DDX3X role characterisation in colorectal cancer: An oncogene or tumour suppressor?

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Abstract

Introduction. The prognosis of colorectal cancer (CRC) is currently unsatisfactory, with high rates of relapse and subsequent metastatic spread. Thus, new combinatorial targeted therapies are needed to accomplish long-term control of CRC. DDX3X emerges as a potential target for the treatment of CRC, however, it has both oncogenic and tumour suppressor functions described by enhancing β-catenin stability and inhibiting Snail, respectively. Objectives. This final degree project consists of the elaboration of a research proposal to investigate the role of DDX3X in CRCs harbouring APC-mutations in Wnt/β-catenin signalling pathway. Methods. A lentiviral vector containing an inducible shRNA will be used to knock-down DDX3X expression in CRC cell lines for the study of the protein's function. Relevance of the study. This study would be relevant to determine if an alteration in the mutational background of CRC cells can establish inclusion and exclusion criteria for the treatment with a DDX3X inhibitor in combination with other targeted therapies.

***Keywords**: Colorectal cancer, DDX3X, dual-role protein, β-catenin, Snail, targeted therapy.*

CONTENTS

1. Research proposal elaboration

This final degree thesis will consist of the design of a research proposal on a relevant topic in the current biomedical research field.

The objectives of this work are listed below.

- To find in pre-existing bibliography a current topic to stablish a hypothesis.
- To plan an experimental design for a research proposal to contrast the proposed objectives.
- To learn to choose the most suitable method to answer each research question.
- To learn to calculate the estimated cost of a research project.

Bibliographic research of recent reviews and original articles on the topic of study will be the method used to elaborate this final degree thesis.

2. Introduction

2.1. Colorectal cancer (CRC)

CRC is the third most common cancer and the second most frequent cause of cancer related deaths worldwide(1). Although screening, early detection, and removal of neoplastic lesions are being major strategies to reduce incidence and mortality from CRC(2), the incidence in young patients is increasing(2). Moreover, 20% of CRC patients are initially diagnosed with metastases and up to 50% of patients with localized disease eventually develop metastasis(1).

Actual first-line treatments are chosen based on tumour-related molecular characteristics, and usually comprise surgical resection followed by chemotherapy combined with molecular targeted therapies(1). Even though actual targeted therapies have increased CRC disease control and patient survival, the prognosis of CRC is currently unsatisfactory, with high rates of relapse and subsequent metastatic spread(3). Furthermore, using molecular targeted monotherapies causes the emergence of resistant clones that escape single-target inhibition due to high heterogeneity of CRC and the complex interplay between different signalling pathways(1)(4).

For these reasons, new combinatorial therapies are needed to overcome therapy resistance and accomplish long-term control of CRC(4). Besides, molecular characterization of CRC tumours is also crucial to find out new targets. All in all, this would allow to treat a wider range of patients, moving towards a more personalized treatment, with a specific therapeutic plan for each patient(4).

2.2. DEAD-Box RNA-Helicase-3 X-linked (DDX3X) controversial role in CRC

DDX3X is a highly conserved protein in the DEAD-box RNA-helicase family that is ubiquitously expressed in human tissues. It regulates almost all stages of RNA metabolism having remarkable effects on many biological processes(5). Recently, some studies started elucidating the implications of DDX3X dysfunction in cancer, among other diseases, and its potential as a therapeutic target(6).

DDX3X has implications in several hallmarks of cancer(7), however, its role as an oncogene or tumour suppressor between different cancers, and within the same cancer type(8), remains controversial(5)(6).

Despite appearing to be antithetical gene categories, dual roles of the same protein may arise from different cellular conditions, resulting in a wide range of pathological outcomes(9)(10).

One of these cancers where DDX3X role remains unclear is CRC, where oncogenic(11)(12) and tumour suppressor functions(8) have been described. Some studies report DDX3X as an activator of Wnt signalling(11)(12). DDX3X has been found to contribute to β-catenin stabilization in CRC by being an allosteric activator of CKIε, which phosphorylates Disheveld protein(*Figure 1*)(11). Moreover, DDX3X inhibition by a small molecule inhibitor led to cell cycle arrest and decreased Wnt/β-catenin pathway activity in Adenomatous Polyposis Coli (APC)-wild-type CRC cells harbouring mutations in β-catenin gene(12), reinforcing DDX3X oncogenic potential.

