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THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE FRANCHE- COMTE PREPAREE AU CENTRE LNC, INSERM U1231

NILTON DE FRANÇA JUNIOR

GALECTIN-9 BINDS TO TRAIL AGONIST RECEPTORS AND REGULATES TRAIL-INDUCED APOPTOSIS

CURITIBA

MARCH/2021

NILTON DE FRANÇA JUNIOR

Galectin-9 binds to TRAIL agonist receptors and regulates TRAIL-induced apoptosis

Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Pontifícia Universidade Católica do Paraná, como requisito à obtenção do título de Doutor em Ciências da Saúde.

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Nilton de França Junior

Galectin-9 binds to TRAIL agonist receptors and regulates TRAIL-induced apoptosis

Thèse présentée et soutenue à Curitiba, Paraná, Brésil le 26 mars 2021.

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Dedication

I dedicate this work
To my parents Nilton de França e Joraci de França
For the support they always gave me, for the incentive to go further and further
I also dedicate to my advisors, Olivier and Selene, source of inspiration
Likewise, I dedicate to my wife, Amanda, example of wisdom.

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ABSTRACT

APO2L/TRAIL (TNF-related apoptosis-inducing ligand) arouses great interest in cancer therapy. This protein induces apoptosis in tumor cells through DR4 or DR5, two transmembrane glycoproteins that harbor N- and O- glycosylations, respectively. The glycosylation of DR4 or DR5 receptors is likely to allow unforseen protein/protein interactions. For instance, galectins, owing to their ability to bind to oligosaccharides, can interact with glycoproteins, and are therefore potentially able to regulate TRAIL pro-apoptotic machinery in tumor cells. Given in addition that conventional chemotherapeutic drugs such as 5-fluorouracil (5FU), often restore or increase TRAIL-induced apoptosis, but that the molecular mechanism underlying this gain of function remains elusive, we investigated the possibility that galectins may link restoration of TRAIL sensitivity upon sequential chemotherapy. We provide evidence, here, that 5FU sensitizes tumor cells to TRAIL-induced apoptosis through its ability to induce the secretion of galectin-9 into the extracellular medium. Mechanistically, the binding of secreted galectin-9 with the extracellular domains of DR4 and/or DR5, increased apoptosis triggered by TRAIL. This gain of function and interaction was strictly related to the sugar moiety harbored by DR4 and DR5. Likewise, point mutations of the glycosylation sites or production of the receptors in prokaryotic cells not only abrogated galectin-3 and 9 binding, but also restoration of TRAIL sensitivity by 5FU. However, when the receptors are properly glycosylated, overexpression of galectins or addition of soluble recombinant of galectin-3 or -9 alone, is sufficient to increase tumor cell sensitivity to TRAIL-induced cell death. Overall, our results suggest that galectins are novel components of TRAIL's DISC whose regulation by 5FU helps explain, at least in part, how this conventional chemotherapy increases the therapeutic potential of TRAIL.

Keywords: Glycosylation, TRAIL, receptor, galectin

RESUMO

APO2L/TRAIL (ligante indutor de apoptose relacionado ao TNF) desperta grande interesse na terapia do câncer. Ao contrário da maioria dos tratamentos comumente usados clinicamente, esta proteína induz apoptose em células tumorais através de DR4 ou DR5, duas glicoproteínas transmembrana que abrigam N- e O- glicosilações, respectivamente. A glicosilação dos receptores DR4 ou DR5 permite que algumas moléculas, como as galectinas, reconheçam a sequência formada pelo oligossacarídeo e, portanto, se liguem a essa glicoproteína, podendo definir a via apoptótica da célula tumoral. Além disso, o 5-fluorouracil (5FU) é uma quimioterapia conhecida por restaurar ou aumentar a apoptose induzida por TRAIL, mas o mecanismo molecular subjacente a esse ganho de função permanece indefinido. Nós fornecemos evidências, de que 5FU sensibiliza as células tumorais à apoptose induzida por TRAIL por meio de sua capacidade de induzir a liberação de galectina-9 para o meio extracelular. Assim como no DISC (Sinal de Indução de Morte Complexo) componentes pró-apoptóticos, proteínas de ligação a carboidratos, como galectina-3 ou 9, por meio de interações diretas com receptores DR4 e DR5 são capazes de contribuir para apoptose induzida por TRAIL. Mecanisticamente, descobriu-se que a galectina-3 ou 9 interage especificamente com o domínio extracelular de DR4 e DR5, aumentando à apoptose desencadeada por TRAIL. A interação com DR4 ou DR5 foi estritamente relacionada ao estado de glicosilação dos receptores. A perda da glicosilação dos receptores DR4 e DR5 induzida por local dirigido a mutação ou produção em células procarióticas prejudicou a ligação da galectina-3 e 9. Os resultados obtidos também mostram que a superexpressão ou adição de recombinante solúvel de galectina-3 ou -9 por si só foi suficiente para aumentar a sensibilidade do TRAIL. Ao todo, nosso resultados revelam as galectinas como novos componentes TRAIL DISC, cuja regulação por 5FU, seja provavelmente responsáveis por sua capacidade de sinergia com TRAIL.

Palavras Chaves: Glicosilação, TRAIL, receptor, galectina.

RESUMÉ

APO2L/TRAIL (ligand inducteur d'apoptose lié au TNF) suscite un grand intérêt dans le domaine de l'oncologie. Contrairement à la plupart des traitements couramment utilisés en clinique, cette protéine induit l'apoptose dans les cellules tumorales par DR4 ou DR5, deux glycoprotéines transmembranaires qui hébergent des sites de N- et O-glycosylations, respectivement. La glycosylation des récepteurs DR4 ou DR5 est susceptible de permettre des interactions protéines/protéines imprévues. En raison de leur capacité à se lier aux oligosaccharides, les galectines peuvent, par exemple, interagir avec les glycoprotéines, et sont donc potentiellement capables de réguler la machinerie pro-apoptotique TRAIL dans les cellules tumorales. Étant donné en outre que les chimiothérapies conventionnelles, telles que le 5-fluorouracile (5FU), rétablissent ou augmentent souvent l'apoptose induite par TRAIL, sans que le mécanisme moléculaire sous-jacent à ce gain de fonction soit connu, nous avons cherché à savoir si les galectines pouvaient expliquer le rétablissement de la sensibilité des cellules tumorales à TRAIL au cours des chimiothérapies séquentielles. Nous apportons la preuve que le 5FU sensibilise les cellules tumorales à l'apoptose induite par TRAIL grâce à sa capacité à induire la sécrétion de la galectine-9 dans le milieu extracellulaire. D'un point de vue mécanistique, la liaison des galectines-3 ou -9 sécrétées avec les domaines extracellulaires des DR4 et/ou DR5, accroît l'apoptose déclenchée par TRAIL. Ce gain de fonction et d'interaction est strictement dépendant de l'état de glycosylation et donc des sucres présents sur DR4 et DR5. Ainsi, des mutations ponctuelles des sites de glycosylation ou de la production de récepteurs solubles dans les cellules procaryotes abrogent la liaison des galectines-3 et -9 aux récepteurs DR4 et DR5, ainsi que la restauration de la sensibilité à TRAIL par le 5FU. Cependant, lorsque les récepteurs sont correctement glycosylés, la surexpression des galectines ou l'ajout de versions recombinantes soluble de la galectine-3 ou -9, seules, suffisent à augmenter la sensibilité des cellules tumorales à la mort cellulaire induite par TRAIL. Dans l'ensemble, nos résultats suggèrent que les galectines sont de nouveaux composants du DISC de TRAIL dont la régulation par le 5FU permet d'expliquer, au moins en partie, comment cette chimiothérapie conventionnelle accroît le potentiel thérapeutique de TRAIL.

Mots clés: Glycosylation, TRAIL, récepteur, galectine.

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LIST OF ABBREVIATIONS

5FU: 5-Fluorouracil

7-AAD: 7-Aminoactinomycin D

A1/BFL-1: Bcl-2-related protein A1

AAL: *Aleuria aurantia lectin*

AKT: Activated kinase protein

Apaf-1: Apoptotic protease activating factor- 1

ATCC: American type culture collection

Bad: Bcl-2 antagonist of cell death

Bak: Bcl-2 antagonist killer 1

Bax: Bcl-2 associated x protein

Bcl-2: B-cell lymphoma 2

Bcl-W: Bcl-2 like 2

Bcl-xL: Bcl-2-related gene X, long isoform

Bid: BH3 interacting domain death agonist

Bid: BH3-interacting domain death agonist

Bik: Bcl -2 interacting killer

Bim: Bcl-2 interacting mediator of cell death

Bmf: Bcl-2-modifying factor

Bok: Bcl-2 related ovarian killer

CARD: Caspase recruitment domain

CAS9: CRISPR associated protein 9

CerS6: Ceramide synthase 6

cFLIPL/S: Cellular Flice inhibitory protein long/short

cIAP: Cellular Inhibitor of apoptosis

cl-CD95L: Cleaved CD95 ligand

Con A: *Concanavalin A*

CRD: Carbohydrate recognition domain

CRD: Cysteine-rich domain

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

dATP: Deoxyadenosine triphosphate

DD: Death domain

DED: Death effector domain

DIABLO: Direct IAP binding protein with

DISC: Death-inducing signaling complex

DNA: Deoxyribonucleic acid

EDAR: Ectodysplasin A receptor

EDARADD: Ectodysplasin-A receptor-associated adapter protein

EGFR: Epidermal growth factor receptor

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

FADD: Fas-Associated death domain

FasL/CD95L: Fas ligand/CD95 ligand

FCS: Fetal calf serum

FdUMP: Fluorodeoxyuridine monophosphate

FdUTP: Fluorodeoxyuridine triphosphate

FITC: Fluorescein isothiocyanate

FUTP: Fluorouridine triphosphate

Gal-1: Galectin-1

Gal-3: Galectin-3

Gal-8: galectin-8

Gal-9: Galectin-9

GALNT14: UDP-N-acetyl-alpha-D-galactosamine:N- cetylgalactosaminyltransferase polipeptide 14

GBPs: Glycan-binding proteins

GC: Golgi complex

GLcNAC: N-acetylglycosamine

GMDS: GDP-mannose-4,6-dehydratase

GPI: Glycosyl-Phosphatidyl-Inositol

gRNA: Guide RNA

HCT116: Colorectal cancer cells

His-TRAIL: Polyhistidine trail

HRK: Harakiri

IZ-TRAIL: Isoleucine zipper

IκB: Inhibitor of NF-κB

JNK: c-Jun NH2 terminal Kinase

LGALS9: Gene galectin -9

LZ-TRAIL: Leucine zipper residues

MAL I: Maackia amurensis lectin I

MAL II: Maackia amurensis lectin II

Man: Mannose

MAPK: Mitogen-activated protein Kinase

Mcl1: Myeloid cell leukemia 1

MCMV: Mouse cytomegalovirus

MDA-MB-231: Triple-negative breast carcinoma

MGAT4: mannosyl (alpha-1,3) –glycoprotein beta-1,4-acetylglucosaminyltransferase

MOI: Multiplicity Of Infection

mRNA: Messenger ribonucleic acid

NF-κB: Nuclear factor-κB

NK: Natural killer

NOXA: Bcl-2-modifying factor

OPG: Osteoprotegerin

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate Buffer Saline

PHA: *Phaseolus vulgaris*

PI3K PhosphatidylInositol-3-Kinase

PNA: *Peanut agglutinin*

PTEN: Phosphatase and tensin homolog

PUMA: Bcl-2-modifying factor

PYD: Pyrin Domain

RCA: *Ricinus communis agglutinin*

rh-TRAIL: Version of recombinant human TRAIL

RIPK1: Receptor-interacting serine/threonine-protein kinase 1

RNA: Ribonucleic acid

siRNA: Interference RNA

Smac: Second mitochondrial activator of caspases

SNA: *Sambucus nigra allgutinin*

ST6Gal-I: ST6 beta-galactoside alpha-2,6-Sialyltransferase

TALEN: Transcription Activator

tBid: Truncated Bid

TNF: Tumor necrosis factor

TRADD: TNFR1-associated death domain protein

TRAIL-R1/DR4: TRAIL receptor 1/Death Receptor 4

TRAIL-R2/DR5: TRAIL receptor 2/Death Receptor 5

TRAIL-R3/DcR1: TRAIL receptor 3/Decoy Receptor 1

TRAIL-R4/DcR2: TRAIL receptor 4/Decoy Receptor 2

TRAIL: TNF-related apoptosis-inducing ligand

TS: Inhibit the action of thymidylate synthase

VAL: B cell lymphoma

VVL: *Vicia villosa lectin*

WGA: *Wheat germ agglutinin*

XIAP: X-linked Inhibitor of Apoptosis Protein

FOREWORD

This work was carried out as part of the fight against cancer and was made possible thanks to the financial support of the Brazilian Ministry of Research, Coordenação de aperfeiçoamento de pessoal de nível superior (CAPES), together with the Comitê Francês de Avaliação da Cooperação Universitária (Cofecub).

This project focused on the study and understanding of the molecular mechanisms involved in triggering programmed cell death through the interaction of TRAIL receptors with galectin-3 and -9. Cell death is a biological process used and studied by the scientific and medical community in oncology. A better understanding of the mechanisms that control cell death, and particularly the death of cancer cells, would allow us to consider new treatments or make existing treatments more effective and better tolerated by patients.

1. INTRODUCTION

1.1. CANCER

Currently, cancer represents the second-largest single cause of mortality worldwide. According to the World Health Organization (WHO), cancer was responsible for the death of 9.6 million people worldwide in 2019. By 2030 it is predicted to be the leading cause of death globally, affecting about 12 million people per year (Plummer et al., 2018). This increase can be explained in part by population growth and aging, and recent disease screening advances (Adami et al., 2019).

Cancer, or malignant tumor, is a generic name for a group of more than 100 diseases characterized by uncontrolled cell proliferation of abnormal cells, resulting from pleiotropic and multifactorial events (Parkin et al., 2002). Each tumor presents its characteristics making each cancer a unique disease (Baylin and Jones, 2016). During carcinogenesis, normal cells accumulate genetic and epigenetic changes leading to oncogenes' activation and inhibition of tumor suppressor genes (Beckman and Loeb, 2017; Gillies and Gatenby, 2007). At this stage, loss of homeostasis occurs as tumor cells can acquire the capacity to unlimited proliferation (immortality), insensitivity to antiproliferative mechanisms, invasion and metastasis production, stimulation of angiogenesis, and resistance to programmed cell death. Additional genomic instability and, to a lesser extent, tissue inflammation are two characteristics that favor the acquisition of other abilities. Modification of energy metabolism and the ability to prevent or resist immune system attacks are also proposed as cancer cells' inherent capabilities (Figure 1) (Hanahan and Weinberg, 2011).

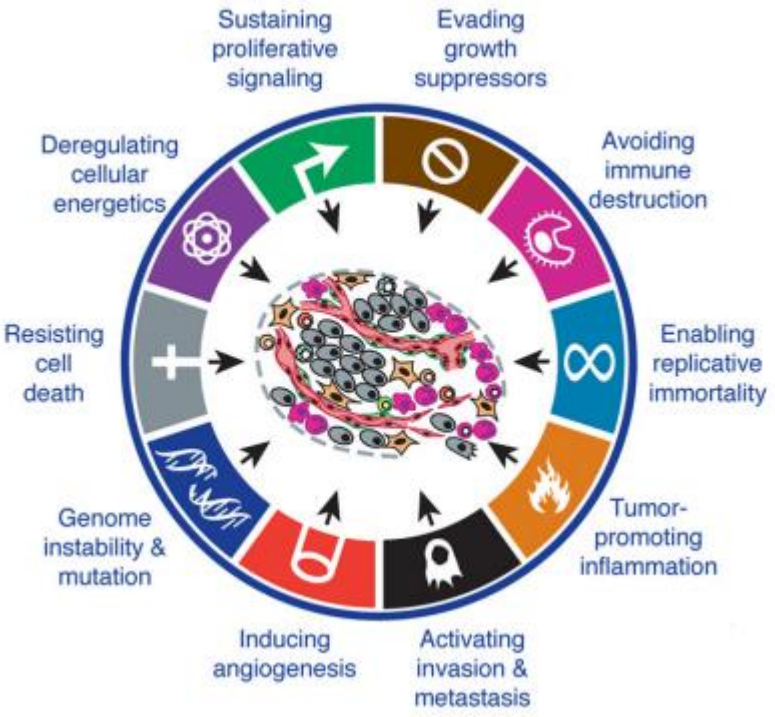


Figure 1: Essential ten capacities acquired by cancer cells during carcinogenesis. Essential ten capacities acquired by cancer cells during carcinogenesis. **Source:** Adapted from (Hanahan and Weinberg, 2011).

1.2. APOPTOSIS

Programmed cell death "apoptosis" is an essential process in maintaining body homeostasis (Degterev et al., 2003), resulting from the evolution of multicellular living beings. The death of one or more cells maintains the other's physiological function, leading to the organism's survival and development (Lockshin and Zakeri, 2001).

It is characterized by reduced cytoplasmic volume, bubble projections on the cell membrane, and phosphatidylserine positioning to the membrane's outer side (Feng et al., 2018). Characteristic changes in cell nuclei also occur, with the activation of endonucleases that fragment DNA (D'Arcy, 2019; Zhang and Xu, 2000). The resultant apoptotic bodies are then removed by phagocytes what contributes to the non-inflammatory profile of apoptosis (Elmore, 2007; Rathmell and Thompson, 2002).

Two distinct pathways interconnected by molecular signaling can initiate apoptosis (Figure 2). The intrinsic pathway is usually activated through severe damage to DNA, hypoxia, or other cellular stresses, such as those caused by chemotherapy or ionizing radiation, and the extrinsic pathway triggers apoptosis through death receptors

(DR) arranged on the cell surface. Activated DRs recruit intracellular adapter proteins, leading to the activation of cysteine proteases, known as caspases.

In mammals, eight DR members have been identified (Table 1): tumor necrosis factor receptor 1 (TNFR1), CD95 (Fas), death receptor 3 (TRAIL-R3, DcR3), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1, DR4), TRAIL-R2 (DR5), DR6, ectodysplasin A receptor (EDAR), and the p75 neurotrophin receptor (p75NTR) (Park, 2011). Their cognate ligands are cytokines belonging to the tumor necrosis factor (TNF) protein family (Guicciardi and Gores, 2009), most often referred to as death ligands.

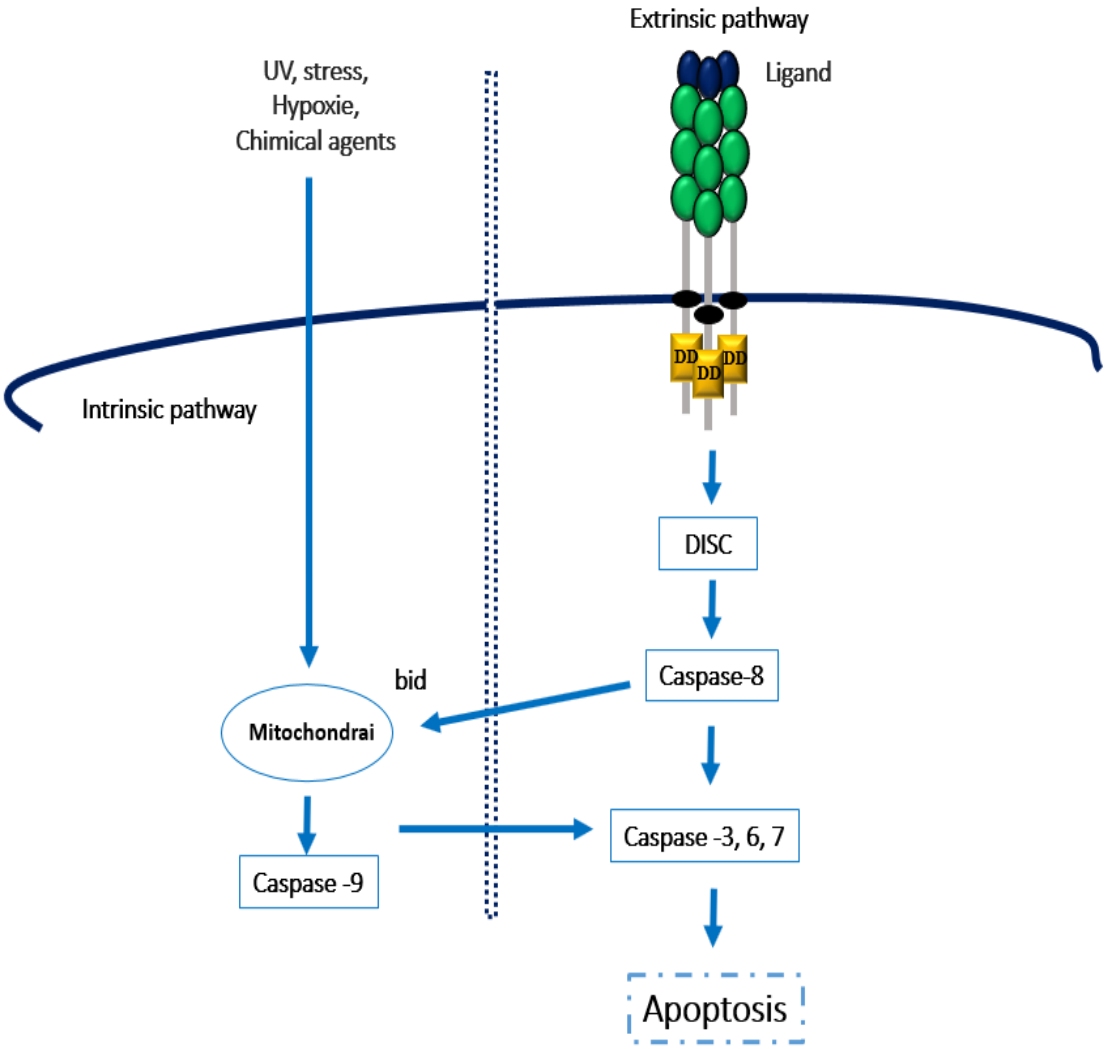


Figure 2: Simplified diagram of the intrinsic and extrinsic pathways of apoptosis. The intrinsic or mitochondrial pathway is activated after stress (chemical or physical) and is characterized by depolarization of the mitochondrial membrane, which also induces the cascade. The extrinsic pathway or the death domain receptor pathway is activated after binding of its ligand, which induces trimerization of death domain receptors and leads to activation of initiation caspases such as caspase-8 after assembly of the DISC complex (death-inducing signaling complex). Caspase-8, in turn, activates caspases effector (caspases-3, -6 and -7). **Source:** Adapted from (Fulda and Debatin, 2006).

