Study of the intracellular mechanism implicated in the resistance to apoptotic cell death in glioblastoma multiforme

María Sánchez Osuna

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DIRECTOR

Dr. Victor José Yuste Mateos (Departament Bioquimica i Biologia Molecular, Universitat Autònoma de Barcelona)

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María Sánchez Osuna

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Director	**PhD Student**
Victor José Yuste Mateos	María Sánchez Osuna

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A la personita más bonita del mundo,
porque sin tí, nada tiene sentido.

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Contents

Summary

Glioblastoma multiforme, one of the most aggressive and lethal tumors, is characterized by a high apoptosis resistance. This doctoral thesis addresses the biochemical behavior of diverse glioblastoma cells, including primary cultures, after cytotoxic insult. Despite the tumoral heterogeneity a limited activation of the caspase-activated DNase (CAD) is a common trait among glioblastoma cells. The results obtained demonstrate that glioblastoma cells cannot correctly degrade DNA during cell death. This should be taken into consideration in order to design more efficient therapies because glioblastoma cells can release their genomic content during cell death.

Resum

El glioblastoma multiforme, un dels tumors més agressius i letals que es coneixen, es caracteritza per una elevada resistència a l'apoptosi. En aquest treball s'ha caracteritzat el comportament de diferents línies cel·lulars i cultius primaris derivats de glioblastoma enfront d'una gran varietat d'estímuls citotòxics. Tot i la variabilitat tumoral, la limitada activació de la endonucleasa apoptòtica DFF40/CAD és un tret comú a totes les cèl·lules de glioblastoma. Els resultats obtinguts demostren que les cèl·lules d'aquest tumor no són capaces de produir una correcta degradació de l'ADN durant el procés de mort cel·lular. Això s'ha de tenir en present a l'hora de dissenyar teràpies més efectives ja que les cèl·lules de glioblastoma poden alliberar el seu material genètic a l'exterior durant el procés de mort cel·lular.

Resumen

El glioblastoma multiforme, uno de los tumores más agresivos y letales que se conocen, se caracteriza por una elevada resistencia a la apoptosis. En este trabajo, se ha caracterizado el comportamiento de diferentes líneas celulares derivadas de glioblastoma y cultivos primarios, frente a una gran variedad de estímulos citotóxicos. A pesar de la variabilidad tumoral, la limitada disponibilidad de la endonucleasa apoptótica DFF40/CAD parece ser un rasgo común a todas las células de glioblastoma. Los resultados obtenidos demuestran que las células de este tumor no son capaces de producir una correcta degradación del ADN durante el proceso de muerte celular. El tener presente este resultado podría ayudar a diseñar terapias más eficaces ya que las células de glioblastoma son capaces de liberar su material genético al exterior durante el proceso de muerte.

Preface

All the work presented henceforth was conducted in the Cell Death, Senescence and Survival Group, placed at the Institut de Neurociències (Universitat Autònoma de Barcelona). This dissertation represents a culmination of work and learning that has taken place during the last four years (2010-2014).

The first chapter of results has been published in The Journal of Biological Chemistry with myself as the first author. Other authors contributed to this work with technical support and helpful criticisms. Victor J Yuste, the supervisor of this thesis, was involved throughout the project in concept of formation and manuscript composition.

In this first chapter, we described the incomplete apoptosis of glioblastoma LN-18 cells after different cytotoxic stimuli. Glioblastoma cells have basal impairments to undergo both apoptotic nuclear alterations and oligonucleosomal DNA degradation. Moreover, the transfection of the major apoptotic endonuclease DFF40CAD showed, for the first time, that oligonucleosomal DNA degradation does not guarantee chromatin deposition into de apoptotic nuclear bodies.

The second chapter of results is currently in preparation for publication as "María Sánchez-Osuna, Fina Martínez-Soler., Mercè García-Belinchón, Sònia Pascual-Guiral, Victoria Iglesias-Guimarais, Elisenda Casanelles, Gerard Plans, Jordi Bruna, Avelina Tortosa, and Victor J. Yuste. *Absence of oligonucleosomal DNA fragmentation after caspase-dependent cell death is a common signature of glioblastoma-derived multiforme cells due to improper activation of DFF40/CAD endonuclease*". Fina Martínez-Soler, Gerard Plans, Jordi Bruna and Avelina Tortosa were involved in the generation of the primary glioblastoma cultures. Mercè Garcia-Belinchón, Sònia Pascual-Guiral, Victoria Iglesias-Guimarais, and Elisenda Casanelles offered me technical support. Victor J Yuste, as supervisor, was involved throughout the project in experimental design.

During this second chapter, we demonstrated that the absence of oligonucleosomal DNA degradation is a common trait in glioblastoma cells. Despite differences in caspase activation or ICAD (inhibitor of CAD) degradation, glioblastoma cells lack apoptotic oligonucleosomal

DNA degradation due to low levels of cytoplasmic DFF40/CAD. As far as we know, this is the first time that a common trait is described among glioblastoma cells.

The last chapter of this dissertation has been performed by me with the supervision of Victor J Yuste after the observation of Elisenda Casanelles about gossypol effects in glioblastoma cells.

In this third chapter, we used a natural phenolic compound, gossypol, together with the death ligand TRAIL, to induce apoptotic nuclear morphology in glioblastoma cells. The absence of oligonucleosomal DNA degradation during the treatment supported previous results indicating that nuclear alterations and DNA hydrolysis are separately regulated in injured glioblastoma cells. We confirmed that CAD protein levels are determinant to allow the hydrolysis of the DNA into low molecular weight fragments but not for nuclear morphology, where besides CAD, other players are needed. Importantly, the combination of TRAIL plus gossypol induced the formation of closed apoptotic nuclear bodies which avoided the release of the genomic content observed during cell death induced by other apoptotic insults. Both TRAIL and gossypol have been proved safe but inefficient when employed separately in clinical trials with glioblastoma patients. Here, we propose that their combination maybe result in a more efficient treatment of glioblastoma since they induce complete nuclear apoptosis that abolish the release of DNA during cell demise.

I. INTRODUCTION

1. Tumors of central nervous system

The central nervous system consists of neurons and neuroglial cells. There are four types of neuroglial cells: astrocytes, oligodendrocytes, ependymal cells and microglia.

At the beginning of 19th century, neuroglial cells, usually referred as glial cells or glia, were described as a connective tissue which held neurons together, hence the name from the Greek word meaning "glue". Later on, it was described that glia act not only as a connective but also as a supportive and protective tissue that provided nutrients, oxygen, mechanical support, guidance in development, immune functions, and waste disposal to neurons. Moreover, since the beginning of the 21st century, it is widely accepted that glial cells function as neurons partners, being involved in complex processes including signal transduction (Gourine et al. 2010) and neurotransmission (Wolosker et al. 2008).

1.1. Definition and classification of gliomas

Although neoplasms can arise from either the neuronal or glial components of the central nervous system, beyond all doubt, the glial tumors or gliomas are more important in terms of frequency and clinical aggressiveness. One of the most accepted theories postulates that glial tumors origin by transformation of a neural progenitor during an asymmetric division (Hadjipanayis & Van Meir 2009a; Hadjipanayis & Van Meir 2009b). However, other theories support a mutation-driven dedifferentiation of mature cells as the initial event driving gliomagenesis (Bachoo et al. 2002).

Glial tumors are classified into three main groups: astrocytomes, oligodendrogliomes and ependymomas (according to morphological similarities with astrocytes, oligodendrocytes and ependymal cells, respectively). Additionally, there is a fourth group called "mixed gliomas" that refers to those tumors containing more than one type of morphologically distinguishable cells (Louis et al. 2007; Fuller 2008).

The World Health Organization (WHO) subdivides astrocytomas into different groups according to tumor malignancy (I to IV in order of increasing malignancy) (Louis et al. 2007; Fuller 2008). Commonly,

the term low-grade non anaplastic[1] astrocytoma is used for grade I and II; high grade anaplastic astrocytoma, for grade III; and glioblastoma multiforme, for grade IV (Burger 1995; Huse et al. 2011).

1.2. Glioblastoma multiforme

Glioblastoma multiforme (GBM), is one of the most aggressive and incurable human cancers. Glioblastoma represents the most common primary intrinsic malignant brain tumor diagnosed each year in developed countries. According to the Central Brain Tumor Registry, 64,000 new cases of primary central nervous system tumors were diagnosed in 2011, 24,000 of which were malignant. Gliomas represented around 80% of all malignant brain tumors registered, with GBM as the most common. In 2012, GBM had an annual incidence rate of 3.2 cases per 100,000 persons, accounting for over 50% of all diagnosed gliomas (CBTRUS 2012). Clinical symptoms include progressive headaches, focal neurologic deficits and seizures (Kleihues & Ohgaki 1999; Huse et al. 2011).

1.2.1. Detection

Observed by magnetic resonance imaging (MRI), GBM appears as a mass with partial contrast enhancement due to partial disruption of the blood-brain barrier. Besides irregular contour, these neoplasms present a peripheral zone of enhanced contrast surrounding a darker necrotic center and a peritumor hypodense area (Van Meir et al. 2010a; Faria et al. 2006).

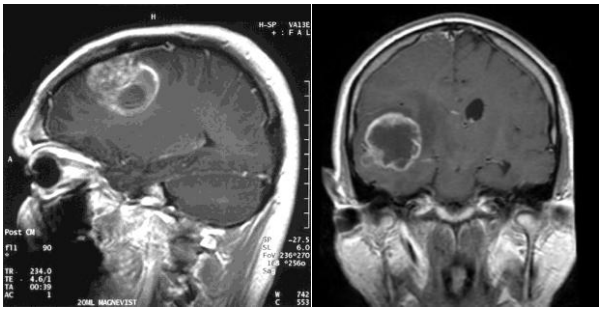

Figure I1. Glioblastoma multiforme images obtained by MRI. GBM appears as a large mass surrounded by vasogenic oedema with a necrotic centre (from Wikipedia and Radiopaedia).

[1] Anaplasia: change in the structure and orientation of cells that undergo dedifferentiation and results in a loss of functionality. Anaplasia is characterized by nuclear atypia, cell pleomorphism, high mitotic activity and endotelial hyperplasia (Louis et al. 2007).

Two variants of GBM have been described: giant cell glioblastoma and gliosarcoma (Lai, Tran, et al. 2011). Despite little differences, all GBM are histologically recognized by nuclear atypia, highly mitotic activity and diffusely infiltrating glial anaplasia that demonstrates marked nuclear and cytoplasmic pleomorphism. Other defining histological traits of GBM are the presence of microvascular proliferation and central areas of tumor necrosis that are often, but not necessarily, associated with perinecrotic nuclear pseudopalisading (Brat & Van Meir 2004; Stupp et al. 2005; Van Meir et al. 2010).

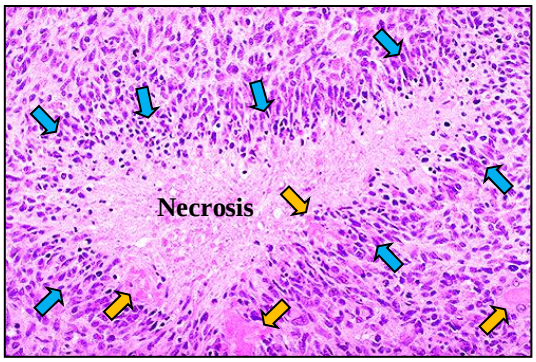

Figure I2. Histological view of glioblastoma multiforme. GBM is characterized by a central area with no or low concentration of cells (purple), new vessels (orange arrows) and pseudopalisading (blue arrows) (from Pathology Outlines).

1.2.2. Location

GBM can be found at any part of the central nervous system including cerebellum, brainstem and spinal cord. However, it has been reported a preferential location at the frontal or temporal lobe (Kanu et al. 2009; Lai, Kharbanda, et al. 2011). Indeed, it has also been found a preference for the frontal lobe in grade II astrocytoma carrying IDH1/2 mutation, which will eventually evolve to glioblastoma or oligodendroglial tumors (Zlatescu et al. 2001; Laigle-Donadey et al. 2004; Stockhammer et al. 2012). These observations suggest that oligodendrogliomas, astrocytomas, and glioblastomas may preferentially originate from precursor cells located in or migrating to the frontal lobe (Ohgaki & Kleihues 2013).

1.2.3. Genetics

Gliomagenesis is an extremely unstable genetic process with activation of oncogenes, inactivation of tumor suppressor genes and many different genetic mutations. In fact, malignant gliomas held the greatest range of genetic abnormalities among tumors. These multiple alterations affecting several signaling pathways, result in enhanced cellular proliferation and apoptosis resistance (Sathornsumetee et al. 2007).

Most of GBMs develop *de novo* in a period of 3-6 months (Ohgaki & Kleihues 2005). However, among 5-10% of GBMs, are diagnosed after resection of a previous low-grade astrocytoma. The former ones are classified as primary GBMs while the latter ones are termed secondary GBMs (Ohgaki & Kleihues 2005; Van Meir et al. 2010a; Lima et al. 2012). Indeed, the classification in primary and secondary GBMs was suggested by Hans-Joachim Scherer forty years before the first edition of the WHO Classification of Tumors of the Nervous System (Burger 1995; Peiffer & Kleihues 1999). Although it was recognized in clinical practice that some glioblastomas were preceded by a low-grade or anaplastic astrocytomas, Scherer's distinction remained conceptual because of these subtypes could not be histopathologically distinguished.

At the end of the 20th century, genetic analysis made possible to molecularly distinguish primary and secondary GBMs. TP53 mutations, very rare in primary GBMs, were very common in secondary glioblastomas (Watanabe et al. 1996; Kleihues & Ohgaki 1999; Yan et al. 2009). Alternatively, epidermal growth factor receptor (EGFR) overexpression prevailed in primary glioblastomas but had low incidence in secondary GBMs (Watanabe et al. 1996). Interestingly, Watanabe and collaborators described that less than 2% of glioblastomas showed TP53 mutation and EGFR overexpression, suggesting that these alterations, should be mutually exclusive and define different genetic pathways in the evolution of primary and secondary glioblastomas (Watanabe et al. 1996). Soon, several studies supported that primary and secondary glioblastomas develop through distinct genetic pathways. While primary glioblastomas typically present EGFR amplification, PTEN mutation, entire loss of chromosome 10 or TERT promoter mutations (Fujisawa et al. 2000; Maher et al. 2001; Ohgaki et al. 2004; Ohgaki 2005; Maher et al. 2006; Ohgaki & Kleihues 2007; Nonoguchi et al. 2013), the most common genetic alterations in secondary glioblastomas are TP53 mutations and 10q and 19q loss of heterozygosis (LOH) (Nakamura et al. 2000; Ohgaki et al. 2004; Ohgaki & Kleihues 2007; Yan et al. 2009). Besides these, other alterations related to secondary GBMs include autocrine loops of TGF-β cytokine, PDGFR overexpression or retinoblastoma protein (RB)-mediated uncontrolled progression of the cell cycle (Nakamura et al. 2000; Hilton et al. 2004; Golestaneh & Mishra 2005). Nevertheless, until the identification of IDH1 mutation as a molecular marker of secondary glioblastoma, the unequivocal separation of the

two subtypes was not possible, at least, from a genetic point of view (Ohgaki et al. 2004; Ohgaki & Kleihues 2007; Balss et al. 2008; Yan et al. 2009; Watanabe et al. 2009).

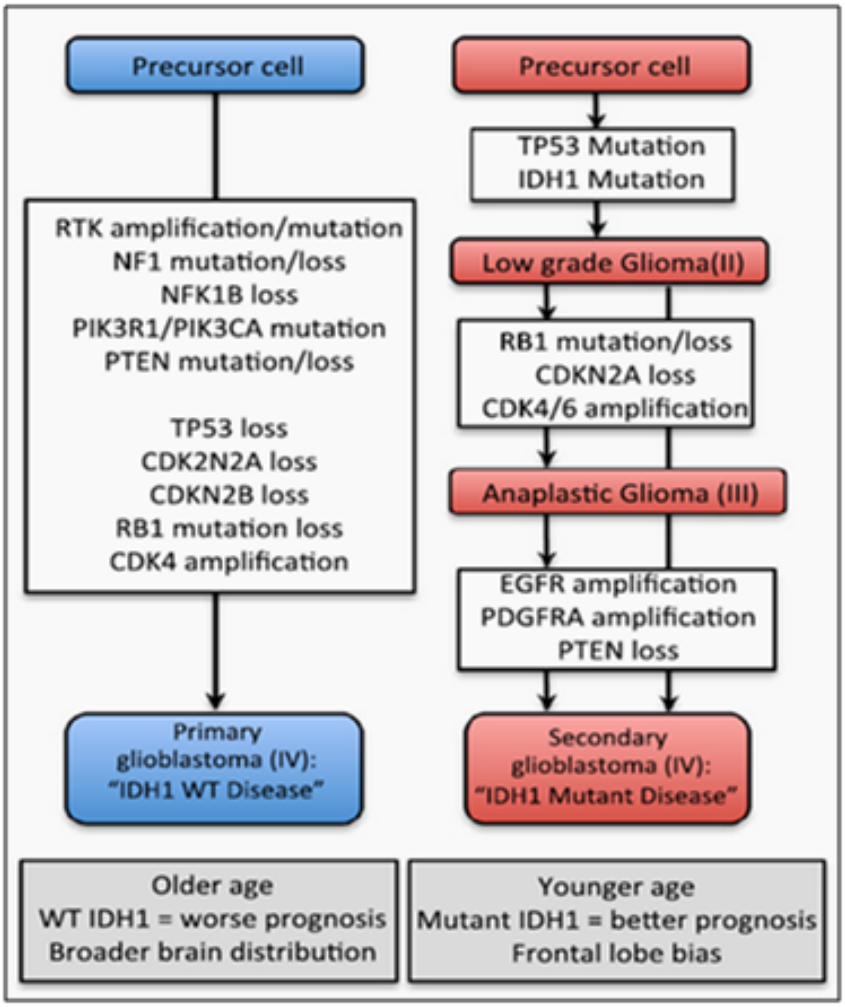

Figure I3. Genetic alterations mostly founded in primary and secondary glioblastoma. Primary and secondary GBM distinguished by the absence or presence, respectively, of mutations affecting the *idh* gene. Moreover, primary and secondary GBM differs in the age of apparition and location of the tumor (Dunn et al. 2012).

Based on the genetics, four subtypes of GBM have been identified: proneural, neural, classical and mesenchymal (Verhaak et al. 2010).

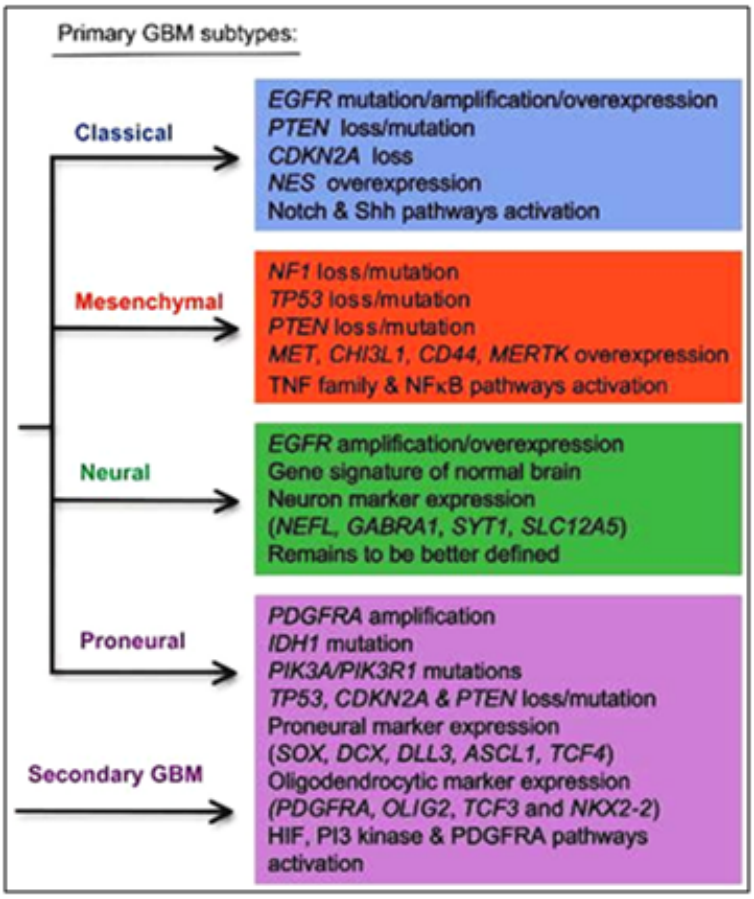

Figure I4. Genetic classification of glioblastoma subtypes. Regarding genetics, GBM have been classified in four different subtypes: classical, mesenchymal, neural and proneural (modified from Van Meir et al. 2010a).

The most relevant genetic alterations found in GBM are explained below.

IDH1. IDH1 enzyme catalyzes the $NADP^+$-dependent oxidation of isocitrate. However, mutant IDH1 catalyzes the NADPH-dependent reduction of α-ketoglutarate (α-KG) to (R)-enantiomer of 2-hydroxyglutarate (2-HG). Although *wild-type* IDH1 can also catalyze this particular reaction (Pietrak et al. 2011), mutant enzyme performs this reaction with much higher efficiency (Dang et al. 2010; Pietrak et al. 2011). Therefore, the major downstream biological effect of IDH1 mutation is the inhibition of α-KG-dependent dioxygenases by 2-HG. α-KG-dependent dioxygenases are involved in a broad range of physiological processes, including histone and DNA demethylation, hypoxic sensing and fatty acid metabolism, among others (Loenarz &

Schofield 2008; Xu et al. 2011; Chowdhury et al. 2011; Lu et al. 2012). Indeed, IDH1 mutant gliomas exhibit a global DNA hypermethylation state (Noushmehr et al. 2010) also observed in IDH1 mutant acute myeloid leukemia (AML) (Figueroa et al. 2010). Interestingly, it is known that mutant IDH1 impairs cell differentiation, suggesting that hypermethylation can contributed to a persistent dedifferentiated state (Figueroa et al. 2010). Moreover, global expression profiles of IDH1 mutant glioblastomas resembled more closely to lineage-committed neural precursors, whereas *wild-type* counterparts resemble neural stem cells (Lai, Kharbanda, et al. 2011).

IDH1 can also play a role in gliomagenesis by regulating cellular hypoxic responses. It has been described that *wild-type* IDH1 drives lipogenesis by reducting glutamine to α-KG in hypoxic cells (Metallo et al. 2012). Intriguingly, the (R)-enantiomer of 2-HG stimulates HIF degradation, which implies that in IDH mutant cells, the HIF-1 response to hypoxia is attenuated (Koivunen et al. 2012). Hence, should be a physiological selection pressure to maintain a copy of the *wild-type* IDH1 gene in cancer cells. However, the study of sequential biopsies from patients with diffuse astrocytoma or oligoastrocytoma suggests that IDH1 mutation is an early event in IDH1 mutant gliomas. Samples from patients who carried only IDH1 mutations at the first biopsy acquired either TP53 mutation or 1p19q loss at the second biopsy, suggesting a temporal sequence of mutation acquisition (Lai, Kharbanda, et al. 2011). This would be consistent with a strand asymmetry mechanism (Rodin & Rodin 1998) in which mutations took place on the transcribed strand in IDH1 but on the non-transcribed strand in p53.

Despite our poor understanding of mutant IDH biology, the mutant status of the IDH1/2 genes may serve as an important prognostic indicator. In fact, patients with grade II glioma, anaplastic astrocytoma or glioblastoma, harboring mutant IDH1 are younger and demonstrate a significantly longer overall survival (Parsons et al. 2008; Yan et al. 2009; Sanson et al. 2009; Hartmann et al. 2010). Moreover, similar survival benefit was observed in patients with tumors that held hypermethylated profile (Noushmehr et al. 2010). In addition, genomic and clinical analysis of glioblastomas harboring mutant and *wild-type* IDH1 suggests that, while histopathologically similar, these tumors may represent disparate disease processes. IDH1 mutant glioblastomas display less contrast enhancement and peritumoral edema, larger initial

size and a higher preference for a frontal lobe location compared with *wild-type* tumors (Lai, Kharbanda, et al. 2011).

Based on these data, several methods to assess IDH1 mutant protein status (Capper et al. 2009) or the 2-HG subproduct (Sahm et al. 2012) have been developed. Although the correlation between 2-HG in serum and IDH mutation status is little specific in glioma (Capper et al. 2012), it is possible to monitor the presence of 2-HG using non-invasive magnetic resonance spectroscopy (MRS) imaging of the brain (Pope et al. 2012).

TP53. Alterations in the tumor suppressor protein TP53 occurs in 30-40% of GBM increasing till 65% in secondary GBM (Ohgaki & Kleihues 2005; Parsons et al. 2008). Besides direct mutation of TP53, other genetic and somatic alterations affecting TP53 pathway have been described in 78% of GBM samples (Anon 2008). Moreover, since 74% of GBM harbor aberrations in all RB, TP53 and receptor tyrosine kinase (RTK) pathways, it is believed that deregulation of these three pathways is a requirement for glioblastoma pathogenesis (Anon 2008). TP53 mutation and expression of mutant TP53 have negative correlation with overall survival in patients with GBM. Indeed, it has been recently suggested that TP53 mutation may decrease the chemosensitivity of glioblastoma to temozolomide by increasing MGMT expression (Wang et al. 2014).

RB. During a normal cell cycle, retinoblastoma (RB) protein is inhibited by hyperphosphorylation, which disrupts RB transcriptional repression complexes allowing G1 to S phase transition (reviewed in Zhu 2005). However, after DNA damage, RB is not phosphorylated, which induces cell cycle arrest by the intra S-phase checkpoint (Knudsen et al. 2000). RB also participates in other cellular processes, such as terminal cell differentiation, maintenance of genetic stability (reviewed in Sherr & McCormick 2002; Burkhart & Sage 2008 and Viatour & Sage 2011) or apoptosis inhibition (Haas-Kogan et al. 1995; Tan et al. 1997; Boutillier et al. 2000; Knudsen et al. 2000; Fattman et al. 2001; Chau et al. 2002; Borges et al. 2005). Although mutations and homozygotic deletions of the RB gene itself appear in only 11% of the cases, gliomas can circumvent RB-mediates cell cycle inhibition through several genetic alterations (Schmidt et al. 1994; Jen et al. 1994; Reifenberger et al. 1994; Costello et al. 1996; Fueyo et al. 1996; Costello et al. 1997). Indeed, RB pathway is altered in 78% of GBM (Anon 2008).

NF1. Neurofibromin (NF1) is a potent tumor suppressor that negatively regulates Ras and mTOR signaling in astrocytes. NF1 inactivation can occur by mutation or proteasomal degradation (Parsons et al. 2008; McGillicuddy et al. 2009). Around 23% of GBM exhibiting inactivated NF1 (Anon 2008) being most commonly found in the mesenchymal subtype (Verhaak et al. 2010).

NF1 loss has been related to Ras/mTOR-mediated increased cell proliferation and migration but also to glioma formation in a manner that is independent of the tuberous sclerosis complex/Ras homolog enriched in brain (TSC/Rheb) control of mTOR (Banerjee et al. 2011). Moreover, Stat3, a potential downstream target of NF1 (Banerjee et al. 2010) non-regulated by mTORC1 or Rac1, also increases pro-proliferative cyclin D1 expression. In fact, it remains yet unclear whether NF1-deficient gliomas could be susceptible to proteotoxicity through mTOR inhibition like other NF1-deficient cancers (reviewed in De Raedt et al. 2011).

Genetically engineered mouse models have demonstrated that homozygous loss of NF1 in astrocytes, while sufficient to increase cell growth, is not enough to induce glioma formation (Bajenaru et al. 2002). Interestingly, NF1-/- astrocytes develop optic gliomas when exists a NF1+/- brain (Bajenaru et al. 2003; Zhu et al. 2005; Daginakatte & Gutmann 2007; Warrington et al. 2010). Moreover, NF1 loss in glial cells, in combination with a germline p53 mutation, results in fully penetrant malignant astrocytomas (Zhu et al. 2005), which progress to glioblastoma upon deletion of PTEN (Kwon et al. 2008). More recent work has revealed that the same combination of genetic alterations in these tumor suppressor genes in neural progenitor cells is necessary and sufficient to induce astrocytoma formation (Alcantara Llaguno et al. 2009).

Other common genetic alterations such as PTEN, EGFR or PDGFR, with clinical potential in GBM, are addressed in the next chapter *'Glioblastoma multiforme: treatment and resistance'*.

1.3. Glioblastoma multiforme: treatment and resistance

In 1920s the neurosurgeon Walter Dandy decided to remove the entire affected hemisphere from two patients with extensive glioblastoma. Despite these radical surgeries, both patients died by development of new glioblastomas in the contralateral hemispheres (Gardner 1933). Unfortunately, almost a century later, this catastrophic panorama has not substantially changed. Its aggressive growth and inexorable recurrence made glioblastoma extremely lethal (reviewed in Siebzehnrubl et al. 2011). After multimodal therapy with surgery followed by radiation and adjuvant chemotherapy, the mean of progression-free survival (PFS) is 7 months, being 14 months the median overall survival (Stupp et al. 2002; See & Gilbert 2004; Stupp et al. 2005; Noda et al. 2009; Florio & Barbieri 2012).

1.3.1. Surgical resection

Maximal resection with preservation of neurological function is the main goal of neurosurgeons in the management of GBM. Many evidences indicate that extensive resection, as long as neurological functioning is maintained, increases effectiveness of adjunct chemo- and radiotherapy and are associated with better outcomes (Lamborn et al. 2004, Stummer et al. 2011). Maximum resection has been recently improved due to advanced imaging modalities including functional MRI, intraoperative stereotactic navigation or fluorescent tumor markers (Stummer et al. 2006).

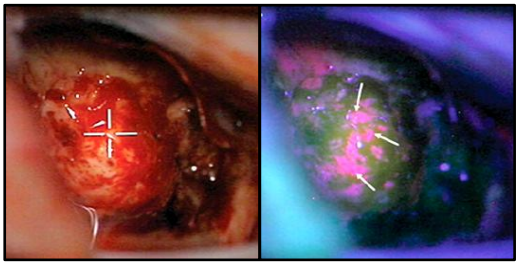

Figure I5. Fluorescence-guided tumor resection. Intraoperative microscopic view of a tumor resection cavity using white light (left) or blue light (right) (modified from Van Meir et al. 2010a).

Nevertheless, one distinctive trait of astrocytic tumors is their diffuse infiltrative nature. Their particular growth, as masses without clear margins intermingled with normal brain cells, makes total resection almost impossible. Indeed, this trait is believed to underlie, more than any other, the surgical incurability of these tumors (Jackson et al. 2001).

1.3.2. Radiotherapy

Radiotherapy has been of key importance to the treatment of glioblastoma for decades (Tofilon & Fike 2000; Belka et al. 2001; Monje et al. 2002; Zhao & Robbins 2009). However, a wide spectrum of non-desirable radiation-mediated injuries has been described including pseudoprogression, occurring within 2-5 months after radiation therapy (Graeb et al. 1982; de Wit et al. 2004) and radiation necrosis, appearing after the third month up to the first year (Watne et al. 1990; Chamberlain et al. 2007). Despite of the risks, efficacy of radiation has been proven in randomized trials (Stupp et al. 2005). Indeed, in conjunction with surgery and medical management, radiation therapy doubles median survival and extends 2-years survival to 10% (Shapiro et al. 1989; Fine et al. 1993; Stewart 2002).

Although the ionizing radiation itself has not significantly changed during the last 25 years, its delivery has greatly improved. Nowadays, a more focused intensity-modulated beam together with the use of image-guided techniques, minimize the radiation dose to nearby critical structures (Stieber & Mehta 2007).

1.3.3. Chemotherapy

Glioblastoma holds significant intratumoral heterogeneity at the transcriptional and genomic levels. Fortunately, the knowledge about gene mutations and expression profiles of GBM has notably increased in the last decade. Indeed, the description of the key signaling pathways altered in gliomas has catapulted lots of new compounds with potential chemotherapeutic suitability to clinical trials. Desolately, although some of the new drugs have shown little impact in progression free survival, most of them have completely failed and almost none of them have shown clear effect in overall survival.

From 2005, oral temozolomide (Temodal) is the first-selected chemotherapy for treatment of GBM (Wick et al. 2012). Temozolomide is an alkylating agent that kills cells by inducing DNA damage. After surgery, temozolomide, given concurrently with radiation and continued thereafter, extends median survival from 12 to 15 months and increases 2-years survival to 27% (Stupp et al. 2005; Stupp et al. 2009). Unfortunately, the survival benefit of temozolomide administration was largely restricted to those patients whose tumors

showed epigenetic silencing of the DNA repair gene *mgmt* (methylguanyl methyltransferase) by methylation (Hegi et al. 2005).

Although lots of different compounds have been proposed for the treatment of GBM, the most important biological functions chemically addressed are tumoral cellular growth and angiogenic dependence.

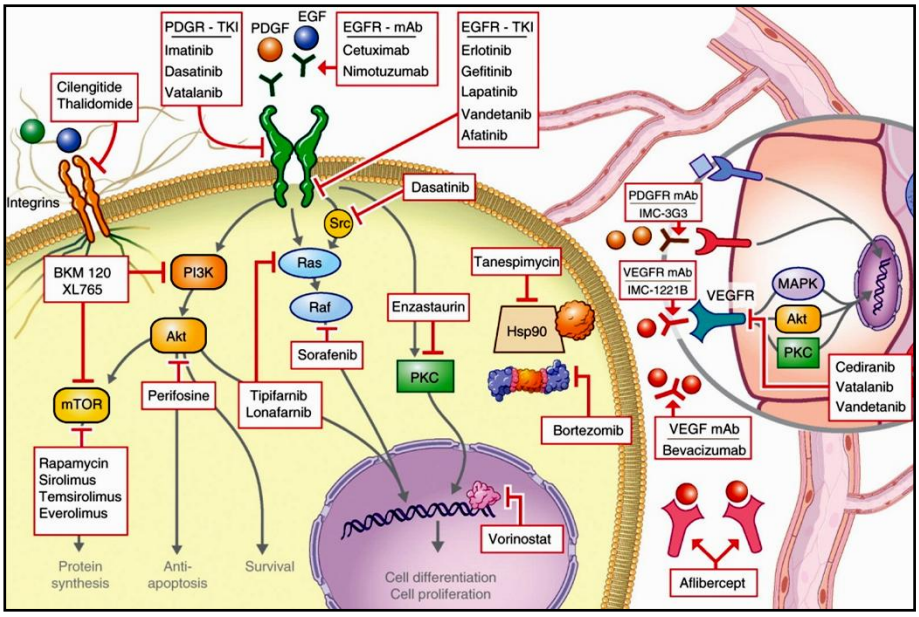

Figure I6. Overview of the latest agents employed in clinical trial for GBM treatment. Chemotherapies currently under investigation in GBM include inhibitors of angiogenesis, cellular growth or histone deacetylases among others (Patel et al. 2012).

a) Growth inhibitors

In the majority of malignant gliomas, cell metabolism, growth, proliferation and survival are deregulated.

EGFR. Epidermal growth factor receptor (EGFR) is considered one of the most attractive therapeutic targets in GBM. The *egfr* gene is amplified and overexpressed in approximately 50% of primary GBMs and associates with poor prognosis (Hurtt et al. 1992; Jaros et al. 1992; Schlegel et al. 1994). Unfortunately, although well tolerated, small-molecules inhibiting EGFR showed little effectiveness in GBM patients (Neyns et al. 2009; Thiessen et al. 2010; Kreisl et al. 2012; Chakravarti et al. 2013; Gallego et al. 2014). Conversely, in combination with radiation, nimotuzumab, an humanized monoclonal antibody against EGFR, has shown significant survival benefit besides

an excellent safety profile in high grade glioma patients (Solomón et al. 2013).

Nearly 50% of tumors with *egfr* amplification also have the constitutively active EGFRvIII receptor which confers a worse prognosis than *wild-type* EGFR expression alone (Shinojima et al. 2003; Heimberger et al. 2005). Interestingly, this truncated form of EGFR has a unique codon, not found in the *wild-type* receptor which creates a therapeutically relevant tumor-specific epitope. CDX-110 or rindopepimut, a peptide vaccine against this epitope, has a favorable toxicity profile and is currently being studied in combination with temozolomide (Del Vecchio et al. 2012; Babu & Adamson 2012).

Interestingly, ectopic overexpression of EGFRvIII in glioma cell lines not only induces cell proliferation (Huang et al. 1997; Narita et al. 2002), but also resistance to apoptosis through modulation of Bcl-X_L expression (Nagane et al. 1998).

PDGF. Nearly 30% of human gliomas show hyperactivation of platelet-derived growth factor (PDGF) signaling, which promote tumor cell proliferation through autocrine and paracrine loops (Brennan et al. 2009; Verhaak et al. 2010). Indeed, the PDGFR inhibitor imatinib mesylate (Gleevec) was reported to have significant antitumor activity both *in vitro* and in orthotopic glioma models (Geng et al. 2006; Benny et al. 2009). Unfortunately, in clinical trials, imatinib showed little responsiveness with no prolongation of PFS of GBM patients (Wen et al. 2006; Razis et al. 2009).

PI3K pathway. In many cases, cellular growth of malignant gliomas is increased due to deregulation of the positive and negative players taking part of the signaling downstream of growth receptor activation. RTK signaling can recruit PI3K to the cell membrane, where mediates the phosphorylation of the lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-bisphosphate (PIP3). Then, PIP3 together with the phosphoinositide dependent kinase 1 (PDK1), allow the recruitment of AKT to the membrane where becomes phosphorylated and active (Sarbassov et al. 2005). PTEN, the phosphatase that directly antagonizes PI3K signaling by reconverting PIP3 to PIP2, is one of the most frequently altered genes in cancer. It undergoes genomic loss, mutation or epigenetic inactivation in 50% of gliomas (Mellinghoff et al. 2005; Mellinghoff et al. 2007). Indeed, elevated AKT phosphorylation has been observed in up to 85% of glioblastoma cell lines and patient samples (Wang et al.

2004). Unfortunately, AKT inhibitors showed limited benefit in the clinics (Kreisl et al. 2009; Rampling et al. 2012).

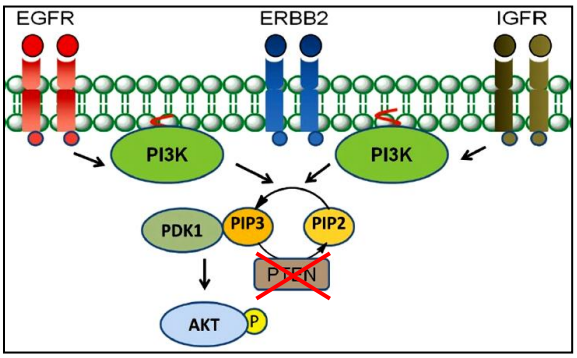

Figure I7. Signaling network promoting growth and survival in cancer cells. In normal cells growth factor receptors activate PI3K which converts PIP2 to PIP3. Then, PDK1 phosphorylates AKT. In cancer cells, overexpression of receptors or loss of PTEN results in constitutively activated AKT which lead to enhanced growth, survival and invasiveness (modified from Dasgupta et al. 2012).

Even though ligand binding to receptor tyrosine kinases ultimately activates mTOR, mTOR inhibitors showed only minimal results in clinical trials with glioblastoma patients (Galanis et al. 2005; Chang et al. 2005). Indeed, effectively inhibiting the PI3K signaling pathway is challenging because the cascade and its feedback regulation is not completely understood. Interestingly, the phosphatase PTEN has been also related to other AKT-independent downstream effects such as the regulation of the half-life of the antiapoptotic protein FLIPs, which affects TRAIL sensitivity (Panner et al. 2009).

