation and the accumulation of p18^{Hamlet}, we transfected U2OS cells with a low amount of p18^{Hamlet} (to limit apoptosis induction, see below) together with increasing amounts of p38α and a constant amount of its activator MKK6DD (Fig. 3F). In this experiment, we observed a direct correlation between p38α activation levels and the amount of p18^{Hamlet} protein expressed. We also detected maximal p18^{Hamlet} phosphorylation on Thr103 with the lowest concentration of p38α used, suggesting that this residue might be efficiently phosphorylated in situations of poor p38 MAPK activation. However, the T103A mutant protein was still able to accumulate upon p38α activation (Supplementary Fig. 5), suggesting that Thr103 phosphorylation may contribute to but is not essential for p18^{Hamlet} accumulation.

Finally, we confirmed that p18^{Hamlet} was an unstable protein with a half-life of less than 3 h in cycloheximide-treated U2OS cells (Fig. 4A). Interestingly, specific activation of p38α was sufficient to significantly increase the half-life of p18^{Hamlet} (Fig. 4B and 4C). In contrast, we could detect no changes in p18^{Hamlet} mRNA levels when cells were treated with cisplatin or UV (Fig. 4D). These results indicated that the stress-induced accumulation of p18^{Hamlet} was mainly regulated at the level of protein stability. To determine the importance of p38 MAPK-mediated phosphorylation in p18^{Hamlet} protein stability we performed cycloheximide-chase experiments in U2OS cells transfected with p18^{Hamlet} wt, T103A or the quadruple mutant 4xT/A and then UV irradiated (Fig. 4E). While the T103A and wt proteins behaved similarly and were both significantly accumulated in response to UV, the 4xT/A mutant expression levels were not affected by this treatment. Moreover, the 4xT/A mutant was expressed at lower levels than wt p18^{Hamlet}, supporting the idea that phosphorylation of these sites could be important for the regulation of p18^{Hamlet} protein stability.

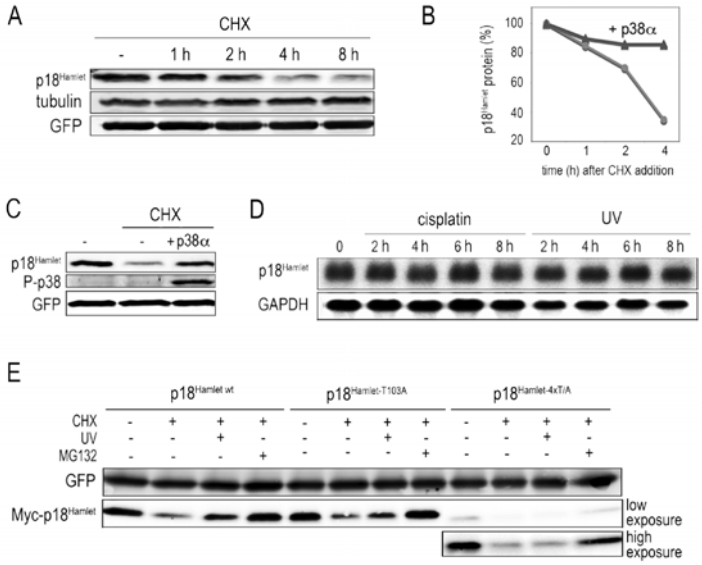

Fig. 4 Stabilization of p18^{Hamlet} protein in response to p38α activation

(A) U2OS cells were co-transfected with p18^{Hamlet} (1 μg) and GFP (500 ng) and incubated with cycloheximide (CHX, 30 μg/ml) for the indicated times. Total cell lysates were analyzed by Western blotting.

(B and **C)** U2OS cells were transfected with p18^{Hamlet}, either alone or together with p38α and MKK6DD, and 24 h later were incubated with CHX for up to 4 h. Expression of p18^{Hamlet} protein was determined by Western blotting. The blots corresponding to cells untreated or treated with CHX for 4 h, in the presence or absence of active p38α are shown in **(C)**.

(D) Total RNAs were obtained from UV or cisplatin-treated SK-Mel-103 cells and were analyzed by Northern blotting with a p18^{Hamlet} probe. GAPDH was used to confirm equal RNA loading.

(E) U2OS cells were transfected with 6 μg of p18^{Hamlet} wt, T103A or 4xT/A, as indicated. 24 h after transfection cells were treated with CHX alone or in combination with MG132 for 4 h. Where indicated, cells were also UV irradiated 1 h before collection. A GFP-expression vector (200 ng) was co-transfected to ensure equal efficiency of transfection.

Accumulation of p18^{Hamlet} induces apoptosis

Once we established that p18^{Hamlet} levels increased in response to DNA damage, we investigated the biological significance of this accumulation. U2OS cells were transfected with either GFP-p18^{Hamlet} or GFP alone and then sorted to analyze the cell cycle profile in the fluorescent cell population. We found that around 6% of the GFP-expressing cells were apoptotic (sub G0/G1 population), whereas this amount increased to 18% in the GFP-p18^{Hamlet} positive cells, indicating that p18^{Hamlet} overexpression was sufficient to induce apoptosis. Interestingly, GFP-p18^{Hamlet} overexpression did not affect the apoptosis levels in SAOS cells, a p53-deficient human osteosarcoma cell line (Fig. 5A).

Given the key role of p53 in the apoptotic response induced by DNA damage, we investigated if p18^{Hamlet} could regulate p53. In U2OS cells, GFP-p18^{Hamlet} overexpression did not affect p53 total levels (Fig. 5B) but it did result in higher levels of the pro-apoptotic p53 target gene NOXA, while it had no effect on other p53-dependent pro-apoptotic genes such as PUMA or Bax. In contrast, NOXA levels were not affected by GFP-p18^{Hamlet} overexpression in SAOS cells. To study the induction of apoptosis by p18^{Hamlet} in more detail, we generated a tetracycline-inducible system in which p18^{Hamlet} protein expression peaked at about 16 h after the addition of tetracycline to U2OS cells (Supplementary Fig. 6). Consistent with the above results, tetracycline-induced p18^{Hamlet} expression was accompanied by an increase in both NOXA mRNA and protein levels (Fig. 5C), while we could detect no significant changes in the expression of other p53-dependent targets, such as PUMA, Bax, p21 or Hdm2 (Fig. 5C and data not shown). We also confirmed that p18^{Hamlet} induction was sufficient on its own to significantly increase the early and late apoptotic populations. In addition, p18^{Hamlet} cooperates with cisplatin treatment in apoptosis induction (Fig. 5D). To confirm the pro-apoptotic function of p18^{Hamlet} in a more physiological system, we overexpressed p18^{Hamlet} in primary MEFs. As shown in Fig. 5E, p18^{Hamlet} overexpression was sufficient to increase apoptosis levels in non-stressed cells, and it also strongly promoted apoptosis induced by cisplatin or UV. Taken together, these results support a role for p18^{Hamlet} in p53-mediated apoptosis induction.

To confirm the role of endogenous p18^{Hamlet} as an apoptosis mediator, we designed siRNA oligonucleotides that efficiently downregulated p18^{Hamlet} (Fig. 6A and Supplementary Fig. 7 and 8). Previous work has shown that p53 contributes to the apoptosis induced by UV or cisplatin in both U2OS and MCF7 cells (Bergamaschi et al., 2003). Using a highly sensitive, quantitative method that detects apoptotic nucleosomes (see Materials and methods), we found that p18^{Hamlet} downregulation did not affect basal apoptosis levels but significantly impaired apoptosis induced by either UV or cisplatin in U2OS cells (Fig. 6A). The same effect was observed in MCF7 cells treated with cisplatin (Supplementary Fig. 7). We then analyzed the effect of p18^{Hamlet} downregulation on the expression of p53-dependent target genes upon cisplatin and UV treatment. Interestingly, only certain p53 target genes were induced in response to these two types of stress. In particular, NOXA and Hdm2 responded to cisplatin, while p21, Bax and PUMA remained unchanged. In the case of UV, only NOXA, and also slightly PUMA, protein levels were upregulated, while p21, Hdm2 and Bax did not increase (Fig. 6B). This result suggests that specific programs of gene expression account for the p53-dependent apoptosis in response to each particular type of stress. Downregulation of p18^{Hamlet} prevented NOXA and, in the case of UV irradiation, also PUMA protein accumulation, but had no effect on Hdm2 protein induction. Taken together, the results support an important role for p18^{Hamlet} in stress-induced apoptosis.

