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Insights into the molecular mechanisms of apoptosis induced by glucose deprivation

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"INSIGHTS INTO THE MOLECULAR MECHANISMS OF APOPTOSIS INDUCED BY GLUCOSE DEPRIVATION"

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RESUMEN

Las células cancerosas se caracterizan por una reprogramación metabólica que está altamente regulada por oncogenes y genes supresores de tumores que podrían ser objeto de una posible terapia tumoral (Iurlaro et al., 2014). Definir los mecanismos moleculares de la muerte inducida por privación de glucosa es muy importante porque todas las células tumorales dependen de la glucosa, no sólo para producir energía, sino también para construir todas las moléculas necesarias para proliferar rápidamente como nucleótidos, proteínas y ácidos grasos (DeBerardinis et al., 2008). El estudio de dichos mecanismos nos puede ayudar también a comprender otras condiciones fisiológicas caracterizadas por la privación de glucosa y oxígeno, tales como la isquemia.

Quitar la glucosa a las células tumorales o tratarlas con medicamentos antiglucolíticos puede dar como resultado diferentes tipos de muerte celular, tales como necrosis o apoptosis, a través de la vía mitocondrial (altamente regulada por las proteínas de la familia Bcl-2) o a través de la vía extrínseca de la apoptosis (El Mjiyad et al., 2011). Nuestro grupo describió un tipo particular de muerte celular que se produce en fibroblastos embrionarios de ratón (MEFs, del inglés: Mouse Embryonic Fibroblasts) deficientes en Bax y Bak sometidos a privación de glucosa. Esta línea celular no podía morir por la vía mitocondrial de la apoptosis, pero moría de una forma dependiente de la caspasa-8 e independiente de los ligandos de muerte que inician la vía extrínseca de la apoptosis (Caro-Maldonado et al., 2010). El objetivo principal de esta tesis es aclarar el mecanismo molecular de la muerte celular por privación de glucosa, con un enfoque particular en la descripción de las posibles plataformas implicadas en la activación de la caspasa-8, en diferentes tipos de células.

Previamente demostramos que MEFs deficientes en Bax y Bak no pueden morir a través de la vía mitocondrial y también que células HeLa que sobreexpresan Bcl-XL no están protegidas de la muerte. Se ha mostrado también que las HeLa mueren de una forma dependiente de caspasa-8 cuando se someten a privación de glucosa, ya que el silenciamiento de esta proteína protege de la muerte (Caro-Maldonado et al., 2010). Además, estos datos indican que las células con una ruta mitocondrial funcional pueden sufrir muerte dependiente de caspasa-8 tras quitarles la glucosa.

Primero se realizó el análisis de la muerte celular mediante incorporación de yoduro de propidio (IP) y se observó que células HeLa sometidas a privación de glucosa mueren en un 40-50% a las 48 horas de tratamiento, mientras que están protegidas de la muerte al tratarlas con el inhibidor de caspasas Q-VD. Para asegurarse de que esta muerte se debió a las caspasas apoptóticas y no a las caspasas inflamatorias, las células fueron tratadas con Y-VAD, un inhibidor de la caspasa-1, que no pudo evitar la muerte celular, lo que confirma nuestras afirmaciones. También confirmamos la activación de caspasas por western blot observando el corte de la caspasa-3 y de PARP.
Para tener resultados más consistentes, hemos comprobado si la privación de glucosa podría inducir también muerte celular dependiente de caspasa-8 en otras líneas celulares. Realizamos los mismos experimentos en células HCT116 deficientes en Bax y Bak que no pueden morir por la vía mitocondrial. Esta línea celular también fue protegida por el inhibidor de caspasas Q-VD y por el silenciamiento de la caspasa-8.

Para definir qué plataforma está activando la caspasa-8 bajo privación de glucosa, buscamos en HeLa si algún componente del sistema inmunitario innato podría estar involucrado. En concreto, algunas proteínas implicadas en la detección del ARN de doble cadena de virus como la

RIG-I, MDA5 y MAVS, han sido descritas por activar la caspasa-8 durante infección viral y dar como resultado la producción de citoquinas y la activación de la respuesta inflamatoria (Rajput et al., 2011). Recientemente, otro grupo ha demostrado que células humanas o de ratón deficientes en Bax y Bak estaban protegidas de la infección por el virus Semliki Forest (SFV) cuando MAVS y la caspasa-8 se silenciaban (El Maadidi et al., 2014). Nos centramos en el estudio de MAVS basándonos en este estudio. Para ello, silenciamos MAVS en células HeLa utilizando diferentes secuencias de siRNAs. Sin embargo, los resultados no fueron interesantes ya nunca pudimos observar protección de la muerte por privación de glucosa. Estos datos nos llevaron a descartar esta hipótesis.

Otra plataforma posiblemente involucrada en la activación de la caspasa-8, en nuestro modelo, podría ser la plataforma del Ripoptosoma, descrita recientemente en la literatura. Dos grupos diferentes han demostrado que tanto el estrés genotóxico inducido por etopósido como la estimulación del TLR3 inducen la formación de una plataforma de alto peso molecular formada por la caspasa-8 o la 10, RIPK1, FADD y FLIP, la cual puede inducir apoptosis dependiente de la caspasa-8 o necroptosis dependiente de RIPK1 y RIPK3 (Feoktistova et al., 2011; Tenev et al., 2011). La actividad de la quinasa RIPK1 es esencial para la formación del Ripoptosoma, así como la inhibición de las proteínas cIAP, observada en ambos modelos. Esta plataforma se forma de manera espontánea en el citoplasma y no es dependiente de los ligandos de muerte.

Anteriormente, habíamos demostrado que MEFs deficientes en Bax y Bak morían de manera independiente de los ligandos de muerte y que el silenciamiento de RIPK1 no impedía la muerte debida a la privación de glucosa (Caro-Maldonado et al., 2010).

Como se ha comentado antes, una característica esencial para la formación del Ripoptosoma es la inhibición de las cIAPs. Se verificó por westren blot que, en células HeLa, la privación de glucosa induce una

clara bajada de los niveles de cIAP1 y cIAP2, lo que nos llevó a pensar en un posible papel de esta plataforma en la inducción de la muerte en nuestro sistema. En experimentos de silenciamiento de RIPK1 no se pudo proteger de la muerte celular tras la retirada de glucosa. Además, no se pudo demostrar la interacción de la caspasa-8 con RIPK1 en experimentos de inmunoprecipitación, tanto en HeLa como en los fibroblastos murinos deficientes en Bax y Bak. Estos datos sugieren que esta plataforma no es la causa de la activación de la caspasa-8 en nuestro modelo de privación de glucosa.

Otra hipótesis a estudiar era si la plataforma autofagosomal o iDISC (recientemente descrita en la literatura) podría tener un papel en la activación de la caspasa-8 bajo privación de glucosa.
Diferentes grupos han descrito que tras la inhibición del proteasoma, una plataforma de membranas autofagosomales está involucrada en el reclutamiento y activación de la caspasa-8 en diferentes tipos celulares (Laussmann et al., 2011; Pan et al., 2011; Young et al., 2012). La caspasa-8 puede formar un complejo con Atg5, FADD, LC3 y p62, siendo LC3 y p62 esenciales para la activación de la caspasa-8 y la consiguiente apoptosis. Anteriormente habíamos demostrado que, tanto en MEFs deficientes en Bax y Bak como en células HeLa sometidas a retirada de glucosa, se producía una acumulación de las proteínas de la autofagia, p62 y la forma lipidada de LC3, LC3-II, debido a una inhibición del flujo autofágico por privación de glucosa (Ramírez-Peinado et al., 2013). También demostramos que el silenciamiento de p62 en MEFs deficientes en Bax y Bak no impedía la muerte debida a la retirada de glucosa, lo que sugería que esta plataforma no es esencial para la inducción de la muerte celular en esas condiciones (Ramírez-Peinado et al., 2013). Decidimos comprobar si estas proteínas autofágicas podrían tener un papel en el reclutamiento y la activación de la caspasa-8 en condiciones de privación de glucosa, en células humanas. Para ello, realizamos experimentos de inmunoprecipitación de la caspasa-8 en condiciones normales o bajo

retirada de glucosa, en las células HeLa, y pudimos observar una clara interacción de LC3 y p62 con la caspasa-8 sólo cuando la glucosa había sido retirada del medio. Para aclarar la función real de esta plataforma en células HeLa, se llevaron a cabo experimentos de silenciamiento de p62 utilizando dos secuencias diferentes. En ambos casos el silenciamiento de la proteína no pudo prevenir la muerte debida a la privación de glucosa.

Estos resultados sugieren que algo más, diferente de la plataforma autofagosomal, está induciendo la muerte celular debida a la privación de glucosa en nuestros modelos celulares.

Todavía existe la posibilidad de que componentes clásicos del DISC, como FADD o FLIP, puedan ser esenciales en la activación o inhibición de la caspasa-8. Hemos investigado si FADD o TRADD podrían tener un papel activo en el reclutamiento de caspasa-8 en HeLa sometidas a privación de glucosa. Experimentos de inmunoprecipitación de la caspasa-8 indicaron una clara interacción con FADD en células HeLa, en dichas condiciones. Además, en situaciones de privación de glucosa y de muerte inducida por el ligando de Fas, el silenciamiento de FADD pudo restablecer la viabilidad celular de forma similar. Sin embargo, el silenciamiento de TRADD no bloqueó la muerte celular por retirada de glucosa, lo que sugiere que FADD, pero no TRADD, es esencial en la inducción de la caspasa-8 y la consecuente apoptosis, en células HeLa sometidas a privación de glucosa.

Tratamos de aclarar el papel de FLIP, el inhibidor específico de la caspasa-8, en la muerte celular debida a privación de glucosa. Anteriormente, nuestro grupo había demostrado que la sobreexpresión de FLIP en MEFs deficientes en Bax y Bak, no inhibía la muerte inducida por la eliminación de glucosa, lo que sugiere que FLIP no tiene ningún papel en la inhibición de la apoptosis en nuestro sistema (Caro-Maldonado et al., 2010). En este sentido, expresamos constitutivamente FLIP murino en células HeLa y no pudimos observar una reducción de la muerte por falta

de glucosa. Sin embargo, se observó por western blot que la eliminación de glucosa induce una bajada en los niveles de FLIP, indicando que la desregulación de FLIP podría jugar un papel en la activación de la caspasa-8 en nuestro modelo. Este dato nos lleva a reconsiderar cuidadosamente nuestras primeras conclusiones.

Por otra parte, se ha descrito que la privación de glucosa puede inducir estrés del retículo endoplásmico en diferentes líneas celulares (León-Annicchiarico et al., 2015) debido probablemente a una acumulación de proteínas mal plegadas, en particular las que no han sido bien glicosiladas. Estas proteínas mal plegadas son detectadas por GRP78, que libera y activa tres receptores localizados en el retículo endoplásmico: ATF6, IRE1 y PERK, involucrados en la respuesta de proteínas mal plegadas (del inlgés: *unfolded protein response*) y que tratan de superar dicho estrés. En el caso de que este sea demasiado grande o persistente, también puede derivar en muerte celular.
Hemos detectado por western blot que en todas las líneas celulares analizadas, HeLa, fibroblastos murinos y HCT116 deficientes en Bax y Bak, hay un aumento considerable de ATF4 y CHOP, dos marcadores de estrés reticular, tras la privación de glucosa. ATF4 es un factor de transcripción importante que induce y regula la expresión de proteínas chaperonas necesarias para superar el estrés, pero puede resultar en muerte celular mediada por CHOP.

Recientemente, diferentes grupos han demostrado que los inductores del estrés del retículo endoplásmico pueden inducir apoptosis debida a la sobreexpresión del receptor TRAIL-R2, conocido también como receptor DR5, siendo regulado directamente por CHOP en su promotor (Yamaguchi and Wang, 2004). Se mostró el año pasado que los inductores del estrés del retículo endoplásmico inducen la trascripción del receptor DR5 mediada por CHOP, mientras que el sensor IRE1α induce la degradación de su mensajero, permitiendo que las células se adapten

al estrés (Lu et al., 2014). También la expresión de TRAIL-R1 o DR4 se ha relacionado con la muerte inducida por estrés reticular en células de cáncer humano (Li et al., 2015), por lo que nos preguntamos si también en nuestro modelo de privación de glucosa podría existir una inducción de receptores de muerte debida al persistente estrés del retículo endoplásmico.

Realizamos western blot de los receptores de muerte DR4, DR5, Fas y TNFR1 en células HeLa y observamos que los receptores DR4 y DR5 están claramente inducidos tras la privación de glucosa a las 24 horas de tratamiento. Los receptores Fas y TRNF1 no están claramente sobreexpresados pero podemos ver un cambio en su tamaño debido, tal vez, a una diferente glicosilación que podría estar involucrada en su activación. También quisimos hacer análisis de q-PCR para definir si los niveles de los mensajeros de dichos receptores de muerte se incrementaban bajo privación de glucosa. Hemos detectado un aumento en los niveles del mensajero del receptor DR5 a solo 8 horas de la retirada de glucosa, pero no se detectaron cambios significativos en los demás receptores de muerte.

Dado que diferentes grupos han demostrado que fármacos inductores del estrés reticular como la tunicamicina o la tapsigargina, inducen muerte celular debido a la sobreexpresión del receptor DR5 dependiente de CHOP, quisimos investigar si en nuestro modelo el estrés del retículo endoplásmico pudiera ser la causa de la inducción del receptor DR5. Por esa razón, decidimos silenciar ATF4 y CHOP en células HeLa para comprobar si estas proteínas estaban realmente implicadas en la inducción de los receptores de muerte y por consiguiente, en la muerte celular. El silenciamiento de ATF4, pero no el de CHOP, bloquea de forma significativa la muerte celular por privación de glucosa. La inducción del receptor DR5 también se vió afectada en esas condiciones. Estos datos indican que ATF4 es un inductor clave del receptor DR5 y de la apoptosis observados en situaciones de falta de glucosa.

Por otra parte, investigamos si el receptor DR5 es esencial en la muerte celular por privación de glucosa en células HeLa. Por lo tanto, decidimos silenciar el receptor DR5 utilizando diferentes secuencias dirigidas a la isoforma larga o a la isoforma corta de la proteína. Pudimos obtener una protección significativa solo con una de las secuencias contra la isoforma larga del receptor DR5, que podría ser la que está jugando un papel importante en la muerte debida al estrés reticular, como se ha descrito anteriormente (Lu et al., 2014). El mismo experimento se hizo en células HeLa que sobreexpresan de forma estable Bcl-xL, de manera que tengan bloqueada la ruta mitocondrial. En estas células, tres secuencias distintas frente a la isoforma larga del receptor DR5 pudieron proteger de la muerte, sugiriendo que la ruta mitocondrial puede jugar un papel importante en la muerte debida a la falta de glucosa en células HeLa. Una vez bloqueada esta ruta, las células se hacen más susceptibles a la muerte por los receptores de muerte. También silenciamos el receptor DR5 en células HCT116 deficientes en Bax y Bak donde una secuencia frente a la isoforma larga y una frente a la corta impidieron la muerte por privación de glucosa, indicando que la importancia de una isoforma frente a la otra en la muerte por falta de glucosa puede depender del tipo celular.

También realizamos experimentos de inmunofluorescencia del receptor DR5 en células HeLa sometidas a privación de glucosa para estudiar su localización durante el tratamiento. Previamente se había demostrado que en condiciones de estrés reticular, el receptor DR5 se localiza en el retículo endoplásmico o en el aparato de Golgi (dependiendo del tipo celular), donde podría estar reclutando el DISC para su activación (Lu et al., 2014). Nuestros experimentos de inmunofluorescencia en células HeLa mostraron que el receptor DR5 se localiza en el aparato de Golgi después de la retirada de glucosa y forma estructuras más compactas donde tal vez puede estar acumulándose y activándose.

Por otra parte, decidimos silenciar los receptores de muerte DR4, TNFR1 y Fas para comprobar un posible papel de estos receptores en la inducción de la muerte celular por privación de glucosa. Datos preliminares indican que estos receptores no juegan un papel importante en nuestro modelo, aunque su relevancia esté todavía por aclarar.

Por último, para asegurarnos de que la inducción de los receptores DR5 y DR4 no es dependiente del ligando de muerte, silenciamos TRAIL en células HeLa y ninguna de la secuencias utilizadas pudo evitar la muerte celular, lo que sugiere que la inducción de los receptores se produce independientemente de su ligando.

Diferentes grupos han demostrado que el tratamiento con TRAIL y ligando de Fas en diferentes tipos celulares puede inducir apoptosis dependiente de caspasa-8, donde la proteasa foma agregados entre sus dominios DED, los cuales son esenciales para su activación (Dickens et al., 2012; Schleich et al., 2012).

Observamos en experimentos de inmunofluorescencia que la caspasa-8 en fibroblastos murinos deficientes en Bax y Bak cambia su patrón después de la privación de glucosa. En primer lugar, el tratamiento induce un aumento en la intensidad de la señal de la caspasa-8 y, además, forma unos filamentos similares a los descritos en la literatura por Siegel, en 1998 y recientemente, por Dickens y Schleich. Los agregados de caspasa-8 observados tras la retirada de glucosa no co-localizan con los filamentos de actina. Sin embargo, hemos detectado un gran porcentaje de co-localización de estos filamentos de muerte con la β-tubulina, siendo mayor en las muestras tratadas que en los controles.

Experimentos de inmunoprecipitación de la caspasa-8 en esta línea celular también indican una débil interacción con la tubulina solo en las muestras privadas de glucosa. Todo este conjunto de datos muestra que la caspasa-8, al menos en esta línea celular, podría ser reclutada sobre un andamio de microtúbulos, que podría ser importante para su oligomerización y completa activación.

Finalmente, decidimos realizar un análisis de espectrometría de masas para detectar de forma cuantitativa cuáles eran las proteínas que interactúan con la caspasa-8 inmunoprecipitada y FADD tras privación de glucosa, tratando de definir los componentes de la plataforma involucrada en la activación de la caspasa-8 en nuestro modelo.

Hemos diseñado las mejores condiciones para precipitar la caspasa-8 y FADD en células HeLa, en condiciones normales y en condiciones de falta de glucosa, y las muestras se han sometido a un análisis de espectrometría de masas en la plataforma de proteómica IDIBELL.

