



**HAL**  
open science

## Identification of the couple GSK3 $\alpha$ /c-Myc as a new regulator of hexokinase II in benzo[a]pyrene-induced apoptosis.

Béatrice Dendelé, Xavier Tekpli, Odile Sergent, Marie-Thérèse Dimanche-Boitrel, Jørn A. Holme, Laurence Huc, Dominique Lagadic-Gossmann

### ► To cite this version:

Béatrice Dendelé, Xavier Tekpli, Odile Sergent, Marie-Thérèse Dimanche-Boitrel, Jørn A. Holme, et al.. Identification of the couple GSK3 $\alpha$ /c-Myc as a new regulator of hexokinase II in benzo[a]pyrene-induced apoptosis.. *Toxicology in Vitro*, 2012, 26 (1), pp.94-101. 10.1016/j.tiv.2011.11.001 . hal-00682104

**HAL Id: hal-00682104**

**<https://hal.science/hal-00682104>**

Submitted on 26 Sep 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



## Identification of the couple GSK3 $\alpha$ /c-Myc as a new regulator of hexokinase II in benzo[a]pyrene-induced apoptosis

Béatrice Dendelé<sup>a,1</sup>, Xavier Tekpli<sup>a,1,2</sup>, Odile Sergent<sup>a</sup>, Marie-Thérèse Dimanche-Boitrel<sup>a</sup>, Jørn A Holme<sup>b</sup>, Laurence Huc<sup>a,3</sup>, Dominique Lagadic-Gossmann<sup>a,\*</sup>

<sup>a</sup> EA SeRAIC, Equipe labellisée Ligue contre le Cancer, IRSET, Université de Rennes 1, IFR 140, Rennes, France

<sup>b</sup> Division of Environmental Medicine, Norwegian Institute of Public Health, Oslo, Norway

### ARTICLE INFO

#### Article history:

Received 22 August 2011

Accepted 3 November 2011

Available online 12 November 2011

#### Keywords:

GSK3

Hexokinase II

c-Myc

Apoptosis

Mitochondria

Benzo[a]pyrene

NHE1

### ABSTRACT

The early apoptotic events induced by environmental pollutants with carcinogenic properties are poorly understood. Here, we focus on the early cytotoxic effects of benzo[a]pyrene (B[a]P). In F258 rat hepatic epithelial cells, B[a]P induces intrinsic apoptosis *via* a mitochondrial dysfunction characterized by the release of hexokinase II (HKII) from the mitochondria. Cancer cells often have an anomalous cell energy metabolism; since HKII dysfunction regulates B[a]P-induced apoptosis in F258 cells, but may also alter cell energy metabolism, HKII release from the mitochondria may represent an important B[a]P-related carcinogenic issue. Thus in the present study, we aimed at deciphering the mechanisms underlying HKII dysfunction upon B[a]P exposure.

We show that while glycogen synthase kinase 3 beta (GSK3 $\beta$ ) regulated the expression of HKII at the transcriptional level, glycogen synthase kinase 3 alpha (GSK3 $\alpha$ ) was involved in B[a]P-induced apoptosis *via* a decrease in c-Myc expression. The reduced level of c-Myc caused the relocation of HKII from the mitochondria to the cytosol, thereby being involved in the formation of reactive oxygen species and apoptosis.

In conclusion, we show that the couple GSK3 $\alpha$ /c-Myc plays a key role in B[a]P-induced early apoptotic cell signaling *via* HKII dysfunction.

© 2011 Elsevier Ltd. All rights reserved.

### 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (B[a]P) are major environmental contaminants, found in ambient air, cigarette smoke and smoked food. B[a]P exhibits carcinogenic effects, due to its metabolism by cytochrome P450 into reactive molecules which may form DNA adducts (Zedeck, 1980). Such a genotoxic action along with non-genotoxic effects of B[a]P may be important for the final outcome: cell survival and possible mutations, or apoptosis (Huc et al., 2006; Salas and Burchiel, 1998; Solhaug et al., 2004). Thus, a better characterization of the early non-genotoxic events induced by B[a]P may add important knowledge to the understanding of the mechanisms of

\* Corresponding author. Address: EA 4427 SeRAIC/IRSET, Université Rennes 1, Faculté de Pharmacie, 2 avenue Professeur Léon Bernard, 35043 Rennes cedex, France. Tel.: +33 (0) 223234837; fax: +33 (0) 223234794.

E-mail address: [dominique.lagadic@univ-rennes1.fr](mailto:dominique.lagadic@univ-rennes1.fr) (D. Lagadic-Gossmann).

<sup>1</sup> Equal contribution.

<sup>2</sup> Present address: National Institute of Occupational Health, Gydas vei 8, N-0033, Oslo, Norway.

<sup>3</sup> Present address: INRA, TOXALIM (Research Centre in Food Toxicology), 180 chemin de Tournefeuille, F-31027 Toulouse, France.

mutagenesis or cytotoxicity. In our previous works, we showed that following B[a]P treatment, rat epithelial F258 cells underwent intrinsic apoptosis *via* a translocation of hexokinase II (HKII) from mitochondria to cytosol and/or reduction of HKII mRNA levels, both events being prevented by p53 or Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 1 (NHE1) silencing (Huc et al., 2006, 2007). Since HKII dysfunction regulates B[a]P-induced apoptosis in F258 cells but may also alter cell energy metabolism, this event may represent an important B[a]P-related carcinogenic issue; thus in the present study, we aimed at deciphering the mechanisms underlying HKII dysfunction upon B[a]P exposure.

HKII is a glycolytic enzyme that mediates the phosphorylation of glucose, and couples intra-mitochondrial ATP synthesis to glucose metabolism. HKII favors aerobic glycolysis, cell proliferation and the maintenance of a low level of reactive oxygen species (Ahn et al., 2009). HKII has been shown to be over-expressed in highly malignant tumors (Mathupala et al., 2001; Peng et al., 2008); and its translocation from mitochondria has been suggested to play a role in cell death (Pastorino and Hoek, 2003; Plas et al., 2002). Glycogen synthase kinase 3 (GSK3) has been reported to be involved in HKII translocation during the mitochondrial apoptotic pathway (Pastorino et al., 2005).

GSK3 $\alpha$  and GSK3 $\beta$  are serine/threonine kinases with enzymatic activity regulated by many signaling pathways (Ding et al., 2000; Kim and Kimmel, 2000). The two GSK3 isoforms are not redundant; indeed, only GSK3 $\beta$  deficiency is lethal in embryogenesis (Hoefflich et al., 2000). Thus GSK3 isoforms have different functions depending on cell models and/or the physiological pathway involved (Beurel and Jope, 2006; Markou et al., 2008). GSK3 is involved in multiple cellular processes, including metabolism, proliferation, and cell differentiation (Frame and Cohen, 2001). Recent studies identify GSK3 as a regulator of the balance between survival and apoptosis (Hernandez-Davies et al., 2011; Spokoini et al., 2010). GSK3 can elicit pro-apoptotic effects by regulating anti-apoptotic transcription factors ( $\beta$ -catenin, HSF-1, Myc) or proteins involved in mitochondria function (p53, CREB) (Bijur et al., 2000; Ciani and Salinas, 2005).