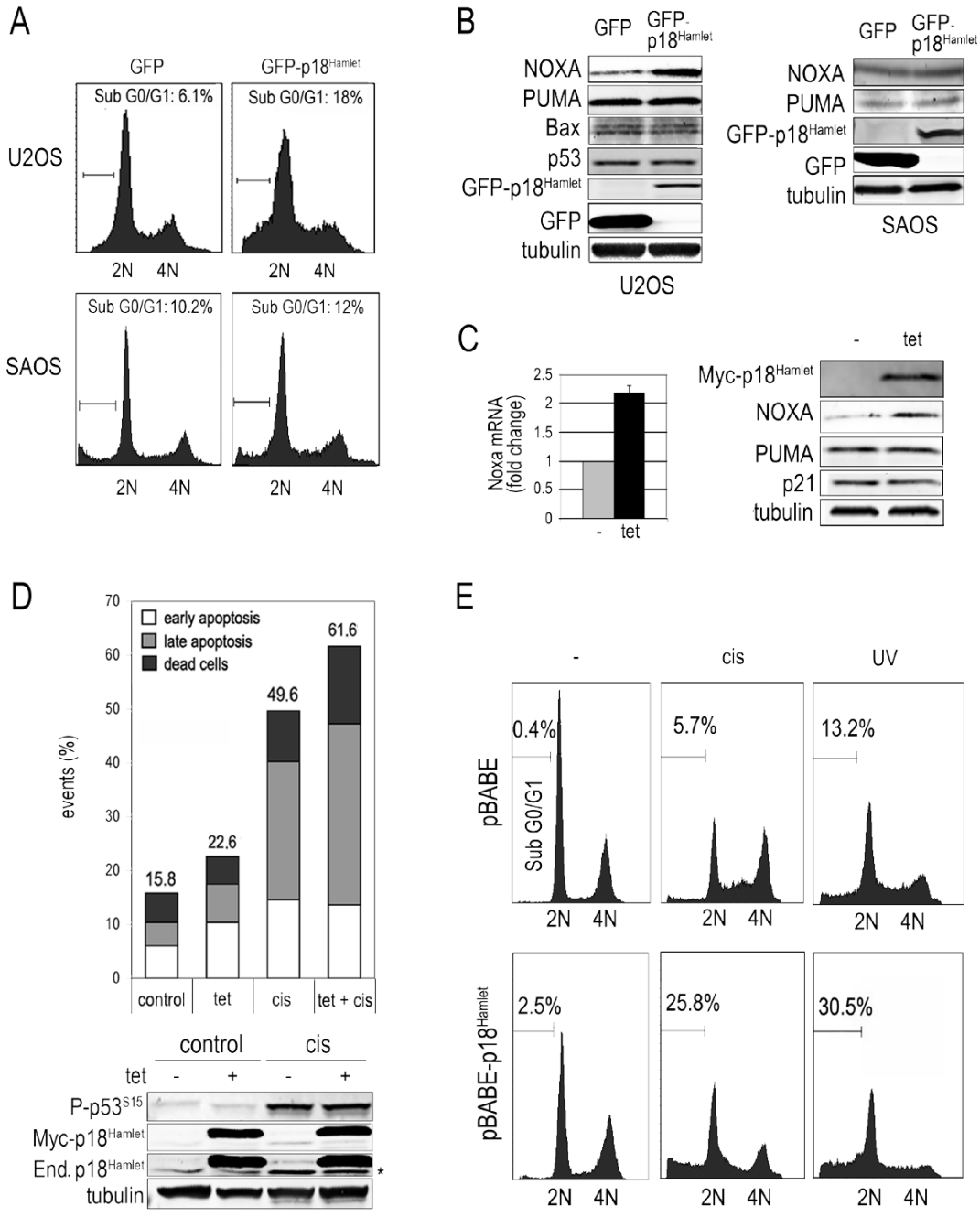

Fig. 5 Induction of apoptosis by p18^{Hamlet} overexpression

(A) U2OS and SAOS cells were transfected with GFP or GFP-p18^{Hamlet} and analyzed by flow cytometry. The percentage of cells with a subG0/G1 DNA content in a representative experiment is shown.

(B) U2OS and SAOS cells were transfected as indicated in **(A)** and the expression of the indicated proteins was analyzed by Western blotting.

(C) U2OS cells expressing inducible p18^{Hamlet} were treated with tet for 24 h before RNA and protein extraction. Samples were analyzed by quantitative RT-PCRs (Left) and Western blotting (Right).

(D) U2OS cells expressing tet-inducible p18^{Hamlet} were incubated with tet for 24 h and then treated with cisplatin for another 24 h, before analyzing apoptosis by annexin V staining. Numbers on top of the bars indicate total percentage of early and late apoptotic events as well as dead cells. Lower panel show the protein levels of overexpressed Myc-p18^{Hamlet}, endogenous (End.) p18^{Hamlet} (marked with an asterisk) and phospho-Ser15-p53.

(E) Primary MEFs were infected with pBABE puro or p18^{Hamlet}-expressing pBABE puro retroviruses. After puromycin selection, cells were treated for 24 h with cisplatin or UV as indicated, and the percentage of subG0/G1 cell population, as a measure of apoptosis levels, was analyzed by FACS.

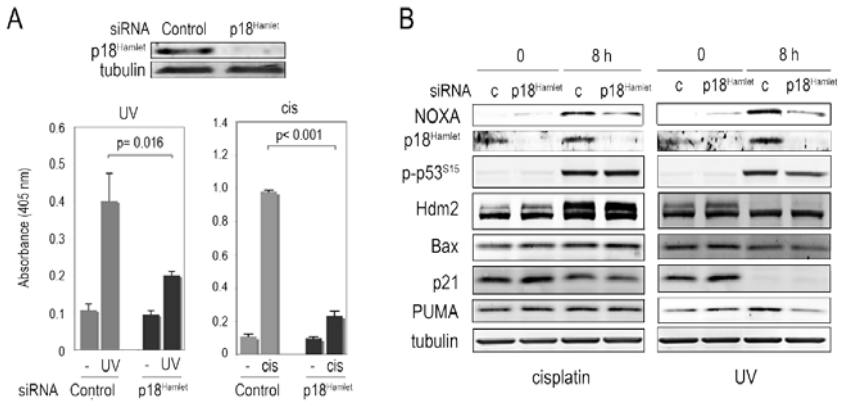

Fig. 6 p18^{Hamlet} is required for apoptosis induction in response to DNA Damage
(A) U2OS cells were transfected with p18^{Hamlet} or control siRNAs and the levels of endogenous p18^{Hamlet} were analyzed by Western blotting (*Upper*). 48 h after transfection, cells were treated with UV or cisplatin for 24 h, and apoptosis was quantified by measuring DNA fragmentation in a colorimetric assay. Means ± standard deviations of three independent experiments are represented. Statistical significance was evaluated with the Student's t-Test (P values are shown).
(B) U2OS cells were treated with siRNAs and UV or cisplatin as described in **(A)**. Expression of the indicated proteins was detected by Western blotting.