Los puntajes más altos obtenidos con este análisis, para ambas proteínas inmunoprecipitadas, eran de componentes del citoesqueleto, como la espectrina, miosina y actina, cuyos niveles aumentan en los inmunoprecipitados después de la eliminación de la glucosa. Estos datos indican que también en células HeLa una plataforma del citoesqueleto podría jugar un papel importante en el reclutamiento y la activación de la caspasa-8.

Otro hallazgo importante en el análisis de espectrometría de masas es la presencia de un efector importante del estrés del retículo endoplásmico en ambas muestras inmunoprecipitadas, el GRP78, que se incrementa mucho después de la privación de glucosa. Son necesarios más análisis para confirmar estas observaciones y verificar si la interacción de estas proteínas con la caspasa-8 y FADD es real o por el contrario, es resultado de un artefacto debido a un aumento general en la abundancia de estas proteínas tras la retirada de glucosa.

Todos nuestros datos indican que diferentes plataformas podrían tener un papel fundamental en la activación de la caspasa-8 en células tumorales tras la privación de glucosa. La comprensión del mecanismo molecular de la muerte debida a la falta de glucosa podría ser esencial para desarrollar nuevas estrategias terapéuticas frente al cáncer.

I. ABBREVIATIONS

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2-DG: 2-Deoxy-D-glucose

AIF: Apoptosis-inducing factor

AMP: Adenosine monophosphate

AMPK: AMP-activated protein kinase

APAF-1: Apoptotic protease activating factor-1

ATF4/6: Activating transcription factor 4 or 6

Atg: Autophagy-related gene

ATP: Adenosine monophosphate

Bad: Bcl-2-associated agonist of cell death

Bak: Bcl-2-antagonist/killer

Bax: Bcl-2-associated x protein

Bcl-2: B-cell lymphoma 2

BcL-xL: B-cell lymphoma-extra large

BH: Bcl-2 homology

Bid: BH3 interacting domain death agonist

Bim: Bcl-2 interacting mediator of cell death

CARD: Caspase recruitment domain

c-FLIP: C-FLICE inhibitory protein

CHOP: CCAAT/enhancer-binding-protein homologous protein

CRD: Cysteine rich domain

CUL3: Cullin 3

DAPI: 4', 6-Diamidino-2-phenylindole

DED: Death effector domain

DISC: Death-inducing signaling complex

DMEM: Dulbecco’s Modified Eagle’s Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DR: Death receptor

eIF2α: Eukaryotic initiation factor 2α

ER: Endoplasmic reticulum

ERAD: ER-associated degradation

FADD: Fas associated death domain

FBS: Fetal bovine serum

GRP78: Glucose regulated protein 78

HIF-1: Hypoxia-inducible factor 1

IAP: Inhibitor of apoptosis protein

IP: Immunoprecipitation

IRE1α: Inositol-requiring protein-1α

IRF3: Interferon refulatory factor 3

JNK: Jun N-terminal kinase

LC3: Microtubule-associated protein 1 light chain 3

MAVS: Mitochondrial antiviral signaling protein

Mcl-1: Myeloid cell leukemia 1

MDA5: Melanoma differentiation associated gene-5

MEFs: Mouse embryonic fibroblasts

mTOR: Mammalian target of rapamycin

NF-kB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells

OXPHOS: Oxidative phosphorylation

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PERK: Protein kinase RNA-like endoplasmic reticulum kinase

PI: Propidium iodide

PI3K: Phosphoinositide 3-kinase

PTEN: Phosphatase and tensin homolog

Puma: p53 upregulated modulator of apoptosis

RIG-I: Retinoic acid inducible gene-I

RIPK1: Receptor interacting protein kinase 1

RNA: Ribonucleic acid

SDS: Sodium dodecyl sulfate

siRNA: Small interfering RNA

SMAC: Second mitochondria-derived activator of caspase

TBS: Tris-buffered saline

TLR3/7/8: Toll-like receptors 3/7 or 8

TNF: Tumor necrosis factor

TNFR1: Tumor necrosis factor receptor 1

TNFSF: Tumor necrosis factor superfamily

TRADD: TNFR associated death domain

TRAF2/5: TNFR associated factor 2 or 5

TRAIL: TNF related apoptosis inducing ligand

TRIS: Trishydroxymethylaminomethane

UPR: Unfolded protein response

UPS: Ubiquitin proteasome system

XBP-1: X-box binding protein-1

Y-VAD-fmk: Tyr-Val-Ala-Asp(OMe) fluoromethylketone

Z-VAD-fmk: Z-Val-Ala-Asp (OMe) fluoromethylketone

II. SUMMARY

II. SUMMARY

Tumor cells undergo a complete metabolic reprogramming which allows them to grow and proliferate fast. The metabolic changes are driven by oncogenes and tumor suppressors and are aimed to increase glycolysis and biosynthetic pathways and to reduce oxidative phosphorylation.

Particularly, cancer cells rely on glucose not only to produce energy but also to synthesize bio-blocks necessary for their proliferation. They prefer to perform glycolysis instead of oxidative phosphorylation even in presence of oxygen, making them more sensitive to glucose deprivation. Treatment with anti-glycolytic drugs, such as 2-deoxyglucose, or glucose withdrawal have been shown to sensitize to radiotherapy or chemotherapy or to TRAIL stimulation, making these treatments good candidates for cancer therapy.

Glucose deprivation can induce different types of cell death, such as apoptosis, generally through the mitochondrial pathway, or necrosis. Tumor cells have developed several mechanisms to escape the mitochondrial pathway of apoptosis. For instance, mutations in anti or pro-apoptotic Bcl-2 family proteins are highly frequent in cancers. This would allow them to be more resistant to nutrient starvation.

Previously, we described that Bax/Bak deficient MEFs subjected to glucose withdrawal die in a caspase-8 dependent manner and independently of the mitochondrial pathway and of death receptor/ligand interaction. Knockdown of caspase-8 prevented cell death due to glucose starvation also in HeLa cells.

This thesis focuses on understanding the molecular mechanisms of apoptosis induced by glucose deprivation in different cell lines. We aimed at defining what platform is activating caspase-8 under glucose

deprivation, which seems to be different from the canonical DISC formed upon death ligand initiation.

Firstly, we described that HeLa cells subjected to glucose deprivation die by caspase-dependent apoptosis because co-treatment with the pan-caspase inhibitor Q-VD but not the inflammatory caspase inhibitor Y-VAD protects from this death. We also showed that Bax/Bak deficient HCT116 cells are protected from death due to glucose withdrawal by Q-VD and caspase-8 silencing, although in this cell line the protection is partial.

We tried then to clarify if some components of the canonical DISC are involved in recruitment and activation of caspase-8. FLIP overexpression did not protect from cell death under glucose deprivation. However, we cannot discard a contribution of FLIP in our system because when we overexpressed FLIP in HeLa cells we observed a clear downregulation of this caspase-8 inhibitor due to the treatment.

Then, we studied the role of the Ripoptosome in caspase-8 activation in HeLa cells subjected to glucose removal. As we already described in Bax/Bak deficient MEFs, in HeLa the knockdown of RIPK1 did not prevent cell death. However, we demonstrated that FADD is essential for cell death execution under glucose deprivation.

Recently, it has been shown that an intracellular DISC (iDISC) can be formed upon proteasome inhibition or endoplasmic reticulum stress on autophagosomal membranes, independently of death ligands. We show by immunoprecipitation and immunohistochemistry experiments that after glucose removal caspase-8 interacts with p62, LC3-II and Atg5, all proteins that associate with the autophagosome. However, we could not demonstrate significant translocation of caspase-8 to these organelles or an essential role of p62 to activate caspase-8 and induce apoptosis after glucose removal.

We observed that glucose deprivation induces endoplasmic reticulum stress in different cell lines, as shown by ATF4 and CHOP induction, as well as death receptors. We describe that ATF4 but not CHOP is responsible for TRAIL-R2 (DR5) induction after glucose withdrawal.

We show by immunoprecipitation that DR5 interacts with caspase-8 and localizes mostly at Golgi apparatus before and after the treatment, where maybe it could be accumulating and recruiting caspase-8. Moreover, the knockdown of DR5 in HeLa cells protects from apoptosis due to glucose deprivation, an effect that is more significant when Bcl-xL is stably expressed in these cells, suggesting that a component of death upon glucose deprivation is dependent on the mitochondrial pathway. However, we demonstrated that apoptosis induced by glucose removal is not dependent on DR5 ligand, as knockdown of TRAIL does not prevent cell death.

Finally, we describe that caspase-8 is located to filament-like structures in Bax/Bak deficient MEFs subjected to glucose deprivation. We show that those filaments co-localize with β-tubulin, suggesting that a cytoskeleton scaffold could be involved in caspase-8 aggregation and activation under glucose deprivation.

All our data indicate that different platforms may have a key role in the activation of caspase-8 in tumor cells after glucose deprivation. Understanding the molecular mechanism of cell death due to glucose withdrawal may be essential to develop new therapeutic strategies against cancer.

III. INTRODUCTION

III. INTRODUCTION

1. CELL DEATH

Defining when a cell is dead or alive is very complex. Previously, the Nomenclature Committee on Cell Death (NCCD) proposed three criteria for defining a dead cell (Galluzzi et al., 2015):
(1) the loss of plasma membrane integrity,
(2) the breakdown of cell into fragments,
(3) the engulfment of cell by neighbor phagocytes.
However, it has been reported that some engulfed cells can be released from the phagocytes and preserve their viability, so nowadays NCCD recommends considering as dead only cells that have undergone complete fragmentation or an irreversible breakage of the plasma membrane.
Many forms of cell death have been described. Apoptosis and Autophagy-dependent cell death are carefully regulated and morphologically well defined. Conversely to apoptosis and autophagic cell death, necrosis was thought to be an accidental and not regulated type of cell death for a long time, but recent discoveries showed that a regulated necrosis, called necroptosis, can also occur. This thesis will focus on the first type of programmed cell death: apoptosis.

1.1 APOPTOSIS

Apoptosis is a regulated cell death that occurs in multicellular organisms with the aim to eliminate superfluous, infected or damaged cells. It plays important physiological roles during embryonic development and in the

immune system. Inappropriate apoptosis can trigger several diseases such as neurodegeneration, autoimmune disorders, ischemia or cancer.
This type of programmed cell death was called apoptosis (from Ancient Greek meaning 'falling off') (Kerr et al., 1972) for its morphological features: cytoplasmic shrinkage, nuclear fragmentation or karyorrhexis, chromatin condensation and the typical membrane blebbing with the development of apoptotic bodies which preserve the plasma membrane integrity.
The entire process of apoptosis can be divided in four phases: induction, initiation, execution and disposal. During the induction several stimuli activate the apoptotic process, divided in stimuli of the extrinsic or intrinsic apoptotic pathway. In the initiation stage, aspartate proteases, called caspases, are activated and in the execution phase they induce all the morphological changes typical of apoptosis. For instance, DNA is fragmented by endonucleases (Cohen et al., 1994) and the phosphatidylserine is externalized to the plasma membrane in order to be recognized by the macrophages (Martin SJ, 1995), which will phagocytize apoptotic cells in the last phase of disposal.

The process of apoptosis is classified, depending on the stimuli, in the intrinsic or mitochondrial pathway and the extrinsic or death receptors pathway.
The intrinsic pathway is highly regulated by the Bcl-2 family proteins among which Bax and Bak form pores on the outer mitochondrial membrane, resulting in the release of cytochrome c. Cytochrome c binds seven adaptor proteins APAF-1, forming the so-called apoptosome, which recruits and activates the initiator caspase-9.
The extrinsic pathway is initiated by death ligands. Once bound to their receptors, they trigger formation of the death-inducing signaling complex (DISC), generally containing FADD and the initiator caspase-8. (Ashkenazi and Dixit, 1998).

Both pathways culminate in the activation of effector caspases, being the most important 3 and 7, which will execute the apoptotic process (Figure 1). A link between the intrinsic pathway of apoptosis and the extrinsic one has been reported. Upon death receptor initiation, active caspase-8 can cleave the BH3-only protein Bid, which in turn translocates to mitochondria to initiate the mitochondrial pathway (Li et al., 1998).

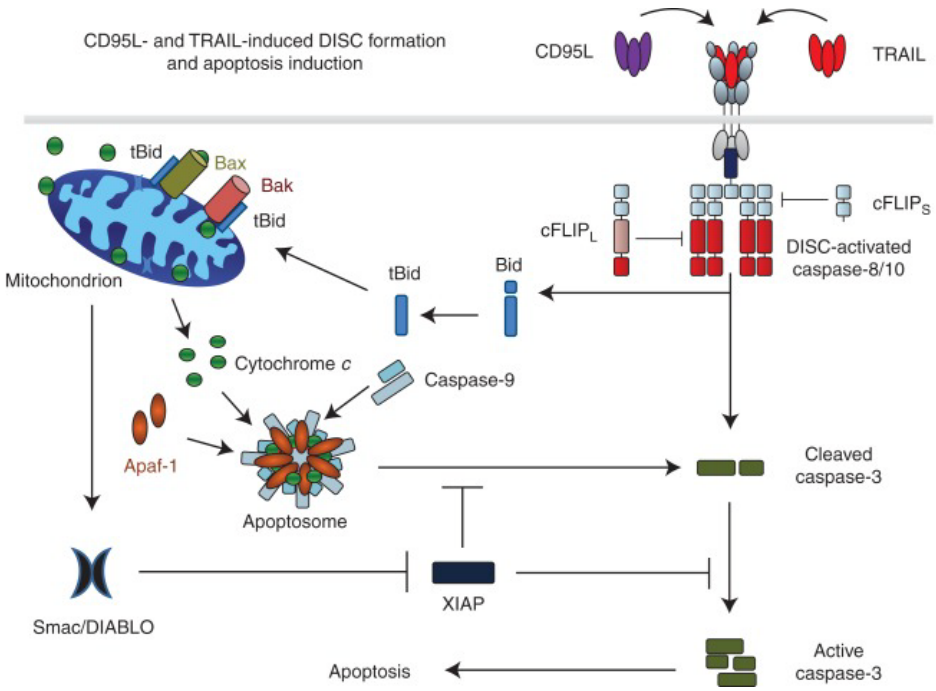

Figure 1: Apoptotic cascades. The extrinsic pathway and the intrinsic pathway of apoptosis. (Walczak, 2013)

1.2 CASPASES

The apoptotic process is orchestrated by the aspartic acid proteases called caspases which are responsible of cell dismantling (Logue and Martin, 2008; Slee et al., 1999). Caspases are initially synthetized as

inactive zymogens to avoid unwanted cell lethality. They have specificity for aspartic acid substrates and need proteolysis at internal aspartic acid residues to be activated by autoproteolysis or by other caspases (Slee et al., 1999). At least 12 human caspases have been identified which present sequence homology among them (Nicholson, 1999) and can have function in apoptosis or in inflammation and skin differentiation (Figure 2). They can have an N-terminal or pro-domain, a large subunit and a small subunit and can be grouped in initiator and effector caspases. Caspases 2, 8, 9 and 10 are the initiator or apical caspases. They have long N-terminal pro-domains that contain caspase recruitment domains (CARDs), necessary for protein-protein interaction, or death effector domains (DEDs), also present in the adaptor molecules involved in apoptosis initiation. Caspases 3, 6 and 7 are the effector caspases involved in the demolition of the cell (Figure 2). In the next chapter, we will focus on caspase-8 structure and on its activation pathways.

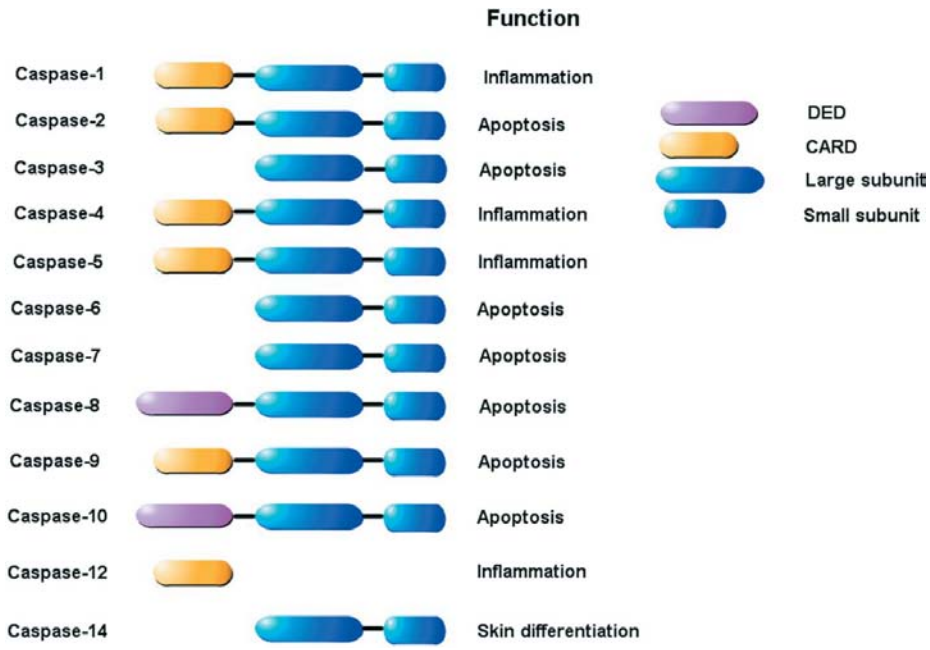

Figure 2: Human caspases: structure and function. (Logue and Martin, 2008)

1.3 THE INTRINSIC PATHWAY OF APOPTOSIS

The intrinsic pathway of apoptosis is initiated by intracellular signals such as absence of grow factors, hormones, cytokines or by hypoxia, DNA damage induced by radiations and free radicals. All of those signals culminate on initiating events that are orchestrated by the major player of the pathway: the mitochondria. The apoptotic mitochondrial events are highly regulated by the Bcl-2 family proteins (Youle and Strasser, 2008). These are generally classified as pro-apoptotic or pro-survival proteins and they are divided in three groups depending on the presence of one, three or four Bcl-2 homology (BH) domains. The anti-apoptotic proteins contain four BH domains (BH1-4) such as Bcl-2, BcL-xL and Mcl-1. The pro-apoptotic proteins can be divided into two groups: the BH1-3 proteins, such as Bax, Bak and Bok, and the BH3-only proteins, among them the most representative are Bim, Bad, Bid, Puma and Noxa (Lomonosova and Chinnadurai, 2008) (Figure 3).