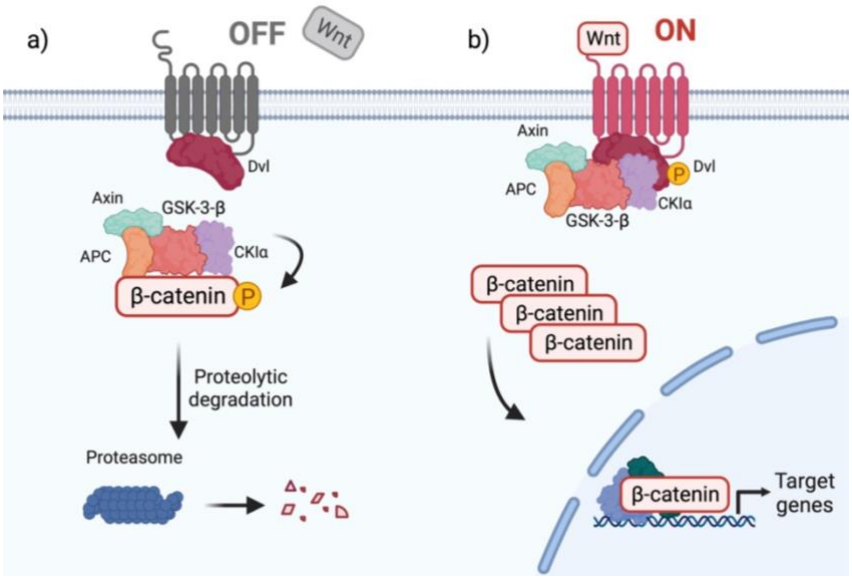

Figure 1. a) *Wnt-off. In the absence of Wnt stimulation, β-catenin is phosphorylated and sent to proteolytic degradation by casein kinase 1α (CKIα) and glycogen synthase kinase 3β (GSK-3-β).* ***b)*** *Wnt-on. Upon Wnt stimulation, the complex that sends β-catenin to proteolytic degradation is sequestered by phosphorylated Disheveld protein (Dvl). This leads to β-catenin cytoplasmatic accumulation, which will translocate into the nucleous and act as a transcription factor(3).*

However, in the same study, it is found that DDX3X is not having a strong oncogenic role in the presence of Wnt/β-catenin constitutive activation due to APC loss(12). Additionally, in a parallel study, *Su SY, et al.* found that low DDX3X expression levels were correlated with poor prognosis and more frequent distant metastasis. It was demonstrated that DDX3X downregulation in CRC promotes cell migration and invasiveness through the upregulation of Snail. This indicates that in some contexts, DDX3X may also have an anticarcinogenic effect mediating the Snail/E-cadherin pathway in CRC(8).

2.3. Epithelial to mesenchymal transition (EMT) related pathways in CRC

EMT is a process in which epithelial cells lose their apical polarity and acquire a mesenchymal phenotype(13)(14), thus enhancing migration abilities and cancer cells invasive and metastatic potential(15).

The two pathways that are involved in the expression of oncogenic and tumour suppressor activities of DDX3X are well-known activators of the EMT-program and play relevant roles during CRC carcinogenesis(15). Snail is a zinc-finger transcription factor(14) that downregulates epithelial associated genes (e.g. E-cadherin), and enhances mesenchymal ones (e.g. vimentin)(14)(15). Further, Wnt/β-catenin not only drives early tumourigenesis(3), but also contributes to maintenance and progression of malignancies by promoting EMT-related genes(16).

Additionally, there is a potent interconnection between these two EMT activators that creates a positive loop that enhances EMT. The activation of Wnt/β-catenin pathway enhances Snail expression, stabilization, and

nuclear localization through the inactivation of GSK-3-β(3), which phosphorylates and sends Snail to proteolytic degradation(17), and through the transcriptional activity of β-catenin. So, in tumours where there is a constitutive activation of Wnt signalling, Snail will probably be overactivated(16). Snail, in turn, upregulates Lymphoid enhancer-binding factor 1 (LEF1), a transcription factor that can act together with β-catenin modifying Wnt/β-catenin target genes, thus redirecting its transcriptional output towards pro-invasive components of the EMT program(16).

All these changes lead to a mesenchymal and less proliferative phenotype of CRC cells, which promote cell invasion and chemoresistance, respectively. Therefore, the interaction of Wnt/β-catenin pathway with Snail is a key step for the initiation of EMT in CRC, which has been associated with increased rate of cancer recurrence and decreased survival of CRC patients(13)(18).

Taking everything into account, it would be worth elucidating if DDX3X is having its described tumour suppressor role through the repression of Snail(8) in the context of Wnt/β-catenin pathway constitutive activation due to APC loss, since 70% of sporadic CRC emerge from APC inactivating mutations(19). If this was the case, in these patients the administration of a DDX3X inhibitor therapy could worsen its prognostic since DDX3X would be braking EMT in CRC cells, diminishing its invasive potential and acquisition of chemoresistance.

3. Hypothesis and objectives

The aim of this work is to investigate the role of DDX3X in CRCs harbouring APC mutations in Wnt/β-catenin signalling pathway. It is hypothesized that in CRCs with this genetic background DDX3X can express its tumour suppressor role through the repression of Snail.

The specific objectives of this research project are listed below.

- To determine the role of DDX3X in APC-mutated CRCs.
- To elucidate the molecular mechanism behind DDX3X role in APC-mutated CRCs.
- To evaluate DDX3X role in APC-mutated CRCs using an in-vivo orthotopic model.

4. Materials & Methods

4.1. Cell lines

CRC cell lines used will be HCT116, DLD-1, SW480, and Colo205. Genetic alterations of commercial cell lines were consulted in CanSAR database(20) and are presented as follows: HCT116 (CTNNB1-S45del), DLD-1 (APC-I141X), SW489 (APC-Q1338X), and Colo205 (APC-T1556fsX3). Cell lines of choice have good engraftment rate in mouse models and metastatic potential. Additionally, HEK293FT cells will be used for lentiviral production(21).

Cells will be maintained in DMEM supplemented with 10% Fetal Bovine Serum, 2 mM Glutamine, penicillin (100 unit/ml), and streptomycin (100μg/ml) according to supplier indications(12).