p18^{Hamlet} activates p53-dependent gene promoters

The ability of p18^{Hamlet} to upregulate p53-dependent pro-apoptotic genes prompted us to investigate whether both proteins could interact. We found that tetracycline-induced p18^{Hamlet} co-immunoprecipitated with endogenous p53 from U2OS cells (Fig. 7A, upper panel) and we also managed to co-immunoprecipitate the two endogenous proteins (Fig. 7A, lower panel). The interaction was also observed in pull-down assays using recombinant GST-p53 and ^{35}S-labelled p18^{Hamlet} and it involves the Zinc-Finger domain of p18^{Hamlet} and the C-terminal region of p53 (see below, Fig. 9D, and Supplementary Fig. 9). In contrast, the overexpression of p18^{Hamlet} did not affect phosphorylation of p53 on Ser15 and Ser46, two common p53 phosphorylation events in response to stress (Fig. 7B).
We then analyzed whether p18^{Hamlet} could regulate the transactivation function of p53. For these experiments, we expressed the p53-regulated promoters of Hdm2, Bax, NOXA, and PUMA in U2OS cells, which contain wild-type p53 protein. As shown in Fig. 7B, p18^{Hamlet} stimulated the transcription of the three p53-regulated pro-apoptotic genes (only a minor effect was observed in the case of Bax), but had no effect on the Hdm2 promoter. We confirmed that both Bax and Hdm2 promoters were indeed able to respond to p53 (Supplementary Fig. 10). Importantly, the stimulation of p53 transcriptional activity by p18^{Hamlet} was dependent upon the integrity of its C-terminal Zinc-Finger HIT domain, as a p18^{Hamlet} derivative lacking the last 37 amino acids (p18$^{Hamlet(1-117)}$) had no significant effect on any of the p53-regulated promoters (Fig. 7C). The C-terminally truncated p18$^{Hamlet(1-117)}$ protein was still able to bind to p38α and localized to the nucleus, as the full length p18^{Hamlet}, but it failed to interact with p53 (Fig. 9D and Supplementary Fig. 9). It therefore appears that p38α and p53 interact with different parts of the p18^{Hamlet} protein and that the C-terminal domain of p18^{Hamlet} is required for p53 transactivation.
To confirm that the effect of p18^{Hamlet} was indeed p53-dependent, we used a reporter plasmid with the PUMA minimal promoter (PUMA 4xBS2) consisting of four tandem repeats of the p53 binding site or a mutant version of this reporter, which does not bind to p53 (Yu et al., 2001). Full-length p18^{Hamlet} increased about four-fold PUMA 4xBS2 transcription whereas the mutant p18$^{Hamlet(1-117)}$ had no effect. In contrast, neither full-length nor truncated p18^{Hamlet} proteins were able to stimulate transcription of the mutated PUMA 4xBS2 promoter (Fig. 7D). Based on these

results, we conclude that p18^{Hamlet} can activate, through its C-terminal domain, the transcription of several p53-dependent genes.

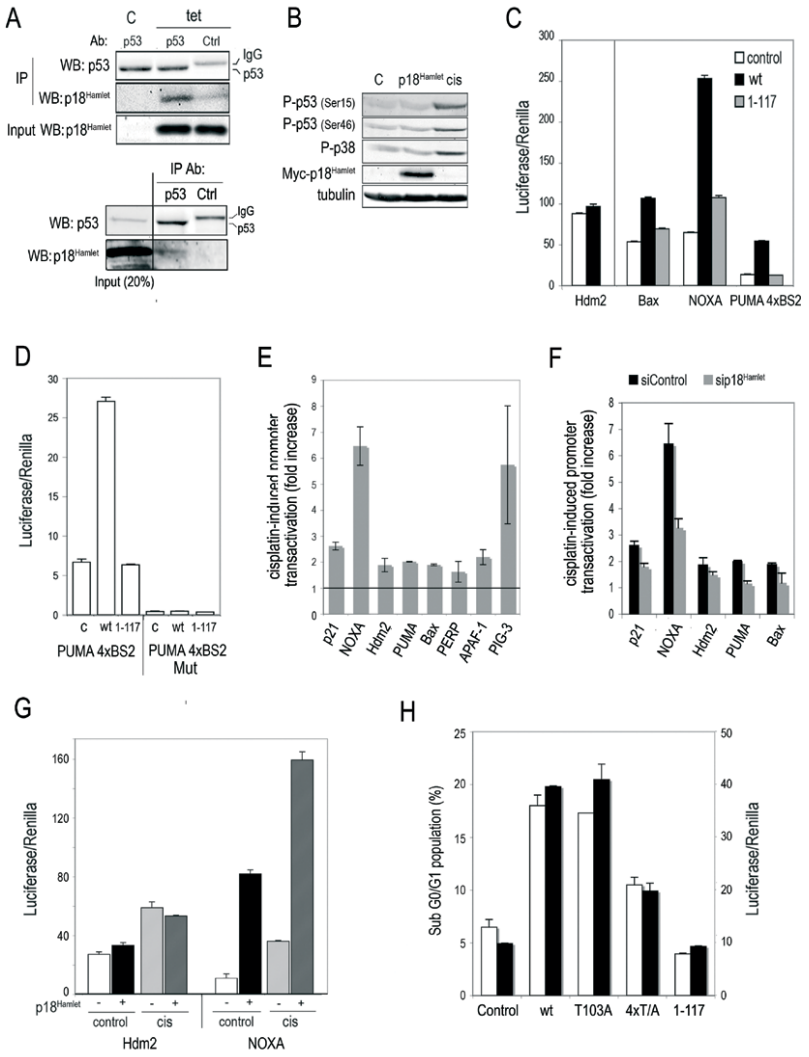

Fig. 7 p18^{Hamlet} stimulates some p53-regulated genes
(A) *Upper*: U2OS cells with inducible p18^{Hamlet} were treated with tetracycline (tet) for 24 h and total cell lysates were immunoprecipitated with p53 or control antibodies and then blotted with p18^{Hamlet} antibodies. *Lower:* Total lysates of MG132-treated U2OS cells were immunoprecipitated and blotted as above to detect interaction between to endogenous p18^{Hamlet} and p53. **(B)** U2OS cells expressing tet-inducible p18^{Hamlet} were treated with tet or cisplatin and then analyzed by Western blotting. **(C)** U2OS cells were transfected with empty vector (control) and either p18^{Hamlet} wt or p18$^{Hamlet(1-117)}$, together with reporter constructs containing different p53-responsive promoters upstream of the luciferase gene, as indicated. Luciferase activity was analyzed 16 h later and transfection efficiency was normalized to Renilla activity. Means $\pm$ s.d. of three independent experiments are represented. **(D)** Transfections and Luciferase assays were performed exactly as in **(C)** to test the wt and mutant PUMA 4xBS2 reporters. **(E)** U2OS cells were transfected with the indicated reporter constructs. 24 h later, cells were treated with cisplatin and after 16 h luciferase activity was measured and normalized to Renilla. **(F)** U2OS cells were transfected with p18^{Hamlet} or control siRNAs and 24 h later co-transfected with the indicated luciferase reporters. 24 h after transfection, cells were incubated with cisplatin for 16 h and luciferase activity was measured. **(G)** U2OS cells were transfected with p18^{Hamlet} or empty vector (control) in combination with Hdm2 and NOXA promoter reporters. 16 h after transfection, cells were mock-treated or treated with cisplatin and luciferase activity was measured 10 h later. Means $\pm$ s.d. of three independent experiments are represented. **(H)** U2OS cells were transfected with GFP alone (control) or the indicated GFP-tagged p18^{Hamlet} proteins and 48 h later the sub G0/G1 percentage in the fluorescent population was determined by FACS (white bars). U2OS cells were transfected with empty vector (control) or Myc-tagged wt and mutant p18^{Hamlet} proteins together with the PUMA 4xBS2 reporter and luciferase activity was measured 16 h later (black bars). Expression levels of Myc- and GFP-tagged p18^{Hamlet} proteins is shown in Supplementary Fig. 12.

Consistent with the results shown in Fig. 6B, cisplatin was able to differentially transactivated several p53-dependent promoters, being more effective in the cases of NOXA and PIG-3 (Fig. 7E) when compared with other genes such as Bax or PUMA. The downregulation of $p18^{Hamlet}$ inhibited about 50% the ability of cisplatin to transactivate the NOXA and PUMA promoters, while it had a more moderate effect on the p21 and Bax promoters (Fig. 7F). In addition, when $p18^{Hamlet}$ overexpression was combined with cisplatin treatment, we observed an additive effect on the NOXA promoter, without affecting the Hdm2 promoter, leading to an imbalance that was clearly favorable to the transcription of the pro-apoptotic gene NOXA (Fig. 7G). Of note, overexpression of $p18^{Hamlet}$ in the p53-deficient SAOS cells had no effect on the PUMA 4xBS2-reporter construct, unless the cells were co-transfected with p53 (Supplementary Fig. 11).
To determine the importance of p38 MAPK-mediated phosphorylation in $p18^{Hamlet}$ function, we analyzed the ability of different $p18^{Hamlet}$ mutants to transactivate the PUMA 4xBS2 promoter and to induce apoptosis. The three $p18^{Hamlet}$ mutants were able to bind to p38α to a similar extent, but phosphorylation was significantly reduced in $p18^{Hamlet\text{-}T103A}$ and specially in the quadruple mutant $p18^{Hamlet\text{-}4xT/A}$ (Supplementary Fig. 2). However, only the $p18^{Hamlet\text{-}T103A}$ mutant recapitulated the activity of the wt protein regarding PUMA promoter transactivation and apoptosis induction, consistent with the idea that the phosphorylation of this residue was not essential for $p18^{Hamlet}$ function and accumulation (Fig. 7H). In contrast, truncated $p18^{Hamlet(1\text{-}117)}$ was not able to induce cell death, supporting the importance of p53 interaction for the induction of apoptosis by $p18^{Hamlet}$. Finally, $p18^{Hamlet\text{-}4xT/A}$ showed a significant decrease in both PUMA transactivation and apoptosis induction, suggesting that $p18^{Hamlet}$ phosphorylation is essential for its activity (Fig. 7H).