Bcl-2, Bcl-xL, Mcl-1 and their siblings protect cells from apoptosis and are bound and neutralized by BH3-only protein interaction. Moreover, it has been shown that pro-survival Bcl-2 proteins are deregulated in many cancers (Frenzel et al., 2009).

The main effect of the pro-apoptotic proteins is to induce the release of apoptogenic factors from the mitochondria, such as cytochrome c and the second mitochondria-derived activator of caspase (SMAC), which is a direct inhibitor of apoptosis protein inhibitors (IAPs). All these events result in caspase-9 recruitment on the apoptosome complex and apoptosis initiation. The BH3-only proteins such as Bim, Bid, Puma or Noxa induce the proapoptotic Bcl-2 family proteins Bak or Bax which trigger the mitochondrial outer membrane permeabilization, resulting in the initiation of the apoptotic process. However, how the BH3-only proteins activate Bax and Bak is still controversial. According to the indirect model, Bax and Bak are sequestered by antiapoptotic proteins, which are displaced by

BH3-only proteins after apoptosis induction. The direct model postulates that BH3-only proteins directly engage and activate Bax and Bak (Villunger et al., 2011).

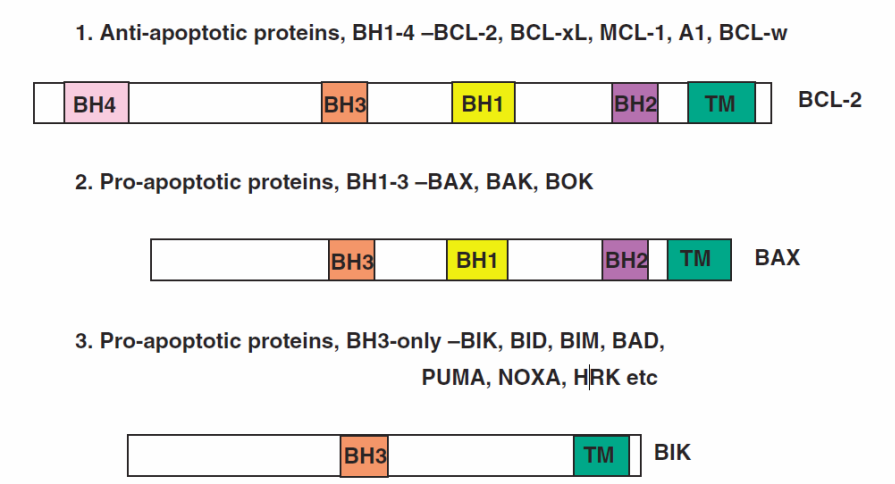

Figure 3: Bcl-2 family proteins. (Lomonosova and Chinnadurai, 2008)

1.4 THE EXTRINSIC PATHWAY OF APOPTOSIS

The extrinsic pathway of apoptosis is initiated upon the binding of death ligands on their receptors. Six human death receptors (DRs) of the tumor necrosis factor superfamily (TNFSF) have been identified. TNF-R1 is bound by TNF; DR3 (TRAMP/APO-3) is bound by TL1A and CD95 (Fas/APO-1) by CD95L (FasL). TRAIL-R1 (DR4) and TRAIL-R2 (DR5) have TRAIL as ligand. It is still not clear if DR6 could be bound by N-APP and what is the signalization induced (Walczak, 2013).

All death receptors are transmembrane proteins that contain cysteine-rich repeats in their extracellular domain, necessary for ligand binding. They also contain an intracellular death domain (DD) required for recruiting

specific adaptors, such as TRADD and FADD which in turn recruit caspase-8 as apical caspase (Ashkenazi and Dixit, 1998).

While TNF-R1 and DR3 induce gene activation as their primary signal, Fas, DR4, and DR5 induce preferentially apoptosis (Figure 4). We will focus on the most important and well characterized death receptors: TNFR1, Fas, DR4 and DR5.

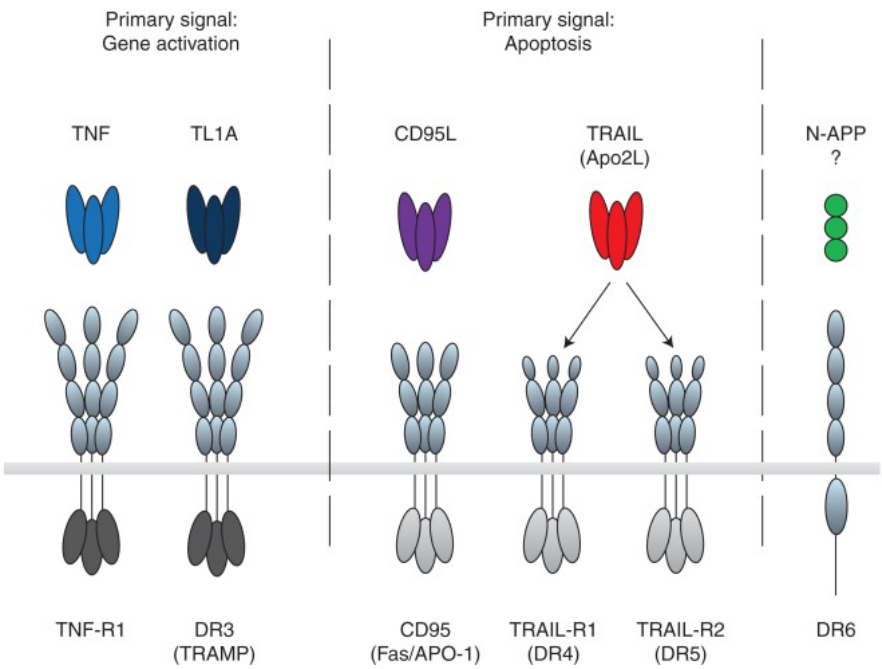

Figure 4: Death ligands and their receptors. (Walczak, 2013)

TNFR1

Tumor necrosis factor (TNF) is a pleiotropic cytokine that regulates several processes such as immunity, inflammation, cell proliferation, and apoptosis.

After binding TNF to TNFR1, the receptor trimerizes and recruits, through its death domain, the adapter molecule TRADD. This, in turn, recruits the TNFR associated factor 2 or 5 (TRAF2/5), the cellular inhibitor of apoptosis 1 and 2 (cIAP1/2) and the receptor interacting protein kinase 1 (RIPK1), forming the so-called complex I. This complex stimulates the NF-kB pathway, inducing pro-inflammatory and anti-apoptotic genes, such as the c-FLICE inhibitory protein (FLIP), a direct inhibitor of caspase-8 (Ashkenazi and Dixit, 1998).

Under FLIP and cIAPs low level conditions, TRADD associates to the adapter protein FADD and the concomitant de-ubiquitination of RIPK1 results in recruitment of the pro-caspase-8, forming the intracellular complex II (Micheau and Tschopp, 2003). This complex initiates the apoptotic process. When caspase-8 is inhibited, RIPK1 can recruit and phosphorylate RIPK3 leading to necroptosis (Christofferson and Yuan, 2010) (Figure 5).

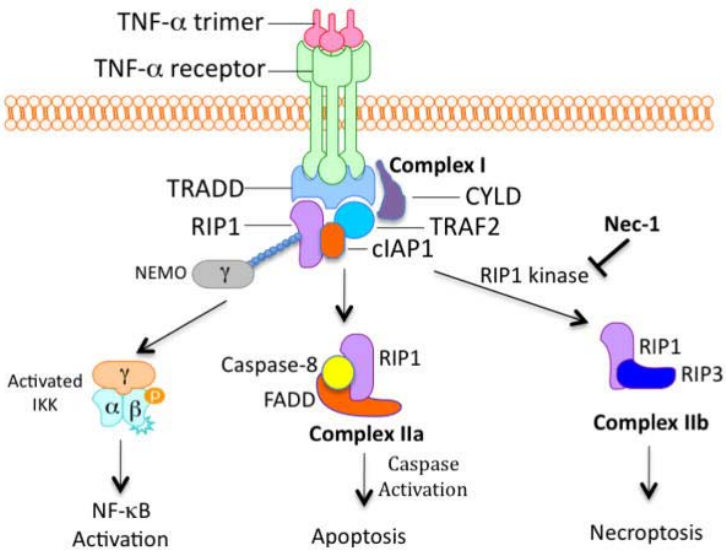

Figure 5: TNFR1 induced pathways. (Christofferson and Yuan, 2010)

Fas

Fas receptor has been identified in 1989 as a cell-surface killing agent. (Yonehara et al., 1989). In spite of existing also as a soluble protein, it is generally synthesized as transmembrane protein and constitutively expressed in several tissues. When bound by its natural ligand, FasL or by agonistic antibodies, it generally induces caspase-8 mediated apoptosis, playing critical roles in the immune system (Strasser et al., 2009).
It has also been reported that Fas can induce non apoptotic pathways, such as ERK, JNK, p38 and NF-κB, resulting in cell proliferation (Peter et al., 2007).
Post-translational modifications seem to regulate Fas activation. It has been shown that palmitoylation on cysteine 199 favors Fas internalization in lipid rafts where it can recruit and activate caspase-8 (Feig et al., 2007).

Tissues and cells are classified in type I or type II depending on whether, upon Fas stimulation, the mitochondrial pathway amplificates the extrinsic pathway of apoptosis. In type I cells, caspase-8 is activated at the DISC, triggering the activation of caspases 3 and 7 and the mitochondrial pathway is not engaged. In type II cells, caspase-8 is activated at the DISC but also it cleaves and activates the pro-apoptotic Bcl-2 family protein Bid, which in turn triggers the intrinsic pathway of apoptosis. The element which distinguishes the two types of cells could be the activity of XIAP (X chromosome-linked inhibitor of apoptosis protein), the inhibitor of caspase-3 (Jost et al., 2009). In type II cells, the release from the mitochondria of SMAC (second mitochondria-derived activator of caspase), inhibitor of XIAP, is essential to determine caspase-3 activation and apoptosis execution.

DR4/TRAIL-R1 and DR5/TRAIL-R2

TRAIL, also known as Apo2 ligand (Apo2L) is a cytokine that, as Fas and TNF, plays physiological roles in the immune system (Kelley and Ashkenazi, 2004). TRAIL is expressed as a transmembrane glycoprotein and can be proteolytically cleaved to form soluble TRAIL. Upon binding of TRAIL to DR4 or DR5, FADD and pro-caspase-8 are recruited through homotypic interactions, resulting in DISC initiation.

DR4 and DR5 are membrane proteins encoded by two genes located on chromosome 8p. Two different isoforms of DR5 have been identified, long DR5 (L) and short DR5 (S), resulting from differential splicing. They only differ in 29 amino acids located between their cysteine-rich domains (CRDs) and the transmembrane domain of the extracellular region. It is still unknown if the two isoforms have functional differences (Figure 6).

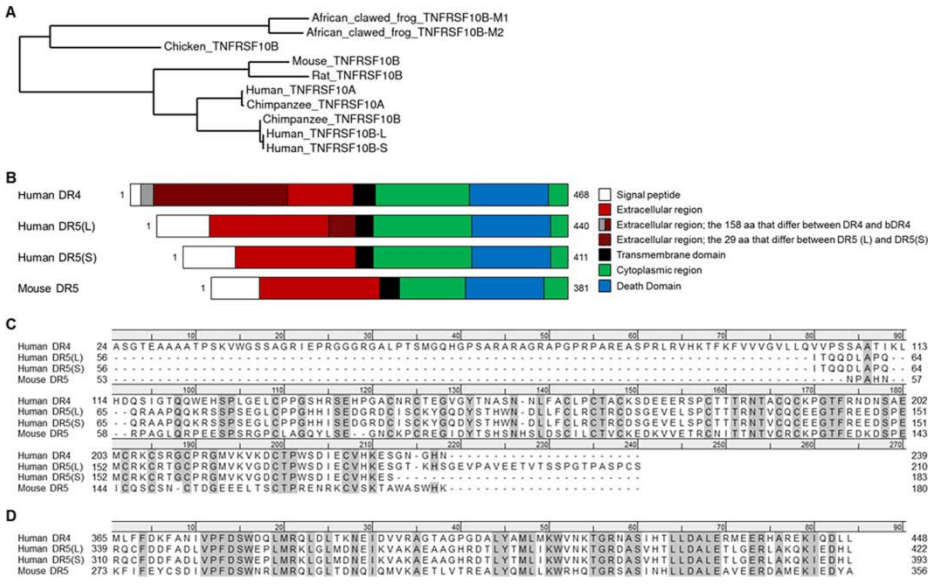

Figure 6: Comparison of TRAIL receptors in different species. (van Roosmalen et al., 2014)

Recently, several chimeric, humanized and human monoclonal antibodies against DR4 and DR5 have been produced and seem promising against different tumors (Mahalingam et al., 2009). Whether cancers prefer to use DR4 or DR5 for TRAIL-induced apoptosis is still not clear. It has been shown that leukemic cells preferentially engage DR4 to drive apoptosis upon TRAIL stimulation, while solid tumors do not present a clear preference.

Post-translational modifications are very important in regulating TRAIL expression and TRAIL sensitivity in cancers.
For instance, S-palmitoylation of DR4, in a region absent in DR5, is essential for its constitutive localization in lipid rafts (Rossin et al., 2009). Another study also reported that S-nitrosylation of DR4 is essential to determine TRAIL sensitivity in several tumor cells (Tang et al., 2006), as well as the O-glycosylation of both receptors, DR4 and DR5 (Wagner et al., 2007). Only DR4 N-glycosylation it has been reported.

2. CASPASE-8

Caspase-8 has essential roles in embryonic development and tissue homeostasis. Conversely to the other caspases, caspase-8 knockout mice die in early embryonic days with defect in hematopoietic progenitors and cardiac development (Varfolomeev et al., 1998). It has been shown that caspase-8 can be also mutated in several cancers such as breast cancer (Cox et al., 2007) or deleted in neuroblastoma (Teitz et al., 2000), depicting a very important role of this caspase in physiology.

Caspase-8 is a monomeric cytoplasmic protein of two often coexpressed isoforms, p55 and p53, both of them contain two tandem DEDs in the N-terminal domain, the large subunit, p18, and the small catalytic subunit, p10 (Boldin et al., 1996; Muzio et al., 1996). In spite of existing predominantly as a monomer, after its recruitment at the DISC, caspase-8 dimerizes in a concentration-dependent way (Donepudi et al., 2003). After dimerization it undergoes two internal cleavage events, first between the large and small subunits, generating the p43 fragment, and then between the large subunit and the adjacent DED, producing p18 (van Raam and Salvesen, 2014). Dimerization and the internal cleavage events of caspase-8 are not enough for its full activation. More aggregation events and post-translational modifications are required for caspase-8 to be completely active and to initiate apoptosis (Nair et al., 2014).

2.1 CASPASE-8 ACTIVATION

Chain assembly model

Conventional models suggest that core DISC components are present at a ratio 1:1 stoichiometry. One ligand trimer binds to one death receptor trimer. Then the receptor binds two or three molecules of FADD and the same number of caspas-8 molecules.

Recently, two different studies have shown by quantitative mass spectrometry and mathematical modeling that in hematopoietic cells the amount of caspase-8 is higher than FADD by several-fold.

Both groups have shown that after TRAIL stimulation or Fas DISC formation, caspase-8 aggregates through its DED domains, starting a caspase-8 chain assembly process (Dickens et al., 2012; Schleich et al., 2012). The aggregation of many caspase-8 molecules through its DED chains is essential for full caspase-8 activation and apoptosis execution.

DISC based on caspase-8 chain assembly has been also shown to require actin cytoskeleton as scaffold for its aggregation. (Chaigne-Delalande et al., 2008).

Moreover, It has been shown that upon stimulation, TRAIL receptors stably interact with E-cadherin which in turn binds F-actin filaments of cytoskeleton in a α-catenin dependent way. Those interactions facilitates the recruitment of FADD and caspase-8 molecules at the DISC, in high molecular weight fractions at lipid-rafts (Lu et al., 2014). Recently, it has also been shown that Paclitaxel, a microtubule stabilizing agent, induces caspase-8 dependent apoptosis in which the DED domains of caspase-8 were found associated with perinuclear microtubules and the centrosomes in both primary and tumor cell lines (Mielgo et al., 2009). So, a microtubule scaffold platform could contribute to caspase-8 activation in different models.

Posttranslational modifications

Caspase-8 also needs post translation modifications, such as ubiquitination, to be fully activated.

Death receptor ligation induces polyubiquitination of caspase-8 on both K63 and K48 residues, through the cullin3 (CUL3)-based E3 ligase, in human carcinoma cell lines. CUL3 interacts with DISC on a cytoskeletal platform in a detergent-insoluble cellular compartment. The ubiquitin-binding protein p62/sequestosome-1 translocates caspase-8 in ubiquitin-enriched foci, triggering a full activation of the protease. This modification

is reversed by the deubiquitinase A20, which results in a decrease of caspase-8 activity (Jin et al., 2009). It has been shown that also in epithelial cells CUL3 ubiquitination of caspase-8 can be essential for its activation (Dickens et al., 2012).

Conversely to ubiquitination that is required for activation of caspase-8, phosphorylation inhibits it. Tyrosine 380 and 465 residues are phosphorylated by Src-family tyrosine kinases, resulting in its suppression (Cursi et al., 2006). It remains still unclear if phosphorylation of caspase-8 regulates its ubiquitination.

FLIP

One of the most important regulators of caspase-8 activation is the FLICE-inhibitory protein (FLIP). FLIP is mainly expressed in two spliced isoforms, the long one, FLIPL and the short one, FLIPS. FLIPL shares high homology with caspase-8 but it is enzymatically inactive, due to the lack of a key cysteine residue in the catalytic domain. FLIPS contains the two DEDs repeats at its N-terminal, but lacks the catalytic domain (Irmler et al., 1997). Both isoforms can interact with FADD through homotypic DED binding and then dimerize with themselves or with caspase-8. Interaction of FLIPS with caspase-8 blocks caspase-8 homodimerization, resulting in apoptosis inhibition (Thome and Tschopp, 2001).

