Convocatoria de ayudas de Proyectos de Investigación Fundamental no orientada

TECHNICAL ANNEX FOR TYPE A or B PROJECTS

1. SUMMARY OF THE PROPOSAL (the summary must be also filled in Spanish)

PROJECT TITLE: Q-METABOLISM IN CELL GROWTH AND SURVIVAL: IMPLICATIONS FOR ANTI-TUMOUR THERAPY.
PRINCIPAL INVESTIGATOR: JAVIER MÁRQUEZ GÓMEZ

SUMMARY
(brief and precise, outlining only the most relevant topics and the proposed objectives):

This proposal is designed to pursue our studies of the relevance of glutamine (Q, Gln) metabolism in cancer growth and proliferation. Pertinent to this goal, we have found that inhibition by antisense technology of K-type glutaminase (KGA), an enzyme linked to neoplastic transformation, allows reversion of the transformed phenotype *in vitro* and makes tumour cells unable to grow *in vivo* due to their inability to evade the host immune response. Furthermore, we demonstrated a similar tumour regression by overexpressing the L-type GA isozyme in human glioblastoma, supporting a role for this isozyme in transcriptional regulation. Interestingly, recent findings showed that c-Myc controls enhanced glutaminolysis in human leukemia and prostate cancers through a microRNA (miRNA) mechanism, and that Gln itself exerts influence over a number of signaling pathways that contribute to tumour growth. However, at present there are no molecular tools of potential therapeutic significance to interfere with the tumoural glutaminolytic process, because anti-Q metabolites in chemotherapy have proven to be quite unspecific and with many side effects. Accordingly, specific Aim 1 will characterize the miRNAs controlling Q-metabolism in KGA-knockdown [KGA (–)] glioma and breast cancer cells and their conexions with known oncongenes and tumour suppresor genes. Specific Aim 2 will undertake a detailed functional genomic analysis to identify miRNAs targeting L-type GA expression in human tumour cell. These studies will be validated in human cancer specimens. Specific Aim 3 will determine the role of L-type GA in the regulation of gene expression and its implication in cell differentiation. Specific Aim 4 will identify the mitochondrial Gln carrier as another useful target for anti-Q therapy. Overall, this proposal should (i) shed light into the tumourigenesis by identifying molecular events that are critical to the metabolic reprogramming shown by cancer cells, and (ii) lay the ground for the development of novel therapeutic strategies aimed at interfering with Q-catabolism.

TITULO DEL PROYECTO: METABOLISMO DE GLUTAMINA EN EL CRECIMIENTO Y SUPERVIVENCIA CELULAR: IMPLICACIONES PARA LA TERAPIA ANTITUMORAL.

RESUMEN
(breve y preciso, exponiendo sólo los aspectos más relevantes y los objetivos propuestos):

Este proyecto continúa nuestros estudios sobre la relevancia del metabolismo de glutamina (Q, Gln) en el crecimiento y la proliferación tumoral. En relación con este objetivo, hemos demostrado que la inhibición de glutaminasa (GA) tipo K (KGA) mediante tecnología antisentido induce reversión del fenotipo transformado *in vitro* e impide su crecimiento *in vivo* en ratones al activar el sistema inmune del animal. Una reversión similar del fenotipo tumoral se obtuvo al sobreexpresar la isoenzima tipo L de GA en glioblastoma humano, sugiriendo un papel de esta proteína en la regulación de la expresión génica. Recientemente, se ha demostrado que la glutaminolisis, activada en leucemias humanas y cáncer de próstata, está controlada por el oncogen c-Myc a través de un mecanismo regulador basado en microRNA (miRNA). En paralelo, numerosas evidencias experimentales indican que la Gln regula de forma esencial rutas de señalización implicadas en el crecimiento y la proliferación tumoral. Sin embargo, no se dispone actualmente de herramientas moleculares con potencial terapéutico para interferir con la glutaminolisis tumoral, dado que los metabolitos anti-Gln presentes en formulaciones de quimioterapia son inespecíficos y, a menudo, presentan indeseables efectos secundarios. De acuerdo con estos antecedentes, nuestro objetivo específico 1 caracterizará los miRNAs que regulan el metabolismo de Gln en células de glioma y cáncer de mama con silenciamiento génico de KGA [KGA (–)], y sus conexiones con oncogenes y genes supresores tumorales. El objetivo específico 2 contempla un análisis de genómica funcional para identificar los miRNAs relevantes para la expresión de la GA tipo L en líneas tumorales humanas. Estos estudios se intentarán validar empleando especímenes de cáncer humano. El objetivo específico 3 elucidará el papel de la GA tipo L en la regulación de la expresión génica y su implicación en la diferenciación celular. El objetivo específico 4 identificará el transportador mitocondrial de Gln, como otra diana potencial para la terapia anti-Gln. Globalmente, este proyecto pretende (i) arrojar luz sobre el proceso de tumorigénesis mediante la identificación de eventos moleculares críticos en la reprogramación metabólica de las células cancerosas, y (ii) establecer las bases para el desarrollo de nuevas estrategias terapéuticas dirigidas a interferir con el catabolismo tumoral de la Gln.

2. INTRODUCTION
(maximum 5 pages)

- The introduction should include: the aims of the project; the background and the state of the art of the scientific knowledge, including the essential references; the most relevant national and international groups working in the same or related topics.

The long-term research objective of this proposal is to shed light into glutamine (Q, Gln) functions in cancer and, hence, being able to support future efforts to manipulate Gln metabolism in tumours and other diseases that may become useful therapeutic strategies. The interest in Gln metabolism has been heightened by recent findings showing that c-Myc controls Gln uptake and degradation in human cancers through a microRNA (miRNA) mechanism (1), and that Gln itself exerts influence over a number of signaling pathways that contribute to tumour growth (2,3). The research programme consist of four main milestones: (i) the characterization of the miRNAs controlling Q-metabolism in KGA-knockdown [KGA(–)] glioma and breast cancer cells, and their conexions with known oncongenes and tumour suppresor genes; (ii) the functional genomic analysis to identify miRNAs targeting L-type GA expression in human tumour cell; (iii) the elucidation of the role of L-type GA in the regulation of gene expression and its implication in cell differentiation and; (iv) the identification of the mitochondrial Gln carrier. These studies will be validated in human cancer specimens. The work outlined below is a multidisciplinary state-of-the-art research that combines genetic, functional genomic and proteomic approaches to determine the potential therapeutic impact of abolishing Q-catabolism in certain types of tumours.

Gln acts as a nitrogen donor for purine and pyrimidine nucleotide synthesis for new DNA, mRNA repair, synthesis of amino acids, carbamoylphosphate, amino sugars, and other metabolites. Gln and glucose are two main substrates for tumour and proliferant cells and their rates of consumption are far higher than the energy and biosynthetic requirements (4). The major degradative pathway for Gln is carried out in mitochondria and is initiated by the enzyme phosphate-activated glutaminase (GA; EC 3.5.1.2). The breakdown of Gln to pyruvate, known as glutaminolyis, drives both a source of biosynthetic precursors and NADPH production in cancer cells. Thus, complete Gln oxidation via the oxidative phosphorylation boasts ATP, widely appreciated for rapid proliferation of cancers. Multiple lines of evidence indicate that the process of tumourigenesis is often associated with altered Q-metabolism. In cancer cell lines, Q-supplementation stimulates proliferation, and promotes a less differentiated phenotype (5). In addition, Gln can serve as an alternative substrate for the Krebs cycle, as well as an redox modulator: Gln, via glutamate (Glu), is precursor for the synthesis of glutathione (GSH), the major endogenous antioxidant in mammalian cells (6). A recent study has revealed the necessity of Gln for the well-known stimulatory effect of essential amino acids (EAA) on the *mammalian target of rapamycin* (mTOR) pathway in Hela cells (7).

The GA protein family members are encoded by two paralogous genes, *Gls* and *Gls2*, presumably derived by gene duplication of a common ancestor. In humans, *Gls* is located in chromosome 2 and encodes GA isozymes classically referred to as kidney-type (K-type or K), while the *Gls2* gene is located in chromosome 12 and codes for liver-type (L-type or L) isozymes (8). Two isoforms derived from each GA gene have been so far identified. The transcripts known as KGA and GAC arise by alternative splicing of the *Gls* gene: the KGA isoform is expressed in all the tissues except liver, whereas the GAC isoform is expressed in heart, pancreas, kidney, lung, and breast and colon cancer (9). Two L-type transcripts have been identified from the *Gls2* gene: the canonical long transcript termed GAB, formed by joining the full 18 exons of the gene, and the short transcript LGA that lacks exon 1 and was originally identified in rat liver (10). Human GAB transcript was isolated by our group as a cDNA clone from ZR-75 breast cancer cells encoding a protein of 602 amino acids, which is 67 amino acids longer than rat liver LGA protein (11). Experimental evidences supporting GAB as a novel L-type GA isoform, different from the classical LGA liver isozyme, have been recently published (10). L-type transcripts derived from the *Gls*2 gene were originally thought to be present in adult liver tissue and absent in extrahepatic tissues. This restricted pattern of expression was generally accepted until quite recently, when results from our laboratory demonstrated L-type GA expression in extrahepatic tissues like brain, pancreas and breast cancer cells (11).