4.2. Animals

All animal care and in-vivo experimental procedures will be conducted in accordance with a protocol approved by the Ethics Committee on Animal Experiments of the Autonomous University of Barcelona.

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (4-weeks old, male/female, n=56(22)) will be purchased from The Jackson Laboratory. NSG mice will be maintained under specific pathogen-free conditions. Submucosal injection in the distal rectum of luciferase-expressing CRC cell lines previously transduced with the inducible DDX3X shRNA construct (in media with Matrigel) will be performed(23).

Tumour volume will be monitored through bioluminescence imaging(24). All mice will be euthanized when the largest tumour volume reaches 1cm^3 (experimental endpoint) using anesthesia with ketamine followed by cervical dislocation(11).

4.3. Methodology

4.3.1. Lentiviral-based Dox-inducible DDX3X short hairpin RNA (shRNA) production and transfection

A lentiviral vector containing an inducible shRNA will be prepared to knock down DDX3X expression in HCT116, DLD-1, SW480, and Colo205 cells.

Three different DDX3X targeting sequences will be tested to find out an efficient shRNA construct able to repress the protein of interest(21). DDX3X targeting sequences will be purchased from Sigma-Aldrich (RNAi consortium ID: *TRCN0000000001*, *TRCN0000000002*, and *TRCN0000314545*). pLKO.1 plasmid will be used to clone and express DDX3X shRNA sequences. Restriction digest of AgeI and EcoRI sites in pLKO.1 plasmid will be performed using Rapid DNA Ligation Kit to allow shRNA ligation(21). A scramble shRNA will be used as a negative control(25). Each shRNA plasmid will be transferred to HEK293FT cells to evaluate which DDX3X targeting sequence induces DDX3X knockdown more efficiently.

Lentiviral vector EZ-Tet-pLKO-Puro (*Figure 2*) with inducible expression of shRNA will be purchased from AddGene. The most efficient DDX3X shRNA oligo will be annealed using a previously described program in the Thermalcycler(21). Restriction digest of NheI and EcoRI sites in EZ-Tet-pLKO-Puro lentiviral vector will be performed using Rapid DNA Ligation Kit to allow shRNA ligation to produce the final lentiviral plasmid(21). Lentiviral plasmid with Green Fluorescence Protein (GFP) will be generated to be used as a positive control(25).

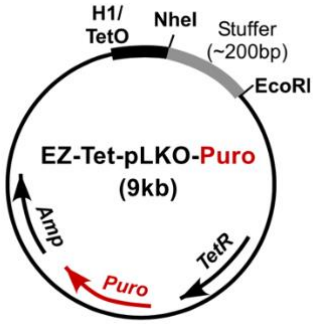

Figure 2. Basic vector map for EZ-Tet-pLKO-Puro lentiviral vector(21).

Lentiviral vector will be cloned using One Shot™ Stbl3™ Chemically Competent E. coli. E. coli will be seeded on LB-agar plates with 100µg/mL Ampicillin (selection of resistant populations). To screen positive shRNA clones and background colonies, GeneElute™ Plasmid Miniprep Kit will be used(21).

ViraPower™ Lentiviral Packaging Mix together with Lipofectamine™ 2000 Transfection Reagent will be used to produce lentivirus in HEK293FT cells. Puromycin (0.5µg/ml) will be added for selection of successfully transduced cells. Viral media with lentiviral particles, containing DDX3X shRNA or GFP vectors, will be collected, filtered, and used to transfect CRC cell lines and HEK293FT cell line, respectively.

HEK293FT cell line and CRC cell lines will be cultured in media with Puromycin (0.5µg/ml) to select GFP or shRNA transduced clones(21). 3 to 5 clones will be selected to perform DNA sequencing to check which clone has incorporated the complete version of the lentiviral vector(25).

4.3.2. Bioluminescence imaging (BLI)

pGL4.51[luc2/CMV/Neo] vector will be used for the generation of luminescent cell lines to be utilized for the *in-vivo* imaging studies. The vector includes a synthetic firefly luciferase (Luc2) under the control of cytomegalovirus (CMV) promoter. The vector will be amplified using One Shot™ Stbl3™ Chemically Competent E. coli and plasmid DNA will be extracted and purified using GeneElute™ Plasmid Miniprep Kit. Successfully transfected cells will be selected using Neomycin to create stable luciferase-expressing cell lines. After intraperitoneal injection of luciferin, signal will be acquired through IVIS Spectrum *in-vivo* imaging system(24)(26).

4.3.3. Western blot (WB)

RIPA lysis buffer will be used to lysate culture or tumour cells for protein extraction. Total protein concentration will be quantified by Pierce BCA Protein Assay Kit. Protein samples will be separated by SDS-polyacrylamide gel electrophoresis and transferred in a PVDF membrane. After blocking, to avoid antibody unspecific bonds, membranes will be incubated at 4ºC overnight with primary antibodies, followed by 1-hour incubation with horseradish peroxidase (HRP)-conjugated antibody. Protein bands will be visualized using chemiluminescence(27)(28).

Primary antibodies (Rabbit Polyclonal) used could be anti-DDX3X, anti-E-cadherin, anti-Vimentin, anti-Snail, anti-GFP and anti-β-actin. Secondary antibodies used will be HRP-conjugated Goat Anti-Rabbit IgG.