PKC. Due to its role in glioma cell proliferation, invasion and angiogenesis, protein kinase C (PKC) also harbors a therapeutic interest. However, in clinical trials, PKC inhibitors alone did not result effective (Galanis & Buckner 2010; Wick et al. 2010). Nevertheless, PKC-β inhibition showed synergistic anti-glioma effects with radiotherapy (Tabatabai et al. 2007; Kreisl et al. 2010) and temozolomide (Butowski et al. 2011).

b) Antiangiogenic therapy

Antiangiogenic therapy, initially proposed by Folkman in 1971, consists in the disruption of tumor blood vessels (Folkman 1971). The early small tumors can feed off the rich glial vasculature by 'co-opting'[2] along the capillaries (Ahluwalia & Gladson 2010). However,

[2] Co-opt: to take or assume for one's own use

once the tumor grows beyond 2-3 mm in size, aggressive tumors like GBM require their own vascular supply to maintain oxygenation and nutrition (Jain et al. 2007). Then, growth is sustained through sprouting angiogenesis or intussusception (Burri et al. 2004; reviewed in Hillen & Griffioen 2007). The former, refers to the formation of new capillaries out of existing ones; the latest, to the split of a capillary into two by interposition of a group of cells. Alternatively, angiogenesis can occur through vasculogenesis, which is the recruitment and integration of systemically circulating progenitor endothelial cells, or 'vascular mimicry'. This last term, introduced in 1999 by Maniotis and collaborators, refers to the de-differentiation of tumor cells that become blood vessels coating cells (Maniotis et al. 1999).

VEGF family. The upregulation of vascular permeability factor A (VEGF-A) in GBM is of outmost importance due to its involvement in angiogenesis. It can be detected in all grades of gliomas and plays a critical role in endothelial cell proliferation in malignant gliomas (Huang et al. 2005; Miyagami & Katayama 2005). In this sense, the anticoagulant ixolaris, which decreased VEGF expression, resulted effective to block tumor growth and angiogenesis, suggesting that anticoagulants could be used as adjuvant therapy for GBM treatment (Carneiro-Lobo et al. 2009).

Bevacizumab is a humanized monoclonal antibody that targets VEGF-A. Bevacizumab received US FDA approval as a single-agent for the treatment of recurrent GBM in May 2009. Nowadays, treatment with bevacizumab has been improved with a superselective intra-arterial cerebral infusion (SIACI) administration which considerably reduces the associated side effects (Riina et al. 2010; Shin et al. 2012). However, bevacizumab does not benefit all malignant glioma patients (Schneider et al. 2009; Ahluwalia & Gladson 2010; Rahman et al. 2010; Chinot et al. 2011; Vredenburgh et al. 2011; Vredenburgh et al. 2012; Narayana et al. 2012; Hainsworth et al. 2012). Indeed, although VEGF-A is described as the major positive modulator in malignant glioma angiogenesis, others ligands and receptors, not blocked after bevacizumab administration, can also play a role in GBM progression (Tammela et al. 2008; Nilsson et al. 2010).

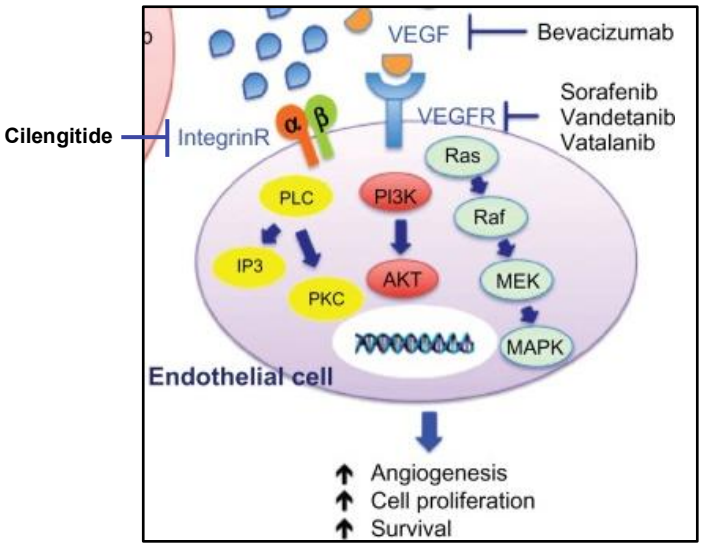

Figure I8. Schematic representation of the main agents targeting angiogenic pathways in endothelial cells. Antiangiogenic compounds can target VEGF angiogenic ligands (e.g. bevacizumab) as well as VEGFR or integrins (e.g. sorafenib, vandetanib, vatalanib and cilengitide) (modified from Scaringi et al. 2013).

Cediranib (AZD2171) is an oral VEGFR inhibitor that binds to the intracellular domain of all three VEGF receptors. A phase II clinical trial in patients with recurrent GBM showed that cediranib therapy reduced blood vessel size and permeability (Batchelor et al. 2007). These were the first clinical data supporting the hypothesis that antiangiogenic therapy may "normalize" the dilated, abnormally permeable tumor vasculature. However, the effect of anti-angiogenic therapy on tumor vessels is just transient, which results in a limited window of opportunity for optimally combining chemotherapy and radiation (Jain et al. 2007). That may explain why anti-angiogenic drugs are usually ineffective as monotherapies (Miletic et al. 2009). Although cediranib showed no PFS prolongation in a phase III trial, it did show clinical activity on some secondary end points including time to deterioration in neurologic status (Batchelor et al. 2013). More interestingly, it has been observed that cediranib can reduce radiation-induced pseudoprogression (Pinho et al. 2014).

In addition to VEGF family inhibition, there are other approaches that could have antiangiogenic activity. αVβ3 and αVβ5 integrins are cell adhesion molecules overexpressed in both glioma and endothelial cells placed at the tumor periphery (Bello et al. 2001). Unfortunately,

although αVβ3 and αVβ5 integrins are involved in angiogenesis, immunity invasion and metastasis (Jin & Varner 2004; Avraamides et al. 2008; Silva et al. 2008), therapies targeting these molecules have failed in the clinic (Eisele et al. 2014).

Interestingly, a study made in intracranial GBM xenografts, showed that after irradiation, bone-marrow-derived cells from fields outside the radiated area are recruited into the tumor to stabilize vasculature which allows the growth of the remaining tumor cells (Kioi et al. 2010). Although PDGFR can mediate vasculogenesis (Guo et al. 2003), sunitinib, a multiple RTK inhibitor that also inhibits PDGFR, showed no antitumor activity when administered to patients with non-resectable glioblastoma prior to radiotherapy (Balaña et al. 2014). Besides PDGFR, the interaction between the HIF-1-dependent stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4 (Ceradini et al. 2004; Jin et al. 2006) can also regulate vasculogenesis. Interestingly, an inhibitor of SDF-1/CXCR4 interaction resulted in a significant reduction in tumor growth and invasion in an animal model of human GBM (Ali et al. 2013). However, the results of this study highlighted the intricate regulation promoted by CXCR4 receptor since its inhibition reduced the expression of several antiangiogenic factors without significantly affecting vascular density. Indeed, the use of antiangiogenic drugs has also been recently associated with the generation of more resistant, invasive and aggressive tumors (Lucio-Eterovic et al. 2009; di Tomaso et al. 2011).

1.3.4. Immune therapy

The central nervous system has mechanisms to restrict the function of the immune system to protect itself against damage. This "immune privilege" makes more difficult to elicit immune responses towards antigens in central nervous system compared to the rest of the body. The suppression of general immunity in the brain is achieved through different mechanisms such as paucity of dendritic cells (DCs), production of anti-inflammatory mediators such as TGF-β or reduced major histocompatibility (MHC-II) expression on infiltrating microglia (Fabry et al. 2008).

In brain tumors, adenoviral immunotherapy has success in generating a tumor microenvironment to which dendritic cells migrate and mature. It was achieved by the expression of thymidine kinase (TK) and fms-like tyrosine kinase-3 ligand (Flt3L). TK is an enzyme that phosphorylates the prodrug ganciclovir which triggers cell death

of tumor cells by inducing DNA crosslinking (Ali et al. 2005). Flt3L is a potent dendritic cell growth factor that serves to increase the number of infiltrating DCs within the tumor microenvironment (Ali et al. 2004; Ali et al. 2005). Cell death caused by TK induces the release of tumor antigens, which are phagocyted by DCs and transported to the lymph nodes where T cells elicit a cytotoxic and antigen specific anti-tumor immune response. This adenoviral immunotherapy, when tested in several mouse and rat GBM models, induced toll-like receptor 2 (TLR2)-dependent tumor regression, long term survival and immunological memory (Ali et al. 2005; Curtin et al. 2009). High-mobility-group box 1 (HMGB1) has been identified as the TLR-2 ligand stimulating dendritic cell maturation during this particular adenoviral immunotherapy (Curtin et al. 2009). Interestingly, HMGB1, part of the chromatin structure in viable cells, is lost in GBM nuclei in response to cytotoxic stimuli (reviewed in Kang et al. 2013), suggesting that other proapoptotic drugs may also be suitable adjuvant molecules for immunotherapeutic strategies.

An important fact during any adaptive immune response is the proper activation of cytotoxic T lymphocytes (CTLs). Indeed, recombinant vaccinia virus expressing cytokines that mediate growth, survival and maturation of antigenic T cells (i.e. IL-2 or IL-12), resulted in inhibition of tumor growth in a rat glioma model (Chen et al. 2000). Moreover, when intracranially delivered in xenograft mouse models, adenoviral vectors expressing the immune-stimulatory cytokines interferon-gamma (IFNγ) and tumor necrosis factor alpha (TNFα), led to a statistically significant increase of survival (Ehtesham et al. 2002; Chatzidakis & Mamalaki 2010).

Interestingly, cytotoxic T lymphocyte-associated antigen 4 receptor (CTLA-4), programmed death-ligand 1 (PD-L1) and the immunosuppressive enzyme indoleamine 2,3-dioxygenase, are overexpressed in GBM-infiltrated lymphocytes as well as in GBM cells. Recently, it has been published that administration of a combination of an indoleamine 2,3-dioxygenase inhibitor and monoclonal antibodies targeting CTLA-4 and PD-L1 receptors, increased survival of mice with orthotopic implanted glioma cells (Dixit 2014). Currently, a clinical trial combining these two antibodies is ongoing.

2. Cell death

The development and homeostasis of multicellular organisms depends on the balance between cell survival and cell death. The first evidences of physiological cell death were described by Karl Vogt in 1842 while studying the metamorphosis of amphibians (Kerr et al. 1972). However, the concept of 'programmed cell death' (PCD) was introduced more than a century later (Lockshin & Williams 1965).

2.1. Cell death classification: from origin to nowadays

In 1973, Schweichel and Merker reported the presence of three distinct cell death morphologies in rat embryos after exposure to toxins. They classified as type 1 cell death associated with heterophagy ('eating of another'); as type 2, cell death associated with autophagy ('eating of itself'); and as type 3, the process that did not involve cell digestion (Schweichel & Merker 1973).

Histologically, the **type 1 cell death** described by Schweichel and Merker coincided with the characteristics described by Kerr, Wyllie and Curie under the term 'apoptosis' one year before (Kerr et al. 1972). That was cellular shrinkage followed by the formation of small, roughly spherical cytoplasmic fragments, some of which contain pyknotic remnants of nuclei. Later on, it was described a low molecular weight (LMW) DNA degradation also named oligonucleosomal DNA fragmentation due to the correspondence in size with multiples from 180 base pair (bp). Soon, this DNA laddering became a distinctive trait of apoptosis (Skalka et al. 1976; Zhivotovsky et al. 1981; Yamada et al. 1981; Wyllie et al. 1984). Importantly, during the last stage of the type 1 cell death, the cellular fragments from the injured cells were eliminated by other cells.

In the **type 2 cell death** proposed by Schweichel and Merker, the damaged cell eliminated its own cytoplasm through the generation of multiple vacuoles containing lysosomal enzymes. In this cell death, the heterophagic component was unusual and restricted to late stages (Krstić & Pexieder 1973). The alterations observed during this process were restricted to the cytoplasm and the cellular membrane, being almost imperceptible at the nuclear level (Hornung et al. 1989; reviewed in Beaulaton & Lockshin 1982). Type 2 cell death was related to diverse physiological processes such as insect metamorphosis, microvilli renewal, gap junction regulation or

development (Krstić & Pexieder 1973; Hornung et al. 1989; Clarke 1990).

The **type 3 cell death** described by Schweichel and Merker characterized by the presence of swelled organelles followed by the appearance of 'empty' spaced in the cytoplasm connected with the extracellular space. During this process, plasma membrane was fragmented and nuclear disintegration was delayed (Schweichel & Merker 1973).

Schweichel and Merker's first classification referring to type 1, 2 and 3 was later renamed by Clarke, settling the concepts of apoptosis, autophagic cell death and vesicular nonlysosomal degradation respectively (Clarke 1990). Moreover, Clarke distinguished two different sub-groups in the type 3 cell death: sub-type 3A presenting nuclear disintegration and sub-type 3B displaying karyolysis (Clarke 1990; Beaulaton & Lockshin 1982).

Since the introduction of Clarke's classification, the concept of programmed cell death was intrinsically associated to apoptosis. However, in 2003, Jaättela and co-workers established a new classification based on nuclear changes observed during cell death. Then, cell death was classified into classical apoptosis, apoptosis-like PCD, necrosis-like PCD and accidental necrosis (Jäättelä & Tschopp 2003).

Classical apoptosis was limited to those processes of cell death in which cells presented very specific nuclear alterations that included those previously described by Kerr and colleagues during apoptotic processes (Kerr et al. 1972). The nuclear changes consisted in chromatin condensation and nuclear fragmentation into different masses round or 'half-moon' shape (Jäättelä & Tschopp 2003). This specific nuclear morphology, actually known as a type II nuclear morphology or stage II chromatin condensation (Susin et al. 2000), was intimately related to the internucleosomal DNA fragmentation and dependent on caspase-3-mediated activation of caspase-activated DNase (CAD) (Sakahira et al. 1998).

The **apoptosis-like PCD**, actually known as caspase-independent apoptosis, referred to cell death in which partially condensed chromatin was peripherally arranged in a ring-shape under the nuclear envelope (reviewed in Leist & Jäättelä 2001). These morphological alterations, currently known as type I nuclear morphology or stage I

chromatin condensation (Susin et al. 2000), were related to cell death processes dependent on apoptosis-inducing factor (AIF), endonuclease G (endoG) and cathepsins (Jäättelä & Tschopp 2003).

Following Jaättela's classification, the term **necrosis-like PCD** was used to define cell death in which the chromatin was either only slightly granulated or not condensed at all (Leist & Jäättelä 2001; Jäättelä & Tschopp 2003). This term, included the type 2 cell death described by Schweichel and Merker, the autophagy from Clarke's classification, as well as other processes with morphological changes similar to necrosis. That included abortive apoptosis, in which caspase activation was impaired or inhibited (Sancho-Martínez et al. 2011), and death receptor-mediated necrosis (Chi et al. 1999; Holler et al. 2000). The necrosis-like PCD have been related to ROS production, activation of poly-(ADP-ribose) polymerase (PARP), receptor interacting proteins (RIP) and serin proteases (Vercammen, Brouckaert, et al. 1998; Ha & Snyder 1999; Denecker et al. 2001).

Finally, the term **accidental necrosis** referred to those chaotic, unexpected and non-regulated cell death processes associated to necrotic morphologies due to membrane breakdown.

Latest recommendations for cell death classification. Due to the intricate and evolving classification of the cell death processes, in 2005, was created the Nomenclature Committee on Cell Death (NCCD). Since then, this committee of experts has revised three times the terminology employed in cell death. The first time, in 2005, cell death classification was based only on morphological criteria (Kroemer et al. 2005). The second one, both morphological and biochemical criteria were taken into consideration (Kroemer et al. 2009). In the latest, the NCCD proposed a new classification referring only to biochemical events (Galluzzi et al. 2012). Accordingly to this latest classification, there are five main types of cell death: extrinsic apoptosis, caspase-dependent or -independent intrinsic apoptosis, regulated necrosis, autophagic cell death and mitotic catastrophe.

2.2. Apoptosis: a programmed cell death

Apoptosis is essential to maintain homeostasis in multicellular organisms. Indeed, its implication in pathologies seemed clear from the beginning (Kerr et al. 1972; Wyllie et al. 1980; Wyllie 1987; Golstein et al. 1991; Williams 1991; Arends & Wyllie 1991).

Under the light microscope, apoptosis is characterized by cellular shrinkage and nuclear pyknosis (Kerr et al. 1972). That means that cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. After hematoxylin and eosin stain, apoptotic cell appears as a round mass with dark eosinophilic cytoplasm and dense violet nuclear chromatin fragments (Häcker 2000). By electron microscopy, the subcellular apoptotic changes are better defined. Early during the chromatin condensation phase, the electrodense nuclear material aggregates peripherally under the nuclear envelope. Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called "budding". Apoptotic bodies of cytoplasm and tightly packed intact organelles, containing or not nuclear fragments, are subsequently phagocyted by macrophages or parenchymal cells and degraded within phagolysosomes (Kerr et al. 1972). Since the engulfing cells do not produce anti-inflammatory cytokines, no inflammatory reaction is associated with apoptosis (Savill & Fadok 2000)

Biochemically, apoptosis comprises three distinct phases: first, the initiation phase; second, the commitment phase; and finally, the execution phase, when the characteristic morphological changes including membrane blebbing, cell shrinkage, chromatin condensation and nuclear fragmentation become obvious (Wyllie et al. 1980; McCarthy et al. 1997; Brunet et al. 1998).

2.2.1. Apoptotic biochemical pathways

a) Apoptotic initiators

There are two main apoptotic pathways: the extrinsic or death receptor-triggered pathway and the intrinsic or mitochondrial pathway. Although clearly different, these two pathways are linked and several molecules from one pathway can influence the other one (Igney & Krammer 2002). In T-cells an additional apoptotic pathway, initiated by either granzyme A or granzyme B, has been described (Martinvalet et al. 2005). While the extrinsic, intrinsic and granzyme B pathways converge on the same terminal or execution pathway initiated by the

cleavage of caspase-3, the granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (Martinvalet et al. 2005).

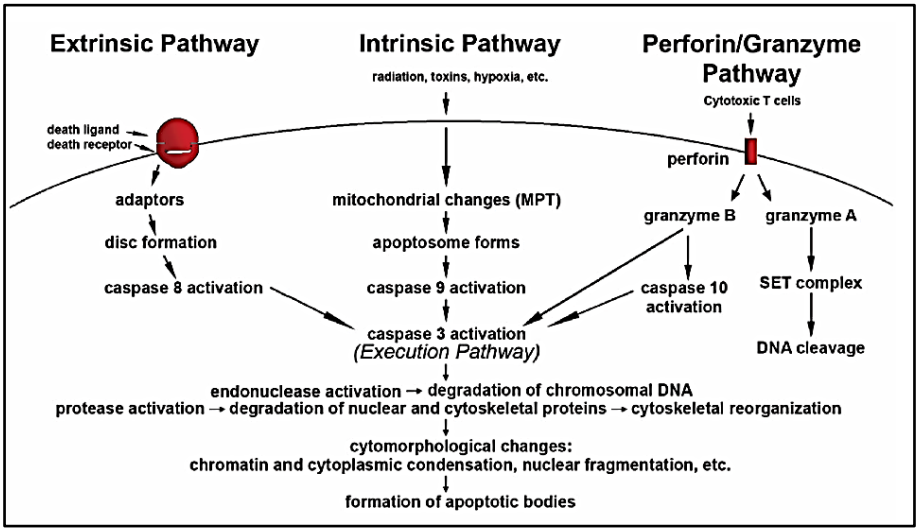

Figure I9. Summary of the different apoptotic pathways. Apoptosis initiates through extrinsic (receptor-mediated), intrinsic (mitochondria-mediated) or granzyme (perforin-mediated) pathway. The three signals converge in the activation of the execution phase mediated by caspase-3 activation (Elmore 2007).

Extrinsic pathway. The extrinsic apoptotic signaling pathway is triggered by transmembrane receptors named death receptors (DR). DR are members TNF receptor (TNFR) gene superfamily (reviewed in Locksley et al. 2001). They share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called death domain (Ashkenazi & Dixit 1998). This death domain (DD) plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. To date, the best-characterized ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, TRAIL/TRAILR1 and TRAIL/TRAILR2 (Chicheportiche et al. 1997; Ashkenazi & Dixit 1998; Peter & Krammer 1998; Suliman et al. 2001; Rubio-Moscardo et al. 2005).

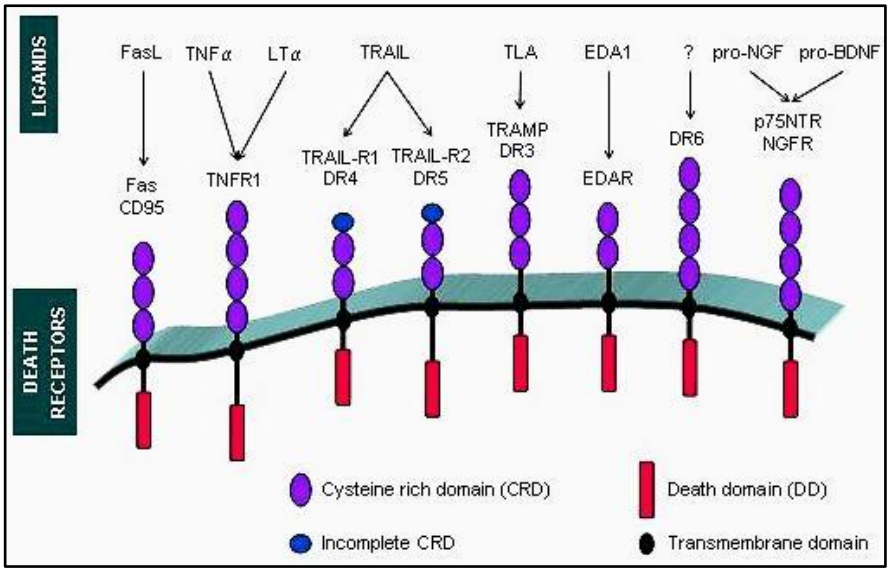

Figure I10. TNF receptor superfamily. The eight human death receptors and their ligands are represented. A representative domain composition is indicated in the legend (Lorz & Mehmet 2009).

The extrinsic phase of apoptosis initiates with a cluster of three death receptors that binds with an homologous trimeric ligand (Wajant et al. 2003). Upon ligand binding, the receptors recruit cytoplasmic adapter proteins through death domains (Boldin, Mett, et al. 1995; Chinnaiyan et al. 1995) creating the death-inducing signaling complex (DISC). Although the exact composition of the DISC depends on the receptor, it always includes the adaptor protein FADD (Chinnaiyan et al. 1995; Hsu et al. 1995; Grimm et al. 1996; Chaudhary et al. 1997; Wajant 2002; Micheau & Tschopp 2003; Lavrik et al. 2005). FADD then associates with initiator pro-caspase-8[3] via dimerization of the death effector domain (DED) which results in the autocatalytic activation of the initiator caspase (Kischkel et al. 1995).

DR-mediated apoptosis can be inhibited by FLIP, a protein that binds to FADD and caspase-8, rendering them ineffective (Kataoka et al. 1998; Scaffidi et al. 1999). Another potential regulator protein is FAIM (Fas apoptotic inhibitory molecule), also named Toso, which

[3] The exact iniciator caspase (-8 or -10) that is recruited by the DISC depends on the ligand and death receptor that mediate the apoptotic signal.

can block Fas-induced apoptosis via inhibition of caspase-8 processing (Hitoshi et al. 1998).

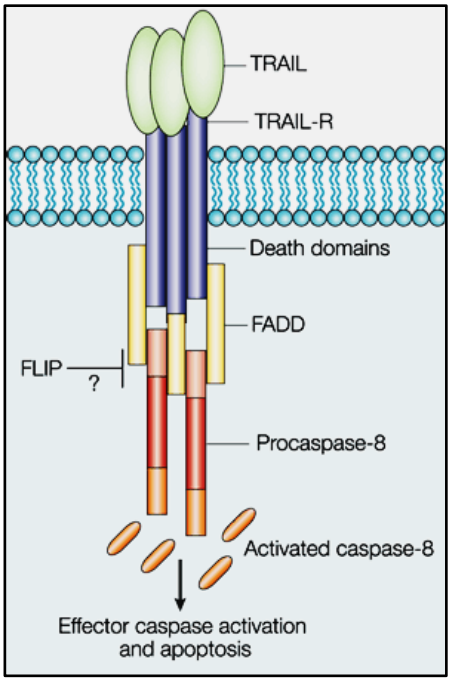

Figure I11. First steps in the activation of the extrinsic apoptotic pathway. Binding of TNF-related apoptosis-inducing ligand (TRAIL) and trimerization of TRAIL death receptors (TRAILR) lead to the recruitment of FAS-associated death domain (FADD). This adaptor molecule recruits and activates caspase-8 which, in the absence of FLICE-like inhibitory protein (FLIP), initiates the caspase cascade that leads to apoptosis (Hersey & Zhang 2001).

Attending to the efficacy of the extrinsic apoptotic pathway, cells can be classified as type I or type II[4]. In type I cells, after the DR-mediated activation of initiator caspase-8 or -10, the executioner caspases-3, -6 and -7 become effectively activated. Nevertheless, type II cells achieve only limited levels of activated caspases after DISC formation and need a mitochondrial amplification loop to finally trigger apoptosis (Scaffidi et al. 1998). This cross-talk between the extrinsic and intrinsic apoptotic pathways in type II cells, occurs by the caspase-8-mediated processing of the proapoptotic protein called Bid. Truncated Bid (tBid) starts a biochemical signaling that leads to activation of the intrinsic apoptotic pathway through mitochondria membrane permeabilization. Only in these conditions, levels of activated caspases are sufficient to proceed with the apoptotic program (Luo et al. 1998; Li et al. 1998).

[4] Do not confuse with the type I and II used for classification of chromatin condensation or the type 1 and 2 used for the first classification of cell death.

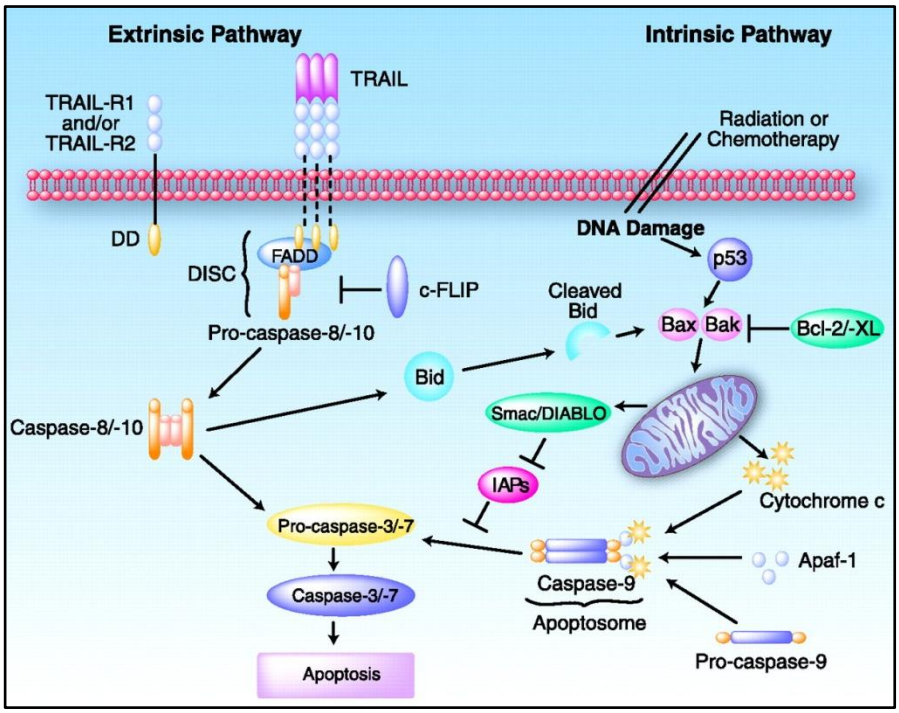

Figure I12. Crosstalk between apoptotic signaling pathways following activation of death receptors. In type I cells, death receptor-mediated activation of caspase-8 or -10 is sufficient for commitment to apoptosis. However, type II cells require signal amplification through the activation of the intrinsic pathway. To achieve that, caspase-8 or -10 cleaves BID which, by interacting with Bax and Bak, induces caspase-9 activation (Carlo-Stella et al. 2007).

Intrinsic pathway. The intrinsic apoptotic signaling pathway, orchestrated mainly by the mitochondria, involves a diverse array of non-receptor-mediated stimuli (reviewed in Joza et al. 2002). The activation of the intrinsic pathway can be the result of both the presence of a positive stimuli or the absence of an inhibitory signal (e.g. radiation, toxins, hypoxia, free radicals and absence of positive growth signals, among others).

There are two differentiated mechanisms activated by intrinsic signals. One, mediated by caspase-12, is triggered by endoplasmic reticulum stress due to accumulation of unfolded or misfolded proteins (Rao, Castro-Obregon, et al. 2002; Rao, Peel, et al. 2002). The other, is mediated by the mitochondrial outer membrane permeabilization

(MOMP) after the formation of the mitochondrial permeability transition (MPT) pore. This process, although not completely elucidated, is regulated by the B-cell lymphoma (Bcl-2) family members. (Marzo et al. 1998; Letai et al. 2002; Kuwana et al. 2002; Scorrano & Korsmeyer 2003). Besides causing loss of the mitochondrial transmembrane potential, the MPT allows the release of proapoptotic proteins from the intermembrane space (IMS) into the cytosol thus activating the caspase-dependent mitochondrial pathway (Saelens et al. 2004).

The first group of proteins released from the mitochondria are cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi (Du et al. 2000; van Loo et al. 2002; Saelens et al. 2004; Garrido et al. 2006). Cytochrome C or Apaf-2 (Liu et al. 1996) is an essential component of the respiratory chain that operates in the inner mitochondria membrane (IMM). When outer mitochondria membrane (OMM) becomes permeable, cytochrome C is partially released to the cytosol where it binds to Apaf-1. This interaction induces a conformational change that exposes the nucleotide-binding site of Apaf-1. If available, dATP binds to Apaf-1 inducing a second structural change that exposes both its oligomerization and its CARD domain. Then, active Apaf-1 assembles into a heptameric protein complex to which procaspase-9 is recruited via interaction with its CARD domain. The resulting protein assemblage, the center of which contains caspase-9, forms the apoptosome which leads to caspase-9 activation (Li et al. 1997; Acehan et al. 2002; Cain et al. 2002). At this point, apoptosis may be still blocked and reverted by targeting caspases (Deshmukh et al. 2000). However, later on in the apoptotic process, a positive feedback on permeabilized mitochondria, partially mediated by caspases and reactive oxygen species (ROS), induced a sustained release of cytochrome C that finally leads to mitochondrial potential loss (Cai & Jones 1998; Cosulich et al. 1999; Waterhouse et al. 2001; Scorrano et al. 2002; Ricci et al. 2003). That may represent a point of no return in the apoptotic process (Deshmukh et al. 2000).

When released through the MPT pore, Smac/DIABLO and HtrA2/Omi facilitate caspase activation by blocking the inhibitors of apoptosis proteins (IAP) (van Loo et al. 2002; Schimmer et al. 2004; Hill et al. 2004). Additionally, both Smac/DIABLO and HtrA2/Omi trigger caspase-independent cytotoxicity (Saelens et al. 2004).

The second group of proapoptotic proteins that are released from the mitochondria comprises apoptosis-inducing factor (AIF) and endonuclease G (endoG) (Li et al. 2001). AIF and endoG have been related to caspase-independent rather than caspase-dependent apoptosis. Indeed, during caspase-dependent apoptosis, AIF and endoG release is a late event that occurs when the cell is already committed to die (Arnoult et al. 2003). However, during caspase-independent cell death, direct activation of PARP-1 allows early translocation of AIF to the nucleus, a required step for cell death (Yu et al. 2002). Nuclear AIF causes DNA fragmentation into 50–300 kilobase pair (kbp) pieces, also known as high molecular weight DNA fragmentation, and stage I chromatin condensation (Susin et al. 2000; Joza et al. 2001). Endonuclease G, when in the nucleus, can also cleave chromatin to produce oligonucleosomal DNA fragments independently of caspases (Li et al. 2001). Therefore, AIF and endonuclease G are key molecules executing caspase-independent apoptotic pathway initiated from the mitochondria.

b) Mitochondrial pathway regulators: Bcl-2 family

The control and regulation of the majority of the apoptotic mitochondrial events occur through members of the Bcl-2 family (reviewed in Cory & Adams 2002). To date, a total of 25 genes either with pro- or antiapoptotic functions have been identified in the Bcl-2 family. Antiapoptotic proteins include Bcl-2, Bcl-X_L, Bcl-w, Mcl1, BAG, among others. Proapoptotic members include Bcl-X_S, Bax, Bak, Bid, Bad, Bim, Bik and Blk. These proteins have special significance since they can determine whether the cell commits to apoptosis or aborts the process.

Bcl-2 members contain different Bcl-2 homology (BH) conserved domains that serve to classify them. The antiapoptotic members are multidomain proteins that contain four BH domains (BH1, BH2, BH3 and BH4). Among proapoptotic members, there are multidomain proteins containing three BH domains (BH1, BH2 and BH3); and those called ‘BH3-only’ proteins (Kelekar & Thompson 1998). Noteworthy, the BH3 domains of proapoptotic members are slightly different from those founded in antiapoptotic members (Hunter & Parslow 1996). The BH4 domain, only present in antiapoptotic members, is implicated in the interaction with proteins not belonging to the Bcl-2 family such as Bag-1 and Raf-1 (Zamzami et al. 1998).

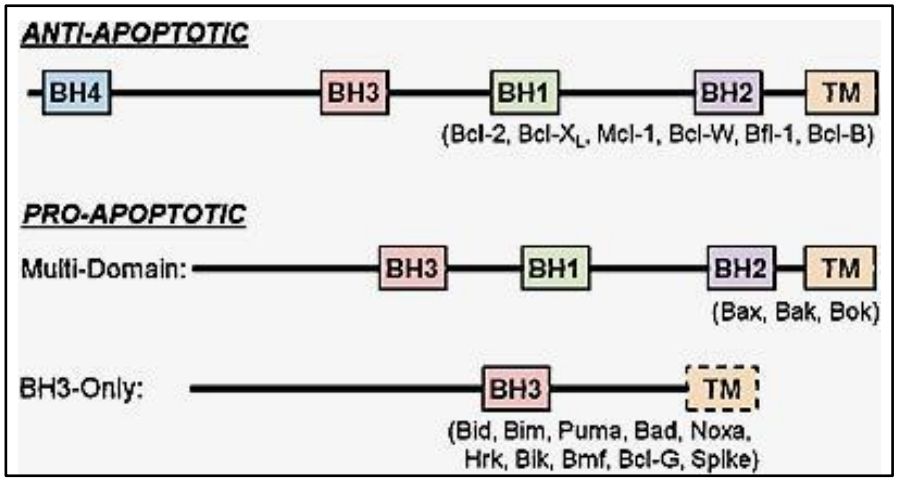

Figure I13. Functional and structural classification of the Bcl-2 family members. Antiapoptotic members are multidomain proteins composed by four BH domains. Proapoptotic members are divided into multi-domain proteins, composed by three different BH domains and BH3-only proteins (Yip & Reed 2008).

There is a complex interaction network among Bcl-2 members to control mitochondria membrane permeabilization. Proapoptotic Bcl-2 members carrying the three BH1, BH2 and BH3 domains can directly induce mitochondria permeabilization. In turn, antiapoptotic members dimerize with these Bcl-2 proapoptotic members to impede their function. In turn, BH3-only proteins can either inhibit antiapoptotic members or directly activate multidomain proapoptotic proteins (Letai et al. 2002; Galonek & Hardwick 2006).

The presence of Bax or Bak is required for cell death (Zong et al. 2001; Cheng et al. 2001). Indeed, when overexpressed, Bax translocates to mitochondria inducing direct caspase-independent cytochrome C release through a mechanism that can be blocked by Bcl-2 overexpression (Rossé et al. 1998). Puma and Noxa are also involved in the Bax-mediated mitochondria permeabilization. During p53-mediated apoptosis, overexpression of Puma increases Bax expression and conformational change, resulting in cytochrome C release and reduction in the mitochondrial membrane potential (Liu et al. 2003). Meanwhile, Noxa localizes to the mitochondria where interacts and inhibits antiapoptotic Bcl-2 family members (Oda et al. 2000). Since both Puma and Noxa are induced by p53, they mediate the apoptosis triggered by genotoxic damage or oncogene activation.

In growth conditions, Bad is phosphorylated and then sequestered by a multifunctional phosphoserine binding molecule called 14-3-3 in the cytosol. Conversely, when unphosphorylated, Bad can exert a proapoptotic function by neutralizing the protective effect of Bcl-X_L and Bcl-2 (Zha et al. 1996; Yang et al. 1997; Kluck et al. 1997; F. Li et al. 1997; Brustugun et al. 1998). Intriguingly, antiapoptotic functions have also been reported for Bad. Yang and co-workers suggest that cell susceptibility to apoptosis is determined by a complex regulation of the interactions of Bad and Bax with Bcl-2 and Bcl-X_L (Yang et al. 1995). Moreover, there are evidences that overexpression of either Bcl-2 or Bcl-X_L downregulates the other, indicating a reciprocal regulation between these two proteins (Elmore 2007).

c) Caspases: a common apoptotic executers

Since their discovery, caspases have been considered key effectors of apoptotic cell death (Ellis & Horvitz 1986; Thornberry et al. 1997; Nicholson & Thornberry 1997; Salvesen & Dixit 1997; Thornberry & Lazebnik 1998; Wolf & Green 1999). Caspases are a family of cysteine-dependent aspartate-directed proteases (c-asp-ases). Caspases contain a conserved QACXG[5] pentapeptide surrounding the cysteine residue in the active site. According to their functions, human caspases can be grouped into three main categories: initiators (including caspases-2, -8, -9 and -10), effectors or executioners (such as caspases-3, -6 and -7) and inflammatory caspases (caspase-1, -4, -5 and 12) (Cohen 1997; Rai et al. 2005; McIlwain et al. 2013). However, other caspases have been described. Caspase-11, for example, is a murine homolog of human caspase-5 (Kang et al. 2002). Interestingly, although caspase-12 is an inflammatory caspase poorly expressed in humans, also exists a shorter variant of caspase-12 that mediates apoptosis under endoplasmic reticulum stress (Nakagawa et al. 2000; Saleh et al. 2004). Caspase-13 is a bovine homolog of human caspase-4 (Koenig et al. 2001) and caspase-14 is a special caspase involved in ontogenesis and skin physiology (Hu et al. 1998; Ahmad et al. 1998; Van de Craen et al. 1998; Lippens et al. 2000). In the last years, other caspases (caspase-15, -16, -17 and -18) have been found in different mammals suggesting that caspase expression may be regulated during

[5] X is Q in caspase-8 and -10, G in caspase-9 and R in the rest of caspases (Cohen 1997).

evolution (Eckhart et al. 2005; Eckhart et al. 2006; Eckhart et al. 2008).