Table 1: Members of the death receptors family and their cognate ligands.

Death Receptor	Ligand	
	Name	**Abbreviation**
TNFR1	Tumor necrosis factor/ Lymphotoxin alpha	TNF/ LTα
Fas	Fas ligand	FasL
DR3	TNF-like protein 1	TL1A
DR4/DR5	TNF related apoptosis-inducing ligand	TRAIL
DR6	alpha-amyloid precursor protein	APP
EDAR	Ectodysplasin A	EDA
p75NTR	nerve growth factor/brain-derived neurotrophic factor/ neurotrophic factor 4,5	NGF/ BDNF/ NT4,5

Source: Adapted from (Lee et al., 2019).

1.3. TRAIL AND THE AGONIST RECEPTORS

The cytokine APO2L/TRAIL (TNF-related apoptosis-inducing ligand) induces apoptosis in cancer cells through DR4 or DR5 (MacFarlane et al., 1997; Pan et al., 1997b; Walczak et al., 1997). This promising antitumor compound has attracted much interest in oncology due to its ability to trigger selective cell death in a wide variety of cancers (Ashkenazi et al., 2008). TRAIL is naturally expressed on the surface of immune cells, particularly monocytes, dendritic cells, natural killer cells, and T cells, all activated by interferons or interleukin-2 (IL2) (Almasan and Ashkenazi, 2003; Smyth et al., 2001). Due to the restricted expression in the immune cells, TRAIL plays an essential role in tumor immune surveillance (Rossin et al., 2019).

TRAIL is a homotrimeric protein with an affinity to the cysteine-rich domain (CRD) of TNF family receptors. The activation of the agonist DR4 or DR5 by TRAIL leads to receptor trimerization and conformational changes that promote the recruitment of adapter proteins through homotypic death domain (DD) interactions (Anees et al., 2015), triggering pro-apoptotic signaling (Figure 3). On the other side of the spectrum, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and OPG receptors cannot induce an apoptotic signal due to the lack of functional DD and, therefore, are called decoy

receptors or TRAIL receptor antagonists. DcR1 has no intracellular domain and is anchored to the membrane through a GPI anchor, while DcR2 has a truncated and non-functional DD. OPG is a soluble protein that has a low affinity for TRAIL (Figure 3) and seems to be more involved in the activation of NF-kB (Nuclear Factor-kB) signaling (Anees et al., 2015). Unlike DR4 and DR5, which are usually expressed in tumor tissues (Ganten et al., 2009; Omran and Ata, 2014), antagonist receptors are present in normal tissues, only occasionally expressed in tumor cells (Daniels et al., 2005) .

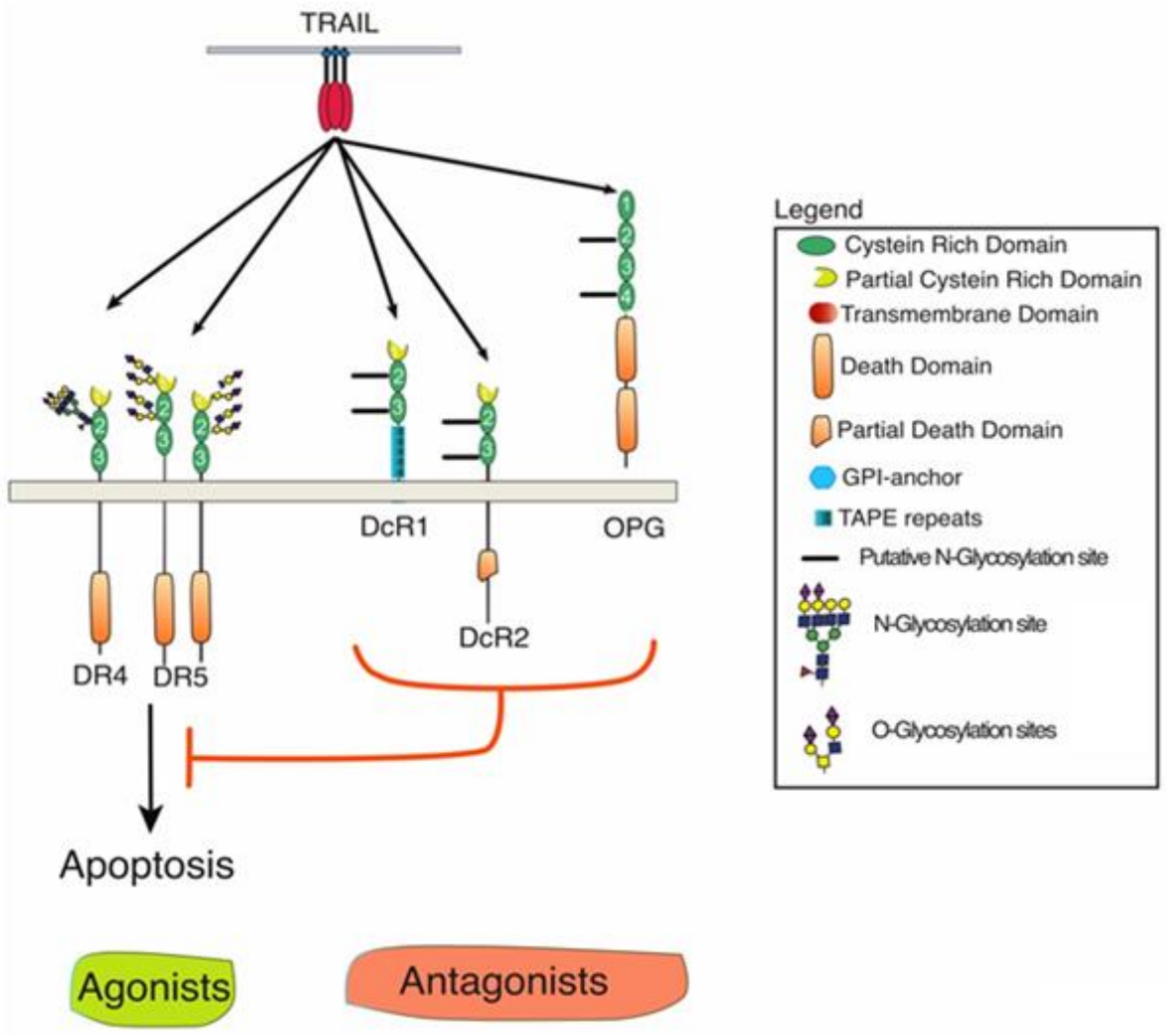


Figure 3: Schematic representation of TRAIL and its receptors. TRAIL and its agonist (DR4 and DR5) or antagonist (DcR1, Dcr2, or OPG) receptors are membrane-bound glycoproteins of the TNF superfamily. DR stands for Death Receptor, DcR for Decoy Receptor. Specific domains of putative and described O- and N-glycosylation sites are depicted in the legend. **Source:** (Micheau, 2018).

The DD protein superfamily includes about 100 members belonging to four subfamilies: death domain (DD), caspase recruitment domain (CARD), pyrin domain (PYD), and death effector domain (DED). Around 30 DD-containing proteins have been identified; however, only a small number of them, like FADD, TRADD, RIPK1, and EDARADD, have been identified to be consistently recruited to interact with members of the DR family (Sessler et al., 2013).

Adapter protein Fas-associated with the death domain (FADD; Figure 4) presents a C-terminal DD that interacts with DRs, and an N-terminal DED, which activates and recruits other DED-containing proteins (Bodmer et al., 2000). The recruitment of FADD allows the binding of the primary caspases, caspase-8 and 10, resulting in the formation of the death-inducing signaling complex (DISC) (Scott et al., 2009). Once grouped in the DISC, the interaction between two procaspases-8 leads to the dimerization of their catalytic C-terminal domains and the prodomain autoproteolysis in the aspartic acid residues 216, 374, and 384. The active caspase-8 dimer (p18-p10) is then released in the cytosol, cleaving effector caspases, thus, starting the caspase cascade recruitment (Hengartner, 2000). For caspase-10, the prodomain autoproteolysis interferes with aspartic acid residues 219 and 415 (Wang et al. 2001), which generates an active dimer of caspase-10 (p23-p12). Caspases-3, -6, and -7 are then dimerized, triggering the apoptotic process (Nuñez et al., 1998; Dickens et al., 2012). According to the activation level, caspase-8 can stimulate the intrinsic pathway, amplifying caspase activation through the mitochondria (Elmallah and Micheau, 2015; Li et al., 1998).

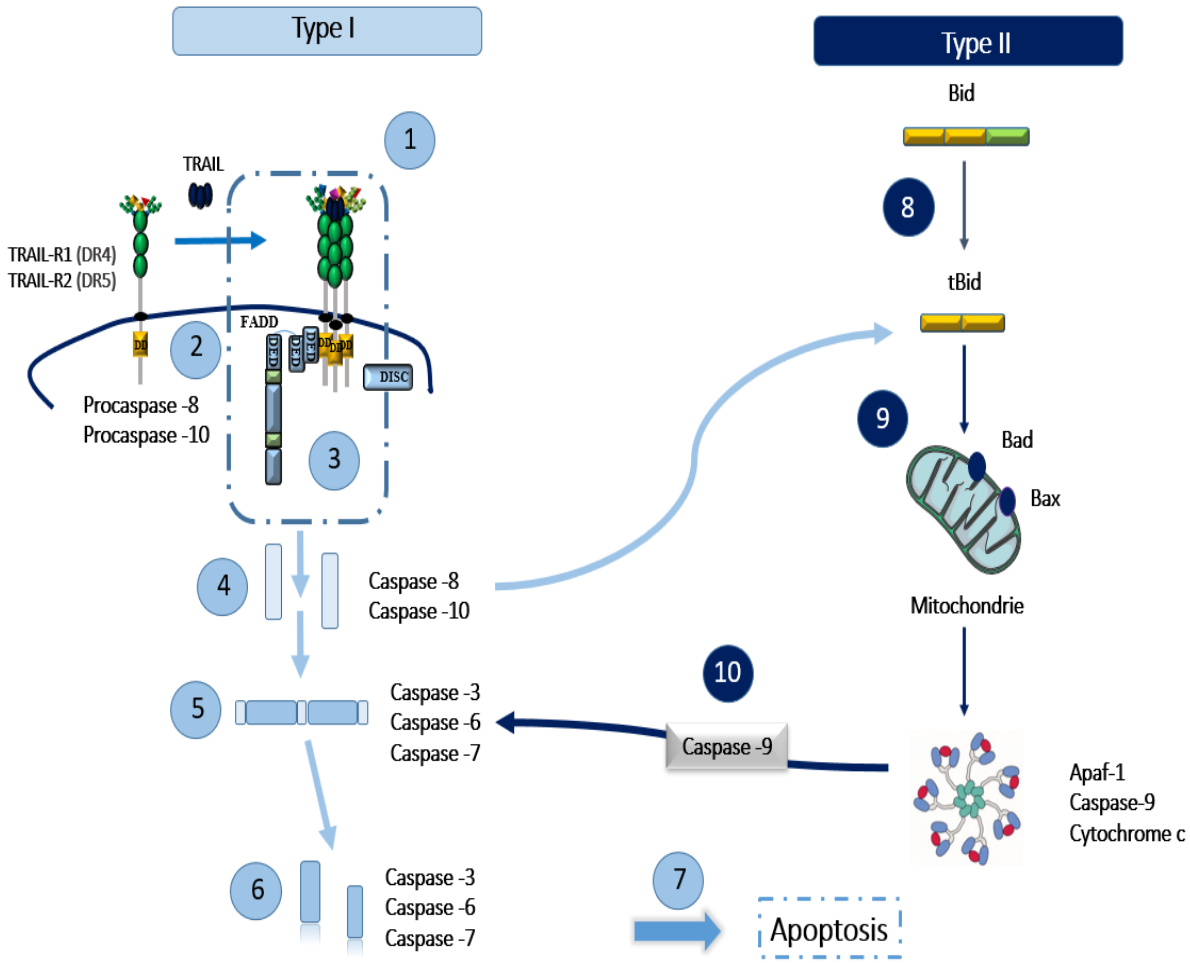


Figure 4: Type I and II apoptotic pathways. Binding of TRAIL to its agonistic receptors (DR4 and/or DR5) leads to its oligomerization (1) and subsequent recruitment of the FADD cytosolic adapter protein through DD homotypic interactions (2). Type I. FADD, in turn, allows the recruitment of initial pro-caspase-8/-10 through interactions with DED, leading to the formation of the so-called death-inducing signaling complex (3). In DISC, chains of caspase-8/-10 (4) are assembled, allowing the self-processing of caspase-8/10 and the release of its active fragments in the cytosol (5), where they activate, by proteolytic cleavage, effector caspases (6) to execute the apoptotic program (7). Type II Signal amplification via the intrinsic pathway is sometimes necessary when caspase-8 is not sufficiently activated. In this scenario, mitochondrial activation is induced by caspase-8/-10 through cleavage of the BH3 Bcl-2 family protein, Bid (8), whose truncated version (tBid) can translocate to mitochondria and induce an alteration in its outer membrane permeability through Bak and Bax interactions (9), allowing the release of cytochrome c, activation of caspase-9, another caspase initiator capable of activating caspase-3/-7 performer (10). **Source:** Adapted from (Elmallah and Micheau, 2015).

1.3.1. APOPTOSIS INTRINSIC PATHWAY ACTIVATION BY TRAIL

Cells sometimes require additional signal amplification through the mitochondrial pathway in order to undergo TRAIL-induced apoptosis. This phenomenon initially described for the Fas signaling pathway (Scaffidi et al., 1998) led to the classification of cells into two types, namely, type-I and type-II cells. In type-I cells, caspase-8 is activated and processed in the DISC in amounts sufficient to activate effector caspases and thus promote apoptosis directly. In contrast, in type II cells, the amounts of active caspase-8 generated in the DISC are limited. Therefore, cells initiate an amplification loop for further transduction of the apoptosis signal by the cleavage of the Bid protein (BH3 interacting domain death agonist), a member of the family Bcl-2 (Elmallah and Micheau, 2015) (Figure 4).

The truncated form of Bid protein (tBid) has a robust pro-apoptotic activity. It is found extensively on the outer mitochondrial membrane and can bind and inhibit Bcl-2 anti-apoptotic proteins (Li et al., 2008; Luo et al., 1998). All members of the Bcl-2 family are characterized by the presence of 1 to 4 conserved domains called the BH (Homology Bcl-2), numbered 1 to 4. These proteins can exert pro- or anti-apoptotic activity and are divided into three classes (Adams and Cory, 2018). The BH3-only family consists of eight members of apoptosis initiating proteins: BID (BH3-interacting domain death agonist), BAD (Bcl-2 antagonist of cell death), BIM (Bcl-2 interacting mediator of cell death), BIK (Bcl -2 interacting killer), BMF (Bcl-2-modifying factor), NOXA, PUMA (Bcl-2-modifying factor), and HRK (Harakiri), all of which promote apoptosis when overexpressed (Warren et al., 2019; Yip and Reed, 2008). The family of effector proteins includes Bax (Bcl-2 associated x protein), Bak (Bcl-2 antagonist killer 1), and Bok (Bcl-2 related ovarian killer).

When activated by tBid, effector proteins promote the mitochondrial outer membrane permeabilization (MOMP), resulting in the release of crucial pro-apoptotic proteins, such as cytochrome c. Cytochrome c induces the formation of a multiprotein complex, similar to DISC in function, which is the platform for the activation of caspase in the intrinsic apoptotic pathway (Roufayel, 2016). This complex, known as apoptosome, is composed of cytochrome c, factor 1 protease activator (Apaf-1), dATP, and pro-caspase-9 (Garrido et al., 2006). In the apoptosome, the apical caspase of the cascade of caspases (pro-caspase-9) becomes activated, leading to the onset of running caspases, pro-caspases-3, -6, and -7, which are essential to complete apoptosis (McIlwain et al., 2013).

The members of the anti-apoptotic Bcl-2 family, Bcl-xL (B-cell lymphoma, long isoform), Bcl-W (Bcl-2 like 2), Mcl1 (Myeloid cell leukemia 1), and A1/BFL-1 (Bcl-2-related protein A1), have four BH domains and similar three-dimensional structures (Strasser, 2005). The BH1, BH2, and BH3 domains interact to form a hydrophobic groove (Yin et al., 1994), allowing the bind of pro-apoptotic partners (Adams and Cory, 2007; Giam et al., 2008). Overexpression of any of these proteins results in resistance to multiple apoptotic stimuli showing some redundancy between them; it is unlikely that under physiological conditions, only one of these proteins is responsible for the survival of a population of cells (Delbridge and Strasser, 2015).

Like the Bcl-2 anti-apoptotic family, the IAPs (apoptosis protein inhibitors) are cell death regulators that, among other functions, bind caspases and interfere with apoptotic signaling through death receptors or intrinsic cell death pathways (Obexer and Ausserlechner, 2014). All IAPs share from one to three common structures, the so-called IAP baculovirus (BIR) repeating domains, which allow them to bind to caspases and other proteins. XIAP (X-linked inhibitor of apoptosis protein) is the most potent and best-defined member of the anti-apoptotic IAP family that directly neutralizes caspase-9 through its BIR3 domain and effector caspases-3 and -7 through its BIR2 domain (Galbán and Duckett, 2010). SMAC/Diablo, a natural XIAP inhibitor released by mitochondria in apoptotic cells, displaces bound caspases from XIAP domains BIR2/BIR3, thus reactivating the execution of cell death (Obexer and Ausserlechner, 2014).

1.3.2 TRAIL REGULATION MECHANISMS

Given TRAIL's ability to induce cell death, it is clear that this signaling must be precisely regulated to maintain the organism's homeostasis. Mutations found at domains such as DD (McDonald et al., 2001) or the ligand-binding site (Fisher et al., 2001) were observed in breast, head and neck cancers, and non-Hodgkin's lymphoma. All of them correlated with loss of receptor functionality (Bin et al., 2006; Lee et al., 2001). Interruptions in membrane traffic and the lack of exposition of receptors in the cell surface are also possible resistance mechanisms. Other studies reported that in breast cancer models, the receptor's internalization was the reason for TRAIL resistance. The blockade of clathrin-mediated endocytosis reversed the sensitivity to apoptosis measured by TRAIL (Austin et al., 2006; Zhang et al., 2009).

Additionally, the overexpression of decoy receptors, DcR1, or DcR2, leads to

apoptosis inhibition (LeBlanc and Ashkenazi, 2003; Pan et al., 1997a). TRAIL receptor antagonists are located at membrane sphingolipids and cholesterol-enriched microdomains, known as lipid rafts. Within lipid rafts, DcR1 "kidnap" TRAIL and compete with DR4 and DR5 for ligand binding. DcR2 receptor acts in a more complex way as it inhibits the activation of caspase-8 without preventing the formation of DISC. Merino and colleagues demonstrated that DcR2 ectopic expression inhibited TRAIL-induced apoptosis by forming a heterocomplex with DR5, leading to inhibition of caspase-8 activation at the DISC (Merino et al., 2006).

Some evidence revealed that the overexpression of the anti-apoptotic protein c-FLIP (cellular inhibitory protein similar to FLICE) induces TRAIL resistance (Dolcet et al., 2005; Geserick et al., 2008). Three isoforms of c-FLIP expressed in humans have been described, a long variant, c-FLIP$_L$, and two short isoforms, c-FLIP$_R$, first isolated from the human Burkitt B-cell line of Raji lymphoma, and c-FLIP$_S$ (Djerbi et al., 2001; Golks et al., 2005; Irmler et al., 1997). c-FLIP is homologous to caspases and can prevent DISC formation (Irmler et al., 1997). Like c-FLIP, changes in the expression of Bcl-2 or XIAP members have often been observed in many types of cancer (Fulda et al., 2002).

Variations in the expression of Bcl-2-family proteins are found in several types of cancer. Bcl-2, Bcl-XL, or Mcl-1 overexpression prevents TRAIL-induced apoptosis in several tumor types, including prostate, breast, lung, and pancreatic tumor cells (Fulda and Debatin, 2004; Hari et al., 2015; Takahashi et al., 2013). In contrast, TRAIL-induced apoptosis can be impaired in HCT116 colorectal cancer cells deficient in pro-apoptotic proteins such as Bax (Deng et al., 2002; Gillissen et al., 2010). The Bax deficiency does not affect TRAIL-induced caspase-8 activation and subsequent Bid cleavage. However, it leads to partial processing of caspase-3 due to XIAP inhibition. The release of Smac/DIABLO from mitochondria through TRAIL-caspase-8-tBid-Bax cascade is essential to remove the XIAP inhibitory effect and allow apoptosis to continue (Deng et al., 2002). Besides, a Bax deficiency may be responsible for inefficiency in cytochrome c release after TRAIL stimulation, as demonstrated in Bax depleted embryonic mouse fibroblasts (Sinicrope et al., 2004).