4.3.4. Bottom-up proteomics

Protein extraction and quantification will be performed as previously described for WB analyses. Protein mixtures will be digested by trypsin and tagged using Tandem Mass Tag (TMT) labelling reagents. TMT-peptides will be loaded into C18 HPCL Columns for subsequent liquid chromatography/tandem mass spectrometry analysis. Proteome Discoverer Software will be used for raw data analysis and TMT-6plex method for protein quantification according to TMT precursors intensity(29)(30).

4.3.5. Immunohistochemistry (IHC)

Culture cells and paraffin-embedded tissues, previously cut into 5µm-think sections by microtome, will be mounted onto adhesion microscope slides. Tissue sections will be deparaffinized and rehidratated. After application of antigen retrieval and blocking with peroxidase, slides will be incubated at 4ºC overnight with primary antibodies. Next day, samples will be incubated with a biotinylated secondary antibody. Signal will be revealed using DAB Substrate Kit, and nuclei will be stained using hematoxylin. Some sections will be stained with hematoxylin-eosin reagents. Samples will be visualized using light microscopy(31)(32).

Primary antibodies (Rabbit Polyclonal) used could be anti-β-catenin, anti-E-cadherin and anti-Ki67. Secondary antibodies used will be Biotin Secondary Antibody Goat Anti-Rabbit IgG.

4.3.6. Quantitative real-time PCR (qRT-PCR)

To evaluate expression, total cellular RNA will be extracted using PureLink™ RNA Mini Kit. Reverse transcription reaction to obtain cDNA will be performed with AMV Reverse Transcriptase and random primers(33).

Target genes will be measured using SYBR-Green in a 7500 Real-Time PCR Thermal Cycler. The expression of GADPH will be used to normalize target genes expression(31)(33).

Primers used for qRT-PCR analysis will be designed using Primer3web and obtained from Integrated DNA Technologies. Evaluated genes could be DDX3X, β-catenin, Snail, E-cadherin, Vimentin.

4.3.7. Proliferation, migration, and invasion assays

BrdU Cell Proliferation Assay Kit is the colorimetric immunoassay that will be used for the quantification of cell proliferation(27). Briefly, 5-bromo-2'-deoxyuridine (BrdU) incorporates into newly synthesized DNA in place of thymidine and can be detected with an anti-BrdU antibody(34). Absorbance will be measured at 450 nm using an ELISA plate reader(27).

Transwell polycarbonate membrane cell culture inserts will be used to perform cell migration and invasion assays. In brief, these assays use two chambers, one containing additional 10% FBS to create a gradient, separated by a porous membrane(35). In the migration assay, cells in serum free DMEM are seeded in the upper chamber and the ones with the ability to direct cell movement towards the gradient will reach the bottom chamber(36). In the invasion assay, polycarbonate membranes will be pre-coated with Matrigel Matrix that will create a thin layer that occludes the porous(33), thus, only cells able to penetrate tissue barriers, such as basal membrane, will accomplish to get to the bottom chamber(36). Cells on the lower surface will be fixed with methanol and stained with Hoechst for fluorescence microscopy visualisation. Quantification will be performed using ImageJ software(33).

4.3.8. Flow cytometry

Circulating tumour cells (CTCs) will be detected by flow cytometry analysis of Cell-Surface Vimentin (CSV) Monoclonal-Antibody. After sample processing, cells will be incubated with CSV-antibody and labelled with Alexa Fluor-405 secondary antibody for data acquisition using Flow Cytometer. Flowjo Software will be used for data analysis(37).

4.3.9. Statistical analysis

Statistical analysis will be performed using GraphPad Prism 9.5.0. Differences between control and silence groups will be tested using two-paired T-tests. Differences between cell lines will be studied using ANOVA followed by Tukey test. $p < 0.05$ will be considered statistically significant(27).

All in-vitro data will be obtained from three independent experiments and expressed as mean ± SEM(31). For the in-vivo experiments, sample size will be estimated according to previous studies(22)(24).

5. Chronogram

This project is divided into 3 main objectives that will be developed during 24 months(*Table 1*).

Table 1. Chronogram that summarizes the timings for the principal experiments that will be performed to contrast each objective.

		2024												2025											
		01	02	03	04	05	06	07	08	09	10	11	12	01	02	03	04	05	06	07	08	09	10	11	12
Workflow	**OBJECTIVE 1: To determine the role of DDX3X in APC-mutated CRCs**																								
	O.1.1. Generation of DDX3X knockdown CRC clones with an inducible lentiviral-based shRNA																								
	O.1.2. In-vitro proliferation, migration and invasion assays																								
	OBJECTIVE 2: To elucidate the molecular mechanism behind DDX3X role in APC-mutated CRCs																								
	O.2.1. Proteomics screening																								
	O.2.2. Validation of EMT markers at transcriptomic and proteomic levels																								
	OBJECTIVE 3: To evaluate DDX3X role in APC-mutated CRCs using an in-vivo orthotopic model																								
	O.3.1. Validation of DDX3X knockdown with the Dox-inducible shRNA in-vivo																								
	O.3.2. In-vivo experimental procedures																								
	O.3.3. In-vivo proliferation, invasion, and metastatic assays																								
	O.3.4. Detection of EMT markers in tumor samples																								

6. Workflow

Experiments planned to accomplish the proposed objectives(*Figure 3*) will be explained in this section.