The present study aimed at looking for the possible involvement of GSK3 in B[a]P-induced apoptosis. Our data identified GSK3, and more precisely GSK3 $\alpha$ , as a regulator of c-Myc expression which controls B[a]P-induced mitochondrial dysfunction via HKII, thus leading to apoptosis.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

Benzo[a]pyrene (B[a]P), lithium chloride (LiCl),  $\alpha$ -naphthoflavone ( $\alpha$ -NF) and DEVD-AMC (Asp-Glu-Asp-7-amino-4-methylcoumarin) were purchased from Sigma Chemicals Co (St Louis, MO, USA). Peptidic inhibitor of GSK3 (IGSK) was purchased from Calbiochem (France Biochem, Meudon, France). All these products were used as a stock solution in dimethyl sulfoxide; final concentration of this vehicle in culture medium was <0.05% (v/v); control cultures received the same concentration of vehicle as treated cultures. Hoechst 33342 and mouse monoclonal Cytochrome C Oxidase 4 (COX IV) antibody were purchased from Invitrogen (Invitrogen, France). Rabbit polyclonal anti-c-Myc, goat polyclonal anti-hexokinase II and mouse monoclonal anti- $\beta$ -actin were from Santa Cruz Biotechnology (Tebu-bio SA, Le Perray en Yvelines, France). Rabbit polyclonal anti-GSK3 $\alpha$ , anti-GSK3 $\alpha$  phospho Serine 21, anti-GSK3 $\beta$ , and anti-GSK3 $\beta$  phospho Serine 9 antibodies were purchased from Cell Signaling (Ozyme, St Quentin-en-Yvelines, France). Secondary antibodies conjugated to horseradish peroxidase were from Dako A/S (Glostrup, Denmark).

### 2.2. Cell culture, apoptosis measurement and Western blotting immunoassays

The F258 rat liver epithelial cell line was cultured in Williams'E medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5 IU/ml penicillin, and 0.5 mg/ml streptomycin at 37 °C under a 5% CO<sub>2</sub> atmosphere and treated 24 h following seeding as previously described (Huc et al., 2004). Microscopical detection of apoptosis was performed in both floating and adherent cells, using Hoechst 33342 labeling, as previously described (Huc et al., 2004). Caspase 3/7 activity assay has been previously described (Huc et al., 2004). Western blot analysis was performed as previously described (Huc et al., 2006).

### 2.3. Transfection of small interfering RNAs (Si RNA)

The following Si RNAs were used: Si RNA oligonucleotides directed against GSK3 $\alpha$  (Si Genome SMART pool, NM\_080107\_00); GSK3 $\beta$  (Si Genome SMART pool, NM\_080108\_00); Si RNA negative control (Si Neg, Si control non-targeting Si RNA#1) (all from Thermo scientific Dharmacon, Colorado); Si RNA against c-Myc (SASI

Rn01\_00089127, SASI Rn01\_00089127\_AS) (Sigma Aldrich, France). Transfections of Si RNA were performed in 60-mm dishes on 90% confluent F258 cells; per dish, Si RNA (100 nM) and 12.5  $\mu$ l TransFectin lipid reagent were applied in a final volume of 2 ml Opti-MEM for 4 h. Transfection rate was evaluated with Si Fluo and corresponds to 86  $\pm$  5% (n = 4) transfected cells. Transfected cells were seeded in order to be treated the day after with B[a]P (5  $\mu$ M). Previous analysis showed that F258 cells exposure to B[a]P 5  $\mu$ M during 36 h, underwent the same apoptotic events as cells exposed to B[a]P 50 nM, during 72 h (Huc et al., 2004; Tekpli et al., 2010). Thus, to keep a good rate of protein down-regulation when transfection with Si RNA was applied, cells were exposed to B[a]P 5  $\mu$ M during 36 h.

### 2.4. Transfection of plasmids

Transfection of vector pExpress-1 with c-Myc gene (Rat MGC Verified FLcDNA, MRN1768\_98079299) (ThermoScientific Open Biosystems, France) was performed according to manufacturer's recommendations. pExpress-1 plasmid control was purchased from Express Genomics (Express Genomics, Maryland/USA). Transfections of plasmids were performed in 60-mm dishes on 90% confluent F258 cells, in presence of lipofectamine™ 2000 transfection reagent (Invitrogen, France). Per dish, plasmid (2  $\mu$ g) and 10  $\mu$ l of lipofectamine™ 2000 reagent were applied in a final volume of 2 ml Opti-MEM for 4 h. Transfection rate was evaluated with pEGFP-N1 vector (clontech, USA) and corresponds to 74% transfected cells. Transfected cells were treated the day after with B[a]P (5  $\mu$ M), as for the protocol with Si RNA.

### 2.5. RNA isolation and analysis

Total RNA extraction and reverse transcription-real time quantitative polymerase chain reaction (RT-PCR) were carried as previously described (Tekpli et al., 2010). The specificity of each gene amplification was checked at the end of PCR reactions through the analysis of dissociation curves of the PCR products. The amount of target cDNA in each sample was established by determining a fractional PCR threshold cycle number (Ct). The expression levels of target genes were normalized to the expression of the 18S gene. The sequences of the primers used for RT-PCR analysis were as follows: 18S sense: GGAAGCATTGCCAAGAAT and antisense: AGTCGGCATCGTTTATGGTC; Hexokinase II sense: CAACATCGTCACTGCCATCT and antisense: GGGACCACTTGCTTCCATTA.

### 2.6. Mitochondrial fractionation

Mitochondrial fractionation was carried as previously described (Huc et al., 2007).

### 2.7. Statistical analysis

All data are quoted as mean  $\pm$  standard error of mean along with number of observations, n, corresponding to separate cultures. ANOVA followed by a Student–Newman–Keuls post-test was used to compare means. In cases of non-normal distributions, ANOVA followed by Dunn post-test was used to compare medians.  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. GSK3 is involved in B[a]P-induced apoptosis

Our first set of experiments was carried out to determine a possible implication of GSK3 in B[a]P-induced apoptosis. F258 cells