GA overexpression seems to be a hallmark exhibited by many tumours (12). Studies on experimental and human tumours looking at changes in enzymatic activity and relative mRNA levels of both Gln synthetase (GS) and GA revealed a similar pattern in many cases: a knockdown of GS expression along with an overexpression of GA (12). The pattern of expression of GA isozymes in tumour cells has been investigated to clarify its role in the malignant transformation and the prospect of its use as a clinically relevant factor. Using leukaemia cells from medullar blood of human patients and several established breast cancer cell lines, coexpression of both transcripts were always found using a competitive RT-PCR assay (13). However, mature lymphocytes from the medullar blood of patient suffering aplasia did not express the K-type transcript and showed a 15-fold increase of L-type transcript. Coexpression was also confirmed at the protein level using isoform-specific antibodies, although the protein data suggest that K-type isoform would account for the majority of GA activity in these human tumour cells (13). These results confirm that simultaneous expression of both K- and L-type GA isozymes in the same cancer cell type is more frequent than previously believed: it has been found in human colorectal tumour cells (14), human hepatoma HepG2 cells, medullar blood mononuclear cells from patients suffering from leukaemia, KU812F human

myeloid cells and human breast cancer cells MCF7 and ZR-75 (13). Furthermore, in human colorectal tumour cells most GA activity was K-type despite of the fact that these cells expressed both GAC and L-type transcripts (14). Taken together, the data suggest that K-type isoform up-regulation correlates with increased rates of proliferation, whereas prevalence of the L-type isoform seems to be related with resting or quiescent cell states. This hypothesis was confirmed by experiments using the mouse Ehrlich ascites tumour cell (EATC) as a model of KGA: our group demonstrated that EATC transfected with antisense KGA cDNA constructs (0.28AS-2 cell line) decreased tumour cell proliferation, showed a reversion of their transformed phenotype *in vitro* (15), and were unable to grow *in vivo* in the peritoneal cavity of mice due to their inability to reject the host immune response (16). Moreover, the antisense GA expression induced apoptosis in 0.28AS-2 cells, caused oxidative stress and sensitised the cells to methotrexate (17).

Recently, it has been reported that oncogenes influence Q-metabolism and that tumour genetics can dictate cellular dependence on Gln for survival. Enhanced c-Myc activity was sufficient to drive Q-metabolism and to impair cell survival in low-Gln conditions (18, 19). c-Myc regulates Q-metabolism in part by stimulating the expression of surface transporters (19). Oncogene c-Myc also indirectly regulates the protein expression of the *Gls* gene through effects on the miRNAs miR23a and miR23b. Normally, these miRNAs bind to the 3'-untranslated region (3'-UTR) of the *Gls* gene and prevent translation of the message. However, c-Myc suppresses miR-23a/b expression and thus derepressed *Gls* translation, facilitating Gln oxidation in the mitochondria (1). Under apoptotic conditions, Gln can also regulate the Wnt and NFKB signalling pathways, as well as controlling expression of the PI3K/Akt pathway (20). Further studies will be required to clarify the definitive picture regarding multifaceted effects of Gln in cancer. These observations stimulate a renewed efforts to understand metabolic reprogramming in tumours and to develop anticancer strategies targeting Q-metabolism (2).

We have previously detailed the 0.28AS-2 tumour cell line as a KGA (-) model (15). Changes at the level of their transcriptome were characterized by functional genomics analysis using the differential display technique. Four genes were significantly down-regulated in KGA-inhibited cells: high-mobility group HMGA2 protein, FMNL3 or formin-like protein 3, NEDD-4 ubiquitin-protein ligase, and ubiquitin carboxyl-terminal hydrolase USP-15 (21). It is worth mentioning that HMGA2 is a member of the *Hmga* gene family of nonhistone chromosomal proteins, often referred to as architectural transcription factors, which has been clearly implicated in cell proliferation and tumourigenesis: overexpression of HMGA2 has been reported in various malignant tumours, including breast cancer. Further phenotypic changes were observed in KGA (-) 0.28AS-2 cells, including a diminished expression of mucin MUC-1, cytokine TGF-β, and reduced activity of N-acetyl-β-D-galactosaminidase, that were in part responsible of the activation of the host immune system that rejected *in vivo* growth of 0.28AS-2 cells (16). Furthermore, the hexosamine pathway and the O-glycosylation of proteins was also altered by KGA inhibition. Surprisingly, Gln: F6P amidotransferase activity could be suppressed by inhibiting KGA in mammary tumours. Impairment of glucosamine-6-phosphate production is predicted to reduce cell growth and to interfere with cell signaling. This resulted in disturbances of O-linked glycosylation pathways, altering the glycosylation status of the transcription factor Sp-1 and increasing its transcriptional activity (22). The mechanism by which loss of KGA activity influences glycosylation is unclear, but recent findings suggest that Gln:F6P amidotransferase, and perhaps other components of the glycosylation machinery, are responsive to intracellular Gln availability which, in turn, is determined largely by KGA activity. Thus, KGA knockdown has dramatic consequences on cell growth, proliferation and survival; therefore, it is worth considering KGA as an effective target for anti-cancer strategy.

Little is known about the molecular mechanisms explaining the KGA (-) phenotype of tumour cells. In an effort to uncover the effects of KGA knockdown, the expression profile of miRNAs was recently screened in wild-type EATC cells and KGA (-) 0.28AS-2 cells, using miRXplore™ Microarrays (unpublished results). There were 9 miRNAs that significantly changed their pattern of expression. Relevant to this proposal were the following miRNAs: mir-125A (0.61x), mir-16 (1.6x), mir-26A (1.7x) and mir-145 (2.7x) (number in parenthesis indicated the fold variation as compared with the wild type cell line). These data, taken together with others recently published in the scientific literature, suggest that changes in miRNA expression would lead to changes in the p53 / c-Myc balance in KGA (—) cancer cells (Fig. 1).

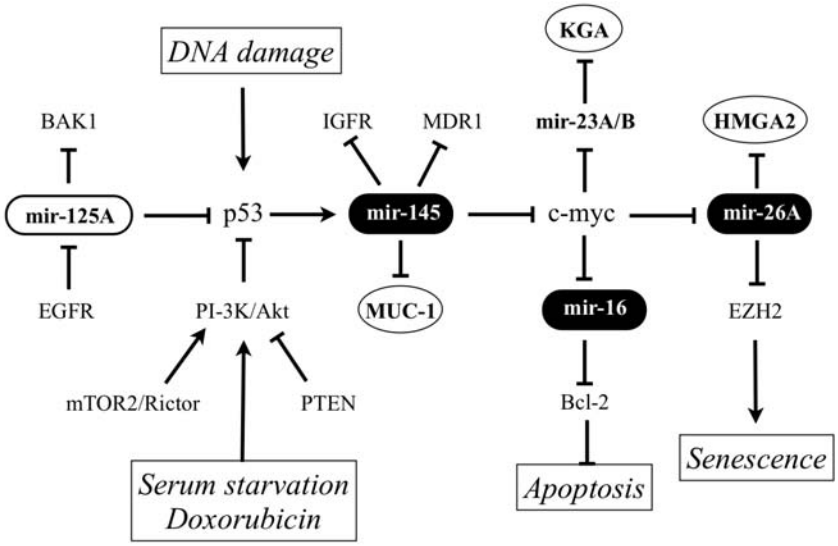

Figure 1. Changes in miRNAs and signalling pathways associated to GA inhibition in cancer cells. Up-regulated miRNAs are shown in black boxes while the down-regulated one is shown in a white box. Muc-1 and Hmga2 genes were shown to be down-regulated in the KGA (—) 0.28AS-2 tumour cell line. These changes would partially explain the phenotype of KGA knockdown cells and points to mir-145 as a key intermediate in the mechanisms underlying KGA inhibition by signalling through PI-3K/Akt and p53 pathways. Arrows denote activation and broken lines inhibition.

Contrary to KGA, the role of L-type isozyme in tumour cells is greatly unknown. Although coexpression of both isozymes is the most common situation in human tumours, we do not know, for example, why in some gliomas, like T98G, expression of LGA is repressed (23). Previous studies also revealed that L-type GA mRNA expression was hardly detectable or low in postoperative samples of glia-derived human tumours (23,24). A crucial difference between GAB and the rest of GA isoforms so far described is that GAB has been found in extramitochondrial localizations, while KGA and LGA have been always exclusively confined in mitochondria (25). The first report of an extramitochondrial localization for a mammalian GA came from immunocytochemistry studies employing anti-GAB antibodies in rat and monkey brain: L-type GA was reported to be present in brain cell nuclei (26). Moreover, the ability of the C-terminus of GAB to interact with PDZ domain-containing proteins has been shown (27). These observations suggest that L-type GA may play a role in the regulation of transcription besides its Gln-hydrolyzing function (25, 26).

Furthermore, in view of the presumed role of L-type GA in modulation of gene transcription, we hypothesized that its deficit has implications for the physiology of glia-derived tumours, perhaps driving them toward malignant phenotype. To address this question, we stably transfected T98G cells with the full GAB cDNA coding sequence and assessed the effects of transfection on basic physiological parameters: proliferation, migration and survival. Next, we used the microarray technique to search for potential differences in gene expression. T98G cells with a vector carrying human GAB sequence increased the expression of GAB mRNA and protein, and the ability of the cells to degrade Gln, as manifested by a three-fold reduction of their steady-state Gln content and a 2.5-fold increase of their Glu content. The transfected cells (T98-GAB cells) showed a 40% decrease of cell survival, well correlated with significant reduction of mitochondrial activity. Also, a 45% reduction of cell migration and a 47% decrease of proliferation index were found as compared with sham-transfected cells. Microarray analysis, which included over 47,000 transcripts, revealed a significantly altered expression of 85 genes in T98-GAB, but not in sham-transfected or control cells ($P < 0.005$). Microarray data were confirmed with real-time PCR analysis for eight genes potentially relevant to malignancy: S100A16, CAPN2, FNDC3B, DYNC1LI1, TIMP4, MGMT, ADM, and TIMP1. Of these changes, decreased expression of S100A16 and MGMT can be best reconciled with the current views on the role of their protein products in glioma malignancy (24).

The mechanism by which GAB enters the nucleus at present is unclear because GAB lacks any known nuclear targeting signal. Therefore, it is tempting to speculate that GAB may reach the nucleus through interactions with other proteins, although this hypothesis remains to be explored. Nevertheless, translocation of a mitochondrial enzyme, lacking a specific nuclear targeting signal, to the nucleus is not without precedent. Mitochondrial 3-hydroxy-3-methyl-glutaryl-CoA synthase has been detected in nuclei. Nuclear translocation of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase seems to involve interaction with nuclear hormone receptors through the L*XX*LL motif present in the mitochondrial enzyme (28). The L*XX*LL motif is a signature sequence that facilitates the interaction of different proteins with nuclear receptors. Strikingly, human GAB contains one such consensus motif, LGDLL, at positions 72-76 of its protein sequence; whether this short motif is necessary and sufficient to mediate the interaction between GAB and some nuclear receptor is an issue that we want to address here. Furthermore, we have recently found that GAB has a consensus site for O-glycosylation, a reversible

modification that takes place in serine or threonine residues and consists in the addition of a single residue of O-linked N-acetylglucosamine (O-GlcNAc). The O-glycosylation of GAB has been confirmed in our laboratory by preliminary immunoprecipitation experiments (unpublished results). Protein modification by O-GlcNAc has been shown to play a key role in fundamental processes like signal transduction regulation, nuclear protein import and cytoskeletal organization. In the present project, we will investigate whether O-glycosylation is a mechanism to target GAB to the nucleus.