It is still controversial whether FLIPL acts as an antiapoptotic or as a proapoptotic molecule. At high levels of expression, it competes with caspase-8 for FADD binding, blocking apoptosis activation (Irmler et al., 1997). Moreover, it has been shown that embryonic fibroblasts derived from FLIP$^{-/-}$ mice are more sensitive to Fas-induced apoptosis and those mice die at early stages of embryonic development, similar to caspase-8$^{-/-}$ mice (Yeh et al., 2000).

When expressed at low levels, FLIPL has been shown to facilitate caspase-8 recruiting and activation at the DISC, functioning as a proapoptotic molecule (Chang et al., 2006).

3. ALTERNATIVE PLATFORMS ACTIVATING CASPASE-8

3.3 VIRAL INFECTION PROTEINS

During a viral infection, once entered into the host cell, the viruses replicate their double-stranded RNA (dsRNA) which are recognized by the pathogen associated molecular pattern (PAMP) receptors (Dixit and Kagan, 2013; Kawasaki et al., 2011). Among PAMP receptors, Toll-like receptors, TLR3, 7, and 8 recognize extracellular dsRNA or single-stranded RNA that are internalized into the endosomes, while the receptor that detects intracellular dsRNA are the retinoic acid inducible gene-I (RIG-I) and melanoma differentiation associated gene-5 (MDA5), which lead to the transcriptional activation of NF-KB and IRF3, resulting in interferons and cytokines production (Akira and Takeda, 2004). Moreover, it has been shown that these receptors can induce caspase-8 dependent apoptosis (Rajput et al., 2011). RIG-I and MDA5 contain two N-terminal CARDs which facilitate their interaction with other CARD containing molecules.

As RIG-I and MDA5, the mitochondrial antiviral signaling, (MAVS) protein contains an N-terminal CARD domain and it functions downstream of RIG-I to induce NF-KB and IRF3 under viral infection (Seth et al., 2005). MAVS is of particular interest since is localized on the external membrane of the mitochondria where it could be playing some pivotal functions in apoptosis as well as immunity.

Recently, it has been shown that MAVS and caspase-8 play key role in apoptosis induced by Semliki Forest virus (SFV) in both mouse and human Bax/Bak deficient cells. In that model, MAVS interacts with caspase-8 and recruits it on mitochondria in a FADD-independent way, resulting in caspase-3 activation and Bax/Bak independent apoptosis (El Maadidi et al., 2014).

3.2 RIPOPTOSOME

Recently, a new platform that activates caspase-8, independently of death receptors, has been identified: the Ripoptosome (Bertrand and Vandenabeele, 2011; Schilling et al., 2014). This complex consists of the core components FADD, caspase-8, cFLIP, and RIPK1. It forms spontaneously in the cytosol and independently of TNF, CD95L/FASL, TRAIL ligands.

This high molecular weight complex is formed upon genotoxic stress in epithelial cells (Tenev et al., 2011) or under TLR3 stimulation in keratinocytes and squamous cell carcinoma (Feoktistova et al., 2011).
The depletion of inhibitors of apoptosis proteins (cIAPs) is essential for its formation; it was obtained using small-molecule mimetics of SMAC, an endogenous IAP antagonist, or the genotoxic compound etoposide (Imre et al., 2011). IAPs are E3 ubiquitin ligases which act as negative regulators of RIPK1, marking it for proteasomal degradation. The depletion of cIAP1 and 2 by IAP antagonists increase the stability of RIPK1, inducing a spontaneous formation of the ripoptosome (Feoktistova et al., 2011).

Both groups have shown that ripoptosome requires RIPK1 kinase activity to be functional and can result in caspase-8 dependent apoptosis or RIPK3 dependent necroptosis. It seems that the stoichiometry of cFLIP isoforms in the complex regulates the prevalence of one type of cell death versus the other (Feoktistova et al., 2012). While cFLIPL-caspase-8 heterodimers completely block cell death, high levels of cFLIPS block apoptosis and result in necroptosis in the absence of cIAPs. When procaspase-8 levels are high, apoptosis is promoted. Once activated, caspase-8 cleaves directly RIPK1, leading to the dissolution of the complex.

3.3 AUTOPHAGOSOMAL PLATFORM/iDISC

The ubiquitin proteasome system (UPS) and autophagy are the two main cellular processes involved in protein degradation. They are strictly connected in order to guarantee the removal of unwanted or damaged proteins and to maintain cellular homeostasis (Buchberger et al., 2010; Korolchuk et al., 2010). Suppression of these pathways can result in several pathological situations, such as aging, cancer and neurodegeneration. Moreover, inhibition of the UPS and autophagy is clinically used for treating tumors (Adams, 2004; Amaravadi and Thompson, 2007).

Once UPS is engaged, misfolded proteins are marked with ubiquitins in an ATP consuming process. The enzymes involved in this process are a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin protein ligase (E3), which cooperate with molecular chaperones to complete correctly the process (Hershko and Ciechanover, 1998; Hochstrasser, 1996). Ubiquitinated aggregates are then transferred to the 26S proteasome to be degraded (Figure 7).

In contrast to UPS, autophagy can degrade not only proteins but also damaged or superfluous organelles such as entire mitochondria. It starts with the formation of a double membrane vesicle called phagophore which sequesters portions of cytosol or damaged proteins to form autophagosome. Then, the autophagosome fuses with lysosomes to degrade the content with acidic lysosomal hydrolases (Arias and Cuervo, 2011; Pattingre et al., 2008; Singh and Cuervo, 2011). The entire process is regulated by autophagy-related genes (Atgs), among them, Atg8, also known as microtubule-associated protein 1 light chain 3, (LC3) which is lipidated to LC3-II form, is used as a reliable autophagy flux marker. While UPS recognizes K48-linked polyubiquitin chains as substrates, autophagy seems to prefer K63-linked chains or maybe monoubiquitylated proteins.

Ubiquitylated aggregates are bound and directed to autophagy degradation by different proteins, including Sequestosome-1 or p62, the neighbour of BRCA1 gene 1 (NBR1) or the histone deacetylase 6 (HDAC6). p62 has also been shown to bind K48 ubiquitinated substrates, functioning as a link between UPS and autophagy (Long et al., 2008).

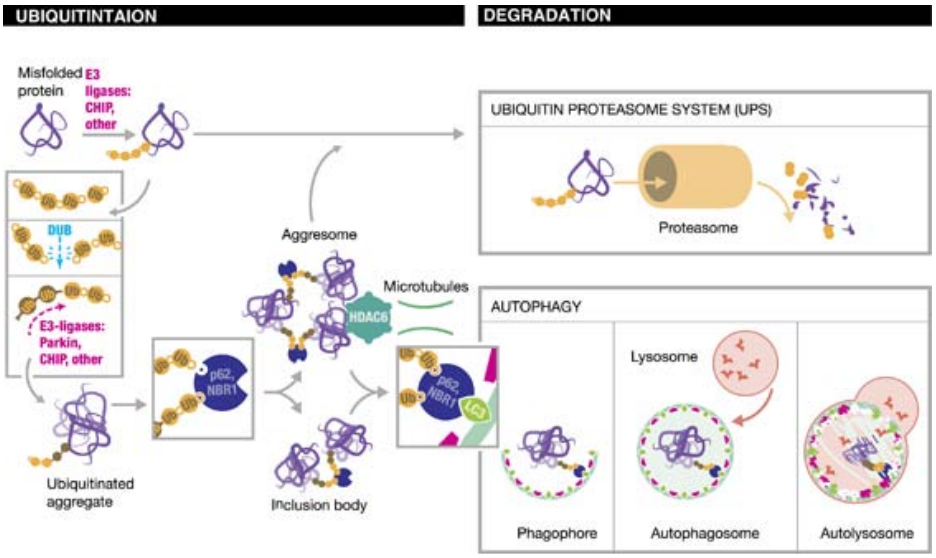

Figure 7: The two major protein degradation systems: the ubiquitin proteasome system (UPS) and autophagy. (Shaid et al., 2013)

Recently, several groups have shown that proteasome inhibition induces caspase-8 dependent apoptosis on a novel intracellular platform, where the main components are autophagy proteins such as Atg5, LC3 and p62 (Laussmann et al., 2011; Young et al., 2012) (Figure 8). Moreover, when proteasome inhibition is coupled with 3-methyl adenine, an inhibitor of autophagy, or with Atg5 deletion, apoptosis and caspase-8 activation is drastically reduced. In those models caspase-8 requires FADD to be activated, in a death ligand-independent manner (Laussmann et al., 2011; Young et al., 2012).

Previously, different groups have already shown that a possible interaction between FADD and Atg5 can occur in vitro and in vivo in response to

interferon γ (Pyo et al., 2005) or in proliferative T cells (Bell et al., 2008). Not only FADD and Atg5 seem to be essential for caspase-8 activation upon proteasome inhibition, but also LC3 and p62. Silencing of LC3 and p62 by shRNAs blocked caspase-8 recruitment on autophagosomal membranes and prevented cell death (Pan et al., 2011).

As previously described, p62 has been shown to promote caspase-8 aggregation after CUL3-mediated polyubiquitination of the enzyme, upon TRAIL stimulation (Jin et al., 2009).

A recent study, investigating apoptosis induced by ABT-263, a BH3 mimetic agent, showed that the up-regulation of p62 promotes caspase-8 aggregation and activation, confirming its important role in the recruitment of the enzyme (Huang et al., 2013). To conclude, p62 can participate in caspase-8 activation in the extrinsic pathway of apoptosis or under proteasome inhibition and autophagy induction.

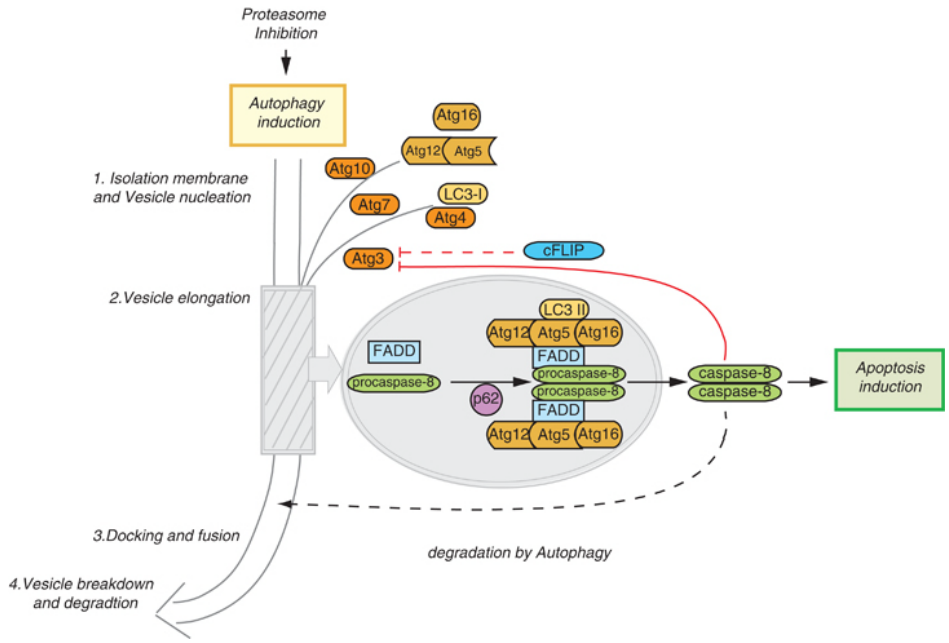

Figure 8: Intracellular autophagosomal platform activating caspase-8 under proteasome inhibition. (Delgado et al., 2014)

4. ER STRESS AND CELL DEATH

4.1 The unfolded protein response

The endoplasmic reticulum (ER) plays important cellular functions in protein synthesis, folding and their post-translational modification. It is also involved in biosynthesis of phospholipids and cholesterol and the maintenance of calcium homeostasis. Several stresses, such as starvation or viral infection, can cause an accumulation of misfolded proteins into the ER, a condition referred as “ER stress”, which induce the unfolded protein response (UPR), aimed at restoring protein homeostasis (Hetz et al., 2015; Ron and Walter, 2007).

The three major sensors of the UPR are the inositol-requiring protein-1α (IRE1α), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6), which reside in the lumen of ER. Under physiological conditions, these proteins are associated with the chaperone BiP/GRP78, which keep them inactive. Under ER stress conditions, they are released and activate several signaling pathways that promote cellular repair and survival, inhibiting protein synthesis. If the ER stress is prolonged and persistent it can also result in cell death (Tabas and Ron, 2011).

PERK is a kinase that phosphorylates the transcription factor NRF2 which induces antioxidant proteins. It also phosphorylates the translation initiation factor eIF2α which blocks protein translation, allowing cells to overcome the stress. eIF2α upregulates specifically the transcription factor ATF4 which induces a set of genes involved in amino acid metabolism and antioxidant response but also a second transcription factor, C/EBP-homologous protein (CHOP) which has been shown to induce ER stress-dependent apoptosis (Harding et al., 2003) (Figure 9).

IRE1α is a kinase and an endoribonuclease which once activated by trans-phosphorylation, regulates the messenger RNA of several genes. Among them, the transcription factor XBP-1 is spliced by IRE1α and

activated to regulate the synthesis of chaperones or genes involved in the ER-Associated Degradation (ERAD) process (Acosta-Alvear et al., 2007). ATF6 is an inactive transcription factor which when released form GRP78 is translocated into the Golgi where it is cleaved and activated. ATF6 can then induce the expression of chaperones but also of CHOP and XBP1 (Yoshida et al., 2000) (Figure 9).

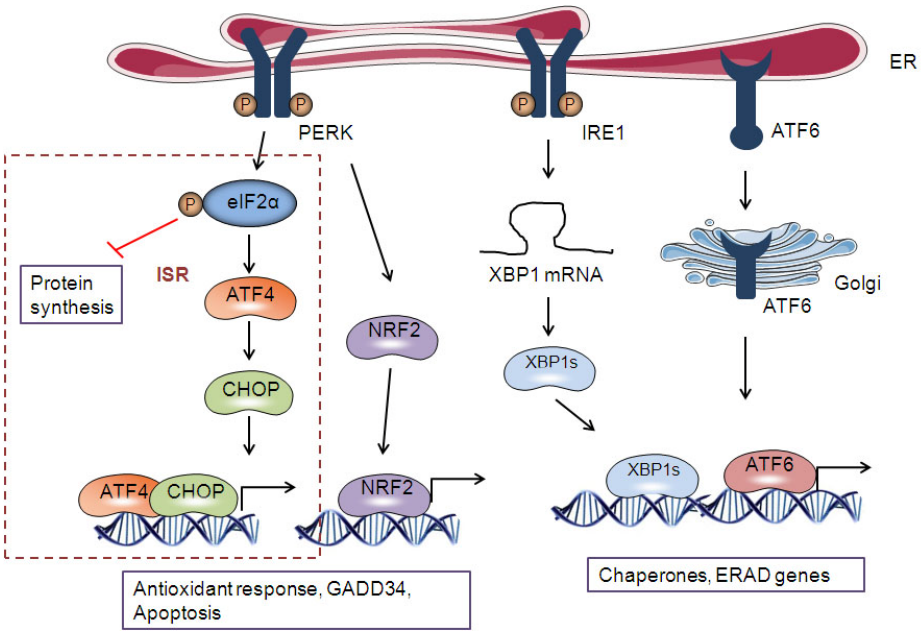

Figure 9: Unfolded protein response (UPR).
(Iurlaro R. and Munoz-Pinedo C., In press, FEBS Journal, 2015)

4.2 ER stress and the mitochondrial pathway of apoptosis

Under persistent ER stress, if cells are not able to restore homeostasis, the UPR signalization can trigger cell death (Cell death induced by endoplasmic reticulum stress, Raffaella Iurlaro and Cristina Muñoz Pinedo, FEBS journal 2015, in Press).

It has been known for decades that ER stressors, such as tunicamycin and thapsigargin, trigger caspase activation generally through the mitochondrial pathway of apoptosis.
Actually, all the three main sensors of the UPR, IRE1α, PERK and ATF6 can trigger pro-apoptotic signals regulating directly or indirectly the expression of Bcl-2 proteins. CHOP has been shown to downregulate the expression of anti-apoptotic Bcl-2 proteins (McCullough et al., 2001) or to upregulate the pro-apoptotic BH3-only protein Bim (Puthalakath et al., 2007). It has been shown that also the BH3-only protein Puma is upregulated after tunicamycin treatment in neurons. However, the mechanism of its regulation is still unclear (Reimertz et al., 2003).

Recently, it has been shown that IRE1α can associates with tumour necrosis factor receptor-associated factor 2 (TRAF2) resulting in c-JUN N-terminal kinase (JNK) activation (Urano et al., 2000). Once activated JNK can regulate the activity of Bcl-2 family proteins though their phosphorylation, inhibiting for instance anti-apoptotic Bcl-2 and Bcl-xL (Fan et al., 2000; Yamamoto et al., 1999) or activating pro-apoptotic Bid and Bim.

4.3 ER stress induces expression of TRAIL receptors

ER stress can induce cell death through TRAIL receptors upregulation in a death ligand independent way. Recently, a study has reported that thapsigargin induces ER-stress-dependent DR5 induction in different cell lines, resulting in cell death (Lu et al., 2014). In this study it has been shown that DR5-upregulation is dependent on CHOP while its degradation is regulated by the sensor IRE1α, allowing cells to adapt to the stress or decide to die if they cannot deal with it. Moreover, they showed that under ER stress, DR5 accumulates in the ER or at the Golgi apparatus depending on the cell type, where maybe it could be recruiting components of the DISC to initiate apoptosis.

It was already shown that CHOP directly regulates the promoter of DR5 gene resulting in cell death, upon thapsigargin treatment, in human carcinoma cell lines (Yamaguchi and Wang, 2004). A recent report shows that also the stress response gene ATF3 is required for ER stress-mediated DR5 induction and the consequent cell death in colorectal cancer cells (Edagawa et al., 2014) (Figure 10).

Moreover, constitutively active HER2/Neu/ERBB2 has recently proved to sensitize human breast epithelial cells to thapsigargin treatment through PERK-ATF4-CHOP dependent up-regulation of DR5 (Martín-Pérez et al., 2014). Both thapsigargin and tunicamycin have been previously shown to sensitize both non transformed and transformed human cell lines to TRAIL treatment by inducing up-regulation of DR5 in a PERK dependent way (Martín-Pérez et al., 2011).