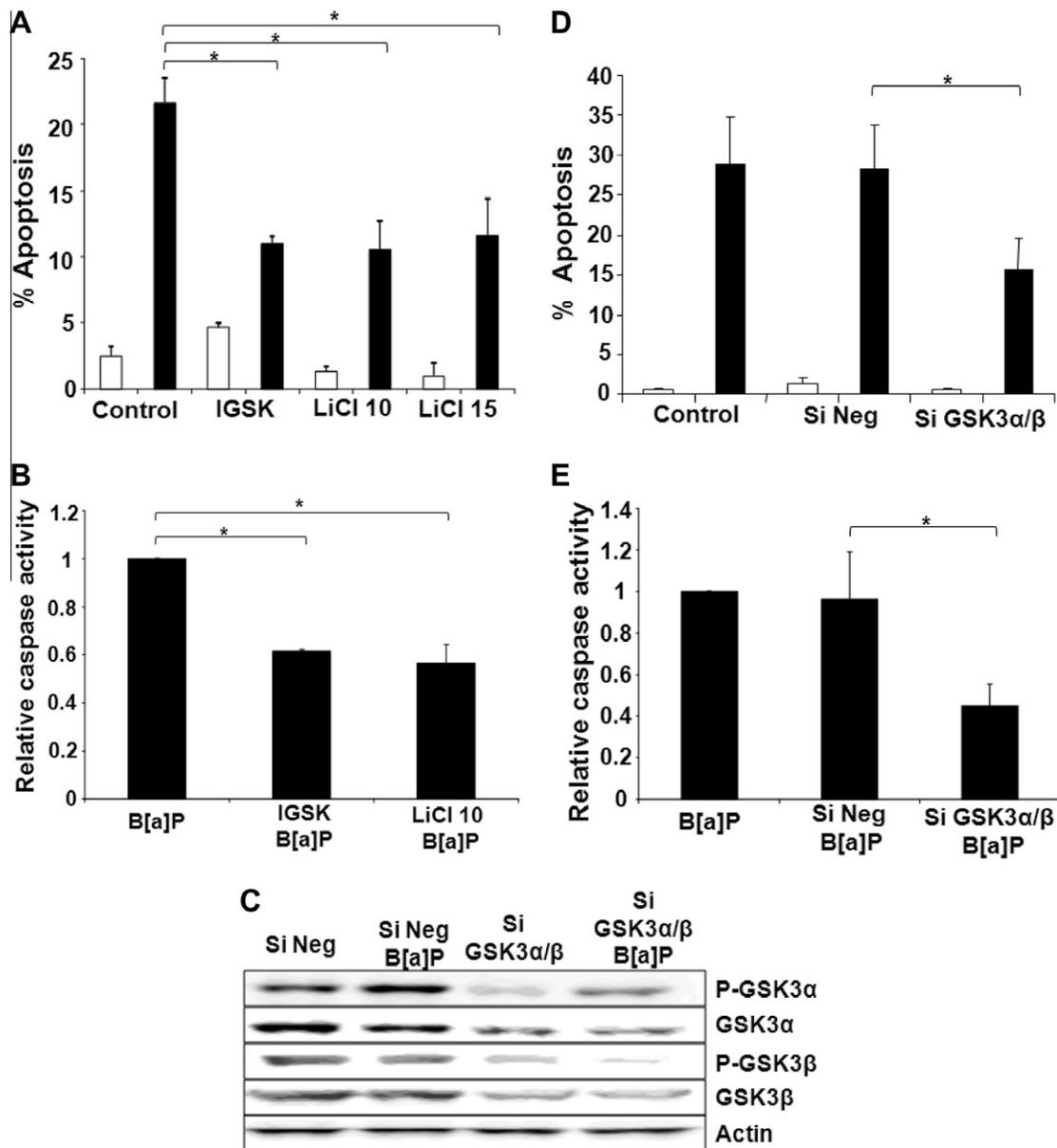
were pre-treated 1 h with two different inhibitors of GSK3, i.e. a peptidic inhibitor (IGSK) and lithium chloride (LiCl) (Feyt et al., 2005), and subsequently co-treated 72 h with B[a]P (50 nM). Analysis of cells with apoptotic morphology (condensed or fragmented nuclei), and measurement of caspase 3/7 activity demonstrated that both inhibitors reduced B[a]P-induced apoptosis (Fig. 1A and B). The two concentrations of LiCl tested gave similar results; further experiments were thus carried out with the lowest concentration. Small interference RNA directed against both GSK3 isoforms (Si GSK3 $\alpha/\beta$ ) efficiently reduced the protein expression of GSK3 isoforms compared to Si negative controls (Si Neg) (Fig. 1C), and significantly decreased B[a]P-induced apoptosis determined by fluorescence microscopy and caspase 3/7 activities (Fig. 1D and E). Note also that, whereas no changes in the phosphorylation state of Ser 9 in GSK3 $\beta$  was observed, B[a]P induced a phosphorylation of GSK3 $\alpha$  at Ser 21 (Fig. 1C).

### 3.2. GSK3 $\alpha$ rather than GSK3 $\beta$ is involved in B[a]P-induced apoptosis

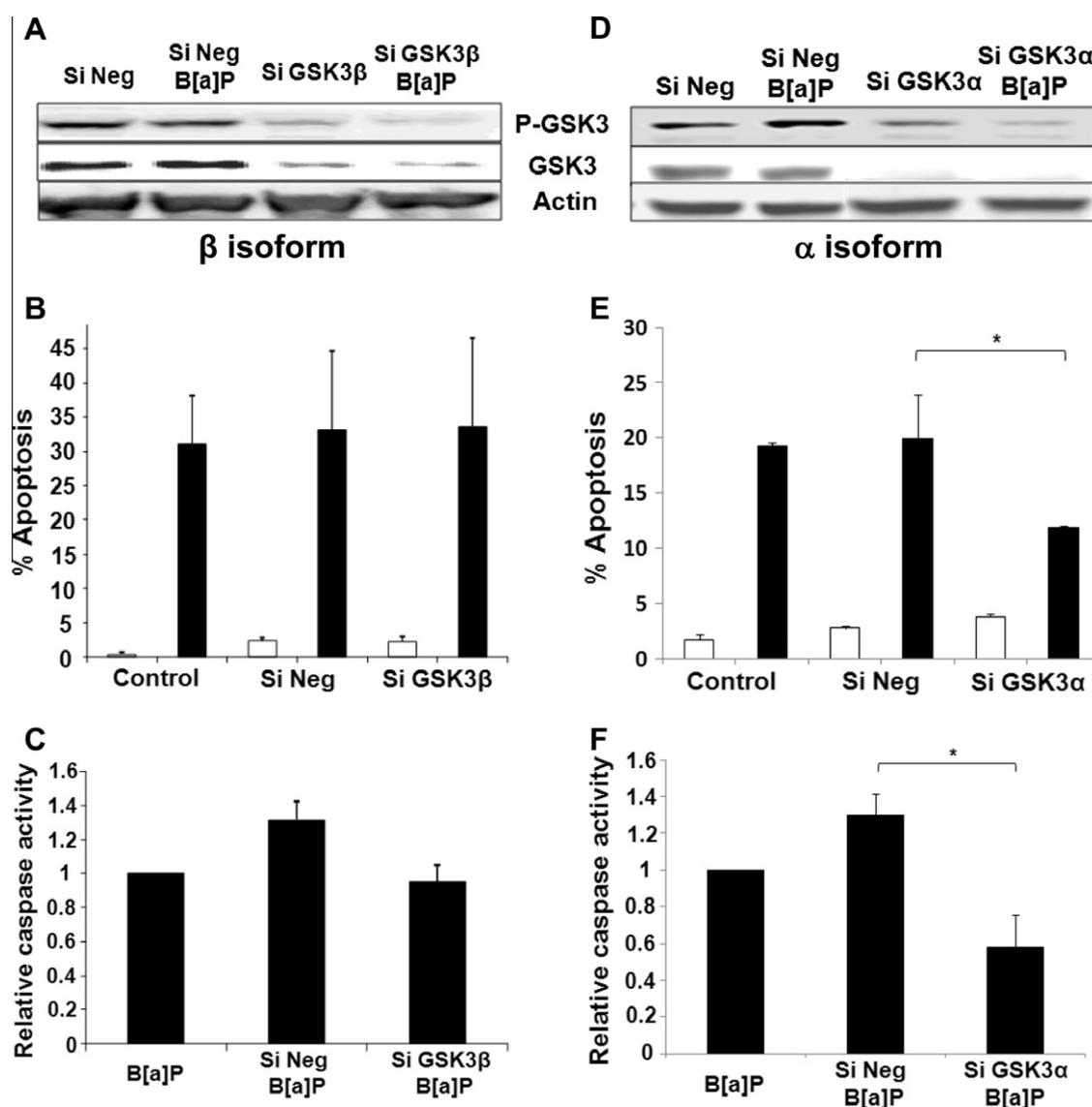
Si RNAs targeting either GSK3 isoform were next tested on B[a]P-induced apoptosis. Even though the Si RNAs directed against GSK3 $\beta$  (Si GSK3 $\beta$ ) were efficient regarding the silencing of this isoform (Fig. 2A), they had no effect on B[a]P-induced apoptosis (Fig. 2B and C). In contrast, regarding the Si RNAs directed against GSK3 $\alpha$ , besides specific reduction of the protein expression (Fig. 2D), they also significantly reduced the apoptotic effects of B[a]P (Fig. 2E and F).