Future analysis of inherited defects of glutaminolytic enzymes, molecular characterization of mitochondrial Gln carriers (MGC) and GA, as well as generation of transgenic animal models will provide further insights into the specific actions of Gln and give further rationale for considering Gln as a therapeutic tool (4). Gln has to be transported through both plasma and inner mitochondrial membranes before glutaminolysis can take place. The MGC is another rational strategy to limit tumour Gln uptake and thus reducing the glutaminolytic pathway which seems to be essential for proliferation. Therefore, in this proposal we will undertake the identification and preliminary characterization of the MGC in human cancer cells, which at present has not been identified at the molecular level. Finally, the molecular portrait of GA expression in human tumour specimens would be of interest in clinical studies if a pervasive pattern would be associated with proliferation, degree of malignancy, response to chemotherapy or clinical outcome of the patients. With this goal in mind, the results obtained in this proposal will be validated by studying the pattern of GA and gene interacting networks in real human tumour samples.

OTHER NATIONAL AND INTERNATIONAL RESEARCH TEAMS WORKING IN THIS FIELD OR IN HIGHLY RELATED TOPICS (the existence of previous or current collaborations is indicated).

-Dr. Ralph deBerardinis, University of Texas Southwestern Medical Center, Dallas, TX (USA), Present Collaboration: Targeting Gln metabolism in human brain tumours. Four human glioma cell lines used in this proposal have been generously donated by Dr. deBerardinis, in the framework of current ongoing collaborations between both research groups.

-Dr. Jan Albrecht, Dept. of Neurotoxicology, Varsow (Poland), Present Collaboration: GA expression in human brain tumours. The T98G glioblastoma cell line overexpressing GAB enzyme is a scientific outcome of our current collaboration.

-Dr. Jorgina Satrústegui, CBM Severo Ochoa, Universidad Autónoma de Madrid, Present Collaboration: Characterization of the mitochondrial Gln carrier (MGC) in tumour cells. Her experience in mitochondrial carriers and their gene silencing using shRNA will be another invaluable collaboration in this proposal.

-Dr. Enrique Calvo, Servicio de Proteómica, CNIC, Madrid, Present Collaboration: Proteomics studies on human glutaminases and related interactors.

-Dr. Ana Pérez Castillo. Instituto de Investigaciones Biomedicas (Madrid), Previous Collaboration: Regulation of gene expression in tumour cells by PPAR nuclear receptors and CEBP proteins.

-Dr. Fernando Rodríguez de Fonseca, Hospital Carlos de Haya (Málaga, Spain), Present Collaboration: Glutamatergic enzymes and cocaine addiction: Functional genomics and proteomics analyses.

-Dr. Antonia Gutiérrez, Dpto. Biología Celular, University of Málaga (UMA) (Spain), GABA receptors in brain, animal models of Parkinson disease. Present Collaboration: GA neurochemistry studies.

-Dr. Ole P. Ottersen, Director, Centre for Molec. Biol. and Neuroscience, University of Oslo (Norway). Previous Collaboration: KGA and LGA expression in brain of in patients with mesial temporal lobe epilepsy.

-Dr. Linda M. Castell, University of Oxford (U.K.), Previous Collaboration: functional and cellular localization studies of LGA in human polymorphonuclear neutrophils (PMN).

-Dr. Vicente Felipo, Department of Neurobiology, Instituto de Investigaciones Citológicas (Valencia, Spain). Previous Collaboration: Expression of glutamatergic enzymes in animal models of hyperamonemia.

-Dr. Benjamín Gaston, University of Virginia (USA), New paradigm of lung disease: airway acidification underlies many of the symptoms of asthma, emphysema and respiratory failure. Previous Collaboration : Glutaminase expression in human pulmonary airway ephitelium.

-Dr. Rosalie McCauley, University of Perth (Australia), Previous Collaboration: gene therapy with antisense GA cDNAs and oligonucleotides in rat mammary and prostate tumours.

-Dr. Smita Mohanty, State University of New York, Stony Brook (USA), Previous Collaboration: RMN studies of purified GIP to determine the three dimensional structure of its PDZ domain.

-Dr. J.M. Estrela, Dept. of Physiology, University of Valencia, Previous Collaboration: transcriptional regulation, glutathione and redox status.

-Dr. M. Yuneva, University of San Francisco, (USA), Present Collaboration: KGA expression on immortalized human kidney epithelial cells and in primary human lung fibroblasts.

-Dr. Norman P. Curthoys, Colorado State University (USA), Regulation of renal glutamine metabolism.

-Dr. John D. McGivan, Bristol University (UK), Regulation of hepatic glutamine metabolism.

-Dr. Dieter Haüssinger, Universität Düsseldorf (Alemania), Glutamine as a cellular signal.

-Dr. Ruben J. Boado, University of California Los Angeles (USA), Assesment on RNAi design and applications.

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3. OBJECTIVES
(maximum 2 pages)

υ 3.1 Describe the reasons to present this proposal and the initial hypothesis which support its objectives (maximum 20 lines)

Since the 1950's cancer biologists have recognized the importance of Gln as a tumour nutrient. Metabolic imbalance is a hallmark of cancer and Gln-dependence is a common phenotype in the altered cancer cell-intrinsic metabolism, either as a consequence or as a cause. Since the 1980's our research team and others have provided solid arguments showing that cancer cells revert their transformed phenotype by interfering selectively with Q-catabolism, particularly with GA isoenzymes. The up-regulationof Q-metabolism is a phenotypic characteristic exhibited by many types of cancer. However, the molecular mechanisms underlying the Gln avidity in carcinomas were not known, until quite recently when an association with c-Myc oncogene through a miRNA-mediated regulatory mechanism has been reported. Therefore, our initial hypothesis states that interfering Gln avidity in tumours by targeting GA and the mitochondrial Q-carrier is an effective anti-cancer strategy for therapeutic purposes. The signals and mechanisms underpinning this metabolic shift are largely unknown and not fully understood, but they are certainly involved in the regulation of cell growth, proliferation and apoptosis. Disrupting this tumour cell's unorthodox metabolism with regard to Gln is the main goal of this project. Characterization of genetically-engineered tumour cells targeting GA and Q-carriers will help to identify tumour-specific regulators, which can be exploited to develop selective therapeutics against cancer. Overall, this proposal should (i) shed light into the tumourigenesis by identifying molecular events that are critical to the metabolic reprogramming shown by cancer cells, and (ii) lay the ground for the development of novel therapeutic strategies aimed at interfering with Q-catabolism.

υ 3.2. Indicate the background and previous results of your group or the results of other groups that support the initial hypothesis

The proposed project builds on our previous research work, which resulted in the discovery of a novel GA isoform (GAB) and the adscription of different roles to GA isozymes in cell proliferation and differentiation. There is a recent body of evidence which strongly support our working hypothesis for anti-Q therapy as an anticancer strategy, namely the inhibition of tumour growth, proliferation and migration by either KGA repression or GAB overexpression. In addition, preliminary data of our group and sound results recently published by other scientists in this area, some of them in collaboration with our group, confirm the feasibility of reaching the stated aims within a three-year framework. Established collaborations and unique research tools enhance the feasibility of the proposed project. The fundamental groundwork knowledge enabling development of this proposal can be briefly summarized as follows:

-The molecular portrait of GA expression in human leukemia cells and several tumour cell lines confirmed that K-type GA expression is up-regulated with increased rates of proliferation, whereas repression of K-type and prevalence of L-type GA transcripts were related to quiescent or resting states.

-The strikingly different subcellular locations of GA isoforms: K-type GA located in mitochondria and L-type GA in nuclei.

-The reversion of the malignant phenotype achieved after knocking down KGA in mammary tumour cell lines, as demonstrated by a marked inhibition of cancer growth and proliferation *in vitro* along with lack of tumourigenicity *in vivo*.

-The recent evidence demonstrating a cross-talk between oncogene c-Myc up-regulation and enhanced glutaminolysis in human leukemia and prostate cancer cells: increased expression of Q-carriers and K-type GA were elicited through miRNA 23a/b repression.

-The mi-RNA expression profiles obtained for KGA (–) 0.28AS-2 cells *vs.* their wild-type EATC cells showed altered expression of miRNAs that are key regulators of p53/c-Myc, MUC-1 and HMGA2. The miRNA signature obtained was dominated by an up-regulation of mir-145, mir-222, mir-26A and mir-16, and down-regulation of mir-125A. This result points to mir-145 and PI-3K/Akt pathway as key mediators of KGA (–) induced phenotype.
-The discovery of protein interacting partners for GAB and the presence of signature sequences and protein motifs on its sequence suggest its role as a multifunctional protein.
-The existence of posttranslational modifications (O-glycosilation) in L-type GA that might be relevant for subcellular targeting and/or regulation of its function.
-The overexpression of human GAB in glioblastoma T98-G cells (T98-GAB cells) promoted a change in the cell's transcriptome conducive to significantly lower proliferation, cell survival and migration rates, characteristics of a less aggressive, less malignant, more differentiated phenotype.
-A cDNA array of T98-GAB glioblastoma cells vs. wild-type T98G cells revealed down-regulation of many genes related with glioma malignancy after GAB overexpression, including several transcription factors, which suggests a role for GAB in transcriptional regulation.
-The characterization of the MGC in mammary tumours (EATC) indicated that Gln transport may represent the rate-limiting step in cancer glutaminolysis.

υ 3.3. Describe briefly the objectives of the project.

1. Characterization of the miRNAs controlling Q-metabolism in KGA (–) glioma and breast cancer cells and their conexions with known oncogenes and tumour suppresor genes.

2. Functional genomic analysis to identify miRNAs targeting L-type GA expression in human tumour cells.

3. Elucidation of the role of L-type GA in the transcriptional regulation of cancer cells and its implication in cell differentiation.