1.4. TARGETING TRAIL TO TUMOR CELLS

In the past few decades, TRAIL has sparked a growing interest in oncology because of its ability to induce tumor cell death while sparing normal cells selectively. Thanks to this peculiarity, many recombinant molecules have been generated to optimize TRAIL properties as an effective antitumoral molecule.

Early versions of recombinant TRAIL included an exogenous sequence called *tag*, essential to ligands' oligomerization and activity. Different *tags* included polyhistidine tails (His-TRAIL) (Pitti et al., 1996), leucine zipper residues (LZ-TRAIL) (Rozanov et al., 2009; Walczak et al., 1997), isoleucine zipper (IZ-TRAIL) (Ganten et al., 2006), and FLAG (Schneider, 2000a). Nonetheless, these variants exhibited toxicity, especially for human liver and brain cells (Ganten et al., 2006; Nitsch et al., 2000), probably by the high degree of oligomerization of these ligands. Another possible explanation is the amount of zinc in the preparations, which affects the adequate TRAIL oligomerization (Hymowitz et al., 2000; Lawrence et al., 2001).

To overcome the presence of *tags*, an "unmarked" version of recombinant human TRAIL (rh-TRAIL), called dulanermin, was selected for clinical investigation (Herbst et al., 2010; Yee et al., 2007). Preliminary results showed patients good tolerance, with mild side effects. However, as the rh-TRAIL half-life is very short, around 30 minutes (Wu et al., 2001), it rarely leads to complete tumor eradication (Valldorf et al., 2016).

New generations of agonistic antibodies from TRAIL receptors are being evaluated, such as the APG350, which showed an extended half-life in mice and monkeys, showing promising activity in pancreatic cancer xenograft models (Gieffers et al., 2013). Another promising antibody is MEDI3039, a potent DR5 agonist in breast cancer cells in vitro and in vivo, but its effectiveness is not yet in clinical trials (Greer et al., 2019).

Conventional chemotherapy uses several compounds, classified according to their function: alkylating agents, antimetabolites, intercalating agents, antimitotics, or topoisomerase inhibitors, as presented in Table 2. A treatment modality that combines two or more therapeutic agents is a cornerstone of cancer therapy. It enhances efficacy compared to monotherapy because it targets key pathways in a synergistic or additive manner (Mokhtari et al., 2017). This approach potentially reduces drug resistance while simultaneously providing therapeutic anti-cancer benefits, such as reducing tumor growth and metastatic potential, arresting mitotically active cells, and inducing apoptosis (Mierzwa et al., 2010).

Table 2: Chemotherapeutic compounds classified according to their function.

Agents	Action	Compound
Alkylating	Create covalent bonds at nucleic acids, impairing DNA replication and transcription.	Cisplatin Oxaliplatin Cyclophosphamide
Topoisomerase inhibitors	Prevents topoisomerase action, inducing DNA breakdown	Etoposide Irinotecan
Intercalating	Prevents DNA replication and transcription	Doxorubicin Actinomycin-D
Antimitotic	Interrupts the mitotic spindle	Paclitaxel/vincristine
Anti-metabolites	Inhibits incorporation of folic acid Inhibits thymidylate synthetase	Methotrexate FluoroUracil (5FU)

Source: The author

5-FU is an aromatic heterocyclic organic compound with a structure similar to that of the DNA (thymine) and RNA (uracil) pyrimidine molecules. It is a uracil analog with a fluorine atom at the C-5 position in place of hydrogen (RUTMAN et al., 1954) (Figure 5). Based on its structure, 5FU can bind to both RNA and DNA, interfering with nucleosides' metabolic synthesis, causing cytotoxicity and cell death. In mammalian cells, 5FU is converted into several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), acts inhibiting the action of thymidylate synthase (TS), fluorodeoxyuridine triphosphate (FdUTP), is incorrectly incorporated into DNA, and fluorouridine triphosphate (FUTP), that interrupt RNA synthesis (FUTP) (Wyatt and Wilson, 2009).

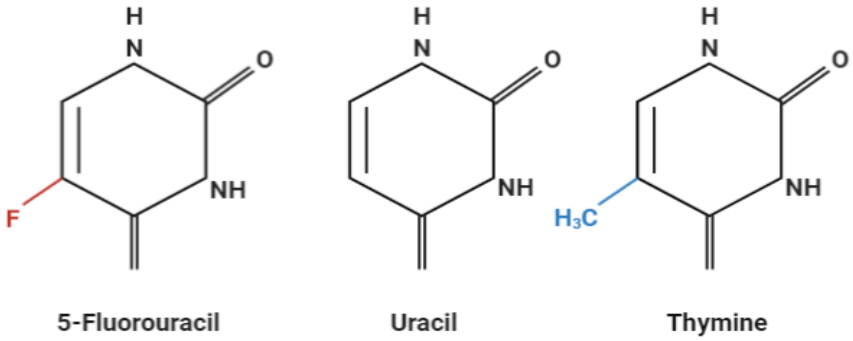


Figure 5: Chemical Structure of 5-FU, Uracil, and Thymine. **Source:** Adapted from (Van Laar et al., 1998).

The use of chemotherapeutic agents combined with TRAIL has proved to increases apoptosis activation *in vitro* and *in vivo* (Anan and Gores, 2005; Ciuleanu et al., 2016). The combined use of fluorouracil (5FU), cisplatin, and etoposide restored tumor cells' sensitivity to TRAIL-induced apoptosis. Treatment with 5FU increases the expression of DR4 and DR5, improves the recruitment of proteins of the DISC complex, and reduces c-Flip protein level (Ganten et al., 2004; Kondo et al., 2006; Morizot et al., 2011). In some cases, previous treatment with 5FU influenced the reduced expression of anti-apoptotic proteins that modulate the intrinsic and/or extrinsic pathways of apoptosis, such as Bcl-2, Bcl-xL, c-FLIP, or XIAP (Mühlethaler-Mottet et al., 2004; Nazim et al., 2017; Song et al., 2003). A study in primary cells showed that a treatment that combines 5FU and TRAIL does not exert toxicity in healthy hepatocytes (Meurette et al., 2006).

Specific agonist antibodies against DR4 or DR5 have been generated and some of them effectively induced apoptosis in a wide range of tumor cell lines, with no toxicity to normal cells (Lin et al., 2003; Pukac et al., 2005), either as a single agent or in combination with chemotherapeutic drugs. Apomab, TRA-8, and LBY135 showed inhibition of tumor cell growth in xenograft mice models (Adams et al., 2008; Lin et al., 2003). TRA-8 also induced cell death in primary human cervical and ovarian cancer (Estes et al., 2007; Kendrick et al., 2008). DS-8273a was tested in 16 advanced cancer patients enrolled in a phase I study. Treatment with DS-8273a resulted in a reduction of MDSCs (Myeloid-derived suppressor cells) without affecting myeloid or mature lymphoid cells (Dominguez et al., 2017). In leukemic and lymphoma cells, bortezomib was combined in vitro with HGSETR1 and HGS-ETR2, which resulted in synergistic effects on cell death (Georgakis et al., 2005; Smith et al., 2007). Bortezomib also sensitized a panel of non-small-cell lung carcinoma (NSCLC) cells to HGS-ETR1 and

HGS-ETR2, while cells were resistant to treatment with the antibodies alone (Luster et al., 2009). In primary cancer cells, doxorubicin has been shown to sensitize lymphoma cells to HGS-ETR1 and HGS-ETR2 *in vitro*, and breast cancer cells to TRA-8 *in vitro* as well as *in vivo*. However, despite promising results from tumor models in mice, the TRAIL death receptor targeting failed in clinical studies, showing no satisfactory antitumor efficacy. These disappointing results can be explained mainly by poor pharmacokinetics and the development of death receptor-induced apoptosis resistance (Brin et al., 2018; Ciuleanu et al., 2016; Dominguez et al., 2017; Wajant, 2019)

1.5. PROTEIN GLYCOSYLATION

Glycosylation is one of the most important post-translational modification processes determining the final protein structure and function. Unlike nucleic acids and polypeptides, oligosaccharide chains are often mounted on nonlinear branched structures by glycosyltransferases and glycosidases using specific sugar-donor substrates (Gabius et al., 2011; Reis et al., 2010). This process varies among species and seems to differ significantly among cell types (Brutschin and Brutschin, 2016). Carbohydrate units on the cellular surfaces are the first layer of interaction between cells and the extracellular matrix components (Taylor et al., 2015), and in general, carbohydrate residues act as signal molecules for the recognition, adhesion, immune response, cellular differentiation, and metastasis (Cohen, 2015; Li et al., 2008; Wu et al., 2007).

Oligosaccharides can form two types of glycosidic bonds with proteins: N-glycosylation and O-glycosylation (Potapenko et al., 2015). In N-glycosylation, an oligosaccharide chain is linked to the hydroxyl group of the asparagine side chain contained in the consensus sequence Asn-X-Ser/Thr, due to an N-glycosylamine bond. In rare cases, the sequence Asn-X-Cysteine is also used (Munkley et al., 2016; Potapenko et al., 2015). It is governed by a series of steps inside the endoplasmic reticulum (ER) and Golgi complex (GC). The first stage occurs in the ER membrane by incorporating an oligosaccharide nucleus, composed of three glucose (Glc), nine mannose (Man), and two N-acetyl glucosamine (GlcNAc) residues (Freeze and Aebi, 2005), into the protein-specific amino acid residue. The second stage involves a reorganization starting from the removal of Glc and Man residues followed by the

incorporation of other saccharides, depending on the protein's function (Pinho and Reis, 2015).

O-linked glycosylation is very common in secreted or plasma membrane glycoproteins (Reis et al., 2010). The first step is transferring the uridine diphosphate N-acetylgalactosamine (UDP-GalNAc) to the serine or threonine residues, catalyzed by enzymes of the UDP-N-acetylgalactosaminyl transferases (ppGalNAc-Ts) family. PpGalNAc-Ts control the first level of complexity of glycosylation, that is, O-glycans' locations and density. The second level of O-glycosylation complexity highlights the lengthening of the carbohydrate chains by other glycosyltransferases. After the first residue of N-acetylgalactosamine (GalNAc) is added, a galactose residue (Gal) is added. These carbohydrate residues can then be sialyzed by sialyltransferases, forming sialic acid antigens (Pinho and Reis, 2015) (Reily et al., 2019).

1.5.2 DR4 AND DR5 Glycosylation

In 2007, Wagner and colleagues published the first article correlating TRAIL-DISC's arrangement with the glycosylation of TRAIL receptors (Figure 9). They demonstrated that cellular sensitivity to TRAIL is closely associated with elevated levels of N-acetylgalactosamine transferases (GALNT14), increasing the apoptotic potential of the DR5 receptor. It was found that inhibition of O-glycosyltransferase by siRNA impaired tumor cells' death after stimulation with TRAIL. In this same study, the authors performed mutagenesis, changing serines and threonines in alanine residues that prevented DR5 O-glycosylation and limited the apoptotic signal transduction capacity (Wagner et al., 2007).

In another study, Moriwaki and colleagues described that DR5 O-glycosylation is necessary to better aggregation and activation of the DISC complex. Besides, the deficiency of GDP-mannose-4,6-dehydratase (GMDS), a fundamental enzyme for fucosylation, can lead to TRAIL resistance in colorectal carcinoma cells (Moriwaki et al., 2011). Despite the evidence showing that O-glycosylation is necessary for the activation of apoptosis in tumor cells via the DR5 receptor, little data describe whether glycosylation is involved in regulating DR4-induced apoptosis.

Our team recently demonstrated that the DR4 receptor is N-glycosylated and that this post-translational modification increases the receptor's ability to trigger apoptosis. Cells with mutations in N-glycosylation alter the distribution of DR4 and/or its arrangement on the cell surface and reduce apoptosis response through TRAIL

stimulation. The fact that DR4 is N-glycosylated while DR5 is O-glycosylated may provide potential clues for the differential promotion of apoptosis by these two receptors in some types of tumor cells (Figure 9) (Dufour et al., 2017b). Also, proteins may undergo additional modifications, including sialylation, fucosylation, or terminal glycan branching. Recent studies have reported that terminal modifications have the potential to affect TRAIL receptor pro-apoptotic signaling (Moriwaki et al., 2011).

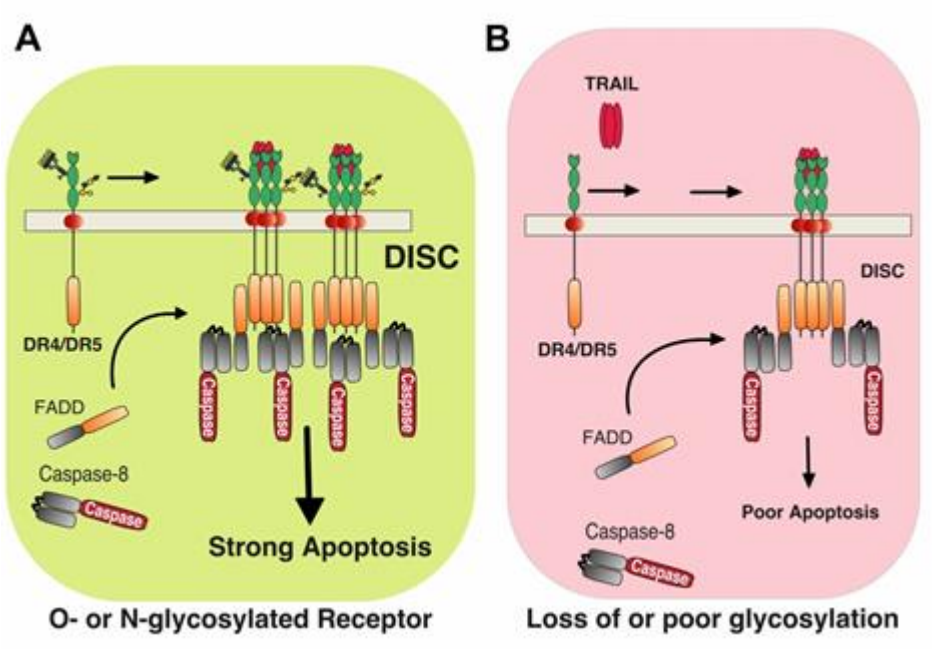


Figure 6: Schematic representation of TRAIL-induced DISC formation. TRAIL-induced apoptosis via DR4 and DR5 in tumor cells is closely associated with the glycosylation of their receptors. **(A**) The stimulation of DR4 or DR5 glycosylated by TRAIL induces the recruitment of the adapter protein FADD and caspase-8, thus forming the so-called TRAIL DISC (Death-Inducing Signaling Complex), where caspase-8 is processed, allowing the triggering of apoptosis. Carbohydrate transferases, including N-acetylgalactosaminyl, fucosyl- or sialyltransferases, as well as galectins, could act directly at the receptor level to regulate the formation and activation of TRAIL DISC. **(B)** In cells that exhibit low N-acetylgalactosaminyltransferase activity or express a non-glycosylated receptor, the binding of TRAIL to the receptors is not altered. However, DISC's formation and the processing of caspase-8 are restricted, impairing the ability of TRAIL to trigger. **Source:** (Micheau, 2018)**.**

1.6. Lectins

In nature, there is a diversity of glycan-binding proteins (GBPs) that selectively recognize specific carbohydrates, being responsible for diverse physiological and pathological processes (Mulloy and Linhardt, 2001; Taylor et al., 2015). Lectins are GBPs of non-immunological origin, without catalytic capacity, which presents two or more binding sites capable of interacting, reversibly, with carbohydrates, precipitating glycoconjugates and agglutinating cells (Ohtsubo and Marth, 2006; Sharon and Lis, 2004).

They are vastly distributed, found in viruses, bacteria, fungi, plants, and animals. They can be used, for example, as tools in the detection, isolation, and characterization of glycoconjugates involved in cellular processes, identifying changes on the cell surface during physiological and pathological events (Reily et al., 2019).

Lectins bind to their ligands through weak bonds such as hydrogen bridges, Van der Walls interaction, and hydrophobic interactions (del Carmen Fernandez-Alonso et al., 2013). The amino acid sequence analysis involved in the protein-carbohydrate recognition showed a polypeptide segment called the carbohydrate recognition domain or CRD (Park and Baenziger, 2004). Through this domain, lectins can be classified according to their structure and function (Loh et al., 2017; Vasta et al., 2011) (Table 3).

Table 3: Animal Lectins based on the structure of the carbohydrate-recognition domain (CRD)

Lectins	Specificity	Location	Role
S-type Lectins (galectins)	β-Galactosides	Cytoplasm/Extracellular membrane	Cell adhesion, cell migration, growth regulation, immune responses, apoptosis
C-type Lectins (Calcium-dependent)	(Mannose, Galactose, Fucose)	Extracellular, transmembrane	Recognition of pathogens, endocytosis, adhesion, immune-modulation
I-type	Sialic acid	Membrane	Regulation of myeloid cell inter-action, differentiation of myeloid cells, adhesion signaling
F-type	L-Fucose	Extracellular	Innate immunity
L-type	High-mannose Glycan	Intracellular, Golgi, Endoplasmic reticulum, extracellular membrane	Protein sorting, assisting secretion of specific glycoproteins
P-type	Man-6-P	Cytoplasm/Extracellular membrane	Endocytosis, intracellular targeting of lysosomal enzymes (lysosome biogenesis)
R-type	Mannose, Galactose,	Cytoplasm/Extracellular membrane	Glycoprotein homeostasis
Pentraxins	α-β/Galectosides	Cytoplasm/Extracellular membrane	Innate immunity
Tachylectin	GlcNAc MurNAc	Membrane	Innate immunity

Source: Adapted from (Loh et al., 2017; Vasta et al., 2011) .

1.6.1 GALECTINS

Type S-type lectin or galectin family have highly conserved amino acid sequences (Barondes et al., 1994), with binding affinity for galactosides. Members of this family are widely distributed in nature, present in sponges, fungi, nematodes, insects, and vertebrates (Barondes et al., 1994; Leffler et al., 2002). Galectins remained conserved during evolution, and the presence of several galectins in a single species may indicate they diverged to specifically perform a variety of functions (Cooper, 2002).

They are often found in the cytoplasm, but depending on cell type and proliferative state, significant amounts of this protein can be detected in the nucleus and cell surface. Secreted galectins can interact with surface glycosylated partners (Brinchmann et al., 2018; Hughes, 2001), translating information encoded by glycans into immune cell activation, differentiation, and homeostasis (Rabinovich et al., 2007). Additionally, galectins may interact with intracellular ligands contributing to mRNA processing, immune regulation, and inflammation (Ilarregui et al., 2005).

The interactions with glycans can be highly complex because of the galectins multivalence and oligomerization and the multivalence of the ligands. They are then classified into three categories based on structural and binding profiles (Figure 10). Prototype galectins contain a single CRD and form non-covalent homodimers. Chimeric, have a CRD and an amino-terminal domain rich in proline, glycine, and tyrosine residues, which is sensitive to metalloproteinases and contributes to the oligomerization of these lectins. Tandem-Repeat galectins are unique polypeptides composed of two distinct CRDs connected by a peptide linker of 5 to 50 amino acid residues (Kamili et al., 2016).

To date, 15 galectins have been characterized, 11 of which are found in humans (Gitt et al., 1995; Sakthivel et al., 2015). Galectin-5 and -6 are expressed only in rats and mice, respectively, while galectins-11 and -15 are exclusively found in ruminants. Galectin transcripts may generate different isoforms. For example, at least seven different mRNAs have been identified for human galectin-8, differently expressed depending on the tissue (Bidon-Wagner and Le Pennec, 2002). It is the same for galectin-9, with three isoforms already identified (Demers et al., 2005).

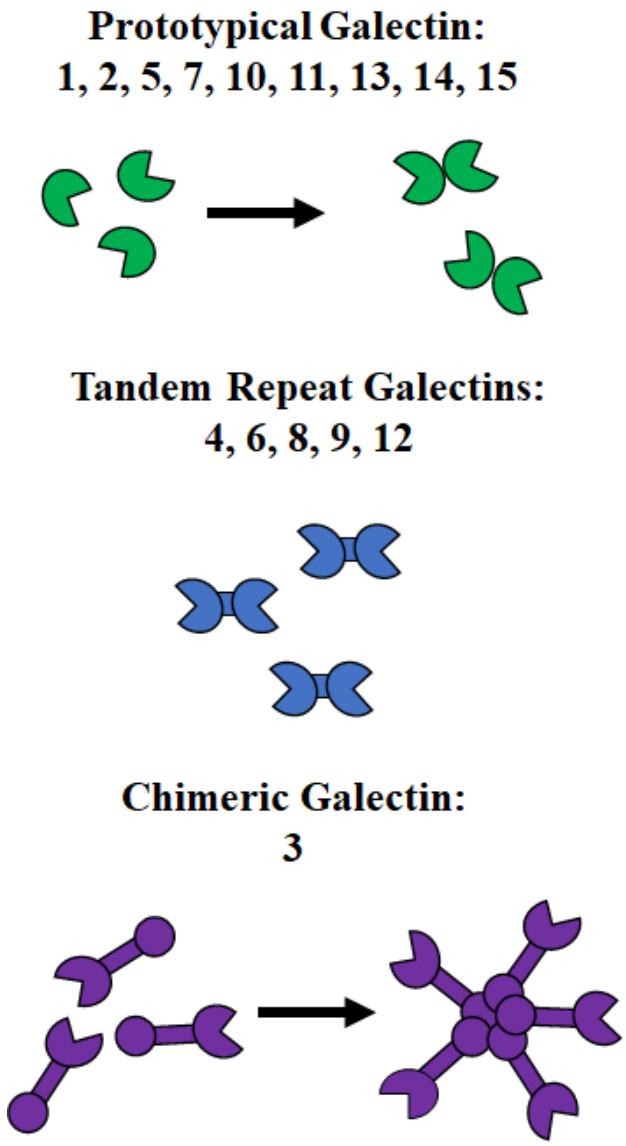


Figure 7: The three subtypes of galectins. Prototypical galectins have only one CRD, while the tandem repeat galectins have two CDRs. Galectin-3 is the only chimeric galectin found in mammals. **Source:** Adapted from (Boscher et al, 2011).