DR4 expression has also been associated to ER stress (Li et al., 2015). It was shown that CHOP interacts with phospho-JUN in a complex that binds to the AP-1 binding site within the DR4 promoter region, mediating apoptosis triggered by thapsigargin and tunicamycin in human lung cancer cells.

4.4 ER stress induces apical caspase-8 activation on the iDISC

Recently, several groups have shown that persistent ER stress can trigger apoptosis independently of death ligands but dependent on caspase-8 activation and its recruitment on an intracellular DISC (iDISC) or autophagosomal platform.

Under ER stress, K63 poly-ubiquitinated caspase-8 molecules are recruited by p62 and LC3 on a FADD/ Atg5 containing platform, similar to that described upon proteasome inhibition (Laussmann et al., 2011; Pan et al., 2011; Young et al., 2012) (Figure 10).

A recent study showed that tunicamycin induces caspase-8 dependent apoptosis in breast cancers and in human colon tumor cells (Tomar et al., 2013). TRIM13, a RING-E3 ubiquitin ligase, has been shown to be essential for K63 caspase-8 ubiquitination and its recruitment and activation on autophagosomal membranes. Moreover, the translocation of caspase-8 on this platform seems to be dependent on its interaction with p62 and LC3-II.

In addition, another group has recently shown that tunicamycin and thapsigargin can trigger caspase-8 dependent apoptosis in cells lacking caspase-9 or Bax and Bak (Deegan et al., 2014). Knockdown of autophagic genes such as Atg5 and Atg7 protected from caspase-8 dependent cell death. They described that ER stressors induce the formation of an apoptotic complex on autophagosomal membranes which functions as scaffold for Atg5, FADD and caspase-8 recruitment and cell death initiation.

In conclusion, ER stress as well as proteasome inhibition, can induce a proteotoxic stress inside the cell which if not solved can trigger apoptosis on the iDISC.

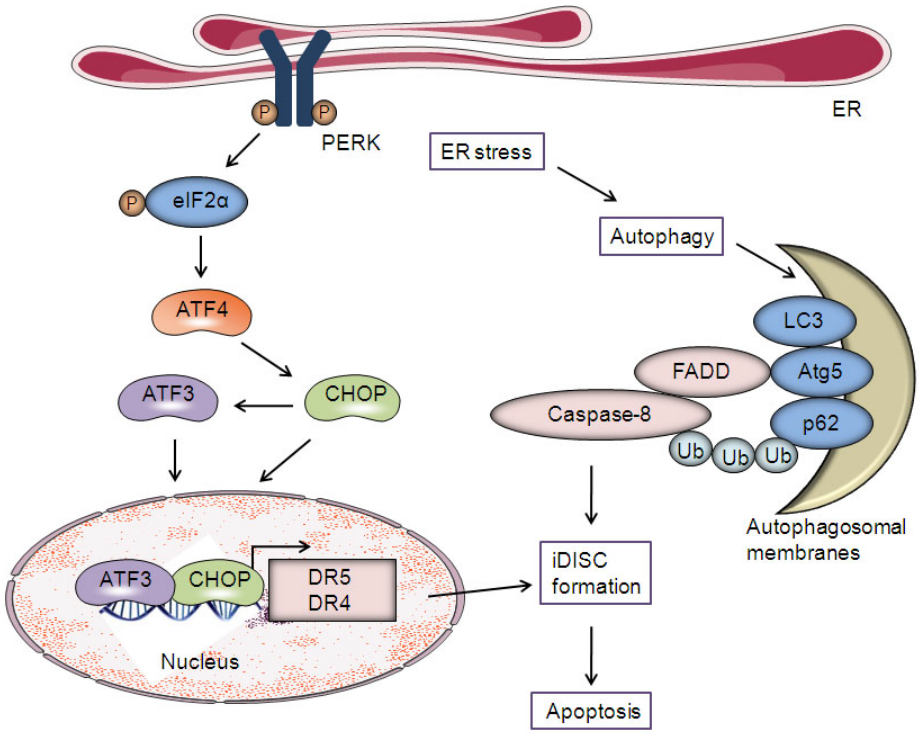

Figure 10: ER stress and cell death.
(Iurlaro R. and Munoz-Pinedo C., In press, FEBS Journal, 2015)

5. CANCER METABOLISM

All cells need nutrients and oxygen not only to produce energy but also to grow, proliferate and perform their cellular functions. It has been known for decades that cancer cells have a special metabolism due to an increased need of nutrients, necessary for their fast proliferation. The most relevant fact is that, even in presence of oxygen, cancer cells rely on glucose as source of energy and carbon, preferring to perform glycolysis instead of respiration. This phenomenon has been called “the Warburg effect” and many theories tried to explain it, such as possible defects in mitochondrial respiration.

Recently, the most accredited theory is that cancer cells increase the uptake and utilization of glucose not only to produce energy, but also all metabolites necessary for their grow, such as nucleic acids and fatty acids (DeBerardinis et al., 2008; Vander Heiden et al., 2009). This glycolytic switch allows cancer cells to deal with their fast rate of proliferation that is more effective for their growth, than to produce adenosine monophosphate (ATP) through respiration.

This metabolic transformation is promoted by signaling pathways that trigger proliferation and it is linked to oncogenic transformation, because it is promoted by oncogenes and inhibited by tumor suppressors, generally mutated in cancers (Jones and Thompson, 2009).
Cancer cells undergo a real metabolic reprogramming that is highly regulated by oncogenes and tumor suppressors (Iurlaro et al., 2014).

5.1 Glycolysis in proliferating cells and its oncogenic regulation

Generally in quiescent cells, once imported into the cell, glucose can be used for glycogen or fatty acids synthesis or can enter glycolysis to produce pyruvate. Then, pyruvate is imported into mitochondria to generate acetyl-CoA that is oxidized through TCA cycle to generate ATPs through the *oxidative phosphorylation* (OXPHOS) (Figure 11A).

On the contrary, proliferating cells or cancer cells prefer to direct all the imported glucose into glycolysis and convert it into pyruvate and then lactate. The remaining pyruvate is used to generate acetyl-CoA which is directed to de novo fatty acids synthesis. As glucose, the imported fatty acids are not stored but converted, through the β-oxidation, into acetyl-CoA and then citrate, which is directed toward de novo fatty acids synthesis, instead of the OXPHOS (Fritz and Fajas, 2010) (Figure 11B).

The uptake of glucose and its catabolism is driven and increased by oncogenes in order to promote cellular proliferation. On the contrary, tumor suppressors up-regulate OXPHOS and TCA cycle (Iurlaro et al., 2014).

c-Myc is one of the oncogenes regulating proliferation and tumor transformation. It is de-regulated in many human cancers (Dang, 2013; Li and Simon, 2013) and it promotes the glycolytic switch through the up-regulation of glycolytic enzymes such as hexokinase-2 and pyruvate dehydrogenase kinase 1 (PDK1), a negative regulator of pyruvate dehydrogenase (PDH), or the lactate dehydrogenase A subunit (LDH-A), stimulating the conversion of pyruvate into lactate (Shim et al., 1997).

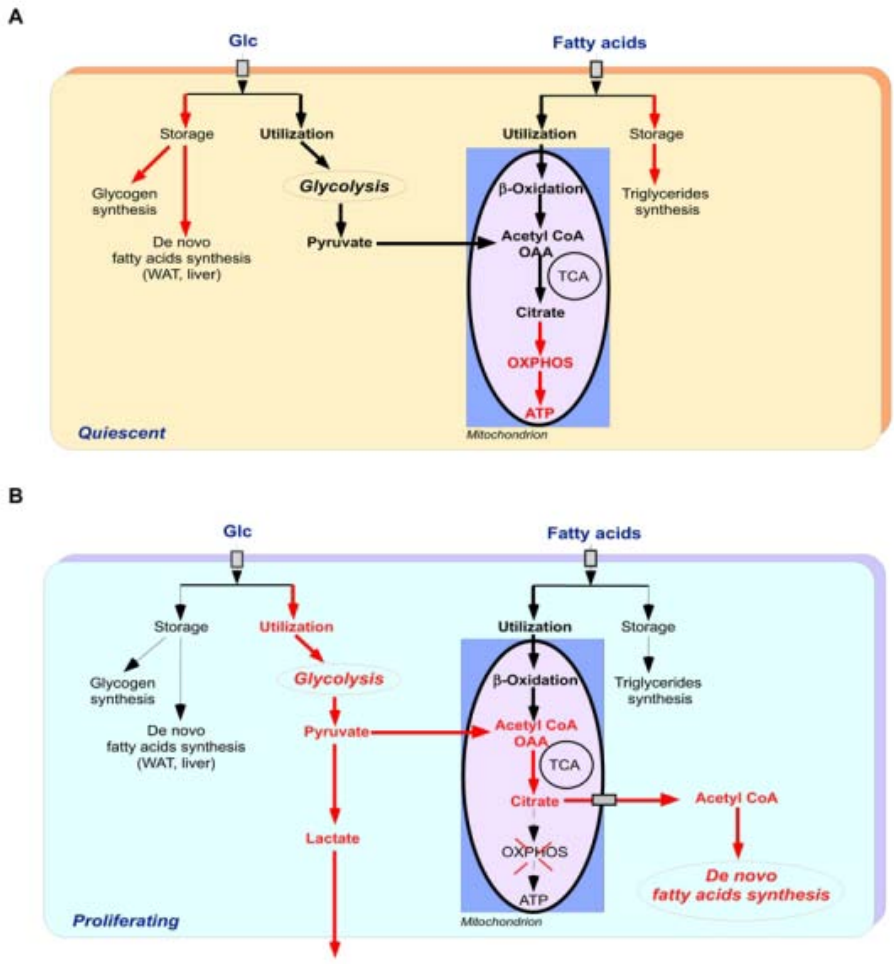

Figure 11: Quiescent and proliferating cell metabolism. Red arrows indicate preferred metabolic pathways. (Fritz and Fajas, 2010)

HIF-1, a transcription factor induced by the hypoxic microenvironment of tumors (Semenza, 2009), triggers the switch from OXPHOS to anaerobic glycolysis. It induces the expression of glucose transporters (GLUT-1, GLUT-3) and as c-Myc, it up-regulates glycolytic enzymes such as LDH-A (Brahimi-Horn et al., 2007; Semenza, 2011) and PDK1 (Kim et al., 2006; McFate et al., 2008).

Moreover, the PI3K/AKT pathway, once activated, can stimulate glycolysis, as well as oncogenic Ras (El Mjiyad et al., 2011). AKT induces

up-regulation and membrane translocation of glucose transporters and stimulates glycolytic enzymes such as phosphofructokinase-1 (PFK1) (Gottlob et al., 2001; Rathmell et al., 2003). K-Ras has been shown to regulate the expression of many glycolytic enzymes (Chiaradonna et al., 2006).

Conversely, tumor suppressors do exactly the other way around, they promote a switch from glycolysis to oxidative phosphorylation.

PTEN, an inhibitor of PI3K/AKT pathway, or p53 are the best candidates. PTEN has been shown to increase OXPHOS and its loss promotes glycolysis (Garcia-Cao et al., 2012; Tandon et al., 2011). P53 up-regulates cytochrome c oxidase 2 gene (SCO2), promoting oxidative phosphorylation (Vousden and Ryan, 2009) and TP53-induced glycolysis and apoptosis regulator (TIGAR), which inhibits the glycolytic enzyme fructose-2,6-bisphosphatase (Bensaad et al., 2006). It can also down-regulate glucose transporters (GLUT1 and GLUT4) (Schwartzenberg-Bar-Yoseph et al., 2004) or decrease glycolytic flux indirectly by activating PTEN (Stambolic et al., 2001).

In addition to glycolysis, other metabolic pathways are reprogrammed in tumors, such as glutaminolysis, pentose phosphate pathway (PPP), lipid synthesis and fatty acids oxidation.

For instance, it has been reported that c-Myc can also control glutamine metabolism, through the increase of mitochondrial glutaminase GLS1 (Gao et al., 2009) and the glutamine transporters SLC1A5 and SLC7A5. Also p53 can regulate glutaminolysis inducing the expression of another isoform of glutaminase, GLS2, under glucose deprivation (Hu et al., 2010; Suzuki et al., 2010).

c-Myc can also re-direct glycolysis to the synthesis of serine and glycine contributing to nucleotide building (Mannava et al., 2008) or stimulate lipid synthesis, up-regulating many fatty acid synthetases (Lovén et al., 2012). In addition, mTOR has been shown to trigger lipid synthesis through the transcription factor SREBP1, (Duvel et al., 2010), or nucleotide synthesis through regulation of PPP (Ben-Sahra et al., 2013).

Although all these metabolic changes are an advantage for tumors to grow and proliferate, on the other hand, they make them more sensitive to nutrient deprivation.

5.2 Nutrient starvation: how cells sense it

Nutrient starvation can occur in both physiological and pathological conditions, such as fasting, ischemia or cancer growth, in all living beings. Non-transformed cells and cancer cells try to survive after nutrient deprivation with different mechanisms. First of all, they stop cell cycle in order to have time for recycling structural components and reducing energy consumption. In that condition, in order to have an energetic storage, cells switch all anabolic pathways to the catabolic ones. However, if cells cannot overcome the energetic stress, several death pathways could be engaged, such as apoptosis, necrosis or autophagic cell death. As highly proliferative tumors are more dependent on food and availability of nutrients for their growth, they are also more susceptible to nutrient deprivation-induced cell death (El Mjiyad et al., 2011).

Cells sense lack of nutrients by detecting changes in ATP levels, resulting on AMP-activated protein kinase (AMPK) activation (Hardie, 2007). Changes in AMP: ATP ratio are detected by the kinase AMPK which, once activated, allows cells to adapt to the energetic stress, through different mechanisms. It induces the translocation to the membrane of glucose transporters and downregulates biosynthetic pathways. AMPK can also block cell cycle through p53 activation and stabilization of the cell cycle inhibitor p27 (Jones and Thompson, 2009; Liang et al., 2007).

In addition, the mammalian target of rapamycin (mTOR) plays an important role in detection of energetic stress. mTOR is highly active in proliferative cells were it is activated by hormones, growth factors and amino acids. Its mayor role is promoting protein synthesis. It is still

controversial whether mTOR is activated directly or indirectly by intracellular amino acids, but MAPK3, Rag A and Rag B proteins have been shown to play a role in this pathway (Wang X. et al., 2009; Findlay et al., 2007; Sancak et al., 2008). Amino acids starvation results in inhibition of protein synthesis through mTOR inactivation and a block in cell cycle, through mTOR-dependent inhibition of cdc2. The inactivation of mTOR can also result in autophagy induction. Autophagy is a physiological process in which damaged or unwanted proteins and organelles are first sequestered into double membrane structures, called autophagosomes, and then degraded into lysosomes. This mechanism allows cells to recycle useless components and to overcome the energetic stress (Figure 12).

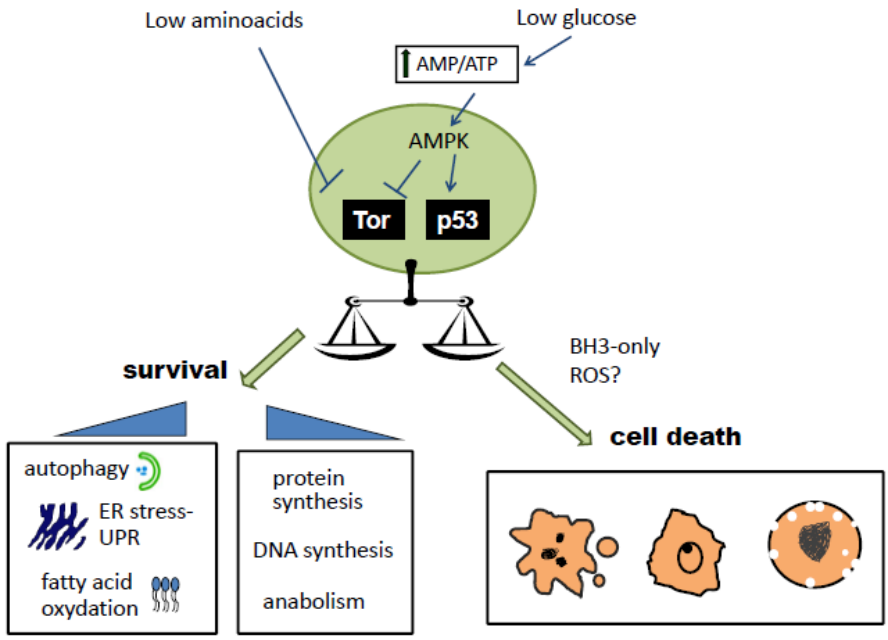

Figure 12: Survival or cell death pathways under nutrient starvation. (Caro-Maldonado et al., 2011).

5.3 Glucose deprivation induces different types of cell death

As discussed earlier, if cells are not able to overcome the energetic stress due to nutrient deprivation, they can die by different pathways. Here we will focus on cell death induced by glucose starvation.

Several reports have shown that glucose deprivation can induce different types of cell death. In several cell lines, antiapoptotic Bcl-2 family proteins prevented this death, suggesting that glucose deprivation promotes the mitochondrial pathway of apoptosis (Table 1). For instance, MCF-7, a breast carcinoma cell line subjected to glucose withdrawal undergoes apoptosis which was prevented by Bcl-2 overexpression (Lee et al., 1997). Also the Bcl-2 homolog Bcl-xL was shown to protect Ba/F3 hematopoietic cells from apoptosis, when overexpressed under IL-3 and glucose starvation (Gonin-Giraud et al., 2002).