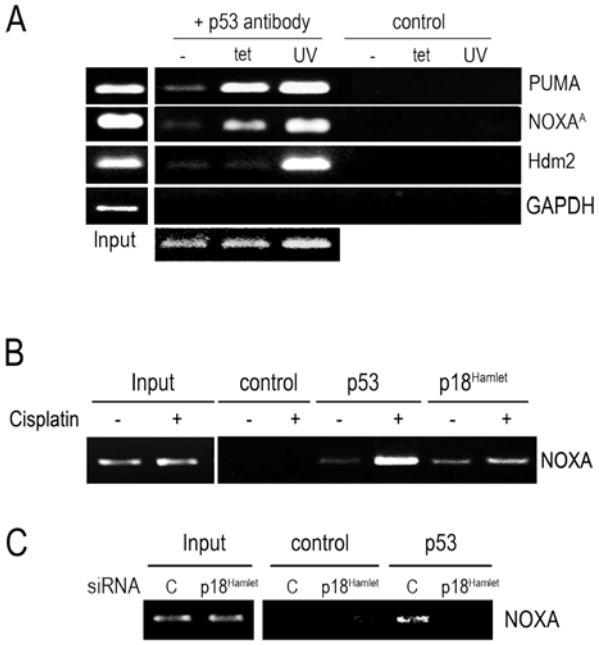

Fig. 8 Recruitment of p53 and $p18^{Hamlet}$ to p53-regulated promoters
(A) U2OS cells expressing tet-inducible $p18^{Hamlet}$ were treated with tetracycline (tet) for 24 h or UV irradiated for 8 h and then subjected to ChIP analysis. The DNA associated with the p53 immunoprecipitates was subjected to PCR with primers specific for the Hdm2, PUMA and NOXA promoters.
(B) U2OS cells were treated with cisplatin for 6 h and then subjected to ChIP analysis using both p53 and $p18^{Hamlet}$ immunoprecipitates and NOXA primers.
(C) p53 recruitment to NOXA promoter was analyzed by ChIP assay in U2OS cells 72 h after incubation with control and $p18^{Hamlet}$ siRNAs.

Our results indicated that the ability of $p18^{Hamlet}$ to stimulate p53-induced transcription was not related to p53 phosphorylation or stabilization. We therefore performed chromatin immunoprecipitation (ChIP) experiments to investigate the recruitment of p53 to the promoter of its target genes. We found that $p18^{Hamlet}$ overexpression was sufficient to enhance p53 binding to both the PUMA and NOXA promoters, whereas it had no effect on the ability of p53 to bind to

the Hdm2 promoter (Fig. 8A). In addition, p18^{Hamlet} itself was also bound to the NOXA promoter, even in unstimulated U2OS cells, and the binding was increased in response to cisplatin treatment (Fig. 8B). Interestingly, p18^{Hamlet} knockdown had a profound effect on p53 loading onto NOXA promoter (Fig. 8C), supporting a key role for p18^{Hamlet} in the recruitment of p53 to certain target promoters.

p18^{Hamlet} levels can be regulated by cyclin G1 in normally proliferating cells

The ability of p18^{Hamlet} to induce apoptosis suggests that the levels of this protein should be strictly regulated under normal growing conditions in order to avoid improper biological responses. We analyzed the subcellular localization of endogenous p18^{Hamlet} protein by immunofluorescence and found a nuclear pattern with a clear and well-defined peri-nucleolar distribution (Fig. 9A, upper panels). The same pattern was observed for transfected Myc-p18^{Hamlet} using either Myc or p18^{Hamlet} antibodies (Supplementary Fig. 13). Interestingly, p18^{Hamlet} was expressed at higher levels in p53-deficient MEFs than in their wt counterparts, whereas cyclin G1 was downregulated (Fig. 9B) and p18^{Hamlet} expression was more disorganized in the absence of p53, being uniformly distributed all over the nuclear compartment (Fig.9A, lower panels). This suggested that p53 downstream effectors could be normally required to maintain p18^{Hamlet} protein at low levels and in the right sub-cellular localization. In fact, p53 overexpression resulted in a decreased accumulation of p18^{Hamlet} protein in U2OS cells (Fig. 9C). A potential candidate regulator of p18^{Hamlet} was the p53 target gene cyclin G1, which has been reported to interact *in vitro* with p18^{Hamlet} (Xu et al., 2000). It has been reported that p53$^{-/-}$ MEFs express lower levels of cyclin G1 protein than wt MEFs (Reimer et al., 1999). In contrast, as mentioned above, p18^{Hamlet} was expressed at higher levels in p53-deficient than in wt MEFs (Fig. 9B), suggesting that both proteins could be subjected to opposite regulation. We confirmed that cyclin G1 and p18^{Hamlet} proteins interacted *in vitro* (Fig. 9D) and co-localized *in vivo* (Fig. 9G). Interestingly, co-transfection of increasing amounts of cyclin G1 with a constant amount of p18^{Hamlet} efficiently decreased the expression of p18^{Hamlet} (Fig. 9E), and this effect could be mediated by the ubiquitin-proteasome system (Fig. 9F). In contrast, the subcellular localization of p18^{Hamlet} was not affected by cyclin G1 overexpression (data not shown). Thus, cyclin G1 induces the degradation of p18^{Hamlet} and can potentially control its expression levels in normally growing cells.

Discussion

Mammalian cells have evolved a complex network of DNA-damage responses to ensure the integrity of their genomes. These mechanisms enable injured cells either to arrest the cell-cycle and establish a DNA repair program or to undergo cell death by apoptosis, depending on the severity of the damage. We have identified p18^{Hamlet} as a new protein regulated by the stress-activated p38 MAPK pathway and have established its implication in p53-induced apoptosis. Specifically, p18^{Hamlet} protein accumulates in response to genotoxic agents and behaves as a p53 transcriptional co-activator that promotes the expression of genes such as NOXA and PUMA, helping cells to undergo apoptosis.

p18^{Hamlet} links the p38 MAPK and p53 pathways

Our results indicate that p38 MAPK plays an important role in the regulation of p18^{Hamlet} half-life. In particular, p38 MAPK activation is required for the accumulation of p18^{Hamlet} induced by DNA damage-inducing agents such as UV. We also showed that several sites that are phosphorylated by p38α in vitro are also important for p18^{Hamlet} protein stability in cells. However, the exact contribution of specific phosphorylation sites to p18^{Hamlet} protein stability

remains to be elucidated. It is also possible that p38 MAPK might regulate the stabilization of the p18^{Hamlet} protein by other mechanisms, in addition to direct phosphorylation.