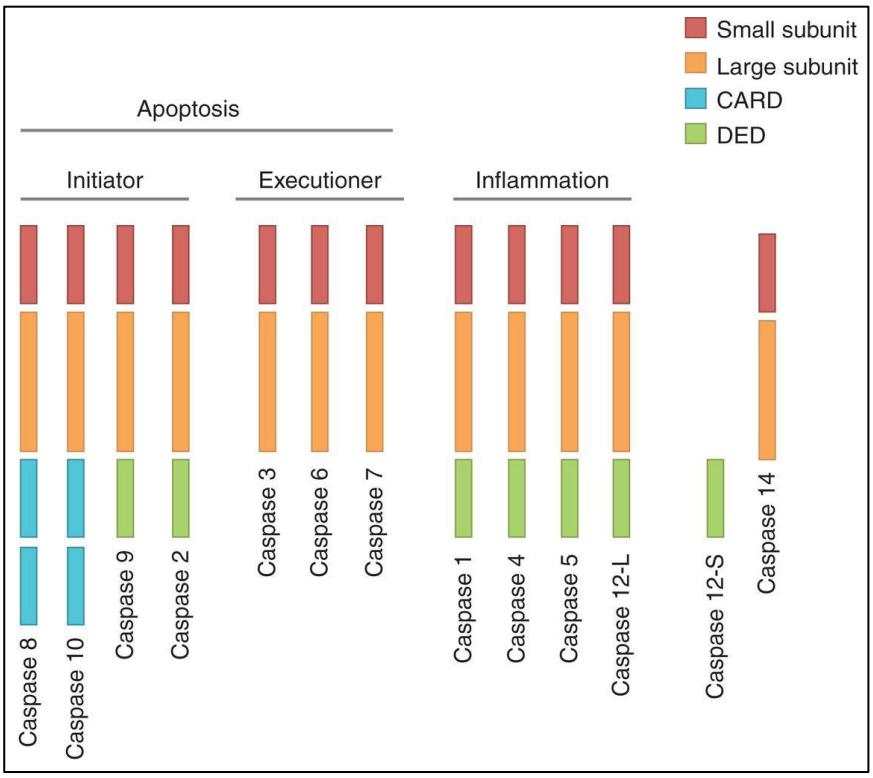

Figure I14. Different domains present in human caspases and its classification. All caspases are formed by small and large subunits. Moreover, caspase-1, -2, -4, -5, -9 and 12 have a death effector domain (DED). Caspase-8 and -10 have a an additional caspase recruitment domain (CARD) (modified from McIlwain et al. 2013).

When synthesized, caspases are inactive proenzymes or zymogens composed by three different subunits: a variable-length amino-terminal prodomain, a large subunit (p20) containing the active site in the middle and a small subunit (p10) at the C-terminus (reviewed in Cohen 1997 and Thornberry & Lazebnik 1998). Initiator caspases exhibit large prodomains (more than 90 amino acids) that include caspase-recruitment domains (CARD) and death effector domains (DED). These motifs allow caspases to interact with death receptors, to join to adaptor molecules or to aggregate and autoactivate (Boldin, Varfolomeev, et al. 1995; Chinnaiyan et al. 1995; Martinon & Tschopp 2007). Executioner caspases, that are directly activated through

proteolysis by initiator caspases, always contain small prodomains (20-30 amino acids) (Cryns & Yuan 1998).

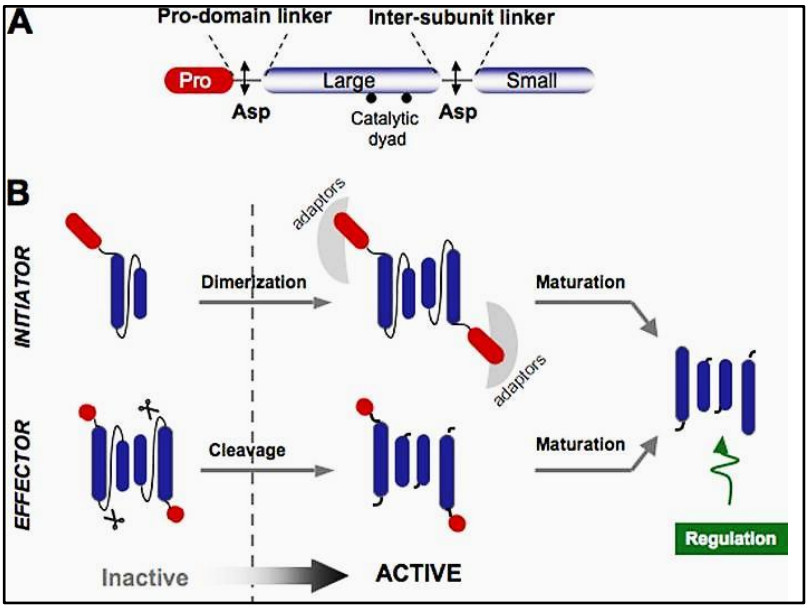

Figure I15. Caspase activation mechanisms. Through adaptor molecules, initiator caspases become recruited and dimerize. Then, autoproteolysis allows the cleavage at the aspartic residues (Asp) which separates large from small subunits and eliminates prodomains. Executioner caspases, which exist as dimers, are activated by initiator caspases through proteolysis at the aspartic residue between large and small subunits. Then, by autocatalytic proteolysis, prodomains are removed (Pop & Salvesen 2009).

Independently of the activation mechanism, the mature caspase is an heterotetramer formed by two p20 and two p10 subunits (Nicholson & Thornberry 1997; Thornberry & Lazebnik 1998). Therefore, to become active, procaspases should dimerize and be sequentially proteolyzed at the internal aspartic residues (reviewed in Shi 2004). The "induced proximity model", accepted for caspase-8 and -9, proposes that initiator caspases exist as monomers prior to apoptosis induction and become active after dimerization. Dimers, structurally similar to mature caspases, harbor strong proteolytic activity leading to interdimer processing. The cleavages at the two aspartic sites of each monomeric initiator caspase stabilize the mature caspase (Muzio et al. 1998; Boatright et al. 2003; Donepudi et al. 2003; Chang et al. 2003). By contrast, the activation of effector caspases is a combination of both trans- and autoactivation (Ramage et al. 1995; Yamin et al. 1996; Martin et al. 1996; Fernandes-Alnemri et al. 1996). Due to the strong

hydrophobic nature of their surface, effector caspases constitutively form dimers (Bose & Clark 2001). However, these precursor dimers are inactive because of structural constrains. The cleavage of the covalent link between large and small subunit by initiator caspases allows the reorganization of catalytic and substrate-biding residues to form proper active sites. This enzymatically competent intermediate undergoes autoproteolytic processing to separate the prodomains from the mature caspase (Chai, Wu, et al. 2001; Riedl, Fuentes-Prior, et al. 2001).

Caspase substrates. Many different cellular proteins have been described as suitable substrates for caspases. Although caspase-mediated processing often results in substrate inactivation, it may also generate new signaling molecules that actively participate in the apoptotic process.

Gelsolin is one of the key substrates of caspase-3. Gelsolin is an actin binding protein that acts as a nucleus for actin polymerization. After caspase-3-mediated cleavage, the resulting fragments from gelsolin cleave actin filaments, hence altering cell division, cytoskeleton, intracellular transport and signal transduction (Kothakota et al. 1997).

Lamins, together with the lamin-associated membrane proteins, are components of the nuclear lamina. A-type lamins are encoded by a unique gen from which four alternative splicing isoforms are generated: lamin A, AΔ10, C and C2. A-type lamins are exclusively cleaved by caspase-6 (Orth et al. 1996; Takahashi et al. 1996). However, the processing of B-type lamins (B1 and B2) remains controversial. While Slee and co-workers demonstrated that lamin B is specifically cleaved by caspase-3 but not by caspase-6 or -7 in cell-free assays (Slee et al. 2001), Broers and collaborators found that lamin B1 transfected into CHO-K1 cells was cleaved by caspase-6 during apoptosis (Broers et al. 2002). Conversely, studies performed in modified chicken DT40 cell line showed that lamin B cleavage was delayed in the absence of caspase-7 (Korfali et al. 2004).

PARP, although a substrate for all executioner caspases, is preferentially cleaved by caspases-3 and -7 (Lazebnik et al. 1994; Takahashi et al. 1996). PARP is a repair enzyme that functions in the DNA damage surveillance network. Despite being a substrate for caspases, PARP-1 has been related to caspase-independent cell death and regulated necrosis (Yu et al. 2002; Moubarak et al. 2007).

Nuclear mitotic apparatus protein (**NuMA**) is specifically degraded at the initial stages of apoptosis. *In vitro*, NuMA can be differentially processed by caspase-3, -4, -6 and -7 (Hirata et al. 1998). Nevertheless, granzyme B can also cleave NuMA independently of caspase activation (Andrade et al. 1998).

α-fodrin or nonerythroid spectrin, is a cytoskeletal protein (reviewed in Bennett & Gilligan 1993). During apoptosis, α-fodrin is processed by caspase-3 and calpains (Harris & Morrow 1990; Harris et al. 1988; Cryns et al. 1996; Jänicke et al. 1998) which has been related to apoptotic membrane blebbing (Martin, O'Brien, et al. 1995).

Other caspase substrates include chaperone p23 (an specific substrate for caspase-7) (Walsh et al. 2008), cytokeratins, DNA repair enzyme DNA-PK, the inhibitor of CAD (ICAD), RB protein, PKCδ, topoisomerases and vimentin (Nicholson & Thornberry 1997; Cohen 1997; Slee et al. 2001). For more information, see the online database CASBASH (reviewed in Lüthi & Martin 2007).

Caspase regulators. The first caspase regulation step is their activation, which means dimerization in the case of apical caspases and proteolysis in the case of the executioners (Boatright et al. 2003; Boatright & Salvesen 2003a; Boatright & Salvesen 2003b). Likewise, once activated, the catalytic activity of caspases can be directly inhibited. Members of the IAP protein family such as XIAP, cIAP1, cIAP2, survivin and NAIP counteract both the activation and the activity of caspases. IAPs are characterized by the presence of one or more baculoviral IAP repeat (BIR) domains, an evolutionarily conserved motif of 70-100 amino acids. cIAP1 and cIAP2 specifically inhibits caspases-3 and -7 through the BIR domains (Roy et al. 1997). Intriguingly, despite possessing three of these domains, NAIP does not inhibit caspase-1, -3, -6, -7 or -8, pointing to a completely different mechanism of action for NAIP (Roy et al. 1997). Caspases-3 and -7 inhibition by XIAP, the most efficient IAP member, occurs after binding of the linker region between BIR1 and BIR2 with the caspase catalytic site (Chai, Shiozaki, et al. 2001; Huang et al. 2001; Riedl, Renatus, et al. 2001). In the case of the apical caspase-9, XIAP can block both its activation and its activity. When the BIR3 domain of XIAP binds to caspase-9, the caspase-9 homodimerization site is overlapped (Shiozaki et al. 2003), which keeps caspase-9 into an inactive monomeric state that prevents its functional assembly (Renatus et al. 2001).

IAPs are regulated by proapoptotic IAP-binding proteins such as Smac/DIABLO and HtrA2/OMI. To counteract with IAP, all IAP-binding proteins share a conserved four-residue IAP-binding motif (IBM) at their N-terminus. Noteworthy, IBM from Smac/DIABLO has been exploited for sensitizing cancer cells to cytotoxic drug treatment (Arnt et al. 2002; Jia et al. 2003; Schimmer et al. 2004).

Moreover, most of IAPs harbor a C-terminal RING domain (reviewed in Salvesen & Duckett 2002; Srinivasula & Ashwell 2008). The RING domain (founded in XIAP, cIAP1 and cIAP2 but not in NAIP) displays ubiquitin ligase activity allowing IAPs to target proteins for proteasomal degradation. Target proteins include caspase-3 and Smac/DIABLO, through which IAPs may contribute to their antiapoptotic function (Suzuki et al. 2001; MacFarlane et al. 2002; Hu & Yang 2003). However, it is not clear to what extent the RING domain contributes to the antiapoptotic nature of IAPs since XIAP, cIAP1 and cIAP2 can result autoubiquitinated and degraded as well (Yang et al. 2000). Intriguingly, XIAP becomes processed during Fas-triggered apoptosis and the resulting fragments possess both anti- and proapoptotic effects (Deveraux et al. 1999).

Relevance of caspases during cell death. Caspases are key biochemical effectors triggering cellular events during apoptosis. Executioner caspases-3 and -6 have major roles in the shrinkage and fragmentation of nuclei. Caspase-3, considered the most important executioner caspase, mediates DNA fragmentation and chromatin condensation through CAD activation. Moreover, caspase-3 is also involved in extranuclear apoptotic events such as the formation of apoptotic bodies or the exposure of phosphatidylserine on the cell surface (Hirata et al. 1998). Indeed, broad-spectrum caspase inhibitors abolished apoptotic biochemical hallmarks. However, the ability of caspase inhibitors to completely block the apoptotic process remains controversial. In many cases, caspase inhibition failed to recover cell growth demonstrating that caspase activation is not the only determinant of life or death decisions (Xiang et al. 1996; Miller et al. 1997; Lavoie et al. 1998; Déas et al. 1998; Trapani et al. 1998; Tainton et al. 2000). Moreover, certain cytoplasmic apoptotic traits may be triggered by enzymes other than caspases (Vanags et al. 1996; McCarthy et al. 1997; Déas et al. 1998; Belmokhtar et al. 2001).

2.2.2. Hallmarks of apoptosis

Biochemically, apoptotic cells are characterized by a reduction in the mitochondrial transmembrane potential, intracellular acidification, production of reactive oxygen species, externalization of phosphatidylserine, selective proteolysis of a subset of cellular proteins, degradation of DNA into internucleosomal fragments and nuclear pyknosis (Wyllie et al. 1984; Hockenbery et al. 1993; Martin, Reutelingsperger, et al. 1995; Lazebnik et al. 1994; Gottlieb et al. 1996; Zamzami et al. 1996). Among all these alterations, nuclear changes regarding chromatin condensation and DNA degradation are considered the hallmarks of apoptosis (Lecoeur 2002).

a) DNA degradation

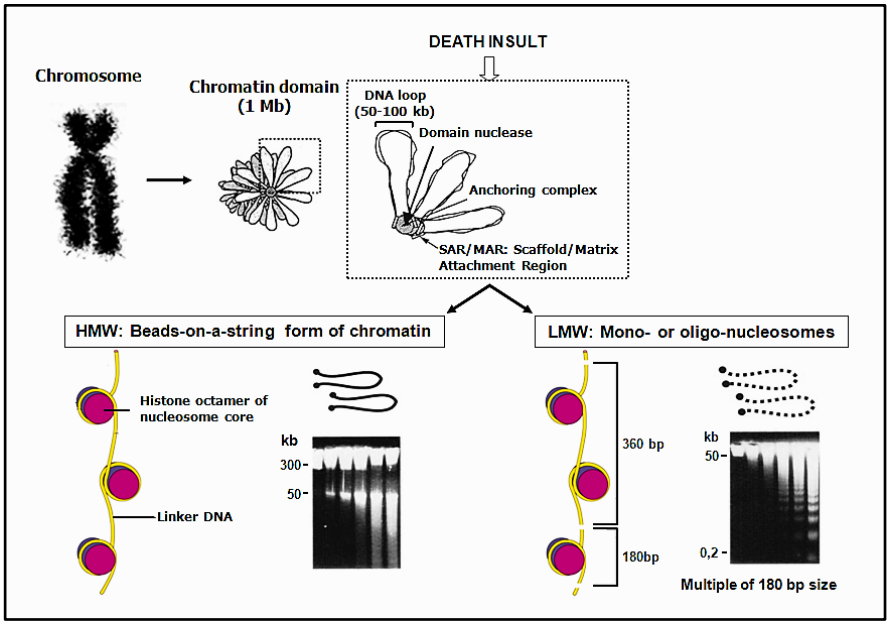

Figure I16. During apoptosis, hydrolysis of DNA takes place in two steps. First, chromatin loops (50 kbp) released from the scaffold/matrix attachment regions (SAR/MAR) in a process that is caspase-independent. Later on, these fragments are hydrolyzed in lower fragments of oligonucleosomal size (Solovyan et al. 2002).

DNA degradation, one of the first biochemical events described during apoptosis (Wyllie 1980), is considered one of the hallmarks of apoptotic cell death (Lecoeur 2002). Genomic DNA cleavage occurs in at least two stages during apoptosis: initial cleavage at intervals of 50 kbp, consistent with the size of chromatin loop domains, followed by a

second stage of internucleosomal DNA cleavage or DNA laddering (Oberhammer et al. 1993). AIF (Susin et al. 1999) and topoisomerase II (Li et al. 1999) have been implicated in the higher-order DNA cleavage reaction. On the other hand, DNA laddering has been associated with DNA fragmentation factor (Liu et al. 1997; Enari et al. 1998; Sakahira et al. 1998; Halenbeck et al. 1998; Liu et al. 1998), endonuclease G (Parrish et al. 2001; Li et al. 2001) and DNase I (Oliveri et al. 2001).

DNA fragmentation factor 40-kDa (DFF40) and endonuclease G (endoG) are considered the two major apoptotic nucleases, but these nucleases possess completely different cellular locations in normal cells and are regulated in vastly different ways.

DFF40/CAD. DNA fragmentation factor 40-kDa (DFF40), caspase-activated nuclease (CPAN) (Halenbeck et al. 1998; Liu et al. 1998) or caspase-activated DNase (CAD) (Enari et al. 1998), is the main endonuclease associated to oligonucleosomal DNA degradation during apoptosis.

CAD belongs to the ββα-Me-finger superfamily of nucleases that share an active site motif (Scholz et al. 2003). CAD is formed by a N-terminal domain called C1 or CIDE-N, a C2 or homodimerization domain and a C-terminal catalytic domain or C3 (Inohara et al. 1999; Sakahira et al. 2001). The analysis of the active site of CAD revealed four completely and two partially conserved histidine residues important for the catalytic endonuclease activity (Meiss et al. 2001). Four of these histidines together with other lysine and tyrosine residues have been proven determinant for the catalytic activity of the endonuclease but irrelevant for the ability of CAD to form oligomers or binding to DNA (Korn et al. 2002).

CAD is an Mg^{2+}-dependent Ca^{2+}-independent endonuclease that is inhibited by Zn^{2+} or Cu^{2+}. CAD activity is maximum at 50-125 mM of K^{+}, which is the range of the cytoplasmic K^{+} concentration for cells undergoing apoptosis (Widlak & Garrard 2001). Intriguingly, Mg^{2+} is required for the catalytic function of CAD but not for the assembly of the endonuclease to DNA (Widlak & Garrard 2001). Indeed, the catalytic activity of the endonuclease is not needed for its binding to DNA, suggesting that activation of CAD can take place in a DNA-bound state (Korn et al. 2005).

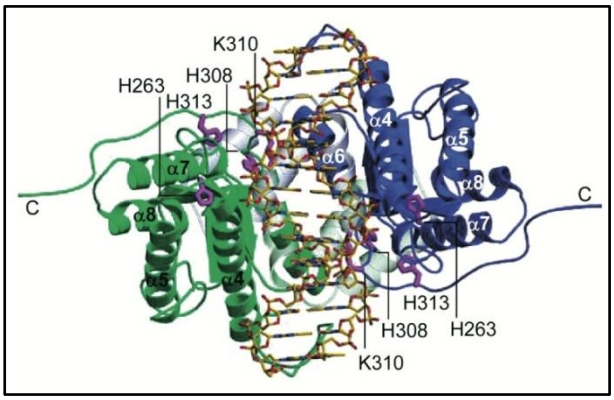

Figure I17 Dimeric structure of functional CAD. The catalytic residues (H263, H308, K310 and H313) are shown. After dimerization, the crevice between the C3 domains allows the interaction with DNA. Of note, the longest helix α4 fits into the major groove of the DNA (Woo et al. 2004).

CAD homodimer exhibits a pair of molecular scissors shape with a deep active site crevice which seems ideal for distinguishing internucleosomal from nucleosomal DNA (Woo et al. 2004). The DNA binding region is a positively charged surface with two functionally distinct parts: the active site that faces the DNA minor groove and the distal helix α4, which together with other close residues, binds to DNA major groove (Reh et al. 2005).

Double-stranded DNA (dsDNA) is the only suitable substrate for CAD (Hanus et al. 2008). Interestingly, all types of oligonucleotides that are not cleaved by the endonuclease, including single-stranded DNA, single- and double-stranded RNA, and RNA-DNA heteroduplexes, inhibit the CAD-mediated cleavage of dsDNA (Hanus et al. 2008).

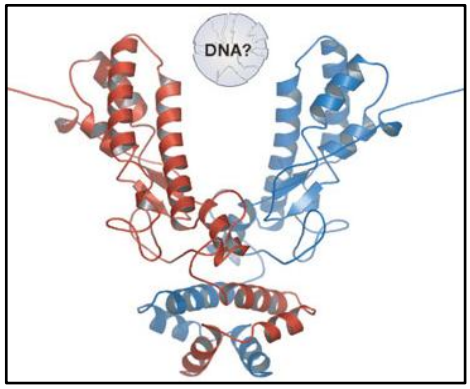

Figure I18. Active CAD has a scissors-like structure. The two monomers are represented in blue and red, and the position of the DNA is indicated (modified from Samejima & Earnshaw 2005).

Inhibitor of CAD (ICAD). In proliferating cells CAD is complexed with its inhibitor, $ICAD_L$ (Enari et al. 1998; Sakahira et al. 1998), also known as DNA fragmentation factor, 45 kDa subunit

(DFF45) (Liu et al. 1997). ICAD harbors two caspase-3-recognition sites at the aspartic residues 117 and 224 (Liu et al. 1997; Sakahira et al. 1998). Based on these proteolytic sites, ICAD can be divided into three different domains: the N-terminal D1 or CIDE, a central D2 and the C-terminal D3 domain. It has been reported a shorter alternative splicing form of ICAD, named $ICAD_S$ or DNA fragmentation factor, 35 kDa subunit (DFF35) (Sakahira et al. 1999). In $ICAD_S$ mRNA, the intron 5 is not removed which results in a truncated protein with a different C-terminus (Sakahira et al. 1999).

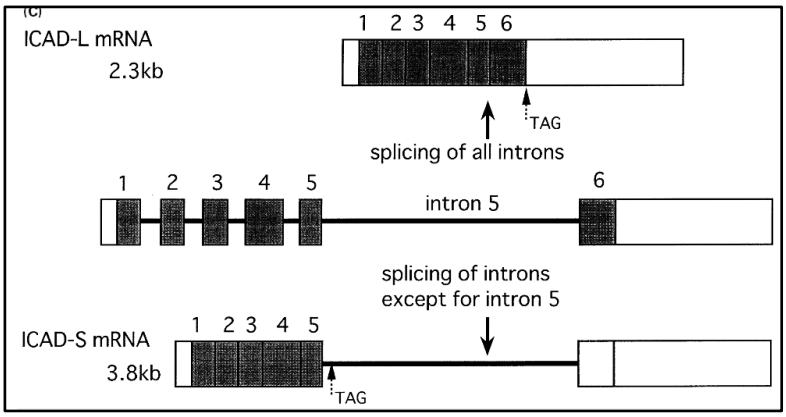

Figure I19. Generation of two isoforms of ICAD, $ICAD_L$ and $ICAD_S$. Boxes represent exons. The black and white areas represent the coding and non-coding regions, respectively. When intron 5 is removed, mRNA generates $ICAD_L$ but when intron 5 remains unspliced, a new stop codon is introduced (black arrow) generating the truncated form $ICAD_S$ (Kawane et al. 1999).

In vivo, DFF40/45 interaction is mediated by binding of three functional domains (D1, D2, and D3) of DFF45 (McCarty et al. 1999a). Although double domain fragments D1-2 and D2-3, as well as full-length DFF45, are more effective inhibitors of CAD, either D1 or D2 domain is capable of inhibiting the endonuclease independently, which means that both long and short isoforms of ICAD are able to inhibit CAD (Sakahira, Enari & Nagata 1999; McCarty et al. 1999a; McCarty et al. 1999b; Ageichik et al. 2007). Moreover, $ICAD_L$ not only sequesters the non-functional CAD monomer but also disassembles the functional homodimer (Woo et al. 2004). Thus, the assembly of DFF40 into the active dimer after caspase-activation is the consequence of both the binding of DFF45 to active caspase-3 instead of to the endonuclease and the cleavage of ICAD from DFF45/40 complex (McCarty et al. 1999a; Woo et al. 2004).

Besides inhibitor, $ICAD_L$ acts a chaperone of CAD preventing aggregation of the endonuclease (Sakahira et al. 1999; McCarty et al. 1999b; McCarty et al. 1999a; Sakahira et al. 2000). Indeed, Sakahira and collaborators demonstrated that $ICAD_L$ not only avoids CAD aggregation, but also refolds unfolded CAD *in vitro* when added at stoichiometric concentrations in a highly reducing environment. The chaperone properties of $ICAD_L$, exclusive for CAD, can be explained by specific recognition and binding to the partially folded CIDE domain from the endonuclease through homodimeric interactions (Sakahira et al. 2000).

Initially, D1 domain of DFF45 was found to be critical for the expression of active DFF40 *in vivo*, suggesting a role for ICAD in binding nascent DFF40 (McCarty et al. 1999b). However, the function of $ICAD_S$ as a chaperone for CAD was reminiscent, being between one and two orders of magnitude less effective than $ICAD_L$ (Sakahira et al. 1999; Scholz et al. 2002). Later on, it was reported the relevance of the C-terminal domain of DFF45 for its chaperone-like activity (Fukushima et al. 2002). The structure of this domain, consisting of four alpha helices arranged in a novel helix, generated a large cluster of negatively charged residues. The chaperone activity of DFF45 depended on the ability of this negative C-terminal cluster to interact with the positively charged catalytic domain of DFF40 through charge complementation (Fukushima et al. 2002). This model provided a reason for the loss of the chaperone activity in DFF35 (Sakahira et al. 1999; Ageichik et al. 2007).

ICAD/CAD location. ICAD/CAD complex was first isolated from the cytoplasm of growing cells (Sakahira et al. 1998). Despite other authors provided evidences of the exclusive location of this complex to the cytosol, (Sabol et al. 1998), soon appeared controversial data pointing nuclear location of ICAD/CAD complex in different cell lines from pork, chicken and human (Samejima & Earnshaw 1998; Samejima & Earnshaw 2000; D. Chen et al. 2000). Since first analysis of ICAD revealed no nuclear localization signal (NLS), it was postulated that should be co-transported with other proteins that do possess NLS (Samejima & Earnshaw 1998). Just two years later, the same scientists reported a sequence rich in basic amino acids at the C-terminus of $ICAD_L$ that could function as independent NLS. Indeed, they observed nuclear $ICAD_L$, while $ICAD_S$ was located diffusely throughout the cell (Samejima & Earnshaw 2000). The same distribution was also observed when ICAD isoforms are complexed

with the endonuclease CAD (Scholz et al. 2002). Interestingly, co-expression of active CAD with human or murine $ICAD_L$ variants lacking the NLS leads to exclusion of CAD from the nuclei in approximately 50% of cells (Scholz et al. 2002).

Few years ago, the initial idea that the nuclear translocation of the complex ICAD/CAD is externally regulated was strengthened by the discovery of the interaction of the C-terminal regions of both CAD and ICAD with the importin α/β-heterodimer. Interestingly, those interactions seem essential for nuclear accumulation of the complex (Neimanis et al. 2007).

Nevertheless, location and regulation of ICAD/CAD complex have resulted cell specific. In thymocytes, for instance, this complex is located in the cytosolic, microsomal and nuclear fractions. After thymocyte stimulation through TCR engagement, CAD from cytosol and microsomes but not from nucleus becomes activated in a caspase-3-dependent manner (Nagata et al. 2002). Interestingly, in rodent brain cells, the predominant isoform of ICAD is the shorter one. Data obtained in neuron-differentiated PC12 using DFF35/40 complexes from rat showed that these heterodimers localize mainly in the nucleus. Indeed, activation of CAD during apoptosis in these cells takes place in the nucleus suggesting that caspases, rather than the ICAD/CAD complex, should translocate to the nucleus in neurons after apoptotic signal (Chen et al. 2000).

CAD activation. After apoptotic stimuli, caspase-3 or -7 cleaves ICAD releasing CAD which in turns leads to oligonucleosomal DNA fragmentation (Liu et al. 1997; Sakahira et al. 1998; Liu et al. 1998; Liu et al. 1999; Yuste et al. 2005). Since CAD shows weaker nuclease activity on naked DNA than chromosome DNA from intact nuclei, additional nuclear factors may be involved in stimulating its activity. To date, several chromatin-associated proteins including histone H1 (Liu et al. 1998; Liu et al. 1999; Widlak et al. 2000), topoisomerase II (Widlak et al. 2000; Durrieu et al. 2000), the high mobility group protein 1 and 2 (HMG-1 and HMG-2) (Liu et al. 1998; Toh et al. 1998; Widlak et al. 2000) and phosphorylated H2AX (Rogakou et al. 2000; Lu et al. 2006) have been described as stimulators of CAD activity. Additionally, CAD activation is negatively regulated by multiple pre- and post-activation steps, that include an appropriate folding during translation and the synthesis of $ICAD_{S/L}$ in stoichiometric excess (Widlak & Garrard 2005).

Interestingly, although CAD has been traditionally related to the generation of blunt-end double-stranded DNA breaks (Widlak et al. 2001), at high ionic strengths CAD induces single-stranded nicks rather than double-stranded breaks (Widlak & Garrard 2001). Moreover, CAD-mediated single-stranded DNA breaks have been recently observed during incomplete apoptosis where oligonucleosomal DNA fragmentation is not detected (Iglesias-Guimarais et al. 2013).

Endonuclease G. The observation that under certain conditions apoptotic stimuli can trigger cell autonomous oligonucleosomal DNA cleavage in CAD-defective mice (Li et al. 2001), led to the discovery of another apoptotic nuclease. Endonuclease G (endoG), that resides in the mitochondrial intermembrane space in normal cells, is released and translocated into the nucleus upon disruption of the outer mitochondrial membrane (Parrish et al. 2001; Li et al. 2001). Then, as CAD, activated endoG can hydrolyze chromatin into fragments of 50 kbp. Moreover, endoG also induces inter- and intranucleosomal DNA cleavages that results in oligonucleosomal DNA fragments (190 bp) with internal single-strand nicks (10 bp periodicity).

EndoG requires either Mg^{2+} or Mn^{2+} but not Ca^{2+} as its divalent cation (Ruiz-Carrillo & Renaud 1987; Li et al. 2001) and is inhibited in the presence of Fe^{2+} or Zn^{2+} (Widlak et al. 2001). Both single-stranded DNA and RNA are preferred substrates over double-stranded DNA. Indeed, at physiological ionic strength, endoG ability to process DNA is 15-fold reduced while cleavage of RNA is not inhibited, which makes endoG a better RNase than DNase (Widlak et al. 2001).

Interestingly, endoG digestion of isolated nuclei did not completely recapitulate the DNA laddering pattern seen during apoptosis in DFF40-knockout cells (Li et al. 2001). Moreover, under certain apoptotic conditions that block CAD activation, DNase I was required for DNA laddering (Oliveri et al. 2001). Altogether, these data suggested that maybe other proteins facilitate endoG activity during apoptosis. In fact, *in vitro* digestion of chromatin substrates with exonuclease III or DNAse I, before addition of endoG at physiological ionic strength, led to more than additive DNA processing (Widlak et al. 2001). Therefore, endoG must participate with DNase I-like enzymes along with exonucleases *in vivo* for apoptotic DNA processing. Nevertheless, in the presence of its nuclear co-activator apoptosis inducing factor (AIF), endoG effectively cleaved duplex

DNA even at physiological salt conditions (Wang et al. 2002; Widlak & Garrard 2005).

Table I1. Comparison of the most relevant characteristics of CAD and endoG (modified from Widlak & Garrard 2005).

	DFF40/CAD	Endonuclease G
Location	Nucleus, cytoplasm	IMS
Inhibitors	DFF45, DFF35	-
Mechanism of activation	Cleavage of specific inhibitor by caspases	Translocation to nucleus
Active form	Dimer/oligomer	Monomer/dimer
Co-activators	H1, HMGB, TopoII	FEN1, AIF
Metal co-factors	Mg, Zn traces	Mg, Mn
pH optimum	Neutral	Neutral
Salt requirements	Optimum at physiological $[K^+]$	Inhibited at physiological $[K^+]$
Substrates	dsDNA	RNA, ssDNA >>dsDNA
Mechanism of DNA cleavage	Blunt-end double strand breaks, 5′-P and 3′OH	Single strand nicks with 5′-P and 3′OH
Sequence preferences	AG/TC blocks with rotational symmetry	5′ of G > C/A residues
Preferential cleavage sites in chromatin	Internucleosomal linker DNA, borders of chromatin loops	Internucleosomal linker DNA, borders of chromatin loops
Intranucleosomal cleavage	None	10 base periodicity

b) Chromatin condensation and nuclear fragmentation.

Nuclear alterations are characteristic traits of apoptosis. Initially, chromatin condenses around the nuclear membrane which is known as stage I chromatin condensation or nuclear morphology. Later, chromatin condenses into highly packed round masses, also known as stage II chromatin condensation. While stage I chromatin condensation is a caspase-independent AIF-dependent event, stage II chromatin condensation requires caspase-mediated activation of CAD (Yuste et al. 2005).

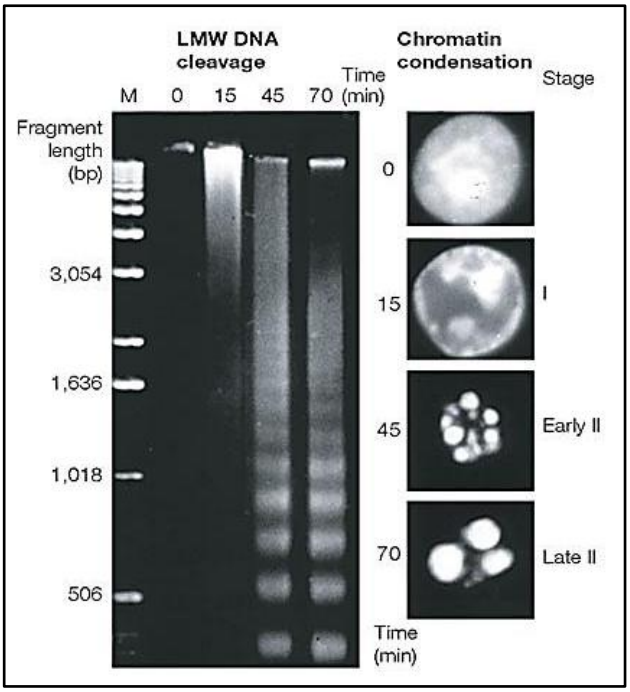

Figure I20. Coordinated chromatin condensation and DNA cleavage during apoptosis. First, chromatin condenses in a ring-like shape below the nuclear envelope. That stage I chromatin condensation coincides with the degradation of DNA into fragments of high molecular weight. Later on, chromatin condenses into smaller masses (early stage II) that finally form the apoptotic nuclear bodies (late stage II). This second step, dependent on CAD activation correlates with the hydrolysis of DNA into oligonucleosomal size fragments (Samejima & Earnshaw 2005).

AIF. Apoptosis inducing factor (AIF) is a mitochondrial intermembrane flavoprotein ubiquitously expressed in mammals (Susin et al. 1999). The AIF precursor, synthesized in the cytosol, is imported to the mitochondria thanks to two mitochondrial localization sequences (MLS). There, the precursor is processed to a mature form through cleavage and binds to a prosthetic group of flavine adenine dinucleotide (FAD). FAD-bound AIF can be reduced by NAD(P)H, state in which AIF can exert its function as a mitochondrial oxydoreductase (Churbanova & Sevrioukova 2008).

Confined to IMS in growing cells, AIF is removed from the IMM during apoptosis by calpain- or cathepsin-mediated cleavage (Polster et al. 2005; Yuste et al. 2005). This truncated form of AIF (tAIF) is then released to the cytosol during transient mitochondrial permeabilization

(Otera et al. 2005). Interestingly, antiapoptotic proteins Bcl-2 and Bcl-X_L are able to avoid AIF processing and tAIF release (Susin et al. 1999; Otera et al. 2005).

From the cytosol, tAIF translocates to the nucleus in a process positively regulated by cyclophilin A (CypA) (Zhu et al. 2007) and inhibited by Hsp70 (Gurbuxani et al. 2003). Once in the nucleus, tAIF binds to DNA in a sequence-independent manner, allowing nuclear alterations characteristic of apoptotic stage I as well as HMW DNA fragmentation (Susin et al. 1999; Ye et al. 2002; Candé et al. 2004). Unexpectedly, redox changes in the active site of the molecule affect both nuclear transport of tAIF and its binding to DNA, meaning that not only normal but also apoptogenic functions of AIF are controlled by NADH (Sevrioukova 2009).

Besides caspase-independent events during apoptosis, AIF is also responsible of morphological changes during necrosis (see chapter 2.3.1. Regulated necrosis).

c) Other characteristics of apoptotic cells

Other characteristics reported during apoptosis include: extensive protein cross-linking (Nemes et al. 1996); cytoskeletal reorganization, which allows disintegration of the cell into apoptotic bodies; expression of surface cellular markers such as annexin I[6] (Arur et al. 2003) and calreticulin (Gardai et al. 2005), that results in the early phagocytic recognition of apoptotic cells; and phospholipid asymmetry with the externalization of phosphatidylserine (Bratton et al. 1997; Ferraro-Peyret et al. 2002; Mandal et al. 2005), which also facilitates the phagocytic uptake of apoptotic cells (Fadok et al. 2001).

2.2.3. Apoptosis: implication in diseases.

In 1972, Kerr and collaborators implied a role for apoptosis in the pathogenesis of cancer (Kerr et al. 1972). Since then, multiple disorders have been attributed, directly or indirectly, to defective cell death. Several diseases including autoimmunity, metabolic disorders and cancer are due to excessive cell accumulation because of cell death blockade. Conversely, atherosclerosis, ischemic injury and

[6] Should not be confused with annexin V, a recombinant phosphatidylserine-binding protein used for the detection of apoptosis (reviewed in van Engeland et al. 1998).

neurodegenerative disorders are the most important diseases associated with extensive cell death (reviewed in Zhivotovsky & Orrenius 2010).

a) Apoptosis in cancer.

That proapoptotic genes can act as tumor suppressors has been largely demonstrated (Fischer et al. 2007; Ho et al. 2008; Krelin et al. 2008; Vakifahmetoglu-Norberg & Zhivotovsky 2010; reviewed in Zhivotovsky & Orrenius 2010). Indeed, invalidation of the cell death program is one of the six essential alterations transforming a normal cell to a tumoral one (Hanahan & Weinberg 2011).

Bcl-2 family. Bcl-2 family includes many proteins with either anti- or proapoptotic functions. Therefore, the balance among them, rather than the overexpression of one particular member of the family, underlies the resistance or sensitivity of tumors to cell death (Zhivotovsky & Orrenius 2010). Several approaches targeting the Bcl-2 family have already been proven in tumoral cells including neutralization of antiapoptotic Bcl-2 proteins by antisense technology (Julien et al. 2000), small molecules that block Bcl-2 interactions with other families (Fesik 2005), or viral-mediated delivery of other proapoptotic members (Naumann et al. 2003).