Inductor	**Response**	**Implicated molecules**	**References**
Glucose withdrawal	Apoptosis	Bax, Bcl-2, Bcl-xL, Mcl-1	Vander Heiden et al (2009) Vander Heiden et al (2001), Lee et al (1997), Gonin-Giraud et al (2002), Alves et al (2006)
		Caspase-8	Caro-Maldonado et al (2010)
		Bad, PUMA, Bim Noxa, p53	Danial et al (2009) Zhao et al (2007), Alves et al (2006)

	Necrotic phenotype		Yuneva et al (2007) Suzuki et al (2003), León-Annicchiarico et al (2015)

Table 1: Glucose starvation induces cell death. (adapted from: Caro-Maldonado et al, 2011)

Many of the Bcl-2 proteins are regulated by glucose metabolism. For instance, a reduction of the antiapoptotic Mcl-1 sensitized Jurkat cells to glucose deprivation (Alves et al., 2006). In addition, glucose deprivation or treatment with the antiglycolytic drug, 2-deoxy-D-glucose (2-DG), has shown to inhibit the translation of Mcl-1, due to mTOR inhibition (Pradelli et al., 2010) while in hematopoietic cells, where glycolysis in enhanced, Mcl-1 is stabilized (Zhao et al., 2007). These data show a clear regulation of Mcl-1 by glucose metabolism.

Among the BH3-only proteins, the most related to glucose metabolism is Bad. Bad forms a complex with the glycolytic enzyme glucokinase (hexokinase IV) and can phosphorylate glucose and contribute in ATP mitochondrial production (Danial, 2009; Danial et al., 2003). It has also been shown that Bad promotes apoptosis in Bad-deficient mouse hepatocytes (Danial et al., 2003) and Bax activation, with consequent mitochondrial apoptosis in human leukemic cells treated with a glycolytic inhibitor (Xu et al., 2005). It is still controversial if Bad induces apoptosis under glucose deprivation, directly, through inhibition of antiapoptotic Bcl-2 proteins or indirectly, through glycolysis regulation.

Recently, it has been shown that also PUMA, a BH3-only protein generally induced after grow factor withdrawal and DNA damage, is induced by glucose deprivation in murine hematopoietic cells in a p53-dependent way (Zhao et al., 2008). In addition, it has been shown that in both primary and tumor cells, glucose deprivation induces apoptosis through p53-

dependent induction of Noxa, in a model where Noxa could be neutralizing its homolog antiapoptotic Mcl-1 (Alves et al., 2006).
Finally, also Bim, generally induced by grow factor withdrawal or ER stress (Puthalakath et al., 2007) has been related to glucose deprivation, in murine hematopoietic cells. In this model Bim is induced maybe due to ER stress generated by glucose withdrawal and the consequent accumulation of not well glycosylated proteins in the ER (Zhao et al., 2008).

Generally, cells subjected to glucose deprivation die by mitochondrial pathway of apoptosis. However, tumors have developed several strategies to bypass the mitochondrial pathway. For instance, antiapoptotic Bcl-2, Bcl-xL or Mcl-1 are often overexpressed, while pro-apoptotic BH3-only proteins or Bax and Bak are mutated or downregulated.

It has been reported that cells lacking Bax and Bak can die by necrosis when subjected to some apoptotic signal. For instance, glucose deprivation under hypoxia, in mouse baby kidney cells overexpressing Akt, induces necrosis only in the Bax- Bak deficient cells and not in the wild-type cell line (Degenhardt et al., 2006).

It has been previously reported that glucose deprivation can induce an atypical chromatin condensation in different cell lines that was defined as necrotic cell death (Suzuki et al., 2003; Yuneva et al., 2007).

Our group recently showed that while 2-DG treatment induces apoptosis, glucose deprivation results in necrosis in several rhabdomyosarcoma cell lines (León-Annicchiarico et al., 2015).

However, we described a particular type of cell death that occurs in mouse embryonic fibroblasts (MEFs) deficient in Bax and Bak, subjected to glucose deprivation. They do not die by necrosis but in a caspase-8 dependent manner and independently of death ligands interaction with

death receptors (Caro-Maldonado et al., 2010). We showed that caspase-8 was required to induce glucose deprivation-dependent cell death also in HeLa cells, showing that caspase-8 can be engaged also in cell lines with functional mitochondrial pathway.

In this thesis we will focus on the characterization of caspase-8 dependent apoptosis induced by glucose deprivation in different cell lines and on the molecular mechanisms involved in its activation.

5.4 Glucose deprivation: a good strategy against tumors

As tumor cells rely on glucose for proliferating fast, targeting glucose metabolism could be a good strategy against cancer (El Mjiyad et al., 2011). Many efforts have been done in the last decade trying to develop recombinants TRAIL able to kill specifically tumor cells. In spite of its low adverse effects on normal cells, TRAIL is not able to induce cell death in different types of tumors. Different studies have shown that glucose deprivation or the antiglycolytic drug 2-DG can improve the effect of TRAIL, TNF and Fas, overcoming the resistance of several cell lines to these death ligands (Muñoz-Pinedo et al., 2003; Pradelli et al., 2010). The main important effect of glucose withdrawal is the inhibition of the antiapoptotic proteins FLIP and Mcl-1, which contribute to the sensitization to death ligands.

Moreover, glucose deprivation has been shown to slightly affect normal cells. A study reports that an inhibitor of glucose uptake called fasentin sensitizes tumors cells to death ligands but not normal peripheral blood cells (Wood et al., 2008).

The glucose analog 2-DG has been reported to sensitize to chemotherapy and radiotherapy in several clinical trials (Singh et al., 2005). As mentioned before, this antiglycolytic drug can modulate the expression of

Bcl-2 family proteins such as Mcl-1, PUMA, Noxa and the tumor suppressor p53, all associated with sensitivity to chemotherapy (Frenzel et al., 2009). In vivo it has been shown that 2-DG sensitizes to radiation in sarcoma mouse models, increasing cell death (Dwarkanath et al., 2001). Although it has been shown that glucose deprivation induces resistance to cell death induced by certain anti-metabolites (Muñoz-Pinedo et al., 2004), combination of glucose deprivation and 2-DG with other drugs should be explored in order to improve anti-cancer therapy.

IV. OBJECTIVES

IV. OBJECTIVES

We previously showed that glucose deprivation induces caspase-8 dependent apoptosis in Bax/Bak deficient MEFs independently of the mitochondrial pathway of apoptosis and of death ligands. The main aim of this thesis is to clarify the molecular mechanisms of apoptosis induced by glucose deprivation with a particular focus on the identification of the platforms involved in the activation of caspase-8 and the resulting apoptosis in different cell types.

1. The first objective of this thesis was to study if glucose deprivation induces caspase-8 dependent apoptosis in different human cell lines and if the canonical DISC formed by death receptors, FADD or TRADD and FLIP could be involved in caspase-8 activation.

2. The second objective was to clarify if some components of the innate immune system with caspase recruitment domains, such as MAVS, could be involved in the activation of caspase-8 under glucose deprivation.

3. The third objective was to study the role of the Ripoptosome in death observed under glucose withdrawal.

4. The fourth objective was to define the role of autophagic proteins, such as p62 and LC3, in the recruitment and subsequent activation of caspase-8 on a possible autophagosomal membrane platform or iDISC.

5. The last aim of this thesis was to study the role of endoplasmic reticulum stress in determining cell death and death receptor up-regulation under glucose deprivation.

V.MATERIALS AND METHODS

V. MATERIALS AND METHODS

1. Cell culture and treatments.

Cell line	Source
HeLa, human cervical adenocarcinoma	Douglas Green, 2006
HeLa FLIP	A. Caro-Maldonado, 2010
HeLa Bcl-xL	A. Caro-Maldonado, 2010
Bax/Bak -/- HCT116, human colorectal tumors	Markus Rehm, 2014
Bax/Bak -/- MEFs, SV-40 immortalized mouse embryonal fibroblasts	Wei, Zong et al. 2001

Table 2: Cell lines used in this project are listed with their reference.

HeLa cells and Bax/Bak -/- MEFs (referenced in table 2) were cultured in pyruvate-free high glucose (25mM) DMEM (Dulbecco's Modified Eagle's Medium; Gibco Life Technologies) and Bax/Bak -/- HCT116 (referenced in table 2) were cultured in RPMI medium, supplemented with 10% FBS (fetal bovine serum; Invitrogen), 200 mg/ml of penicillin (Invitrogen), 100 mg/ml of streptomycin (Invitrogen) and 2mM glutamine (Invitrogen). Cells were maintained at 37 ºC and in a 5% CO_2 atmosphere. Cells were cultured in 10 cm dishes (BD) and splitted 3 times per week using 0.05% trypsin EDTA-Solution (Invitrogen).

For the treatments, HeLa and Bax/Bak-/- MEFs were plated at a concentration of 150,000/ml in 6 well plates or 60 mm dishes (BD) and treated 24 hours later, when they reached the concentration of 500, 000/ml corresponding to an 80% confluence. Bax/Bak-/- HCT116 cells were plated at a concentration of 200,000/ml in 6 well plates and treated 24 hours later at a 500,000/ml concentration.

Q-VD-OPH (apeXbio) and Y-VAD (Sigma) are used at 10µM, Z-VAD-fmk (apeXbio) is used at 20µM and all are added at the moment of the

treatment. The same amount of DMSO was added in the control wells. Fas human activating CH11 antibody (Millipore) was used at 50nM.

Glucose deprivation.

Before treatment cells were washed twice with FBS-free, pyruvate-free, glucose-free DMEM (Gibco life technologies) and then treated with DMEM medium without glucose, completed with 200 mg/ml penicillin, 100 mg/ml streptomycin, 2mM glutamine and 10% dFBS (dialyzed FBS) that has been previously inactivated and dialyzed. The same procedure has been used for treating Bax/Bak -/- HCT116 by using glucose-free RPMI (Gibco life technologies).

Serum dialysis.

FBS was dialyzed using a dialysis membrane with a pore diameter of 25 Å (SERVA Electrophoresis GmbH) that has been before washed with milli-Q water by warming and moving. Then the serum is added inside the membrane and washed twice with 1x PBS for one hour each, and a third time overnight at 4 ºC. The day after the serum is filtered with 0.22 µm filters and aliquoted.

2. Small interfering RNAs (siRNAs) transfection.

To silence genes in a sequence-specific manner, we used small double-stranded RNAs, siRNAs of 19-23 nucleotides (nt) fragments with 2-nt unpaired and unphosphorylated 3' ends which, once transfected into the cells, lead to the endonucleolytic cleavage of the target mRNAs.

HeLa and Bax/Bak-/- HCT116 were plated in 6 well plates (BD) at a confluence of 50%, let them attach and transfected 5 hours later. The transfecting solution of 50nM each siRNA and 1 µl/ml Dharmafect1 (Fisher-Thermo Scientific) was incubated 30 minutes in DMEM without serum and antibiotics and then added to the cells that have been

previously changed in DMEM without antibiotics. The transfection medium was kept for 24 hours or 48 hours in the case of FADD siRNAs before performing treatments. All siRNA sequences are from Sigma apart from caspase-8 and RIPK1 siRNAs and their control that are from Dharmacon. All sequences used in this project are listed in table 3.

Human protein target	siRNA sequence
ATF4 (1)	5' GCC UAG GUC UCU UAG AUG A 3'
ATF4 (2)	5' CCAGAUCAUUCCUUUAGUUUA 3'
Caspase-8	unknown, ON-TARGET plus siRNA pools of 4 oligos
CHOP	5' AAGAACCAGCAGAGGUCACAA 3'
DR4 (1) (total TRAIL-R1)	5' CACCAAUGCUUCCAACAAU 3'
DR4 (2) (total TRAIL-R1)	5' AACGAGAUUCUGAGCAACGCA 3'
DR5L (1)	5' GCUGUGGAGGAGACGGUGAUU 3'
DR5L (2)	5' CCUGUUCUCUCUCAGGCAUUU 3'
DR5L (3)	5' UGUGCUUUGUACCUGAUUCUU 3'
DR5S	5' UAUGAUGCCUGAUUCUUUGUG 3'
DR5 (total TRAIL-R2)	5' GACCCUUGUGCUCGUUGUC 3'
FADD (1)	5' GAUUGGAGAAGGCUGGCUC 3'
FADD (2)	5' GAACUCAAGCUGCGUUUAU 3'
FAS (1)	5' GAGAGUAUUACUAGAGCUU 3'
FAS (2)	5' AAGGAGUACACAGACAAAGCC 3'
MAVSa	5' CCACCUUGAUGCCUGUGAA 3'
MAVSb	5' CAGAGGAGAAUGAGUAUAA 3'
P62 (1)	5' GAUCUGCGAUGGCUGCAAUUU 3'
P62 (2)	5' GCAUUGAAGUUGAUAUCGAU 3'
RIPK1	unknown, ON-TARGET plus siRNA pools of 4 oligos
TNFR1 (1)	5' GGAACCUACUUGUACAAUGAC 3'
TNFR1 (2)	5' GCUGUGGACUUUUGUACAU 3'
TRADD (1)	5' GGAGGAUGCGCUGCGAAAUUU 3'
TRADD (2)	5' CUGGCUGAGCUGGAGGAUG 3'
TRAIL(1)	5' AACGAGCUGAAGCAGAUGCAGdTdT 3'
TRAIL(2)	5' UUGUUUGUCGUUCUUUGUGUU 3'
Control (non targeting sequence)	5' UAAGGCUAUGAGAGAUAC 3' (Sigma control)
ON-TARGET control	unknown (Dharmacon control)

Table 3: siRNA sequences used in this project are herein listed.

3. Western blot.

Protein extract preparation.

After treatments cells were collected by trypsinization and were washed with PBS and centrifuged at 500 g. The pellet was resuspended in RIPA buffer (Thermo Scientific, 25mM Tris HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% Sodium Deoxycholate, 0.1% SDS) completed with protease inhibitors (Roche) and phosphatase inhibitors (Roche) and the lysates were stored at -20 ºC. Then, the lysates were sonicated to degrade DNA and quantified using BCA colorimetric kit (Pierce-Thermo Scientific) following manufacturer's instructions. Briefly, 2 mg/ml BSA (bovine serum albumin) was used to create a standard curve that was taken as reference to calculate the concentration of our samples based on their absorbance at 562 nm. The procedure was performed in 96 well plates (Nirco) and the absorbance was measured at *BioTek's PowerWave XS microplate spectrophotometer*.

Gel electrophoresis and protein transfer.

40 µg of proteins per condition were prepared in 40 µl of final volume of 4x Laemmli buffer (63mM Tris-HCl; 10% glycerol; 2% SDS; 0.01% bromophenol blue; 5% 2-mercaptoethanol) and warmed at 95 ºC for 10 minutes. The proteins were loaded in a SDS-PAGE (Sodium Dodecyl Sulphate- *PolyAcrylamide* Gel Electrophoresis, Mini-protean, Bio-Rad) of different polyacrylamide percentage according to the size of the studied proteins. Generally a 8% resolving gel was used for 80-130 kDa proteins, a 10% or 12% resolving gel for 40-60 kDa proteins and 14% resolving gel was used for proteins smaller than 30 kDa. The gels were run at 120 V for 1 h 30 min in 500 ml of 1x Running Buffer (0.24M Tris-HCl; 1.92M glycine; 34.67mM SDS; pH 8.3). Then, the proteins were transferred to nitrocellulose (Bio-Rad) or Polyvinylidene Fluoride (PVDF, Millipore) membrane using Trans-Blot SD (Bio-Rad) semy-dry system for 1h at 200

mA each membrane in 1x Transfer Buffer (48mM Tris-HCl; 39mM glycine; 0.035% SDS; 20% methanol). The transfer of the proteins was checked by Ponceau S. staining (5% Acetid acid, 0.1% Ponceau) before proceeding with the blocking step.

Detection of the proteins with ECL system.

The membranes were blocked with 5% nonfat dry milk, 0.1% Tween, 1X TBS (10X TBS: 6 g Tris, 43.85 g NaCl; pH 7.5) blocking solution for 1 hour at room temperature. Then, the membranes were incubated with primary antibodies generally diluted 1:1,000 in blocking solution for 1-2 hours at room temperature or overnight at 4 ºC in agitation. The day after, we washed the membranes 3 times for 5 minutes each with 0.1% Tween-1x TBS and then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies diluted 1:5,000 in blocking buffer for 1 hour at room temperature by moving. After 3 washes in 0.1% Tween-1x TBS for 5 minutes each, the membranes were developed with an enhanced chemiluminescence (ECL) reaction using a freshly prepared ECL reagent (Promega).

Detection of the proteins with Odyssey system.

The membranes were blocked in Odyssey blocking solution (Li-Cor Biosciences) for 1 hour at room temperature by moving. The primary antibodies were prepared as before, while the secondary antibodies were diluted 1:15,000 in a solution of 1:1- TBS:Odyssey blocking buffer. The last wash was performed in a solution of TBS without Tween to avoid interfering with the detection system. The membranes were developed at the *Odyssey Infrared Imaging System*. Primary and secondary antibodies used in western blot analysis are listed in tables 4 and 5 respectively.

Primary antibody α:	produced in	MW (kDa)	brand	cat number
Actin (C-4)	mouse	42	ICN	8691001
human Atg5 (DRFU)	rabbit	55	Cell	12994

			signaling	
human ATF4/Creb2 (C-20)	rabbit	38	Santa Cruz	sc200
human c-IAP1 (B75-1)	mouse	72	Pharmigen	556533
human c-IAP2	rabbit	66	R&D	af817
human Caspase-3	rabbit	35	Cell Signaling	9662
human Caspase-3	rabbit	17-19	Cell Signaling	9661
human Caspase-8 (5F7)	mouse	55	MBL	M032-3
human Caspase-8 (C20)	goat	55-18	Santa Cruz	SC-6136
mouse Caspase-8 (IG12)	rat	55-18	Enzo	ALX-804-447
human CHOP/GADD153 (F-168)	rabbit	30	Santa Cruz	SC-575
human DR4-TRAIL-R1 (B-N28)	mouse	43-46	Diaclone	852.980.000
human DR5-TRAIL-R2 (D4E9)	rabbit	40-48	Cell Signaling	8074S
human FADD	rabbit	27	Santa Cruz	SC-5559
human FAS (CH11)	mouse	43	Millipore	05-201
human FLIP (NF6)	mouse	52-27	Enzo	ALX-804-428
mouse FLIP (Dave-2)	rat	55-28	Enzo	ALX 804-127
GRP78/Bip	goat	78	Santa Cruz	sc1050
LC3	rabbit	18-16	Abcam	ab48394
human MAVS	rabbit	70-57	Abcam	ab25084
PARP	rabbit	89-116	Cell Signaling	9542
human p62	rabbit	62	Enzo	BML-PW9860
RIPK1 (C38)	mouse	74	Pharmigen	610458
human TNF-R1 (H-5)	mouse	55	Santa Cruz	sc-8436
TRADD (H-278)	rabbit	34	Santa Cruz	sc-7868
β-Tubulin	mouse	55	Sigma	T4026

Table 4: Specifications for primary antibodies used in western blot analysis.