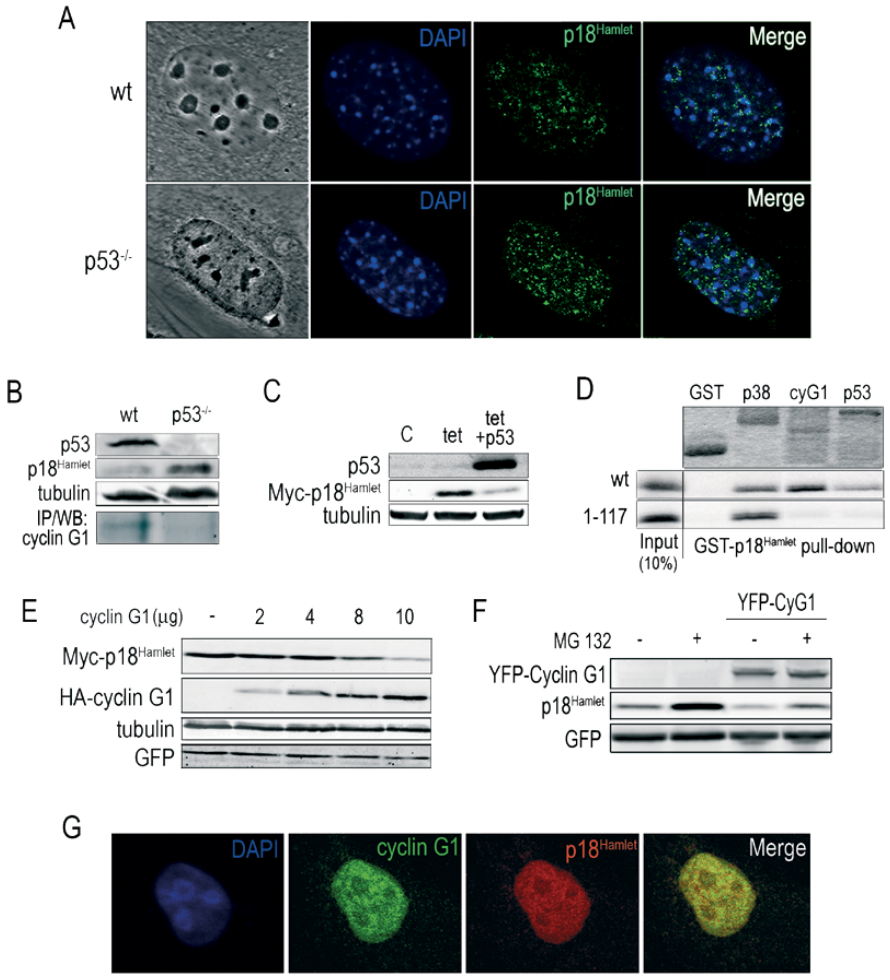

Fig. 9 The p53 target gene cyclin G1 controls p18^{Hamlet} protein levels under normal growing conditions
(A) MEFs (wt and p53$^{-/-}$) were immunostained with p18^{Hamlet} antibodies. Nuclear localization was confirmed by DAPI staining.
(B) Expression of the indicated proteins was analyzed by Western blotting in wt and p53$^{-/-}$ MEFs.
(C) U2OS cells with inducible p18^{Hamlet} were treated with tetracycline (tet) and 24 h later were transfected with p53 and analyzed by Western blotting.
(D) GST-pull down assay was performed by incubation of ^{35}S-labeled p18^{Hamlet} wt and 1-117 with GST and GST-fused p38α, cyclin G1 or p53, as indicated.
(E) HEK-293 cells were co-transfected with Myc-p18^{Hamlet} (5 μg) and increasing amounts of HA-cyclin G1 (0-10 μg). 24 h after transfection, the expression levels of the indicated proteins was analyzed by Western blotting using HA and Myc antibodies. Transfection efficiency was evaluated by co-transfection with GFP (500 ng).
(F) HEK-293 cells were co-transfected with p18^{Hamlet} (1 μg) and YFP-cyclin G1 (9 μg) and 16 h after transfection, cells were treated for 5 h with MG132. The expression levels of the indicated proteins was analyzed by Western blotting.
(G) U2OS cells expressing tet-inducible p18^{Hamlet} were transfected with HA-cyclin G1. Cellular localization was analyzed by immunostaining with HA and p18^{Hamlet} antibodies. Co-localization areas are indicated in yellow (Merge).

Previous studies have documented that activation of the p38 MAPK pathway may lead to p53-induced apoptosis (Bulavin and Fornace, 2004). The connection between p38 MAPK activation and increased transcription from p53-regulated promoters has been classically attributed to the ability of p38 MAPK to directly phosphorylate p53 on Ser33 and Ser46 (Bulavin et al., 1999; Sanchez-Prieto et al., 2000). Our findings provide a new mechanism by which p38 MAPK can contribute to p53-induced apoptosis, namely by contributing to the stabilization of $p18^{Hamlet}$, a p53 co-activator that stimulates p53-dependent transcription.

We have shown that the stimulatory effect of $p18^{Hamlet}$ on p53-regulated genes is mediated by the p53 binding sites in the promoters. Nevertheless, we cannot rule out that $p18^{Hamlet}$ could also have p53-independent functions. The presence of $p18^{Hamlet}$ homologues in yeast, which lack p53-related proteins, is as yet of unclear biological significance but might support this possibility.

$p18^{Hamlet}$ as a determinant for p53 response specificity

There are several mechanisms by which $p18^{Hamlet}$ could provide specificity to p53-regulated stress responses. First, $p18^{Hamlet}$ does not seem to be an ubiquitously-expressed protein, in contrast with p38 MAPK and p53, and might therefore provide tissue-specific responses. Second, $p18^{Hamlet}$ accumulates only in response to certain types of stresses, such as UV and cisplatin, but not in response to γ-irradiation (not shown). Finally, $p18^{Hamlet}$ can specifically stimulate the transcription of certain p53-dependent promoters. In particular, in response to cisplatin, $p18^{Hamlet}$ contributes to the p53-dependent transcriptional activation of NOXA, but not Hdm2, suggesting that the effects of $p18^{Hamlet}$ on p53-mediated transcription are promoter specific. This property of $p18^{Hamlet}$ is shared by other p53 co-activators. For example, ASPP proteins can stimulate the transcription of the pro-apoptotic genes Bax and PIG3, but not of Mdm2 or $p21^{Cip1}$ (Samuels-Lev et al., 2001), whereas the protein hDaxx specifically represses p53-mediated induction of genes involved in cell cycle arrest such as $p21^{Cip1}$ (Gostissa et al., 2004). Other p53 co-activators, such as the p300/CBP cofactor JMY, can efficiently upregulate Bax but not $p21^{Cip1}$ (Shikama et al., 1999).

It has been clearly established that $p21^{Cip1}$ has a key role in p53-induced cell cycle arrest, but the molecular pathways involved in p53-mediated apoptosis are not fully understood. Several pro-apoptotic molecules can be transcriptionally induced by p53, but the contribution of each factor to the apoptotic response depends on both the cell type and the nature of the stress. We have found that $p18^{Hamlet}$ can stimulate the recruitment of p53 to the $p21^{Cip1}$ promoter (not shown), but we could not observe $p21^{Cip1}$ protein induction in response to UV or cisplatin, most likely due to posttranslational down-regulation (Fotedar et al., 2004). Thus, the contribution of $p18^{Hamlet}$ to the regulation of the $p21^{Cip1}$ promoter needs to be further investigated.

The function of $p18^{Hamlet}$ as a transcriptional co-activator is further supported by recent work, which has identified this protein as a potential subunit of the SRCAP (SNF2-related CBP-activating protein) complex (Cai et al., 2005). Interestingly, SRCAP may contribute to the recruitment of the histone acetyltransferase CBP to certain promoters (Eissenberg et al., 2005). The p300/CBP proteins are well-established regulators of the p53 response that control p53 acetylation and its DNA binding activity (Barlev et al., 2001; Espinosa and Emerson, 2001). It is therefore tempting to speculate that p53 modulation by $p18^{Hamlet}$ could involve the regulation of p300/CBP.

Control of $p18^{Hamlet}$ expression in proliferating cells

The accumulation of $p18^{Hamlet}$ can potently trigger apoptosis suggesting that its expression should be tightly controlled. We have identified a p53-dependent negative feed-back loop that normally maintains $p18^{Hamlet}$ at low steady-state levels. Regulatory loops are a common feature of the p53 pathway. The best characterized one involves the E3 ubiquitin ligase Hdm2, a p53

target gene that is responsible for maintaining low basal levels of p53 activity under normal proliferating conditions (Haupt et al., 1997; Kubbutat et al., 1997). Our results show that $p18^{Hamlet}$ levels are increased in p53-deficient cells but downregulated when p53 is overexpressed. This negative effect of p53 on $p18^{Hamlet}$ expression may be mediated by cyclin G1, a p53 target gene whose overexpression suffices to interfere with $p18^{Hamlet}$ accumulation and that can associate with this protein *in vitro* as well as co-localize in cells.
In summary, our results support a link between $p18^{Hamlet}$ and p53 function at two different levels. On the one hand, the half-life of $p18^{Hamlet}$ increases in response to DNA damaging agents and this is mediated at least in part by p38 MAPK. Accumulation of $p18^{Hamlet}$ leads to apoptosis, by increasing the ability of p53 to bind to specific promoters such as the pro-apoptotic genes NOXA and PUMA. In addition, low steady-state levels of $p18^{Hamlet}$ are maintained by a p53-dependent mechanism, probably mediated by cyclin G1. Therefore, $p18^{Hamlet}$ functions as a new cell-fate regulator, which contributes to the implementation of p53-regulated cellular responses.

Materials and methods

DNA cloning and mutagenesis

The human $p18^{Hamlet}$ cDNA was obtained from a Gal4 fusion-expressing clone identified in the yeast two-hybrid screenings using p38α as a bait (Cheung et al., 2003). Expression constructs for *Escherichia coli* and mammalian cells are described in Supplementary data. All $p18^{Hamlet}$ mutants were prepared using the QuickChange® site-directed mutagenesis kit (Stratagene) and were verified by DNA sequencing.