TP53. Besides its relevance as a metabolic modulator (Zhou et al. 2003; Nutt et al. 2005; Matoba et al. 2006; Bensaad et al. 2006), TP53 regulates tumor development by inhibiting the growth of damaged or stressed cells (Polyak et al. 1997). Under stress conditions, a fraction of cellular p53 migrates to the mitochondria and initiates apoptosis by enhancing ROS formation (Marchenko et al. 2000; Zhao et al. 2005). This early translocation of p53 to mitochondria precedes the later translocation to the nucleus where p53-triggered transcriptional activity takes place. Once in the nucleus, TP53 can activate the transcription of a variety of proapoptotic genes (CD95, Bax, Puma or Noxa among others), as well as genes involved in cell cycle regulation (p21, 14-3-3 σ, GADD45, among others) and autophagy (e.g. Dram, Sestrins) (reviewed in Zhivotovsky & Orrenius 2010).

Due to the elevated ratio of tumors harboring p53 mutations, many attempts to restore the activity of mutant p53 have been described (reviewed in Hainaut & Wiman 2009). PRIMA-1(MET) is a low-molecular weight compound that restores DNA binding activity of mutant p53 to promoters of proapoptotic genes. Interestingly, PRIMA-

1(MET) induces mitochondria-mediated apoptosis that inhibits tumor growth *in vivo* (Shen et al. 2008).

Glycolysis. One of the important characteristics of tumor cells is a predominant glycolytic production of ATP, also under aerobic conditions, known as the "Warburg effect" (reviewed in Zhivotovsky & Orrenius 2009). Indeed, the most glycolytic tumor cells are the most aggressive (Simonnet et al. 2002). In a variety of cancer cells, inhibition of glycolysis with a non-metabolizable glucose analogue, 2-deoxyglucose (2-DG) caused a marked decrease in ATP level, especially in clones where mitochondrial ATP supply was compromised (Halicka et al. 1995). ATP depletion led also to rapid dephosphorylation of Bax which, in turns, leads to OMM permeabilization and subsequent massive cell death (Xu et al. 2005). Combining inhibitors of glycolysis with conventional chemotherapeutic drugs might provide an efficient therapeutic strategy to overcome drug resistance under hypoxic conditions (reviewed in Zhivotovsky & Orrenius 2010). Currently, several glycolytic inhibitors are in preclinical and clinical development.

b) Proapoptotic drugs for GBM

As previously described, a characteristic trait of malignant glioma cell is an intense resistance to death. In addition to those affecting growth and survival, gliomas harbor genetic alterations affecting regulatory and effector molecules involved in apoptotic networks.

BH3 mimetic compounds. The role of the Bcl-2 family in gliomagenesis has been extensively studied. There is a correlation between tumor grade and expression of several antiapoptotic Bcl-2 proteins (Weller et al. 1995; Krajewski et al. 1997). Moreover, during the transition from primary to recurrent GBM, a shift toward an antiapoptotic balance is also observed (Strik et al. 1999). Indeed, NFκB, one of the major antiapoptotic mediators that is overexpressed in gliomas, can directly induce the expression of the antiapoptotic proteins such as IAPs, Bcl-2 and Bcl-X_L (reviewed in Van Meir et al. 2010). Additionally, overexpression of EGFRvIII can also upregulate Bcl-X_L in glioma cells (Nagane et al. 1998).

Besides their classical roles, Bcl-2 family members may also contribute to gliomagenesis through enhancement of migration and invasion by altering the expression of mesenchymal traits and metalloproteinases (Wick et al. 1998; Wick et al. 2001; Wick et al.

2004; Lee et al. 2013). Indeed, several Bcl-2 inhibitors known as BH3 mimetic compounds, due to its resemblance to the proapoptotic BH3-only proteins, have been tested in glioma. ABT-737 is a promising small molecule that binds to the antiapoptotic proteins Bcl-2, Bcl-X_L and Bcl-w, that has been proved effective in prolonging survival of intracranial xenograft GBM model (Tagscherer et al. 2008). Other studies with ABT-737 suggest that this compound may also trigger autophagy by blocking Bcl-2-mediated inhibition of beclin 1 in the endoplasmic reticulum (Maiuri et al. 2007). However, it has been shown that, at least in glioblastoma cells, beclin 1 can act as a proapoptotic effector by blocking Bcl-2 and Bcl-X_L antiapoptotic activity (Huang et al. 2014).

Gossypol is another BH-3 mimetic that inhibits mainly Bcl-2, Bcl-X_L but also Mcl-1. In primary chronic lymphocytic leukemia cells, treatment with gossypol rapidly increased activity of phospholipase A2 (PLA2) leading to an increase in cytoplasmic Ca^{2+}, endoplasmic reticulum stress and upregulation of the BH3-only protein NOXA (Soderquist et al. 2014). Interestingly, gossypol efficiently induced cell death in glioma cells resistant to other BH3 mimetics including HA14-1, BH3I-2' and ABT-737. Likewise, AT-101, a derivative of gossypol used in the clinic, overcame resistance to other Bcl-2 inhibitors in patients. Furthermore, gossypol potentiated cell death induced by temozolomide in MGMT-negative cells and, to a lesser extent, in MGMT-expressing cells (Voss et al. 2010). Interestingly, gossypol-triggered cell death resulted dependent on the autophagic proteins beclin 1 and atg5. Indeed, it has recently been reported that gossypol also enhances radiation-induced phagocytosis in glioblastoma cells (Keshmiri-Neghab et al. 2014). Data from a clinical trial in 1999 indicated that gossypol was well tolerated and had a low, but measurable, response rate in a heavily pretreated, poor-prognosis group of patients with recurrent glioma (Bushunow et al. 1999).

Death ligands. Several evidences support important roles of death receptors in glioma pathogenesis (Weller et al. 1994; Roth et al. 1997; Shinoura et al. 1998; Nagane et al. 2000; Röhn et al. 2001; Maleniak et al. 2001). For instance, the decoy receptor for CD95 ligand (CD95L), soluble decoy receptor 3 (DcR3), is expressed on malignant glioma cell lines correlating with the grade of malignancy (Roth et al. 2001).

Lately, the TRAIL death receptor system has gained considerable interest. Highly malignant tumors express higher amounts of TRAIL

receptors in comparison with less malignant tumors or normal tissue. Moreover, TRAIL expression has been positively correlated with survival of patients with primary GBM (Kuijlen et al. 2006). Loco-regional administration of TRAIL inhibited growth of human glioma cell xenografts (Roth et al. 1999) and acted synergistically with chemotherapeutic drugs (Nagane et al. 2000; Röhn et al. 2001; Arizono et al. 2003; Song et al. 2003).

The expression levels of agonist and antagonistic death receptors does not always correlate with susceptibility of glioma cells to death ligand-induced apoptosis (Knight et al. 2001; Hetschko et al. 2008). In many cases, inactivation of TRAIL-induced apoptosis occurs downstream of receptor activation (LeBlanc et al. 2002; Murphy et al. 2014). Peptides derived from Smac/DIABLO, a potent IAPs inhibitor, acted synergistically with TRAIL to induce tumor cell apoptosis both *in vitro* and *in vivo* without demonstrable neurotoxicity (Fulda et al. 2002). Likewise, the Bcl-2 and Bcl-X_L inhibitor, ABT-737, as well as the Akt-mTOR inhibitor rapamycin have shown synergistic effects when administered with TRAIL in GBM (Panner et al. 2006; Tagscherer et al. 2008; Cristofanon & Fulda 2012). Of particular interest for GBM is the combination of TRAIL with the proteasome inhibitor bortezomib (Brooks et al. 2005; Mani & Gelmann 2005). Notably, bortezomib sensitizes to treatment primary TRAIL-resistant GBM cells (Koschny et al. 2007). More importantly, although bortezomib induces growth arrest in both normal and cancer cells, normal cells restart division after treatment whereas cancer cells die by apoptosis (Fernández et al. 2005).

2.3. Necrosis: regulated and not regulated cell death

Necrosis has been described as a consequence of extreme physicochemical stress, such as heat, osmotic shock, mechanical stress, or freeze-thawing, which kills cells quickly and directly. Therefore, necrotic cell death has been traditionally considered an uncontrolled and accidental process.

Regardless of the primary insult, during necrosis, ATP levels, or more critically ATP/ADT ratio, drop to almost zero in seconds. That leads to failure of ATP-dependent ion channels and pumps that initiates a massive cell volume increase through Na^+ influx (Barros et al. 2001). The high intracellular Na^+ concentration results in the activation of the (Na^+/K^+)-ATPase, which further decreases cellular ATP levels. ATP depletion leads to the opening of non-selective Ca^{2+} channels, resulting in elevated intracellular Ca^{2+} levels and activation of the (Ca^{2+})-ATPase with eventual mitochondrial depolarization (Barros et al. 2001; Padanilam 2003). In addition, with severe ATP depletion, ionic homeostasis is no longer maintained by K^+ efflux, leading to further Na^+ and water influx that precipitate cellular swelling and collapse (reviewed in Barros et al. 2001; Padanilam 2003).

Morphologically, necrosis is characterized by volume increase with cell rounding and swelling; disrupted organelle membranes; formation of cytoplasmic vacuoles and blebs; distended endoplasmic reticulum; condensed, swollen or ruptured mitochondria; disaggregation and detachment of ribosomes; swollen and ruptured lysosomes and eventually bursting of the cytoplasmic membrane (Laster et al. 1988; Kerr et al. 1972; Majno & Joris 1995; Trump et al. 1997). However, despite cellular collapse, during necrosis nuclei remain largely intact (Krysko, Vanden Berghe, D'Herde, et al. 2008; Krysko, Vanden Berghe, Parthoens, et al. 2008; Vanden Berghe et al. 2010).

Loss of membrane integrity and release of intracellular content grant necrotic cells the ability to induce an inflammatory response. The immunogenic endogenous molecules, collectively termed damage-associated molecular patterns (DAMPs), include HMGB1, IL-1a, uric acid, DNA fragments, and mitochondrial content among others (Eigenbrod et al. 2008; Kono et al. 2010; Krysko, Vanden Berghe, D'Herde, et al. 2008; Sauter et al. 2000). Interestingly, several DAMPs stimulate pattern-recognition receptors (PRRs), that are initial sensors of infection that coordinate the inflammatory response mediated by

pathogen-associated molecular patterns (PAMPs), which highlight the similarities between pathogen-induced and necrosis-triggered inflammatory responses (Kaczmarek et al. 2013).

2.3.1. Regulated necrosis

For a long time, necrosis has been considered as a merely accidental cell death. Nevertheless, in the late 1980s, several studies described a TNF-induced necrosis (Laster et al. 1988; Grooten et al. 1993) negatively regulated by caspases (Vercammen, Beyaert, et al. 1998). Since then, several authors have suggested that necrosis could be partially regulated (Colbourne et al. 1999; Barkla & Gibson 1999; Chautan et al. 1999; Holler et al. 2000; Chan et al. 2003; Vanden Berghe et al. 2004; Moubarak et al. 2007).

The definition of regulated necrosis remains primarily descriptive. Regulated necrosis is characterized by partial/stage I nuclear chromatin condensation, absence of dilation of intracellular organelles and lack of caspase activation. From a biochemical point of view, it is not possible to distinguish a programmed necrotic process by the activation of a specific protein because the proteins implicated are also involved in other cell death programs. The best method to recognize programmed necrosis is the analysis by flow cytometry of cellular integrity and phosphatidylserine exposure. When externally exposed, phosphatidylserine binds to annexin V. Moreover, when membrane integrity is lost, injured cells stains with propidium iodide (PI). During apoptosis, the delay between annexin V staining and PI-labeling allows the initial detection of annexin V-positive/PI-negative cell population before the detection of double positive cells (Boujrad et al. 2007). In contrast, in programmed necrosis, there is a very short delay between phosphatidylserine exposure and plasma membrane breakdown meaning that only a double-positive annexin V/PI cell labeling is normally detected (Boujrad et al. 2007; Moubarak et al. 2007; Artus et al. 2010). Strikingly, cells undergoing programmed necrosis can be efficiently phagocyted, suggesting that the annexin V-labeling is not due to the passive diffusion of the dye (Baritaud et al. 2010).

a) Effectors of regulated necrosis

The first molecular characterization of necrotic PCD was done after induction of DNA damage with high doses of alkylating agents, such as N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (MNU) (Yu et al. 2002; Alano et al. 2004; Wsierska-

Gadek et al. 2003; Moubarak et al. 2007). The most relevant proteins related to regulated necrosis are explained below.

PARP-1. Poly(ADP-ribose) polymerases (PARP) are enzymes catalyzing the production of poly(ADP-ribose) or PAR polymers. After DNA damage, the activation of PARP-1 results in poly(ADP-ribosyl)ation of key DNA-repair proteins. This enzymatic reaction is realized at the expense of NAD^+ that is cleaved into ADP-ribose and nicotinamide (reviewed in Shall & de Murcia 2000 and Haince et al. 2005). Then, NAD^+ levels are restored by recycling nicotinamide with two ATP molecules. However, extensive DNA-damage depletes NAD^+ and ATP, driving the cell to necrosis (Yu et al. 2002; Alano et al. 2004; Kang et al. 2004; Yu et al. 2006).

Calpains. The calpain family comprises a heterogeneous group of cysteine proteases with a large expression pattern. The most studied calpains are the μ-calpain (calpain I) and m-calpain (calpain II). Both μ- and m-calpains are heterodimeric enzymes sharing a common small regulatory subunit protein encoded by the *capn4* gene. Besides its implication in caspase-dependent (Nath et al. 1996; Toyota et al. 2003; Cao et al. 2003; Cartron et al. 2004) and caspase-independent (Artus et al. 2006; Sanges et al. 2006; Cao et al. 2007) apoptosis, calpains are a key component of regulated necrosis (Moubarak et al. 2007). Calpains are needed to process AIF which, after Bax-mediated mitochondrial permeabilization, is released to the cytosol and transported into the nucleus (Moubarak et al. 2007).

RIP1/RIP3. In 2005, Degterev and collaborators coined the term necroptosis (Degterev et al. 2005). Necroptosis can be triggered by members of the TNF family (through TNFR1, TNFR2, TRAILR1, and TRAILR2), Fas ligand, toll-like receptors, lipopolysaccharides and genotoxic stress (Laster et al. 1988; Vercammen et al. 1997; Vercammen, Brouckaert, et al. 1998; Holler et al. 2000; Matsumura et al. 2000; Moriwaki, Chan et al. 2003). Likewise, different stimuli inducing physical-chemical stress can initiate necroptosis, including anticancer drugs, ionizing radiation, photodynamic therapy, glutamate, and calcium overload (reviewed in Fulda 2013).

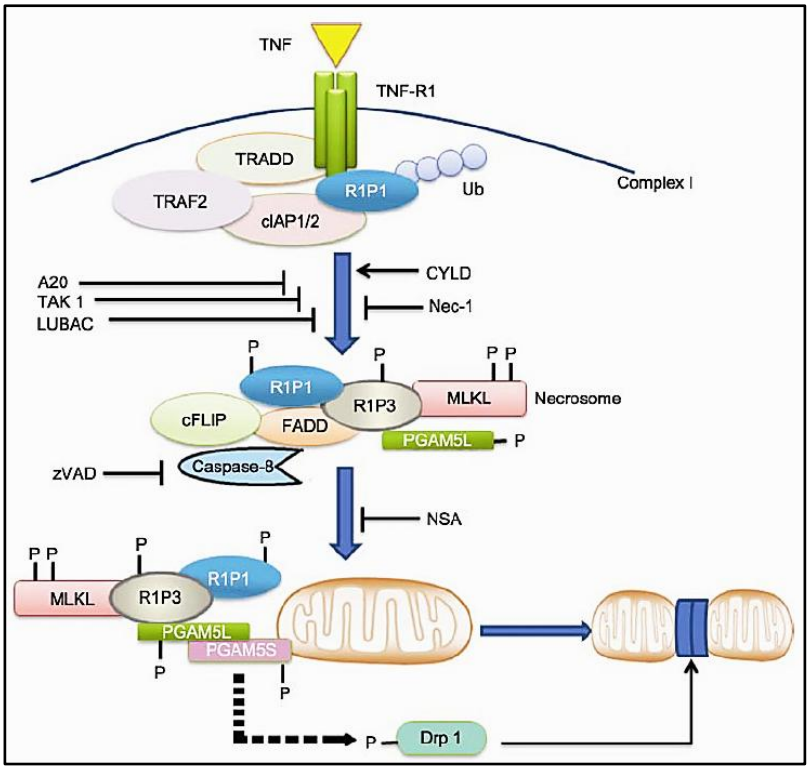

Figure I21. TNF-induced programmed necrosis pathway. After ligand binding, TNFR1 recruits TRADD, RIP1, TRAF2 and IAP1/2. When RIP is ubiquitinated, a prosurvival signal is generated through complex I. However, when CYLD deubiquitinates RIP1, necrosome is formed by the recruitment of caspase-8, FADD and RIP3. Autophosphorylation of RIP1 and RIP3, allows the recruitment of MLKL that, after phosphorylation, activates the necrosome. Finally, through interaction with PGAM5, necrosome activates Drp1, thus promoting mitochondrial fragmentation and necrosis (Zhou et al. 2012).

After TNFα-TNFR1 interaction, TNFR1 recruits TRADD, RIP1 and TNFR-associated factor 2 (TRAF2) (Harper et al. 2003; Micheau & Tschopp 2003). TRAF2 binding to IAP1 and IAP2 allows the recruitment of the linear ubiquitin chain assembly complex (LUBAC). If RIP1 remains ubiquitanated, the resultant complex I promotes cell survival but, after deubiquitination of RIP1 by cylindromatosis (CYLD) (Wright et al. 2007), complex I is internalized to endosomes where TNFR1 is released (Kaczmarek et al. 2013). Then, RIP1 recruits FADD, caspase-8 and RIP3 (Zhang et al. 2009; He et al. 2009) leading to necrosome formation (Holler et al. 2000; Cho et al. 2009). Finally, RIP3 mediates the recruitment of mixed lineage kinase like protein (MLKL) and the mitochondrial phosphatase 5 (PGAM5), key players

for necroptosis execution (Sun et al. 2012; Zhao et al. 2012; Murphy et al. 2013; Cai et al. 2014). Indeed, RIP3-mediated MLKL phosphorylation is essential for necrosome activation and cell death. More interestingly, both long and short isoforms of PGAM5 are required not only for cell death but also for ROS production during intrinsic necroptosis. Indeed, PGAM5-mediated activation of the mitochondrial fission factor Drp1 is a crucial event promoting mitochondrial fragmentation needed for necrosis execution (Wang et al. 2012).

b) Necrotic regulators

Histone H2AX. H2AX is a member of the histone H2A family. Besides its contribution to DNA packaging and genomic stability, H2AX is involved in DNA damage repair (Redon et al. 2002). Ser_{139}-phosphorylated H2AX (γH2AX) accumulates at sites of DNA double-strand breaks (Rogakou et al. 1998) where restructures chromatin and assists in the recruitment of DNA repair and signaling factors (Celeste et al. 2003; reviewed in Pilch et al. 2003 and Fernandez-Capetillo et al. 2003). Indeed, after MNNG-induced DNA damage, γH2AX prevented both nuclear alterations and cell death through direct interaction with tAIF (Artus et al. 2010; Baritaud et al. 2010). This inhibitory interaction, occurring also during apoptosis (Lu et al. 2006), demonstrates that chromatinolysis promoted by AIF is commonly regulated by γH2AX during both apoptosis and necrosis.

Flip. Flip molecules have been originally described as regulators of caspase-8-mediated apoptosis (Irmler et al. 1997; Giampietri et al. 2008; Quintavalle et al. 2010). Nevertheless, the role of Flip is quite complex. In the absence of Flip, procaspase-8 can homodimer within the ripoptosome (RIP1-FADD-caspase-8) leading to caspase-8 activation and apoptosis. However, either long ($Flip_L$) and short ($Flip_S$) isoforms can form heterodimers with caspase-8 thus inhibiting apoptosis. Moreover, caspase-8/$Flip_L$ heterodimers induce RIP cleavage which results in both apoptosis and necroptosis inhibition. Conversely, since caspase-8/$Flip_S$ heterodimers lack proteolytic activity necessary for RIP1 degradation, the presence of $Flip_S$ promotes RIP3-mediated necroptosis (Oberst et al. 2011; Feoktistova et al. 2011).

IAP. As aforementioned, IAPs are well known caspases regulators (see chapter 2.2.1. Apoptotic biochemical pathways: caspases). IAP1 and IAP2 can inhibit caspase-8 activation thus favoring necrosome

formation and necroptosis (Wang et al. 2008; Tenev et al. 2011; Feoktistova et al. 2011; He et al. 2014). However, IAP proteins are also able to block necroptosis by inducing proteasomal degradation of RIP1. Moreover, when IAPs are absent or inhibited, RIP1 kinase activity leads to ripoptosome formation (Tenev et al. 2011; Feoktistova et al. 2011) resulting in an apoptotic cell death that can be inhibited by necrostatin-1.

c) Physiopathological implications of regulated necrosis

The most studied form of regulated necrosis is necroptosis. Necroptosis occurs physiologically during development as well as in adult life (Roach & Clarke 2000) and plays a key role in the regulation of T cell proliferation and survival (Zhang et al. 1998; Salmena et al. 2003; Kang et al. 2004; Kaiser et al. 2011; Ch'en et al. 2011). One suggested model states that, in physiological conditions, active caspase-8 abolishes T cell necroptosis, whereas, in pathological conditions such as viral infection, inactivation of caspase-8 allows T cells death via necroptosis (Martinon et al. 2000; Ma et al. 2005; Feng et al. 2007; Upton et al. 2010; Ch'en et al. 2011).

Necroptosis has been associated with different pathological conditions such as ischemia in brain (Degterev et al. 2005; Northington et al. 2011) and myocardial tissues (Smith et al. 2007; Oerlemans et al. 2012), infections (Cho et al. 2009; Upton et al. 2010), neurodegenerative diseases, pancreatitis (He et al. 2009) or photoreceptor cell loss (Trichonas et al. 2010). Moreover, increasing evidences point that necroptosis could be impaired during oncogenesis (Alameda et al. 2010; Liu et al. 2012). Since necroptosis represent an alternative form of cell death when caspase activation is blocked, necroptosis may be an important therapeutic strategy to target tumor cells, particularly those resistant to apoptosis (Chautan et al. 1999; Han et al. 2007; Bonnet et al. 2011; Jiang et al. 2011; Wu et al. 2013).

II. OBJECTIVES

Both apoptosis and necrosis are observed in glioma. Although these two kinds of cell death can be independently regulated, it is unclear whether in glioblastoma apoptosis and necrosis can develop in parallel or influence each other. Interestingly, previous studies made in our laboratory pointed that glioblastoma-derived U87-MG cells do not display apoptotic morphology after apoptotic insult. Therefore, the main objective of this work was to study the cell death signaling pathways in glioblastoma multiforme cells. The project had three parts.

First, to analyze the apoptotic signaling pathways in human glioblastoma multiforme-derived LN-18 cells. That meant:

- To determine whether apoptotic insults can trigger cell death in LN-18 cells.
- To characterize the biochemical and morphological changes in these glioblastoma-derived cells during cell death.
- To discern the molecular clues impeding LN-18 cells to undergo classical apoptosis.

Second, to extend the results obtained in the first part to other glioblastoma cells, including primary cells. Here, the objectives were:

- To study whether the behavior of LN-18 cells is also observed in other glioblastoma cells, including primary samples.
- To ultrastructurally analyze the morphological nuclear changes of apoptotic glioblastoma cells and to define whether they correspond to apoptotic or necrotic alterations.
- If nuclear alterations do not clearly correspond to a complete apoptotic or necrotic process, study the mechanisms involved in this particular kind of cell death.

Third, if possible, to manipulate the behavior of glioblastoma cells to reach a complete apoptotic cell death phenotype. In this part, the ideas were:

- To induce a pre-apoptotic phenotype allowing glioblastoma cells to undergo complete apoptosis.
- To achieve a complete apoptotic cell death by combining different apoptotic inductors and inhibitors.
- To determine the major impairments of glioblastoma cells to undergo canonical apoptosis.

III. RESULTS

1. DFF40/CAD is necessary and sufficient for oligonucleosomal DNA breakdown, but not for chromatin disassembly during caspase-dependent apoptosis of LN-18 glioblastoma cells.

Sánchez-Osuna M, Garcia-Belinchón M, Iglesias-Guimarais V, Gil-Guinon E, Casanelles E, Yuste VJ. Caspase-activated DNase Is Necessary and Sufficient for Oligonucleosomal DNA Breakdown, but Not for Chromatin Disassembly during Caspase-dependent Apoptosis of LN-18 Glioblastoma Cells. J. Biol. Chem. 2014 Jul 4;289(27):18752-18769.

Sánchez-Osuna M, Garcia-Belinchón M, Iglesias-Guimarais V, Gil-Guiñón E, Casanelles E, Yuste VJ. Caspase-activated DNase is necessary and sufficient for oligonucleosomal DNA breakdown, but not for chromatin disassembly during caspase-dependent apoptosis of LN-18 glioblastoma cells. J Biol Chem. 2014 Jul 4;289(27):18752-69. doi: 10.1074/jbc.M114.550020

2. Absence of oligonucleosomal DNA fragmentation after caspase-dependent cell death is a common signature of glioblastoma-derived multiforme cells due to improper activation of DFF40/CAD endonuclease.

María Sánchez-Osuna, Fina Martínez-Soler, Mercè Garcia-Belinchón, Sònia Pascual-Guiral, Victoria Iglesias-Guimarais, Elisenda Casanelles, Gerard Plans, Jordi Bruna, Avelina Tortosa, and Victor J. Yuste. **Absence of oligonucleosomal DNA fragmentation after caspase-dependent cell death is a common signature of glioblastoma-derived multiforme cells due to improper activation of DFF40/CAD endonuclease.** *Manuscript in preparation.*

Absence of oligonucleosomal DNA fragmentation after caspase-dependent cell death is a common signature of glioblastoma-derived multiforme cells due to improper activation of DFF40/CAD endonuclease.

María Sánchez-Osuna[1, 2], Fina Martínez-Soler.[3], Mercè Garcia-Belinchón[1, 2], Sònia Pascual-Guiral[1], Victoria Iglesias-Guimarais[1, 2], Elisenda Casanelles[1, 2], Gerard Plans [4], Jordi Bruna [4], Avelina Tortosa [3], and Victor J. Yuste[1, 2].

Authors Affiliations

1 Cell Death, Senescence and Survival group, Departament de Bioquimica i Biologia Molecular & Institut de Neurociencies, Facultat de Medicina, Universitat Autonoma de Barcelona, Barcelona, Spain.

2 Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Spain.

3 Department of Basic Nursing, Institut d'Investigació Biomèdica de Bellvitge-Universitat de Barcelona, Barcelona, Spain;

4 Unit of Neuro-Oncology, Services of Neurology and Neurosurgery, Hospital Universitari de Bellvitge, Barcelona, Spain.

Running title: *Inherent resistance of glioblastoma cells to apoptosis.*

Keywords: apoptosis, DFF40/CAD, oligonucleosomal DNA fragmentation, glioblastoma.

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Corresponding Author

To whom correspondence should be addressed: Victor J. Yuste, Departament de Bioquímica i Biologia Molecular, Facultat de Medicina, Universitat Autònoma de Barcelona, Campus de Bellaterra, 08193 Bellaterra, Barcelona, Spain. Tel.: (34) 93 581 3762; Fax: (34) 93 581 1573. E-mail: victor.yuste@uab.cat

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Abstract

Glioblastoma multiforme is one of the most aggressive and lethal human cancers. Although many alterations affecting apoptotic pathways have been described in glioblastoma cells, the ultimate reason underlying the necrotic phenotype of this tumor remains unknown. Here, we present a morphological and biochemical analysis of different glioblastoma cells, including primary samples, treated with staurosporine, a strong apoptotic inductor. Glioblastoma cells display high heterogeneity in terms of caspase activation and nuclear alterations during apoptosis. Nevertheless, all glioblastoma cells are unable to degrade DNA into nucleosome-size pieces, one of the hallmarks of caspase-dependent apoptosis. Interestingly, glioblastoma cells show limited levels of the main apoptotic endonuclease DFF40/CAD in the cytosol, the subcellular compartment in which caspase activation takes place. The sole transfection of the endonuclease was sufficient to induce oligonucleosomal DNA degradation in apoptotic glioblastoma cells which points to the activation of the endonuclease as the key final bottleneck regulating this apoptotic hallmark. Intriguingly, staurosporine-mediated nuclear changes are not affected by higher levels of the endonuclease, indicating differential regulation for these two traditionally linked apoptotic hallmarks. Moreover, nuclear alterations in injured glioblastoma cells include the release of the genomic content to the extracellular media. In summary, glioblastoma cells display an incomplete apoptosis characterized by the absence of apoptotic DNA degradation which may play a role in the high resistance of these tumors.

Introduction

Glioblastoma multiforme (GBM) is the most frequent and aggressive tumor that arises from neuroepithelial tissue. Glioblastoma is characterized by microvascular hyperplasia and necrosis, traits that pathologically distinguish it from lower grade tumors (Rong et al. 2006). During necrosis, the rupture of the plasma membrane induces the release of intracellular content to the external media. Therefore, the damage of surrounding cells and tissues together with the consequent localized inflammatory responses are unavoidable consequences

associated to necrosis (Kroemer et al. 1998). Conversely, apoptotic cell death is a tightly regulated process often disabled in glioblastoma cells. Apoptosis is characterized by a specific biochemical cascade, during which executioner caspases, including caspase-3, caspase-6 and caspase-7, become activated. Caspase activation leads to the hydrolysis of the internucleosomal DNA together with very specific nuclear changes including chromatin condensation and nuclear fragmentation (Lecoeur 2002; Timmer & Salvesen 2007; Dix et al. 2008). The main players involved in these alterations are the endonuclease DFF40/CAD (Liu et al. 1998; Enari et al. 1998; Halenbeck et al. 1998) and its inhibitor, ICAD (Liu et al. 1997; Sakahira et al. 1998), that resulted processed after the activation of caspase-3 (Liu et al. 1997; Sakahira et al. 1998; Liu et al. 1998). Although cells can be eliminated by different mechanisms including necrosis, the disposition of "eat-me" signals on the surface allows the rapid engulfment and removal of apoptotic cell bodies by phagocytic cells without eliciting surrounding tissue inflammation (Henson et al. 2001).

Here, we analyze the outcomes of different human glioblastoma cells (six well-established cell lines and primary cells obtained from five patient samples) after challenging with a strong apoptotic insult such as staurosporine. None of the glioblastoma cells used in this study display oligonucleosomal DNA degradation after staurosporine treatment even in those cells in which caspases are correctly activated. The absence of DNA laddering seems to be caused by low levels of cytoplasmic DFF40/CAD rather than improper caspase-3 activation or ICAD processing since the sole transfection of the endonuclease is sufficient to enable DNA laddering in staurosporine-treated glioblastoma cells. Intriguingly, we show that apoptotic nuclear morphology, very heterogeneous among apoptotic glioblastoma cells, is not affected by the endonuclease CAD. Indeed, data obtained through electron microscopy points to others players, maybe those controlling the anchorage of chromatin to the inner membrane of the nuclear envelope, as key regulators of apoptotic nuclear alterations in glioblastoma. Interestingly, ultrastructural analysis demonstrated that injured glioblastoma cells release their genomic content to the extracellular media which, together with the absence of DNA degradation, may explain the extreme aggressiveness of these tumors.

Material and Methods

Reagents - All chemicals were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise indicated. The pan-caspase inhibitor q-VD-OPh was from MP Biomedicals Europe (Illkirch, France). Antibodies against caspase-3 (9662; 1:2,000) and caspase-7 and cleaved poly(ADP-ribose) polymerase (PARP) (9542; 1:5,000) (9492; 1:2,000) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against DFF40/CAD (AB16926, 1:500) and α-Fodrin (clone AA6) (MAB1622; 1:40,000) were obtained from Millipore Iberica S.A.U. (Madrid, Spain). Antibodies against caspase-6 (clone 3E8) (M070-3; 1:2,000) and DFF45/ICAD (clone 6B8) (M037-3; 1:40,000) were from MBL (Naka-ku Nagoya, Japan). Anti-PKM2 antibody (H00005315-A01; 1:1,000) was from Abnova (Jhongli, Taiwan). Anti-Lamin A/C (clone JOL2) (Ab40567; 1:2,000) was from Abcam (Cambridge, UK). Anti-p23 antibody (clone JJ3) (NB300-576; 1:10,000) was obtained from Novus Biological Europe, Inc (Cambridge, UK). Horseradish peroxidase-conjugated secondary antibodies against mouse IgG (A9044; 1:20,000) or rabbit IgG (A0545; 1:20,000) were purchased from Sigma.

Cell Lines and Culture Procedures –Human glioblastoma-derived cells A172, LN-18, LN-229, T98G, U251-MG, and U87-MG, as well as human neuroblastoma-derived SH-SY5Y cells were routinely grown in 100-mm culture dishes (BD Falcon, Madrid, Spain) containing 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively) and 10% of heat-inactivated fetal bovine serum (FBS) (Invitrogen S.A, Barcelona, Spain). Cells were maintained at 37°C in a saturating humidity atmosphere containing 95% air and 5% CO2. Primary glioblastoma cells named as #04, #30, #35, #45 and #52, were obtained as previously described by Tortosa's lab (Villalonga-Planells et al. 2011). All experiments were performed before passage 20.

Chromatin Staining with Hoechst 33258 and Trypan Blue Exclusion Assays - Nuclear morphology staining with Hoechst 33258 and Trypan blue assays were performed as previously established in our laboratory (Gozzelino et al. 2008). Stained nuclei were scored as stage I (peripheral chromatin condensation), stage II (more than two different masses of condensed chromatin), stage III (condensed condensation without signs of karyorrhexis) nuclear morphology or non-condensed.

Low Molecular Weight (LMW) or Oligonucleosomal DNA Degradation Analysis - LMW analysis was carried out as previously described (Ribas et al. 2008). Extracted DNA was analyzed in 1.8% agarose gel in 1 mM ethylene diamine tetraacetic acid (EDTA), 40 mM Tris acetate, pH 7.6, stained in 0.5 μg/ml of ethidium bromide and then visualized using a Syngene Gene Genius UV transilluminator equipped with a photographic camera.

Transmission Electron Microscopy - Untreated cells or cells treated with staurosporine for 10 h were fixed and processed as described in (Sánchez-Osuna et al. 2014). Ultrathin sections were evaluated using a transmission electron microscope (Jeol JEM-1400) equipped with a CCD GATAN ES1000W Erlangshen camera.

DEVD-directed Caspase Activity - Quantitative DEVD-like activities in cell lysates were performed as previously described (Yuste et al. 2001). The resulting 96-multiwell microplates were incubated during 10 hours at 35ºC. The data were obtained by using a BIO-TEK Synergy HT fluorimeter, under an excitation filter of 360 nm (40 nm bandwidth) and an emission filter of 530 nm (25 nm bandwidth).

Protein Extractions and Western Blotting - Cells were detached, pelleted at 500 x g for 5 minutes and washed once with PBS. Cells were then lysed with Igepal buffer or with SET buffer as previously described (Sánchez-Osuna et al. 2014) to obtain cytosolic or total protein extracts respectively. Alternatively, C, N1 and N2 fractions were obtained as previously established in our laboratory (Iglesias-Guimarais et al. 2012). Protein extracts were quantified by a modified Lowry assay (DC protein assay, Bio-Rad, Barcelona) and fractionated on SDS-PAGE. Once the specific antibodies were blotted, membranes were stained with Naphthol blue, allowed to dry and scanned.

Transfection of DFF40/CAD - The eukaryotic expression vector pcDNA3 (Invitrogen) carrying the open reading frame cDNA of DFF40/CAD and the empty vector were used to transfect glioblastoma cells. Cells were transfected by using Lipofectamine 2000 Reagent (Invitrogen), for established cell lines, or Attractene Transfection Reagent (Qiagen), for primary cells, according to manufacturer's instructions. To obtain stably transfected cells, 24h after transfection, cells were grown in selective media containing 0.5-1mg/ml of G-418 (Invitrogen) during 25-30 days (until non-transfected cells died in selective media) and used as a pool.

Results

Glioblastoma-derived cells do not display apoptotic hallmarks during the cell death triggered by staurosporine.

We have recently described the behavior of glioblastoma-derived LN-18 cells when challenged with apoptotic stimuli (Sánchez-Osuna et al. 2014). Although we found that DFF40/CAD-independent mechanism was governing the nuclear changes during cell death in injured human glioblastoma-derived LN-18 cells, we demonstrated that DFF40/CAD is sufficient to induce DNA laddering after cytotoxic insult (Sánchez-Osuna et al. 2014). Here, we extend the data obtained in LN-18 cells to other glioblastoma-derived cells and primary samples.

First, we analyzed whether staurosporine was able to induce cell death in other well-established glioblastoma-derived cells such as A172, LN-229, T98G, U87-MG and U251-MG cells. After 24 h of treatment, cell death was measured in staurosporine-treated and non-treated cells by trypan blue exclusion assay. Values obtained in non-treated cells were lower than 10% in all cases. In staurosporine-treated cells, the percentages obtained were: 89.86 ± 4.88% in A172, 81.95 ± 1.80% in LN-18, 82.47 ± 2.82% in LN229, 98.42 ± 0.19% in T98G, 79.55 ± 2.02% in U87-MG and 86.18 ± 5.27% in U251-MG (Fig. 1A).

Since nuclear alterations are distinctive traits of apoptosis, we analyzed the nuclear morphology in staurosporine-treated glioblastoma-derived cells after bisbenzimide of Hoechst staining. As classical apoptotic cellular model we used staurosporine-treated neuroblastoma-derived SH-SY5Y cells (Boix et al. 1997). As shown in Fig. 1B, injured SH-SY5Y cells displayed several smaller-size masses of compacted chromatin as a result of both chromatin compaction and nuclear fragmentation, which is classically known as stage II nuclear morphology (Susin et al. 2000). In staurosporine-treated glioblastoma cells, nuclear morphology was very dissimilar. In A172 and LN229 cells staurosporine provoked a non-homogeneous response composed by a mix of fragmented (50.66. ± 10.55% in A172 and 54.34 ± 9.23% in LN229 cells) and non-fragmented nuclei (38.58 ± 9.25% in A172 and 36.88 ± 9.69% in LN229 cells) (Fig. 1B). These non-fragmented pyknotic nuclei, were compatible with stage III chromatin compaction (Yuste et al. 2005).