HCT116 cell line, which has a wild-type version of APC, will be used as a positive control. DLD-1, SW489, and Colo205, which have a non-functional version of APC, will be the subjects of study. DDX3X basal expression levels will be assessed on each CRC cell line at transcriptomic (qRT-PCR) and proteomic (WB) levels before starting the experimental procedures.

6.1. OBJECTIVE 1: To determine the role of DDX3X in APC-mutated CRCs

The aim of the following set of experiments is to determine whether DDX3X is expressing its oncogenic or tumour suppressor potential in CRCs harbouring a truncated version of APC.

6.1.1. Generation of DDX3X knockdown CRC clones with an inducible lentiviral-based shRNA

To elucidate DDX3X role, lentiviral particles containing a Dox-inducible shRNA for DDX3X knockdown will be prepared and used to create a DDX3X knockdown clone for each cell line. A lentiviral particle containing Dox-inducible GFP will be used to generate a control in HEK293FT cells.

Upon the addition of Docycycline (Dox), Tetracycline-repressor gene (TetR) in the EZ-Tet-pLKO-Puro vector is sequestered, and Tetracycline-resistance protein (TetO) can drive DDX3X shRNA expression since its repression is relieved(21). This inducible system will allow the use of the same pool of cells for controls (no shRNA induction) and study group (plus Dox)(21)(25).

A functional assay (Dox dose-response) will be performed before starting the experiments to check which Dox dose accomplishes to induce GFP expression or DDX3X knockdown in the clones that have incorporated the complete version of the lentiviral vector. Generally, 10ng/ml of Dox are sufficient to induce target knockdown

at 72 hours(21), however, the dosage and timing also depend on protein stability and basal expression levels. Therefore, doses of 5ng/ml, 10ng/ml, and 20ng/ml will be tested.

The inducible system will be evaluated after Dox administration by qRT-PCR to check mRNA levels, at 48 h, and by immunoblot to check protein levels, at day 5(21). GFP expression will also be visualized with a fluorescence microscopy(25).

For each CRC stable clone, a knock down group will be generated by the addition of the proper dose of Dox according to the dose-response functional assay. HEK293FT cells with inducible expression of GFP will be used as a control. All experiments will be performed in both expressing and knockdown groups.