1.6.3. GALECTIN AND CANCER

Increasing evidence points out the roles of glycosylation in tumor progression stages. As tumor cells progress, the composition of glycans may change in parallel with changes in cell metabolism. This phenomenon includes incomplete synthesis and neosynthesis, which refers to abnormal glycosylation patterns (Kannagi et al., 2008).

Some tumors, such as melanoma, prostate, breast cancer, and ovarian cancer, show overexpression of galectins, which correlate with the stage of malignancy, aggressiveness, or metastatic potential (Blidner et al., 2015). For example, in endometrial tumors, galectin-1 was overexpressed compared to non-tumor endometrial cells, while silencing galectin-1 reduced tumor proliferation (Griffioen and Thijssen, 2014). In murine lung cancer, the autocrine secretion of galectin-1 caused tumor progression (Kuo et al., 2012). In human bladder cancer, increased expression of galectin-1 mRNA was correlated with tumor histological grade and advanced clinical stage. In this same study, the effects of galectin-3 were evaluated, with no correlation with tumor progression (Cindolo et al., 1999). Another study reported the activity of galectin-3 in tumor cells, depending on its location. Nuclear galectin-3 was correlated with antitumor responses (Califice et al., 2004); in contrast, the strong cytoplasmic expression of gal-3 was associated with tumor aggressiveness (Wang et al., 2018). Galectin-8 demonstrates different abilities to modulate migration, mainly in glioblastoma and colon cancer cells (Nagy et al., 2002). A similar scenario exists for galectin-9, which increases the adhesion of melanoma, colon cancer, in contrast, reduces the adhesion of melanoma cells, breast cancer, and the formation of colon cancer cell metastases (Hirashima et al., 2008; Irie et al., 2005).

Many glycans, glycosyltransferases, and glycosidases play critical roles in programmed cell death, preventing ligand-receptor interactions, which influence the formation of signaling complexes and modulating ligand secretion (Lichtenstein and Rabinovich, 2013; Wagner et al., 2007). TRAIL ligand promotes tumor cells' apoptosis through the DR5 death receptors, whose O-glycosylation status determines their sensitivity to the ligand (Wagner et al., 2007). In addition, N-glycosylation also plays an important regulatory role in DR4-mediated apoptosis, but not for DR5, which is devoid of N-glycans. In this context, defective apoptotic signaling by TRAIL receptors deficient in N-glycan was associated with less aggregation of the TRAIL receptor and reduced formation of DISC death-inducing signaling complex, but not with reduced TRAIL binding affinity (Dufour et al., 2017b). Gal-3 is, for example, capable of binding

directly to membrane receptors such as CD45 (a transmembrane phosphatase) to induce apoptosis in lymphomas of the type T. Other studies have also shown that galectin-3 can induce or inhibit TRAIL-induced apoptosis (Mazurek et al., 2007). Until now, the underlying molecular mechanisms have been associated with the regulation of signaling downstream of TRAIL DISC and not at the level of the receptor itself. The sensitivity of tumor cells to apoptosis induced by TRAIL and the resistance of healthy cells can be explained in part by the profound changes in glycosylation. Galectin-3 can also interact with the intracellular FasL receptor domain to induce apoptosis (Dumic et al., 2006; Kageshita et al., 2002). Since CD95L is part of the TNF receptor family, it is interesting to question whether some galectins can also interact with the death of domain receptors for TRAIL and, more particularly for DR4, since it is N-glycosylated.

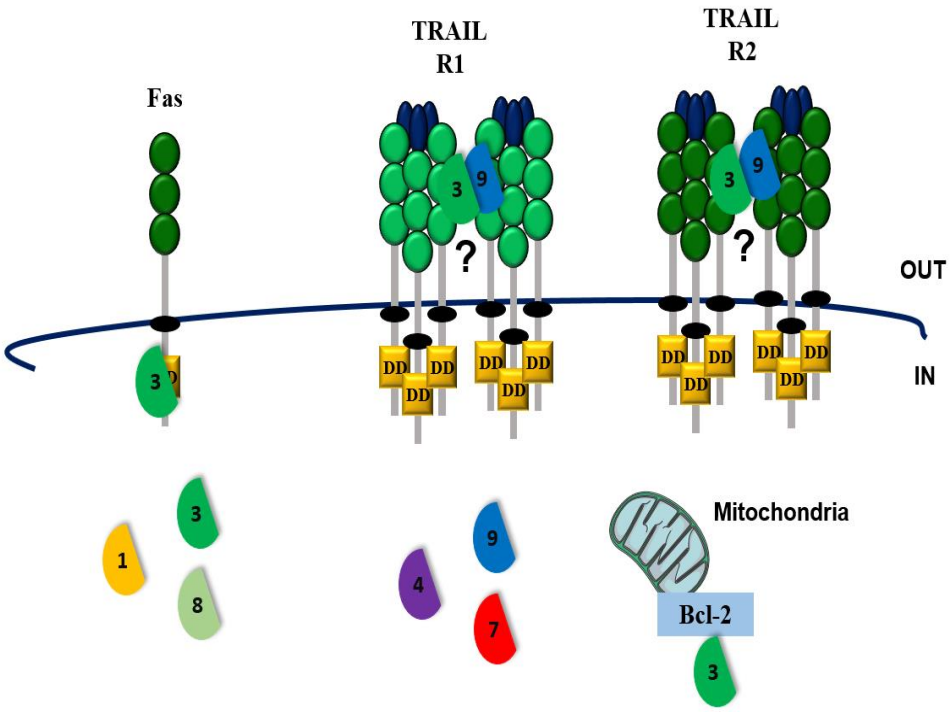


Figure 8: Interactions between galectins-3, -9, and TRAIL-R1/TRAIL-R2.

Source: The Author

2. JUSTIFICATION

Transduction of the apoptotic signal by death receptors involves different ligands of the TNF superfamily. Among these, TRAIL is the only real therapeutic interest since it effectively induces most tumor cells' death without destroying healthy ones. Therefore, it is valid to look for new strategies that potentialize TRAIL action and, mainly, reverse the resistance acquired by some tumor cells.

It is already known that some conventional chemotherapeutics sensitize tumor cells to apoptosis induced by TRAIL, but the mechanisms have not yet been completely elucidated. Considering that the importance of glycosylation for the proper functioning of the death receptors has already been described, we investigated whether chemotherapy could affect the receptors' glycosylation profile on the cell surface and stimulate their activation via galectins. The direct binding of galectin to glycosylated TRAIL receptors has not been widely studied; however, some evidence suggests that these interactions are eventually more constant and significant than expected.

3. GENERAL AIM

To investigate the interaction of galectin-3 and -9 with the extracellular domain of TRAIL receptors DR4 and DR5 and their contribution to TRAIL-induced apoptosis.

3.1. SPECIFICS AIMS

- To evaluate the ability of a conventional chemotherapeutic drug to sensitize tumor cells to TRAIL-induced apoptosis and to induce the release of galectin 3 and 9 to the extracellular medium;
- To analyze the modulation of TRAIL-induced apoptosis by galectin -3 and -9 expression;
- To analyze the modulation of TRAIL-induced apoptosis by exogenous galectin -3 and -9;
- To investigate the participation of galectin-3 and -9 as components of the TRAIL-DR4/5-DISC complex;

4. MATERIAL AND METHODS

4.1. CELL LINES AND VIRUSES

The human B Lymphoma cell line VAL and the triple-negative breast carcinoma cell line MDA-MB-231 were provided by Dr. Thierry Guillaudeux, Rennes (France), and Dr. Patrick Legembre, Rennes (France), respectively. HCT116 colon carcinoma cells were from the ATCC. TRAIL-receptor-deficient HCT116 and MDA-MB-231 (DKO) cells were generated using the TALEN approach as described by (Dufour et al., 2017b). HCT116 and MDA-MB-231 cells were cultured in DMEM medium (Dutscher, Brumath, France), supplemented with 10% fetal calf serum (Dutscher, Brumath, France). VAL cells were cultured in RPMI 1640 medium (Dutscher) containing ultraglutamine, 10% heat-inactivated calf serum (Dutscher, Brumath, France). Isogenic DKO (deficient for both DR4 and DR5) derivative MDA-MB-231 cells, reconstituted for DR4 or DR5 expression, and CRISPR/CAS9-mediated deletion of galectin-9 were grown in the presence or absence of puromycine (2.5 µg/mL) and blasticidin (10 µg/mL) from Fisher Scientific (Illkirch, France). Cells infected with the retroviral vector encoding full-length galectin-3 and -9 were either selected using puromycine as above or sorted by flow cytometry for GFP positivity. All these cell lines were grown in 5% CO2 at 37°C. Cells were stimulated or not with 5FU (1µg/mL) for 24 to 72h and treated with TRAIL (at the indicated concentration) for 6 to 24 hours before analysis (apoptosis, flow cytometry, Elisa, or Western blot). When indicated, recombinant galectins were added simultaneously with TRAIL.

4.1.2. CHEMICALS AND ANTIBODIES

For western blot analysis, the following antibodies were used. The anti-DR4 (Cat# AB16955) and anti-DR5 (Cat# AB16942) antibodies were purchased from Chemicon (Millipore, Molsheim, France). The anti-actin (C4) and anti-GAPDH (0411) antibodies were from Santa Cruz Biotechnology (Clinisciences, Montrouge, France). The anti-FADD (Cat# 610400) from Transduction Laboratories (BD biosciences, Le Pont de Claix, France). The anti-caspase-8 (clone 5F7) and anti-caspase-10 (clone 4C1) were from Medical & Biological Laboratories (Clinisciences, Nanterre, France). Anti-GAPDH (clone 0411) and anti-HSC70 (clone B-6) and Actin (clone I-9) antibodies were obtained from Santa Cruz Biotechnology (CliniSciences, Nanterre, France). The

mouse anti-galectin-9 (9S2-1) was from Biolegend (Ozyme, Saint-Cyr-l'Ecole, France). The rabbit anti-galectin-3 (Cat# 250503) and -9 (D9R4A) were from Abbiotec (CliniSciences, Nanterre, France) and Cell Signaling (Ozyme, Saint-Cyr-l'Ecole, France), respectively. Biotinylated lectins, *Aleuria Aurantia Lectin* (AAL, B-135), *Concanavalin A* (Con A, B-1005), *Maackia Amurensis* Lectin I (MAL I, B-1313), *Maackia Amurensis* Lectin II (MAL II, B-1265), Peanut *Agglutinin* (PNA, B-1155), *Ricinus Communis Agglutinin* I (RCA I, B-1085), *Sambucus Nigra Lectin* (SNA, B-1305), *Vicia Villosa Lectin* (VVL, B-1235), *Wheat Germ Agglutinin* (WGA, B-1025) were from Vector labs (CliniSciences, Nanterre, France). The lectin *Phaseolus vulgaris* (PHA-L, cat# L11270) was purchased from Thermo Fisher Scientific. Streptavidin-HRP (P0397) was from Dako (Les Ulis, France). Secondary antibodies: HRP-conjugated anti-rabbit was obtained from Jackson ImmunoResearch (Interchim, Montluçon, France), HRP-conjugated anti-mouse IgG1-, Ig2a- and Ig2b-specific antibodies were from Southern Biotech (Clinisciences, Nanterre, France). The following antibodies were used for Flow cytometry. Mouse anti-DR4 and anti-DR5 antibodies were from Covalab (Villeurbanne, France) (Dubuisson et al., 2019). Anti-galectin-9-APC (BLE348908) was from (Ozyme, Saint-Cyr-l'Ecole, France). *Phaseolus vulgaris* (red kidney bean) L-PHA- Alexa Fluor™ 488 Conjugate (10592893) was from Fisher Scientific. Other lectins are described above. The Alexa-488-coupled goat anti-mouse antibody was from Molecular Probes (Invitrogen, Cergy Pontoise, France). For apoptosis experiments, FITC-Annexin V (556420), PE-Annexin V (556422), APC-Annexin V (550475) and 7AAD (559925) were from (BD Biosciences, France). Puromycin (cat# ant-pr-1) was purchased from Invitrogen (Toulouse, France). Measurement of galectin-9 in the supernatants was performed using the following Elisa Kit (cat # SEA309Hu, Costar, Cambridge, MA, USA).

4.1.3. PLASMID CONSTRUCTIONS

pMIGR-Gal3 vectors (OM1329 and OM1330) were kindly provided by Dr. Heisterkamp (Children Hospital of Los Angeles, CA). pMIGR-Galectin-9 (OM1480) retroviral vector and pRSET-Galectin-9 were obtained from a pUC57 vector encoding the full-length sequence of LGALS9 (OM334, obtained from Genscript) digested with BamHI and EcoRI and subcloned into pMIGR (El Fajoui et al., 2011) or after a BglII-EcoRI restriction digest, to obtain pMIGR-Gal-9 (OM1480) and pREST-Galectin-9 (OM1361). pRSET-Galectin-3 (OM1360) was also obtained as above from a pUC57 vector encoding the full-length sequence of LGALS3 (OM333, obtained from Genscript). Retroviral vectors encoding full-length DR4, DR5, or DR4-156A (N-glyc-deficient) were described in (Dufour et al., 2017b). Lentiviral CRISPR vectors encoding gRNAs targeting LGALS9 were generated as described by (Ran et al., 2013). using pLenti-CRISPR V2 (Addgene 52961) and the following primers: 5'- CAC CGA GTC CAG CTG TCC CCT TTT C-3' and 5'- AAA CGA AAA GGG GAC AGC TGG ACT c-3' to generate OM 1221; 5'-CAC CGG AAC GGA AGC TGG GGG CCC GAG G -3' and 5'- AAA CCC TCG GGC CCC CAG CTT CCG TTC-3' to generate OM 1479. Sequences of all constructs were confirmed by sequencing.

4.1.4. RECOMBINANT PROTEIN PRODUCTION

His-hTRAIL, galectin-3, and galectin-9 were produced as described previously (Schneider, 2000). The insoluble fraction of galectin-3 and -9 was solubilized with guanidine (6M) for one hour at 30°C, the cell extracts were centrifuged at 27000 g and the supernatant was loaded onto a Nickel (Ni-NTA) column. Soluble galectins were eluted with glycine and the sample was neutralized by the addition of Tris-base (1M) prior to dialysis in PBS containing β-mercaptoethanol.

4.1.5 GENE DELETION OR DOWNREGULATION

For siRNAs experiments, HCT116 cells were transfected either with a GAL3 siRNA (L-010606-00-0005) or an ON-TARGETplus non-targeting pool D-001810-10-20 (Fisher Scientific, France) as follows. Cells ($5x10^5$) were added in each well of a 6-well plate and allowed to adhere overnight at 37°C. The following day, cells were transfected with indicated siRNA (1 nM) using INTERFERin, according to the provider's protocol (Polyplus, Strasbourg, France), and analyzed 72 h after transfection for expression by Western blotting and apoptosis. For CRISPR/CAS9 experiments, MDA-MB-231-DKO cells were infected with a lentiviral CRISPR vector encoding a gRNA encompassing the 5'UTR and the exon 1 or a gRNA located in exon 2 of LGALS9, OM1221 (pLenti-CRISPR-LGALS9-H4) or OM1479 (pLenti-CRISPR-LGALS9-G4), respectively. Briefly, Lenti-X™ 293T cells (Ozyme) were seeded in 10 cm2 dishes and co-transfected with 1 µg/mL pVSV-G, 10 µg/mL psPAX2 (Addgene #12260), and 10 µg/mL OM1221 or OM1479 using polyethyleneimine (PEI, Cliniscience) according to the manufacturer's instructions. Viral particles were generated as described previously (Morgenstern and Land, 1990) and cells were transduced for 16 h in 6-well plates in the presence of polybrene (Sigma-Aldrich, Lyon, France) (8 µg/mL). Cells were then washed in phosphate-buffered saline, harvested, plated in a complete medium containing puromycin (2.5 µg/mL), blasticidin (10 µg/mL), or both. Serial clonal dilutions or populations were next amplified for at least 14 to 20 days before the analysis of galectin-9-deficiency by PCR and Western blot.

4.1.6. EVALUATION OF APOPTOSIS BY ANNEXIN-V LABELING

Apoptosis was determined by detecting phosphatidylserine externalization after co-labeling with Annexin V-FITC and 7-AAD. Analyzes were performed using a flow cytometer (FACS Calibur, France). Apoptosis is presented as the percentage of positively staining cells compared to untreated cells as a control. Each experiment was performed independently at least three times. Briefly, $1x10^5$ cells were cultured in 24-well plates overnight in complete medium. The following day, cells were trypsinized, centrifuged at 1500 rpm for 5 min, washed in PBS (1X), centrifuged at 1500 rpm for 5 min, and Annexin-V and 7-AAD staining was performed according to the manufacturer's instructions. The acquisition included a minimum of 10,000 cells.

4.1.7. CHEMOTHERAPY AND TRAIL TREATMENTS

For sequential treatments, cells were cultured in 24-well plates ($1x10^5$) or 75 cm^2 flasks ($5x10^5$) overnight in complete medium. The next day, cells were treated or not with 5FU (1 µg/mL) in a medium containing 1% FBS for 24, 48, and 72 hours. After treatment with 5FU (Sigma-Aldrich, Lyon, France), his-TRAIL at concentrations of (200; 500 and 1000 ng/mL) was added or not for 6 h.

4.1.8 ANALYSIS OF TRAIL RECEPTOR EXPRESSION BY FACS

Cells were incubated with indicated antibodies or control mouse IgG1 at 10 µg/ml for 30 minutes at 4 °C, followed by goat anti-mouse secondary antibody Alexa-488 or Alexa-680 for 30 minutes at 4° C. Antibodies and conjugates were diluted in PBS (1X) containing 3% BSA. After each incubation, the cells were washed three times with PBS (1X). Surface staining was analyzed on a FACS calibur flow cytometer.

4.1.9. LECTIN EXPRESSION ANALYSIS ON THE CELL SURFACE BEFORE AND AFTER 5FU TREATMENTS

Cells were incubated with the indicated lectins at 10µg/ml for 30 minutes at 4°C, followed by Alexa-680 anti-steptavidin secondary antibody (Invitrogen S21378 – Lot 57591A) for 30 minutes at 4 °C. Antibodies and conjugates were diluted in PBS (1x) containing 3% BSA. After each incubation, cells were washed three times with PBS (1x). Surface staining was analyzed by FACS calibur flow cytometer.

4.1.10. ANALYSIS OF MDA-MB-231 EDITED FOR GALECTIN-9 AND CORRESPONDING TRAIL RECEPTOR AFTER CELL PERMEABILIZATION

Cells were permeabilized or not with 1% saponin for 15 minutes and then incubated with APC-conjugated anti-Galectin-9 or APC control, mouse IgG1 at 10 µg/mL for 30 minutes. Antibodies and conjugates were diluted in PBS (1X) containing 3% BSA). After each incubation, cells were washed three times with ice-cold PBS (1x). Surface staining was analyzed on the FACS calibur flow cytometer.

4.1.11. DETECTION OF GALECTIN-9 IN THE SUPERNATANTS BY ELISA

Determination of secreted galectin-9 in the supernatant of MDA-MB-231 WT, HCT116 WT, and VAL WT cells was performed after treatment of the cells with 5FU (1 µg/mL) for 24; 48 or 72 h. Supernatants (10 mL) were collected and concentrated (1000 µL) in the Speed Vac (Thermo ScientificTM), for 6 hours in high centrifugation. An enzyme-linked immunosorbent assay (ELISA) kit for galectin-9 (SEA309Hu, Lot L190205427, Costar, Cambridge, MA, USA) was used to detect galectin-9 in the supernatant. In summary, supernatants and standards were added in triplicates (100 µL per well) to each well of the ELISA plate. The plate was sealed and incubated for 1 h at 37 °C. Soon after, the wells were washed (300 µl PBS-Tween three times), blocked, and emptied and the biotinylated detection antibody was added for 1 h at 37 °C, followed by 100 µL avidin peroxidase for 30 min. TMB substrate was added to each well. After 10 min at 37 °C, 50 µL stop solution was added and absorbance was measured at a wavelength of 450 nm. This kit allows for the detection of Gal-9 with a dynamic range of 7.8 to 500 pg/mL.

4.1.12. IMMUNOPRECIPITATIONS

For TRAIL DISC analysis $8x10^7$ cells were stimulated in 1 mL of complete medium with 5 µg His-TRAIL for 15 to 60 minutes at 37°C. Cells were then washed with PBS before lysis in NP40 lysis buffer containing a protease inhibitor cocktail. Cell lysates were pre-cleared with Sepharose 6B (Sigma-Aldrich) for 1 hour, then the DISC was immunoprecipitated overnight with protein G-coated beads (Amersham Biosciences, Les Ullis, France) at 4°C in the presence of an anti-His antibody (clone AD1.1.10) from AbD serotec (Bio-rad, Marnes-la-Coquette, France) (Morlé et al., 2015). Beads were washed four times with NP40 lysis buffer, and then immunoprecipitated complexes were eluted in loading buffer (63 mM Tris-HCl pH 6.8, 2 % SDS, 0.03 % phenol red, 10% glycerol, 100 mM DTT), then boiled for 5 min before analysis by western blot.

4.1.13. PREPARATION OF CELL LYSATES

Cells were harvested by centrifugation at 1500 rpm for 5 minutes at 4°C, washed once with ice-cold PBS, and lysates were prepared by resuspending the resulting cell pellets in 50 µL lysis buffer per 5x106 cells (1% of NP40, 20mM Tris-HCl pH 7.5, 150mM NaCl and 10% glycerol) supplemented with complete protease inhibitor cocktail (Roche Diagnostic, France) according to the manufacturer's instructions. After 20 minutes of incubation on ice, the lysates were centrifugated at 20000 g for 15 minutes at 4°C to remove cell debris and transferred to a fresh tube.