4. Identification and preliminar characterization of the mitochondrial Gln carrier (MGC).

υ 3.4. For Coordinated projects only, the coordinator must indicate (maximum 2 pages):

- the global objectives of the coordinated project, the need for coordination, and the added value provided by this coordination
- the specific objectives of each subproject
- the interaction among the objectives, activities and subprojects
- the mechanisms of coordination for an effective execution of the project.

4. METHODOLOGY AND WORKING PLAN
(in the case of coordinated projects this title must include all the subprojects)

Detail and justify precisely the methodology and the working plan. Describe the working chronogram.

- The working plan should contain the tasks, milestones and deliverables. The projects carried out in the Hesperides or in the Antarctic Zone must include the operation plan.
- For each task, it must be indicated the Centre and the researchers involved in it.
- If personnel costs are requested, the tasks to be developed by the personnel to be hired must be detailed and justified. Remember that personnel costs are eligible only when personnel is contracted, fellowships are not eligible as personnel costs.

TUMOUR CELL MODELS AND PATIENT SPECIMENS.

The proposal will focus in established human tumour cell lines currently in use in the lab of the research team and where the team members have already demonstrated a wide experience in functional genomics and metabolic studies. The selection of gliomas and breast cancer cells as the prevalent tumour models in this proposal is well justified by several reasons. First, essential background and previous results have been obtained by our group in mammary tumours (EATC) and breast cancer cells (MCF7, ZR-75, etc.). Therefore, it is expected that the initial hypothesis about the regulation of K-type GA by a concrete set of miRNAs in the proposed models of human tumour cell lines can be corroborated. Second, gliomas and breast cancer cells usually display c-Myc overexpression, a oncogene related to Q-metabolism in tumours and with implications in miRNA regulation. Third, four stably-transfected tumour cell lines knock-out for KGA [KGA (—)] and one cell line knock-in for GAB [GAB (+)] are available in our lab and, all of them, are gliomas and breast cancer cell lines. Fourth, the participation of two neurosurgeons (Dr. Arráez and Dr. Ibáñez) and two medical oncologists (Dr. Benavides and Dr. Ales) in the project assures the supply of tumour specimens from human patients, mostly gliomas (the most malignant brain tumour) and breast cancers. And fifth, gliomas release Glu as the molecular mechanism to kill surrounding neuronal cells in brain and thereby vacating room for tumour growth and invasion (Sontheimer, H., 2008, J. Neurochem. 105, 287-295); the source of Glu is unknown but K-type GA might be a plausible supplier of released Glu, related with the invasive properties of these brain tumours.

1.- miRNAs AND SIGNALLING CASCADES in KGA (-) TUMOUR CELL MODELS.

1.1.- Down-regulationof genes expression by RNAi technology.

Gene silencing in cultured cells by introduction of small interfering RNAs (siRNAs) will provide a fast and efficient means of knocking down gene expression in short-term experiments. We will acquire siRNAs targeting KGA and the Q-carrier Slc25A44 from Thermo Scientific Dharmacon (ON-TARGET plus, SMART pool). We will follow manufacturer's recommendations about siRNA controls and transfection methods. For long-term gene silencing short hairpins (sh) RNA offer an opportunity to potently and stably silence gene expression. This RNAi strategy is based on cell transfection with plasmids that are able to express primary sh-miRNA. After an endogenous maturation process, the miRNA will generate the small interfering RNA (siRNA) effector molecules. We wil acquire KGA- and Slc25A44-specific sh-miRNA cDNA clones from Sigma (MISSION shRNA). These clones are sequence-verified shRNA lentiviral plasmids for gene silencing in mammalian cells. The parental vector (pLKO.1-puro) allows for transient transfection or stable selection via puromycin resistance. In addition, the plasmids may be

used to generate lentiviral transduction particles in packaging cell lines. We will follow essentially the manufacturer's recommendations to carry out these experiments. The validation of KGA or Q-carrier down-regulationfor each construction will be assessed by qRT-PCR and Western blot. Cell growth rates will be determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method described in the literature.

1.2.- Phenotypic characterization of KGA(-) protein expression profile by Western blot.

We have first described that inhibition of KGA expression leads to a reduced expression of key proteins, like MUC-1 and HMGA-2, associated with tumour transformation. In addition, those changes has been linked to a change in miRNA profiles that modulates p53/c-Myc balance (Fig. 1). Therefore, the aim here is to characterize the expression level of key proteins implicated in p53/c-Myc signalling in our KGA(-) models. Using whole cell extracts, we will analyze expression of the following proteins by Western blot: p53, c-Myc, HMGA-2, MUC-1, EGFR, Bcl-2 and EZH2. Commercial antibodies for all the proteins will be employed, except for HMGA-2 that can be detected by antibodies raised previously in our lab.

1.3.- Apoptosis and oxidative stress analyses.

Ln229, Ln229 [KGA (-)], SFxl, SFxl [KGA (-)] glioma cell lines (kindly donated by Prof. R.J. DeBerardinis) will be cultured as described (Yang et al., 2009, Cancer Res. 69, 7986-93). Human glioblastoma T98G and T98-GAB cell lines will be used as previously described (ref. #24). Apoptotic rate will be detected using Annexin V staining kit (Sigma). Measurement of reactive oxygen species (ROS) generation, total glutathione, enzymatic activity of catalase, superoxide dismutase, and glutathione peroxidase will be quantified as described previously by authors (ref. #17). Analysis of caspase-3, caspase-9, Bid and Bcl-2 will be carried out using Western blot experiments.

1.4.- Analysis of mir-145 expression and function in mammary tumour cells.

As stated in the introduction, we first characterized the phenotype of KGA (–) cells in mouse mammary tumour (0.28AS-2 cells). The miRNA expression pattern observed pointed out to mir-145 as a key mediator of the phenotype of KGA(–) cells in the context of p53/c-Myc regulation (see fig. 1). Mir-145 is usually down-regulated in breast cancer cells, both in culture and *in vivo* (Lee and Dutta. 2009, Annu. Rev. Pathol. Mech. Dis. 4:199–227). However, this miRNA was found to be up-regulated in KGA(–) 0.28AS-2 cells. In human MCF7 breast cancer cells, the antisense expression of KGA provoked the inhibition of both L- and K-type GA and their phenotype closely resembled that of mouse KGA(–) 0.28AS-2 cells (ref. #22). In this project, we will ascertain the role of mir-145 in the reversion of the malignant phenotype, induced by KGA inhibition, dealing with MCF7 cells specifically knockdown for the KGA isoform using RNAi technology (section 1.1).

First, to evaluate quantitatively mir-145 and others miRNAs related to p53/c-Myc pathway (mir-16, mir-26a and mir-125a), we will isolate total miRNAs from cultured MCF7 cells with MirVana RNA Isolation kit (Stratagene); then, miRNAs will be quantify by a real-time PCR technique using NCode™ EXPRESS SYBR-GreenER™ miRNA qRT-PCR Kits (Invitrogene).

Second, we will determine whether the inhibition of mir-145 expression rescue the wild-type phenotype of MCF7 cells. To proof that, we will down-regulate its expression using the lentiviral system miRZip™ Anti-microRNAs (System Biosciences), following manufacturer´s instructions. After that, we will analyze cell growth rate and the expression of phenotypical markers described in section 1.2.

As mention before, the miRNA mir-145 is strategically situated in the p53/c-Myc pathway (Fig. 1). This tumour suppressor miRNA directly controls c-Myc and, indirectly, it also affects to mir-23a/b that targets KGA. Therefore, the overexpression of mir-145 could influence Gln metabolism and, through mir-16 and mir-26a, apoptosis and differentiation procesess as well. To assess this effect, we will transduce MCF7 cells with Lenti-miR-145™ MicroRNA Precursor Clones (System Biosciences) to overexpress mir-145.

1.5.- Influence of Gln and EAA availability in the status of mTORC1 signaling cascade in KGA(-) cells.

The *mammalian target of rapamycin* (mTOR) is a conserved Ser/Thr kinase that regulates cell growth and metabolism in response to environmental cues, like growth factors (insulin/IGF), energy status of the cell, nutrients (amino acids), and stress. In addition, PI-3K/Akt pathway, tightly related to tumour transformation and p53 downregulation, is functionally linked to mTOR signalling. The recent findings that p53 restoration in tumours represses c-Myc through induction of the tumour suppressor miR-145, along with the fact that cellular uptake of Gln and its subsequent rapid efflux in the presence of EAA is the rate-limiting step that activates mTOR, makes this signalling pathway a central target to evaluate the effects of KGA knockdown in tumours.

1.5.1.- Evaluation of the mTOR signalling status in cultured parental and knockdown KGA cells.

We will answer the question of how KGA inhibition affects the intracellular and extracellular Gln and Glu concentrations at the different phases of cell growth, and how the levels of endogenous Gln and the presence of Leu prime mTORC1 activation.

We will study the expression of the Gln transporter SLC1A5 and the bidirectional transporter SLC7A5/SLC3A2 in parental and KGA knockdown cells. Whole cell extracts will be analyzed by Western blots probed with anti-SLC1A5 and anti-SLC7A5 commercial antibodies using whole cell extracts of parental and KGA knockdown cell lines. The concentrations of free amino acids in culture supernatant and cell extracts will be determined by reversed-phase HPLC using pre-column derivatization with Dansyl chloride and UV detection at 254 nm (Márquez et al., 1986. J. Chromatogr. 280:275-83).

mTORC1 signalling will be checked by determining the phosphorylation of S6K1 (causing an SDS-PAGE mobility shift) and ribosomal protein S6, a key downstream target of S6K1. The phosphorylation of $S6K1^{Thr389}$ and $S6^{Ser235/236}$ and $S6^{240/244}$, and total levels of S6K1 (70 kDa cytoplasmic and 85 kDa nuclear isoforms) will be assessed by Western blotting using anti-phospho-S6K1(Thr389) and anti-S6K1 antibodies.

1.5.2.- Evaluation of KGA knockdown on the role of Gln in growth factor signals upstream of mTORC1.