Cell culture

HEK-293, HeLa, SAOS, MCF7, U2OS, and melanoma SK-Mel-103 cells as well as wt and $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. FuGene reagent (Roche Applied Science) was used for cell transfection according to the manufacturer's protocol. Cells were treated with UV (50-100 J/m^2) and cisplatin (5 -10 μg/ml), as indicated.
The generation of stable cell lines expressing inducible $p18^{Hamlet}$ and the retroviral infections were performed as indicated in Supplementary data.

Transfection of siRNA and apoptosis measurement

The siRNA oligonucleotide for $p18^{Hamlet}$ (UGCGGACACUGGAAAGAAAUU) was obtained from Dharmacon (Lafayette, CO). U2OS and MCF7 cells were grown to 50% of confluence, and transfected with Dharmafect Reagent 1 (Dharmacon) according to the manufacturer's protocol. Cells were treated with UV or cisplatin 48 h after siRNA transfection. Human Lamin A siRNA (siGLO™, Dharmacon) was used as a control. Apoptosis was analyzed using the Cell Death Detection ELISA^{PLUS} Kit (Roche Applied Science).

Flow cytometry analysis

Cells were trypsinized, washed with PBS, fixed with chilled 70% ethanol for 30 min at 4º C and then incubated in PBS containing 30 mg/ml of RNAse and stained for 30 min at 37º C with propidium iodide (25 μg/ml). Apoptotic cells were determined by their hypochromic subdiploid staining profiles. To estimate early apoptotic cells, Alexa 488-conjugated annexin V was used together with propidium iodide counter stain following the manufacturer's recommendations (Molecular Probes, Inc).

Luciferase expression analysis

U2OS and SAOS cells ($2x10^5$) were plated 24 h before transfection in 6-multiwell dishes. Transactivation assays contained 30 ng of the Renilla expression construct phRL-TK (Promega), as a transfection control, 10 ng of p53, 300 ng of promoter reporter and 700 ng of full-length or truncated $p18^{Hamlet}$, as indicated. Cells were lysed in reporter lysis buffer 24 h after transfection. In the case of cisplatin treatments, cells were treated with the drug 24 h after transfection and collected 10-16 h later. Luciferase and Renilla activities were measured using Dual-Luciferase® Reporter kit (Promega).

Antibodies, Western blotting, Immunoprecipitation, Pull-down and kinase assays

Western blot analysis was performed using 40-60 μg of the cell lysates prepared in ice-cold IP lysis buffer. Buffers and antibodies are described in Supplementary data.

For the immunoprecipitations, 20 μl of anti-Myc or anti-HA agarose conjugates were incubated with 250-500 μg of protein lysates for 14 h at 4ºC. The beads were then washed 3 times in IP buffer and analyzed by immunoblotting or further washed in kinase buffer and used for kinase assays (Alonso et al., 2000).

GST pull-downs and in vitro kinase assays were performed as described in Supplementary data.

Quantitative RT-PCR, Northern blot, ChIP analysis, Ubiquitination assays, Immunofluorescence and confocal microscopy

These protocols are described in Supplementary data.

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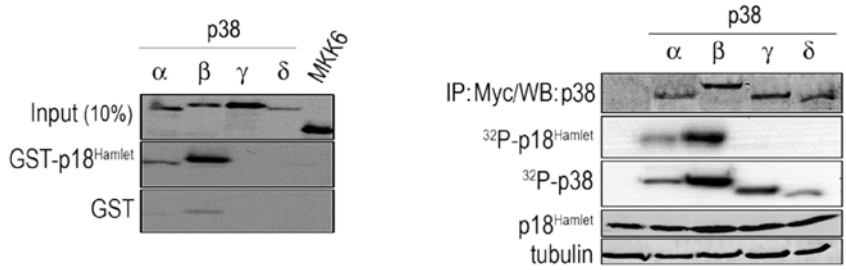
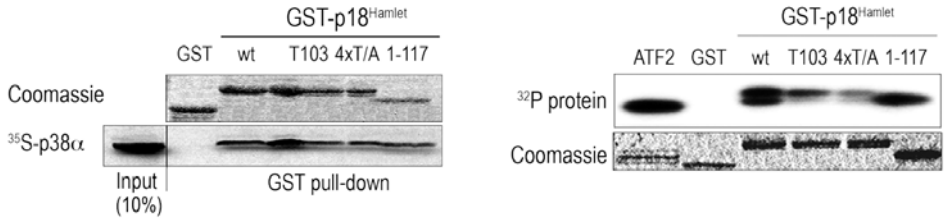
Supplemental Data

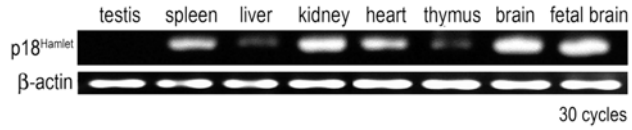
Supplementary Figures



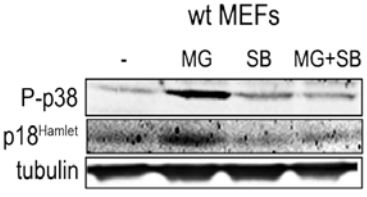
Supplementary Figure 1. p18^{Hamlet} phosphorylation by and binding to p38 MAPK family members
Left: GST-pull down assay with GST or GST-p18^{Hamlet} and ^{35}S-labelled p38 MAPKs (α, β, γ, and δ) and MKK6.
Right: HEK-293 cells were transfected with Myc-tagged p18^{Hamlet} alone or together with the four Myc-tagged p38 MAPKs. 48 h after transfection, p18^{Hamlet} and the p38 MAPKs were immunoprecipitated with Myc antibodies and subjected to a kinase assay in the presence of ^{32}P-γ-ATP and MBP-MKK6DD protein. Total cell lysates and Myc IPs were analyzed by Western blotting.



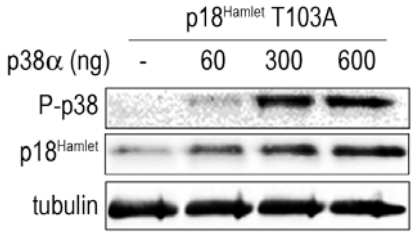
Supplementary Figure 2. Binding to and phosphorylation by p38α MAPK of p18^{Hamlet} proteins
Left: In vitro pull-down assay with recombinant GST-p18^{Hamlet} mutants incubated with in vitro translated ^{35}S-labelled p38α.
Right: Kinase assay was performed with active p38α and the indicated GST-p18^{Hamlet} proteins (1 μg).



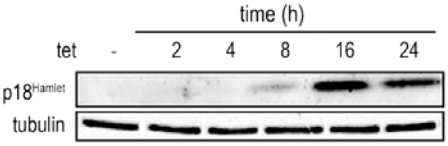
Supplementary Figure 3. Expression of p18^{Hamlet} mRNA in human tissues as determined by semi-quantitative RT-PCR using primers that recognize the full-length p18^{Hamlet} coding sequence. β-actin was amplified as a control.



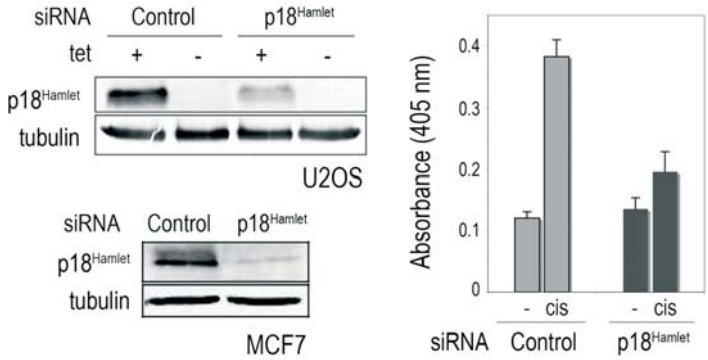
Supplementary Figure 4. MG132-induced p18^{Hamlet} accumulation depends on p38 MAPK activity
MEFs were either untreated or treated with the proteasome inhibitor MG132 (25 μM) in the presence or absence of the p38 MAPK inhibitor SB203580 (10 μM) for 2 h. Total lysates were analyzed by Western blotting with the indicated antibodies.