### 3.3. GSK3 regulates HKII expression and localization

We next investigated a possible role for GSK3 in B[a]P-induced down-regulation of HKII mRNA expression (Huc et al., 2007). Quantitative RT-PCR and SYBR green method showed that GSK3



**Fig. 1.** Effects of GSK3 on B[a]P-induced apoptosis. F258 cells were pre-treated 1 h with GSK3 inhibitors: IGSK (5  $\mu$ M) or LiCl (10 or 15 mM), and subsequently treated or not with B[a]P 50 nM, 72 h. F258 cells were transfected with Si Neg or Si GSK3 $\alpha/\beta$  and subsequently treated or not with B[a]P 5  $\mu$ M during 36 h. (A and D) Apoptotic nuclei were analyzed by Hoechst 33342 staining.  $N \geq 5$  independent experiments. White bars: vehicle; black bars: B[a]P. (B and E) DEVDase (caspases 3/7) activities were measured by spectrofluorimetry, averaged from 5 independent experiments and expressed as Relative Fluorescence Unit (RFU)/ $\mu$ g proteins normalized by the RFU of control cells.  $N \geq 5$  independent experiments. (C) 50  $\mu$ g of whole cell lysate were separated on 10% SDS–polyacrylamide gel electrophoresis. Immunoblots were probed with rabbit polyclonal anti -GSK3 $\alpha$ , -P-GSK3 $\alpha$  (Ser 21), -GSK3 $\beta$ , -P-GSK3 $\beta$  (Ser 9), and mouse monoclonal anti- $\beta$ -actin antibodies.



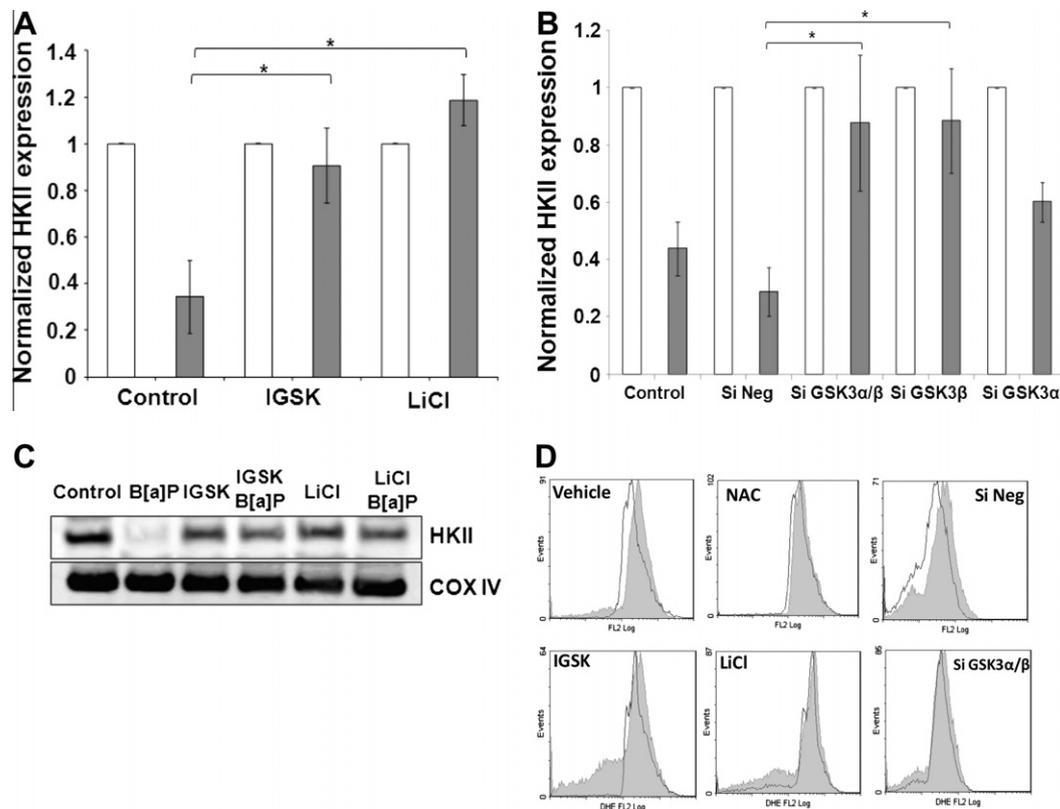
**Fig. 2.** GSK3 isoforms and B[a]P-induced apoptosis. F258 cells were transfected with Si RNA directed against either the  $\alpha$  or the  $\beta$  isoform of GSK3 (Si GSK3 $\alpha$  or Si GSK3 $\beta$ ) or with Si RNA control (Si Neg) and subsequently treated with B[a]P 5  $\mu$ M during 36 h. (A and D) 50  $\mu$ g of whole cell lysate were separated on 10% SDS-polyacrylamide gel electrophoresis. Immunoblots were probed with rabbit polyclonal anti-GSK3 $\alpha$ , -P-GSK3 $\alpha$  (Ser 21), -GSK3 $\beta$ , and -P-GSK3 $\beta$  (Ser 9) antibodies or mouse monoclonal anti- $\beta$ -actin antibody. Blots are representative of at least 5 independent experiments. (B and E) Apoptotic nuclei were analyzed by Hoechst 33342 staining.  $N \geq 5$  independent experiments. White bars: vehicle; black bars: B[a]P. (C and F) DEVDase activities were measured by spectrofluorimetry, averaged from five independent experiments and expressed as RFU/ $\mu$ g proteins normalized by the RFU of control cells.  $N \geq 5$  independent experiments.