This goal will be carried out by checking insulin-regulated activation of Akt- and EGF-regulated activation of ERK1/2 and S6K1 in our KGA(–) cell lines. Starved cells will be preincubated with Gln or starve medium for 1 hr prior to adding EAA, or EAA/Gln and insulin (100 nM), or EGF (25 ng/mL) at different times. Phosphorylation of S6K1, Akt and ERK1/2 will be analyzed by Western blotting using anti-phospho-S6K1(Thr389), anti-phospho-Akt(Ser473) and anti-phospho-ERK1/2(Thr183/Tyr185) antibodies.

1.5.3.- Determination of the O-glycosylation status of mTOR.

Knocking down KGA in mammary tumour cells inhibits Gln:F6P amidotransferase activity and modifies the O-linked glycosylation status of proteins. Since it has been previously described that mTOR α4 phosphoprotein is a target of O-GlcNAc transferase, we will evaluate possible changes in the O-glycosylation degree of mTOR in KGA (–) cells. Whole cell lysates will be incubated with 50 µl of Protein A MicroBeads (Miltenyi Biotec, Germany) and 2 µg of RL2 antibody, that specifically recognizes O-linked GlcNAc modification in proteins, or 10 µg of anti-mTOR antibody. Lysates are then incubated for 30 min on ice and later applied onto a mMACS column (Miltenyi Biotec). Proteins will be eluted with 70 µl of preheated (95ºC) 1xSDS-PAGE sample buffer. The eluted immunoprecipitated proteins will be analyzed by Western blotting using either anti-mTOR or RL2 antibodies, respectively.

2.- FUNCTIONAL GENOMIC ANALYSIS TO IDENTIFY miRNAS TARGETING L-TYPE GA EXPRESSION IN HUMAN TUMOUR CELLS.

Computational analysis by TargetScan prediction program (http://www.targetscan.org/) shows several miRNAs as putative interactors of the 3´-UTR of L-type GA. It is noteworthy that miR-15 and miR-16, which frecuently are associated to apoptotic capacity of tumour cells, appear among those miRNAs. We will ascertain whether L-type GA mRNA is a real target for some miRNAs selected by bioinformatic analysis and, with that purpose in mind, we have planned the following experiments.

2.1.- Ectopic expression of bioinformatically-selected miRNAs in tumour cells.

miRNAs selected by computational analysis as putative interactors of L-type GA mRNA will be ectopically expressed in MCF7 breast cancer cells, and T98G and T98-GAB glioma cells, as experimental models. We will transfect those cells either with double strand-oligo miRNAs (ds-oligo miRNAs) (Dharmacon) or miRNASelect™pEP-has-mir x Expression Vector (Cell Biolabs) by using Lipofectamine 2000 (Invitrogen) or other conventional commercial transfection reagents. The experiments will be carried out in 12- or 24-well plates. After 24-48h, the effects caused by miRNAs on L-type GA expression are analyzed by measuring its protein content by Western blot (using isoform-specific antibodies for the L-type GA) and its mRNA level by qRT-PCR with the following isoform-specific primers: Forward*:* TTCCGAAAGTGTGTGAGCAG; Reverse: CCACAGGTCTGGGTTTGACT. We will also analyze the influence of miRNAs expression on cell growth by a MTT-based assay according to standard procedures.

2.2.- Luciferase reporter assays.

In orther to determine whether the putative miRNAs selected as interactors directly target L-type GA mRNA, we will carry out a luciferase reporter assay. Briefly, 3´-UTR potencial miRNA-binding sequences of L-type GA mRNA will be subcloned downstream of the *Renilla* luciferase gene of Dual Luciferase Reporter Vector psiCHECK-2 (Promega). The resulting constructions (psiCHECK-2-3´UTR-L-type GA) and ds-oligo mirRNAs (Dharmacon) will be co-transfected in HeLa or HEK 298T cells. After that, the reduction of luciferase activity will be measured compared to cells that were transfected only with the psiCHECK-2-3´UTR-L-type GA plasmids by using the Dual-luciferase Reporter Assay System (Promega) and following manufacturers´instructions. Alternatively, these experiments will be carried out by using the pmiR-GLO vector (Promega) and Dual-Glo^{R} Luciferase Assay System or miRNA Select™ pMIR-GFP Reporter System (Cell Biolabs, Inc). In order to check the specificity of the assays, we will measure the luciferase activity in the presence of chemically modified anti-miRNA antisense inhibitor oligonucleotides (AntagomiRNAs), or by using ds-oligo miRs mutated in their seed sequence (by PCR mutagenesis), or by using constructions with specific deletions or mutations in the 3´-UTR-L-type GA mRNA miRNA-binding segments. As AntagomiRNAs we will use commercially available Locks Nucleic Acid (LNA) (Exiqon) or Peptide Nucleic Acid (PNA) (Panagene).

2.3.-Patient specimens studies.

We further investigate if any of the miRNAs targeting L-type GA mRNA could play a role in some aspects related to type of cancer, tumour malignancy, response to treatments or any other parameter of clinical significance for glioma and breast cancer patients. To achieve this goal, freshly frozen patients specimens will be obtained from the “Servicio de Neurocirugía” and “Servicio de Oncología” of the Regional Universitary Hospital “Carlos Haya” from Málaga (Spain). In these samples, we will check for the presence of miRNAs regulating L-type GA mRNA. In brief, we will apply the same procedure described in section 1.4 of this proposal, based on miRNAs purification from total RNA isolated from the tissues (MirVana RNA Isolation kit, Stratagene). Then, Stem-Loop PCR technique will be implemented to quantify specific miRNAs using known primers. The miRNA populations obtained from patients’

specimens will be also employed to evaluate the expression level of miRNAs previously identified and characterized in KGA (–) tumour models. Of course, the molecular portrait of GA isoenzyme expression will be determined in all human specimens by qRT-PCR and Western blot/immunohistochemistry.

3.- L-TYPE GA AND TRANSCRIPTIONAL REGULATION.

To gain insights into the nuclear role of L-type GA in relation with cell's proliferation and differentiation we will take human glioma T98G and SH-SY5Yneuroblastoma cells as models. Preliminary results have shown that human SH-SY5Y neuroblastoma cells behave as a good L-type GA nuclear import model. Moreover, this cell line differentiates *in vitro* and acquires a neuronal phenotype in response to phorbol ester (PMA).

3.1.- Identification of the L-type GA present in cell nuclei and posttranslational modifications.

Nuclear extracts will be isolated from cell nuclei of tumour cells after subcellular fractionation (NE-PERNuclear and Cytoplasmic Extraction Kit, Pierce).The purity of the extract will be analyzed by Western blots using antibodies for constitutive nuclear and mitochondrial proteins (routinely used in our lab to evaluate mitochondrial contamination of nuclear fractions by Western blot analysis). L-type GA will be purified using GIP-affinity purification column (Campos-Sandoval et al., 2007, Int. J. Biochem. Cell Biol. 39, 765-73) or immunoaffinity chromatography with anti-L-type GA antibodies. The intact pure protein will be analyzed by both Edman degradation and MALDI-TOF mass spectrometry (MS) (Enrique Calvo, CNIC's Proteomics Facility, Madrid). Pure protein will be also digested with trypsin and resulting tryptic peptided analyzed by nano-HPLC-ESI MS (Proteomics Facility, University of Málaga). LGA isozyme lacks the first exon (67 amino acids at the amino-terminus) when compared with GAB protein; therefore, the outcome from these proteomics analyses will reveal us the identity of nuclear GA. Furthermore, the presence of posttranslational modifications will be deduced by assessing shifts in the molecular mass of peptide peaks after nanoHPLC-ESI experiments. In this case, MS will be done with Electron Transfer Dissociation (ETD) technique, a softer mode for ion generation that preserves the bonds of posttranslational modifications. When necessary, hybrid systems with triple cuadrupoles that allow selection of specific mass to charge ratios will be used (Enrique Calvo, CNIC's Proteomics Facility, Madrid).

3.2.- ChIP assays.

The plausible interaction of L-type GA with DNA, either directly (as a transcriptional factor) or indirectly (as a transcriptional coregulator) will be evaluated by the Chromatin Immunoprecipitation assay (ChIP). Initial ChIP trials employing anti-L-type GA antibodies have not been satisfactory because the antibodies were not suitable for immunoprecipitation (IP). Now, we will take advantage of our collection of different GA constructs in eukaryotic expression vectors. Thus, we will perform cell transfections with the whole ORF of GA engineered with the c-myc or the HA tags. The presence of GA in nuclei will be first assessed by Western blot. The CHIP *assay kit* of Upstate will be implemented with isolated nuclei from tumour cells. After formaldehyde fixation, the DNA will be cleaved with a BioRuptor, an equipment specially designed for ChIP assays allowing chromatin disruption and breakdown of DNA in a gently and reproducible way. Then, GA/DNA complex will be precipitated using anti-c-myc or anti-HA antibodies conjugated to agarose. The DNA fragments are purified using QIAquick Spin kit (Qiagen) and clone into TOPO vectors by blunt-end cloning or, alternatively, PCR-amplified by using random primers. Sequencing of the DNA will reveal the specific DNA loci regulated by L-type GA. Once regulatory DNA sequences have been identified, EMSA experiments will be planned. Immunoprecipitated proteins will be analyzed by SDS-PAGE and silver staining compatible with MS. Then, proteins are identified by MS employing nano-HPLC-ESI, as detailed before. As a complimentary method, we will design GST *pull-down* assays using the recombinant fusion protein Glutathione-S-transferase (GST)-GAB and the affinity resin GSH-Sepharose to specifically precipitate nuclear transcription partners of L-type GA, as

described previously (see reference #27). Proteins will be submitted to MS analysis for further identification.

3.3.- Transcriptional reporter assays with nuclear receptors.

The full-length GAB cDNA, deletion mutants lacking the LXXLL sequence motif (amino acids 72-76 of the GAB sequence) (Δ1-76), and full-length point mutants with altered LXXLL motif for interaction with nuclear receptors will be cloned into eukaryotic expression vector (pcDNA-3 and fluorescent-tagged vectors of the GFP series). To check whether or not GA is translocated into the nucleus by interaction with nuclear receptors, tumour cells will be cotransfected with the respective GAB constructs and mammalian expression plasmids for nuclear receptors, containing the consensus ligand-binding domain of different nuclear receptors and the luciferase reporter gene. Transfections of human tumour cells have been successfully set up in our lab with Metafectene Pro (Biontex). After transfections, cells will be incubated with concrete ligands for nuclear receptors and luciferase activity measured. In parallel, nuclear translocation of GA will be evaluated by subcellular fractionation and Western blotting or by confocal microscopy in the case of using GFP-tagged vectors.