Secondary antibody α:	conjugated with	brand	cat number
rabbit	HRP	Zymax	81-6120
mouse	HRP	Zymax	81-6520
goat	HRP	Rockland	K915 Kit
rat	HRP	Zymax	61-9520
rabbit	IRDye 800CW	LI-COR Biosciences	926-32213
mouse	IRDye 680CW	LI-COR Biosciences	926-68022
goat	IRDye 680CW	LI-COR Biosciences	926-68024

Table 5: Specifications for secondary antibodies used in western blot analysis.

4. Immunoprecipitation.

Sample preparation.

After the treatment cells were collected using immunoprecipitation (IP) buffer (see specifications in table 7) completed with protease inhibitors (Roche) and lysed on ice for 20 minutes, sonicated and quantified using BCA assay (Pierce).

Bead preparation.

Pure Proteome™ Protein G Magnetic Bead System (Millipore) was used for the immunoprecipitation. In particular, 25 µl of magnetic beads per condition were previously blocked incubating with IP buffer including 1% BSA for 1 hour at 4 ºC under rotation. The beads were then incubated with 1 µg of primary antibodies (table 6) for 3-4 hours at 4 ºC under rotation.

Immunoprecipitation.

The beads were incubated with 1200 µg of lysates in 1 ml of IP buffer completed with protease inhibitors (Roche) overnight at 4 ºC under rotation. The day after, the beads were washed 3-4 times with IP buffer and proteins eluted with IP buffer + 2% SDS and then incubated with laemmli buffer (with freshly added 2-mercaptoethanol) and warmed at 95 ºC for 10 minutes before being loaded in a SDS-PAGE. Western blot was performed as described before.

Caspase-8 is the same size as IgG heavy chain so, in order to evoid interference with denatured IP antibody fragments, when the primary antibody was from rabbit, we used the Clean-Blot IP Detection reagent, HRP-conjugated (Thermo Scientific) as secondary antibody for detection in western blots. Mouse TrueBlot ULTRA HRP-conjugated (Rockland Immunochemicals) was used as secondary antibody when the primary antibody was from mouse.

Primary antibodies α:	produced in	concentration	µl /2µg	brand	cat number
human Caspase-8 (C-20)	goat	200 µg/mL	5	Santa Cruz	SC-6136
mouse Caspase-8	rabbit	1 mg/ml	1	Abcam	ab138485
FADD	rabbit	200 µg/mL	5	Santa Cruz	SC-5559
p62	rabbit	1 mg/ml	1	Enzo	BMLPW9860
GST	goat	1 mg/mL	1	Rockland	K915
GFP	rabbit	1 mg/mL	1	Rockland	K915

Table 6: Specifications for primary antibodies used in immunoprecipitation analysis.

Human IP BUFFER	Mouse IP BUFFER
20mM Tris-HCl (pH 7.5)	30mM TRIS
137mM NaCl	150mM NaCl
1% Triton x-100	10% glycerol
2mM EDTA (pH 8)	1% Triton x-100
	100µM orthovanadate
	200µM PMSF

Table 7: Composition of IP buffers used for human or mouse samples.

5. Immunocytochemistry.

Every cell line was plated in 12 well plates (BD) on 12 mm round sterile coverslips pre-coated with 0.1% Poly-L-Lysine solution (Sigma) at 37 ºC. 24 hours later, cells were treated at a confluence of 70-80% with Glucose deprivation for 24 hours more. Pre-warmed (37 ºC) MitoTracker (Invitrogen) solution was then added to the cells, diluted in DMEM at a final concentration of 200nM. Cells were incubated in this solution for 20 minutes at 37 ºC. The medium was then removed and cells washed with PBS.

Cells were then fixed with 500 µl of a PBS-4% paraformaldehyde solution (Merck) for 20 minutes at room temperature, by moving. Cells were again washed 3 times with PBS 5 minutes each and then blocked with 500 µl of blocking buffer (0.05% Triton, 3% BSA in PBS) for 1 hour, by moving, at room temperature, directly in the 12 well plate.

Then, the cells were incubated with primary antibodies (listed in table 8) diluted 1:200 in blocking buffer. This was performed by adding 40 µl of the antibody solution to a parafilm piece, covering this with the coverslip and placing it at 4 ºC overnight in a humid covered dish. Next morning, the coverslips were washed 3 times with PBS for 5 minutes each and

incubated 1 hour with secondary antibodies (see references in table 9) diluted 1:400 in blocking buffer at room temperature. Cells were then washed 3 times more with PBS and once with DAPI diluted 1:10,000 in PBS for 10 minutes at room temperature. The coverslips were then mounted using 3 µl of Vectashield solution (Vector laboratories) and let dry for at least one day.

Photos at different zooms were acquired using the software application *Suite Advanced Fluorescence* (LAS AF, 2.6.0.7266) at confocal *Leica TSC SP5 Spectral* with *HCX PLAPO lambda blue 63X 1.4* objective and the co-localization analysis was done by using co-localization plugin of the software Fiji/Image J (as shown in figure 13).

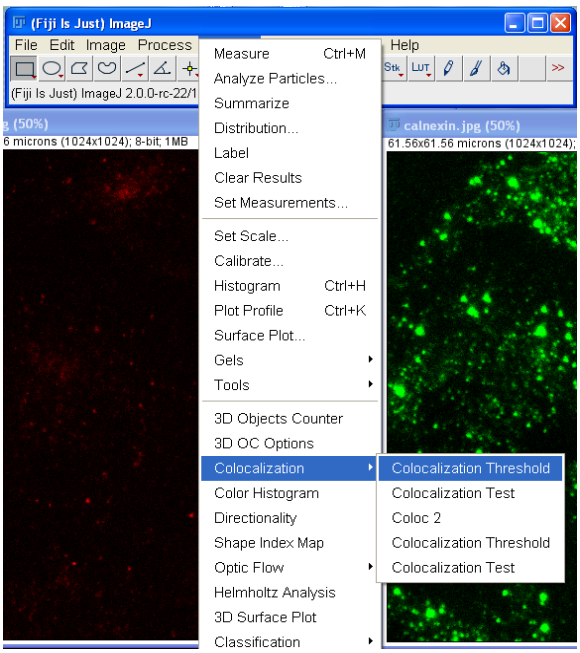

Figure 13: Example of co-localization analysis of two channels, red and green in Fiji/ImageJ software.

Primary antibodies α:	produced in	brand	cat number
calnexin	mouse	Abcam	ab31290
human Caspase-8 (C-20)	goat	Santa Cruz	SC-6136
mouse Caspase-8	rat	Enzo	ALX-804-447
human DR5-TRAIL-R2	rabbit	Cell Signaling	8074S
human FADD	rabbit	Santa Cruz	SC-5559
human FAS	mouse	Millipore	05-201
GRP78/BiP	goat	Santa Cruz	sc1050
GM130	mouse	BD pharmigen	610822
LC3	rabbit	Abcam	ab48394
human MAVS	rabbit	Abcam	ab25084
p62	rabbit	Enzo	BML-PW9860
β-tubulin	mouse	Sigma	T4026
ubiquitin	mouse	Enzo	PW8810-0500

Table 8: Specifications for primary antibodies used in immunofluorescence analysis.

Secondary antibodies α:	conjugated with	brand	cat number
rabbit	Alexa Fluor 568	Life technologies	A21235
mouse	Alexa Fluor 647	Life technologies	A11011
goat	Alexa Fluor 488	Life technologies	A11055
rat	Alexa Fluor 488	Cell Signaling	4416S
phalloidin	Alexa Fluor 568	Life technologies	A12380

Table 9: Specifications for secondary antibodies used in immunofluorescence analysis.

6. Cell death analysis.

Propidium iodide (PI) incorporation.

After the treatment, adherent cells and dead cells in suspension were collected by trypsinization and centrifuged at 450 g for 7 minutes.

Then they were resuspended in a final volume of 300 µl of PBS plus 0.5 µg/ml of propidium iodide (PI) and analyzed at the *Gallios Flow Cytometer Beckman Coulter.* PI is a DNA intercalating agent and a fluorescent molecule that is used to stain dead cells that are more prone to incorporate it. The quantification has been done using the version *7.6.4 of the software FlowJo* as indicated in figure 14.

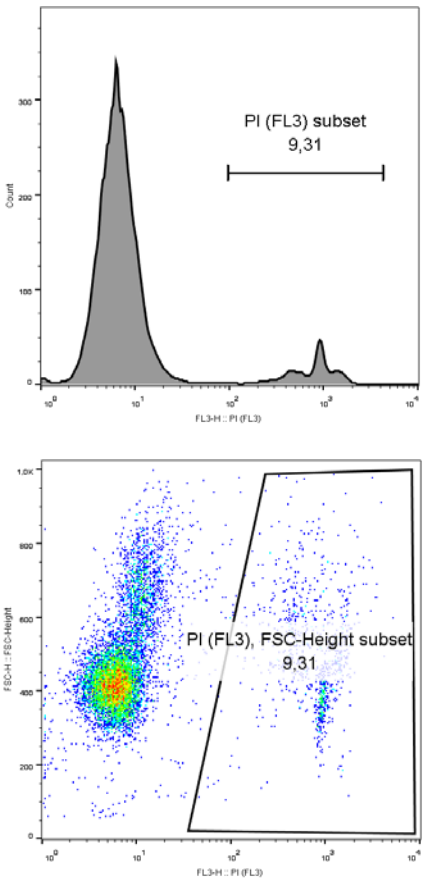

Figure 14: The graphs show an example of percentage of HeLa that incorporated PI after 24 hours of glucose deprivation; represented by histogram or FL3 versus FSC. The lower panel indicates the analysis that we used for the quantification.

Light-scattering changes analysis.

When a cell intersects with the laser light of the flow cytometer it will be scattered in different directions depending on the cell size and structure. The forward light scatter (FS-A) signal gives information on the cell size while the side scatter (SS-A) on the homogeneity of the cell structure, depending on the condensation of the cytoplasm and the nucleus and the granularity of the cell. The forward light scatter is decreased already at the early stages of apoptosis due to the shrinkage of the cell and a decrease in intensity of side scatter is also observed at late stages of apoptosis. The analysis has been performed using the version *7.6.4* of the *software FlowJo* (Figure 15).

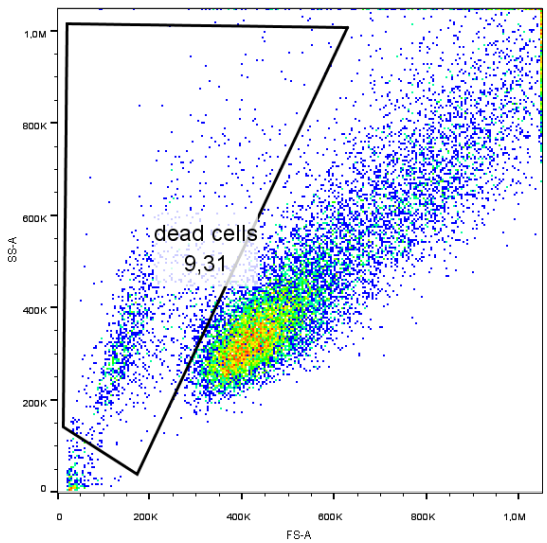

Figure 15: The graph FS-A versus SS-A shows an example of HeLa treated with glucose deprivation for 24 hours that start dying as indicated by the squared population. The second population of cells, bigger and more complex, represents the ones that are alive.

LDH test.

Another way to measure cell death is quantifying the lactate dehydrogenase (LDH), a stable cytoplasmic enzyme that is present in all cells but released in the medium only after membrane damage and cell rupture. The LDH test (Promega) is an easy enzymatic and colorimetric assay where the lactate dehydrogenase creates a formazan dye that is measured at 490 nm.

After the treatment, 50 µl of the conditioned medium per condition were resuspended in the reactive solution and incubated 30 minutes at 37 ºC in a 96 well plate (Nirco). After that, 50 µl of the Stop solution were added at the samples and the absorbance at 490 nm was acquired at *BioTek's PowerWave XS microplate spectrophotometer*.

7. q-PCR.

Reverse transcription.

After the treatment, cells were collected for RNA extraction and q-PCR analysis of death receptors. Cells were collected at room temperature by trypsinization, were washed once with PBS and centrifuged 5 minutes at 300 g. RNA was extracted from the pellets using the RNeasy MINI KIT (Qiagen) according to manufacturer's instructions. Pellets were either resuspended in RTL buffer (Qiagen) completed with 2-mercaptoethanol and stored at -80ºC, or the complete procedure was performed. The

obtained RNA was quantified at the NanoDrop (ND-1000, Thermo Scientific) and 1 µg of RNA per condition was retro-transcribed at cDNA using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. The steps of the reaction are indicated in table 10. At the end of the reaction the cDNA was diluted 1:10 in RNAse free water to a final concentration of 10 ng/µl and stored at -20 ºC.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (ºC)	25	37	85	4
Time (min)	10	120	5	∞

Table 10: Amplification steps of the reverse transcription (Applied Biosystems).

q-PCR.

The q-PCR analysis was performed using the *LightCycler® 480 SYBR Green I Master solution* (Roche) starting with 10 ng of cDNA per reaction. SYBR Green I is a DNA double-strand-specific dye which binds to the amplified PCR products and can be detected by its fluorescence. 5 µl of SYBR Green and 5µM of primers were used per reaction. The master mix conditions and the primers used in the analysis are reported in table 11 and 12, respectively.

	Master Mix I (µl per tube)		**Master Mix II (µl per tube)**		**Master Mix III (µl per tube)**
Sybr Green	5	**Master mix I**	8	**Master mix II**	9
Water	3	**5 µM Primer**	1	**1:10 cDNA**	1
Total	8	**Total**	9	**Total**	10

Table 11: Master mix solutions preparation.

Target gene	Forward	Reverse
hL32	AACGTCAAGGAGCTGGAAG	GGGTTGGTGACTCTGATGG
hTRAIL	GTCTCTCTGTGTGGCTGTAAC	GGGCTGTTCATACTCTCTTCG
hTRAIL-R1 (DR4)	GCTGTGCTGATTGTCTGTTG	TCGTTGTGAGCATTGTCCTC
hTRAIL-R2 (DR5)	TGAGACCTTTCAGCTTCTGC	ATCGTGAGTATCTTGCAGCC
hFas	AAAGGAGCTGAGGAAAGTGG	CATAGGTGTCTTCCCATTCCAG
hTNFR1	TCACCGCTTCAGAAAACCACC	AAGCACTGGAAAAGGTTTT

Table 12: Primer sequences used in the q-PCR analysis.

Amplification was performed in a *Light Cycler* of 96 well plate (Roche) using the protocol reported in table 13. Analysis was performed measuring the ΔCq between the Cq of our sample and the one of the control. The Cq indicates the amount of template present at the start of the amplification. The control used in the analysis was RPL32, L32 ribosomal protein gene. All results were then normalized to their control treatment.

	Cycle (n)	Temperature (ºC)
Pre-incubation	1	95
Amplification	45	95, primer-dependent, 72
Melting Curve	1	95, 65, 97
Cooling	1	40

Table 13: Amplification steps of q-PCR analysis.

8. Statistics.

Error bars in results figures represent the standard error of the mean (S.E.M.).

The significance of data was measured using two tailed, paired Student's t-test. Significant differences are marked as follow: $p<0.05$ is one asterisk (*), $p<0.01$ is two asterisks (**), $p<0.001$ is three asterisks (***).

N.S. means not significant.

VI. RESULTS

VI. RESULTS

1. Glucose deprivation induces caspase-8 dependent apoptosis in different types of human tumor cells.

Previously, our group described that tumor cells subjected to glucose deprivation can die by necrosis (Ramírez-Peinado et al., 2013) or by a caspase-8 dependent apoptosis in Bax/ Bak deficient MEFs, lacking the mitochondrial pathway (Caro-Maldonado et al., 2010). Moreover, the knockdown of caspase-8 in HeLa cells, which are able to undergo apoptosis through the mitochondrial pathway, protected from cell death after glucose withdrawal. On the contrary, the stable overexpression of Bcl-xL in HeLa did not prevent death (Caro-Maldonado et al., 2010). All these findings suggested that caspase-8 dependent apoptosis could be engaged in both kinds of tumor cells, with a functional or not functional mitochondrial pathway, under glucose deprivation.

We decided to better characterize the molecular mechanisms of apoptosis in human tumor cells subjected to glucose removal. For this reason, we performed cell death analysis using Propidium Iodide (PI) incorporation and cytometer detection in glucose deprivation kinetics. HeLa cells were subjected to glucose deprivation for 24, 48 and 72 hours and the percentage of PI incorporation, at the different time points, is shown in figure 16A. HeLa die at around 20% at 48 hours and 80% at 72 hours of treatment and they are protected by co-treatment with the pan-caspase inhibitor Q-VD that inhibits cell death. To be sure that this death was due to apoptotic caspases and not to inflammatory caspases we co-treated cells with Y-VAD, an inhibitor of caspase-1, that could not prevent cell death (Figure 16A and 16B), confirming our statements.

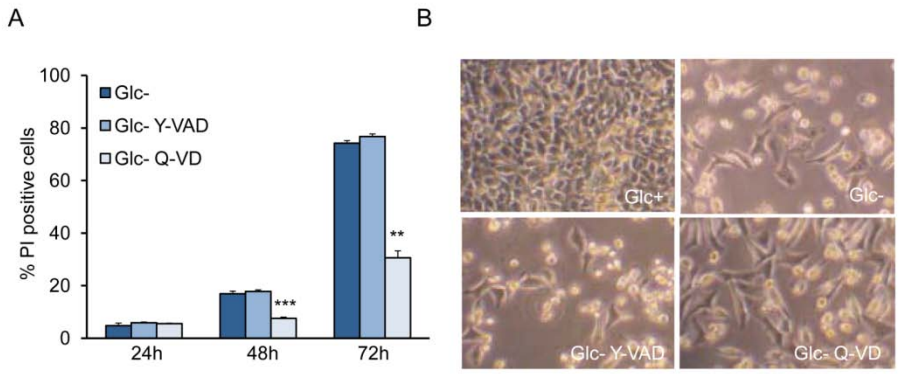

Figure 16: **Q-VD but not Y-VAD is protecting HeLa cells from glucose deprivation.** HeLa cells were plated at 20% confluence in 60 mm dishes and 24 h later they were treated with Glc- completed medium (FBSd+PS-Q) in presence or absence of 10µM Q-VD or 10µM Y-VAD for 24, 48 and 72 h. **A**, cells were collected for PI analysis by FACS. The average and S.E.M. of three experiments are reported. **B**, representative photos, at Olympus inverted microscope, of controls and cells treated for 72 h are shown.