4.1.14. WESTERN BLOTTING

Immunoprecipitates or cell lysates were separated by SDS-PAGE then transferred to PVDF or nitrocellulose membranes. Nonspecific binding sites were blocked by incubation in PBS containing 0.05% Tween 20 (PBST) and 5% powdered milk. For immunoblots aiming at detecting lectin binding profiles, membranes were saturated with PBS containing 0.05% Tween 20 and 5% BSA. Immunoblots were incubated overnight with a specific primary antibody or biotinylated lectins, washed four times in PBST then incubated for 1 hour with an HRP-conjugated secondary antibody. Blots were developed using the Covalight Xtra ECL enhanced chemiluminescence reagent (ref. 00118075) according to the manufacturer's protocol (Covalab, France).

4.1.15. BINDING STUDIES

Binding data processing was performed with ForBio Data Analysis Software version 7.1.0.36 with Savitsky-Golay filtering to reduce noise. Association and dissociation data were fit globally (single-phase exponential decay function) in Prism version 5.0a software (GraphPad Software, San Diego, CA.).

4.1.16. STATISTICAL ANALYSIS

Statistical analysis was performed using the Student's t-test. All statistical analyses were performed using Prism version 5.0a software (GraphPad Software, San Diego, CA.). P values *<0.05 and **<0.01 were considered significant.

5. RESULTS

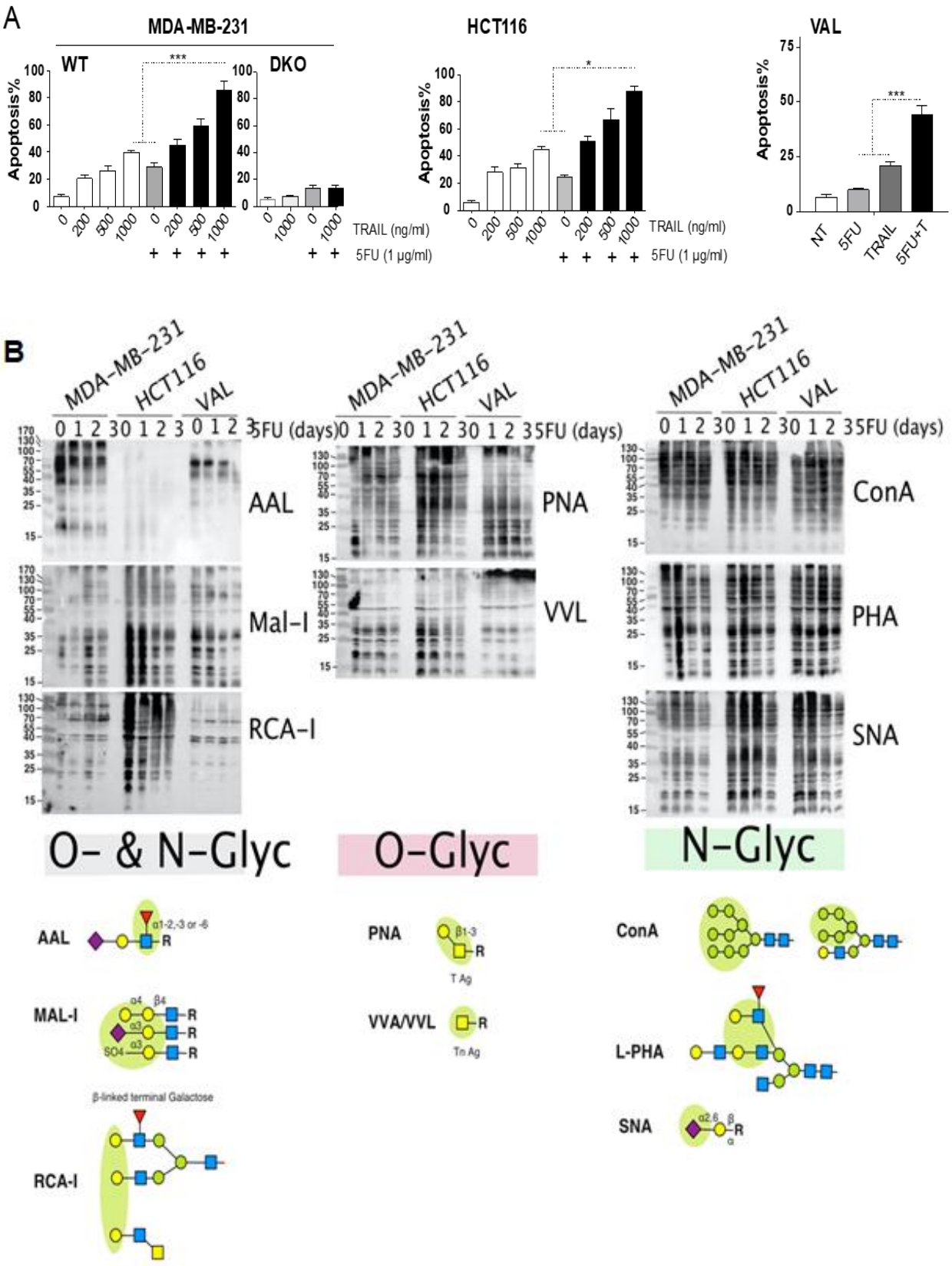
5.1. 5FU-MEDIATED SENSITIZATION OF TUMOR CELLS TO TRAIL-INDUCED CELL DEATH IS ASSOCIATED WITH ALTERATIONS IN GLYCOSYLATION

To investigate if conventional chemotherapeutic drugs such as 5FU could contribute to restoring or sensitizing tumor cells to apoptosis induced by TRAIL, the triple-negative breast cancer MDA-MB-231, the colon carcinoma HCT116, and the B cell lymphoma VAL cell lines were treated sequentially with 5FU and then stimulated with TRAIL (Figure 9A). As expected, all of them showed little sensitivity to TRAIL-induced apoptosis after 72 hours, even when treated with high concentrations (Figure 9A). Pre-treatment with 5FU, at a concentration that induces less than 20% of apoptosis, significantly increased its susceptibility to TRAIL-induced cell death, reaching up to 80% of apoptosis in HCT116 and MDA-MB-231 cells and almost 50% in lymphoma cell line B. The VAL cell line is highly resistant, known to express high levels of c-FLIP and DcR2, a TRAIL receptor antagonist at the steady-state level (Jacquemin et al., 2012).

We then speculated that 5FU could induce qualitative variations in protein glycosylation. Changes in N- and O-glycosylation during 5FU stimulation were analyzed by immunoblot from cell extracts prepared after 24, 48, or 72 hours of stimulation with 5FU (1 µg/mL), using a panel of lectins (Figure 9B). Staining intensities were quantified using Image J software (9C). Specifically, the early increase of PNA and VVL staining on day one, followed by a decrease on days 2 and 3, shows that 5FU probably alters the O-glycosylation profile in the three cell lines (Figure 9B). Likewise, albeit not observed in VAL cells, an increase in MAL-I staining was also observed in the extracts obtained from MDA-MB-231 and HCT116 cells, suggesting that 5FU likely induces an increase in O-glycosylation. Consistent with that, immunoblot changes in N-glycosylation were also detected in MDA-MB-231 and HCT116 cells, as demonstrated by the early increase in L-PHA staining. This lectin, which binds specifically to terminal galactose, N-acetylglucosamine, and mannose residues, is reminiscent of complex N-glycans. However, in VAL cells, the increase in L-PHA staining was found to occur slightly later and to a much lower extent than MDA-MB-231 and HCT116 cells (Figure 9B). High-mannose moieties were also observed in VAL cells, as demonstrated with Con A staining. Finally, changes in sialylation and fucosylation were also found after 5FU stimulation, as reflected by variations in SNA

and AAL staining. SNA binds to sialic acids bound to galactoses at position α2-6 and α2-3, while AAL displays a high affinity for fucose residues at α1-6 or 1-4 position and N-acetylactosamine structures.

To determine whether the qualitative changes described above correspond to glycosylated membrane-bound proteins, lectins binding on intact cells was analyzed by flow cytometry after a 72h treatment with 5FU (Figure 9D). Results showed that 5FU induces an increase in the cellular surface glycosylation evidenced by the staining with WGA, a lectin with affinity for N-acetylglucosamine and sialic acid residues, found in both N- and O-glycosylated proteins, and L-PHA, that exhibits high specificity for N-glycosylated proteins. Staining with the other lectins were either inconsistent or unchanged.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WT
DKO
HCT116
VAL
Apoptosis%
TRAIL (ng/ml)
5FU (1 μg/ml)
NT
5FU
TRAIL
5FU+T
B
MDA-MB-231
HCT116
VAL
5FU (days)
AAL
Mal-I
RCA-I
PNA
VVL
ConA
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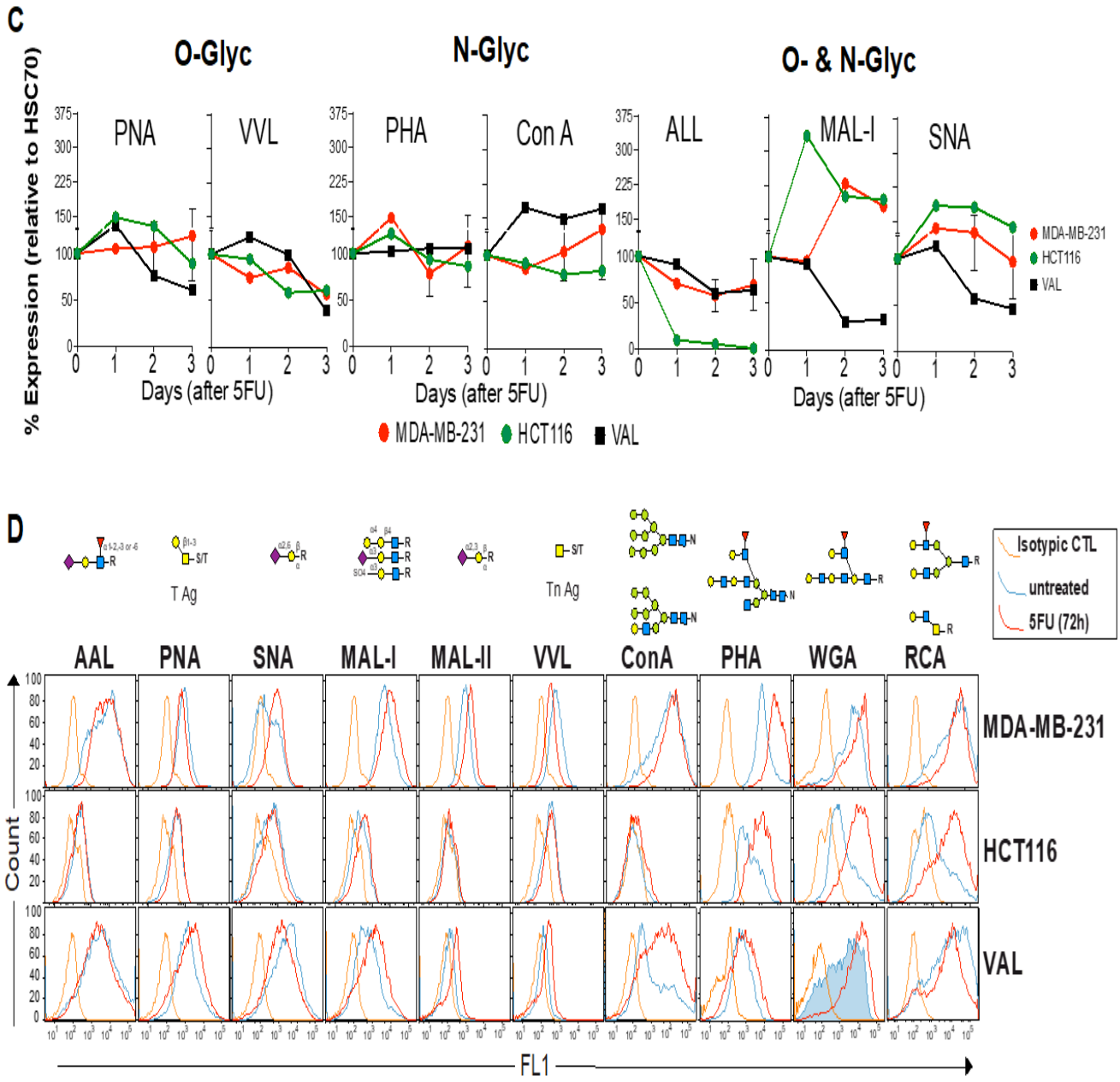


Figure 9: Alteration of glycosylation by 5FU induces apoptosis. **A.** MDA-MB-231, HCT116 and VAL cells were treated or not for 72 h with 1 µg/mL 5FU and stimulated or not with increasing TRAIL concentrations for an additional 6 hours, except VAL and MDA-MB-231 TRAIL-receptor-deficient (DKO) cells which were stimulated with 1 µg/mL TRAIL. Apoptosis was monitored by flow cytometry after Annexin V and 7AAD staining. **B.** Alteration of glycosylation by 5FU analyzed by immunobot. Indicated cells were stimulated with 1 µg/mL 5FU for 24, 48 or 72 hours and cell extracts were analyzed by immunoblot using a panel of lectins recognizing O- and N-glycosylated proteins. Lower part: sugar moieties recognized by each lectin are highlighted in green in each corresponding illustrated glycotype. **C.** Staining intensities were quantified using Image J. **D**. Cells were stimulated with 5FU during 72 has above and lectin binding was assessed by flow cytometry as indicated in the material and method. Isotypic control and specific stainings in unstimulated or stimulated cells are shown in the inlet box. **Source:** The author

5.2. 5FU INDUCES GALECTIN-3 AND -9 SECRETION

Given that 5FU may be involved in changes in glycosylation, including TRAIL DR4/DR5 receptors, we questioned whether 5FU could alter expression levels of galectin or its subcellular location. To answer this question, we first assessed by immunoblot the impact of 5FU on the expression levels of galectins -3 and 9 during treatment (24, 48, and 72 hours). The results observed in MDA-MB-231 cells using NP40, a mild detergent, reveal the loss of galectin-3 and -9 in the soluble fraction and the accumulation in the insoluble fraction in a time-dependent manner after stimulation (Figure 10). A similar result was observed in VAL cells (Figure 10A). However, this loss in the NP40 soluble fraction was not associated with a concomitant accumulation in the insoluble.

We assume after secretion galectins may be able to bind to transmembrane proteins and be detectable in the extracellular medium. To answer this question, we monitor the galectin content in the conditioned medium of cells treated with 5FU for 72 hours by flow cytometry and ELISA. Consistent with this hypothesis, and although to a limited extent, using two different anti-galectin-9 antibodies, we were able to detect an increase in the staining of galectin-9 on the cell surface after 5FU stimulation in MDA-MB-231, HCT116 cells, and VAL (Figure 10C and D). In line with this finding, analysis of galectin-9 secretion by ELISA indicated that 5FU stimulation induces significant galectin secretion by the three studied cell lines (Figure 10E). These results together provide evidence that 5FU not only alters the glycosylation status of tumor cells but also induces the release of galectin-9 and probably other galectins in the extracellular compartment.

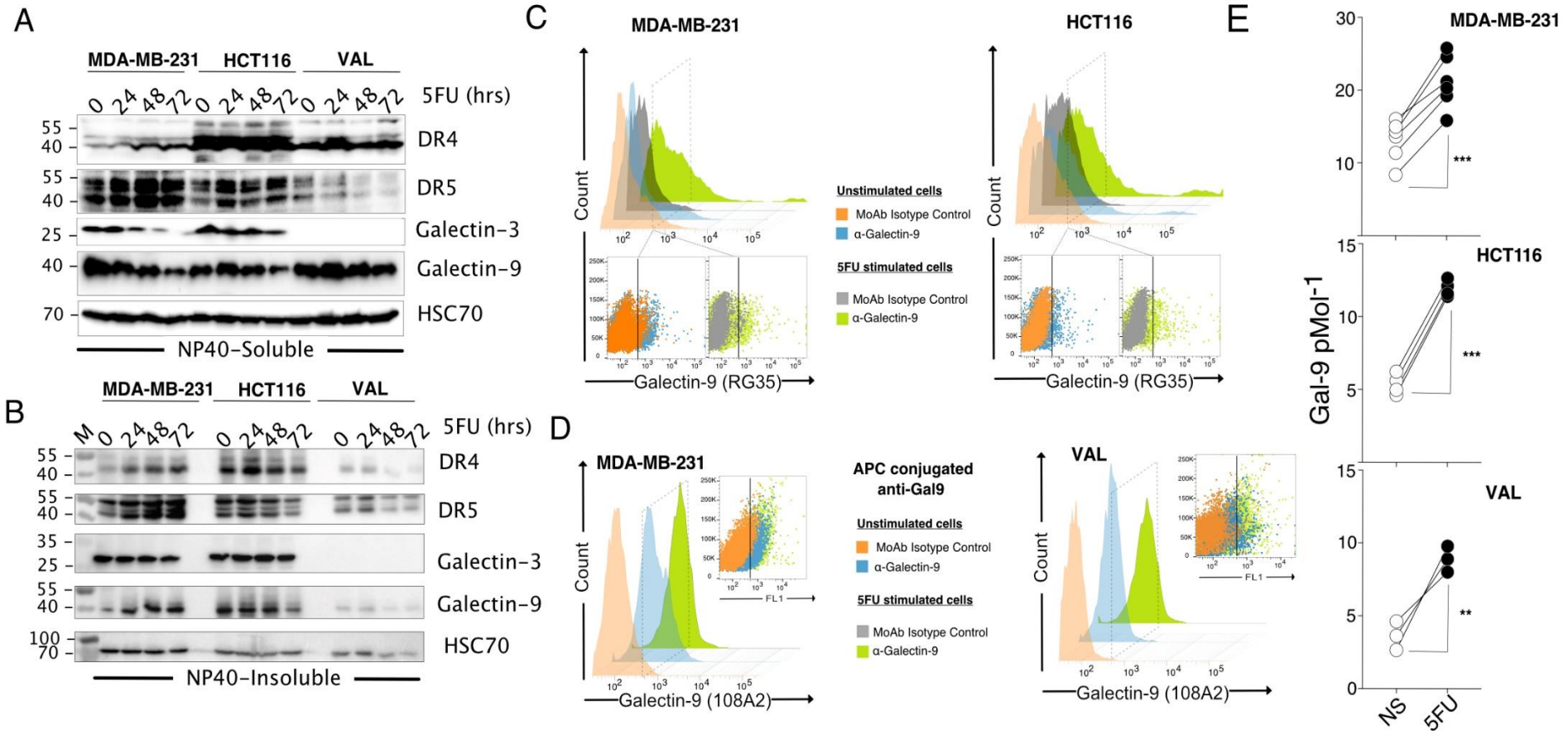


Figure 10: 5FU induces galectin-9 secretion. **A,B** Indicated cells were stimulated with 1 µg/mL 5FU for 24, 48 or 72 hours and cell extracts were analyzed by immunoblot for DR4, DR5, galectin-3 and -9 staining from NP40 soluble or insoluble fractions. HSC70 served here as loading control. **C,D** Analysis of galectin-9 membrane expression after 5FU stimulation. Indicated cells were stained with two commercial Galectin-9 antibodies 72 h after 5FU stimulation. **E** Galectin-9 secretion in cells stimulated for 72 h with 5FU was estimated by ELISA from corresponding conditioned media. **Source:** The author.

5.3. GALECTIN-3 INCREASES TRAIL-INDUCED APOPTOSIS

To investigate the contribution of galectin-3 in TRAIL induced apoptosis, we first inhibited its expression in HCT116 cells using interference RNA (siRNA) and compared it with control (non-galectin-3 siRNA targeting). The reduction in galectin-3 expression, as assessed by RT-qPCR and immunoblotting, was around 70% (Figure 11A), with a 50% reduction in the apoptosis rate. Consistent with these results, overexpression of galectin-3 in the MDA-MB-231 cell line, on the contrary, enhanced TRAIL-induced apoptosis (Figure 11B and C), regardless of its phosphorylation status. Notably, the addition of exogenous galectin-3 together with TRAIL was sufficient to recapitulate the gain of function demonstrated above in MDA-MB-231 cells (Figure 11D), suggesting that 5FU-mediated secretion of galectin-3 is likely to account for the increased sensitivity of the tumor cells to TRAIL-induced cell death.

Since 5FU was found to modify glycosylation and induce the secretion of galectins, we speculated that this change in subcellular localization is likely to account for the gain of function, probably through direct interaction galectin-3 with DR4 or DR5. In agreement with this hypothesis, immunoprecipitation and DISC-forming proteins' detection showed that galectin-3 overexpression leads to an increase in its recruitment in the TRAIL-DISC complex (Figure 11E). Concomitant with this increase in galectin-3 recruitment, DR4, but not DR5, was also more prevalent in the complex, particularly in galectin-3 overexpressing cells compared to control cells (Figure 11E). As expected, overexpression of galectin-3 in MDA-MB-231 cells did not further increase TRAIL-induced cell death after 5FU stimulation (Figure 11F), probably because 5FU is alone able to induce the secretion of galectins, and the amount of secreted galectin is sufficient to induce the increase in apoptosis following TRAIL stimulation.