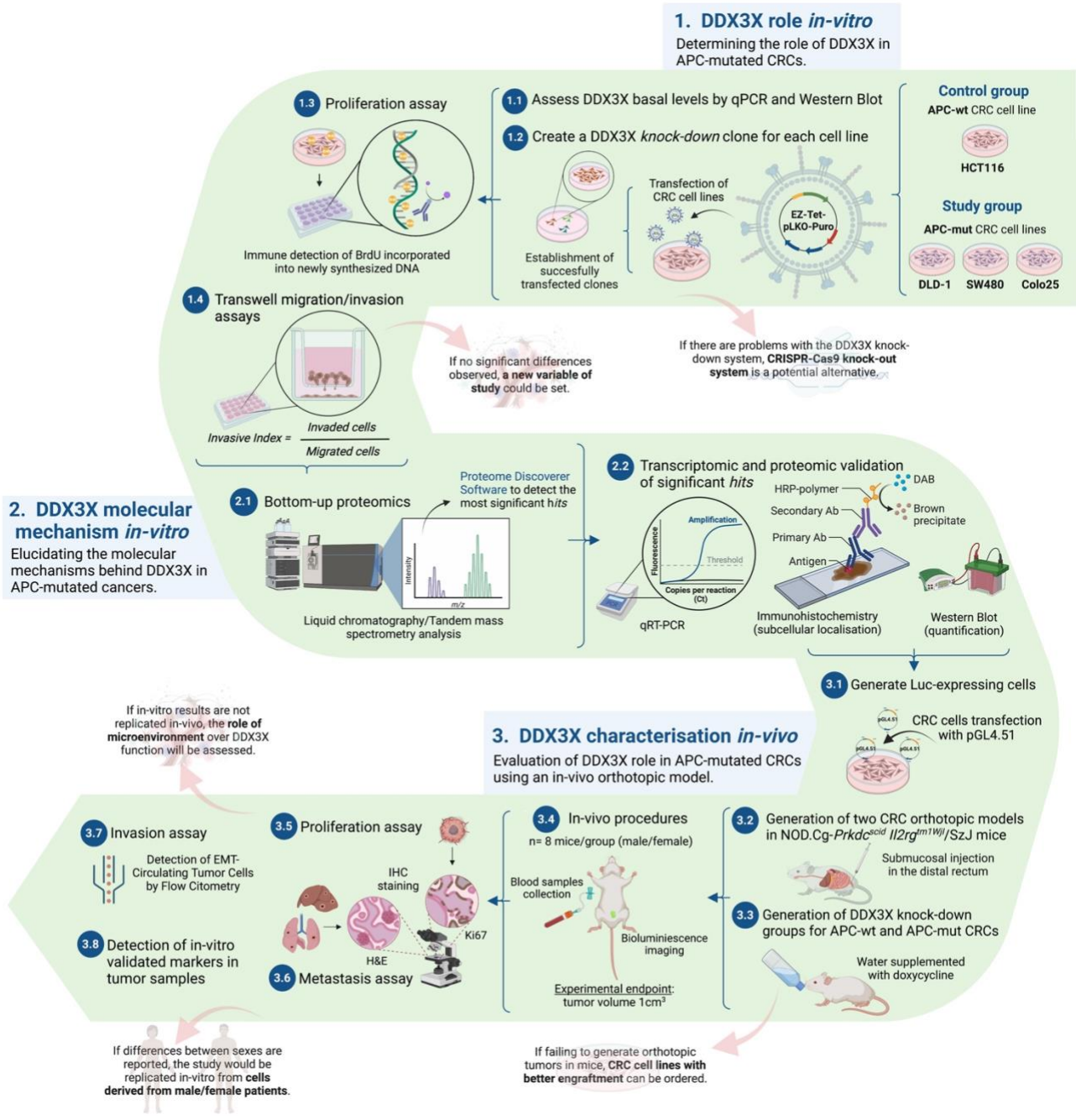

Figure 3. Worflow schematic representation including the proposed contingency plans in some critical steps.

6.1.2. In-vitro proliferation, migration, and invasion assays

Proliferation, migration, and invasion assays(27)(33) will be performed *in-vitro* to assess if these abilities are diminished or enhanced by the knockdown of the protein of interest. HEK293FT cells with inducible expression of GFP will be used as a control to check the proper functioning of the silencing system and to assess if Dox is responsible for any changes observed in the assays(21).

Cell proliferation will be quantified by the immune detection of BrdU incorporated into newly synthesized DNA(34). Cells that migrated to the lower chamber of the migration and invasion assays will be visualised by fluorescence microscopy and quantified using ImageJ software. Invasive index will be calculated "invaded (passed through pre-coated membrane) vs migrated (passed through non-coated membrane) cells ratio", to determine the relative contribution of invasiveness to the general motility ability(35).

6.2. OBJECTIVE 2: To elucidate the molecular mechanism behind DDX3X role in APC-mutated CRCs

The aim of the following set of experiments is to elucidate which are molecular mechanisms behind DDX3X role in APC-mutated CRCs.

6.2.1. Proteomics screening

Since cellular complexity arises from different layers beyond the genome, the protein profile of control and DDX3X knockdown cells will be analysed to assess the functional diversity of both groups(38).

A bottom-up proteomics will be performed for the identification and quantification of proteins that are significantly different between both groups(30). The most statistically significant *hits* will be further validated.

6.2.2. Validation of significant hits at transcriptomic and proteomic levels

Validation of markers where a statistically significant difference between groups has been detected in the proteomics study will be performed.

Following, an example of the validation analysis will be described for some EMT markers that may potentially change between groups(8)(12). Transcriptomic evaluation of β-catenin and Snail, together with epithelial (E-cadherin) and mesenchymal (Vimentin), will be assessed by qRT-PCR. Protein levels of E-cadherin, Vimentin, Snail, and β-catenin, will be assessed by WB. Moreover, IHC staining will be performed to evaluate β-catenin subcellular localization(39) and E-cadherin membrane presence, to assess Wnt/β-catenin signalling pathway and Snail/E-cadherin pathway activation, respectively.

The analyses will be performed in DDX3X control and knockdown groups to validate if there are significant differences in the activation of these pathways.

6.3. OBJECTIVE 3: To evaluate DDX3X role in APC-mutated CRCs using an in-vivo orthotopic model

Since microenvironment has been described as an important component that can influence the function of dual role proteins(10), the following set of experiments aims to evaluate if DDX3X role is conditioned, not only by the genetic background, but by microenvironment interactions in an in-vivo orthotopic model.

CRC orthotopic models will be created with the implantation of two luciferase-expressing CRC cell lines, either HCT116 (control) or one with an APC-mutation (study group) elected according to previous in-vitro results.

6.3.1. Validation of DDX3X knockdown with the Dox-inducible shRNA in-vivo

A proof of concept will be done before starting the experiments to check which Dox dose accomplishes to induce DDX3X knockdown in-vivo. Water supplemented with 1mg/ml Dox has been reported to induce target knockdown in mice(40), for this reason, 0mg/ml, 1mg/ml, and 1.5mg/ml doses will be tested. DDX3X expression levels in tumour samples will be assessed by WB after mice being anesthetized and sacrificed.

6.3.2. In-vivo experimental procedures

For the experimental in-vivo study, mice injected with each luciferase-expressing cell line will be randomly divided into control or silenced group and given standard water or water supplemented with the elected dose of Docycycline to induce DDX3X knockdown, respectively(40). 8 mice/group with the same number of males and females included in each group will be used(22). In all the experiments, differences between sexes will be assessed to determine if sex is a potential variable that can influence the role of the protein.

Blood samples will be collected once per month after anaesthesia with ketamine. Tumour growth and metastasis will be monitored in-vivo by BLI after luciferin intraperitoneal injection every 2 weeks(24)(26). On the experimental endpoint, tumours will be harvested and weighted. Moreover, lungs and liver will be dissected to perform a metastasis assay. DDX3X expression levels in tumour samples will be analysed by WB.

6.3.3. In-vivo proliferation, invasion, and metastatic assays

Cell proliferation will be assessed through IHQ staining of Ki67 marker on tumour samples.

CTCs quantification and characterization by flow cytometry will be performed to evaluate cells invasive potential(13). Instead of epithelial cell adhesion molecule (EpCAM)-based enrichment technique, which fails to detect CTCs that have undergone EMT, CSV will be analysed to detect and quantify those EMT-CTCs which reflect the invasive and metastatic potential(37).