Additionally, other assays will be implemented to assess direct binding of L-type GA to DNA. Transient transfection experiments utilizing L-type GA expression vectors and luciferase reporter plasmids containing promoters sensitive to various different signalling pathways (p53, Myc, Rb, STAT3, SRE, AP1, NFKB, CRE, SP1, CEBPs) will be used in these experiments.The level of reporter activation will be compared between L-type GA vector and empty vector. EMSA and supershift analyses are other experimental approaches to assess direct binding of L-type GA to DNA. Glioma and neuroblastoma cells will be transfected with expression vectors containing the full-length human GAB or LGA sequence, under conditions which maximize nuclear GA translocation. Nuclear extracts will be isolated as described (Segura et al., 2005. Cancer Lett. 218, 91-8). Adequate gel-purified oligonucleotides containing binding sites for known transcription factors will be end-labelled with [γ-^{32}P]ATP and quantitative EMSA and supershift experiments using anti-L-type GA antibodies will be performed as described by authors (Pérez-Gómez et al., 2003. Biochem. J. 370, 771-784).

3.4.- In vitro chromatin condensation assay.

To assess the effect of L-type GA on chromatin remodelling and further significance of nuclear translocation of this protein, nuclei will be prepared from tumour cells as described before. To the nuclear suspension functional L-type GA (GAB or LGA) or bovine serum albumin (BSA) (as a control) will be added. Functionally active L-type GA will be produced from a baculovirus recombinant expression system and affinity purified with a GIP-column (Campos-Sandoval et al., 2007). Experiments with Gln or Glu supplementation will be done in parallel. Samples will be then incubated for 30 min at 25ºC with mild shaking. They are transferred to polylysine-coated glass slides and fixed in 95% ethanol/5% acetic acid at -20ºC for 20 min. Nuclei are stained with DAPI (10ng/ml). Chromatin condensation can be viewed under a fluorescent microscope.

3.5.- Influence of GAB up-regulation on miRNA expression.

We will establish the differentially expressed miRNA in T98G compares to T98-GAB cells using miRXplore™ Microarrays (same technique already employed by us to assess miRNAs profiles in mouse mammary tumours).

4.- MITOCHONDRIAL GLN CARRIER (MGC): IDENTIFICATION AND CHARACTERIZATION.

This will be a collaboration with Dr. J. Satrustegui's lab. Dr. Satrustegui is a world-reference in the area of mitochondrial amino acid carrier proteins. Following her suggestion, we'll undertake this task to

identify and perform a preliminar characterization of the MGC in cancer cells, as another key target in our anti-Q therapy for cancer reversal. The MGC is another rational strategy to limit tumour Gln uptake and thus reducing the glutaminolytic pathway which seems to be essential for proliferation. Therefore, in this proposal we will undertake the identification and preliminary characterization of the MGC in human cancer cells, which at present has not been identified nor characterized at the molecular level. The members of the solute carrier family 25 (SLC25) are known to transport molecules over the mitochondrial membrane. Novel members of this family have been recently cloned and one of them, SLC25A44, has been postulated to be the putative MGC (del Arco and Satrustegui, 2005. New mitochondrial carriers: an overview. Cell Mol. Life Sci. 62, 2204-7).

4.1.- Real-time PCR screening of MGC.

The putative MGC has been adscribed to the Homo sapiens SLC25A44 gene. We already has the full-lenght cDNA clone of this gene in the pOTB7 vector. We will design primers for human, rat and mouse Slc25A44, in order to perform a comprehensive localization and quantitation study in human cancer cells and mammary mouse tumour cells (EATC and 0.28AS-2). The rat and mouse primers represent useful controls for the PCR reactions, because pioner studies on these species have established the tissue distribution of MGC at the protein level. Furthermore, EATC possess a very active MGC previously characterized by our group by kinetic transport assays. Quantitative real-time PCR for Slc25A44 and the appropriate reference genes will be performed using the BioRad MyiQ detection system and SYBR-GREEN as a fluorescent probe. Besides determining the abundance of the MGC mRNA in the tumour models object of this proposal, the main goal of this real-time PCR analysis is to select a suitable cancer model with strong expression of MGC for further knock out of the gene and characterization. It will be also determined by real-time PCR whether there is a compensatory induction of the MGC expression in KGA (—) cancer cells.

4.2.- Gene silencing of MGC in tumour cells.

Gene silencing of the mitochondrial Gln carrier will be achieved using shRNA constructs targeted at the MGC sequence, as described in section 1.1. Constructs will be transfected in tumour cells with apropriate transfection controls; individual transfected cell clones will be isolated by positive selection. The MGC (-) knock out cell line will be validated by RNA isolation and q-PCR.

4.3.- Mitochondria isolation and Gln transport kinetic assays.

To confirm the identity of the MGC, mitochondria from wild-type and transfected MGC (–) cells will be isolated as described previously (Molina et al., 1997, Biochem. J. 308, 629-633). Transport assays using [^{14}C]-Gln will tell us whether or not Slc25A44 is the mitochondrial glutamine carrier, after comparison of the radioactive Gln incorporated in both tumour cell types. After confirming the identity of the carrier, we will conduct studies to characterize growth, proliferation, survival and morphological features of the knock out cells. The reduction of Gln import into the mitochondria is expected to yield a less malignant and more differentiated phenotype, and thus providing convergent evidence supporting our hypothesis for inhibition of cancer growth and proliferation targeting Q-catabolism in cancer cells.

ADEQUACY OF THE WORKING PLAN.

The planning and time table of the project, distributed by concrete tasks, is shown on next page. It has been designed trying to reach all the scientific milestones at the end of the three-year period. To fulfil this goal, we have designated as main responsible of each scientific task the most experienced person in that particular area. At the same time, we have achieved a timely and logical distribution of tasks. It will be also avoided temporal overlapping of several activities or massive use of the same laboratory. In this way, different objectives will be started at the same time.

The first objective is the characterization of the miRNAs controlling Q-metabolism in KGA (–) glioma and breast cancer cells and their conexions with known oncongenes and tumour suppresor genes. In these tasks, Dr. Juan A. Segura (expert in cancer signalling pathways) and Dr. José M. Matés (expert in transcriptional regulation and cell assays for oxidative stress and apoptosis) will lead most of the experimentation involved in this goal. Dr. Segura has already conducted massive miRNA array assays for KGA (–) mammary tumour cells. The medical doctors M.A. Arráez (Chief of the Department of Neurosurgery) and G. Ibáñez (from the Neurosurgery Dept. too), and Dr. M. Benavides, medical oncologist and Chief of the Oncology Department, and Dr. I. Ales, will be the persons in charge to skilfully analyse pathological samples, as well as the physiological interpretation of the results. The superior graduated technician (SGT) will be the person conducting cell transfections and *in vitro* cultures of knock-out cell lines.

The second objective is a functional genomic analysis to identify miRNAs targeting L-type GA expression in human tumour cells. Dr. Francisco J. Alonso, our expert in antisense technology and gene silencing with RNAi methods, will design the strategy to identify the miRNAs affecting L-type GA expression, with the expert assistance of the SGT.

In relation to our third objective, Dr. Javier Márquez (expert in protein chemistry and proteomics) will lead the studies about the nuclear role of L-type GA, being the main responsible of the characterization of its posttranslational modifications. Eventually, nuclear translocation of L-type GA will be leaded by Dr. Javier Márquez, with a large expertise in related topics, whereas the identification and preliminary characterization of the mitochondrial Gln carrier will be a direct responsibility of Dr. J.M. Matés. With regard to proteomics studies, it is worth mentioning that Dr. Márquez is the scientific responsible of the Proteomics Facility of the University of Málaga. This facility, which is now running in its third year, has recently incorporated novel and state-of-the-art instrumentation (see the summary of granted projects and contracts) of great relevance for this proposal, particularly the nano-HPLC-ESI and the SELDI-TOF apparatus.

Finally, we would like to emphasize the strong need of a SGT in this project. This position has been occupied by Mrs. Carolina Lobo in previous SAF projects and she has made an outstanding work. As we discuss in the justification of the budget, this person has become invaluable for our group because of her expertise in cancer cell cultures and basic techniques of cell and molecular biology. SGT will be responsible of essential tasks that require a great expertise, like maintenance and culture of human tumour cell lines, handling and culture of cancer knock out cell lines, purification of DNA, RNA and recombinant proteins, immunological techniques (production of antibodies, Western blots). This figure results indispensable in any laboratory that seeks to be in a leading area of research.

ABBREVIATIONS IN THE WORKING PLAN:

JAVIER MÁRQUEZ GÓMEZ (JM)	FRANCISCO ALONSO CARRIÓN (FA)
JOSÉ M. MATÉS SÁNCHEZ (MS)	JUAN A. SEGURA CHECA (JS)
SUPERIOR GRADUATED TECHNICIAN (SGT)	MERCEDES MARTÍN RUFIÁN (MMR)
MIGUEL ÁNGEL ARRÁEZ (MAA)	MANUEL BENAVIDES ORGAZ (MB)
GUILLERMO IBÁÑEZ (GI)	INMACULADA ALES DÍAZ (AD)

4.1 CHRONOGRAM MODEL (EXAMPLE)

This chronogram must indicate the persons involved in the project, including those contracted with project funds.
Underline the name of the person responsible of each task.