inhibitors as well as Si GSK3 $\alpha/\beta$  and Si GSK3 $\beta$  prevented the B[a]P-induced reduction of HKII mRNA expression (Fig. 3A and B). In order to gain further insight into the involvement of GSK3 in B[a]P-induced apoptosis, we tested the role of GSK3 in the control of mitochondrial HKII localization. After a 72 h-treatment with 50 nM B[a]P, HKII was released from mitochondria into cytosol (Huc et al., 2007), as shown by the decrease in the mitochondrial expression of HKII (Fig. 3C) or by the loss of co-staining of HKII with the specific mitochondrial fluoroprobe mitotracker (red) (Supplementary Fig. 1). GSK3 inhibitors prevented from B[a]P-induced HKII relocation from the mitochondria (Fig. 3C, Supplementary Fig. 1). GSK3 inhibitors or Si RNA directed against both GSK3 isoforms also prevented from the consequences of the mitochondrial dysfunction as estimated by the anion superoxide ( $O_2^-$ ) production (Fig. 3D and Supplementary Fig. 2). Taken together, these results suggest that B[a]P, through a process involving GSK3, affects the mRNA expression of HKII and its protein sub-cellular localization.

### 3.4. GSK3 $\alpha$ regulation of c-Myc expression is involved in apoptosis

The proto-oncogene c-Myc has been reported to be involved in the apoptosis mediated by GSK3 inhibition (Kotliarova et al., 2008), and also to regulate the expression of HKII (Podar and Anderson, 2010). Thus, as B[a]P might affect c-Myc expression (Fields et al., 2004; Zhao and Ramos, 1998), we tested the possible involvement of c-Myc in the B[a]P-induced signaling cascade. We found that B[a]P markedly reduced the level of c-Myc protein. Such an effect was prevented by the cytochrome P450-inhibitor:  $\alpha$ -naphthoflavone ( $\alpha$ -NF) (Fig. 4A). Interestingly, both LiCl and IGSK (Fig. 4A) as well as Si RNA directed against GSK3 $\alpha$  (Fig. 4B) abolished the B[a]P-induced c-Myc down-regulation. Note also that chemical inhibition of Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 1 (NHE1) and p53 pathways also repressed the B[a]P-induced c-Myc down-regulation (Supplementary Fig. 3).

To counteract the effects of B[a]P on c-Myc expression we transfected the cells with a c-Myc-plasmid construct. Over-expression



**Fig. 3.** Effects of GSK3 on HKII expression and sub-cellular localization. F258 cells were pre-treated 1 h with GSK3 inhibitors: IGSK (5  $\mu$ M) or LiCl (10 mM), and subsequently treated or not with B[a]P 50 nM, 72 h. F258 cells were transfected with Si Neg or Si GSK3 $\alpha/\beta$ , Si GSK3 $\alpha$  or Si GSK3 $\beta$ , and subsequently treated or not with B[a]P 5  $\mu$ M during 36 h. (A and B) Quantitative analysis of HKII mRNA levels in F258 cells. White bars: vehicle; grey bars: B[a]P.  $N \geq 5$  independent experiments. (C) 15  $\mu$ g of protein lysate from mitochondrial fractions were separated on 12% SDS–polyacrylamide gel electrophoresis. Immunoblots were probed with goat polyclonal anti-HKII and mouse monoclonal anti-COX IV antibodies. COX IV served as a loading control. Data are representative of three independent experiments. (D) Histogram of superoxide anion production obtained by flow cytometry analysis of cells stained with the superoxide anion sensitive probe DHE. The antioxidant molecule N-acetylcysteine (NAC, 5 mM) prevented the B[a]P-induced, mitochondria-related reactive oxygen species production. Peaks are representative of 3 independent experiments. White histograms: vehicle; grey histograms: B[a]P.

of c-Myc using a plasmid construct (Pl c-Myc), significantly decreased B[a]P-induced apoptosis compared to cells transfected with a control plasmid (Pl Ctr) (Fig. 4C). Furthermore, c-Myc over-expression inhibited the B[a]P-induced reduction of mitochondrial HKII protein level (Fig. 4D). In contrast, silencing of c-Myc expression using Si RNA directed against c-Myc (Si c-Myc) significantly enhanced apoptosis (Fig. 4E).

Therefore, in B[a]P-treated cells, the c-Myc decrease controlled by GSK3 $\alpha$  appears to play a significant role in the related apoptosis, and might constitute the intermediate between GSK3 $\alpha$  and HKII, since c-Myc expression regulated the mitochondrial HKII protein levels.