The presence of EMT-CTCs is only associated with a higher likelihood of metastasis(13). Thus, to assess if metastasis is taking place, CTCs analysis will be complemented with hematoxylin-eosin staining of dissected tissues to count visible metastatic nodules(28).

6.3.4. Detection of in-vitro validated markers in tumour samples

Markers validated in-vitro will be further examined by IHQ in mice tumour samples to elucidate if the molecular mechanism behind DDX3X is maintained in in-vivo conditions.

7. Discussion and impact of the expected results

This research project aims to clarify if an alteration in the genetic background of CRC cells can predict DDX3X role. Thus, it is expected to find out if the mutational status of APC can help to distinguish which patients can benefit from a DDX3X inhibitor.

Although the synthesis of a drug to induce the expression of DDX3X is discarded since this molecule has a clear oncogenic role described in some tumours(5), elucidating the cases where it has a tumour suppressor function can help to stablish inclusion and exclusion criteria for the combination of targeted therapies with a DDX3X inhibitor.

In the context of Wnt/β-catenin signalling pathway constitutive activation, Snail might be overexpressed(16). If DDX3X acts as a tumour suppressor through Snail inhibition in CRCs with APC loss, as hypothesized, in 70% of sporadic CRCs DDX3X expression might be beneficial.

So, if it is found that in DDX3X knock-down group of APC-mutated cell lines *in-vitro* and *in-vivo*, there is a significant reduction in proliferation and increased invasive potential in comparison to control group, together with an increased Snail/E-cadherin activation, this would confirm the hypothesis of this work, having a translational application for the election of the proper combinatorial therapies for this group of patients.

7.1. Contingency plans

Contingency plans that have been considered are summarized in *Table 2* and *Figure 3*.

Table 2. Contingency plans considered for different potential problems that could emerge during the project development.

Potential problems	Contingency plan
OBJECTIVE 1: To determine the oncogenic or tumor suppressive role of DDX3X in APC-mutated CRCs	
P1.1. *Failing to establish DDX3X knockdown CRC clones using the inducible lentiviral-based shRNA or excessive leakage of DDX3X expression.*	***C1.1.*** *CRISPR-Cas9 knock-out of DDX3X is a potential alternative(42).*
P1.2. *Statistically significant alterations in cell viability due to Doxycline toxicity.*	***C1.2.*** *CRISPR-Cas9 knock-out system would also be the alternative(42).*
P1.3. *No significant differences observed between control and study groups.*	***C1.3****. Establishing a new variable that could be determining DDX3X role in colorectal cancer, such as sex or specific interactions with microenvironment(10).*
OBJECTIVE 3: To evaluate DDX3X role in APC-mutated CRCs using an in-vivo orthotopic model	
P3.1. *Failing to generate orthotopic CRC tumors in mice.*	***C3.1.*** *CRC cell lines with a better engraftment rate would be ordered.*
P3.2. *In-vitro results are not repicated in-vivo.*	***C3.2.*** *Perform in-vitro and in-vivo experiments to assess if microenvironment interactions are causing functional changes in DDX3X protein(10).*
P3.3. *Differences between sexes are reported.*	***C3.3.*** *Since DDX3X is an X-linked protein(5), differences between sexes may appear. If this was the case, the study would be replicated in vitro with cells procedent from male or female patients.*

8. Costs

The estimated project cost is 169.881,22€. The items included in the budget are presented in *Table 3*.

Table 3. Justification of the estimated project cost.