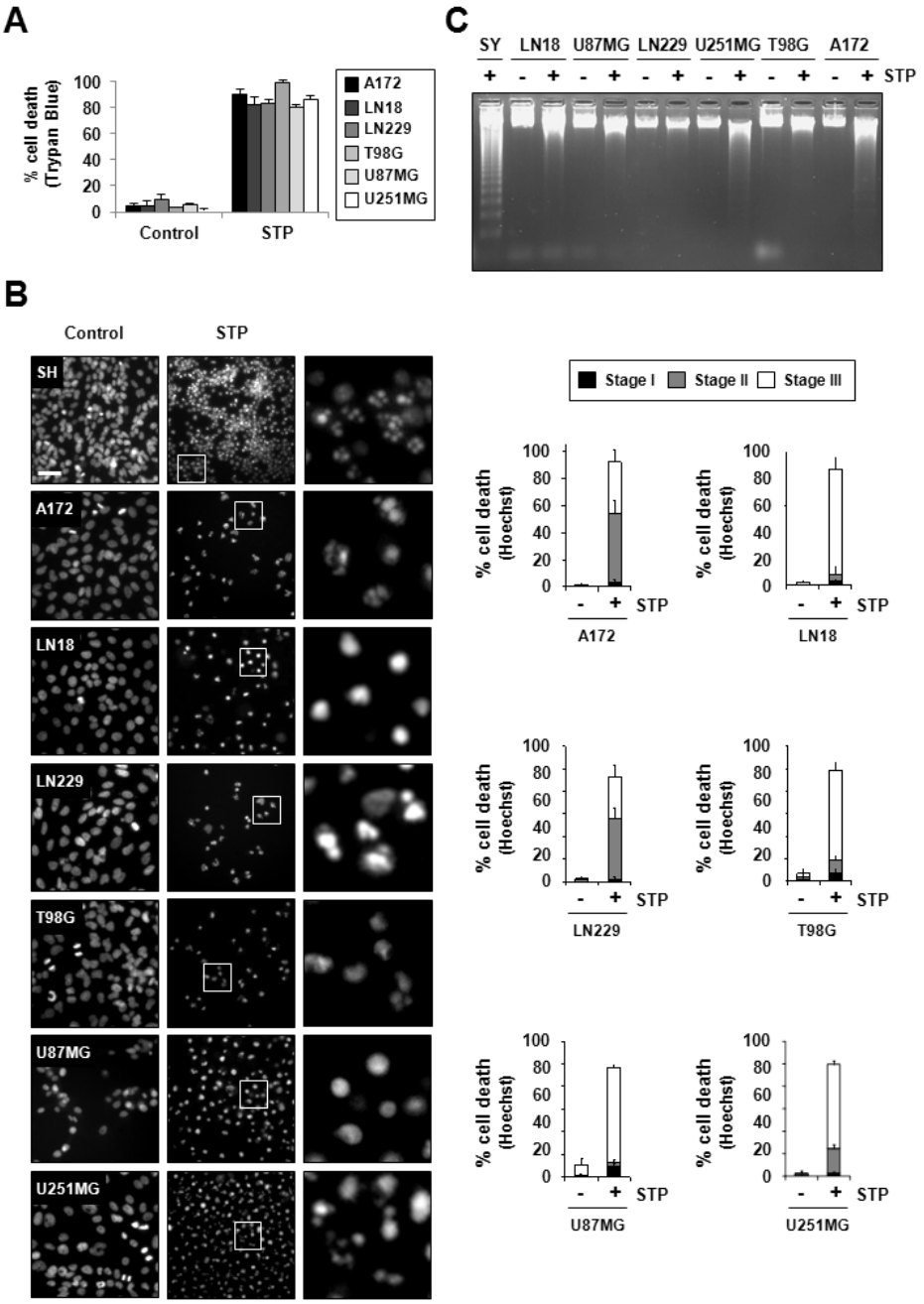

Figure 1. Glioblastoma cells are unable to degrade DNA into low molecular weight fragments during staurosporine-triggered apoptosis independently of the apoptotic nuclear morphology displayed. Glioblastoma cells (A172, LN18, LN229, T98G, U87MG or U251MG) and SH-SY5Y cells were treated with 1 µM staurosporine (STP or +) or left untreated

(control or -). **A,** after 24h, Trypan blue assay was performed. The percentage of cell death was calculated by counting blue (dead) and white (alive) cells. The graphic represents the means ± SD of at least 300 cells from three independent experiments. **B,** after 24 h, cells were fixed with paraformaldehyde, stained with Hoechst 33258 and photographed by using an epifluorescence microscope under UV light. Representative photographs from each condition are shown. From staurosporine-treated cells, magnifications are also shown *(right)*. Scale bar = 40 μm. More than 300 nuclei from glioblastoma cells were counted and scored as stage I, II or III chromatin condensation. Graphic represents means± SD (n=3). **C,** after 6 h of treatment, cells were collected and DNA was extracted and electrophoresed. Note that staurosporine-treated SH-SY5Y cells are used as a positive control for oligonucleosomal DNA degradation.

On the other hand, T98G, U87-MG and U251MG cells together with LN-18 cells, displayed homogeneous nuclear alterations characterized by a unique mass of condensed chromatin without signs of chromatin fragmentation (79.35 ± 6.22%, 84.64 ± 2.51%, 74.77 ± 3.26% and 79.32 ± 8.70% respectively) (Fig. 1B). The caspase-independent stage I of nuclear morphology, characterized by peripheral compaction of the chromatin below the nuclear envelope was uncommon in injured glioblastoma cells, being around 4.6% and less than 9% in all cases (Fig. 1B).

Besides nuclear alterations, oligonucleosomal DNA fragmentation is also considered a hallmark of apoptosis. Thus, we analyzed DNA from injured and healthy glioblastoma cells taking SH-SY5Y cells as a positive control for apoptotic DNA degradation (Boix et al. 1997). Surprisingly, none of the glioblastoma cells tested showed DNA laddering upon staurosporine treatment (Fig. 1C).

Glioblastoma-derived cells activate executioner caspases after staurosporine treatment.

Caspases are important effectors triggering the necessary biochemical responses to dismantle the cell during apoptosis. Since glioblastoma-derived cells failed at displaying the hallmarks of apoptotic cell death, we analyzed caspase activation in protein lysates from both untreated and staurosporine-treated glioblastoma cells. After 6 h of staurosporine treatment, we detected a substantial increase in DEVDase-directed caspase activity in all glioblastoma cells: 3.8 (A172), 7.2 (LN18), 7.8 (LN229), 2.4 (T98G), 4.0 (U87MG) or 4.0 (U251MG) -fold induction when compared to lysates from non-treated cells (Fig. 2A). To analyze the relevance of caspase activation in the cell death triggered by staurosporine in glioblastoma cells, we took

advantage of the pan-caspase inhibitor q-VD-OPh (qVD). As represented in Fig. 2B, the addition of qVD to the culture media was sufficient to avoid the cytotoxicity induced by the alkaloid reducing to less than 20% the blue cells observed in a trypan blue exclusion assay (12.94 ± 7.63% in A172, 4.75 ± 4.59% in LN18, 20.66 ± 2.76% in LN229, 11.90 ± 3.96% in T98G, 19.81 ± 1.86% in U87MG, and 17.15 ± 4.38% in U251MG). Caspase activation was also corroborated by Western blot of PARP (general caspase substrate), fodrin (specific substrate for caspase-3), lamin A/C (specific substrate of caspase-6) and co-chaperone p23 (specific substrate for caspase-7). After 6 h of staurosporine treatment, all glioblastoma cells showed cleaved PARP, p120 fodrin fragment, p47/37 fragments from lamin A/C and p15 fragment from the co-chaperone p23, indicating that executioner caspases -3, -6 and -7 were correctly activated (Fig. 2C). All these four cleavages were correctly inhibited when the pan-caspase inhibitor qVD was added to the culture media. Moreover, the addition of qVD to the culture media avoided the staurosporine-triggered nuclear alterations in glioblastoma cells (data not shown).

The ICAD/CAD endonucleolytic system is correctly activated in glioblastoma-derived cells after staurosporine treatment.

To allow the final steps of apoptosis to proceed, active caspase-3 should cleave the inhibitor of CAD (ICAD) thus allowing DFF40/CAD endonuclease release. As shown in Fig. 2D, staurosporine effectively induced processing of caspase-3 to the p19/17 activation fragments in all glioblastoma cells. Moreover, caspase-3 cleavage correlated with the appearance of the p11 fragment from caspase-3-mediated processing of $ICAD_L$. Then, we analyzed DFF40/CAD protein levels in total protein extracts from staurosporine-treated or non-treated cells. All glioblastoma-derived cells expressed lower amounts of DFF40/ CAD than SH-SY5Y cells (Fig. 2E). More importantly, the endonuclease was not degraded after staurosporine treatment (Fig. 2E).

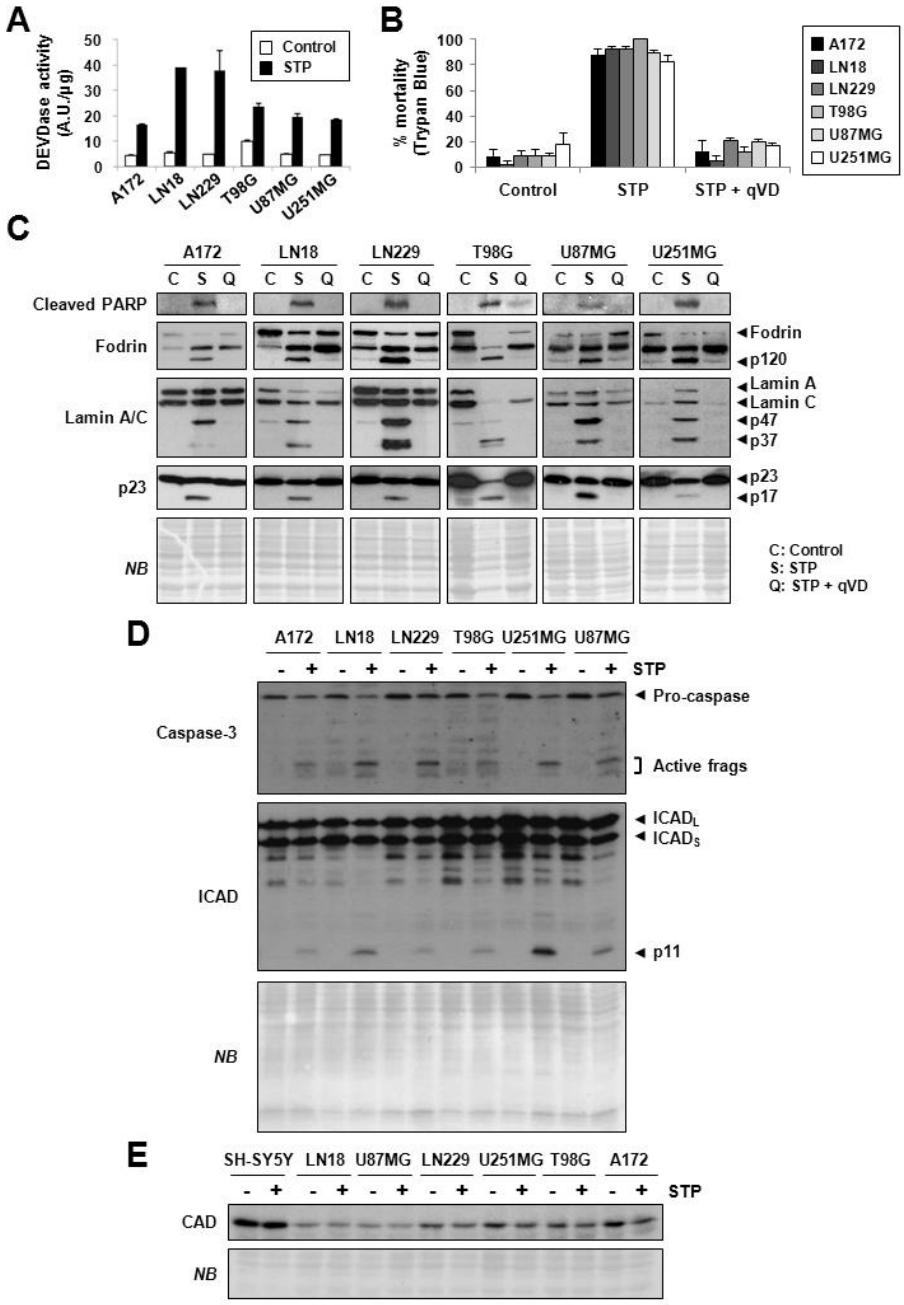

Figure 2. Glioblastoma cells suffer a caspase-dependent cell death and correct DFF40/CAD activation upon staurosporine treatment. Glioblastoma cells were treated with 1 µM staurosporine (STP, S, +), with 1 µM staurosporine plus 10 µM q-VD-OPh (Q) or left untreated (Control, C, -). **A,** after 6h, untreated or staurosporine-treated cells were collected and

protein extracts were obtained. DEVD-like activity was measured and graphed (n=2). **B,** Trypan blue exclusion assay was performed after 24 h treatment. Cell death was expressed as the percentage of blue (dead) cells over total. The graphic represents the means ± SD of more than 300 cells from three independent experiments. **C,** after 6 h treatment, cells were collected and protein extracts were obtained. Immunoblots against cleaved PARP, fodrin, lamin A/C and co-chaperone p23 are shown. **D,** caspase-3 and ICAD immunoblots are shown in staurosporine-treated or untreated cells. **E,** total protein levels of DFF40/CAD are shown in both untreated and staurosporine-treated cells. Naphthol Blue staining is used as loading control in **C, D and E**.

Glioblastoma cells present low levels of cytoplasmic DFF40/CAD.

Based on previous studies made in our group which points out the importance of the cytosolic pool of DFF40/CAD in the nucleosomal hydrolysis of DNA during apoptosis (Iglesias-Guimarais et al. 2012), we decided to analyze the subcellular distribution of DFF40/CAD in glioblastoma cells. We obtained the cytosolic (C), nucleoplasmic (N1) and chromatinic-enriched (N2) fractions from staurosporine-treated or non-treated glioblastoma cells and we focused on caspase-3, ICAD and DFF40/CAD localization. In non-treated cells, procaspase-3 as well as long and short isoforms of ICAD ($ICAD_L$ and $ICAD_S$) located in both cytosolic and nucleoplasmic fraction. Alternatively, and although present to some extent in the cytosol, DFF40/CAD localized mostly in the nucleoplasmic fraction of non-treated cells (Fig. 3). After treatment, active fragments from caspase-3 clearly appeared in the cytosol, being only slightly detected in the nucleoplasm and completely absent in the chromatinic fraction. Similarly to that observed for caspase-3, staurosporine efficiently induced the processing of cytosolic ICAD, only partially observed in the nucleoplasmic fraction (Fig. 3). Moreover, after staurosporine insult, cytosolic DFF40/CAD disappeared; nucleoplasmic DFF40/CAD remained unaltered, whereas certain levels of endonuclease appeared in the chromatinic fraction (Fig. 3). So far, these results indicated that DFF40/CAD subcellular localization in glioblastoma cells (mostly nucleoplasmic) did not coincide with the subcellular compartment (cytosol) in which the activation of caspase-3 and fragmentation of ICAD occurred preferentially.

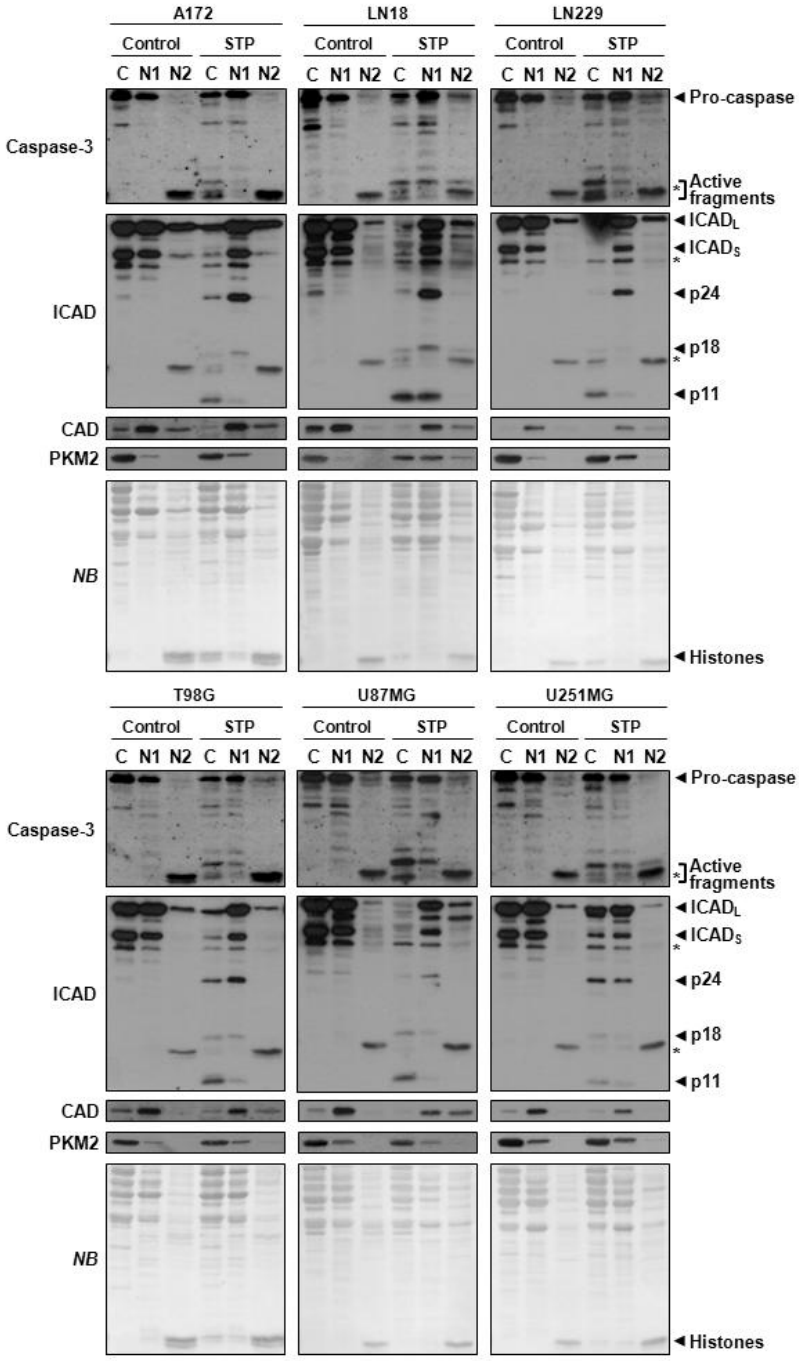

Figure 3. Staurosporine-triggered caspase-3 activation and ICAD degradation colocalize with low levels of cytosolic DFF40/CAD in glioblastoma cells. A172, LN18, LN229, T98G, U87MG and U251MG

glioblastoma cells were treated with staurosporine for 6 h (STP) or left untreated (control). Then, cells were collected and cytosolic (S1), soluble nuclear (N1) and chromatinic-enriched (N2) fractions were obtained. CAD, caspase-3 and ICAD levels were assessed in the three different fractions. PKM2 was used as a marker of cytosolic fraction. Histones can be observed in after Naphthol Blue staining. Note that DFF40/CAD endonuclease mobilizes from the cytosolic S1fraction to the chromatinic N2 fraction after STP treatment.

The over-expression of DFF40/CAD restores the ability of glioblastoma-derived cells to degrade DNA into oligonucleosomal-size fragments.

Previously, we demonstrated that overexpression of DFF40/CAD in LN-18 cells was sufficient to allow injured cells to undergo DNA laddering but insufficient to affect nuclear morphology (Sánchez-Osuna et al. 2014). Then, we decided to overexpress the endonuclease in glioblastoma cells capable to undergo nuclear fragmentation (A172 and LN229 cells). As shown in Fig. 4A, overexpression of the endonuclease was sufficient to allow staurosporine-treated A172 and LN229 cells to hydrolyze their genomic content into oligonucleosome-size pieces. Intriguingly, the nuclear morphology observed in CAD-overexpressing cells was the same observed in mock-transfected (Fig. 4B) or non-transfected cells (Fig. 1B). Ultrastructural analysis revealed that untreated CAD-overexpressing cells did not present any significant difference compared with the mock-transfected cells (Fig. 4C, upper panels). More importantly, as already observed by Hoechst staining, we observed different stages of nuclear morphology in mock- and CAD-transfected glioblastoma cells. Most of staurosporine-treated cells displayed several masses of condensed chromatin within the nucleus (Fig. 4C, middle panels). Nevertheless, some injured cells displayed a unique mass of condensed chromatin (Fig. 4C, lower panels).

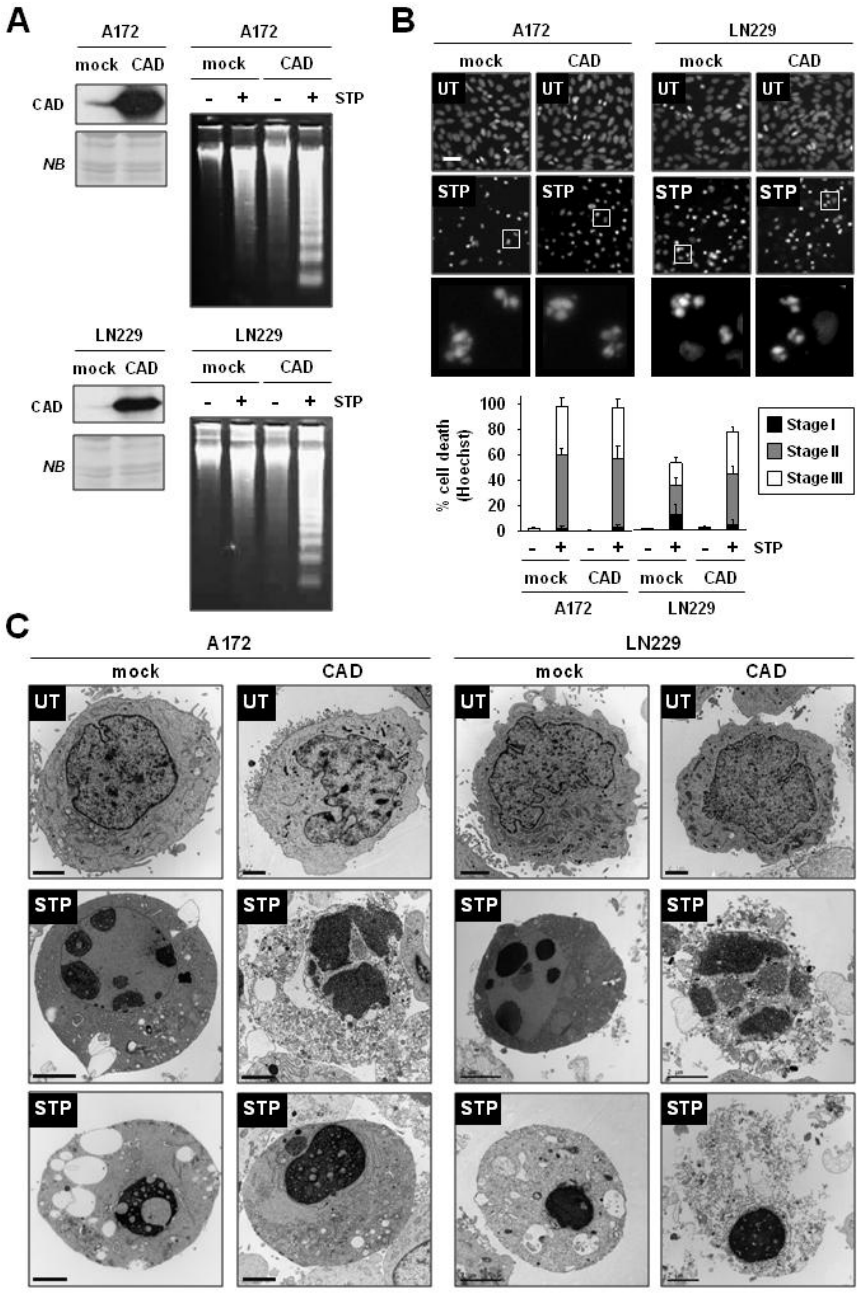

Figure 4. The over-expression of DFF40/CAD allows apoptotic glioblastoma cells to hydrolyze DNA into oligonucleosome-size fragments without altering nuclear morphology. A172 and LN229 cells were transfected with a eukaryotic expression vector carrying the open reading frame of human CAD (CAD) or with the empty vector (mock). 24 h after transfection cells were treated with 1 μM staurosporine (STP or +) or left

untreated (UT or -). **A,** from untreated cells, protein extracts were obtained and western blot against CAD was performed to corroborate the overexpression of the endonuclease **(left)**. Naphthol blue is used as loading control. After 6 h of treatment, DNA was extracted and electrophoresed **(right)**. Note that DNA laddering only appears in staurosporine-treated glioblastoma cells that overexpress DFF40/CAD. **B,** after 24 h, staurosporine-treated or untreated cells were fixed with paraformaldehyde, stained with Hoechst 33258 and photographed by using an epifluorescence microscope under UV light. Representative images from each condition are shown. From staurosporine-treated cells, magnifications are also shown **(lower panels)**. Scale bar = 40 μm. More than 300 nuclei in each condition were counted and scored as stage I, II or III chromatin condensation. The means ± SD are graphed (n=3). **C,** electron microscopy images from the most representative morphologies observed in each condition. Scale bar = 2 μm.

Primary glioblastoma cells die after staurosporine treatment without displaying DNA laddering despite caspase activation.

At that point, we decided to extend our results to primary glioblastoma cells. We obtained the primary cells from patient samples and performed all the experiments before passage 20. First, primary cells were challenged to 1 μM staurosporine and counted after trypan blue exclusion assay. The percentages of cell death reached were 98.93 ±0.18% in #04 cells, 95.70 ± 2.86 % in #30 cells, 88.82 ± 5.03% in #35 cells, 92.29 ± 0.46 % in #45 cells and 94.07 ± 1.69% in #52 cells (Fig. 5A). Despite cell death, none of the primary glioblastoma cells displayed DNA laddering after staurosporine treatment (Fig. 5B). Consequently, we analyzed the activation of the executioner caspases-3, -6 and -7 in these cells. After 6 h of staurosporine treatment, p120 fragment from fodrin (specific substrate of caspase-3), p47/37 fragments from lamin A/C (cleaved by caspase-6) and p15 fragment from co-chaperone p23 (specific caspase-7-meadiated cleavage) were observed in #04, #35 and #52 cells thus demonstrating the correct activation of executioner caspases (Fig. 5C). In #30 cells, staurosporine induced the cleavage of fodrin and co-chaperone p23 but not lamin A/C pointing a putative impairment in caspase-6 activation but a correct activation of caspase-3 and -7 (Fig. 5C). In #45 cells, the cleavage of the three caspase substrates was completely absent.

The key event allowing the correct activation of DFF40/CAD is the processing of its inhibitor ICAD by caspase-3. Therefore, we also checked caspase-3 and ICAD cleavage by western blot. Active fragments from caspase-3 were detected in staurosporine-treated #04, #30, and #35 cells but not in #45 or #52 cells (Fig. 5D, upper panel).

However, staurosporine induced the processing of ICAD not only in #04, #30, #35, but also in #52 cells, depicted by the partial loss of the ICAD isoforms and the detection of p24/p11 fragments from $ICAD_L$ and p18 fragment from $ICAD_S$ (Fig. 5D, second panel).

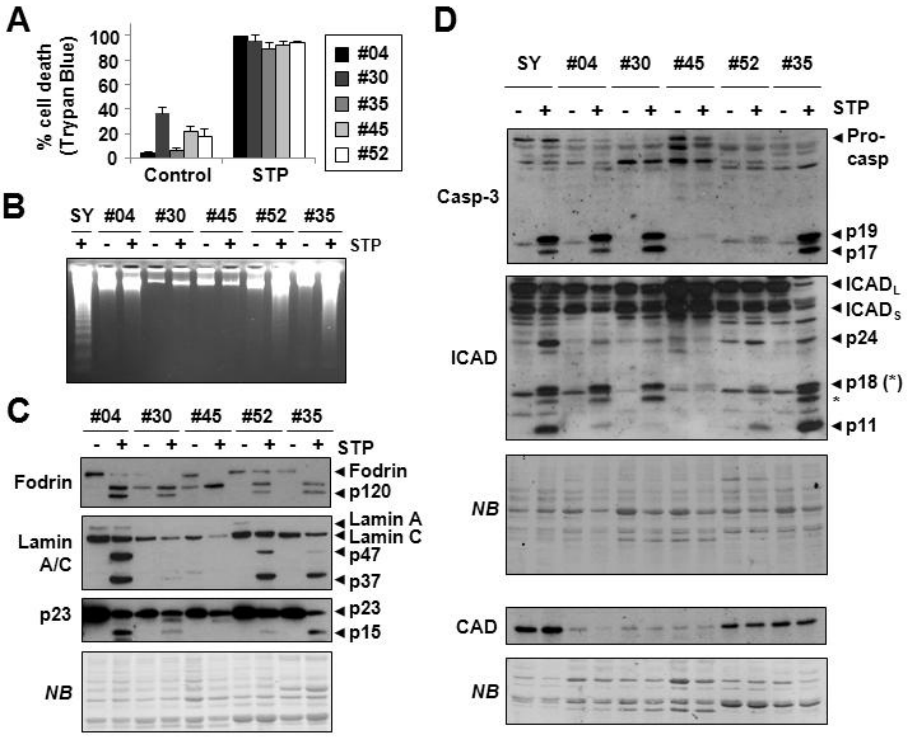

Figure 5. Primary glioblastoma cells die after staurosporine treatment without displaying DNA laddering independently of caspase activation or DFF40/CAD protein levels. Primary glioblastoma cells were treated with 1 μM staurosporine (STP or +) or left untreated (control or -). **A,** after 24 h (#04, #30, #35, #45) or 48 h (#52) treatment, cells were detached and Trypan blue exclusion assay was performed. Cell death is represented as a percentage of the means ± SD of blue cells (died) over total (n=3). **B,** 24 h after treatment, cells were collected and DNA was extracted and electrophoresed. Staurosporine treated SH-SY5Y cells (SH) were used as positive control. **C,** after 6h, cells were collected and protein extracts were obtained. Western blot against fodrin, lamin A/C and co-chaperone p23 were done to assess the activation of caspases-3, -6 and -7 respectively. Naphthol Blue staining is used as loading control. **D,** caspase-3 activation, ICAD cleavage and total protein levels of CAD were assessed in staurosporine-treated or untreated cells after 6 h treatment. Note that SH-SY5Y cells (SH) are used to compare total protein levels in a cellular model fully competent to display apoptotic hallmarks.

Then, we analyzed DFF40/CAD endonuclease expression in total extracts from the different primary cultured cells. The endonuclease was present over the time of treatment showing the same protein levels than in non-treated cells (Fig. 5E). Moreover, although protein expression varied among primary glioblastoma cells, total levels of DFF40/CAD were always lower than those levels found in SH-SY5Y cells being really low in #04, #30 and #45 cells (Fig. 5D, fourth panel).

One of the controversial points about using cell lines or primary samples after several passages is whether the results obtained correctly recapitulate the original characteristics of the tumor. To ascertain whether glioblastoma cells had lost the DFF40/CAD protein expression during cell culture or as a result of the tumorigenic process, we followed the expression of the endonuclease over cell culture. DFF40/CAD protein levels were maintained over cell culture of #04 cells meaning that, for our interests, glioblastoma primary cells can be used at least, up to 20 passages (supplementary Fig. S1).

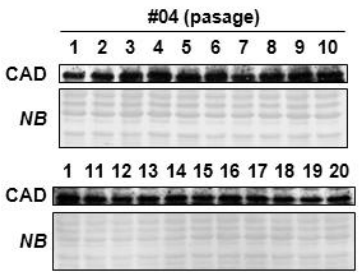

Figure supplementary S1. DFF40/CAD protein expression does not change over cell culture. From passage 1 to passage 20 of the primary glioblastoma cells #04, protein extracts were obtained and western blot against CAD was perform. Naphthol Blue staining is used as loading control. Note that sample from 'passage 1' is in both western blots to make them comparable.

DFF40/CAD overexpression allows injured primary glioblastoma cells to display DNA laddering without affecting nuclear morphology.

At that point, we decided to overexpress DFF40/CAD in #04 and #52 primary cells expressing low or high levels of the endogenous endonuclease, respectively. As shown in Fig. 6A, higher levels of endonuclease were sufficient to induce DNA laddering after staurosporine treatment in both #04 and #52 cells. Regarding to nuclear morphology, CAD-overexpression, as already described for well-established glioblastoma-derived cell lines, had no effect in the nuclear alterations displayed by injured cells. Both mock- and CAD-transfected #04 primary cells showed a unique mass of condensed chromatin without any sign of chromatin fragmentation after exposure to the alkaloid (Fig. 6B). In #52 primary cells, staurosporine induced a

more relaxed chromatin status, compatible with shrank stage I nuclear morphology in both CAD- and mock-transfected cells (Fig. 6B). Therefore, higher levels of DFF40/CAD endonuclease allowed primary cells to hydrolyze their genomic content into oligonucleosomal size pieces without affecting the mechanisms controlling nuclear changes during apoptosis.

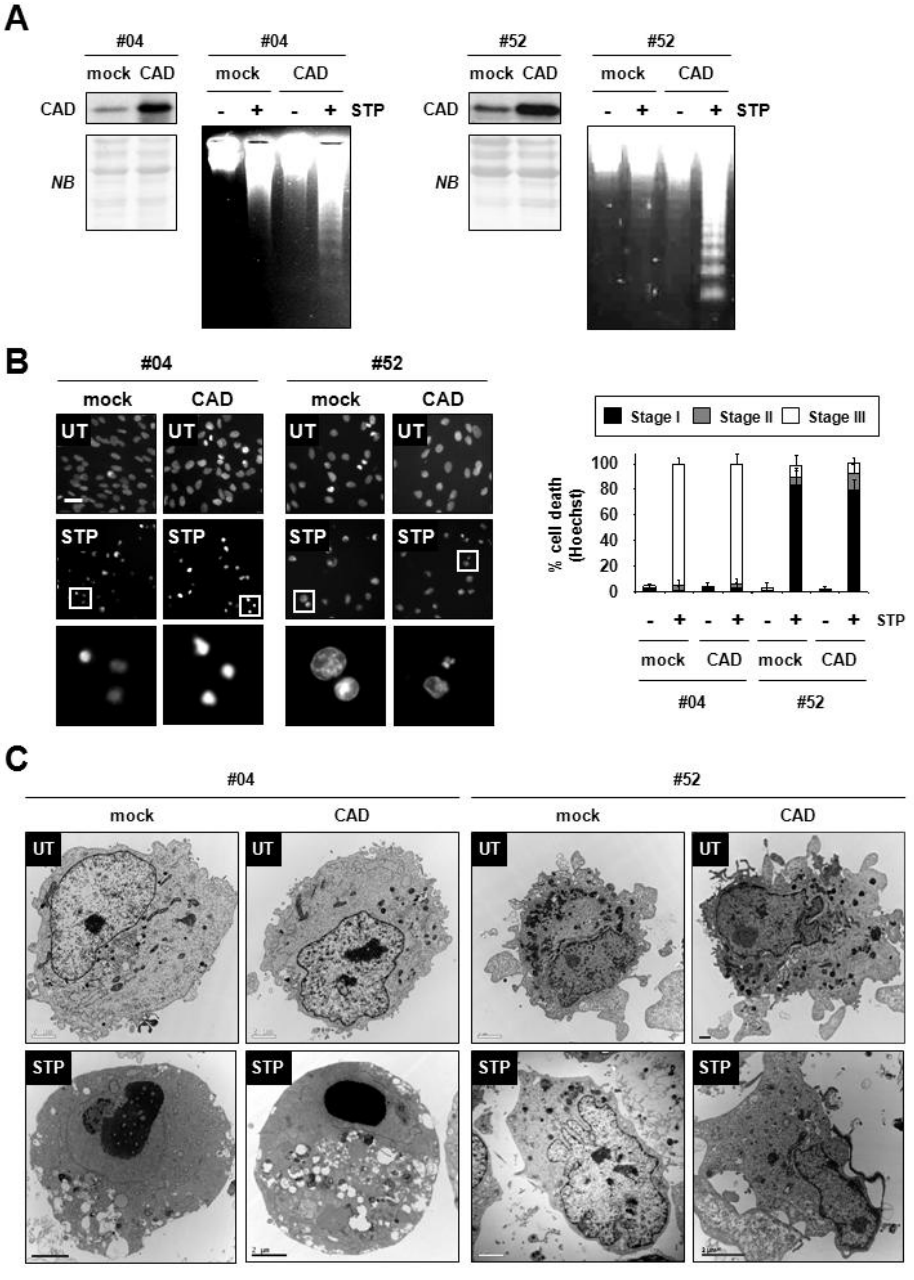

Figure 6. The over-expression of DFF40/CAD in injured primary glioblastoma cells induces DNA laddering without affecting nuclear morphology. Primary glioblastoma cells were transfected with a eukaryotic expression vector carrying the open reading frame of human CAD (CAD) or with the empty vector (mock). After growing in selective media during 4 weeks, transfected cells were treated with 1 μM staurosporine (STP or +) or left untreated (UT or -). **A,** from untreated cells, protein extracts were obtained and western blot against CAD was performed to corroborate the overexpression of the endonuclease. Naphthol blue is used as loading control. After 6 h of treatment, DNA was extracted and electrophoresed. Note that DNA laddering only appears in staurosporine-treated cells that overexpress the endonuclease. **B,** after 24 h, staurosporine-treated or untreated cells were fixed with paraformaldehyde, stained with Hoechst 33258 and photographed by using an epifluorescence microscope under UV light. Representative images from each condition are shown **(left)**. From staurosporine-treated cells, magnifications are also shown **(lower panels)**. Scale bar = 40 μm. More than 300 nuclei in each condition were counted and scored as stage I, II or III chromatin condensation. The means ± SD are graphed (n=3). **C,** electron microscopy images from the more representative morphology observed in each condition. Scale bar = 2 μm.

Apoptotic nuclear morphology is controlled by the attachment of the chromatin to the inner surface of the nuclear envelope

At that point, we decided to analyze nuclear changes in injured glioblastoma cells by electron microscopy. As already observed after bisbenzimide staining, the most representative nuclear morphologies were those resembling type II or III chromatin condensation (Fig 7, frame 4 to 9). In apoptotic A172 cells, chromatin appears condensed into different masses within the same compartment delimited by the nuclear envelope. Interestingly, these masses of condensed chromatin appeared tightly attached to the inner part of the nuclear envelope (Fig. 7, frame 5 and 6, blue arrowheads). Nuclear alterations displayed by apoptotic A172 cells, similar to those observed in staurosporine-treated LN229 cells (data not shown), were clearly different to those observed in neuroblastoma-derived SH-SY5Y cells. Early, apoptotic SH-SY5Y cells displayed half-moon shaped condensation of the chromatin that resulted in nuclear protuberances (data not shown). Later on, these chromatin-filled nuclear protuberances disaggregated and resulted in little rounded masses of chromatin enclosed by continuous nuclear envelope (Fig. 7, frame 2 and 3). In staurosporine-treated U87-MG cells, chromatin condensed into a single mass and nuclear envelope appeared broken (Fig.7, frame 8 and 9). Interestingly, in these cells, points of attachment of the chromatin to the nuclear envelope were rarely detected. Indeed, little masses of condensed chromatin

compatible with broken attachments were really frequent and easy to detect (Fig.7, frame 8 and 9, orange arrowheads). In summary, chromatin, but not nuclear envelope, became fragmented in apoptotic A172 cells and neither chromatin nor nuclear envelope disassembled in U87-MG cells. Therefore, none of the nuclear morphologies found in staurosporine-treated glioblastoma cells completely recapitulated the classical apoptotic alterations displayed by neuroblastoma-derived SH-SY5Y cells.

Interestingly, at late stages during the apoptotic process, when cell dismantling was more obvious, nuclear envelope of glioblastoma cells became fragmented and the nuclear content released (Fig. 7B, middle and right panels). However, even when cytoplasm was completely destroyed and cellular membrane was not preserved in SH-SY5Y cells, genomic content remained condensed and constrained (Fig. 7B, left panel). In conclusion, chromatin from injured glioblastoma cells is not safely packaged and when cellular integrity is compromised, chromatin can disperse throughout the cell and release to the surrounding media.