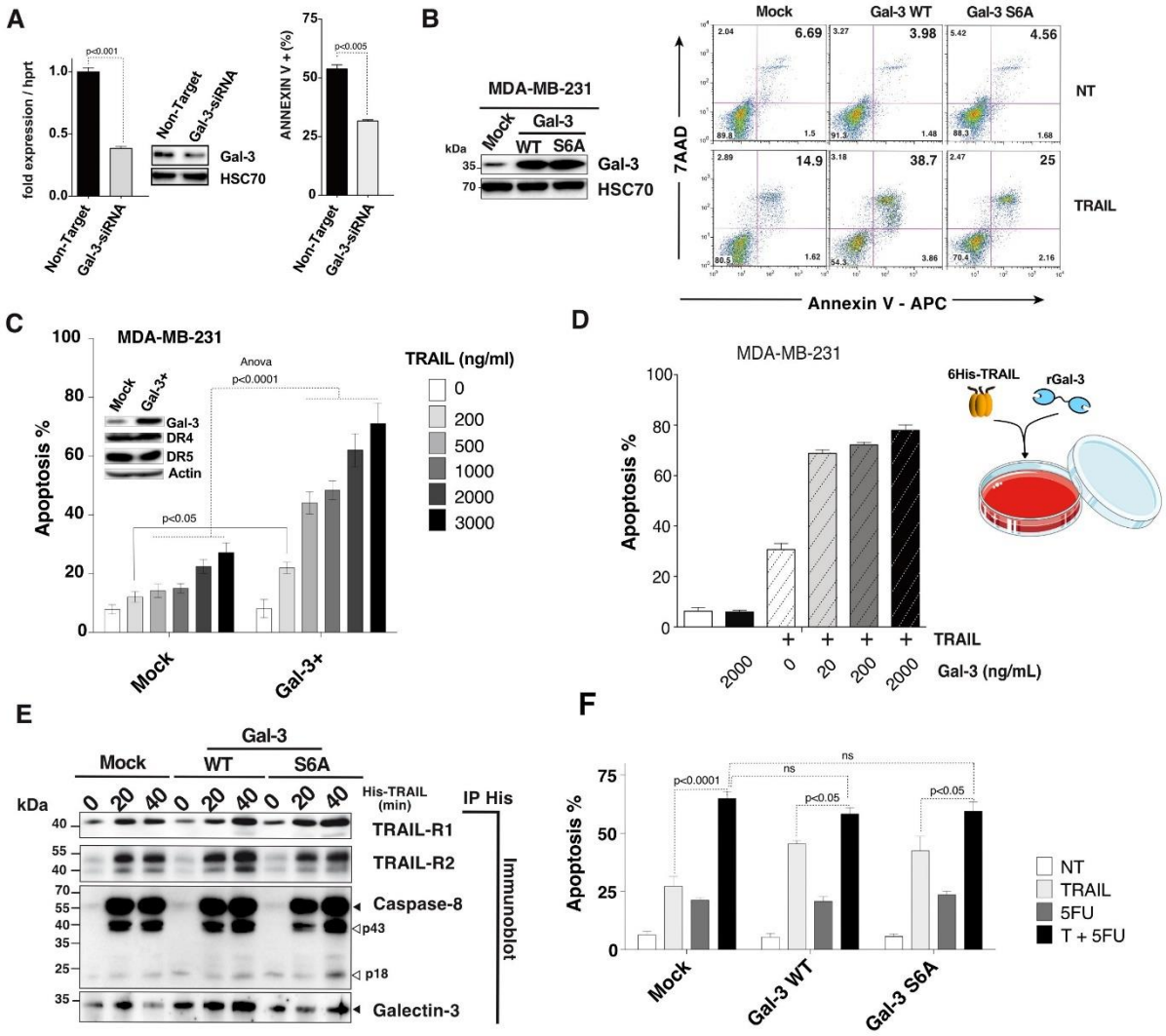
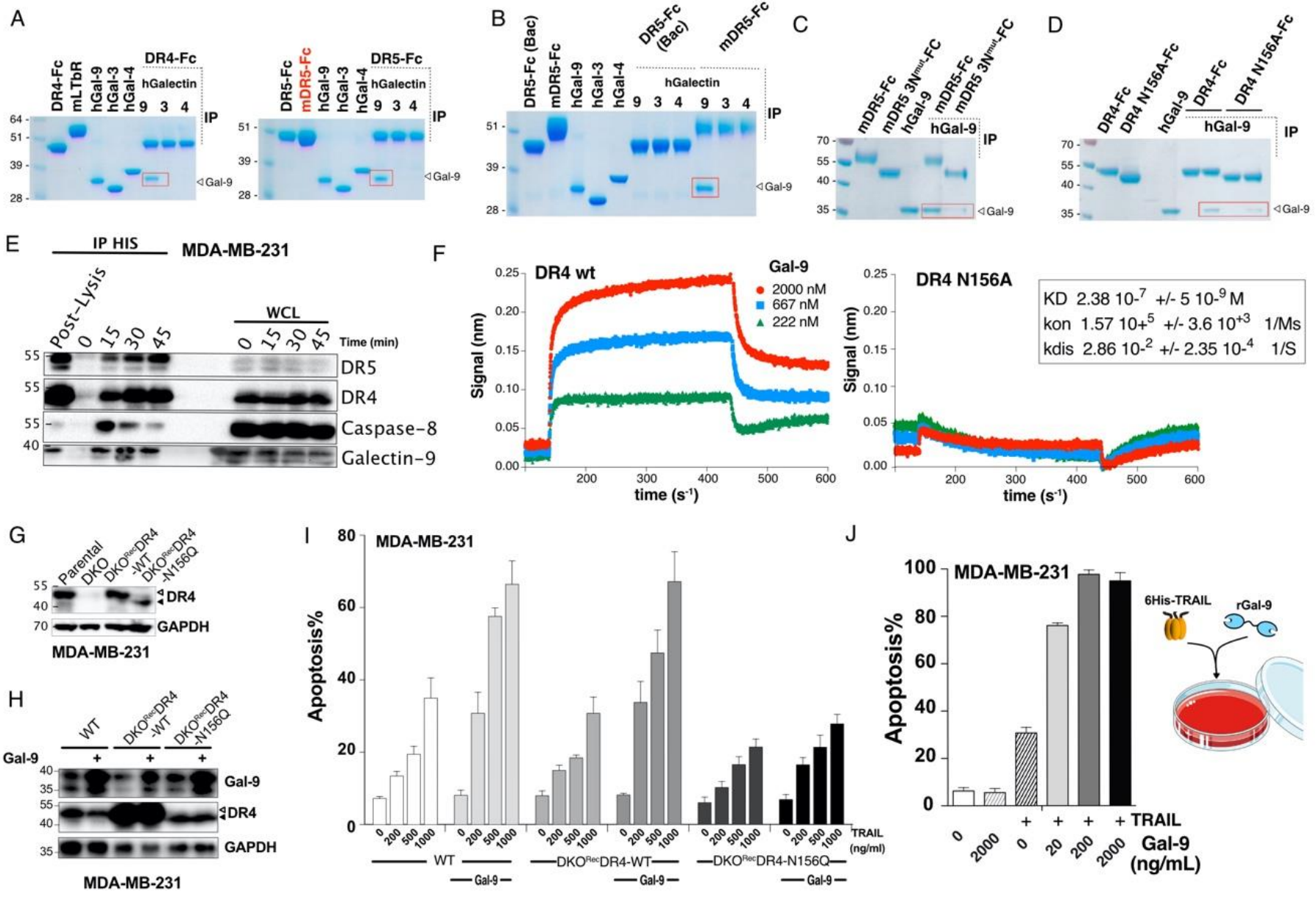


Figure 11: Galectin-3 increases TRAIL-induced apoptosis. **A** Evaluation of galectin-3 protein expression by western blot in the HCT116 line, after siRNA. HSC70 was used here as a loading control. **B** Evaluation of overexpression of galectin-3 and DR4 / DR5 receptors by western blot in HCT116, after the approach of overexpression by lentivirus. Actin was used here as a loading control. Sensitivity of Gal-3 siRNA cell derivatives indicated for TRAIL-induced apoptosis. **C** Analysis of apoptosis induced by TRAIL at different concentrations in MOCK cells and cells overexpressing gal-3. **D** lustration of the experimental scenario and TRAIL-induced apoptosis (1000 ng/ml) and increasing concentrations of recombinant galectin-3. **E** Immunoprecipitation and detection of DISC-forming proteins in MDA-MB-231 cells, whether or not overexpressing wild galectin-3. **F** Sensitivity of cell derivatives indicated for apoptosis induced by 5FU in combination with TRAIL. **Source:** The author

5.4 GALECTIN-9 ENHANCES TRAIL-INDUCED APOPTOSIS

In order to understand how galectin-3 and -9 are likely to interfere with TRAIL-DISC assembly and apoptosis we further investigated the potential interactions of these carbohydrate-binding proteins with DR4 and DR5 by co-immuno-pull-down. For this, recombinant versions of the extracellular domain of DR4 or DR5 fused to the human Fc chain were produced in HEK 293 cells with preservation of their glycosylation status. As shown in figure 12A, DR4 and DR5, as well as the unique mouse TRAIL receptor (mDR5), were able to pull-down galectin-9, but not galectin-3 or galectin-4 (Figure 12A and B). Importantly to note that the interaction of galectin-9 with the receptors used in this experiment, was strictly dependent on glycosylation. The production of the DR5 receptor in prokaryotic cells (Bacteria), unable to glycosylate nascent proteins, as well as the mutation of mDR5 (Figure 12C) or DR4 (Figure 12D) in N-glycosylation sites, severely impaired the interaction of this galectin with these receptors.

To further investigate how galectin-9 regulates TRAIL-induced cell death, we took advantage of the MDA-MB-231 isogenic cell lines, generated previously by Dufour et al. (2017), to generate cells overexpressing or not galectin-9. These cells express either the WT or non-glycosylable form of DR4 (N156A). Cells overexpressing galectin-9 were found to be more sensitive to TRAIL-induced cell death as compared to the parental counterpart or isogenic derivatives (Figure 12I), except for those expressing the non-glycosylable DR4 (DKO-^{rec}DR4-N156A). Noteworthy, the lack of gain of function of galectin-9 was tightly associated with the loss of DR4 glycosylation, but not a loss of DR4 expression (data not shown). Likewise, the sensitivity of the MDA-MB-231-DKO-^{rec}DR4-N156A isogenic cells expressing or not galectin-9 was comparable and clearly weaker than the parental cells expressing both DR4 and DR5, or even that of MDA-MB-231-DKO reconstituted with WT form of DR4 (Figure 12I). Importantly, similar to galectin-3, recombinant galectin-9, added simultaneously with TRAIL, also enhanced, in a dose-dependent manner, the sensitivity of MDA-MB-231cells to apoptosis induced by this cytokine (Figure 12J), clearly demonstrating that galectins can directly interact with and regulate TRAIL receptor signal transduction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Signal (nm)
time (s-1)
DR4 N156A
KD 2.38 10-7 +/- 5 10-9 M
kon 1.57 10+5 +/- 3.6 10+3 1/Ms
kdis 2.86 10-2 +/- 2.35 10-4 1/S
G
Parental
DKO
DKORecDR4 -WT
DKORecDR4 -N156Q
DR4
GAPDH
MDA-MB-231
H
WT
DKORecDR4 -WT
DKORecDR4 -N156Q
Gal-9
Gal-9
DR4
GAPDH
MDA-MB-231
I
MDA-MB-231
Apoptosis%
TRAIL (ng/ml)
WT
DKORecDR4-WT
DKORecDR4-N156Q
Gal-9
J
MDA-MB-231
Apoptosis%
6His-TRAIL
rGal-9
TRAIL
Gal-9 (ng/mL)

Figure 12: Galectin-9 enhances TRAIL-induced apoptosis. **A** Recombinant galectin-3, -4, -9 as well as DR4 and DR5 fused to Ig Fc (DR4-, DR5-Fc) or immuno-pulled downs of indicated combinations were analyzed by Coomassie blue. **B** Similar experiment was performed using a version of DR5-Fc produced in bacteria (non-glycosylated) and compared to the unique mouse agonist TRAIL receptor (mDR5). **C-D** Comparison of galectin-9 pull-down by glycosylated (mDR5 or DR4) or their corresponding non-glycosylable mutated versions, mDR5-3Nmut and DR4-N156A, respectively. **E** TRAIL DISC analysis showing time dependent pull-down of DR4, DR5 and galectin-9. F Galectin-9 affinity for glycosylated or non-glycosylated DR4, analyzed by SPR**. G-H** Analysis of DR4 and Galectin-9 expression in MDA-MB-231 parental and isogenic cell lacking TRAIL receptor expression (DKO) and DKO cells reconstituted for DR4 or DR4-N156A expression and derivatives overexpressing galectin-9. GADPH was used here as a loading control. **I** Sensitivity of indicated isogenic cell derivatives to apoptosis induced by increasing concentrations of TRAIL. **J** llustration of the experimental setting and apoptosis induced by TRAIL (1000 ng/ml) and increasing concentrations of recombinant galectin-9. **Source:** The author

We next questioned whether the loss of galectin-9 expression would impair or compromise the efficacy of the sequential 5FU/TRAIL treatment in our cells. To address this question, we used the CRISPR/CAS9 approach to edit galectin-9 in MDA-MB-231-DKO cells. Next, we reconstituted either DR4 or DR5, using a retroviral approach. Galectin-9 deficiency was monitored by flow cytometry after membrane permeabilization (Figure 13A).

Given that the knockout of galectin-9 in MDA-MB-231 cells did not alter the expression of DR4 and DR5 receptors, we next evaluated its sensitivity to TRAIL-induced cell death. The loss of galectin-9 was associated, regardless of the receptor, with a loss of sensitivity to TRAIL-induced apoptosis (Figure 13B). It is important to emphasize that the rescue of the loss of function of galectin-9 by the addition of recombinant galectin-9 in the culture medium restored the sensitivity of TRAIL. The deletion of galectin-9, however, only partially compromised the synergy of the sequential 5FU/TRAIL treatment, regardless of the receptor considered (Figure 13C). Loss of galectin-9 was associated, regardless of the receptor, with a loss of sensitivity to TRAIL-induced apoptosis (Figure 13B). Importantly, the rescue of the loss of function of galectin-9 by the addition of recombinant galectin-9 in the culture medium restored TRAIL sensitivity. Galectin-9 edit, however, only partially compromised the synergy of the sequential 5FU/TRAIL treatment, regardless of the considered receptor (Figure 13C).

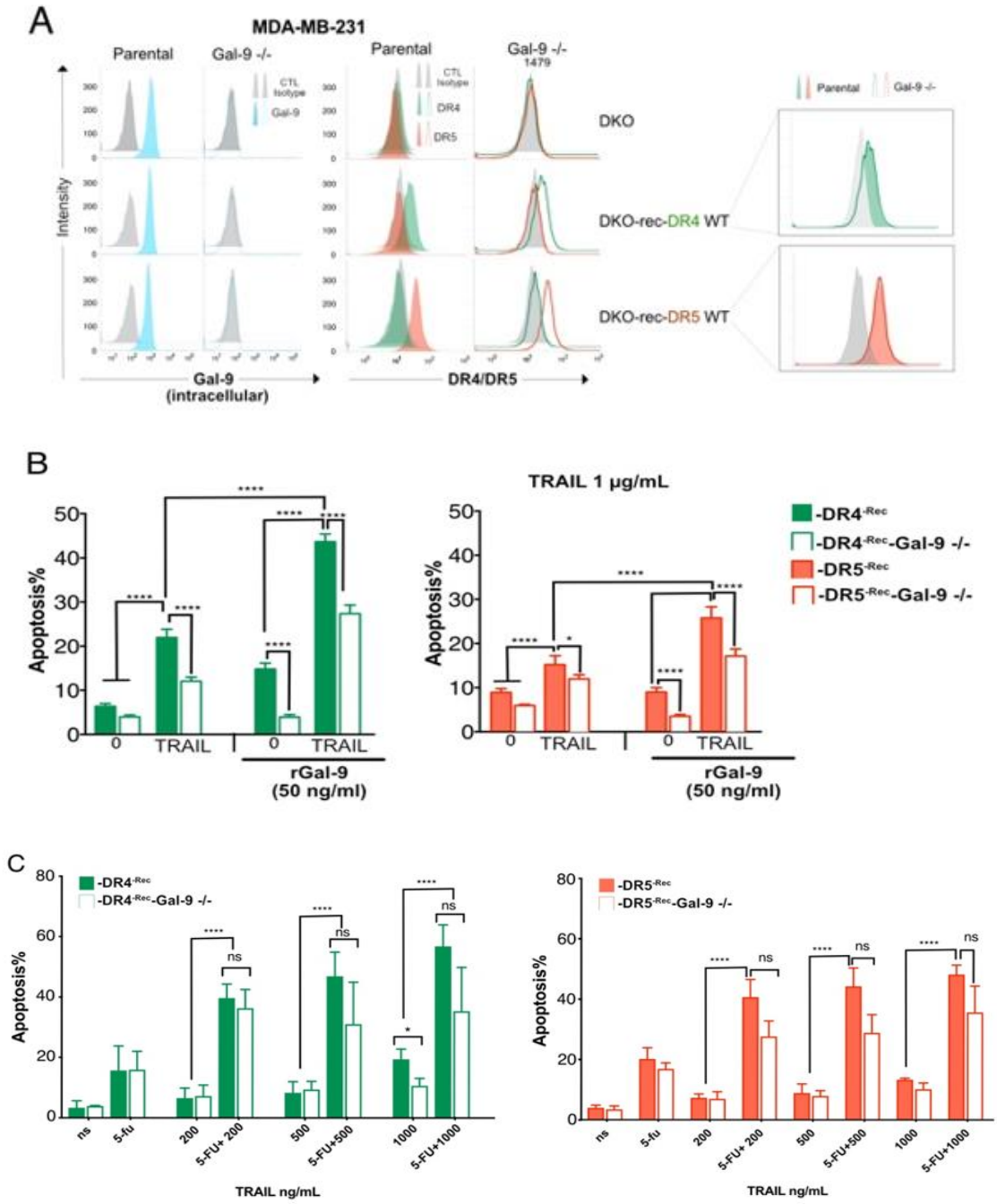

Figure 13: Galectin-9 gene editing impairs TRAIL-induced apoptosis. **A** Analysis of MDA-MB-231 cells edited for galectin-9 and corresponding TRAIL receptor isogenic precursors were analyzed by flow cytometry for galectin-9 expression after cell permeabilization. **B** Sensitivity of these cells, with the exception of the TRAIL receptor DKO isogenic cell line was assessed by flow cytometry for annexin V and 7AAD staining after stimulation with increasing concentrations of TRAIL in the presence or absence of recombinant galectin-9 as indicated. **C** Cells, expressing or not DR4, DR5, and/or galectin-9 were analyzed as in (B) after sequential treatment for 72 h with 5FU (1 µg/mL) followed or not by a 6 h TRAIL stimulation at the indicated concentration. **Source:** The author.

7. DISCUSSION

APO2/TRAIL ligand and its agonist receptors have been studied for cancer treatment for over 20 years (Micheau, Shirley and Dufour 2013). TRAIL receptors 1 and 2 share structural homology (Özören and El-Deiry, 2003). Both receptors recruit FADD and caspase-8, triggering apoptosis through the formation of the DISC complex (Ashkenazi, 2002; Kischkel et al., 2000). However, some tumor cells can escape TRAIL's cytotoxic action by developing resistance mechanisms (Trivedi and Mishra, 2015; Zhang and Fang, 2005). In the present work, we demonstrated that the chemotherapeutic 5FU sensitized the tumor cells to TRAIL-induced apoptosis due to its ability to release galectin-9 that interact directly with TRAIL agonist receptors. The loss of DR4 and DR5 glycosylation caused by site-directed mutation or production in prokaryotic cells impaired galectin-9 binding. Altogether, our results reveal galectins as new components of the TRAIL-DISC complex, whose regulation by 5FU is probably responsible for synergy with TRAIL.

We initially studied the combined effects of 5FU and TRAIL in apoptosis on cells previously resistant to TRAIL. Increased apoptosis was found in MDA-MB-231 triple-negative breast cancer, colon carcinoma HCT116, B cell lymphoma VAL cell lines when compared to cells treated with TRAIL alone (Figura 9A). As previously reported, 5FU can reduce the expression of c-FLIP and XIAP (Ganten et al., 2004; Kondo et al., 2006; Morizot et al., 2011), and can also promote the alteration in the Bcl-2 proteins balance in favor of pro-apoptotic family members (Mühlethaler-Mottet et al., 2004; Song et al., 2003). However, until now, it remains unknown how 5 FU favors the formation of DISC and the recruitment of caspase-8 (Byun et al., 2018; Galligan et al., 2005; Shirley et al., 2011).

We assessed whether treatment with the 5FU could induce qualitative variations in cellular glycoconjugates. In this approach, we used a panel of specific lectins for different ligands that together could show whether the changes caused by 5FU treatment would affect both N- and O-type glycosylations. For all cell types, we verified an increase in the staining density for N-type, while only in the VAL cells were alterations in the O-glycosylation noticed, highlighting the growth after 72h of stimulation. The most evident changes occurred for the stainings with PHA and SNA, which specifically recognize N-glycosylated units.

Since the accumulation of sialyl antigen is a common modification in tumor cells (Pinho and Reis, 2015; Trinchera et al., 2017), we hypothesized that 5FU could remove

sialic acid units, facilitating the binding of lectins that specifically recognize galactose or N-acetylgalactosamine units. It would allow cells to be recognized by macrophages through galactose receptors, therefore degraded (Lu and Gu, 2015). Even not investigated in this study, we could also assume that the observed accumulation of N- and O-glycosylated proteins in the detergent soluble protein fraction may result from the stimulation of increased expression of enzymes acting in glycosylation.

Aiming to better investigate the role of surface glycosylations and assuming the roles of galectins in the activation of several physiological events (Wang et al., 2019), we questioned whether 5FU could alter the levels of galectin expression or its subcellular location. Although it is unclear how galectins are secreted, their retention on the cell surface requires glycosylated plasma membrane receptors (Stewart et al., 2017), forming a network, resulting in cascade signaling events (Brewer et al., 2002; Hsu and Liu, 2002). Our results showed that treatment with 5FU increased the amount of galectin-9 on the cell surface of the three cell lines tested, as detected by flow cytometry and later confirmed by ELISA.