Type description	Units	Cost (€)
Fungible		**39.816,22**
Disposable laboratory material		1.147,2
Pipette tips, Serologic pipettes		*162*
Eppendorffs, PCR Tubes, Falcons		*20*
Nitrile Gloves		*34*
Adhesion microscope slides (#23888-114units, Thermo-Fischer Scientific)	1	*30,2*
96-well ELISA plate (#MSEHNFX-40units, Sigma-Aldrich)	1	*160*
Transwell polycarbonate membrane cell culture inserts (#CLS3412-24EA, Coring)	3	*741*
Cell culture material		12.272,4
HCT116 (#ECACC-91091005) cell line	1	*450*
DLD-1 (#ECACC-90102540) cell line	1	*450*
SW480 (#ECACC-87092801, Sigma-Aldrich) cell line	1	*450*
Colo205 (#ECACC-87061208) cell line	1	*450*
HEK293FT (#R70007, Invitrogen) cell line	1	*1.978*
One Shot™ Stbl3™ Chemically Competent E. coli (#C737303, Thermo-Fisher)	1	*560*
DMEM culture media (#D5030-10L, Sigma-Aldrich)	4	*496*
Fetal Bovine Serum (#F7524-500mL, Sigma-Aldrich)	8	*3.544*
L-Glutamine (#G2150-20mL, Sigma-Aldrich)	20	*960*
Penicillin-Streptomycin solution 100X (#P7539-100mL, Sigma-Aldrich)	4	*326*
LB-agar plates (#L5542-10EA, Sigma-Aldrich)	2	*102*
Cell culture flasks (#CLS431082-25EA, Coring)	4	*1.112*
Selection antibiotics from Sigma-Aldrich: Ampicillin (#A5354-10mL), Puromycin (P9620-10mL), Neomycin (#N1142-20mL)	2	*1.394*
Kits and reagents		11.934,04
Matrigel Matrix (#356234-5mL, Corning)	2	*684,30*
Rapid DNA Ligation Kit (#K1422-50rxns, Thermo-Fisher Scientific)	1	*198*
GeneElute™ Plasmid Miniprep Kit (#PLN70-1KIT, Sigma-Aldrich)	1	*1204*
ViraPower™ Lentiviral Packaging Mix (#K497500-60rxns, Invitrogen)	1	*3.325*
Lipofectamine™ 2000 Transfection Reagent (#11668027-0.75mL, Invitrogen)	1	*636*
Firefly Luciferase Substrate (#LUC100-15mL, Signosis)	1	*694*
Pierce BCA Protein Assay Kit (#23227-1L, Thermo-Fischer Scientific)	1	*285*
PVDF membrane (#IPVH00010-1EA, Millipore)	1	*499*
Trypsin (#T1426-100mg, Sigma-Aldrich)	1	*107*
Tandem Mass Tag (TMT) labelling reagents (#90063-15rxns, Thermo-Fisher Scientific)	1	*830*
C18 HPCL Columns (#504971-1EA, Sigma-Aldrich)	1	*836*
Peroxidase (#P8375-5KU, Sigma-Aldrich)	1	*138*
DAB Substrate Kit (#34002-275mL, Thermo-Fisher Scientific)	1	*133*
Hematoxylin-Eosin Staining Solution (#GHS280-2.5L, Sigma-Aldrich)	1	*228*
PureLink™ Genomic DNA Mini Kit (#K182000-10preps, Invitrogen)	2	*107*
PureLink™ RNA Mini Kit (#12183020-10preps, Invitrogen)	2	*356*
AMV Reverse Transcriptase (#10109118001-1000u, Sigma-Aldrich)	1	*542*
SYBR Green JumpStart™ TaqReadyMix™ (#S4438-100rxns)	1	*313*
BrdU Cell Proliferation Assay Kit (#6813-1KIT, Cell Signalling Technology)	1	*818,74*
Antibodies		4.798
Anti-DDX3X (#09-860, Sigma-Aldrich)	1	*450*
Anti-E-cadherin (#07-697, Sigma-Aldrich)	1	*546*
Anti-Vimentin (#SAB5700782, Sigma-Aldrich)	1	*348*
Anti-Snail (#SAB5700703, Sigma-Aldrich)	1	*348*
Anti-GFP (#SAB4701015, Sigma-Aldrich)	1	*342*
Anti-beta-actin (#PA5-78715, Invitrogen)	1	*455*
HRP-conjugated Goat Anti-Rabbit IgG (#31460, Invitrogen)	1	*244*
Anti-beta-catenin (#C2206, Sigma-Aldrich)	1	*513*

Anti-Ki67 (#SAB5700770, Sigma-Aldrich)	1	*348*
Biotin Secondary Antibody Goat Anti-Rabbit IgG (#65-6140, Invitrogen)	1	*549*
CSV Monoclonal-Antibody clone 84-1 (#H00007431-M08, Thermo-Fisher)	1	*347*
Alexa Fluor-405 secondary antibody (#A-31553, Thermo-Fisher)	1	*308*
Primers, Plasmids and Vectors		2.190,5
DDX3X targeting sequences (RNAi consortium, Sigma-Aldrich)	3	*945*
Green Fluorescence Protein (#14757, Addgene)	1	*85*
pLKO.1 plasmid (#10878, Addgene)	1	*255*
Scramble shRNA (#1864, Addgene)	1	*85*
EZ-Tet-pLKO-Puro vector (#85966, Addgene)	1	*170*
pGL4.51[luc2/CMV/Neo] vector (#E1310, Promega)	1	*618*
Primers from Integrated DNA Technologies	5	*32,5*
In-vivo experimental		7.474,08
NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice (#IMSR_JAX:005557)	56	*5344,08*
Ketamine (#Y0000450)	1	*130*
Animal facilities and mantainment		*2.000*
Facilities and external collaborations		**6.065**
Equipment facilities		2.700
Fume hood (Laboratory Safety Level 2)		*720*
IVIS Spectrum in-vivo imaging system		*440*
Light and fluorescence microscopes		*280*
NanoDrop UV Spectrophotometer		*360*
qRT-PCR Thermal cycler		*750*
Microtome (Room of Pathological Anatomy)		*150*
External collaborations (Analytical services)		1.700
Mass spectometry analysis	10	*600*
DNA sequencing	4	*200*
Flux cytometry analisis	32	*900*
Softwares		1.665
Graphpad prism 9.5.0		*380*
Flowjo		*285*
Proteome Discoverer Software		*1.000*
Personal		**120.000**
Hired scientists		120.000
Post-doctoral researcher	1	*70.000*
Technician	1	*50.000*
Publications and congresses		**3.000**
Indirect costs		**1.000**
Total full economic cost		**169.881,22**

9. Diffusion and formation plan

The experimental results obtained from this project will be presented for publication in Oncology Journals with high impact factor. Additionally, this work will be exposed in national and international congresses.

Finally, a PhD thesis will be planned with the aim of providing more insights of inclusion and exclusion criteria for a treatment with a DDX3X inhibitor. Congresses will be attended to actualize the obtained results and acquired knowledge, while new articles will be presented for journal publication.

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