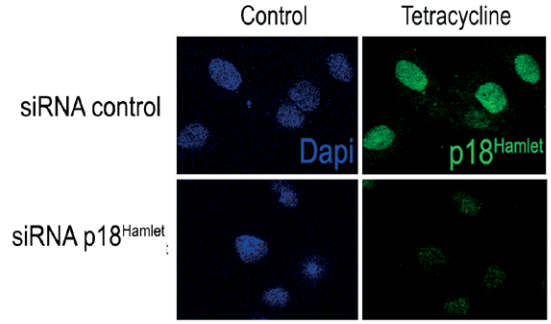
Supplementary Figure 5. U2OS cells were co-transfected with MKK6DD (600 ng), p18$^{Hamlet\text{-}T103A}$ (1 μg) and increased amounts of p38α as indicated. Total lysates were analyzed by Western blotting with the indicated antibodies.



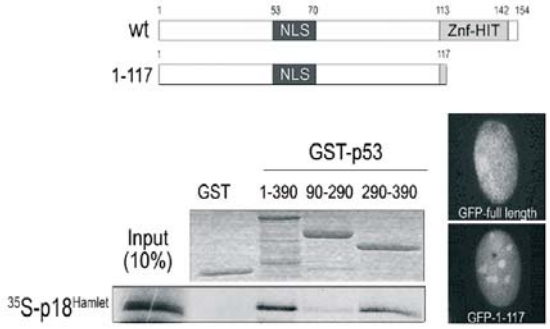
Supplementary Figure 6. Tetracycline-induced p18^{Hamlet} expression in U2OS cells
Cells were treated with 1 μg/ml of tetracycline for the indicated times and p18^{Hamlet} levels were evaluated by Western blotting.



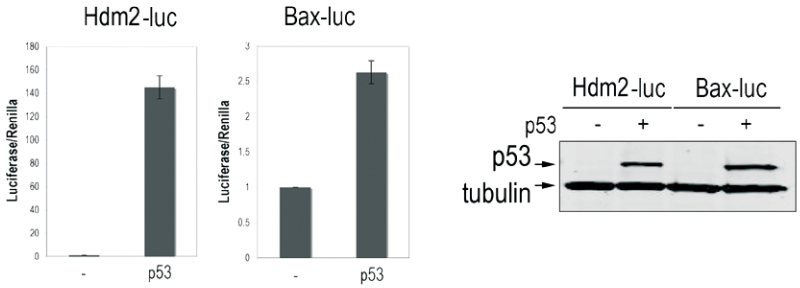
Supplementary Figure 7. Knockdown of p18^{Hamlet} by siRNA
Left: U2OS cells expressing tetracycline-inducible p18^{Hamlet} (upper panel) and MCF7 cells (lower panel) were transfected with p18^{Hamlet} siRNA or lamin A siRNA (as a control). After 48 h, U2OS were treated with tetracycline for 16 h. Levels of p18^{Hamlet} were evaluated by Western blotting three days after transfection.
Right: MCF7 cells were treated with cisplatin (10 μg/ml) for 16 h and apoptosis was quantified by measuring DNA fragmentation in a colorimetric assay. Means ± standard deviations of two independent experiments performed in triplicates are represented.



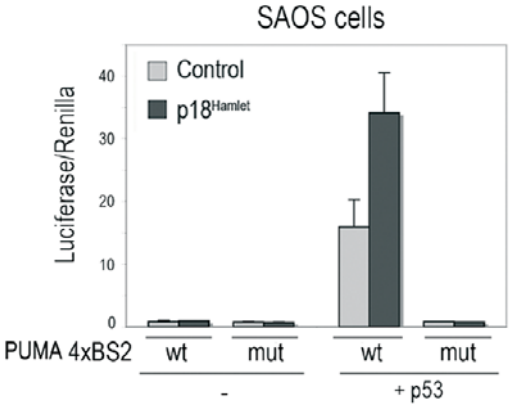
Supplementary Figure 8. The specificity of p18^{Hamlet} antibody was tested by immunofluorescence of tetracycline inducible p18^{Hamlet}-expressing U2OS cells treated with siRNA control or siRNA p18^{Hamlet}.



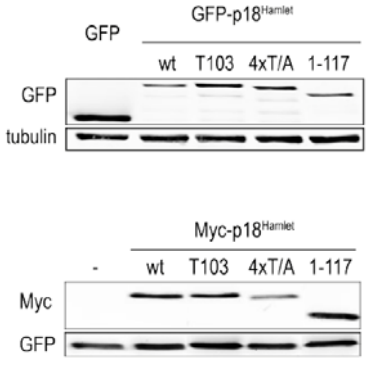
Supplementary Figure 9. GST pull-down assays were performed by incubation of ^{35}S-labelled p18^{Hamlet} proteins with GST, GST-p53 full-length, GST-p53$^{90-290}$, or GST-p53$^{290-390}$, as indicated. Input represents 10% of the ^{35}S-labelled proteins. Localization of transfected GFP-p18^{Hamlet} proteins in U2OS cells is shown.



Supplementary Figure 10. SAOS cells were transfected with Hdm2-luc and Bax-luc reporter constructs alone or in combination with 10 ng of p53 expression vector. Luciferase activity was analyzed 16 h later and normalized to Renilla.



Supplementary Figure 11. SAOS cells were transfected with p18^{Hamlet} or empty vector (control) either alone or in combination with 10 ng of p53 expression vector, together with reporter constructs containing the wt or mutated PUMA 4xBS2 minimal promoter, as indicated. Luciferase activity was analyzed after 16 h and was normalized to Renilla activity.



Supplementary Figure 12. U2OS were transfected with GFP or the indicated GFP-tagged p18^{Hamlet} proteins (upper panel) or with GFP together with the indicated Myc-tagged p18^{Hamlet} proteins (lower panel). Protein level expression was analyzed by Western blotting 48 h after transfection.

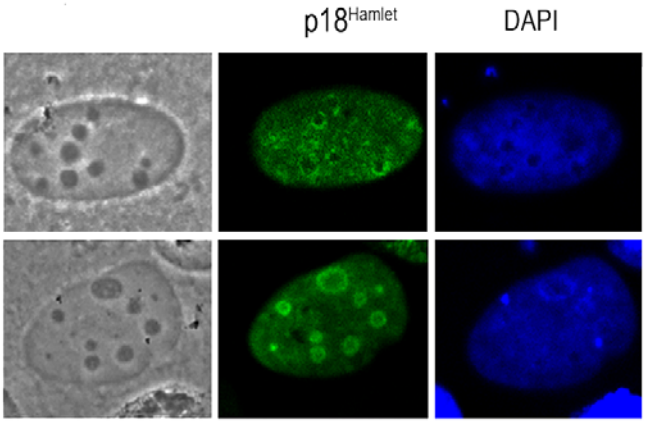


Figure 13. U2OS cells were transfected with Myc-tagged p18^{Hamlet} expression vector and stained with p18^{Hamlet} antibodies or DAPI. Two different nuclear fields are shown.

Supplementary Materials and methods

Expression constructs

For expression in *Escherichia coli* as GST (glutathione S-transferase)-fusion proteins, human p18^{Hamlet}, p53 and cyclin G1 were amplified by PCR, cloned into the pCRII vector (Invitrogen), sequenced and then subcloned into the pGEX-KG vector as NcoI-XhoI (p18^{Hamlet}), EcoRI (p53), and BamHI-XhoI (cyclin G1) fragments. The bacterial expression constructs for GST-p38α and MBP-MKK6DD were already described (Alonso et al., 2000). For expression in mammalian cells of N-terminally Myc-tagged p38α and p18^{Hamlet}, the human cDNAs were cloned into the FTX5 vector as BamHI (p38α) and NcoI-XhoI (p18^{Hamlet}) fragments, and then subcloned into pCDNA3.1 (Invitrogen) as HindIII-XhoI fragments in both cases (HindIII partial digestion was required for p38α subcloning). For the expression of N-terminally HA-tagged cyclin G1, the cDNA was amplified by PCR, cloned into pCRII and then subcloned as an EcoRI fragment into the HA-pCDNA3.1 vector (kindly provided by Giulio Superti-Furga, Research Center for Molecular Medicine, Vienna, Austria). For the expression of GFP-tagged p18^{Hamlet} protein, the pCRII-p18^{Hamlet} vector was digested with EcoRI and p18^{Hamlet} was subcloned into the pEGFP-C2 vector (Clontech Laboratories). For the expression of YFP-tagged human cyclin G1, the pCR2.1TOPO-cyclin G1 plasmid was obtained from the German Resource Center for Genome Research (RZPD), digested with SacI ApaI and the insert was subcloned into the pEYFP vector (Clontech Laboratories), with the correct frame being generated by mutagenesis. The construct for tetracycline-inducible p18^{Hamlet} expression was generated by subcloning Myc-p18^{Hamlet} as a HindIII-XhoI fragment into pCDNA4/TO vector (Invitrogen). The C-terminally deleted mutant p18$^{Hamlet(1-117)}$ was prepared by replacing the residue 118 of p18^{Hamlet} for a stop codon. The HA-Ubiquitin expression construct was a kind gift from Ivan Dikic (Goethe University Frankfurt, Germany). The constructs to express truncated GST-fused p53 proteins were provided by Pedro Lazo (CIC-Universidad de Salamanca, Spain).