Given that that 5FU induces galectin-9 secretion and galectin-3 has been described to confer resistance or to facilitate TRAIL-induced cell death, depending on its subcellular location or phosphorylation state (Mazurek et al., 2011, 2007; Seyrek et al., 2019), we defined a series of experiments to evaluate the effects of galectin-3 expression in 5FU/TRAIL-treated cells. Interestingly, when HCT116 cells (susceptible to TRAIL) are transfected with siRNA targeting Gal-3, TRAIL-induced apoptosis was reduced by a factor of 2. Consistently with these results, we demonstrate that MDA-MB-231 tumor cells transfected with overexpression galectin-3 are more likely to undergo TRAIL-induced apoptosis, regardless of their phosphorylation state. In line with these results, the overexpression of wild-type galectin-3 (WT) or its non-phosphorylable version (S6A) sensitizes MDA-MB-231 cells TRAIL-induced apoptosis. However, overexpression of galectin-3 does not influence the 5FU-induced mortality rate, nor apoptosis induced by the 5FU TRAIL combination.

It is worth noting that the mechanism of apoptotic activity by galectin-3 is not fully understood (Mazurek et al., 2012), and the interaction of galectins with TRAIL receptors has been rarely documented (Pan et al., 1998). However, there is evidence that galectin-3 phosphorylation increases TRAIL-induced apoptosis (Lee et al., 2003), implying that the effect of galectin-3, in this case, occurs in the cytosol. Also, galectin-3 interacts with several molecular components of apoptosis regulatory pathways. Galectin-3 is complexed with the Fas receptor (CD95), whose engagement is known

to induce apoptosis (Takenaka et al., 2004). Galectin-3 also causes activation of mitochondrial apoptosis events, including cytochrome c release and activation of caspase-3, but not caspase-8 (Nakahara et al., 2005). However, to date, no study has verified the presence of galectin-3 with the DISC assembly.

Galectin-3 overexpression in MDA-MB-231 cells showed that this lectin can be recruited into the complex and that its overexpression can lead to an increase in TRAIL DISC recruitment. Notably, TRAIL DISC contains more DR4 receptors in the MDA-MB-231 cells that express galectin-3 than in control cells. The recruitment of DR5, however, is relatively similar in all cell models tested. These results suggest that galectin-3 can increase TRAIL-induced apoptosis, promoting the recruitment of the DR4 receptor to DISC. Since galectin-3 appears to contribute to the sensitization of cells resistant to TRAIL, acting proximally within the DISC, we hypothesized that its deregulation in sensitive cells could inhibit the pro-apoptotic signaling of TRAIL.

We also investigated the protein-protein interactions of DR4 and DR5 receptors with galectin-3 and -9. Furthermore, we generated cell lines deficient and overexpressing galectin-9 to analyze this galectin's relative contribution by triggering apoptosis by the TRAIL ligand. Our results show that the presence of galectin-9 was essential for better TRAIL-induced apoptosis. Therefore, the arrangement and grouping of TRAIL receptors on the cell surface after TRAIL stimulation may represent a crucial step to induce efficient activation of caspase-8, demonstrating that the presence of galectin will probably have a critical regulatory role in the process.

Our results on the potentiation of TRAIL activity by galectin-9 give continuity to this hypothesis. The non-glycosylated version of DR4 overexpressing galectin-9 cannot induce apoptosis after stimulation with TRAIL, despite this galectin's presence. Notably, these results are in line with the functional tests performed with the recombinant extracellular domain of the DR4 and DR5 receptor, fused to the Fc chain. Galectin-9 interacted with the glycosylated receptors. Its binding was drastically reduced or hampered by the lack of glycosylation. These results suggest that DR4 glycosylation is crucial for the formation of DISC after TRAIL stimulation; the direct interaction between galectin-9 and the receptor is necessary for the effectiveness of apoptosis. In line with these results, the regulatory function associated with DR4 N-glycosylation is essential for apoptosis induced by TRAIL (Dufour et al., 2017b).

We also evaluated the participation of galectin-9 in the receptor aggregation and activation within the DISC complex in breast cancer cells. Surprisingly, galectin-9 was found at DISC, along with the recruitment of caspase-8 and DR4 and DR5 receptors.

Likewise, the results generated by the SPR protein-protein interaction analysis revealed that the affinity of the glycosylated Fc-fused-receptor to galectin-9 is an important factor, being drastically reduced when the recombinant receptor was produced with a point mutation preventing N-glycosylation. In addition, we evaluated the addition of exogenous galectins followed by TRAIL treatment. Galectin -3 and -9 were able to restore apoptotic sensitivity to TRAIL, but the addition of galectins alone could not induce apoptosis in breast cancer cells.

By contrast, the activities demonstrated with recombinant proteins added *in cellulo* may not represent the endogenous protein's functions. Additional studies are needed to definitively establish the apoptosis-inducing functions of endogenous galectins in pathological processes. The cell surface receptors responsible for galectins' actions also continue to be clarified (Pace et al., 1999; Stillman et al., 2006). As mentioned earlier, galectins do not have specific individual receptors; galectin-3 and -9 have been shown to bind to many different glycoproteins, regulating critical biological processes during the development of the organism (Díaz-Alvarez and Ortega, 2017; Wada et al., 1997). Despite the vast repertoire of recognition of different ligands, recently, a study with blackberry lectin (Morus nigra, Morniga-G), demonstrated that this lectin induced the cell death of Tn-positive leukemic cells by means of O-glycosylation concomitant, caspase and TRAIL/DR5 dependent pathways (Poiroux et al., 2019).

In consonance with this result, we demonstrate the ability of galectin-3 or -9 to bind to glycosylated extracellular domains of the DR4 and DR5 receptors, increasing apoptosis induced by TRAIL. Knowing that 5FU is responsible for restoring tumor cells' sensitivity through its ability to release galectins in the extracellular compartment, we speculated that the knockout for galectin-9 could interfere with the restoration of sensitivity induced by 5FU. As expected, the 5FU TRAIL combination could not sensitize cells to apoptosis due to the lack of galectin-9. It is worth mentioning that cells expressing the DR4 receptor were more sensitive than cells expressing DR5. The reasons for this selective involvement are still unknown. Still, recent data suggest that DR5 can also exhibit pro-tumorigenic potential and contribute to stress-induced cell death of the endoplasmic reticulum, regardless of its binding to TRAIL (Dufour et al., 2017). The fact that DR4 is N-glycosylated while DR5 is O-glycosylated provides significant clues for the differential promotion of apoptosis by DR4 and DR5 in some types of tumor cells (MacFarlane et al., 2005; Micheau, 2018). In this sense, it would be interesting to generate cells deficient for O-glycosylation and to resume testing

against gene edition of galectin-3 or-9 with 5FU treatments in these cells, in order to better clarify the role of O- versus N-glycosylation in regulating TRAIL or non-apoptotic signal transduction.

In summary, our results demonstrate that the chemotherapeutic agent 5FU induces the release of galectin-9 from tumor cells to the extracellular environment. The release of endogenous galectin allows interaction with the extracellular domain of DR4 and DR5, contributing to increased TRAIL-induced apoptosis. The interaction with DR4 or DR5 was strictly related to the state of glycosylation of the receptors. The mutation of the N-glycosylation sites of DR4 impaired the binding of galectin-9, even after overexpression of this galectin in breast cancer cells. Likewise, overexpression or addition of soluble recombinant of galectin-3 or galectin-9 alone was sufficient to increase the sensitivity of TRAIL, while its deletion impaired TRAIL-induced apoptosis.

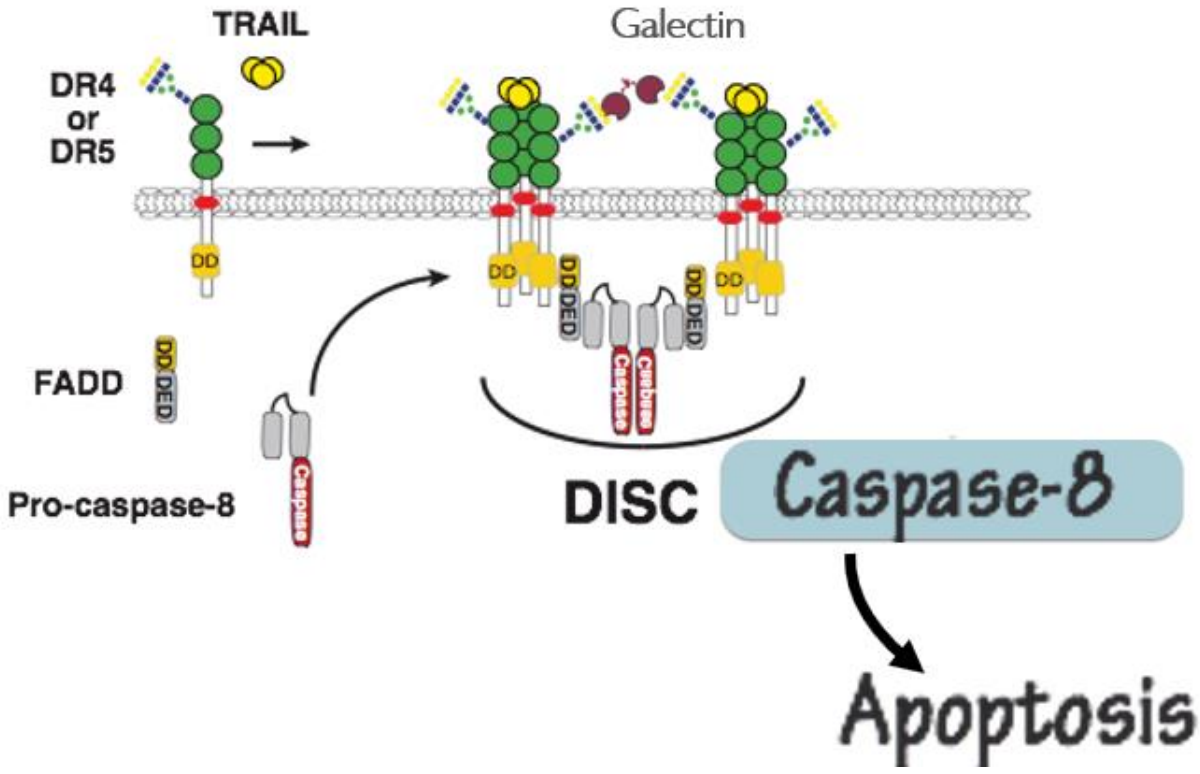

Figure 14: TRAIL-induced apoptosis via DR4 and DR5 in tumor cells is closely associated with the glycosylation of their receptors. The stimulation of DR4 or DR5 glycosylated by TRAIL induces a better oligomerization of receptors in the presence of galectin, allowing the recruitment of the adapter protein FADD and caspase-8, thus forming the so-called TRAIL DISC (Death-Inducing Signaling Complex), where caspase- 8 is processed, allowing triggering of apoptosis. **Source: The authors**

6. CONCLUSIONS

Regulation of APO2L/TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis by carbohydrate-binding proteins at the membrane level has, to date, never been found to occur in a direct manner. We provide, here, strong evidence that galectins can directly interact with TRAIL receptors and increase their pro-apoptotic activity. The binding of galectin-9 to DR4 or DR5, as expected, was found to require proper glycosylation of the receptors. While our immune-pull down experiments fail to show binding of galectin-3 to the recombinant receptors, either DR4 or DR5, our functional study suggests that in vivo, both galectin-9 and galectin-3 are likely to bind to these receptors, as both carbohydrate-binding proteins were able to increase cell sensitivity to TRAIL-induced cell death. However, given that all transmembrane glycoproteins are potentially able to interact with galectins, it cannot be excluded that additional partners may be required for interaction with DR4 or DR5. Notwithstanding, and remarkably, the finding that conventional chemotherapeutic drugs such as 5FU may sensitize tumor cells to TRAIL-induced apoptosis due to their ability to induce galectin release to the extracellular compartment is sustained by our finding that galectins such as galectin-3 and -9 can interact directly with fully glycosylated DR4, and increase its pro-apoptotic potential. While further studies will be required to extend our findings to additional conventional chemotherapeutic drugs and understand how they induce the secretion of galectins, our results uncover galectins as novel TRAIL signaling regulators, whose sugar specificity allow them not only to bind TRAIL receptors, but also any glycosylated protein within their neighborhood, extending the field of investigations to be explored.

7. FUTURE PERSPECTIVES

As next steps, the knockout for galectin-3 and the study of the involvement of other galectins that may be related to the pro-apoptotic process induced by TRAIL will be carried out. In addition, in the coming months, we expect to investigated is the participation of other chemotherapeutic agents, which may be involved in the sensitization of tumor cells by increasing TRAIL-induced apoptosis

8. STUDY LIMITATIONS

The present study has limitations: The regulation of glycosylation after treatment with the chemotherapeutic agent 5FU was inconclusive in this study. Although the results obtained by staining with lectins directed to the recognition of N- and O-glycosylation present differences between the control and cells treated with 5FU, additional studies must be performed. In this case, the participation of enzymes responsible for the glycosylation process could be investigated, for example, overexpression of enzymes involved in the glycosylation of DR4 and DR5, in particular, ST6Gal-I (ST6 beta-galactoside alpha-2,6-sialyltransferase) or MGAT4 (mannosyl (alpha-1,3) -glycoprotein beta-1,4-Nacetylglucosaminyltransferase). These enzymes are respectively involved in protein glycosylation. Another point to be raised, although the extracellular location of galectins has been well established, many issues surrounding unconventional galectin secretion still need to be resolved. There is evidence that these are secreted by direct translocation and using a vesicle-based pathway, but it is not clear how these separate pathways relate to each other. Studies on secretion regulation often depend on drugs that can have multiple effects, therefore, few conclusions about the regulation mechanism can be safely drawn. Therefore, there is a clear need for more work to investigate the mechanism and regulation of galectin secretion regulated by chemotherapy treatment.

9. SUPPLEMENTARY ACTIVITIES

9.1. FINANCING AND COLLABORATIONS OF THIS RESEARCH

This work was carried out with the support of CAPES/COFECUB (COMITÊ FRANCÊS DE AVALIAÇÃO DA COOPERAÇÃO UNIVERSITÁRIA COM O BRASIL), Doctorate Scholarship Abroad No. 16/2015 with CAPES Support process 88887.142643/2017-00, Project No. 88887.130187/2017-01.

This thesis project involves a co-tutela agreement between the Pontifícia Universidade Católica do Paraná (PUC/PR) e a Université de Bourgogne (UNB).

9.1.2 PhD SECONDMENT

The second was held at the National University of Ireland Galway, during the period of 6 months (1st June 2019 - 31st November 2019) for the purpose of collaborative research and sharing of advice and experiences.

This visit was made possible by the Marie Skłodowska-Curie research and innovation team, funded by the European Commission Exchange Project (RISE), DISCOVER (MSCA-RISE project number 777995).

Our joint aim during my visit was to identify the mechanism underlying the functions of TNF receptors DR5- and/or DR4 in ER stress-induced apoptosis.

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ANNEX I - CO-AUTHOR ARTICLE

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Original Article

Antitumoral effects of *Amblyomma sculptum* Berlese saliva in neuroblastoma cell lines involve cytoskeletal deconstruction and cell cycle arrest

Efeito antitumoral da saliva do carrapato *Amblyomma sculptum* Berlese em células de neuroblastoma envolve desconstrução do citoesqueleto e parada do ciclo celular

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Abstract

The antitumor properties of ticks salivary gland extracts or recombinant proteins have been reported recently, but little is known about the antitumor properties of the secreted components of saliva. The goal of this study was to investigate the *in vitro* effect of the saliva of the hard tick *Amblyomma sculptum* on neuroblastoma cell lines. SK-N-SK, SH-SY5Y, Be(2)-M17, IMR-32, and CHLA-20 cells were susceptible to saliva, with 80% reduction in their viability compared to untreated controls, as demonstrated by the methylene blue assay. Further investigation using CHLA-20 revealed apoptosis, with approximately 30% of annexin-V positive cells, and G0/G1-phase accumulation (>60%) after treatment with saliva. Mitochondrial membrane potential ($\Delta\psi m$) was slightly, but significantly ($p < 0.05$), reduced and the actin cytoskeleton was disarranged, as indicated by fluorescent microscopy. The viability of human fibroblast (HFF-1 cells) used as a non-tumoral control decreased by approximately 40%. However, no alterations in cell cycle progression, morphology, and $\Delta\psi m$ were observed in these cells. The present work provides new perspectives for the characterization of the molecules present in saliva and their antitumor properties.

Keywords: Tick saliva, animal toxin, tumor cell death, pediatric cancer.

Minireview

An overview of neuroblastoma cell lineage phenotypes and *in vitro* models

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Impact statement

This review provides an update on the mostly used cell line *in vitro* models for neuroblastoma (NB), a heterogeneous disease with high metastatic potential and resistance to treatment. The genetic and phenotypic profiles of the most used NB cell lines in the last 10 years are presented, considering the molecular markers that are involved in the distinct NB tumor phenotypes, including distinct core regulatory circuitries and non-coding RNAs. This gathered information can assist in the selection of the most appropriate NB *in vitro* model, based on the specific goals and objectives of each study.

Abstract

This review was conducted to present the main neuroblastoma (NB) clinical characteristics and the most common genetic alterations present in these pediatric tumors, highlighting their impact in tumor cell aggressiveness behavior, including metastatic development and treatment resistance, and patients' prognosis. The distinct three NB cell lineage phenotypes, S-type, N-type, and I-type, which are characterized by unique cell surface markers and gene expression patterns, are also reviewed. Finally, an overview of the most used NB cell lines currently available for *in vitro* studies and their unique cellular and molecular characteristics, which should be taken into account for the selection of the most appropriate model for NB pre-clinical studies, is presented. These valuable models can be complemented by the generation of NB reprogrammed tumor cells or organoids, derived directly from patients' tumor specimens, in the direction toward personalized medicine.

Keywords: Pediatric cancer, neural crest tumors, neuroblastoma cell lines, SH-SY5Y, tumor stem cells, *in vitro* models

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Introduction

Neuroblastoma (NB) is an extracranial solid tumor in children and comprehends 8% to 10% of all pediatric cancers.[1] It is a heterogeneous disease that presents a broad spectrum of clinical behaviors; in children aged 18 months or older, it is often unresectable or metastatic, requires intensive multimodal therapy, and is associated with a survival rate lower than 50%.[2] On the other side of the spectrum, NB with spontaneous regression without chemotherapy is seen in low-risk subgroups.[3]

NB is derived from cells within the neural crest, likely sympathoadrenal progenitor cells that differentiate to sympathetic ganglion and adrenal catecholamine-secreting chromaffin cells.[4] The presentation and symptoms at diagnosis reflect the tumor location, commonly in the adrenal medulla or anywhere along the sympathetic ganglia. Metastases are present in about 50% of patients at diagnosis, with bone marrow metastases corresponding to 80% of the cases. Metastases are also found in bones and regional lymph nodes, while the involvement of the central nervous system and lungs is rare.[5]

The International Committee of Pathology of Neuroblastoma classifies NB tumors into distinct subtypes, according to histological findings, which include the amount of Schwannian stroma present in the tumor and the mitosis-karyorrhexis index (MKI).[6] Generally, the poorly differentiated or undifferentiated histology confers a worse prognosis to patients. Age is also an important prognostic indicator; in <18-month-old patients, poorly differentiated NB is still considered a favorable prognosis if the MKI is not high; however, in patients aged >18 months, poorly differentiated NB is invariably unfavorable.[7]

ANNEX II – SUBMITTED ARTICLES/UNDER REVIEW

Submitted for publication in the journal *Cancer Immnunology Research* (ISSN: 2326-6066), in December 2020, and is entitled: Neutral sphingomyelinase 2 heightens anti-melanoma immune response and anti-PD-1 therapy efficacy.

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Manuscript Title: Neutral sphingomyelinase 2 heightens anti-melanoma immune response and anti-PD-1 therapy efficacy.
Corresponding Author: Dr. Ségui
Full Author List: Anne Montfort, Florie Bertrand, Julia Rochotte, Julia Gilhodes, Thomas Filleron, Jean Milhès, Carine Dufau, Caroline Imbert, Joëlle Riond, Marie Tosolini, Christopher Clarke, Florent Dufour, Andrei Constantinescu, Nilton de França, Virginie Garcia, Michel Record, Pierre Cordelier, Pierre Brousset, Philippe Rochaix, Sandrine Silvente-Poirot, Nicole Therville, Nathalie Andrieu-Abadie, Thierry Levade, Yusuf Hannun, Hervé Benoist, Nicolas Meyer, Olivier Micheau, Celine Colacios, and Bruno Ségui

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Cancer Immunology Research uses the CRediT taxonomy for author contributions. Assignment of these contribution terms is optional for initial submissions but required for revised submissions. Any contributions assigned to you by Dr. Ségui for this version of the manuscript are listed below. Any corrections must be discussed with the corresponding author, Dr. Ségui.
Nilton de França:
Investigation

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https://cir.msubmit.net/cgi-bin/main.plex?el=A5IO2Blax6B3FwBS7a6A9ftdjKeg7TMrYV1OVKFvASITHwZ

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https://cir.msubmit.net/cgi-bin/main.plex?el=A2IO5Blax2B3FwBS5F7A9ftdj4FKeyO3eYVIVan0GbkW8gZ

Thank you again for submitting your work to *Cancer Immunology Research*. Please feel free to contact our office with any questions throughout the course of consideration.