#### 4. Discussion

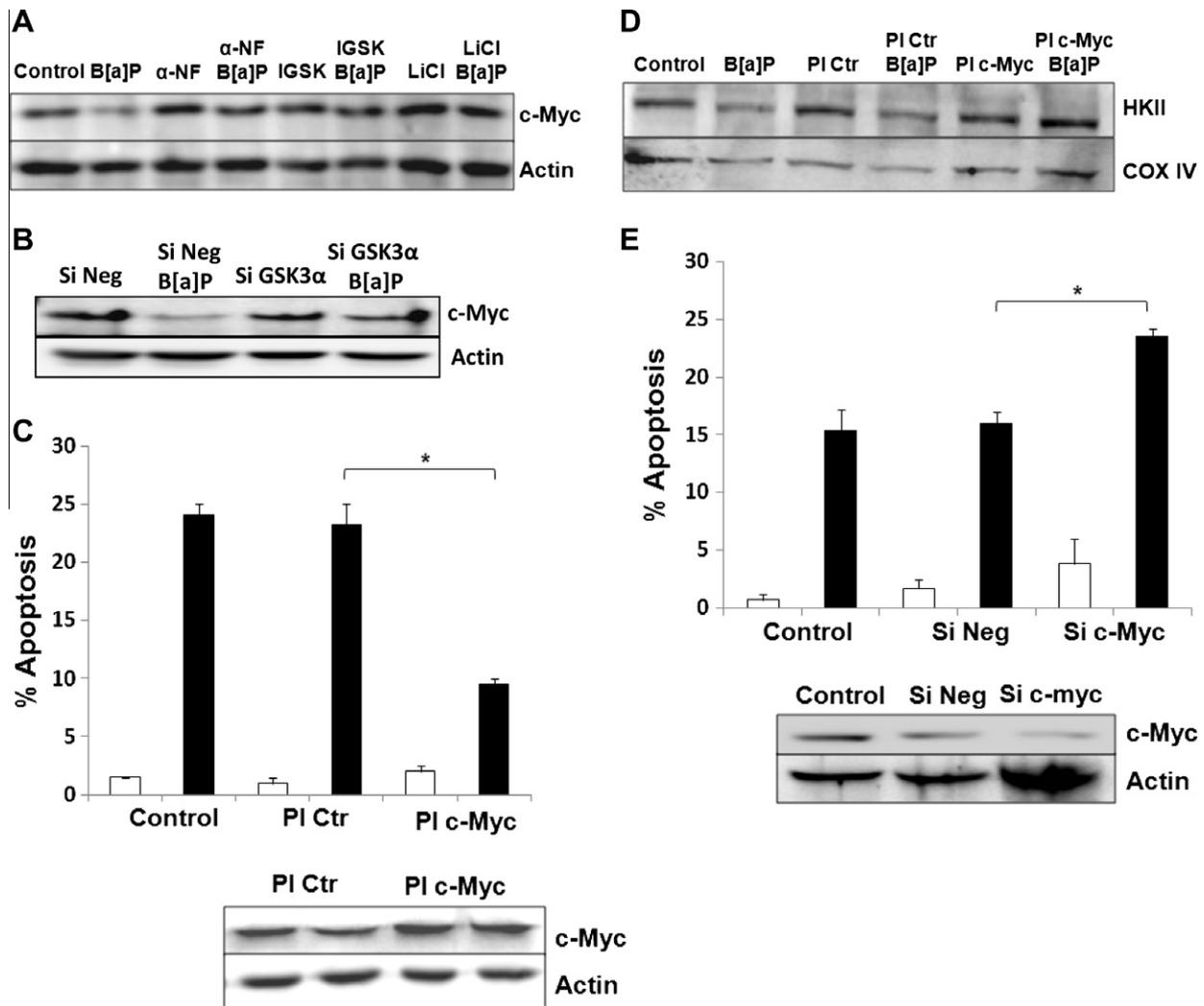
Our previous works demonstrated the prominent role of translocation of HKII from mitochondria to cytosol in B[a]P-induced apoptosis in F258 cells; p53 and NHE1 activations cross-talked to deregulate HKII mitochondrial localization as well as its mRNA expression (Huc et al., 2007). The present study identifies for the first time the couple GSK3 $\alpha$ /c-Myc as a novel target in B[a]P-induced apoptosis (Fig. 5).

Most of the studies regarding the role of GSK3 in apoptosis have reported that the  $\beta$  isoform is preferentially involved in the apoptotic cascade, notably by inducing a mitochondrial dysfunction (Pastorino et al., 2005; Vene et al., 2008). The experiments carried out with Si RNA targeting either isoform pointed to GSK3 $\alpha$  as being the major isoform involved in our apoptotic model. Most of the studies linking GSK3 to HKII translocation have shown an

involvement of the  $\beta$  isoform (Das et al., 2008; Pastorino et al., 2005). In this context, our study is the first one to clearly evidence a role for GSK3 $\alpha$  in the control of HKII sub-cellular localization. The apoptotic role of GSK3 $\alpha$  has also been reported by others, notably in cardiac cells (Zhai et al., 2007); thus, a link between GSK3 $\alpha$  and HKII would deserve more focus in the future.

Our previous work demonstrated that B[a]P down-regulates the mRNA expression of HKII in F258 cells. The present study shed some light on the underlying intracellular mechanism since GSK3 $\beta$  seems to be involved in this regulation. But GSK3 $\beta$  appears not to be involved in B[a]P-induced apoptosis; this might thus indicate that the decrease in HKII mRNA expression would not be essential for apoptosis to occur whereas the GSK3 $\alpha$ -dependent HKII translocation might be the main key event responsible for the mitochondrial dysfunction induced by B[a]P. Nevertheless, it is worth noting that a recent study in mice showed that *in vivo* down-regulation of HKII expression might constitute an efficient therapeutic strategy towards colon cancer development (Peng et al., 2008). It would be interesting to test the consequence of HKII mRNA expression down-regulation on mitochondrial HKII protein level in this latter model.

The present work further illustrates the differential roles for the two GSK3 isoforms. Indeed, it has been shown for example that GSK3 $\alpha$  KO mice develop normally whereas targeted deletion of GSK3 $\beta$  leads to embryonic lethality (Force and Woodgett, 2009); moreover, whereas Ser 9 phosphorylation of GSK3 $\beta$  mediates pathological cardiac hypertrophy, Ser 21 phosphorylation of GSK3 $\alpha$  would play a compensatory role during pressure overload (Matsuda et al., 2008).



**Fig. 4.** c-Myc involvement in apoptosis. F258 cells were pre-treated 1 h with either the GSK3 inhibitors, IGSK (5  $\mu$ M), LiCl (10 mM), or the metabolism inhibitor  $\alpha$ -NF ( $\alpha$ -Naphthoflavone, 10  $\mu$ M), and subsequently treated or not with B[a]P 50 nM, 72 h. F258 cells were transfected with either plasmid allowing over-expression of c-Myc (PI c-Myc) or plasmid control (PI-Ctr), or with Si RNA directed against c-Myc (Si c-Myc), GSK3 $\alpha$  (Si GSK3 $\alpha$ ) or Si Neg, and subsequently treated or not with B[a]P 5  $\mu$ M during 36 h. (A, B and D) Cell lysates were separated on 10% SDS–polyacrylamide gel electrophoresis. Immunoblots were probed with rabbit polyclonal anti-HKII, -COX IV, -c-Myc and mouse monoclonal anti- $\beta$ -actin antibodies. (C and E) Apoptotic nuclei were analyzed by Hoechst 33342 staining. White bars: vehicle; black bars: B[a]P.  $N = 3$  independent experiments in all cases.

Regarding the present study, we found that B[a]P decreased c-Myc expression; this was prevented by inhibition of GSK3. Furthermore, c-Myc was found to be involved in apoptosis *via* the control of HKII mitochondrial localization. Our data therefore suggest a link between GSK3 $\alpha$ , c-Myc and HKII. c-Myc is known to be involved in the regulation of glucose metabolism (Dang et al., 2009; Valera et al., 1995); one might then suppose that the decrease in c-Myc expression may lead to a decrease in intracellular glucose, thereby favoring the disruption of HKII from mitochondria.

It remains to be determined if the decrease of c-Myc expression is involved in the regulation of HKII mRNA expression by GSK3 $\beta$ . In support to this hypothesis, it has been shown that c-Myc can transactivate the *HKII* gene (Kim et al., 2007). When considering the regulation of c-Myc expression by GSK3, it is worth noting that it has been shown that GSK3 is capable of binding c-Myc in nucleus, which would allow the Thr-58 phosphorylation of c-Myc and hence regulation of its stability (Gregory et al., 2003). Finally, a very recent study has evidenced, by using statins, that the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) might be a critical regulator of c-Myc phosphorylation,

activation, and tumorigenic properties, since an inhibition of HMG-CoA reductase blocked the phosphorylation and activation of c-Myc (Cao et al., 2011). As B[a]P has been previously shown to inhibit this enzyme in our apoptosis model (Tekpli et al., 2010), such a link between HMG-CoA reductase and c-Myc expression would deserve further investigation.