Tasks	Centre	Persons	First Year (*)	Second Year (*)	Third Year (*)
1.- mi-RNAs AND SIGNALLING CASCADES in KGA (-) MODELS.					
1.1.- Down-regulationof gene expression by RNAi technology	LBM	FA, SGT	x |x|x|x|x|x|x| | |	| | | | | | | | | |	| | | | | | | | | |
1.2.- Phenotypic characterization of KGA(-) cells.	LBM	FA, SGT	| | | | | | | |x|x| x|x	x|x| | | | | | | | |	| | | | | | | | | |
1.3.- Apoptosis and oxidative stress analyses.	LBM	MS, GI	| | | | | | | | | | |	| |x|x|x| x|x| | |	| | | | | | | | | |
1.4.- Analysis of mir-145 expression and function.	LBM	JS, FA, AD	| | | | | | | | | | |	x|x|x |x|x|x|x|x|x|x|x	| | | | | | | | | |
1.5.- Q and EAA influence on mTORC1 signaling in KGA(-) cells.	LBM	JS, AD, SGT	| | | | | | | | | | |	| | | | | | | | | |	x|x|x |x|x|x|x|x|x|x|x
2.- miRNAS TARGETING L-TYPE GA EXPRESSION					
2.1.- Ectopic expression of miRNAs in tumour cells.	LBM	JS, MAA	x|x|x|x| | | | | | |	| | | | | | | | | |	| | | | | | | | | |
2.2.- Luciferase reporter assays.	LBM	FA, MB, SGT	| | | |x|x|x|x|x|x| x|x	| | | | | | | | | |	| | | | | | | | | |
2.3.- Patient specimens studies.	HCH, LBM	MAA, MB, SGT	| | | | | | | | | | |	x|x|x |x|x|x|x|x|x|x|x	x|x|x |x|x|x|x|x|x|x|x
3.- L-TYPE GA AND TRANSCRIPTIONAL REGULATION.					
3.1.- L-type GA in cell nuclei and posttranslational modifications.	LBM	JM, MS	x|x|x |x|x|x|x|x|x|x|x	| | | | | | | | | |	| | | | | | | | | |
3.2.- ChIP assays.	LBM	JM, JS	x|x|x |x|x|x|x|x|x|x|x	| | | | | | | | | |	| | | | | | | | | |
3.3.- Transcriptional reporter assays with nuclear receptors.	LBM	JM, FA, MMR	| | | | | | | | | | |	x|x|x |x|x|x|x|x|x|x|x	x|x|x|x|x| | | | |
3.4.- In vitro chromatin condensation assay.	LBM	JM, JS	| | | | | | | | | | |	| | | | | | | | | |	| | | | | |x|x|x| | |
3.5.- Influence of GAB up-regulation on miRNA expression.	LBM	JM, MMR	| | | | | | | | | | |	| | | | | | | | | |	| | | | | | | | |x|x|x
4.- MITOCHONDRIAL GLN CARRIER					
4.1.- Real-time PCR screening of MGC,	LBM	MS, FA	| | | | | | | | | | |	| | | | | |x| x|x| x| x|x	| | | | | | | | | |
4.2.- Gene silencing of MGC in tumour cells.	LBM	MS, SGT	| | | | | | | | | | |	| | | | | | | | | |	x|x|x|x|x|x| | | | |
4.3.- Mitochondria isolation and Gln transport kinetic assays.	LBM	MS, JS	| | | | | | | | | | |	| | | | | | | | | |	| | | | | |x| x|x| x| x|x

(*) Mark an X inside the corresponding boxes (months)
HCH: Hospital “Carlos Haya“; LBM: Lab of “Biología Molecular“

5. BENEFITS DERIVED FROM THE PROJECT, DIFUSION AND EXPLOTATION OF RESULTS (maximum 1 page)

The following items must be described:

- Scientific and technical contributions expected from the project, potential application or transfer of the expected results in the short, medium or large term, benefits derived from the increase of knowledge and technology.
- Diffusion plan and, if appropriate, exploitation plan of the results.

After the completion of this proposal, we expect to demonstrate that: (i) interfering with Q-catabolism is a powerful tool to fight against cancer; (ii) knocking down KGA isoform or overexpressing L-type GA isozymes may revert the malignant phenotype of human gliomas and breast cancer cells and; (iii) L-type GA is a multifunctional protein and plays a role in the regulation of gene expression. Furthermore, we expect to discover: (i) the miRNAs governing KGA and L-type GA expression in human tumour cells; (ii) the signalling pathways involved in glutaminolysis and their relationship with known oncogenes and tumour suppressor genes and; (iii) the identity of the MGC. This is a biomedical research project focused in basic areas of cancer. Nevertheless, due to the profound physiological implications of glutaminolysis in cancer, it may be anticipated that the basic knowledge originated in the frame of this project hold the promise of become useful therapeutic strategies for cancer treatments. The discovery of miRNAs regulating the metabolic reprogramming of tumours and the RNAi approaches targeting KGA and MGC in human gliomas and breast cancer cells, may have potential interest in cancer gene therapy considering the promising results already obtained in experimental tumours treated with antisense KGA cDNAs.

The project is a multidisciplinary innovative approach based on a truly collaborative effort between members of the group, medical oncologists, neurosurgeons and reputed national and international scientific partners. The participation of clinical groups belonging to one of the most important regional Hospital from Andalucia (Carlos de Haya) improves qualitatively the proposal, allowing us to validate *in vitro* results with cancer specimens from human patients. The proposal does not have high-risk tasks: most of the techniques and methodologies have been already set up in our lab. Furthermore, the collaboration of experts in the field supposes an additional warranty to reach our objectives. To fulfil our goals we have devised a strategic working plan with the participation of reputed scientists, usual partners of our team, who perfectly complement the group's expertise.

The topics of the present proposal correspond to several of the priority fields of the Spanish National Research Programmes. In particular, the priorities of the National Programme of Medicine and the Strategic Health Action establish as general objectives "to get a deeper knowledge in the molecular, biochemical, cellular and genetic mechanisms underlying the pathogenesis of diseases" and also "to develop and implement rational therapeutic and diagnostic tools to fight against pathologies". Another preferential thematic area is devoted to "the cellular, molecular and genetic basis of neurodegenerative diseases and alterations in the development of the nervous system" (priority area 3.1). Moreover, for neoplastic diseases, the National Programme stimulates research on "molecular and cellular mechanisms in cancer growth and proliferation"(priority area 1.1), "molecular, cellular and structural biology, genetics and epigenetics of tumoural processes" (priority area 1.2), "cancer genomics and proteomics" (priority area 1.3). The present proposal fits also entirely in thematic priorities of European research.

Dissemination of research results during the course of the grant and beyond will be carried out at three levels: scientific, public and commercial. At the scientific level, prompt and timely dissemination will be through presentations in national and international conferences and publication in suitable peer-reviewed scientific journals. Public dissemination will be primarily through the research group's websites, which will provide descriptions of the project and research findings in terms understandable for general audiences as well as for more specialized audiences. The websites are: Instituto Andaluz de Biotecnología (http://www.iab.cica.es/biomedic.htm), Fundación IMABIS of biomedical research (http://www.imabis.org/web/pageView.aspx?sec=1&apt=370&pag=268), Dept. of Molecular Biol. and Biochem. (http://www.bmbq.uma.es/glutaminasa), research profiles of the BiomedExperts web page (http://www.biomedexperts.com/), and the exclusive Glutaminase domain of our group (http://www.glutaminase.org/). In addition, the work will be presented at suitable broader scientific/societal/policy forums in Spain and abroad and also, where appropriate, through press releases that will be widely circulated, through participation in TV and radio shows, through articles in the popular press and through communication with patient interest groups. Communications will be sent to the Sociedad Española de Bioquímica y Biología Molecular (http://www.sebbm.bq.ub.es/), the FEBS web (http://www.febs.unibe.ch/) and to scientific forums of the EU (http://www.elsf.org/). All these media will allow increasing the visibility of our research and the promotion and dissemination of our results.

Finally, the exploitation of our results will be made by contracts, collaboration agreements and patents. There is an extremely high demand for our antibodies against GA isoforms. We have obtained a patent for their application in Biomedicine (Campos, J.A., Aledo, J.C., Olalla, L., Gutiérrez, A., Alonso, F.J., Segura, J.A., Márquez, J. TITULO: Anticuerpos isoenzima-específicos frente a la glutaminasa humana tipos K y L. (2002) Número de REGISTRO: P200201819). We have also signed a collaboration agreement since July 16, 2003 with the company BioVendor Laboratory Medicine Inc. (Brno, República Checa), for several biotech products –recombinant GA proteins, antibodies- that this company needs for development of diagnostic kits (ELISA) based on GA, with special incidence in cancer.

6. BACKGROUND OF THE GROUP
(In the case of a coordinated project the topics 6. and 6.1. must be filled by each partner)
(maximum 2 pages)

- Indicate the previous activities and achievements of the group in the field of the project:

  If the project is related to other previously granted, you must indicate the objectives and the results achieved in the previous project.

  If the project approaches a new research field, the background and previous contributions of the group in this field must be indicated in order to justify the capacity of the group to carry out the project.

Our team has almost 30 years of experience in studies concerning tumour nitrogen metabolism. At the beginning (early 1980's) we use experimental models of mouse mammary tumours (Ehrlich ascites tumour cells) and since 1995 we initiated studies on human cancer cell lines using molecular biology approaches. Furthermore, in 1997 we started a new research line about the cellular and molecular mechanisms of Glu generation in brain, after a short sabbatical leave of the PI in Brandeis University (Waltham, USA), in the lab of Dr. Irwin B. Levitan, to learn the technology of yeast two-hybrid screening. With regard to cancer's molecular biology a key milestone was the cloning of the first human L-type GA enzyme and its identification on human chromosome 12. In the meantime, we have made seminal contributions in understanding the role of Gln and GA isoenzymes in cancer and brain. These findings have allowed to our group becoming a world reference in the field, as can be deduced from examination of recent cancer reviews (see, for example, the review by DeBerardinis and Cheng published this month in Oncogene (ref. #2) and references #1, 3 and 5). Some of our contributions, summarized below, have also challenged established paradigms concerning GA expression in mammalian tissues.

SCIENTIFIC ACHIEVEMENTS OF PREVIOUS RESEARCH IN THE TOPIC OF THE RESEARCH PROJECT

1) Cloning and sequencing of human L-type GA cDNA isolated from ZR75 breast cancer cell library.
2) Chromosomal localization of the human GA genes.
3) Pattern of expression of L- and K-type GA mRNA transcripts in human tissues. Both isozymes are co-expressed in brain and pancreas.
4) Discovery of a novel GA isoform in mammalian tissues: the GAB isoenzyme.
5) Discovery of a novel extramitochondrial localization for a GA isozyme in brain cells and certain human cancer cells: nuclear location of GAB.
6) Isolation of a genomic clone of human GAB comprising the whole ORF and promoter regions (GenBank # AF348119). Characterization of the transcription start site and promoter of human *Gls2* gene.
7) Transfection of Ehrlich ascites tumour cells (EATC) with antisense cDNA constructs of KGA. Selection and characterization of a transfected cell line (0.28AS-2) showing a more differentiated phenotype and unable to grow *in vivo*.
8) Characterization of phenotypic alterations in 0.28AS-2 cells correlated with an effective anti-tumour response of the host immune system.
9) Functional genomics study by Differential Display technique of the transcriptome of 0.28AS-2 cells vs wild-type EATC counterpart. Isolation and cloning of several down-regulated genes related with tumour biology.
10) Long-range immunization against the wild-type EATC cells in mice inoculated with 0.28AS-2 antisense-expressing cells (PATENT).