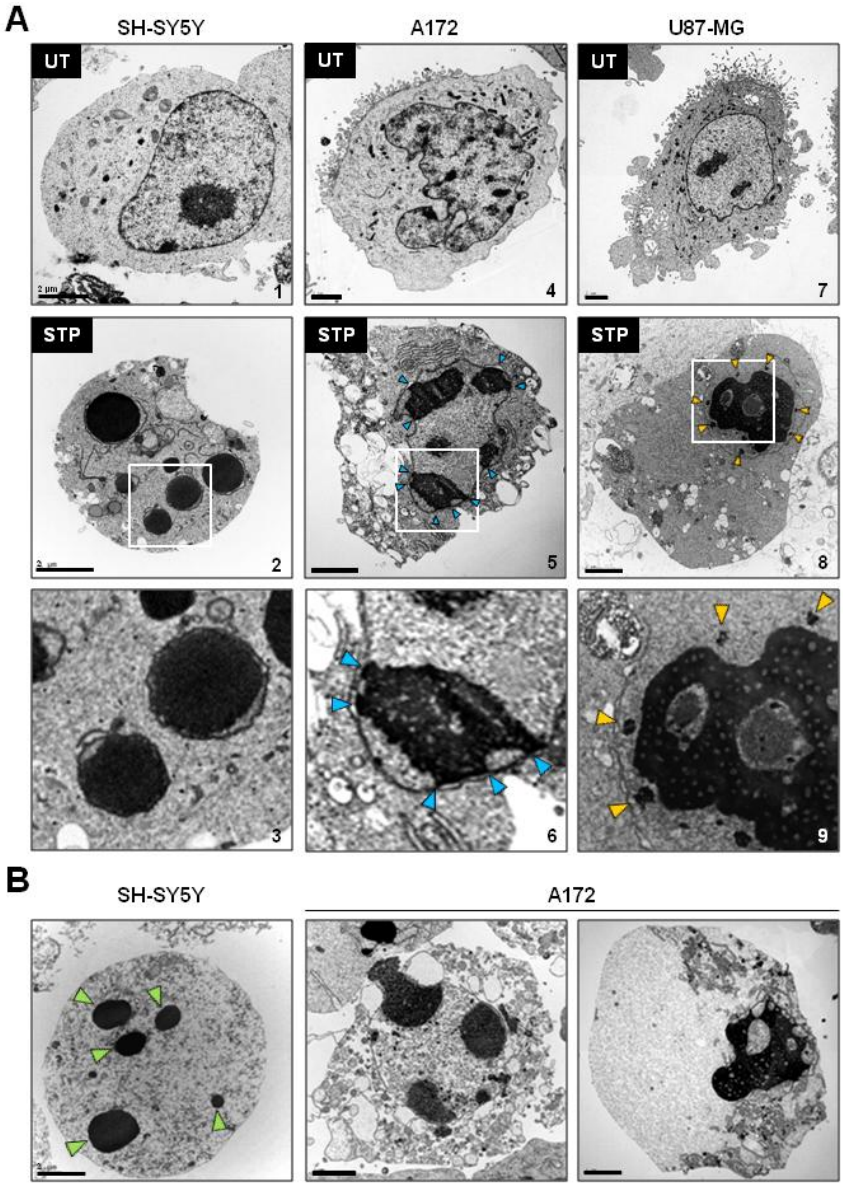

Figure 7. Chromatin anchorage to nuclear envelope determines the nuclear morphology observed during apoptosis but it does not impede the release of DNA during cell death. A, cells treated for 10 h with 1 µM staurosporine **(2, 5 and 8)** or left untreated **(1, 4 and 7)** were visualized by electron microscopy. **1 to 3,** SH-SY5Y cells; **4 to 6,** A172 cells; **7 to 9,** U87-MG cells. Blue arrowheads indicate the attachment points of the chromatin to the inner nuclear envelope. Orange arrowheads mark punctual masses of chromatin compatible with broken points of attachment. **3, 6 and 9** are magnifications of the framed regions. **B,** cells were treated during 24 h with 1 µM staurosporine. Of note, despite of the extreme cytoplasmic destruction

that include membrane disruption, SH-SY5Y cells maintain intact several electrodense structures compatible with apoptotic nuclear bodies (green arrowheads) while glioblastoma-derived A172 cells release their genomic content independently of the nuclear morphology displayed (stage II or III). Scale bar = 2 μm.

Discussion

Here, we describe the apoptotic outcome of several glioblastoma cells when challenged to staurosporine, a strong apoptotic inductor. We found that the alkaloid induced a caspase-dependent cell death with disparate nuclear morphologies. In T98G, U87MG and U251MG, as well as already described for LN-18 cells (Sánchez-Osuna et al. 2014), chromatin condenses but remains unfragmented in a unique mass. Conversely, in A172 and LN229, the chromatin condenses in one or more different clumps. Independently of the apoptotic morphology displayed, none of the glioblastoma cells tested showed oligonucleosomal DNA degradation after apoptotic insult. Interestingly, all glioblastoma cells displayed low levels of the DFF40/CAD endonuclease in the cytosolic fraction, the one in which caspase activation takes place preferentially. Intriguingly, higher levels of the endonuclease allow apoptotic glioblastoma cells to hydrolyze their genomic content into oligonucleosome-size fragments without altering the nuclear morphology observed in non-transfected or mock-transfected cells.

As depicted by the term ‘multiforme’, glioblastoma harbor significant intratumoral heterogeneity on the cytopathological, transcriptional, and genomic levels. Our data perfectly reflect this heterogeneity. Conversely to other glioblastoma cells, #45 primary cells failed at activating caspases after staurosporine challenge. Nevertheless, those cells died after staurosporine treatment, demonstrating that the alkaloid can operate through both caspase-dependent and caspase-independent pathways as previously described (Belmokhtar et al. 2001). Data obtained from primary glioblastoma #52 cells showed slightly confusing results. Direct analysis of caspase-3 cleavage failed at showing the classical activation fragments for the executioner caspase. However, the p120 fragment of fodrin, specifically cleaved by active caspase-3, and ICAD processing, preferentially mediated by caspase-3, were correctly processed at the time of treatment. Therefore, we could assume that probably caspase-3

activation fragments could be detected at different (probably earlier) times of treatment.

Although it has been suggested that inefficient caspase activation contributes to gliomagenesis (Furnari et al. 2007), we detected correct caspase activation after apoptotic insult in most of the glioblastoma cells. Indeed, fluorometric assays showed certain caspase activity already in non-treated cells. That result agrees with previous data pointing to basal caspase activity as a key regulator of glioblastoma motility and invasiveness (Gdynia et al. 2007). Nevertheless, our results demonstrated that functional activated caspases do not guarantee the completion of apoptotic hallmarks. Stage II chromatin condensation was only partially detected in two cell lines (A172 and LN229), whereas DNA laddering was completely absent in apoptotic glioblastoma cells. Moreover, the presence of apoptotic nuclear morphology did not always correlate with caspase activity or CAD protein levels, a possibility previously suggested and fitting in LN-18 cells (Sánchez-Osuna et al. 2014). Subcellular analysis of glioblastoma cells proved that caspase-3 activation occurs preferentially at the cytoplasmic level while CAD was preferentially located in the nucleoplasm, meaning that glioblastoma cells could also regulate late stages of apoptotic cell death by controlling the subcellular distribution of the different apoptotic effectors. That could explain why cells with higher levels of DFF40/CAD such as #52 or A172, after correct caspase activation and ICAD processing, do not display CAD-dependent DNA degradation.

One of the main differences between long and short isoforms of ICAD is the presence or absence, respectively, of a nuclear localization sequence (NLS). It has been suggested that $ICAD_L$ and $ICAD_S$ might work as tissue-specific modulators in the regulation of apoptotic DNA degradation by controlling not only the enzymatic activity but also the amount of CAD available in the nuclei of mammalian cells (Scholz et al. 2002). Interestingly, our results show that both isoforms of ICAD are present in the nucleoplasmic fraction of glioblastoma cells which suggest that, besides the NLS, other factors should regulate nuclear accumulation of the DFF complex in glioblastoma cells.

By electron microscopy, we found a correlation between the attachment of the chromatin to the nuclear envelope and the final apoptotic nuclear morphology of glioblastoma cells. The lack of chromatin fragmentation observed in some glioblastoma cells always

coincided with the detachment of the chromatin from the nuclear envelope. Conversely, those cells condensing chromatin into more than one clump maintained chromatin tightly attached to the inner nuclear membrane. Much to our surprise, those smaller masses of condensed chromatin did not form apoptotic nuclear bodies but remained in the same non-fragmented nuclear compartment. That apoptotic behavior resulted, contrarily to that reported till date, in the release of the genetic content at late stages in which nuclear envelope and cellular integrity cannot be still preserved. Indeed, one of the main differences between apoptosis and other kinds of cell death, such as necrosis, relies on extracellular consequences. Although last steps of apoptosis include the intracellular degradation within the phagocytic cell of the apoptotic bodies, the present study suggests that, in glioblastoma cells, apoptotic cell death may lead to a different end. Incomplete packaging of the nuclear content into apoptotic bodies would elicit inflammatory responses traditionally related to necrosis.

Glioblastoma remains a therapeutic challenge for clinicians and researchers. Despite the current focus on combinatorial therapies against multiple signaling pathways to induce maximal inhibition, these tumors remain completely lethal with recurrence being the rule (reviewed in See & Gilbert 2004; Demuth & Berens 2004; Patel et al. 2012). Here, we demonstrate that injured glioblastoma cells die without degrading DNA into oligonucleosome-size fragments and, in addition, that they release their genomic content into the extracellular media during the process. Since it has been proved that DNA released from dead cells can lead to disparate consequences that range from cytotoxicity to cellular transformation in putative 'receptor' cells (Ermakov et al. 2013; Glebova et al. 2013), our results may explain the intrinsic aggressiveness of this kind of tumors.

This is the first time reporting a common trait among glioblastoma cells. Moreover, it has never been reported a common behavior among all the cells from the same family of tumors. Thus, it would be reasonable to think that this particular finding could be intimately related to gliomagenesis. Further investigation is needed to determine the utility of this finding in the clinic.

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3. Gossypol sensitizes glioblastoma cells to TRAIL-mediated nuclear apoptosis despite of low DFF40/CAD protein levels.

Abstract

Glioblastoma multiforme is an extremely aggressive solid tumor with a huge rate of genomic alterations. Frequently, glioblastoma cells have dysregulated apoptotic pathways that lead to apoptosis resistance. Therefore, therapies targeting Bcl-2 family members are considered promising therapies in the treatment of cancers, especially, in those resistant to apoptosis such as glioblastoma. Here, we show how gossypol, a BH3 mimetic, is able to sensitize TRAIL-resistant glioblastoma cells to extrinsic apoptosis. Both TRAIL and TRAIL plus gossypol but not gossypol treatment induce caspase-dependent cell death. However, only after TRAIL plus gossypol, glioblastoma cells display classical stage II apoptotic nuclear morphology, absent during treatment with TRAIL or gossypol. Moreover, stage II nuclear morphology mediated by gossypol plus TRAIL, but not those induced by TRAIL or gossypol, relies on DFF40/CAD, the major apoptotic endonuclease. Interestingly, as the individual treatments, the cotreatment fails to generate DNA laddering in glioblastoma cells. The transfection of DFF40/CAD reveals that higher levels of the endonuclease allow apoptotic glioblastoma cells to undergo DNA laddering independently of nuclear alterations. Nevertheless, cell free *in vitro* assays suggest that CAD translocation to the nucleus is not the only factor regulating oligonucleosomal DNA degradation in LN-18 glioblastoma cells and indicated the relevance of CAD stoichiometry. Altogether, these results support previous data suggesting that nuclear alterations and DNA degradation are processes independently regulated during apoptosis in glioblastoma cells. While CAD activity seemed the only factor regulating DNA laddering, other factors are needed to trigger classical apoptotic nuclear alterations.

Experimental procedures

Reagents - All chemicals were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise indicated. The pan-caspase inhibitor q-VD-OPh (50 μM) was from MP Biomedicals Europe (Illkirch, France). Caspase-3 antibody (9662; 1:2,000) was from Cell Signaling Technology (Beverly, MA). Antibodies against DFF40/CAD (AB16926, 1:500) and α-Fodrin (clone AA6) (MAB1622; 1:40,000) were obtained from Millipore Iberica S.A.U. (Madrid, Spain). DFF45/ICAD antibody (clone 6B8) (M037-3; 1:40,000) was from MBL (Naka-ku Nagoya, Japan). Anti-Lamin A/C (clone JOL2) (Ab40567; 1:2,000) was from Abcam (Cambridge, UK). Anti-p23 antibody (clone JJ3) (NB300-576; 1:10,000) was obtained from Novus Biological Europe, Inc (Cambridge, UK). Anti-Apoptosis Inducing Factor (A7549; 1:20.000) was from Sigma-Aldrich. Secondary antibodies were mouse IgG (A9044; 1:10,000) and rabbit IgG (A0545; 1:20,000). The cytotoxic drugs used were: ABT-737 (Selleck, 50 μM); chelerythrine (20 μM); EM 20-25 (50 μM); gossypol (Selleck, 100 μM); HA14-1 (100 μM); nocodazole (100 μM); obatoclax (Selleck; 100 μM); paclitaxel (50 μM); staurosporine (1 μM); TW37 (Selleck; 10 μM); vinblastine (50 μM); CH11 (MBL; 0,1 μg/ml); TNFα (0,1 μg/ml); TRAIL (BioTrend; 0,1 μg/ml); Z36 (25 μM).

Cell Lines and Culture Procedures – Human glioblastoma-derived cells A172, LN-18, LN229 and U251-MG, as well as human neuroblastoma-derived SH-SY5Y cells routinely grown in 100-mm culture dishes (BD Falcon, Madrid, Spain) containing 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively) and 10% of heat-inactivated fetal bovine serum (FBS) (Invitrogen S.A, Barcelona, Spain). Medium was routinely changed every 3 days. Cells were maintained at 37°C in a saturating humidity atmosphere containing 95% air and 5% CO2. For the different experiments, cells were grown at the adequate cell densities in culture dishes or multi-well plates (BD Falcon, Madrid, Spain) using the same culture conditions as described above.

Cell-free Assay: preparation of nucleus, cytosolic extracts and reconstitution of reaction - Cytosolic extracts and nuclei were obtained as previously established in our laboratory (Iglesias-Guimarais et al. 2012) and then the cell free *in vitro* reaction was carried out. After 2 hours, a 10% of the total volume of the reaction was separated and

lysed with SET buffer. The rest of the reaction was centrifuged 15 minutes at 16,000 x g (4ºC) and the supernatant was removed. Then, nuclei were resuspended in distilled water. A 20% of the total volume was separated and lysed with SET buffer ('Pelleted nuclei'). From the rest of the nuclei, DNA was extracted according to oligonucleosomal DNA extraction protocol.

Chromatin Staining with Hoechst 33258 and Trypan Blue Exclusion Assay - Nuclear morphology staining with Hoechst 33258 and Trypan blue assay were performed as previously established in our laboratory (Gozzelino et al. 2008). The cell nuclei were visualized with a Nikon ECLIPSE TE2000-E microscope equipped with epifluorescence optics under UV illumination and a Hamamatsu ORCA-ER photographic camera. Stained nuclei were scored as stage I (peripheral chromatin condensation), stage II (nuclear pyknosis and karyorrhexis) or stage III (nuclear shrinkage and highly compacted chromatin) chromatin condensation.

Transmission Electron Microscopy - Cells were treated with TRAIL plus gossypol for 4 h. Then, cells were collected and processed as previously described (Sánchez-Osuna et al. 2014). Ultrathin sections were evaluated with a transmission electron microscope (Jeol JEM-1400) equipped with a CCD GATAN ES1000W Erlangshen camera.

DEVD-directed Caspase Activity - Quantitative DEVD-like activities in cell lysates were performed as previously described (Yuste et al. 2001). The resulting 96-multiwell microplates were incubated at 35ºC and a kinetic measure of activity was performed during 10 hours (one hour per lecture point) in a BIO-TEK Synergy HT fluorimeter with excitation filter of 360 nm (40 nm bandwidth) and emission filter of 530 nm (25 nm bandwidth).

Protein Extractions and Western Blotting - Cells were lysed with Triton buffer (50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1X protease inhibitor cocktail (Sigma)) unless otherwise indicated. To obtain total extracts, SET buffer (10 mM Tris-HCl, pH 6.8, 150 mM NaCl, 1 mM EDTA, 1% SDS) was used as previously explained (Sánchez-Osuna et al. 2014). Alternatively, cytosolic (C), nucleoplasmic (N1) and chromatinic-enriched (N2) fractions were obtained as previously established in our laboratory (Iglesias-Guimarais et al. 2012). The protein concentration in the supernatants was quantified by a modified Lowry assay (DC protein assay, Bio-Rad, Barcelona, Spain) and 5- 35 μg of protein were loaded

in SDS-polyacrylamide gels. Protein lysates were electrophoresed, electrotransferred and probed with the appropriate specific antibodies.

Oligonucleosomal (LMW) DNA Degradation Analysis – DNA was extracted and purified as previously described (Ribas et al. 2008). Then, DNA was electrophoresed in a conventional agarose gel (1.8% agarose in TAE solution), stained in 0.5 μg/ml of ethidium bromide and then visualized using a Syngene Gene Genius UV transilluminator equipped with a photographic camera.

Transfection of DFF40/CAD - LN-18 cells were transfected with the eukaryotic expression vector pcDNA3 containing or not the open reading frame of the human CAD as previously explained (Sánchez-Osuna et al. 2014) and used as a pool.

Transfection of siRNA - siRNA transfection was performed by using DharmaFECT siRNA Transfection Reagent according to manufacturer's instructions. Three (for CAD) or four (for AIF) days after transfection, cells were detached and reseeded in adequate plates. To evaluate the down-regulation of protein expression Western blots of total protein extracts were performed at the initial time of treatment. The sequences employed were: 5′-GGAACAAGAUGGAAGAGAA-3′ for CAD and 5′-GCATCAGGGGGCAAAATCG-3′ for AIF.

Results

We have previously reported the apoptotic outcomes of glioblastoma cells when treated with different apoptotic stimuli. We found that caspase-dependent cell death occurred in the absence of apoptotic hallmarks (Sánchez-Osuna et al. 2014). Despite correct activation of caspases and ICAD degradation, nuclear alterations in apoptotic glioblastoma cells did not depend on CAD, the major endonuclease associated to apoptotic nuclear morphology. Intriguingly, isolated nuclei from glioblastoma-derived LN-18 cells failed to display apoptotic nuclear alterations when combined with proficient cytoplasms (i.e. from staurosporine-treated neuroblastoma-derived SH-SY5Y cells) *in vitro*, pointing to additional mechanisms regulating nuclear changes in glioblastoma cells.

BH3 mimetics induced nuclear heterogeneous responses in glioblastoma-derived LN-18 cells.

Glioblastoma cells can evade apoptosis through a variety of mechanisms. Antiapoptotic Bcl-2 family members have been widely related to this apoptosis resistance in glioblastoma. Therefore, we decided to test the response of glioblastoma-derived LN-18 cells to different BH3-only-like or BH3 mimetics compounds. We used ABT-737 (inhibitor of Bcl-2, Bcl-X_L and Bcl-w) (Oltersdorf et al. 2005), chelerytrine (Bcl-X_L inhibitor) (Chan et al. 2003), EM20- 25 (Bcl-2 inhibitor) (Milanesi et al. 2006); AT-101 or gossypol (Bcl-2, Bcl-X_L and Mcl-1 inhibitor) (Zhang et al. 2003; Kitada et al. 2003), HA14-1 (Bcl-2, Bcl-X_L inhibitor) (Wang et al. 2000); obatoclax (inhibitor of Bcl-2, Bcl-X_L, Bcl-w and Mcl-1) (Pérez-Galán et al. 2007; Trudel et al. 2007; Nguyen et al. 2007), TW37 (Bcl-2, Bcl-X_L and Mcl-1 inhibitor) (Zeitlin et al. 2006).

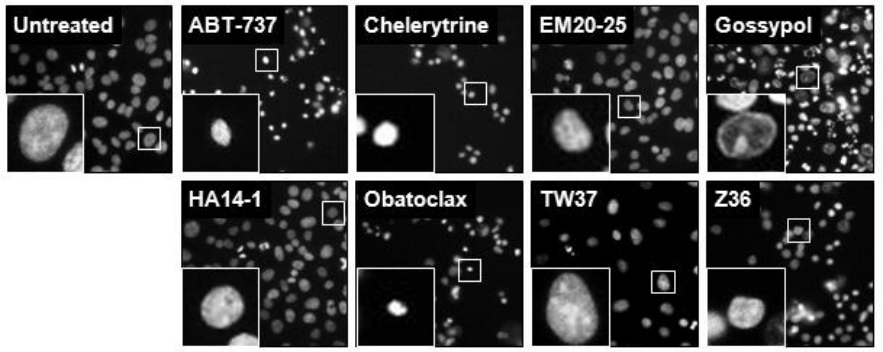

Figure 1. BH3 mimetics induce heterogeneous responses in LN-18 glioblastoma cells. LN-18 cells were treated with 50 μM ABT-737, 20 μM chelerythrine, 50 μM EM 20-25, 100 μM gossypol, 100 μM HA14-1, 100 μM obatoclax, 10 μM TW37 and 25 μM Z36. After 24 h, cells were fixed, stained with bisbenzimide and photographed under UV light. Magnifications show representative morphologies in each condition. *Scale bar = 40 μm.*

As shown in Fig. 1, BH3 mimetics induced different morphological alterations in LN-18 cells. ABT-737 and obatoclax induced type III chromatin condensation, depicted by pyknotic nuclei without signs of nuclear fragmentation. Gossypol induced a peripheral chromatin condensation characteristic of the type I nuclear morphology (Fig. 1). Chelerytrine, EM20-25, HA14-1 and Z36 reduced nuclear size without showing chromatin condensation whereas TW37 did not affect nuclear aspect (Fig. 1).

The combination of gossypol with extrinsic apoptotic ligands induces nuclear fragmentation in LN-18 glioblastoma cells.

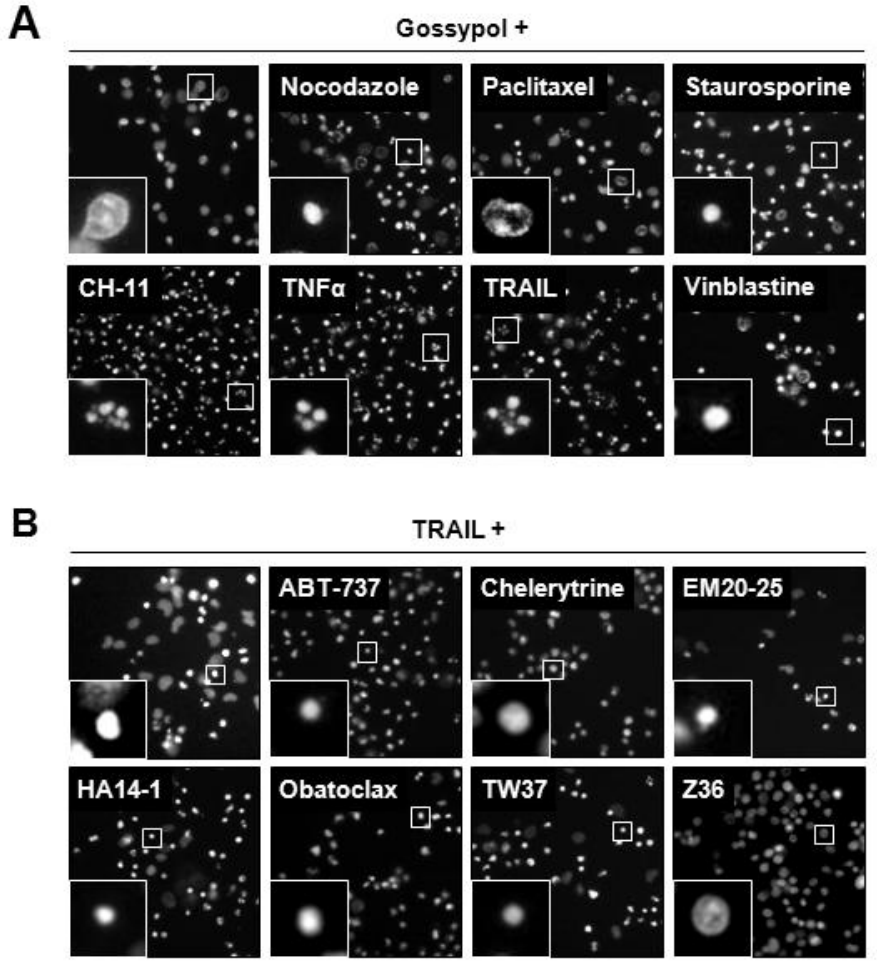

Figure 2. Type II nuclear morphology is observed in LN-18 cells after the treatment with gossypol plus death ligands. A, LN-18 cells were treated with 100 μM gossypol combined with 100 μM nocodazole, 50 μM paclitaxel, 1 μM staurosporine, 50 μM vinblastine, 0,1 μg/ml CH11, 0,1 μg/ml TNFα or 0,1 μg/ml TRAIL. **B,** LN-18 cells were treated with 0,1 μg/ml TRAIL alone or in combination with 50 μM ABT-737, 20 μM chelerythrine, 50 μM EM 20-25, 100 μM HA14-1, 100 μM obatoclax, 10 μM TW37 and 25 μM Z36. **A and B,** cells were fixed with paraformaldehyde, stained with Hoechst and photographed under UV light. Magnifications show representative morphologies in each condition. *Scale bar = 40 μm.*

Since type I chromatin condensation is considered an early step precluding stage II nuclear morphology (Samejima & Earnshaw 2005),

we wondered whether a combination of drugs inducing, on one hand, stage I chromatin condensation and a potent activation of caspases, on the other, can overcome the resistance of glioblastoma cells to apoptosis. To test our hypothesis, we cotreat glioblastoma cells with gossypol plus other proapoptotic drugs inducing cell death in glioblastoma cells (Sánchez-Osuna et al. 2014). The combination of gossypol with intrinsic apoptotic insults (i.e. nocodazole, paclitaxel, staurosporine or vinblastine) induced stage III chromatin condensation (Fig. 2A), meaning that gossypol cannot affect the nuclear alterations induced by these proapoptotic drugs alone (Sánchez-Osuna et al. 2014). However, extrinsic apoptotic insults such as TNFα, TRAIL or CH-11, that provoked stage III chromatin condensation when employed alone (Sánchez-Osuna et al. 2014), efficiently generated apoptotic stage II nuclear morphology in combination with gossypol (Fig. 2A). Noteworthy, other BH3 mimetics unable to generate stage I nuclear morphology on their own, failed to induce apoptotic nuclear fragmentation in combination with TRAIL (Fig. 2B).

Nuclear alterations induced by TRAIL plus gossypol cotreatment were deeper evaluated by electron microscopy. Ultrastructural analysis revealed that the combination of gossypol plus TRAIL generated different masses of chromatin clearly separated by nuclear envelope (Fig. 3). Since those apoptotic nuclear bodies were completely closed, cells cotreated with TRAIL and gossypol died without releasing nuclear content.

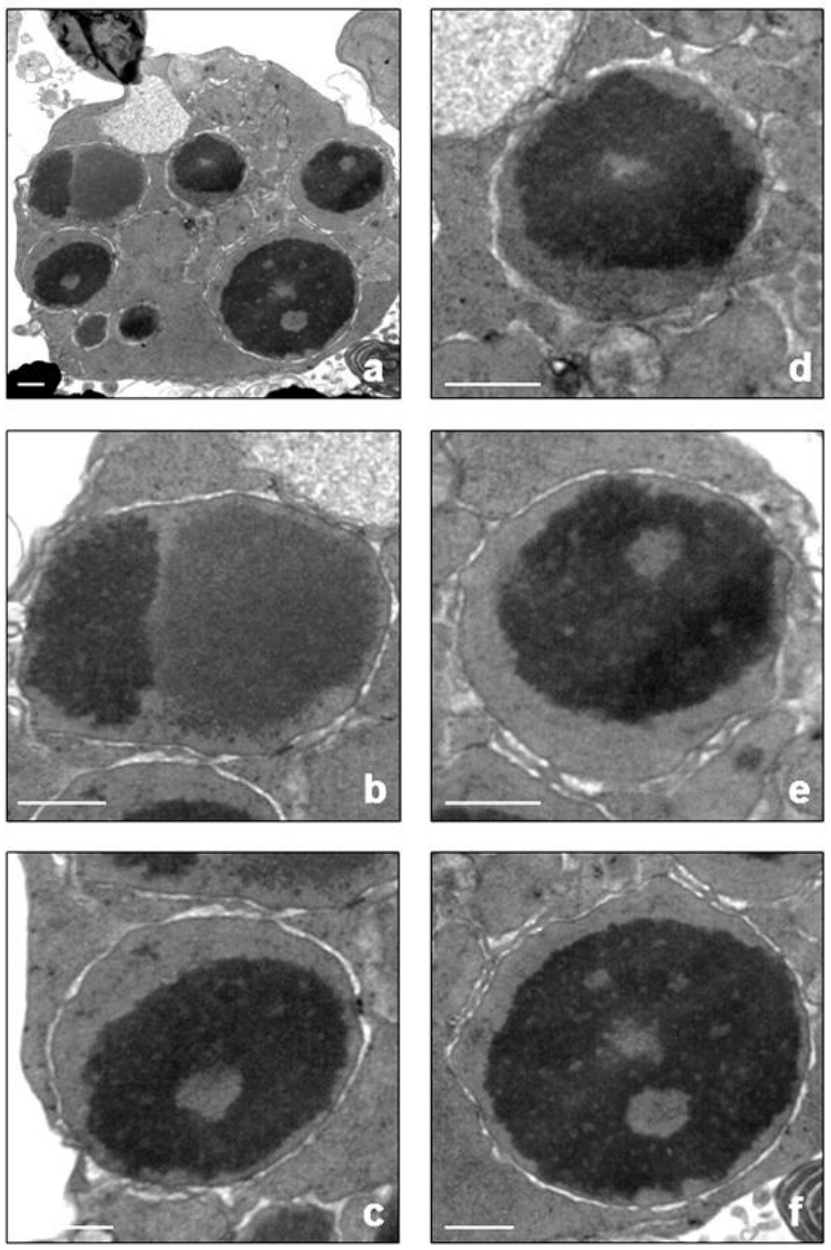

Figure 3. Small pieces of condensed chromatin surrounded by nuclear envelope are observed after TRAIL plus gossypol treatment in LN-18 cells. LN-18 cells were treated with 0,1 µg/ml TRAIL plus 100 µM gossypol during 4 h. **a,** representative image of electron microscopy. **b to f,** magnifications of fragments of chromatin within apoptotic nuclear bodies. *Scale bar: 0.5 µm*

Stage II chromatin condensation in LN-18 glioblastoma cells treated with TRAIL plus gossypol depends on caspase activation and DFF40/CAD.

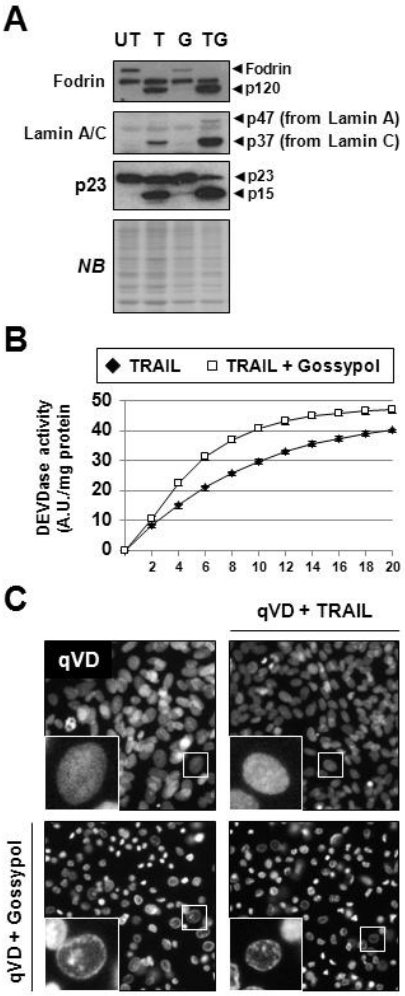

Figure 4. TRAIL plus gossypol and TRAIL but not gossypol alone induce caspase-dependent nuclear morphology in LN-18 cells. A, LN-18 cells were treated with 0.1 µg/ml TRAIL (T), 100 µM gossypol (G), TRAIL plus gossypol (TG) or left untreated (UT). After 6 h, cells were detached and protein extracts were obtained. Immunoblots against fodrin, Lamin A/C and p23 are shown. Naphthol Blue (NB) is used as loading control. **B,** DEVD-directed enzymatic activity was assessed in protein extracts from LN-18 cells treated with TRAIL (filled squares) and TRAIL plus gossypol (empty squares) during 6 h. **C,** LN-18 cells were treated with 50 µM qVD alone or in combination with TRAIL, gossypol or with TRAIL plus gossypol. After 24 h of treatment, cells were fixed, stained with Hoechst and photographed under UV light. Magnifications show representative morphologies in each condition. *Scale bar = 40 µm*.

To explore the biochemical effects of the combination of TRAIL plus gossypol, we analyzed the activation of executioner caspases. We used the processing of fodrin (specific substrate of caspase-3), lamin A/C (specific substrate of caspase-6) and cochaperone p23 (specific substrate of caspase-7) as specific markers of caspase activation. After TRAIL treatment, p120 fragment from fodrin, p47/37 fragments from lamin A/C and p15 fragment from cochaperone p23, appeared (Fig. 4A). Although gossypol did not induce caspase activation, depicted by the absence of these fragments, gossypol plus TRAIL generated the

processing of caspase substrates efficiently (Fig. 4A). Moreover, DEVD-directed caspase like activity assay showed that lysates from LN-18 cells treated with TRAIL plus gossypol contained higher levels of caspase activation compared to lysates from TRAIL-treated cells (Fig. 4B).

To determine whether caspase activation is a later consequence or a cause of the TRAIL plus gossypol-induced cell death, we took advantage of the pan-caspase inhibitor qVD-OPh (qVD). After Hoechst staining, we observed that nuclear morphology induced by TRAIL, but not that mediated by gossypol, was dependent on caspase activation (Fig. 4C). Moreover in the presence of qVD, TRAIL plus gossypol cotreatment induced stage I chromatin condensation like gossypol alone (Fig. 4C). Similarly, the presence of qVD avoided the stage II nuclear morphology induced by the cotreatment of gossypol plus other extrinsic apoptotic inductors such as TNFα or CH11 (Fig. 5). Thus, both type II and III nuclear morphologies induced by TRAIL plus gossypol or TRAIL alone but not gossypol-induced type I are caspase-dependent processes.

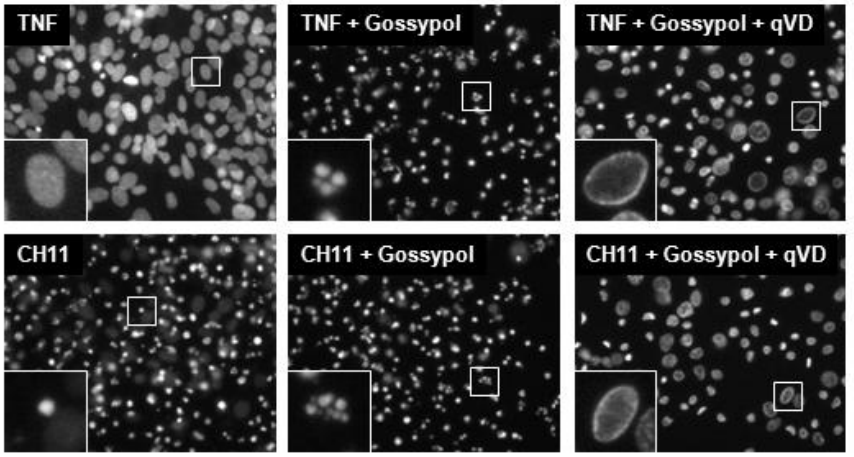

Figure 5. The combination of gossypol with other death ligands such as TNFα or CH11 also induces caspase-dependent stage II nuclear morphology. LN-18 cells were treated with 0.1 μg/ml CH11 or 0.1 μg/ml TNFα combined or not with 100 μM gossypol in the presence or not of qVD. After 24 h of treatment, cells were fixed, stained with bisbenzimide and photographed under UV light. Magnifications show representative morphologies in each condition. *Scale bar = 40 μm.*

DFF40/CAD is involved in the nuclear alterations induced by TRAIL plus gossypol in LN-18 cells.

Caspase-activated DNAse (DFF40/CAD) is the main apoptotic nuclease controlling nuclear fragmentation, during caspase-dependent apoptosis. Previous studies made with staurosporine demonstrated that caspase activation does not guarantee CAD activation in glioblastoma cells. To explore whether DFF40/CAD was actually involved in the morphological alterations observed after TRAIL plus gossypol cotreatment, we knockdown CAD protein levels with a specific siRNA. As shown in Fig. 6, the absence of CAD abolished the stage II chromatin condensation induced by TRAIL plus gossypol, generating a type III nuclear morphology similar to that observed in TRAIL-treated cells. However, the knockdown of the endonuclease did not alter the stage III chromatin condensation induced by TRAIL or the stage I chromatin condensation induced by gossypol (Fig. 6). This result agrees with previous data supporting that CAD is only involved in the type II nuclear morphology but not in type I or III (Samejima, Tone, & Earnshaw, 2001; Yuste et al., 2005; Sánchez-Osuna et al., 2014).

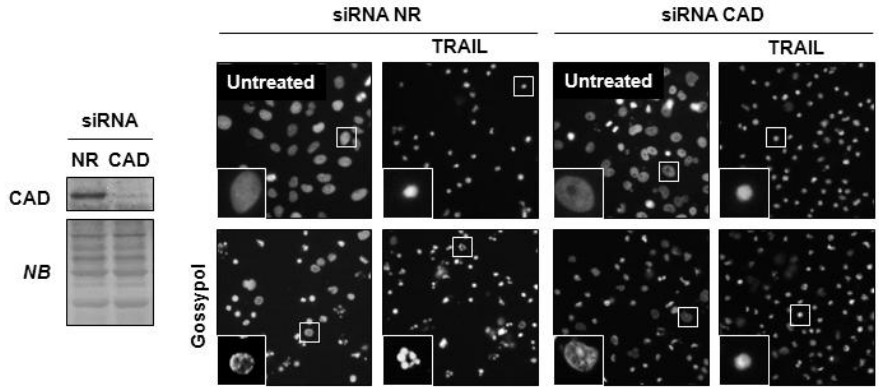

Figure 6. DFF40/CAD mediates nuclear morphology induced by TRAIL plus gossypol in LN-18 cells. LN-18 cells were transfected with the siRNA against CAD (CAD) or against a non-relevant sequence (NR). 3 days after transfection cells were left untreated or treated with 0.1 µg/ml TRAIL, 100 µM gossypol or both during 24h. Cells were then fixed, stained with Hoechst and visualized under UV light. Magnifications show representative morphologies in each condition. *Scale bar = 40 µm.*

Stage I chromatin condensation has been mainly related to the activation of apoptosis inducing factor (AIF) (Susin et al. 1999). AIF is a mitochondrial protein that results cleaved and translocated to the

nucleus early during apoptosis. Once in the nucleus, AIF promotes the characteristic ring-shaped chromatin condensation in a caspase-independent manner. Therefore, we decided to analyze whether gossypol nuclear changes are due to AIF activation. Surprisingly, AIF siRNA affects neither gossypol-triggered stage I nor stage II chromatin condensation observed after cotreatment with TRAIL (Fig. 7). As expected, AIF knockdown demonstrated that this factor is not necessary for the stage III chromatin condensation observed during TRAIL-induced cell death in glioblastoma cells (Fig. 7).