Sincerely,

ANNEX III - INTERNATIONAL THESIS AGREEMENT IN PORTUGUESE VERSION

CONVÊNIO DE COTUTELA INTERNACIONAL DE TESE

Preâmbulo

Considerando:

Pela Université Bourgogne Franche-Comté:
- **O Código da Educação;**
- **O decreto de 26 de maio de 2016, fixação do quadro nacional para a formação e modalidades levando à emissão de doutorado nacional, MEN;**
- **Os estatutos da Université Bourgogne Franche-Comté; a seguir designados UBFC, sediada na 32 Avenue de l'Observatoire 25000 Besançon, representada pelo Senhor Nicolas CHAILLET, Presidente.**

ENTRE

A Université de Bourgogne, sediada na Esplanade Erasme, 21000 Dijon, representada pela Senhor Alain BONNIN, Presidente;

E

A Pontifícia Universidade Católica do Paraná (doravante referida como PUCPR), instituição de educação superior sem fins lucrativos, neste ato representada por seu Reitor, Prof. Waldemiro Gremski, e pelo Presidente da Associação Paranaense de Cultura - APC, mantenedora da PUCPR, Délcio Afonso Balestrin, ambas com sede na Rua Imaculada Conceição, 1155, CEP 80215-901, Prado Velho, Curitiba, Brasil, inscritas no CNPJ 76.659.820/0003-13 e 76.659.820/0001-51, respectivamente.

decidem o que segue a respeito da preparação de uma tese em cotutela por:

Nilton de França Junior

AB
KC

TÍTULO I - MODALIDADES ADMINISTRATIVAS

ARTIGO 1: Dados do Doutorando;

A presente tese será preparada a partir do ano letivo 2018/2019, por:

Nilton de FRANÇA JUNIOR,

***Nascido em* 04/08/1984 em Curitiba, Paraná, Brasil.**

ARTIGO 2: Matrícula e taxas de matrícula

A matrícula do Doutorando para a tese em cotutela inicia-se durante o ano universitário de 2018/2019. A duração prevista dos trabalhos de pesquisa é fixada em 3 anos, segundo a regulamentação em vigor na França, a contar da data da primeira matrícula de tese na Université Bourgogne Franche-Comté. A defesa da tese está prevista para o ano de 2020. Esta duração só poderá ser prolongada a título excepcional após visto favorável dos dois estabelecimentos e por proposição dos orientadores da tese. Esta solicitação deve ocorrer 6 meses antes da data prevista para conclusão da tese e ser objeto de uma alteração.

O Doutorando deverá ser matriculado nos dois estabelecimentos. As taxas de matrícula serão recebidas pela Université de Bourgogne, para 2018/2019 e pela Pontifícia Universidade Católica do Paraná, para 2018/2019 e 2019/2020.

ARTIGO 3: Modalidades especificas

3.1 - Previdência social e Segura de Responsabilidade civil

O doutorando se compromete a contratar um plano de previdência social e uma apólice de seguro para cobrir riscos de responsabilidade civil para todo o período de formação do doutorado, serviços esses que devem valer tanto na França como no Brasil.
O Sr Nilton de França Junior estará coberto por um plano de seguridade social e de responsabilidade civil privada. Será necessário apresentar os comprovantes de ditos serviços no ato da matrícula.

3.2 - Alojamento

Durante sua estadía na França o doutorando permanecerá no seguinte endereço:

49 Rue Jeannin, 21000 Dijon,

Durante sua estadia no Brasil o doutorando permanecerá no seguinte endereço:

Rua Imaculada Conceição, 1155, Curitiba, Paraná, Brasil, CEP 80215-901.

AB KC

Nilton de FRANÇA JUNIOR

UBFC

JURÍDICO Marcela Grupo Marista

2

TÍTULO II - MODALIDADES PEDAGÓGICAS

ARTIGO 4: Trabalhos de pesquisa

Os trabalhos de pesquisa tratarão do tema: « Investigação das vias de morte induzidas pela saliva de Amblyomma sculptum em células tumorais e TRAIL. »

Os orientadores são:

- **na UBFC :**
  Sr Olivier MICHEAU
  Titulo: Directeur de Recherches 2 (DR2) HDR
  Laboratório: INSERM, UMR 1231 , Laboratório de Lipídeos, Nutrição e Câncer.

- **na PUCPR :**
  Srta Selene ELIFIO ESPOSITO
  Titulo: Professora titular
  Laboratório: Laboratório Experimental Multiusuário.

Os dois orientadores cientes do presente convênio, se comprometem a orientar a preparação da tese em cotutela de maneira conjunta e integral.

O trabalho de pesquisa será realizado alternadamente em ambas as instituições, quando necessário, seguindo um cronograma co-decidido a cada ano pelos dois supervisores.

ARTIGO 5: Atividades de formação

Dentro UBFC, o estudante poderá se beneficiar do treinamento oferecido pelo Pôlo de Pesquisa, facilitando seu rastreamento de carreira, cursos de formação oferecidos pela escola de pós-graduação que permite estabelecer a formação através da investigação, formação técnica e científica oferecida pela Unidade de Pesquisa.

Dentro PUCPR, para obter o título de Doutor pela PUCPR, o doutorando deverá completar os módulos de ensino conforme descritos a seguir:

- **disciplinas de formação, pertencentes ao núcleo obrigatório;**
- **disciplinas eletivas, pertencentes ao núcleo de concentração;**
- **disciplinas do núcleo instrumental;**

ARTIGO 6: Redação da tese

A tese será redigida em Inglês;
A redação deverá ser complementada por um resumo substancial em francês e português.

ARTIGO 7: Defesa da tese

A tese deverá ser defendida uma única vez no Brasil. A defesa será reconhecida por ambas as universidades.
O idioma utilizado na defesa da tese poderá ser português ou inglês.
Os relatores serão nomeados conjuntamente pelas duas universidades em conformidade com as normas de cada uma : 2 relatores externos à UBFC e à PUCPR.

AS
KC

Nilton de FRANÇA JUNIOR

UBFC

JURÍDICO Marcela Grupo Marista

ARTIGO 8: Júri

A autorização para defesa da tese será acordada conjuntamente pelos dois estabelecimentos, após visto favorável de suas instâncias competentes. A banca da tese será designada pelas autoridades legais do estabelecimento onde a tese será defendida, após acordo do estabelecimento co-signatário.
A composição do júri tese deve cumprir, na medida do possível, os regulamentos de ambos os países e permitir, tanto quanto possível, uma representação equilibrada de mulheres e homens. Ela será composta equilibradamente por representantes científicos dos dois países e terá ao menos quatro membros. Ela terá:

- **ao menos a metade de personalidades exteriores aos dois estabelecimentos,**
- **ao menos a metade de professores ou equivalentes (pesquisadores).**

O número de membros da banca não poderá exceder à sete. A composição da banca deverá levar em conta as possibilidades de financiamento das despesas de deslocamento e de hospedagem de todos os membros.
O Presidente da banca deverá ser um professor titular ou equivalente. Ele será designado de acordo com o regulamento da universidade em que a tese será defendida e não deve ser nenhum dos orientadores da tese.

ARTIGO 9: Diplomas

A ata de defesa será redigida na língua do país onde ela será defendida. Em seguida, ela será traduzida em francês. Ela deverá permitir apreciar as aptidões do candidato ao expor seus trabalhos e o domínio que tem do seu tema de pesquisa.
Após o pronunciamento da banca, as instituições universitárias – Universidade de Bourgogne Franche-Comté,(UBFC), concederá o Título de Doutor em Bioquímica e Biologia Molecular e a Pontifícia Universidade Católica do Paraná (PUCPR) concederá o título de Doutor em Ciências da Saúde.

ARTIGO 10: Proteção da propriedade intelectual e publicação

A proteção do objeto da tese, assim como das publicações e da utilização dos resultados de pesquisa comuns às duas universidades, deverão ser assegurados de acordo com os procedimentos específicos de cada país envolvido na cotutela.

ARTIGO 11: Entrega, relatório e reprodução das teses

O Doutorando deverá concordar com as normas em vigor nos dois países para o depósito, a descrição e a reprodução de teses.

AB
KC

Nilton de FRANÇA JUNIOR

UBFC

JURÍDICO Marcela Grupo Marista

TÍTULO III - DISPOSIÇÕES FINAIS

ARTIGO 12: Adendo

O doutorando e seus orientadores, cientes do presente convênio, se comprometem a respeitá-lo; se durante a preparação do doutorado, modificações ou adaptações se fizerem necessárias (prorrogação do prazo, programa de pesquisa), um adendo deverá ser conjuntamente acordado por ambas as universidades signatárias do presente convênio de cotutela.

O presente convênio é estabelecido em 4 cópias originais;

Assinaturas com carimbos dos estabelecimentos:

Curitiba, de 2018.

La Directrice de thèse / *Orientador de tese,*
Prof.ª Selene ELIFIO ESPOSITO

Université Bourgogne Franche-Comté

Le Directeur de thèse /
Orientador de tese,
M. Olivier MICHEAU

Le Directeur du LNC /
Diretor da LNC,
M. Laurent LAGROST

Le Directeur de l'Ecole Doctorale ES /
Responsável da Escola Doutoral ES,
M. Thierry RIGAUD

14 JUIN 2018
Le Président de l'UBFC,
Presidente de UBFC,
Monsieur Nicolas CHAILLET

31 MAI 2018
Le Président de l'Université de Bourgogne,
Présidente da Université de Bourgogne,
Monsieur Alain BONNIN

Le Recteur de PUCPR,
Reitor da PUCPR,
Senhor Waldemiro GREMSKI

Lino Alfonso Jungbluth
CPF 454.515.249-04

Pedro João Wolter
Procurador
CPF: 077.560.709-68

Le Président de L´Associação Paranaense de Cultura
Délcio Afonso BALESTRIN

Le Doctorant / *A Doutorando*
Nilton de FRANÇA JUNIOR

Le Directeur de l'internationalisation/
Diretor da Internacionalização
Dr. Marcelo Távora MIRA

ANNEX IV - INTERNATIONAL THESIS AGREEMENT, FRENCH VERSION

CONVENTION DE COTUTELLE INTERNATIONALE DE THESE

Préambule

Vu:

pour l'Université Bourgogne Franche-Comté :

- **Le Code de l'Education ;**
- **L'arrêté du 25 mai 2016 fixant le cadre national de la formation et les modalités conduisant à la délivrance du diplôme national de doctorat, MEN ;**
- **Les Statuts de l'Université Bourgogne Franche-Comté; ci-après dénommée UBFC, sise au 32 avenue de l'Observatoire 25000 Besançon, représentée par son président, Monsieur Nicolas CHAILLET.**

ENTRE

L'Université de Bourgogne, sise Esplanade Erasme, 21000 Dijon, représentée par Monsieur Alain BONNIN, Président,

ET

La Pontifícia Universidade Católica do Paraná, établissement à but non lucratif, ici représentée par son Recteur, Prof. Dr. Waldemiro Gremski, et par le Président de l'Associação Paranaense de Cultura – APC, entreteneur de la PUCPR, Mr. Délcio Afonso Balestrin, les deux avec leurs sièges au-près de la Rue Imaculada Conceição, 1155 – Prado Velho, Curitiba, Paraná, Brésil 80215-901 avec leurs respectifs numéros de référence fiscaux 76.659.820/0003-13 et 76.659.820/0001-51.

arrêtent les dispositions suivantes concernant la préparation d'une thèse en cotutelle par:

Nilton de França Junior

Nilton de FRANÇA JUNIOR

JURÍDICO Marcela Grupo Marista

TITRE I – MODALITÉS ADMINISTRATIVES

ARTICLE 1: Renseignements sur le Doctorant

Cette thèse est préparée à partir de l'année universitaire 2018/2019, par :

Nilton de França Junior,

né le 04/08/1984 à Curitiba (Paraná, Brésil), de nationalité brésilienne.

ARTICLE 2: Inscription et droits d'inscriptions;

L'inscription du Doctorant pour une thèse en cotutelle prend effet pendant l'année universitaire 2018/2019. La durée prévisionnelle des travaux de recherche est fixée à 3 ans à compter de la date de la première inscription en thèse à l'Université Bourgogne Franche-Comté. La soutenance de la thèse est prévue au cours de l'année 2020. Cette durée ne pourra être prolongée qu'à titre exceptionnel après avis favorable des deux établissements et sur proposition des directeurs de thèse. Cette demande doit intervenir 6 mois avant la date prévisionnelle de fin de thèse.

Le doctorant devra être inscrit dans les deux établissements. Les droits d'inscription seront perçus par l'Université de Bourgogne, pour 2018/2019. Les droits d'inscription seront perçus par l'Université Pontificale Catholique du Paraná, pour 2018/2019 et 2019/2020.

ARTICLE 3: Modalités particulières

3.1 - Sécurité Sociale et Assurance Responsabilité civile;

Le doctorant s'engage à souscrire une couverture sociale et une assurance responsabilité civile durant toute la durée de ses études doctorales, le couvrant tant en France qu'en Brésil.
Monsieur Nilton de França Junior est affiliée au régime général de sécurité sociale et souscrit à une Assurance Responsabilité Civile privée.
Les justificatifs correspondants seront produits au moment de l'inscription.

3.2 - Hébergement

Lors de son séjour en France, le doctorant résidera à l'adresse suivante:

49 Rue Jeannin, 21000 Dijon

Lors de son séjour en Brésil, le doctorant résidera à l'adresse suivante :

Rua Imaculada Conceição, 1155, Curitiba, Paraná, Brasil, CEP 80215-901.

AB

Nilton de FRANÇA JUNIOR

UBFC

JURIDICO Marcela Grupo Marista

TITRE II – MODALITÉS PÉDAGOGIQUES

ARTICLE 4: Travaux de recherche

Les travaux de recherche porteront sur : « Recherche sur les voies de la mort induite par la salive Amblyomma dans les cellules tumorales et TRAIL. »

Les directeurs de recherche seront:

- **à l'UBFC:**
  Monsieur / Olivier MICHEAU
  Titre et qualité: Directeur de Recherches 2 (DR2) HDR
  Laboratoire: INSERM, UMR 1231 , Laboratoire Lipides Nutrition Cancer.

- **à la PUCPR:**
  Mademoiselle : Selene ELIFIO ESPOSITO
  Titre et qualité : Professeur titulaire
  Laboratoire : Laboratoire Expérimental Multi-utilisateur.

Les deux directeurs de thèse, qui ont pris connaissance de cette convention, s'engagent à exercer conjointement et pleinement l'encadrement de cette thèse en cotutelle.

Les travaux de recherche seront effectués en alternance dans les deux établissements selon un calendrier élaboré chaque année, en tant que besoin, conjointement par les deux directeurs de thèse.

ARTICLE 5: Activités de formations

Au sein d'UBFC, le doctorant peut bénéficier à la fois de formations proposées par le Pôle Recherche facilitant sa poursuite de carrière, de formations proposées par son école doctorale permettant d'asseoir sa formation par la recherche, et de formations techniques et scientifiques proposées par l'Unité de Recherche.

Au sein de PUCPR, pour obtenir le titre de Docteur de la PUCPR, le doctorant devra valider les modules d'enseignement décrits ci-dessous:

- **disciplines d'éducation, appartenant au cours obligatoires**
- **disciplines au choix, appartenant aux cours de concentration,**
- **disciplines des cours fondamentaux**

ARTICLE 6 : Rédaction de la thèse

Le mémoire de thèse sera rédigé en Anglais. La rédaction devra être complétée par un résumé substantiel en langue française et portugaise.

ARTICLE 7 : Soutenance de la thèse

La thèse donnera lieu à une soutenance unique au Brésil. La soutenance sera reconnue par les deux établissements.
La langue utilisée pour la soutenance sera le Portugais ou Anglais.
Les rapporteurs seront désignés conjointement par les deux établissements et conformément aux règles des deux établissements : 2 rapporteurs extérieurs à l'UBFC et de la PUCPR.

AS

Nilton de FRANÇA JUNIOR

UBFC

JURÍDICO Marcela Grupo Marista

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ARTICLE 8 : Jury

L'autorisation de soutenance de la thèse est accordée conjointement par les deux établissements, après avis favorable de leurs instances compétentes. Le jury de la thèse est désigné par les autorités légales de l'établissement où la thèse sera soutenue, après accord de l'établissement cosignataire.
La composition du jury de soutenance doit respecter, dans la mesure du possible, la réglementation en vigueur des deux pays et permettre, autant que possible, une représentation équilibrée des femmes et des hommes. Il est composé sur la base d'une proportion équilibrée par des représentants scientifiques des deux pays et comprend au moins quatre membres. Il comprend :

- **au moins la moitié de personnalités extérieures aux deux établissements.**
- **au moins la moitié de professeurs ou assimilés (directeurs de recherche).**

Le nombre des membres du jury ne peut excéder sept. La composition du jury devra prendre en compte les possibilités de financement des frais de déplacement et d'hébergement de tous les membres.
Le Président du Jury doit être un Professeur ou équivalent. Il sera désigné selon La réglementation de l'Université où la thèse sera soutenue et ne devra pas être l'un des co-directeurs de thèse.

ARTICLE 9: Diplômes

Le rapport de soutenance sera établi dans la langue du pays où la thèse sera soutenue. Il sera ensuite traduit en français. Il devra permettre d'apprécier les aptitudes du candidat à exposer ses travaux et la maîtrise qu'il a de son sujet de recherche.
Après admission prononcée par le jury, l'UBFC décernera le grade de « Docteur de la Communauté d'Université et d'Etablissements - Université Bourgogne Franche-Comté, *dans* Biochimie Biologie Moléculaire et l'établissement PUCPR cocontractant décernera le titre de Docteur en Science de la Santé.

ARTICLE 10 : Protection intellectuelle et Publication

La protection du sujet de thèse ainsi que les publications, l'exploitation et la protection des résultats de recherche communs aux deux laboratoires d'accueil du doctorant doivent être assurées conformément aux procédures spécifiques à chaque pays impliqué dans la cotutelle.

ARTICLE 11: Dépôt, signalement et reproduction des thèses

Le doctorant devra se conformer aux règles en vigueur dans les deux pays pour le dépôt, le signalement et la reproduction des thèses.

Nilton de FRANÇA JUNIOR

JURÍDICO Marcela Grupo Marista

TITRE III – DISPOSITIONS FINALES

ARTICLE 12 : Avenant

Le doctorant et les directeurs de thèse ayant pris connaissance de cette convention s'engagent à en respecter les termes ; en cas de modifications ou d'adaptations rendues nécessaires (durée, programme de recherche) durant la préparation du doctorat, un avenant devra être adopté conjointement par les deux établissements signataires de la présente convention de cotutelle.

La présente convention est établie en 4 exemplaires originaux,

Visa des autorités pédagogiques de chaque université /
Visto das autoridades pedagógicas de cada universidade

A Pontifícia Universidade Católica do Paraná

La Directrice de thèse / *Orientador de tese,*
Prof.ª Selene ELIFIO ESPOSITO

Université Bourgogne Franche-Comté

Le Directeur de thèse /
Orientador de tese,
M. Olivier MICHEAU

Le Directeur du LNC /
Diretor da LNC,
M. Laurent LAGROST

Le Directeur de l'Ecole Doctorale ES /
Responsável da Escola Doutoral ES,
M. Thierry RIGAUD

14 JUIN 2018
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Presidente de UBFC,
Monsieur Nicolas CHAILLET

31 MAI 2018
Le Président de l'Université de Bourgogne,
Presidente da Université de Bourgogne,
Monsieur Alain BONNIN

Le Recteur de PUCPR,
Reitor da PUCPR,
Senhor Waldemiro GREMSKI
Lino Afonso Jungbluth
CPF 454.515.249-04

Pedro João Wolter
Procurador
CPF: 077.560.709-68

Le Président de L´Associação
Paranaense de Cultura
Délcio Afonso BALESTRIN

Le Doctorant / *A Doutorando*
Nilton de FRANÇA JUNIOR

Le Directeur de l'internationalisation/
Diretor da Internacionalização
Dr. Marcelo Távora MIRA

ANNEX V - LETTER OF INVITATION, PhD SENCONDEMENT.

DISCOVER

OÉ Gaillimh
NUI Galway

Nilton De Franca,
INSERM, UMR1231
Dijon
30th April 2019

RE: Letter of invitation

Dear Nilton De Franca,

On behalf of the National University of Ireland Galway I wish to invite you visit our University for a period of 6 months (1st June 2019 – 31st Nov 2019) for the purpose of collaborative research and sharing of advice and experiences which would be of benefit to our research team/students.

This visit is made possible by the European Commission funded Marie Skłodowska-Curie Research and Innovation Staff Exchange (RISE) project, DISCOVER (MSCA-RISE project number 777995). In the RISE programme, you will remain an employee of your home institution (INSERM) and will return at the end of the visit.

Your visit will bring valuable expertise in Molecular and cellular biology, especially in the field of the molecular pathways regulating the interaction between the immune system and cancer cells. During your visit, my laboratory will benefit from your expertise in genetics, protein biochemistry and cell death assays to progress our project funded by the European Union (H2020 DISCOVER RISE project) aiming at understanding the role of tumour necrosis factor receptors (TNF) and how they can be utilized to eradicate cancer cells. will help be valuable for the RISE project. Our joint aim during your visit is to identify the mechanistic underlying the functions of the TNF receptors DR5- and/ or DR4 in ER-stress induced apoptosis.

NUI Galway will provide you with the necessary required office space, support and access to all University facilities. In addition, you will be introduced to and collaborate with our colleagues across the higher education sector in Ireland.

Your travel and accommodation expenses (2,000 Euro per month) will be provided for as detailed in the DISCOVER RISE grant agreement.

I look forward to your joining us in Galway.

Yours Sincerely,

Eva Szegezdi

Eva Szegezdi, Ph.D.
Research Lecturer in Cancer Biology
PI Cancer Niche Group
Director of Blood Cancer Biobank Ireland Apoptosis Research Centre
National University of Ireland
Galway
T: + 353 (0)91 495037
Email: eva.szegezdi@nuigalway.ie