In conclusion, the present study evidences a new role for GSK3 $\alpha$  in B[a]P-induced cell death *via* changes in c-Myc level and HKII sub-cellular localization. As HKII is over-expressed in various types of cancer cells (Mathupala et al., 2001; Peng et al., 2008), our study might help identifying new anticancer therapeutic targets to modulate HKII tumor effects. Finally, our data provide new information regarding the signaling pathways induced by B[a]P which regulate the delicate balance between proliferation, cell cycle arrest, cell survival and apoptosis.

## 5. Conflict of interest

None declared.

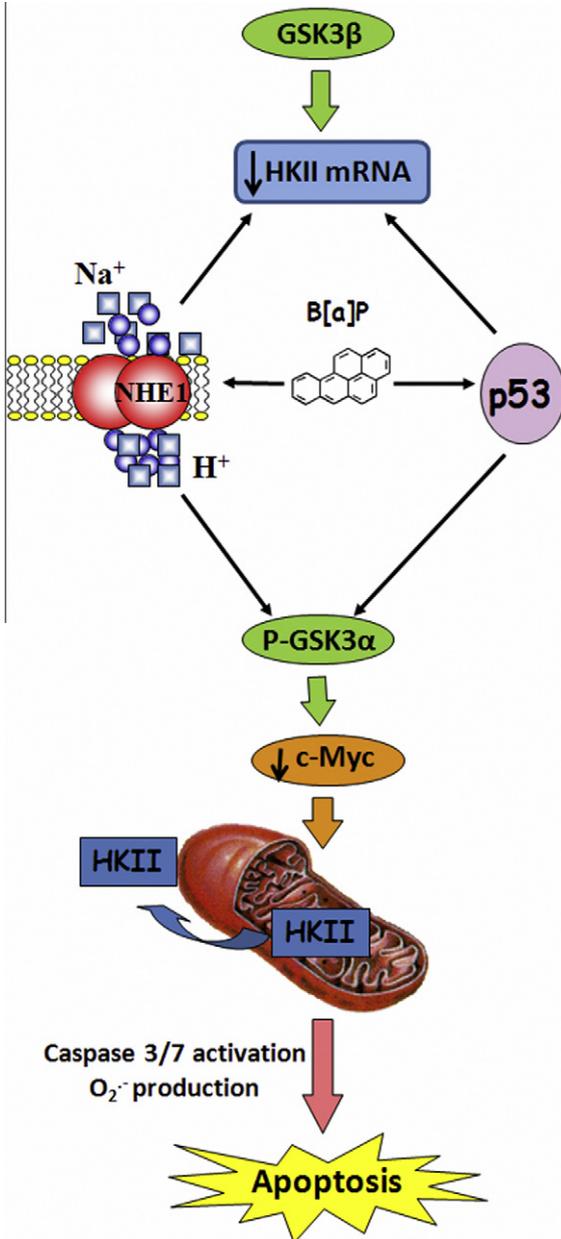


Fig. 5. Schematic summary of the role of GSK3 in the B[a]P-induced effects on HKII in F258 cells.

## Acknowledgements

We wish to thank Mary Rissel for her skillful technical assistance and Olivier Fardel for fruitful discussions. We also wish to thank St phanie Dutertre and the Platform "Microscopie" of IFR140, Universit  Rennes 1. This study was financially supported by the Ligue Nationale contre le Cancer. B atrice Dendel  was a recipient of a fellowship from the R gion Bretagne. Xavier Tekpli was a recipient of a fellowship from the French Ministry of Research and from the Association for Research on Cancer (ARC).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tiv.2011.11.001.