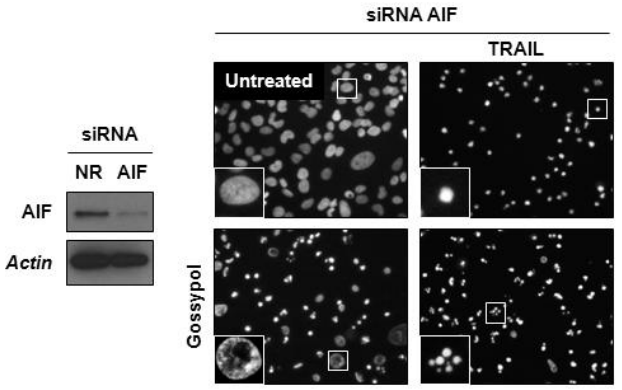

Figure 7. AIF does not mediate nuclear morphology induced by gossypol, TRAIL or their combination in LN-18 cells. LN-18 cells were transfected with the siRNA against AIF (AIF) or against a non-relevant sequence (NR). 4 days after transfection cells were left untreated or treated with 0.1 μg/ml TRAIL, 100 μM gossypol or both during 24h. Cells were then fixed, stained with Hoechst and visualized under UV light. Magnifications show representative morphologies from each condition. *Scale bar = 40 μm.*

The combination of TRAIL plus gossypol fails to generate DNA laddering in LN-18 glioblastoma cells.

Besides mediating nuclear changes, CAD endonuclease is involved in oligonucleosomal DNA degradation during apoptosis. Since CAD was fully competent to mediate morphological nuclear changes after gossypol plus TRAIL cotreatment, we decided to analyze apoptotic DNA degradation. Neither TRAIL nor gossypol alone was able to induce oligonucleosomal DNA degradation in LN-18 glioblastoma cells (Fig. 8A). However, the cotreatment with TRAIL plus gossypol induced just a slight DNA laddering (Fig. 8A).

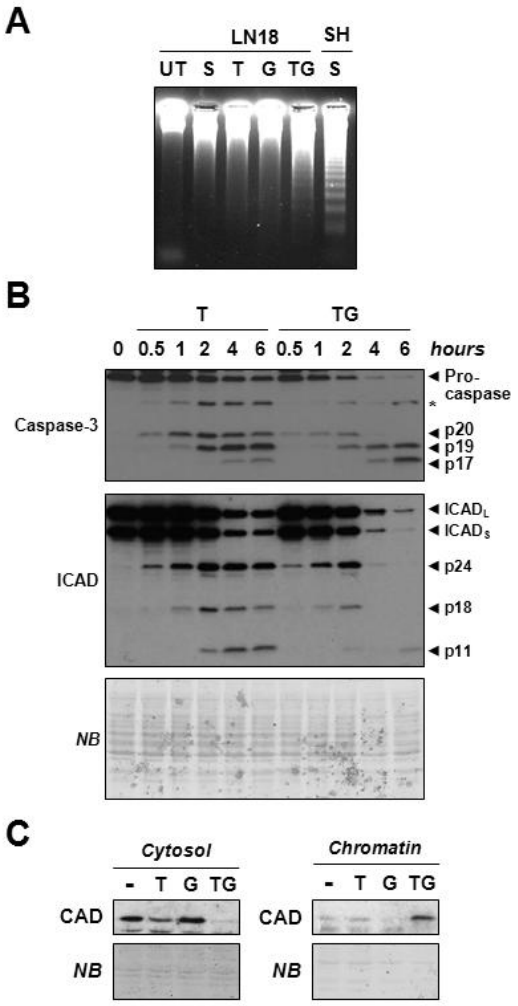

Figure 8. TRAIL plus gossypol cotreatment induces only slight oligonucleosomal DNA degradation on LN-18 cells despite triggering ICAD degradation and CAD translocation to the nucleus. A, cells were treated with 1 μM staurosporine (S), 0.1 μg/ml TRAIL (T), 100 μM gossypol (G), TRAIL plus gossypol (TG) or left untreated (UT). After 6 h, cells were collected and oligonucleosomal DNA degradation was analyzed. Note that neuroblastoma-derived SH-SY5Y (SH) cells are used as a positive control. **B,** LN-18 cells were treated with TRAIL or TRAIL plus gossypol for the indicated times. Then, cells were collected and protein extracts were obtained. Caspase-3 activation and ICAD degradation were analyzed through western blot. **C,** cytosolic and chromatinic-enriched extracts were obtained from untreated LN-18 cells or cells treated with TRAIL (T), gossypol (G) or both (TG) during 6 h. CAD protein levels were assessed by western blot. Naphthol Blue (NB) was used as loading control in **B and C**.

Since the caspase-3/ICAD/CAD axis is the minimal system regulating DNA laddering, we analyzed the activation of caspase-3 and the degradation of ICAD during treatment. Caspase-3-preactivation fragment (p20) was observed already after half an hour of TRAIL treatment whereas caspase-3-activation fragments (p19/17) appeared after 2 and 4 h of TRAIL respectively (Fig. 8B, first panel). Conversely, gossypol plus TRAIL induced a more drastic caspase-3 processing. While, p19 fragment was not detected till 2 h of treatment, procaspase as well as the p20 preactivation fragment completely disappeared after 4 h, being p19/17 the only caspase-3 detected

fragments (Fig. 8B, first panel). Likewise, ICAD, sequentially degraded during TRAIL treatment, was abruptly degraded during TRAIL plus gossypol cotreatment (Fig. 8B, second panel). Interestingly, cytosolic CAD mobilized better to the nucleus after TRAIL plus gossypol cotreatment than after TRAIL treatment (Fig. 8C). These higher levels of CAD in the chromatinic-enriched fraction correlated with a complete depletion of cytosolic CAD in lysates from TRAIL plus gossypol cotreated cells (Fig. 8C).

CAD overexpression is sufficient to generate DNA laddering in apoptotic LN-18 cells and facilitates stage II nuclear morphology after TRAIL plus gossypol cotreatment.

Therefore, we decided to use CAD-overexpressing LN-18 glioblastoma cells. According to previous data (Sánchez-Osuna et al. 2014), CAD-overexpressing but not mock-transfected cells displayed DNA laddering after treatment with staurosporine (Fig. 9A). The same result was obtained after TRAIL treatment (Fig. 9A). Moreover, the slight DNA laddering generated after the cotreatment with TRAIL plus gossypol in mock-transfected cells was clearly detected in CAD-overexpressing LN-18 cells (Fig. 9A). Thus, the low levels of the endonuclease seemed to be the reason why LN-18 cells cannot display DNA laddering during apoptosis.

Regarding nuclear morphology, CAD overexpression did not induce any significant difference after TRAIL treatment. Nevertheless, CAD-overexpressing cells displayed nuclear fragmentation easily after gossypol or gossypol plus TRAIL cotreatment. Around 39% (38.61 ± 8.19%) of mock-transfected cells displayed stage II chromatin condensation during gossypol plus TRAIL cotreatment, whereas the percentage increased up to 58% (58.06 ± 4.28%) for CAD-overexpressing cells (Fig. 9C). Moreover, levels of karyorrhexis after gossypol treatment were also slightly higher in CAD-overexpressing cells (15.79 ± 6.46 %) compared to those observed in mock-transfected cells (6.71 ± 2.07%). These results pointed that, besides TRAIL-promoted CAD activation, other requirements only achieved after gossypol exposure are needed to promote apoptotic type II nuclear morphology.

CAD translocation from cytosol to the nucleus during apoptosis does not correlate with the ability of LN-18 cells to display type II nuclear morphology.

Due to the relevance of CAD translocation to the nucleus during apoptosis, we decided to analyze the specific location of caspase-3 activation and ICAD degradation after the treatments. After 6 h of TRAIL, both mock- and CAD-transfected cells showed similar levels of caspase-3 processing and ICAD degradation (Fig. 9B). However, as already observed for other caspase substrates, caspase-3 and ICAD were more efficiently activated or degraded, respectively, after the combination of gossypol plus TRAIL. Noteworthy, both caspase-3 and ICAD were processed at the same extent in the cytosol of mock- and CAD-transfected LN-18 cells (Fig. 9B). Moreover, like in wild type cells, cytosolic CAD protein levels slightly diminished after TRAIL treatment in both mock- and CAD-overexpressing LN-18 cells. After TRAIL plus gossypol cotreatment, cytosolic CAD strongly diminished in CAD-overexpressing cells whereas completely disappeared in mock transfected cells (Fig. 9B). Accordingly, analysis of the chromatinic-enriched fraction revealed that TRAIL plus gossypol cotreatment induced better translocation of CAD to the nucleus than TRAIL treatment (Fig. 9B, lower panels).

The amount of CAD in the nuclei of TRAIL-treated CAD-overexpressing cells is higher than the amount of endonuclease in the nuclei of mock-transfected cells after TRAIL plus gossypol cotreatment (Fig. 9B). Intriguingly, CAD-overexpressing cells were unable to display apoptotic nuclear fragmentation after treatment with TRAIL whereas the cotreatment with TRAIL plus gossypol generated type II apoptotic nuclear morphology independently of CAD total protein level.

Altogether, our results show that CAD translocation to the nucleus does not correlate with apoptotic nuclear morphology but with the ability of LN-18 cells to undergo DNA laddering during cell death.

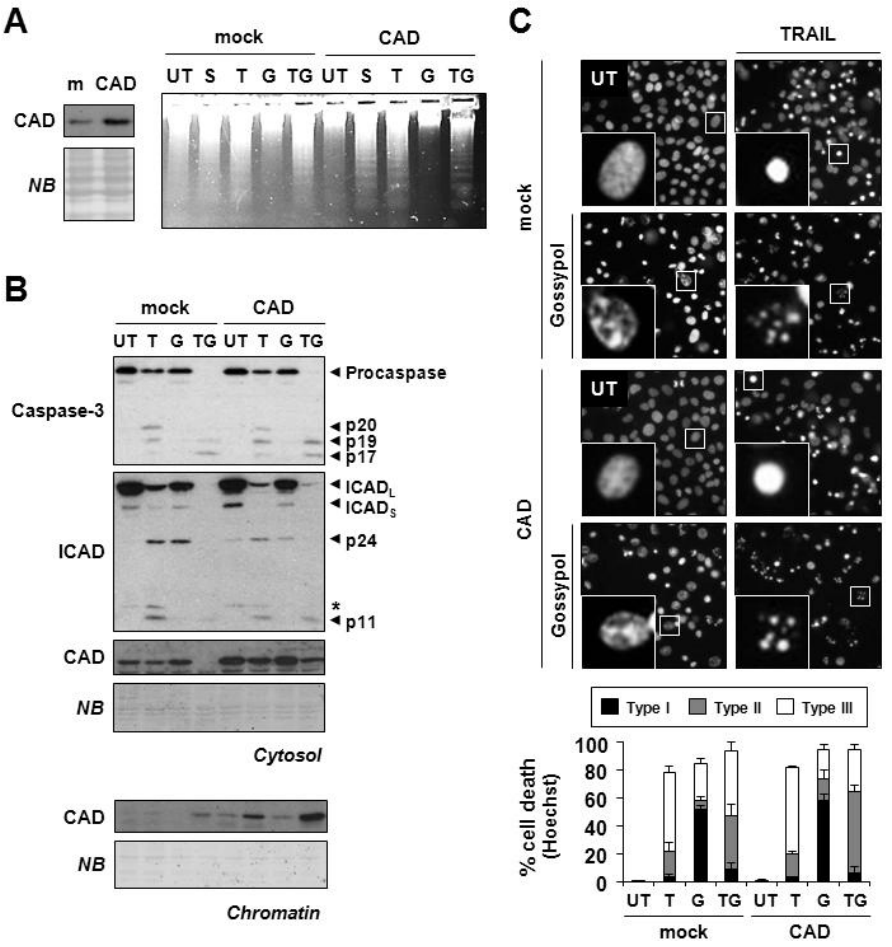

Figure 9. CAD overexpression allows apoptotic LN-18 cells undergoing DNA laddering without altering nuclear morphology. Mock-transfected and CAD-overexpressing LN-18 cells were treated with 1 µM staurosporine (S), 0.1 µg/ml TRAIL (T), 100 µM gossypol (G), TRAIL plus gossypol (TG) or left untreated (UT). **A left,** CAD protein levels were assessed in extracts obtained from mock-transfected or CAD-overexpressing cells. **On the right,** after 6 h of treatment cells were collected and DNA extracted according to 'Oligonucleosomal DNA Degradation Analysis' procedure. **B,** cytosolic and chromatinic fractions were obtained from untreated cells or after 6 h of treatment with TRAIL, gossypol or TRAIL plus gossypol. Protein levels of cytosolic caspase-3, ICAD and CAD were assessed by western blot. CAD protein levels in the chromatinic fraction are also shown. Naphthol Blue (NB) staining was used as a loading control. **C,** cells treated during 24 h were fixed, stained with Hoechst and visualized with an epifluorescence microscope. Representative nuclear morphologies from each condition are augmented. Nuclei were counted and scored as type I, II or III. Cell death is represented by the means ± SD obtained after counting more than 300 cells. *Scale bar = 40 µm.*

CAD translocation from LN-18 cytosolic lysates to the nucleus is insufficient to generate DNA laddering in vitro.

To deeply study the balance between caspase activity, CAD mobilization and DNA laddering, we performed a cell free *in vitro* assay with exogenous caspase-3. The same amount of recombinant caspase-3 was added to lysates from untreated neuroblastoma-derived SH-SY5Y cells (able to undergo DNA laddering during apoptosis) or glioblastoma-derived LN-18 cells (unable to display DNA laddering after apoptotic stimuli). Exogenous caspase-3 induced endogenous caspase-3 cleavage and complete ICAD degradation in lysates from SH-SY5Y and LN-18 cells (Fig. 10A, first and second panels). Moreover, recombinant caspase-3 induced a nuclear translocation of cytosolic CAD in both cases (Fig. 10B). However, while CAD protein levels in the cytosol of LN-18 cells (supernatant of the reaction) were completely depleted after addition of recombinant caspase-3, the endonuclease was still detected in SH-SY5Y supernatants after the reaction (Fig. 10B, first panel). Importantly, since total levels of CAD in glioblastoma cells were lower (Fig. 10A, third panel), CAD protein levels reached in isolated nuclei when incubated with lysates from LN-18 cells were also lower (Fig. 10B, second panel). Accordingly, while lysates from SH-SY5Y cells incubated with recombinant caspase-3 generated DNA laddering, lysates from LN-18 cells were unable to hydrolyze the chromatin from isolated nuclei (Fig. 10C).

At that point, we decided to perform a cell free assay with increasing amounts of protein from LN-18 glioblastoma cells. Higher amounts of protein generated higher amounts of p19/17 caspase-3 activation fragments and p11 ICAD fragment (Fig. 11A), which correlated with better translocation of CAD to the nucleus (Fig 10B). Nevertheless, to reach similar levels of nuclear CAD to those obtained when SH-SY5Y cytoplasms were used, we needed four times more concentrated lysates from LN-18 cells (Fig 11B). Surprisingly, even when 1 μg of cytosolic proteins from LN-18 cells was used, just a slight signal of DNA laddering was detected (Fig. 11C).

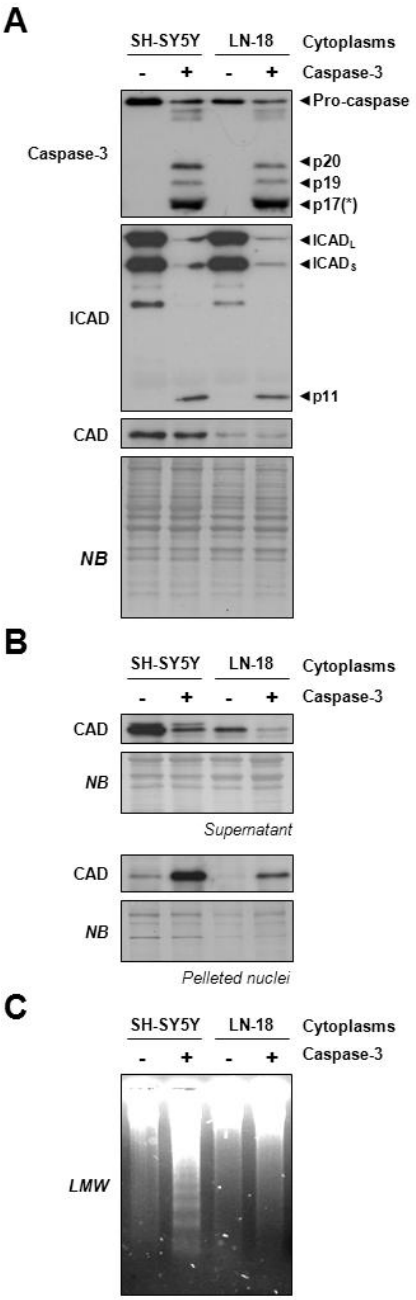

Figure 10. Complete translocation of cytosolic DFF40/CAD from glioblastoma lysates to isolated nuclei is insufficient to trigger DNA laddering *in vitro*. Protein extracts from untreated LN-18 and SH-SY5Y cells and isolated nuclei were obtained as indicated in experimental procedures for 'Cell-free assay'. **A,** after the cell-free *in vitro* reaction, in the presence or not of recombinant caspase-3, caspase-3, ICAD and CAD total protein levels were analyzed through western blot. **B,** CAD protein levels present in the cytosol and in the nuclei after the cell-free *in vitro* reaction are shown. Naphthol Blue (NB) staining is used as loading control in A and B. **C,** DNA from isolated nuclei was extracted and analyzed accordingly to *LMW DNA Degradation Analysis*.

Then, we performed another cell-free assay with decreasing amounts of protein from SH-SY5Y cells. As expected, lower amounts of protein induced lower levels of caspase-3 activation fragments and p11 ICAD degradation fragment (Fig. 11D) and lower levels of CAD translocation to the nucleus (Fig. 11E). Surprisingly, five times less amount of protein from SH-SY5Y cells, and consequently, of nuclear CAD, was sufficient to generate DNA laddering in isolated nuclei (Fig. 11F). Altogether, these results suggested that, despite the release of the endonuclease from its inhibitor, ICAD, and translocation to the nucleus, additional mechanisms might restrict DFF40/CAD activity in LN-18 cells.

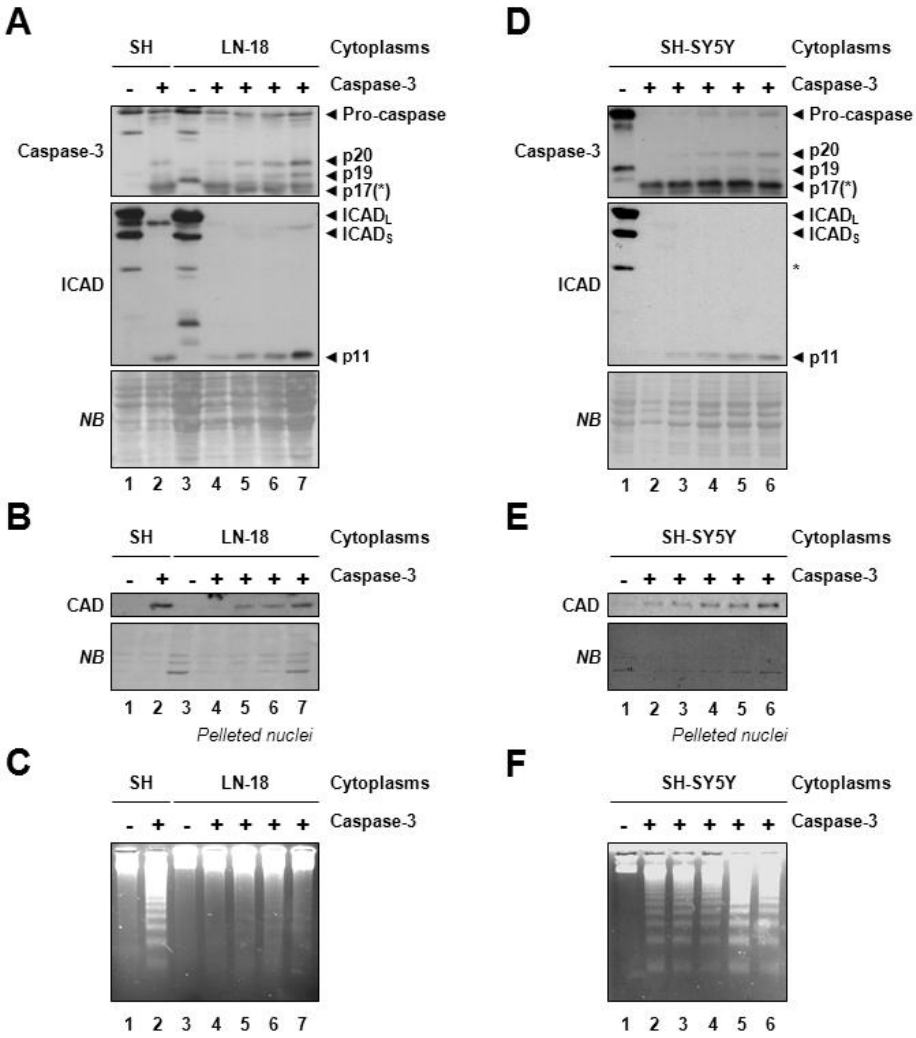

Figure 11. CAD from LN-18 extracts is insufficient to generate DNA laddering *in vitro*. A, cell-free in vitro reaction was performed with 250 µg of SH-SY5Y protein extracts or increasing amounts of LN-18 protein extracts: 250 (4), 500 (5), 750 (6) or 1000 (3 and 7) µg. Total levels of caspase-3 and ICAD are shown. **D,** cell-free in vitro reaction was performed different amounts of SH-SY5Y protein extracts: 250 µg (1 and 6), 50 µg (2), 100 µg (3), 150 µg (4) or 200 µg (5). Total levels of ICAD and caspase-3 are shown. **B and E,** CAD protein levels in the nucleus were analyzed through western blot. **C and F,** DNA laddering was assessed after the in vitro reaction. Naphthol Blue staining is used as loading control in **A, B, D and E**.

Gossypol can sensitize TRAIL-resistant glioblastoma cells to death ligand-mediated apoptosis.

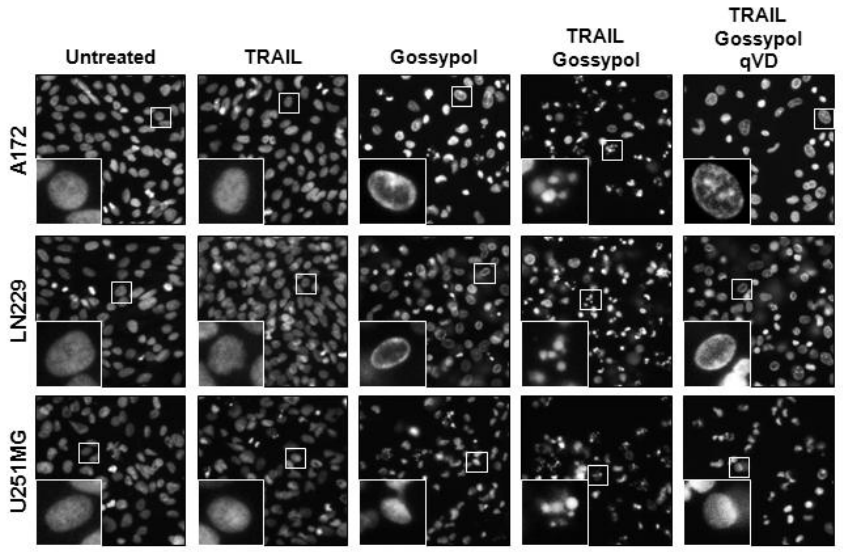

Figure 12. Gossypol plus TRAIL cotreatment efficiently induced caspase-dependent stage II nuclear morphology in TRAIL-resistant glioblastoma cells. A721, LN229 and U251-MG glioblastoma cells were treated during 24 h with 0.1 µg/ml TRAIL, 100 µM gossypol, TRAIL plus gossypol with or without 50 µM qVD or left untreated. Cells were then fixed with paraformaldehyde, stained with bisbenzimide and photographed under UV light. Magnifications show representative morphologies from each condition. *Scale bar = 40 µm.*

Our results showed that gossypol is able to synergize with death ligands to induce extrinsic apoptosis in TRAIL-responsiveness LN-18 glioblastoma cells. Nevertheless, many glioblastoma cells are resistant to TRAIL cytotoxicity, which raised the question whether gossypol can also sensitize TRAIL-resistant cells to apoptosis. We used three different glioblastoma cell lines resistant to TRAIL cytotoxicity: A172, LN229 and U251MG. Similar to LN-18 cells, A172 and LN229 cells displayed stage I chromatin condensation after gossypol treatment. Conversely, gossypol failed to generate peripheral condensation of the chromatin in U251MG cells (Fig. 12). More importantly, although resistant to TRAIL, A172, LN229 and U251MG cells displayed classical stage II chromatin condensation when cotreated with TRAIL plus gossypol. Noteworthy, these apoptotic morphologies were dependent on caspase activation since they were abolished in the presence of qVD (Fig. 12, right panels). Therefore, we concluded that

gossypol not only facilitates apoptotic nuclear changes in TRAIL-sensitive LN-18 cells, but also sensitizes TRAIL-resistant glioblastoma cells to extrinsic apoptosis.

Discussion

We have previously described different nuclear alterations in glioblastoma cells, including type I, II and III chromatin condensation after cytotoxic insult. Moreover, CAD, the main apoptotic nuclease, does not alter any of these nuclear changes occurring during caspase-dependent cell death in glioblastoma cells. Here, we show that gossypol allows glioblastoma cells to undergo classical type II nuclear morphology after extrinsic apoptotic insult. Interestingly, gossypol plus TRAIL cotreatment generates classical apoptotic nuclear bodies, characterized by small masses of condensed chromatin surrounded by unbroken nuclear envelope. That contrasts with the incomplete type II nuclear morphology previously observed, characterized by the presence of chromatin-free apoptotic nuclear bodies just composed by nuclear envelope (e.g. LN-18 cells) or different masses of chromatin within the same nuclear compartment (e.g. A172 cells) (Sánchez-Osuna et al. 2014 and Sánchez-Osuna et al. manuscript in preparation).

Interestingly, gossypol and TRAIL cotreatment induces stronger caspase activation than TRAIL alone. The presence of a preapoptotic complex of procaspase-3, Hsp60 and Hsp10 in mitochondrial fraction of Jurkat cells has previously been described (Samali et al. 1999; Xanthoudakis et al. 1999). Then, if this preapoptotic complex would exist in glioblastoma cells, it may be possible that the combination of TRAIL and gossypol efficiently triggers the activation of this specific population of the executioner caspase. Indeed, it has been proposed that after mitochondrial caspase-3 activation, the release of Hsps accelerates cytosolic caspase-3 maturation (Samali et al. 1999), which might explain the results obtained regarding the abrupt activation of caspase-3 after TRAIL plus gossypol treatment.

Remarkably, gossypol-mediated nuclear morphology was not altered by the pan-caspase inhibitor qVD. These results support previous data pointing that stage I chromatin condensation occurs early during apoptosis in a caspase-independent fashion (Yuste et al. 2005; Toné et al. 2007). Unexpectedly, nuclear morphology induced by gossypol did not depend on AIF, which disagrees with previous data pointing to this flavoprotein as the major mediator of the stage I chromatin condensation (Susin et al. 1999). Whether this unknown

AIF-independent alternative mechanism is due to specific molecular alterations of LN-18 cells or to a general effect of gossypol remains unclear. Interestingly, in U251-MG cells the combination of gossypol plus TRAIL induced classical nuclear fragmentation although gossypol alone did not induce stage I chromatin condensation. This particular behavior suggests that stage I nuclear morphology is, in fact, a non-necessary consequence of gossypol effects. Thus, gossypol would affect apoptotic pathways at different levels and, besides triggering those characteristic nuclear changes, it must generate different and more important changes that allow classical apoptotic signaling after TRAIL-mediated caspase activation.

Gossypol has been essentially described as an inhibitor of Bcl-2, and Bcl-X_L. Moreover, gossypol can also inhibit antiapoptotic Mcl-1, a key protein orchestrating the apoptosis resistance in glioblastoma cells (Li et al. 2013; Murphy et al. 2014; Pareja et al. 2014). Nevertheless, other BH3 mimetics that also inhibit Bcl-2, Bcl-X_L or Mcl-1 failed to reproduce gossypol effects during TRAIL-triggered cell death. Therefore, the effects of gossypol regarding sensitization of glioblastoma cells to classical type II nuclear morphology cannot rely in its ability as BH3-mimetic compound. Interestingly, gossypol directly interacts and inhibits the apurinic/apyrimidinic endonuclease 1/redox enhancing factor-1 (APE1/Ref-1) (Qian et al. 2014). APE1/Ref-1 is an essential multifunctional protein involved in repairing oxidative DNA damage and transcriptional regulation that has been also related to DFF40/CAD-independent apoptotic DNA degradation (Yoshida et al. 2003). Moreover, APE1/Ref-1, commonly overexpressed in cancers, has been related to resistance to radiation- and chemotherapy-triggered apoptosis (Fan et al. 2003; Jiang et al. 2010; Chen et al. 2013; Cun et al. 2013). Specifically, APE1/Ref-1 overexpression contributes to resistance of gliomas to alkylating agents (Silber et al. 2002) whereas its inhibition prevents cell growth and enhances cytotoxicity of temozolomide and methyl methanesulfonate (MMS) (Bapat et al. 2010). Therefore, the effects of gossypol in glioblastoma cells during TRAIL-mediated apoptosis could be partially caused by APE1/Ref-1 inhibition.

Gossypol plus TRAIL cotreatment induces efficient translocation of cytosolic DFF40/CAD to the nucleus. However, increased levels of nuclear CAD during apoptosis do not always correlate with the ability of LN-18 glioblastoma cells to undergo stage II chromatin condensation suggesting that little amounts of CAD are sufficient to

trigger apoptotic nuclear changes under appropriate conditions. Interestingly, it has been suggested that nucleoplasmic rather than cytosolic fraction of CAD triggers chromatin condensation and nuclear disassembly during apoptosis (Iglesias-Guimarais et al. 2013). This hypothesis fits in glioblastoma cells, in which CAD is predominantly nucleoplasmic (Sánchez-Osuna et al. manuscript in preparation). Nevertheless, CAD cannot be the only factor regulating nuclear changes during apoptosis since stage II chromatin condensation is only observed after gossypol plus TRAIL treatment but CAD becomes also activated during TRAIL-mediated cell death. Interestingly, gossypol harbored DNase activity *in vitro* (Srivastava and Padmanaban 1987). Hence, maybe one possibility is that gossypol-mediated DNA nicks can cause the mobilization of nucleoplasmic proteins, including CAD. That might explain why after gossypol treatment, the protein quantification of nucleoplasmic fraction dropped to zero. Another possibility is that gossypol-mediated DNA nicks are directly involved in the generation of nuclear morphology. That may have sense since CAD-mediated single strand DNA breaks have proven to be determinant in the generation of the type II nuclear morphology (Iglesias-Guimarais et al. 2013).

Noteworthy, gossypol only triggers stage II chromatin condensation when combined with death ligands but not with stimuli triggering intrinsic apoptosis. Degradation of lamina proteins is a required step for the nuclear alterations occurring during apoptosis, including nuclear breakdown (Rao et al. 1996; Broers et al. 2002; Ruchaud et al. 2002). Although both intrinsic and extrinsic pathways can trigger degradation of lamina proteins, it has been suggested that the heterochromatin reorganization triggered by intranuclear lamina degradation depends on caspase-8 activation (Raz et al. 2006). Then, maybe both caspase-8 and gossypol-mediated chromatin reorganization are needed to allow apoptotic nuclear changes in glioblastoma cells.

Despite the high efficiency of gossypol and TRAIL cotreatment inducing apoptotic type II nuclear morphology, this combination triggers just a minor DNA laddering in glioblastoma cells, the other hallmark of apoptosis. We have previously demonstrated that low levels of cytosolic CAD impairs DNA laddering during apoptosis in different tumor cells, including glioblastoma-derived cells (Iglesias-Guimarais et al. 2012; Sánchez-Osuna et al. manuscript in preparation). However, cell free assays performed with increasing

amounts of cytosolic extracts from LN-18 cells showed that, as *in vivo*, better DFF40/CAD nuclear translocation induced only residual oligonucleosomal DNA degradation. Intriguingly, lower amounts of nuclear CAD were sufficient to clearly induce DNA laddering in isolated nuclei when cytosolic extracts from SH-SY5Y were used. Since CAD transfection is sufficient to induce oligonucleosomal DNA degradation after apoptotic insult (i.e. staurosporine, TRAIL or TRAIL plus gossypol), endogenous CAD stoichiometry might regulate DNA laddering. In vivo, monomeric forms of $ICAD_L$ and $ICAD_S$ are in excess respect to CAD (Lechardeur, Dougaparsad et al. 2005; Widlak, Lanuszewska et al. 2003). Indeed, it has been proven that CAD activation requires sufficient proteolysis of $ICAD_L$ (Lechardeur, Dougaparsad et al. 2005). Moreover, $ICAD_L$ free molecules can interact and inhibit CAD homoligomers previously activated (Widlak, Lanuszewska et al. 2003). In fact, stoichiometry between CAD and its inhibitor ICAD is an important mechanism to avoid inappropriate activation of the endonuclease in healthy cells (Widlak & Garrard 2005). Therefore, it could be possible that a disproportionate CAD/ ICAD balance restricts CAD endonucleolytic activity in glioblastoma cells.

The effectiveness of TRAIL treatments will depend on the responsiveness of tumor cells to this cell death ligand. Although TRAILR1 and TRAILR2 are expressed in 95% of glioblastoma samples (Frank et al. 1999), the percentage of GBM resistant to TRAIL-induced apoptosis is higher than 5%. Many tumors express higher levels of TRAILR2 compared with TRAILR1, whereas TRAILR2 signaling is only poorly activated by TRAIL (Mühlenbeck et al. 2000). Interestingly, the fusion of TRAIL to an antibody fragment not only improves pharmacokinetics but also converts soluble TRAIL into membrane-bound TRAIL that efficiently signals apoptosis via TRAILR1 but also TRAILR2 (Wajant et al. 2001; Bremer et al. 2004). Nevertheless, for those cases in which the resistance to TRAIL resides downstream the activation of the receptor, the co-administration with gossypol or similar derivatives could help to overcome the impairment, as already demonstrated for A172, LN229 and U251-MG cells.

Since the discovery of extracellular nucleic acids in the circulation in 1948, many researchers have looked for free DNA and its relation with diseases (Mandel 1948). It is known that free DNA can elicit immunological responses and autoimmunity disorders (Tan et al. 1966;

Martins et al. 2000; Miyake & Onji 2013). In cancer, it seems that the concentration of circulating DNA decreases with successful therapy (Leon et al. 1977; Stroun et al. 1989). However, in the case of gliomas, the partially conserved blood-brain barrier hinders large DNA molecules to reach blood, which makes that the free DNA fragments released from tumor cells represent only a tiny fraction of the total circulating nucleic acids in the serum (Lavon et al. 2010; Shi et al. 2012). Nevertheless, the amount of free DNA detected on cerebrospinal fluid is clearly higher in patients with gliomas than in healthy people. Interestingly, while the quantity of short DNA fragments (associated to apoptosis) was only slightly higher, the amount of long DNA fragments in cerebrospinal fluid from glioma patients largely doubles the amount founded in healthy people (Shi et al. 2012). These results support the data obtained through electron microscopy revealing that glioblastoma cells release their genomic content during cell death. Moreover, it correlates with our observations about the refractoriness of glioblastoma cells to display DNA laddering. Noteworthy, the combination of TRAIL plus gossypol avoids the spreading of the genomic content by inducing the formation of closed apoptotic nuclear bodies, which may reduce the negative effects associated to free-DNA during glioblastoma treatment.

Apoptotic oligonucleosomal DNA degradation within the dying cell favors clearance of apoptotic cells and reduces persistent circulating waste (Herrmann et al. 1998). Nevertheless, it has been demonstrated that even not cleaved DNA correctly packaged into apoptosis corpses could be degraded within the phagocytic cell by waste-management nucleases (Krieser et al. 2002; Mukae et al. 2002; Widlak & Garrard 2009). Therefore, although unable to generate DNA laddering, the closed apoptotic nuclear corpses generated after the cotreatment with gossypol plus TRAIL might improve the phagocytosis of the cellular waste generated during the cell death of glioblastoma cells.

Interestingly, free oxidized DNA released from death cancer cells can stimulate adaptative responses in distant still alive tumor cells which augment their survival and resistance to oxidative stress (Ermakov et al. 2013; Glebova et al. 2013). Hence, an incorrect package of genomic content that allows its release during cell death may probably be involved in the extreme aggressiveness of this kind of tumors. Consequently, the efficacy of glioblastoma treatments could be possibly influenced by the specific kind of cell death induced during the treatment.

It seems clear from the available literature that single-agent apoptotic therapies have a limited efficacy in the treatment of glioblastoma multiforme. Due to the inherent heterogeneity of this tumor, combinatorial strategies that sensitize glioblastoma cells to apoptosis on one hand, and trigger cell death on the other, seem more feasible therapies in the glioblastoma management. Therefore, maybe the combination of gossypol, a well-tolerated BH3 mimetic already proved in the clinic (Bushunow et al. 1999) and TRAIL, a death ligand widely used in the treatment of glioblastoma (reviewed in Kuijlen, Bremer, Mooij, den Dunnen, & Helfrich, 2010), might be considered as an alternative therapy for those cases in which other therapies are ineffective.

II. DISCUSSION

Nowadays, glioblastoma multiforme remains a challenge for researchers and clinicians. Extensive resection of the tumor, if possible, is the first step in the management of glioblastoma patients (Lamborn et al. 2004). However, the high infiltrative profile of this tumor makes total resection ineffective despite the huge technical advances made in neurosurgery during the last decade (Jackson et al. 2001; Stummer et al. 2006). Besides surgery, radiation has been of key importance to the treatment of glioblastoma for decades (Shapiro et al. 1989; Fine et al. 1993; Stewart 2002; Stupp et al. 2005). But radiation also contributes to the disruption of the blood brain barrier which breaks the immunoprivilege of the brain and generates neurological damage (Graeb et al. 1982; Watne et al. 1990; Li et al. 2003; de Wit et al. 2004; Chamberlain et al. 2007).

Over the last decade, lots of new chemical compounds have been proposed and tested in glioblastoma-derived cellular cultures and animal models. However, the majority of these new treatments have failed in clinical trials with only few of them showing modest or limited effectiveness. Indeed, the resistance to treatment, often associated to defects in the apoptotic program, is a concern for many types of cancer. In this work, we deeply study cell death mechanisms in glioblastoma multiforme cells. Depending on the treatment, we were able to induce different kinds of apoptotic cell death. Interestingly, distinct types of cell death could generate different immune responses. Traditionally, apoptosis was described as a form of cell death immunologically silent whereas necrosis was always related to potent inflammatory responses (Thompson 1995; Griffith et al. 1996; Steinman et al. 2000; Ferguson et al. 2002; Miyake et al. 2007). However, necrotic cells can be less immunogenic than cells undergoing certain types of apoptosis (Zitvogel et al. 2004; Casares 2005). In fact, subtle differences in the composition of the cell surface or the subproducts secreted by the dying cells determine whether the death of the cell is immunogenic or not (Obeid et al. 2007). These characteristics depend on both internal and external factors such as the intrinsic antigenicity of the cell, the stress before cell death, the inducer, the precise cell death pathway engaged or the ability of the immune system to respond (Green et al. 2009). Our results indicate that glioblastoma cells possess non-classical biochemical signaling pathways that generate incomplete apoptosis with some necrotic aspects such as DNA release after widely known strong apoptotic insults. Therefore, the consequences in the non-tumoral surrounding

parenquima may not be the expected ones from either necrosis or apoptosis.