11) Human leukaemia cells and several human cancer cell lines of breast, hepatocellular carcinoma and colon co-express K- and L-type GA isoforms.
12) Overexpression of GAB in T98G human glioblastoma cell line alters gene expression and reduces survival, migration and proliferation. A cDNA microarray study detected decreased expression of genes related with glioma malignancy.
13) Yeast two-hybrid screening of a human brain cDNA library using the C-terminal end of human GAB as bait. Cloning and sequencing of four positives: GIP ("Glutaminase-Interacting Protein"), SNT, RACK1 and UFD1. GIP and SNT belong to the PDZ family. GAB has a PDZ-recognition domain in its C-terminal half.
14) *In vitro* characterization of the GAB-GIP interaction. Heterologous expression and purification of GIP in bacteria.
15) Heterologous expression of human GAB and KGA in bacteria. Affinity purification. Production of polyclonal isoform-specific anti-GA antibodies (PATENT).
16) Heterologous expression of functional human KGA and GAB protein in Sf9 insect cells infected with recombinant baculovirus. Kinetics and molecular charaterization of purified recombinant proteins.
17) Availability of cDNA GA clones of KGA, LGA and GAB from human and mouse. Genomic clones of human and mouse GAB.
18) Characterization of the MGC in submitochondrial particles of mammary tumour cells (EATC). The existence of a very active, high-affinity and specific Gln carrier in mitochondria was demonstrated. Kinetic and molecular analysis discarded that GA and MGC belong to the same protein and suggested that MGC may be the rate-limiting step in mitochondrial glutaminolysis.

We have worked very hard trying to consolidate a stable research team composed by four professors, avoiding the traditional fragmentation frequently seen in Spanish research groups. At the regional level, we are a group consolidated as excellent team in the Life Sciences area of the Junta de Andalucía. With regard to national issues, if we are lucky enough to get this grant, this will be the sixth consecutive research project awarded to our team (and to the PI) in the "Plan Nacional de I+D+i", concretely in the National Programme of Biomedicine. In our opinion, this clearly reflects a constant trajectory of excellence in research.

As an indication of the international leadership of our team, we organized an International Workshop entitled "Glutamine as a cellular signal" in November, 2001. Sponsored by the Foundation Ramón Areces, the Workshop was a key meeting point for prestigious investigators working in the field of Gln/Glu in cancer and brain. We have submitted an Expression of Interest to the EU trying to involve relevant scientists in the field of Gln-related issues, in order to prepare a proposal for the Seventh Framework Programme of the EU (FP7). The PI has been invited speaker in two prestigious international Workshops held in Wierzba (Poland) in the summer of 2005 and 2008. These meeting were focused on brain Gln/Glu studies, including brain tumours and neurological diseases, and allow the PI to establish new interactions with leading groups in the field.

6.2 PUBLIC AND PRIVATE GRANTED PROJECTS AND CONTRACTS OF THE RESEARCH GROUP

Indicate the project and contract grants during the last 5 years (2005-2009) (national, regional or international)

Include the grants for projects under evaluation

Title of the project or contract	Relationship with this proposal (1)	Principal Investigator	Budget EUROS	Funding agency and project reference	Project period (2)
GLUTAMATO Y ADICCIÓN A COCAÍNA: EVALUACIÓN FUNCIONAL DE LAS ACCIONES DE LA COCAÍNA SOBRE GENES REGULADORES DE LA TRANSMISIÓN GLUTAMATÉRGICA (Subproyecto)	3	FERNANDO RODRÍGUEZ DE FONSECA	110.000	MCYT Proyectos Integrados de Investigación de Genómica y Proteómica GEN2003-20651-C06-02	C
SISTEMA TÁNDEM NANO-HPLC Y ESPECTRÓMETRO DE MASAS DE TRAMPA DE IONES E IONIZACIÓN POR ELECTROSPRAY.	1	JAVIER MÁRQUEZ GÓMEZ	355.000	MEC, PROGRAMA NACIONAL DE INFRAESTRUCTURAS CIENTÍFICAS Y GRANDES EQUIPOS	C
GLUTAMINASA Y FUNCIÓN NEURONAL: ANÁLISIS GENÓMICO Y PROTEÓMICO	2	JAVIER MÁRQUEZ GÓMEZ	174.536,30	CONSEJERÍA DE INNOVACIÓN, CIENCIA Y EMPRESA. JUNTA DE ANDALUCÍA. CVI-01543	C
SISTEMA DE PRODUCCIÓN DE INGREDIENTES FUNCIONALES A ESCALA INDUSTRIAL .	3	JAVIER MÁRQUEZ GÓMEZ	69.000	INSTITUTO ANDALUZ DE BIOTECNOLOGÍA y EMPRESA BIONATURIS, S.A. BIOÁNDALUS 08/23/L5.4	C
ANÁLISIS FUNCIONAL DE LA GLUTAMINASA L EN CEREBRO: IMPLICACIÓN EN NEUROPROLIFERACIÓN Y ALTERACIONES NEUROLÓGICAS.	1	JAVIER MÁRQUEZ GÓMEZ	246.840	MEC SAF 2007-61953	C
INCORPORACIÓN A LA RED RETICS DE TRASTORNOS ADICTIVOS (RTA).	3	JAVIER MÁRQUEZ GÓMEZ	27.832,86	INSTITUTO DE SALUD CARLOS III RD06/0001/1012	C
CORRELACIÓN DE LA EXPRESIÓN TISULAR DE LAS ISOENZIMAS DE GLUTAMINASA CON LA AGRESIVIDAD DE TUMORES HUMANOS DE ALTA INCIDENCIA.	2	FRANCISCO J. ALONSO CARRIÓN	53.015	CONSEJERÍA DE SALUD, JUNTA DE ANDALUCÍA, PI-0316/2008	C
Sistema tándem de Protein-Chip + y espectrómetro de masas SELDI-TOF, HPLC para análisis de aminoácidos y Digestor Automático para aplicaciones de Proteómica.	1	JAVIER MÁRQUEZ GÓMEZ	374.000	PROGRAMA NACIONAL DE INFRAESTRUCTURAS CIENTÍFICAS MICINN	C

(1) Write 0, 1, 2 or 3 according to: 0 = Similar project; 1 = Very related; 2 = Low related; 3 = Unrelated.

(2) Write C or S if the project has been funded or it is under evaluation, respectively.

7. TRAINING CAPACITY OF THE PROJECT AND THE GROUP
(In the case of Coordinated Projects this issue must be filled by each partner)

This title must be filled only in case of a positive answer to the corresponding question in the application form. Justify that the group is able to receive fellow students (from the Suprograma de Formación de Investigadores) associated to this project and describe the training capacity of the group. In the case of coordinated projects, each subproject requesting a FPI fellowship must fill this issue. Note that all necessary personnel costs should be included in the total budget requested. The available number of FPI fellowships is limited, and they will be granted to selected projects as a function of their final qualification and the training capacity of the groups.

In our opinion, the group's formative capacity is plenty proved. As indicatory, we can cite that Dr. Márquez has been the advisor of ten Ph.D. Thesis, some co-directed with other professors of the research team. These graduated students are now in the following professional appointments:

University Professor in Spain	⟶ 2	University Professor in USA	⟶ 1
High-School Professor	⟶ 1	Marie Curie Postdoctoral Fellow	⟶ 1
Biotec/Pharma Companies	⟶ 4	Health & Nutritional Specialist	⟶ 1

Two more PhD students tutored by members of our group are now working as Senior Research Scientist in research projects. The fellowship holders in our research group acquire a solid formation in basic and state-of-the-art- techniques of Biochemistry, Molecular Biology, Cell Biology, Immunology, Protein Chemistry, Proteomics and Functional Genomics. Most of them consider this fact as one of their most important assets in their careers. The capacity to offer a multidisciplinary training is one of the main characteristics of our lab. This has been possible by joining professors with different backgrounds and trainings, and also by keeping them together for a considerably number of years.

We would like to mention that one postdoctoral fellow of our group, Dr. Gerard Bannenberg, is currently appointed as Ramon y Cajal postdoctoral fellow in the National Centre of Biotechnology, CSIC (Madrid). In addition, nine Graduate Thesis were done during the last seven years and obtained the highest mark, between them: Jorge Lora Cabrera: "Study of apoptosis and redox state in Ehrlich ascites tumour cells expressing antisense glutaminase"; Beatriz Fernández Molina: "Expression of glutaminase isoforms in blood of leukaemic patients"; Marta Tosina García: "Determination of the functionality of hexosamine pathways in tumour cells using a HPLC method"; Carolina Cardona Ramírez: "Search and characterization of proteins interacting with GIP by proteomics techniques".

Every professor in our group has been signed for remarkable research centres abroad. Consequently, they have adequate experience on research in very competitive areas, interacting with an important number of fellows and students. Skills acquired by our Ph.D. students are in a high range, as we can infer by opinions of their respective supervisors in their contracts abroad. It is also noteworthy that José M. Matés, Ph.D., and Juan A. Segura, Ph.D. (now Associate Professors and members of the group) won respectively two positions as senior research scientific in the Human and Mobility Program (now renamed Marie Curie Fellowship) of the EU. This fact confirms their excellent preparation in the area of molecular biology and biochemistry.

The Project we are now presenting is innovative and multidisciplinary, which increases its capacity for training in a large number of modern techniques. At present, we do not have any Ph.D. student or predoctoral fellowship in our cancer research line; this fact has a negative impact on the scientific outcome of our group. Even though the four professors of the research team participate full-time in the project, the high teaching load during the academic course makes indispensable this figure in our group.