## References

- Ahn, K.J., Hwang, H.S., Park, J.H., Bang, S.H., Kang, W.J., Yun, M., Lee, J.D., 2009. Evaluation of the role of hexokinase type II in cellular proliferation and apoptosis using human hepatocellular carcinoma cell lines. *J. Nucl. Med.* 50, 1525–1532.
- Beurel, E., Jope, R.S., 2006. The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. *Prog. Neurobiol.* 79, 173–189.
- Bijur, G.N., De Sarno, P., Jope, R.S., 2000. Glycogen synthase kinase-3beta facilitates staurosporine- and heat shock-induced apoptosis. Protection by lithium. *J. Biol. Chem.* 275, 7583–7590.
- Cao, Z., Fan-Minogue, H., Bellocin, D.I., Yevtodiynko, A., Arzeno, J., Yang, Q., Gambhir, S.S., Felsner, D.W., 2011. MYC phosphorylation, activation, and tumorigenic potential in hepatocellular carcinoma are regulated by HMG-CoA reductase. *Cancer Res.* 71, 2286–2297.
- Ciani, L., Salinas, P.C., 2005. WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nat. Rev. Neurosci.* 6, 351–362.
- Dang, C.V., Le, A., Gao, P., 2009. MYC-induced cancer cell energy metabolism and therapeutic opportunities. *Clin. Cancer Res.* 15, 6479–6483.
- Das, S., Wong, R., Rajapakse, N., Murphy, E., Steenberg, C., 2008. Glycogen synthase kinase 3 inhibition slows mitochondrial adenine nucleotide transport and regulates voltage-dependent anion channel phosphorylation. *Circ. Res.* 103, 983–991.
- Ding, V.W., Chen, R.H., McCormick, F., 2000. Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. *J. Biol. Chem.* 275, 32475–32481.
- Feyt, C., Kienlen-Campard, P., Leroy, K., N'Kuli, F., Courtoy, P.J., Brion, J.P., Octave, J.N., 2005. Lithium chloride increases the production of amyloid-beta peptide independently from its inhibition of glycogen synthase kinase 3. *J. Biol. Chem.* 280, 33220–33227.
- Fields, W.R., Desiderio, J.G., Leonard, R.M., Burger, E.E., Brown, B.G., Doolittle, D.J., 2004. Differential c-myc expression profiles in normal human bronchial epithelial cells following treatment with benzo[a]pyrene, benzo[a]pyrene-4, 5 epoxide, and benzo[a]pyrene-7, 8–9, 10 diol epoxide. *Mol. Carcinog.* 40, 79–89.
- Force, T., Woodgett, J.R., 2009. Unique and overlapping functions of GSK-3 isoforms in cell differentiation and proliferation and cardiovascular development. *J. Biol. Chem.* 284, 9643–9647.
- Frame, S., Cohen, P., 2001. GSK-3 takes centre stage more than 20 years after its discovery. *Biochem. J.* 359, 1–16.
- Gregory, M.A., Qi, Y., Hann, S.R., 2003. Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. *J. Biol. Chem.* 278, 51606–51612.
- Hernandez-Davies, J.E., Zape, J.P., Landaw, E.M., Tan, X., Presnell, A., Griffith, D.J., Heinrich, M.C., Glaser, K.B., Sakamoto, K.M., 2011. The multi-targeted receptor tyrosine kinase inhibitor, Linifanib (ABT-869), induces apoptosis through an AKT and Glycogen Synthase Kinase 3(beta)-dependent pathway. *Mol. Cancer Ther.* 10, 949–959.
- Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O., Woodgett, J.R., 2000. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 406, 86–90.
- Huc, L., Rissel, M., Solhaug, A., Tekpli, X., Gorria, M., Torriglia, A., Holme, J.A., Dimanche-Boitrel, M.T., Lagadic-Gossman, D., 2006. Multiple apoptotic pathways induced by p53-dependent acidification in benzo[a]pyrene-exposed hepatic F258 cells. *J. Cell. Physiol.* 208, 527–537.
- Huc, L., Sparfel, L., Rissel, M., Dimanche-Boitrel, M.T., Guillouzo, A., Fardel, O., Lagadic-Gossman, D., 2004. Identification of Na<sup>+</sup>/H<sup>+</sup> exchange as a new target for toxic polycyclic aromatic hydrocarbons. *FASEB J.* 18, 344–346.
- Huc, L., Tekpli, X., Holme, J.A., Rissel, M., Solhaug, A., Gardyn, C., Le Moigne, G., Gorria, M., Dimanche-Boitrel, M.T., Lagadic-Gossman, D., 2007. C-Jun NH2-terminal kinase-related Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 1 activation controls hexokinase II expression in benzo(a)pyrene-induced apoptosis. *Cancer Res.* 67, 1696–1705.
- Kim, J.W., Gao, P., Liu, Y.C., Semenza, G.L., Dang, C.V., 2007. Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Mol. Cell. Biol.* 27, 7381–7393.
- Kim, L., Kimmel, A.R., 2000. GSK3, a master switch regulating cell-fate specification and tumorigenesis. *Curr. Opin. Genet. Dev.* 10, 508–514.
- Kotliarova, S., Pastorino, S., Kovell, L.C., Kotliarov, Y., Song, H., Zhang, W., Bailey, R., Maric, D., Zenklusen, J.C., Lee, J., Fine, H.A., 2008. Glycogen synthase kinase-3 inhibition induces glioma cell death through c-MYC, nuclear factor-kappaB, and glucose regulation. *Cancer Res.* 68, 6643–6651.
- Markou, T., Cullingford, T.E., Giraldo, A., Weiss, S.C., Alsafi, A., Fuller, S.J., Clerk, A., Sugden, P.H., 2008. Glycogen synthase kinases 3alpha and 3beta in cardiac myocytes: regulation and consequences of their inhibition. *Cell. Signal.* 20, 206–218.
- Mathupala, S.P., Rempel, A., Pedersen, P.L., 2001. Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. *J. Biol. Chem.* 276, 43407–43412.
- Matsuda, T., Zhai, P., Maejima, Y., Hong, C., Gao, S., Tian, B., Goto, K., Takagi, H., Tamamori-Adachi, M., Kitajima, S., Sadoshima, J., 2008. Distinct roles of GSK-3alpha and GSK-3beta phosphorylation in the heart under pressure overload. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20900–20905.

- Pastorino, J.G., Hoek, J.B., 2003. Hexokinase II: the integration of energy metabolism and control of apoptosis. *Curr. Med. Chem.* 10, 1535–1551.
- Pastorino, J.G., Hoek, J.B., Shulga, N., 2005. Activation of glycogen synthase kinase 3 $\beta$  disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity. *Cancer Res.* 65, 10545–10554.
- Peng, S.Y., Lai, P.L., Pan, H.W., Hsiao, L.P., Hsu, H.C., 2008. Aberrant expression of the glycolytic enzymes aldolase B and type II hexokinase in hepatocellular carcinoma are predictive markers for advanced stage, early recurrence and poor prognosis. *Oncol. Rep.* 19, 1045–1053.
- Plas, D.R., Rathmell, J.C., Thompson, C.B., 2002. Homeostatic control of lymphocyte survival: potential origins and implications. *Nat. Immunol.* 3, 515–521.
- Podar, K., Anderson, K.C., 2010. A therapeutic role for targeting c-Myc/Hif-1-dependent signaling pathways. *Cell Cycle* 9, 1722–1728.
- Salas, V.M., Burchiel, S.W., 1998. Apoptosis in Daudi human B cells in response to benzo[a]pyrene and benzo[a]pyrene-7, 8-dihydrodiol. *Toxicol. Appl. Pharmacol.* 151, 367–376.
- Solhaug, A., Refsnes, M., Holme, J.A., 2004. Role of cell signalling involved in induction of apoptosis by benzo[a]pyrene and cyclopenta[c, d]pyrene in Hepa1c1c7 cells. *J. Cell. Biochem.* 93, 1143–1154.
- Spokoini, R., Kfir-Erenfeld, S., Yefenof, E., Sionov, R.V., 2010. Glycogen synthase kinase-3 plays a central role in mediating glucocorticoid-induced apoptosis. *Mol. Endocrinol.* 24, 1136–1150.
- Tekpli, X., Rissel, M., Huc, L., Catheline, D., Sergent, O., Rioux, V., Legrand, P., Holme, J.A., Dimanche-Boitrel, M.T., Lagadic-Gossmann, D., 2010. Membrane remodeling, an early event in benzo[a]pyrene-induced apoptosis. *Toxicol. Appl. Pharmacol.* 243, 68–76.
- Valera, A., Pujol, A., Gregori, X., Riu, E., Visa, J., Bosch, F., 1995. Evidence from transgenic mice that myc regulates hepatic glycolysis. *FASEB J.* 9, 1067–1078.
- Vene, R., Larghero, P., Arena, G., Sporn, M.B., Albini, A., Tosetti, F., 2008. Glycogen synthase kinase 3 $\beta$  regulates cell death induced by synthetic triterpenoids. *Cancer Res.* 68, 6987–6996.
- Zedeck, M.S., 1980. Polycyclic aromatic hydrocarbons: a review. *J. Environ. Pathol. Toxicol.* 3, 537–567.
- Zhai, P., Gao, S., Holle, E., Yu, X., Yatani, A., Wagner, T., Sadoshima, J., 2007. Glycogen synthase kinase-3 $\alpha$  reduces cardiac growth and pressure overload-induced cardiac hypertrophy by inhibition of extracellular signal-regulated kinases. *J. Biol. Chem.* 282, 33181–33191.
- Zhao, W., Ramos, K.S., 1998. Modulation of Hepatocyte Gene Expression by the Carcinogen Benzo[a]pyrene. *Toxicol. In Vitro* 12, 395–402.