During oncogenesis, acquisition of multiple mutations allows the generation of new antigenic peptides that might be recognized by the immune system. Consequently, after radiation and chemotherapy, dying tumor cells can elicit immune responses against the tumor antigens rather than normal self-antigens to which the immune system has been tolerized (Obeid et al. 2007; Apetoh et al. 2007). Indeed, the concept of immunogenic chemotherapy has recently emerged to refer to the capacity of a cytotoxic compound to trigger a tumor antigen specific immune response that contributes to the tumoricidal activity and protects the host against relapse. Nevertheless, most anticancer drugs elicit non-immunogenic apoptosis and even the most immunogenic cytotoxic compounds triggering protective immune responses fail to control the disease in most cases (reviewed in Locher et al. 2010). In brain tumors, it is unclear whether immunological consequences from cell death play positive or negative roles. Microglial cells, the resident macrophages of central nervous system, are abundant in brain tumors being a 30% of total glioma tissue (Charles et al. 2012). But, rather than fighting against the tumor, several studies clearly evidence that tumor cells recruit microglia to support their proliferation, migration and invasion (Rao 2003; Platten et al. 2003; Markovic et al. 2005; Erlich et al. 2007; Daginakatte et al. 2008; Alves et al. 2011; Fonseca et al. 2012). Therefore, advantages of immunogenic chemotherapy remain still unproven in the case of gliomas.

Lately, and due to the high apoptosis resistance developed by many tumor cells, other kinds of cell death such as regulated necrosis or autophagy have gained therapeutic interest. In glioblastoma, spontaneous necrosis is mostly initiated due to limited blood supply and anoxia conditions generated after microthrombotic processes (Barker et al. 1996; Pierallini et al. 1998; Homma et al. 2006; Miller et al. 2006). However, it has been suggested that, in some cases, necrosis could be due to a redirection of an incomplete apoptosis (reviewed in Nicotera & Melino 2004). Bcl2-like 12 (Bcl2L12) is a proline-rich protein characterized by a C-terminal sequence with significant homology with the BH2 domain of the Bcl-2 protein family (Scorilas et al. 2001). Bcl2L12, often overexpressed in primary GBMs, is a potent inhibitor of post-mitochondrial signal transduction during apoptosis due to its capacity to neutralize active caspase-3 (Stegh et al.

2008), to inhibit maturation of effector caspase-7 (Stegh et al. 2007) and to inhibit p53 transcriptional activity in the nucleus (Stegh et al. 2010). Interestingly, knockdown of Bcl2L12 sensitizes human glioma cell lines to apoptosis and reduces tumor formation in an orthotopic transplant model *in vivo* (Stegh et al. 2007). Then, upregulation of Bcl2L12 in glial cells may represent an important event in malignant glioma pathogenesis through the regulation of the apoptosis/necrosis balance (Furnari et al. 2007). Nevertheless, the role of Bcl2L12 might be irrelevant during caspase-dependent incomplete apoptosis in which caspase-3 and -7 become correctly processed an activated.

Autophagy is a highly conserved cytoprotective process whereby cytoplasmic contents are sequestered, transported via double-membrane autophagosomes to lysosomes, and degraded. Although considered a particular mode of cell death (Galluzzi et al. 2012), autophagy constitutes a survival mechanism in both normal and tumor cells (Gammoh et al. 2012). Several molecules structurally defined as proapoptotic BH3-only proteins or BH3 mimetics such as gossypol cannot only regulate apoptosis but also promote autophagy (Gao et al. 2010; Zhang et al. 2010; Jang & Lee 2014; Keshmiri-Neghab et al. 2014). Indeed, Beclin 1 is an autophagic signaling protein that has been recently identified as novel BH3-only protein. As other BH3-only proteins, Beclin 1 interacts with antiapoptotic multidomain proteins of the Bcl-2 family, in particular Bcl-2 and Bcl-X_L (Liang et al. 1998; Oberstein et al. 2007). Therefore, BH3-mimetic compounds can competitively disrupt the inhibitory interaction between Beclin 1 and Bcl-2/Bcl-X_L. Interestingly, only Beclin 1-Bcl-2/Bcl-X_L complexes present in the endoplasmic reticulum, but not those present in mitochondria, are disrupted by ABT737, which causes autophagy of mitochondria (mitophagy) but not of the endoplasmic reticulum. Consequently, only reticulum-targeted and not mitochondrion-targeted Bcl-2/Bcl-X_L can inhibit autophagy induced by Beclin 1 which points to a spatial organization of autophagy and apoptosis. In this way, BH3-only proteins exert two independent functions. On the one hand, they can induce apoptosis, directly or indirectly, by activating the mitochondrion-permeabilizing function of proapoptotic Bcl-2 family proteins. On the other hand, they can activate autophagy by liberating Beclin 1 from its inhibition by Bcl-2/Bcl-X_L at the level of the endoplasmic reticulum (Maiuri et al. 2007).

Gossypol, as inhibitor of Bcl-2 and Bcl-X_L (Oberstein et al. 2007) could also interact with Beclin-1 disrupting its interaction with the

antiapoptotic proteins. Indeed, gossypol effects depend on the specific circumstances of the cell, meaning that gossypol might enhance autophagy in a situation in which cells are resistant to apoptosis (Lian et al. 2010). Although it has been suggested that active autophagy itself can prevent tumor development (Levine & Kroemer 2008), remains uncertain whether autophagy represents a mechanism for preventing apoptosis or for enhancing non-apoptotic cell death. Therefore, it is difficult to predict whether autophagy manipulation might become a therapeutic strategy for glioblastoma multiforme in the future.

The study of the different cell death modalities in glioblastoma cells have proven to be a very efficient biological tool to shed light on the biochemical events occurring during apoptosis. Moreover, it may help to understand the consequences of treatments, the evolution of the disease and hopefully, the ultimate reason underlying the extreme aggressiveness of this kind of tumors.

III. CONCLUSIONS

This dissertation addresses the ultimate mechanisms governing cell death in glioblastoma cells. The main conclusions of this work are:

1. Glioblastoma-derived LN-18 cells are resistant to most of the drugs actually used in the treatment of cancer.
2. Glioblastoma-derived LN-18 cells display a caspase dependent cell death lacking oligonucleosomal DNA degradation and apoptotic type II nuclear morphology after apoptotic insult.
3. LN-18 cells generate chromatin-free apoptotic nuclear bodies during staurosporine-triggered cell death.
4. DFF40/CAD overexpression is sufficient to induce oligonucleosomal DNA degradation but not to generate apoptotic nuclear morphology in staurosporine-treated LN-18 cells.
5. The hydrolysis of DNA into oligonucleosome-sized fragments does not guarantee the deposition of the genomic content into nuclear apoptotic bodies.
6. Isolated nuclei from LN-18 cells are unable to become fragmented by fully apoptotic-competent cytoplasms.
7. Lack of oligonucleosomal DNA degradation during caspase-dependent cell death is a common trait in glioblastoma.
8. Caspase activation occurs preferentially in the cytoplasmic fraction of glioblastoma cells.
9. Glioblastoma cells present low levels of cytoplasmic DFF40/CAD.
10. The DFF40/CAD overexpression allows apoptotic glioblastoma cells to hydrolyze their genomic content without altering nuclear morphology observed in mock-transfected cells.
11. Chromatin detachment from the inner membrane of the nuclear envelope impairs apoptotic chromatin fragmentation in U87MG and LN-18 glioblastoma cells.
12. Condensed and fragmented chromatin in A172 and LN229 glioblastoma cells is not packaged into apoptotic nuclear bodies.
13. Glioblastoma cells release their genomic content into the extracellular media during staurosporine-triggered apoptotic cell death.

14. Gossypol induces caspase-independent cell death characterized by an AIF-independent stage I chromatin condensation in LN-18 cells.
15. Gossypol enhances caspase-activation and DFF40/CAD nuclear translocation induced by TRAIL in LN-18 cells.
16. Stage II chromatin condensation induced by the combination of gossypol and TRAIL in LN-18 cells depends on DFF40/CAD.
17. Combination of gossypol and TRAIL is insufficient to induce oligonucleosomal DNA degradation in LN-18 cells.
18. Higher amounts of cytosolic extracts from LN-18 treated with recombinant caspase-3 failed to generate oligonucleosomal DNA degradation in isolated nuclei.
19. Only the overexpression of DFF40/CAD allows glioblastoma cells to undergo DNA laddering, which points to endogenous stoichiometry of the endonuclease as the main problem avoiding oligonucleosomal DNA degradation during apoptosis.
20. Gossypol sensitizes TRAIL-resistant glioblastoma cells to extrinsic apoptosis.
21. The combination of gossypol plus TRAIL in CAD-overexpressing cells allows a complete apoptotic cell death characterized by chromatin condensation and nuclear fragmentation and DNA laddering.

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Abbreviations

AIF: apoptosis inducing factor

AML: acute myeloid leukemia

APAF1: apoptotic protease activating factor 1

APAF2: apoptotic protease activating factor 2

Bcl-2: B-cell lymphoma 2

Bcl-X_L: B-cell lymphoma extra large

BH: Bcl-2 homology

CAD: caspase-activated DNase

CFLAR: caspase-8 and FADD-like apoptosis regulator

CTLA-4: cytotoxic T lymphocyte-associated antigen 4 receptor

CYLD: cylindromatosis

DFF35: DNA fragmentation factor 35-kDa

DFF40: DNA fragmentation factor 40-kDa

DFF45: DNA fragmentation factor 45-kDa

Drp1: dynamin-related protein 1

EGFR: epidermal growth factor receptor

EGR-1: early growth response gene-1

FADD: Fas-associated death domain protein

FDA: food and drug administration

FLIP: FLICE-like inhibitory protein

$FLIP_L$: FLICE-like inhibitor protein long variant

$FLIP_S$: FLICE-like inhibitory protein short variant

Flt3L: fms-like tyrosine kinase-3 ligand

HIF: hypoxia-inducible factor

HMGB1: high mobility group protein B1

HtrA2/OMI: high-temperature requirement protein A2/

IAP: inhibitor of apoptosis protein

IAP1: inhibitor of apoptosis protein 1

IAP2: inhibitor of apoptosis protein 2

IBM: IAP-binding motif

$ICAD_L$: inhibitor of caspase-activated DNase long variant

$ICAD_S$: inhibitor of caspase-activated DNase short variant

IFNγ: interferon-gamma

IKK: IκB kinase

IL: interleukin

IMM: inner mitochondrial membrane

LUBAC: linear ubiquitin chain assembly complex

Mcl-1: induced myeloid leukemia cell differentiation protein

MGMT: methylguanyl methyltransferase

MLKL: mixed lineage kinase domain-like protein

MNNG: N-methyl-N0-nitrosoguanidine

MOMP: mitochondrial outer membrane permeabilization

MPT: mitochondrial permeability transition

MRI: magnetic resonance imaging

MRS: magnetic resonance spectroscopy

mTOR: mammalian target of rapamycin

NEMO: NF-kB essential modulator

NF1: neurofibrin-1

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

OMM: outer mitochondrial membrane

PARP: poly(ADP ribose) polymerase

PCD: programmed cell death

PDGF: platelet-derived growth factor

PDGFR: platelet-derived growth factor receptor

PFS: progression-free survival

PI3K: phosphatidyl inositol 3 kinase

PKC: protein kinase C

PUMA: p53-upregulated modulator of apoptosis

RB: retinoblastoma protein

RIP: receptor-interacting protein

RIP1: receptor interacting protein 1

RIP3: receptor interacting protein 3

RTK: tyrosine kinase receptor

SDF: stromal cell-derived factor

Smac/DIABLO: second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI

tAIF: truncated apoptosis inducing factor

TGF-β: transforming growth factor beta

TNFα: tumor necrosis factor alpha

TNFR: TNF receptor

TNFR1: TNFα Receptor 1

TNFR2: TNFα receptor 2

TRADD: TNFR associated death domain

TRAF2: TNFR associated factor 2

TRAIL: TNF related apoptosis inducing ligand

TRAILR1: TRAIL receptor 1

TRAILR2: TRAIL receptor 2

TSC/Rheb: tuberous sclerosis complex/Ras homolog enriched in brain

VEGF: vascular permeability factor

VEGFR: vascular permeability factor receptor

XIAP: X-linked inhibitor of apoptosis

Annex: *Curriculum Vitae*

Surname, Name: **Sánchez Osuna, María**

Place and date of birth: Córdoba, 19th January 1987

Current address: *Cell death, Senescence and Survival Research group,* Institut de Neurociències – Dpt. Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, 08193, Barcelona.
Phone: +34 93 586 8141
Personal e-mail: **mariasanchezosuna@gmail.com**
Personal phone: **+34 637 971 070**

1. Academic education

Title: **Biotechnology Bachelor's Degree**
University: Universitat Autònoma de Barcelona
Date: September 2005 - July 2009

Title: **Biomedical Research Master's Degree**
University: Universidad Pompeu Fabra
Date: September 2009 – September 2010

2. Research Fellowships

1.
Funding: Centro Superior de Investigaciones Científicas (CSIC).
Name: **Becas JAE de Introducción a la investigación**.
Date: July 2008- September 2008.
Research Institution: Instituto de Biología Molecular de Barcelona (IBMB-CSIC).

2.
Funding: Ministerio de Educación, Política Social y Deporte.
Name: **Beca de colaboración**.
Date: October 2008- June 2009.
Research Institution: Centre de Biotecnologia Animal i Teràpia Gènica (CBATEG), Universitat Autònoma de Barcelona.

3.
Funding: Fundació 'La Caixa' (Obra Social La Caixa).
Name: **Beca de máster en España**.
Date: September 2009- September 2010.
Research Institution: Universidad Pompeu Fabra (UPF).

4.
Funding: Ministerio de Educación.
Name: **Beca del Programa de Formación de Profesorado Universitario (FPU)**.
Date: December 2010 – December 2014.
Research Institution: Neurosciences Institute – Biochemistry and Molecular Biology Department, Universitat Autònoma de Barcelona.

3. Research Projects

1.
Research Project: **Study of the role of chromatin regulators in neural development.**
Research Institution: Instituto de Biología Molecular de Barcelona (IBMB-CSIC).
Date: July 2008- September 2008.
Principal Investigator: Marian Martínez-Balbás.

2.
Research Project: **Genotyping and characterization of a transgenic mouse expressing TNFalpha in the peripheral nervous system.**
Research Institution: Centre de Biotecnologia Animal i Teràpia Gènica (CBATEG).
Date: October 2008- June 2009.
Principal Investigator: Assumpció Bosch Merino.

3.
Research Project: **Absence of oligonucleosomal DNA degradation in glioblastoma cells during apoptosis.**
Research Institution: Neurosciences Institute – Biochemistry and Molecular Biology Department, Universitat Autònoma de Barcelona.
Date: September 2009- September 2010.
Principal Investigator: Victor José Yuste Mateos.

4.
Research Project: **Different forms to die and the same consequences? The relevance of a correct ICAD/CAD system activation during apoptosis.**
Funding: Dirección General de Investigación – Ministerio de Ciencia e Innovación **(ref.: SAF2011_24081)**
Research Institution: Neurosciences Institute – Biochemistry and Molecular Biology Department, Universitat Autònoma de Barcelona.
Date: January 2012- December 2012.
Principal Investigator: Victor José Yuste Mateos.

5.
Research Project: **Regulation of ICAD/CAD system in different cellular paradigms and consequences in tumor cells signaling.**

Funding: Secretaría de Estado de Investigación, Desarrollo e Innovación - Ministerio de Economía y Competitividad **(ref.: SAF2012_31485)**
Research Institution: Neurosciences Institute – Biochemistry and Molecular Biology Department, Universitat Autònoma de Barcelona.
Date: January 2013- December 2015.
Principal Investigator: Victor José Yuste Mateos.

4. Scientific publications.

Sanchez-Osuna M, Garcia-Belinchon M, Iglesias-Guimarais V, Gil-Guinon E, Casanelles E, Yuste VJ. *Caspase-Activated DNase is necessary and sufficient for oligonucleosomal DNA breakdown, but not for chromatin disassembly during caspase-dependent apoptosis of LN-18 glioblastoma cells.* J. Biol. Chem. 2014 Jul 4;289(27):18752-18769

Iglesias-Guimarais V, Gil-Guinon E, **Sanchez-Osuna M**, Casanelles E, Garcia-Belinchon M, Comella JX, Yuste VJ. *Chromatin collapse during caspase-dependent apoptotic cell death requires DFF40/CAD-mediated 3'-OH single-strand DNA breaks.* J Biol Chem. 2013 Mar 29;288(13):9200-15.

Iglesias-Guimarais V, Gil-Guiñon E, Gabernet G, García-Belinchón M, **Sánchez-Osuna M**, Casanelles E, Comella JX, Yuste VJ. *Apoptotic DNA degradation into oligonucleosomal fragments, but not apoptotic nuclear morphology, relies on a cytosolic pool of DFF40/CAD endonuclease.* J Biol Chem. 2012 Mar 2;287(10):7766-79.

Casanelles E, Gozzelino R, Marqués-Fernández F, Iglesias-Guimarais V, Garcia-Belinchón M, **Sánchez-Osuna M**, Solé C, Moubarak RS, Comella JX, Yuste VJ. *NF-κB activation fails to protect cells to TNFα-induced apoptosis in the absence of Bcl-x_L, but not Mcl-1, Bcl-2 or Bcl-w.* Biochim Biophys Acta. 2013 Jan 28;1833(5):1085-1095.

In preparation:

María Sánchez-Osuna, Fina Martínez-Soler, Mercè Garcia-Belinchón, Sònia Pascual-Guiral, Victoria Iglesias-Guimarais, Elisenda Casanelles, Gerard Plans, Jordi Bruna, Avelina Tortosa, Victor J. Yuste. *Absence of oligonucleosomal DNA fragmentation after caspase-dependent cell death is a common signature of glioblastoma-derived multiforme cells due to improper activation of DFF40/CAD endonuclease.*

Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Victoria Iglesias-Guimarais, Elisenda Casanelles, Victor J. Yuste. *Chelerytrine induces necrotic-like features mediated by an early activation of caspases.*

5. Attendance to scientific congress.

1.
Authors: Urresti J, García-Belinchón M, Iglesias-Guimarais V, Gabernet G, **Sánchez-Osuna M,** Comella JX, Yuste VJ.
Title: "Ausencia de degradación oligonucleosomal del ADN durante la apoptosis inducida en células derivadas de glioblastoma humano".
Participation: **Poster P29**
Congress: III Jornadas científicas del Institut de Neurociències
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 9-10/12/2009

2.
Authors: **María Sánchez-Osuna**, Mercè García-Belinchón, Victoria Iglesias-Guimarais, Gisela Gabernet, Jorge Urresti, Joan X. Comella, Victor J. Yuste
Title: "Absence of oligonucleosomal DNA degradation in human glioblastoma-derived cell lines during apoptotic cell death".
Participation: **Poster P57**
Congress: VII Jornada científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Place: Universidad Autónoma de Barcelona, Barcelona.
Date: 10/02/2010

3.
Authors Victoria Iglesias-Guimarais, Gisela Gabernet, Estel Gil-Guiñon, Laia Montoliu-Gaya, Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Elisenda Casanelles, Joan X. Comella, Victor J. Yuste.
Title: "SK-N-AS cells display caspase-dependent nuclear morphology without concomitant oligonucleosomal DNA fragmentation".
Participation: **Poster P53**
Congress: VIII Simposi de neurobiologia experimental
Place: Institut d'Estudis Catalans, Barcelona.
Date: 19-20/10/2010

4.
Authors: **María Sánchez-Osuna**, Carla Giner-Delgado, Mercè Garcia-Belinchón, Inés Guardia-Pena, Victoria Iglesias-Guimarais, Estel Gil-Guiñon, Elisenda Casanelles, Jorge Urresti, Joan X. Comella, Victor J. Yuste.
Title: "LN-18 glioblastoma-derived cell line does not present oligonucleosomal DNA degradation or nuclear fragmentation upon apoptotic stimuli".
Participation: **Poster P54**
Congress: VIII Simposi de neurobiologia experimental
Place: Institut d'Estudis Catalans, Barcelona.
Date: 19-20/10/2010

5.
Authors: Elisenda Casanelles, Victoria Iglesias-Guimarais, Estel Gil-Guiñon, Fernando Marqués-Fernández, Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Joan X. Comella y Victor J. Yuste.
Title: "Bcl-xL, y no Bcl-2, Bcl-W o Mcl-1, controla la apoptosis inducida por la activación de receptores de muerte".
Participation: **Comunicación oral**
Congress: X Aporeunion (APORED)
Place: Cuenca
Date: 06-08/02/2011

6.
Authors: **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Elisenda Casanelles, Estel Gil-Guiñon, Irene Casanova, Joan X. Comella, Victor J. Yuste.
Title: "Glioblastoma-derived cell line LN18 displays a non-classical cell death upon apoptotic insult".
Participation: **Poster P30**
Congress: VIII Jornada científica del Departament de Bioquímica i Biologia Molecular
Place: Universidad Autónoma de Barcelona, Barcelona.
Date: 09/02/2011

7.
Authors: Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Victoria Iglesias-Guimarais, Elisenda Casanelles, Estel Gil-Guiñon, Irene Casanova, Joan X. Comella, Victor J. Yuste.
Title: "Caspase-dependent cell death in the absence of nuclear fragmentation and oligonucleosomal DNA degradation in SHSY5Y human neuroblastoma-derived cells".
Participation: **Poster P46**
Congress: VIII Jornada científica del Departament de Bioquímica i Biologia Molecular
Place: Universidad Autónoma de Barcelona, Barcelona.
Date: 09/02/2011

8.
Authors: Victoria Iglesias-Guimarais, Estel Gil-Guiñon, Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Elisenda Casanelles, Gisela Gabernet, Joan X. Comella and Victor J. Yuste.
Title: "Nuclear morphological changes and oligonucleosomal DNA fragmentation during apoptotic cell death rely on CAD endonuclease activity through dissociated mechanisms".
Participation: **Poster P52**
Congress: VIII Jornada científica del Departament de Bioquímica i Biologia Molecular
Place: Universidad Autónoma de Barcelona, Barcelona.
Date: 09/02/2011

9.
Authors: Victoria Iglesias-Guimarais, Estel Gil-Guiñon, **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Elisenda Casanelles, Nuria Llecha, Joan X. Comella, Victor J. Yuste.
Title: "SK-N-AS cells display caspase-dependent nuclear morphology without concomitant oligonucleosomal DNA fragmentation: independent biochemical apoptotic outcomes but relied on DFF40/CAD activation".
Participation: **Poster P-061**
Congress: 13th International TNF conference
Place: Yumebutai International Conference Center, Hyogo, Japan
Date: 16-17/05/2011

10.
Authors: Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Victoria Iglesias-Guimarais, Elisenda Casanelles, Estel Gil-Guiñon, and Victor J. Yuste.
Title: "Lack of nuclear apoptotic hallmarks during caspase-dependent cell death in SH-SY5Y human neuroblastoma cells".
Participation: **Poster P4**
Congress: XIX Jornades de Biologia Molecular
Place: Institut d'Estudis Catalans, Barcelona.
Date: 7-8/07/2011

11.
Authors: Estel Gil-Guiñon, Irene Casanova, Victoria Iglesias-Guimarais, Elisenda Casanelles, **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Mònica Jara and Victor J. Yuste.
Title: "Expression profile of microRNAs during Actinomycin D-mediated sensitization towards TRAIL-induced cell death in U87MG glioblastoma cells".
Participation: **Poster P5**
Congress: XIX Jornades de Biologia Molecular
Place: Institut d'Estudis Catalans, Barcelona.
Date: 7-8/07/2011

12.
Authors: Victoria Iglesias-Guimarais, Estel Gil-Guiñon, Gisela Gabernet, **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Elisenda Casanelles, Joan X. Comella and Victor J. Yuste.
Title: "DFF40/CAD status determines a new paradigm of caspase-dependent cell death in the absence of oligonucleosomal DNA fragmentation".
Participation: **Poster P6**
Congress: XIX Jornades de Biologia Molecular
Place: Institut d'Estudis Catalans, Barcelona.
Date: 7-8/07/2011

13.
Authors: **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Estel Gil-Guiñon, Elisenda Casanelles, Irene Casanova and Victor J. Yuste.

Title: "LN-18 human glioblastoma-derived cells display incomplete caspase-dependent cell death upon different apoptotic insults".
Participation: **Poster P7**
Congress: XIX Jornades de Biologia Molecular
Place: Institut d'Estudis Catalans, Barcelona.
Date: 7-8/07/2011

14.
Authors: Estel Gil-Guiñon E, Irene Casanova-Salas, Teixidó L, Victoria Iglesias-Guimarais, Elisenda Casanelles, **María Sánchez-Osuna,** Mercè Garcia-Belinchón, Mònica Jara, Victor J. Yuste.
Title: "Actinomycin D-mediated intracellular microRNA environment alteration and sensitization towards TRAIL-induced cell death in U87MG human-derived glioblastoma cells".
Participation: **Comunicación oral**
Congress: XXXIV Congress de la SEBBM
Place: Institut d'Estudis Catalans, Barcelona.
Date: 05-08/09/2011

15.
Authors: Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Victoria Iglesias-Guimarais, Elisenda Casanelles, Estel Gil-Guiñon, Irene Casanova and Victor J. Yuste.
Title: "Lack of nuclear apoptotic hallmarks during caspase-dependent cell death in SH-SY5Y human neuroblastoma cells".
Participation: **Poster P-11**
Congress: Signal rewiring and addition in cancer
Place: Institut d'Estudis Catalans, Barcelona.
Date: 19-21/09/2011

16.
Authors: **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Elisenda Casanelles, Estel Gil-Guiñon, Irene Casanova and Victor J. Yuste.
Title: "Inherent resistance of human glioblastoma cells to degrade DNA into oligonucleosomal fragments upon different apoptotic insults".
Participation: **Poster P-39**
Congress: Signal rewiring and addition in cancer
Place: Institut d'Estudis Catalans, Barcelona.
Date: 19-21/09/2011

17.
Authors: Victoria Iglesias-Guimarais, Estel Gil-Guiñon, Gisela Gabernet, **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Elisenda Casanelles, Irene Casanova, Joan X. Comella and Victor J. Yuste.
Title: "The extent of caspase-induced DFF40/CAD-mediates DNA degradation determines distinct apoptotic cell fates and new cell death paradigms".
Participation: **Poster P-44**

Congress: Signal rewiring and addition in cancer
Place: Institut d'Estudis Catalans, Barcelona.
Date: 19-21/09/2011

18.
Authors: Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Victoria Iglesias-Guimarais, Elisenda Casanelles and Victor J. Yuste.
Title: "Caspase-dependent DNA damage failure to reach the classical apoptotic phenotype in SH-SY5Y human neuroblastoma derived cells".
Participation: **Poster P-11**
Congress: The DNA damage response in human disease
Place: Institut d'Estudis Catalans, Barcelona.
Date: 28-30/05/2012

19.
Authors: Victoria Iglesias-Guimarais, Estel Gil-Guiñon, Gisela Gabernet, Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Elisenda Casanelles, Joan X. Comella and Victor J. Yuste.
Title: "Double-stranded DNA breaks are not detected in SK-N-As cells displaying type II nuclear morphology upon apoptotic stimuli".
Participation: **Poster P-17**
Congress: The DNA damage response in human disease
Place: Institut d'Estudis Catalans, Barcelona.
Date: 28-30/05/2012

20.
Authors: **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Elisenda Casanelles and Victor J. Yuste.
Title: "Absence of double-strand DNA breaks in human glioblastoma-derived cell lines during apoptotic cell death".
Participation: **Poster P-35**
Congress: The DNA damage response in human disease.
Place: Institut d'Estudis Catalans, Barcelona.
Date: 28-30/05/2012

21.
Authors: Victoria Iglesias-Guimarais, Estel Gil-Guiñon, Gisela Gabernet, Mercè Garcia-Belinchón, **María Sánchez-Osuna**, Elisenda Casanelles, Joan X. Comella and Victor J. Yuste.
Title: "Double-stranded DNA breaks are not detected in SK-N-As cells displaying type II nuclear morphology upon apoptotic stimuli".
Participation: **Poster P-17**
Congress: The DNA damage response in human disease.
Place: Institut d'Estudis Catalans, Barcelona.
Date: 28-30/05/2012

22.
Authors: Victoria Iglesias-Guimarais, Gisela Gabernet, Estel Gil-Guiñon, Mercè Garcia-Belinchón, **María Sánchez-Osuna,** Elisenda Casanelles, Joan X. Comella, Victor J. Yuste.
Title: "DFF40/CAD-mediated nuclear morphology does not require oligonucleosomal DNA fragmentation during apoptotic cell death".
Participation: **Poster P-60**
Congress: I ICIBICAT Global Questions in Advanced Biology.
Place: Institut d'Estudis Catalans, Barcelona.
Date: 9-12/07/2012

23.
Authors: **María Sánchez-Osuna,** Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Elisenda Casanelles, Victor J. Yuste.
Title: "The resistance to apoptotic stimuli is a common trait in human glioblastoma multiforme-derived cells".
Participation: **Poster P-75**
Congress: I ICIBICAT Global Questions in Advanced Biology.
Place: Institut d'Estudis Catalans, Barcelona.
Date: 9-12/07/2012

24.
Authors: **María Sánchez-Osuna,** Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Fina Martínez-Soler, Elisenda Casanelles, Gerard Plans, Jordi Bruna, Avelina Tortosa and Victor J. Yuste.
Title: "Low cytosolic levels of DFF40/CAD impair oligonucleosomal DNA degradation in glioblastoma cells".
Participation: **Oral**
Congress: XI Aporeunión (APORED)
Place: IPV Beatriz Palace Hotel, Fuengirola, Málaga.
Date: 5-6/06/2013

25.
Authors: Mercè Garcia-Belinchón, **María Sánchez-Osuna,** Victoria Iglesias-Guimarais, Elisenda Casanelles, and Victor J. Yuste.
Title: "Impairment of apoptotic hallmarks through early cellular events during a caspase-dependent cell death in SH-SY5Y human-derived neuroblastoma cells".
Participation: **Oral**
Congress: XI Aporeunión (APORED)
Place: IPV Beatriz Palace Hotel, Fuengirola, Málaga.
Date: 5-6/06/2013

26.
Authors: Mercè Garcia-Belinchón, **María Sánchez-Osuna,** Victoria Iglesias-Guimarais, Elisenda Casanelles, and Victor J. Yuste.
Title: "Impairment of apoptotic hallmarks through early cellular events during a caspase-dependent cell death in SH-SY5Y human-derived neuroblastoma cells".

Participation: **Poster**
Congress: The International Cell Death Society Meeting: "Mechanism of cell death: The command to die"
Place: IPV Beatriz Palace Hotel, Fuengirola, Málaga.
Date: 6-9/06/2013

27.
Authors: Elisenda Casanelles, **María Sánchez-Osuna,** Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Joan X. Comella and Victor J. Yuste.
Title: "BCL-X_L controls SMAC/DIABLO release from the mitochondria protecting human glioblastoma-derived cells to TRAIL-induced apoptosis".
Participation: **Poster**
Congress: The International Cell Death Society Meeting: "Mechanism of cell death: The command to die"
Place: IPV Beatriz Palace Hotel, Fuengirola, Málaga.
Date: 6-9/06/2013

28.
Authors: **María Sánchez-Osuna,** Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Fina Martínez-Soler, Elisenda Casanelles, Gerard Plans Jordi Bruna, Avelina Tortosa and Victor J. Yuste.
Title: "Low levels of CAD impair low molecular weight DNA degradation in glioblastoma cells after apoptotic stimuli".
Participation: **Poster**
Congress: The International Cell Death Society Meeting: "Mechanism of cell death: The command to die"
Place: IPV Beatriz Palace Hotel, Fuengirola, Málaga.
Date: 6-9/06/2013

29.
Authors: **María Sánchez-Osuna,** Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Fina Martínez-Soler, Elisenda Casanelles, Gerard Plans, Jordi Bruna, Avelina Tortosa and Victor J. Yuste.
Title: "Apoptotic stimuli induce a partial apoptotic cell death in glioblastoma cells due to CAD low levels".
Participation: **Poster P10**
Congress: XX Jornades de Biologia Molecular
Place: Societat Catalana de Biologia (SCB), Barcelona.
Date: 11-12/06/2013

30.
Authors: Elisenda Casanelles, **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Joan X. Comella and Victor J. Yuste.
Title: "The specificity of Bcl-xL protecting human glioblastoma derived cells to TRAIL-induced apoptosis relies on Smac release".
Participation: **Poster P2**
Congress: X Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.

Date: 13/06/2013

31.
Authors: **María Sánchez-Osuna,** Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Fina Martínez-Soler, Elisenda Casanelles, Gerard Plans, Jordi Bruna, Avelina Tortosa and Victor J. Yuste.
Title: "Apoptotic stimuli induce a partial apoptotic cell death due to low CAD levels".
Participation: **Poster P4**
Congress: X Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 13/06/2013

32.
Authors: Mercè Garcia-Belinchón, **María Sánchez-Osuna,** Victoria Iglesias-Guimarais, Elisenda Casanelles and Victor J. Yuste.
Title: "Refractoriness of CAD endonuclease to induce the canonical apoptotic phenotype during caspase-dependent cell death in SH-SY5Y human neuroblastoma derived cells".
Participation: **Poster P51**
Congress: X Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 13/06/2013

33.
Authors: **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Elisenda Casanelles and Victor J. Yuste.
Title: "DFF40/CAD endonuclease is dispensable for chromatin disassembly during caspase-dependent cell death in human-derived glioblastoma multiforme LN-18 cells".
Participation: **Oral O-1**
Congress: IV Scientific Conference
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/04/2014

34.
Authors: Ricard Canals Florit, Laura Martínez-Escardó, Victor J. Yuste and **María Sánchez-Osuna**.
Title: "Estudi de l'efecte del fragment p11 d'ICADL sobre l'endonucleasa CAD en el procés apoptotic en cèl·lules derivades de glioblastoma humà".
Participation: **Poster P-1**
Congress: IV Scientific Conference
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/04/2014

35.
Authors: Laura Martínez-Escardó, Ricard Canals Florit, Victor J. Yuste and **María Sánchez-Osuna**.
Title: "Characterization of the short variant of the Inhibitor of Caspase-activated DNase (DFF35/ICADs) in apoptosis-resistant human-derived glioblastoma multiforme cells".
Participation: **Poster P-2**
Congress: IV Scientific Conference
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/04/2014

36.
Authors: **María Sánchez-Osuna**, Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Elisenda Casanelles and Victor J. Yuste.
Title: "DFF40/CAD endonuclease is not sufficient to promote nuclear apoptotic morphology during caspase-dependent cell death in LN-18 cells".
Participation: **Oral O-8**
Congress: XI Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/06/2014

37.
Authors: Laura Martínez-Escardó, Ricard Canals Florit, Victor J. Yuste and **María Sánchez-Osuna**.
Title: "Characterization of the short variant of the Inhibitor of Caspase-activated DNase (ICADs) in apoptosis-resistant human glioblastoma multiforme-derived cells".
Participation: **Poster P-25**
Congress XI Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/06/2014

38.
Authors: Raquel Larramona-Arcas, **María Sánchez-Osuna** and Victor J. Yuste.
Title: "Caspase-activated DNase allows the release of nuclear histones during apoptosis".
Participation: **Poster P-31**
Congress: XI Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/06/2014

39.
Authors: Sònia Pascual-Guiral, Albert Sanllorente, Mercè Garcia-Belinchón, **María Sánchez-Osuna** and Victor J. Yuste.

Title: “N-acetyl-D-sphingosine induces caspase-independent cell death leading to stage I nuclear morphology in SH-SY5Y cells”.
Participation: **Poster P-48**
Congress: XI Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/06/2014

40.
Authors: Albert Sanllorente, **María Sánchez-Osuna** and Victor J. Yuste.
Title: “Analysis of the subcellular location of Caspase-activated DNase after apoptotic insult in different human tumor-derived cell lines”.
Participation: **Poster P-52**
Congress: XI Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/06/2014

41.
Authors: Olga Almacellas-Rabaiget, Mercè Garcia-Belinchón, **María Sánchez-Osuna** and Victor J. Yuste.
Title: “Chelerythrine induces caspase-dependent non-apoptotic cell death in human neuroblastoma SH-SY5Y cells”.
Participation: **Poster P-54**
Congress: XI Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/06/2014

42.
Authors: Enric Costa Torrents, Mercè Garcia-Belinchón, **María Sánchez-Osuna** and Victor J. Yuste.
Title: “Curcumin promotes the specific degradation of Caspase-activated DNase allowing non-apoptotic cell death in SH-SY5Y cells”.
Participation: **Poster P-55**
Congress: XI Jornada Científica del Departament de Bioquímica i Biologia Molecular
Place: Universitat Autònoma de Barcelona, Barcelona.
Date: 04/06/2014

43.
Authors: **María Sánchez Osuna,** Fina Martinez-Soler, Sònia Pascual-Guiral, Mercè Garcia-Belinchón, Victoria Iglesias-Guimarais, Elisenda Casanelles, Gerard Plans, Jordi Bruna, Avelina Tortosa and Victor J. Yuste
Title: “Lack of oligonucleosomal DNA degradation during caspase-dependent cell death is a common trait of human glioblastoma multiforme-derived cells due to improper activation of DFF40/CAD”
Participation: **Oral and poster P01-8**
Congress:XXXVII Congreso de Bioquímica y Biología Molecular

Place: Palacio de Congresos, Granada
Date: 9-12/09/2014

44.
Authors: Raquel Larramona-Arcas, Laura Martínez-Escardó, **María Sánchez-Osuna** and Victor J. Yuste
Title: "The activation of DFF40/CAD and the cytoplasmic release of nuclear histones during caspase-dependent apoptotic cell death"
Participation: **Poster P01-16**
Congress:XXXVII Congreso de Bioquímica y Biología Molecular
Place: Palacio de Congresos, Granada
Date: 9-12/09/2014

45.
Authors: Granados-Colomina C, **Sánchez-Osuna M**, Vendrell J, Pallarès I, Yuste VJ.
Title: "Latexin enhances retinoic acid-mediated differentiation in SH-SY5Y cell line"
Participation: **Poster**
Congress:XXXVII Congreso de Bioquímica y Biología Molecular
Place: Palacio de Congresos, Granada
Date: 9-12/09/2014

6. Other skills

Language training: Français Langue Etrangère Course. Ecole de Langues et Lycée International de Touraine. Tours (France), July 2004.

Language training: EF International School of English. 72h course (upper-intermediate level). Dublin (Ireland) August – September 2007.

Language training: Language Studies, First Educational Language Training. 45h course, Gloucester (England) August 2008.

TOELF mark: 88/120. Barcelona, March 2009.

Programa Argó 2010: tutor of undergraduate students. Universitat Autònoma de Barcelona, 70h, July 2010.

One of the supervised students, Anna Salamero Boix got two external prizes for her work: *'VIII Premios a Trabajos de Investigación de Estudiantes de Bachillerato y Ciclos Formativos de Grado Superior, Área de Salud y Nutrición'* from Universitat de Lleida and *'Premio Universitat de Vic de Investigación de Bachillerato, ex aequo, apartado Osona Contra el Cáncer'*.

Programa Argó 2012: tutor of undergraduate students, Universitat Autònoma de Barcelona, 70h, June - July 2012.

Tutor of several undergraduate students from Biochemistry and Biomedicine Bachelor's Degrees during their Degree Final Project. Universitat Autònoma de Barcelona, 2013 - 2014.