Generation of a p18^{Hamlet} inducible system

Stable cell lines expressing inducible p18^{Hamlet} were generated using the Invitrogen T-REx^{TM} system. U2OS cells were transfected with a 6:1 ratio of the Tetracycline repressor vector pcDNA6/TR-blasticicin and the inducible expression vector pcDNA4/TO-zeocin containing Myc-p18^{Hamlet}. Positive clones were selected in media containing blasticidin (5 μg/ml) and zeocin (200 μg/ml). Myc-tagged p18^{Hamlet} protein was induced by the addition of tetracycline (1 μg/ml).

Retroviral infections

Retroviruses were produced in HEK-293T cells by transient transfection. Culture supernatants were collected 48 h (first supernatant) and 72 h (second supernatant) post-transfection, filtered and supplemented with 4 μg/ml polybrene (Sigma). MEFs at approximately 10^6 cells per 10-cm dish were infected with 8 ml of the first supernatant and 24 h later with the second supernatant, and incubated 48 h post-infection with 1.5 μg/ml of puromycin for 4 days.

Antibodies and buffers for immunoblotting

GST-$p18^{Hamlet}$ protein was used to generate polyclonal antibodies in rabbits, which were purified by pre-incubation of the antiserum with beads-coupled GST followed by a standard western blot affinity purification on GST-$p18^{Hamlet}$. Phospho-specific antibodies that recognize $p18^{Hamlet}$ phosphorylated on Thr103, were raised in sheep against the synthetic phosphopeptide GPNYLpTACAG corresponding to residues 98-107 of human $p18^{Hamlet}$ and were affinity purified on the same phosphopeptide.

The following commercial antibodies were used for immunoblotting: anti-p38 (Santa Cruz), anti-phospho-p38 (Cell Signaling), anti-α-tubulin (Sigma), anti-phospho-Thr (9381, Cell Signaling), anti-Myc (9E10; Santa Cruz), anti-GFP (Clontech), anti-HA (Roche), anti-human-p53 (DO-1; Santa Cruz), anti-mouse-p53 (CM5; Novocastra), anti-phospho-p53 (Ser 15 and Ser 46; Cell Signaling), anti-cyclin G1 (H-46; Santa Cruz), anti-NOXA (Oncogene Research Products), anti-PUMA (ab 9643, Abcam Ltd), anti-Bax (P-19, Santa Cruz), anti-p21 (C-19, Santa Cruz), anti-Hdm2 (SM-p14, Abcam Ltd).

Immunoblots were developed and quantified using Alexa Fluor 680 (Molecular Probes) or Li-Cor IRDye 800 (Rockland) labeled antibodies with the Odyssey Infrared Imaging System (Li-Cor).

The agarose beads used for immunoprecipitation were anti-Myc (sc-40 AC; Santa Cruz), anti-HA (sc-7392 AC; Santa Cruz) and anti-p53 (sc-126AC; Santa Cruz). For kinase assays, immunoprecipitates were further washed in kinase buffer: 80 mM β-glycerophosphate, pH 7.5, 20 mM EGTA, 15 mM $MgCl_2$, 2.5 mM benzamidine, 1 mM PMSF, 1 mM DTT, and 2 μg/ml each of aprotinin and leupeptin.

To prepare total cell lysates for immunoblotting, we used IP buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 0.1 mM Na Vanadate, 1 mM PMSF, 2 μM microcystin, 2.5 mM benzamidine, and 2 μg each of aprotinin, leupeptin, and pepstatin A per ml.

Quantitative Real-Time PCR

Total RNA was isolated from U2OS cells using Quiagen RNeasy kit and cDNA was synthesized with Superscritpt-II reverse transcriptase using random hexamer primers (Invitrogen) following the manufacturer indications. An Applied Biosystems 7900HT Fast Real-Time PCR System was used to determine the mRNA levels of PUMA, NOXA, BAX, Hdm2, and GAPDH using the following primers:

NOXA: fw 5'-TGTCCGAGGTGCTCCAGTT-3'; rev 5'-TGAAACGTGCACCTCCTG-3'
PUMA: fw 5'-GTGCCCTCGGCAGTGTCC-3'; rev 5'-GTACTGTGCGTTGAGGTC-3'
BAX: fw 5'-CACCAGCTCTGAGCAGATC-3'; rev 5'-GCTGCCACTCGGAAAAAG-3'
HDM2: fw 5'-GTGCTGTAACCACCTCAC-3'; rev 5'-GCTCTTTCACAGAGAAGC-3'
GAPDH:
fw 5'-GACCCCTTCATTGACCTCAAC-3'; rev 5'-GAGGGGCCATCCACAGTCTTC-3'

Data analysis was done by normalizing to GAPDH mRNA levels.

Northern blot

Total RNA was isolated with the RNeasy kit (Qiagen), fractioned in formaldehyde agarose gels and blotted onto nylon membranes. The full-length $p18^{Hamlet}$ cDNA was used as a probe, which was prepared by using the Ready-to-go kit (Amersham-Pharmacia).

ChIP analysis

ChIP analysis was performed exactly as described (Espinosa et al., 2003). After immunoprecipitation and reversal of the cross-linking, DNA was purified and used as template for PCR reactions that were performed in the exponential range of amplification that varied from 30 to 35 cycles. Amplification products were analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide. All PCR products were in the size range of 200-250 bp. Primers used for PCR of HDM2, GAPDH, and PUMA were previously described (Koutsodontis et al., 2005; Zeng et al., 2002). For NOXA promoter amplification we used the following set of primers: NxA fw (58º C) 5'-TTTTCTGGGCTTGTTTACCC-3';
NxA rev 5'-TACAAAACGAGGTGGGAGGA-3'.

Immunofluorescence and confocal microscopy

Cells were rinsed in PBS, fixed in 4% paraformaldehyde for 30 min and washed again with PBS. Non-specific sites were blocked by incubation in PBS containing 1% BSA and 0.5% Triton-X100 for 1 h at RT. Cells were then washed 4 times in PBS and incubated with the following primary antibodies: affinity-purified polyclonal anti-p18^{Hamlet} (1:200), monoclonal anti-HA (1:500) and monoclonal anti-Myc (1:500). After four washes with PBS, cells were incubated with Alexa Fluor 488 and 594 (1:500) secondary antibodies (Molecular Probes), stained with DAPI (0.1 μg/ml) for 10 min, and mounted in mowiol. The samples were examined using both fluorescence and confocal microscopy (Leica Microsystems).

Ubiquitination assay

To detect p18^{Hamlet} ubiquitination, empty vector or the Myc-p18^{Hamlet}-encoding plasmid (4 μg) were co-transfected with HA-Ubiquitin (6 μg). 16 h after transfection, cells were treated with 25 μM MG132 for 5 h. Cells were harvested and used for immunoprecipitation with anti-Myc antibody coupled to beads. Ubiquitinated p18^{Hamlet} forms were visualized with p18^{Hamlet} and HA antibodies.

Pull-down and kinase assays

Recombinant GST-fusion proteins were expressed in *E. coli* BL21 DE3 and purified using standard protocols. For GST pull-down assays, ^{35}S-labelled proteins were generated using the TNT Coupled System (Promega, Madison, WI) and incubated with GST-proteins in IP buffer for 2 h at 4º C. Beads were washed four times with IP buffer and proteins were analyzed by SDS-PAGE followed by autoradiography. p38 MAPK activity assays using as substrates GST-ATF2 (residues 19-96) or recombinant wild type and mutant GST-p18^{Hamlet} proteins were carried out as described (Alonso et al., 2000).

Supplementary References

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