To demonstrate that cell death under glucose deprivation is apoptotic we performed western blot analysis of effector caspase targets, such as PARP and caspase-3 as a substrate of caspase-8. As we can see in figure 17, the removal of glucose results in PARP cleavage after 48 and 72 hours of treatment that is prevented by co-treatment with Q-VD. We can also observe the reduction on procaspase-3 levels and an induction of its cleavage at 48 and 72 hours of treatment that was prevented by Q-VD.

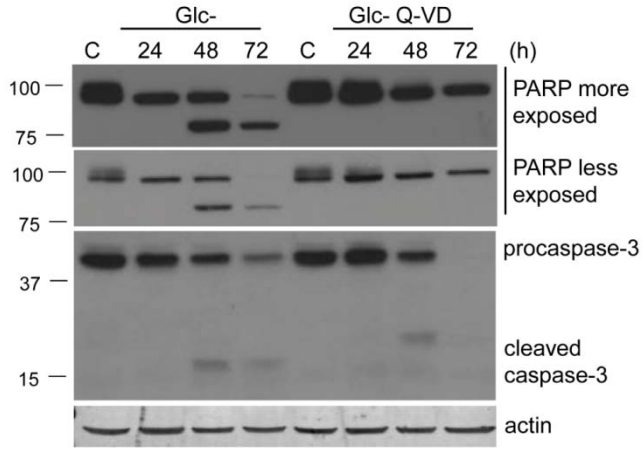

Figure 17: **Glucose deprivation causes caspase activation.** HeLa cells were plated at 20% confluence in 60 mm dishes and 24 h later they were treated in glucose-free medium for 24, 48 and 72 h and collected for western blot. The activation of caspase-3 and cleavage of PARP are shown by immunoblot.

We decided to check if caspase-8 could be activated under glucose deprivation in a human cell line that could not undergo apoptosis through the mitochondrial pathway. For this reason, we deprived Bax-Bak deficient HCT116 of glucose and we analyzed PI incorporation at the cytometer, as indicated in figure 18. This cell line died slowly under glucose deprivation: 40% of cell death is reached only at 72 hours of glucose removal.

As observed in HeLa, also Bax-Bak deficient HCT116 are protected by the co-treatment with the pan-caspase inhibitor Q-VD (Figure 18A and 18B), indicating that apoptotic caspases are activated under the treatment. We detect in this cell line a reduction of the total form of PARP after glucose removal but we could not detect the cleaved form of this caspase target (Figure 18C). We observed a clear reduction of procaspase-3 levels, but a strange pattern of caspase-3 is detected in this cell line, indicating maybe the presence of heterodimers or trimers of this caspase, after glucose withdrawal (Figure 18C).

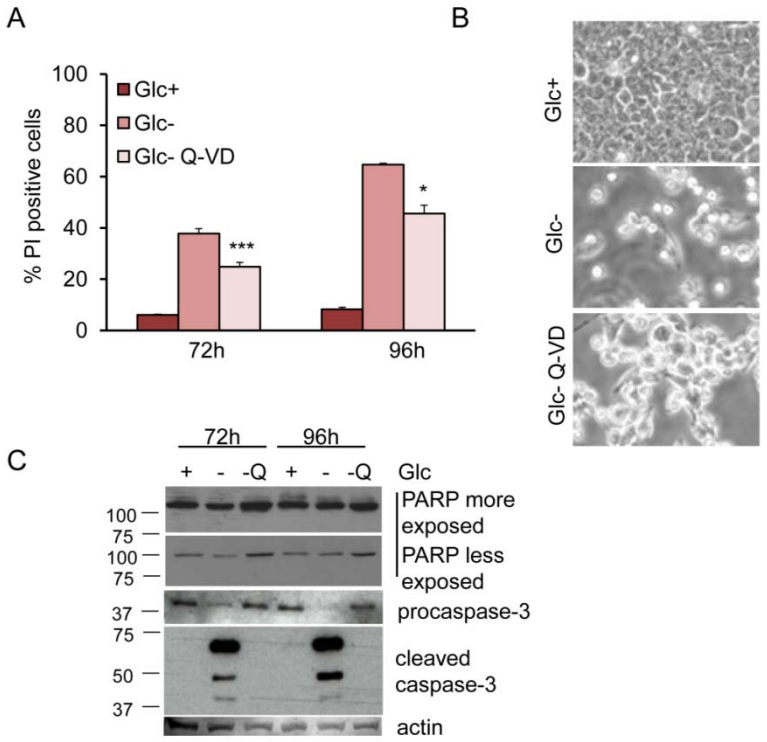

Figure 18: Bax/BaK -/- HCT116 die by apoptosis under glucose deprivation. Cells were treated at 80% of confluence with complete Glc-RPMI plus or minus 10µM Q-VD and collected at 72 h and 96 h. **A**, PI analysis at cytometer. The average and S.E.M. of three experiments are reported. **B**, representative photos of cell death at 72 h are shown. **C,** western blots of caspase 3 and PARP are reported at 72 and 96 h of controls (+), glucose deprived conditions (-) and glucose deprived plus 10µM Q-VD (-Q).

To test whether caspase-8 was involved in death observed in Bax-Bak deficient HCT116 under glucose deprivation, we performed silencing experiments of caspase-8 using the siRNA technique and we could detect a significant reduction of cell death under glucose deprivation when the caspase-8 was knocked-down (Figure 19). All these data suggest that glucose deprivation induces caspase-8 dependent apoptosis in diverse human tumor lines with intact or inefficient mitochondrial pathway.

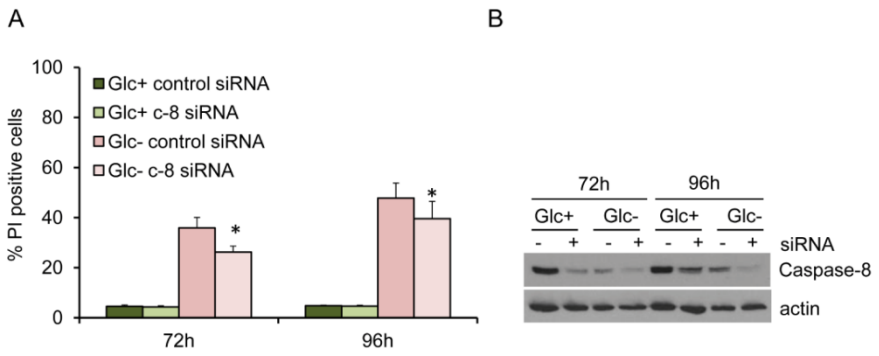

Figure 19: Bax/BaK -/- HCT116 die in a caspase-8 dependent manner under glucose deprivation. Bax/Bak-/- HCT116 cells were plated at 50% confluence in six-well plates and 5 h later they were transfected with 50nM caspase-8 siRNA and the control siRNA. 24 h post transfection, cells were treated without glucose for 72 and 96 h and collected for PI analysis by FACS (**A**). The average and S.E.M. of three experiments are shown. (**B**). Western blot of caspase-8 is reported.

Finally, we tried to clarify a possible role of FLIP in death under glucose deprivation. For this reason, we used WT HeLa and HeLa that were stably transfected with murine FLIPL in a GFP expressing plasmid, as shown in figure 20A. We subjected both cell lines to normal or glucose deprived conditions, in the presence or absence of 10μM Q-VD, and then, they were collected for western bot analysis and PI incorporation at cytometer at the indicated time points (Figure 20B and 20C).

Both cell lines were protected from death by co-treatment with the pan-caspase inhibitor Q-VD, but we could not observe any reduction of cell death under glucose deprivation in cells overexpressing FLIPL (Figure 20C). We were not able to detect FLIP in WT HeLa by western blot, due maybe to its low levels of expression (Inna Lavrik, personal communication), but we observed that glucose deprivation is inducing a down-regulation of the ectopically overexpressed FLIP.

This means that a possible down-regulation of FLIP under glucose deprivation could be contributing to the activation of caspase-8 and the subsequent cell death. However, using this technique we cannot conclude whether FLIP could potentially inhibit the formation of the complex that activates caspase-8.

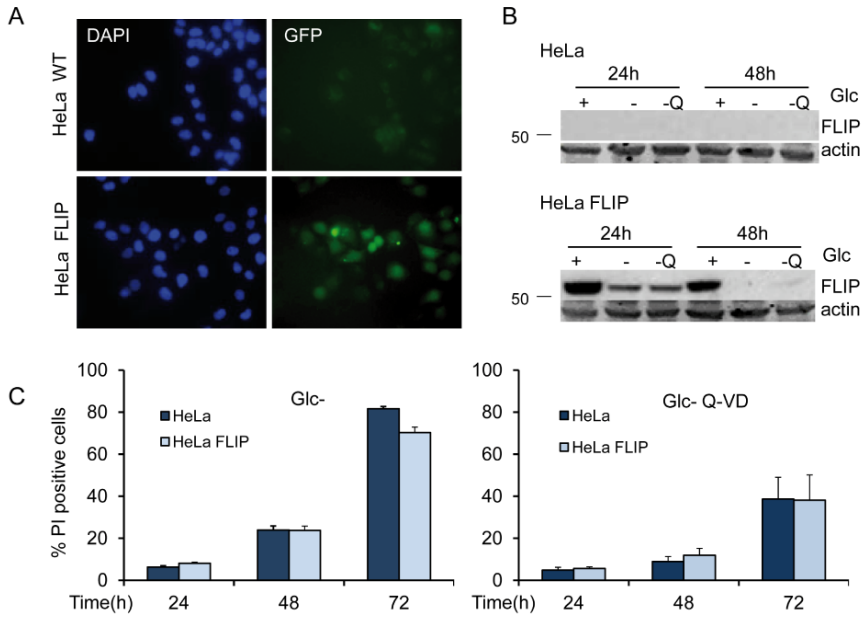

Figure 20: FLIP is downregulated under glucose deprivation. HeLa cells were stably transfected with a vector encoding FLIP and GFP. **A,** epifluorescent microscope photos are shown comparing WT HeLa and HeLa overexpressing FLIP. **B, C,** HeLa and HeLa FLIP were plated at 20% confluence in 60 mm dishes and 24 h later they were treated without glucose in the presence or absence of 10µM Q-VD for 24, 48 h and collected for western blot to detect FLIP (B) and at 24, 48, 72 h for PI analysis at cytometer (C). The average and S.E.M. of three experiments are reported.

2. The viral infection response protein MAVS is not involved in the activation of caspase-8 under glucose deprivation in HeLa cells.

Different groups have shown that some proteins involved in viral infection response such as RIG-I, MDA5 and MAVS can also activate caspase-8 under viral infection and result on cytokines production and inflammatory response (Rajput et al., 2011; El Maadidi et al., 2014). We initially focused on MAVS, a protein localized on the external membrane of the mitochondria, which may have a possible role in the activation of caspase-8 in our model.

We performed silencing experiments of MAVS in HeLa using different sequences of siRNA and we subjected cells to glucose removal for the indicated times, for PI incorporation at cytometer and western blot analysis (Figure 21A and 21B).
We could not observe any protection from cell death due to glucose deprivation. To clarify the possible involvement of MAVS in caspase-8 activation after glucose withdrawal, we performed immunostaining of MAVS in HeLa investigating a possible translocation of this protein after glucose removal. However, we could not detect a clear localization of this protein in the mitochondria, contrary to what we observed in HEK 293 cells (data not shown). For these reasons we discarded these hypotheses.

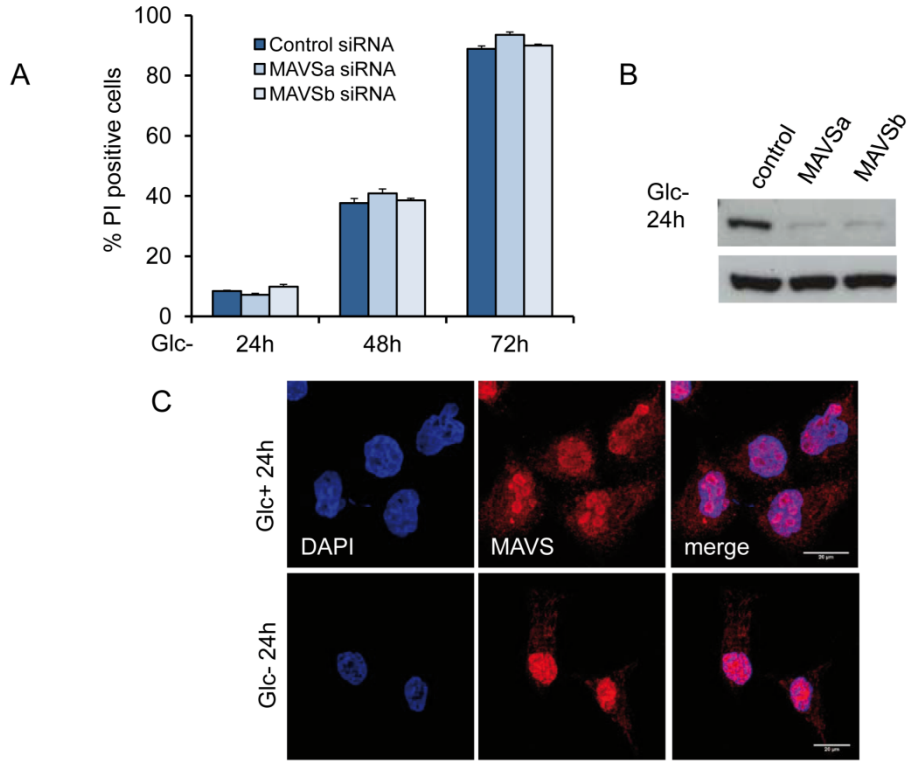

Figure 21: MAVS is not involved in HeLa cell death under glucose deprivation. Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM MAVSa, MAVSb siRNAs and the control siRNA. 24 h post transfection, cells were treated without glucose for 24, 48 and 72 h and collected for PI analysis at cytometer (A). The average and S.E.M. of three experiments are reported. **B**, western blot of cells treated without glucose for 24 h, transfected with control, MAVSa and MAVSb siRNAs are shown. **C**, Hela cells were plated at 20% confluence in 12 well plates on glasses coated with poly-L-lysine and 24 h later they were treated with Glc+ and Glc- for 24 h before performing immunofluorescence of MAVS. Epifluorescent microscope (Nikon) pictures of MAVS and nuclei are shown.

3. Cell death observed under glucose deprivation is not dependent on the Ripoptosome platform in different cell types.

Our second hypothesis was that the so-called Ripoptosome platform could have a role in the activation of caspase-8 under glucose deprivation. Tenev et al and Feoktistova et al (Feoktistova et al., 2011; Tenev et al., 2011) described the formation of this 2 MDa platform upon genotoxic stress and TLR3 stimulation, respectively, which was engaged in apoptotic or necrotic cell death. Both groups showed that the main components of the Ripoptosome are RIPK1, FADD and caspase-8 and that RIPK1's kinase activity as well as the inhibition of the cIAP proteins is essential for the Ripoptosome assembly. The platform is formed spontaneously in the cytosol and independently of TNF, CD95L/FASL, TRAIL interaction with their receptors.

We previously published that Bax-Bak deficient MEFs subjected to glucose deprivation die independently of death ligands and of RIPK1. Silencing of this kinase did not prevent cell death due to glucose deprivation and co-treatment with Necrostatin-1, a RIPK1 inhibitor, did not protect cells from apoptosis (Caro-Maldonado et al., 2010).

To further investigate a possible role of the Ripoptosome in death observed under glucose deprivation in Bax-Bak deficient MEFs, we set up immunoprecipitation experiments of mouse caspase-8 in normal growing conditions or in glucose removal for 24 hours in the presence or absence of the pan-caspases inhibitor Z-VAD. We could not detect pull down of RIPK1 in those conditions (Figure 22), leading us to discard this hypothesis, at least in Bax-Bak deficient MEFs.

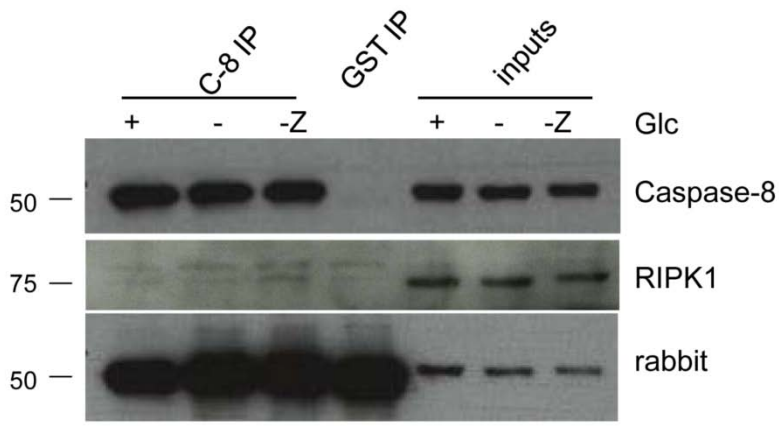

Figure 22: RIPK1 is not co-immunoprecipitated with caspase-8 in Bax/Bak-/- MEFs under glucose deprivation. Bax/Bak-/- MEFs were plated at 20% confluence in 100 mm dishes and 24 h later they were treated with Glc+ (+), Glc- (-), Glc- plus 20µM Z-VAD (-Z) for 24 h before performing immunoprecipitation of caspase-8. Anti-GST antibody was used as an isotype control. Western blot of caspase-8 and RIPK1 are reported.

An essential requirement for Ripoptosome formation is the downregulation or inhibition of cIAPs. We previously observed a decrease in the levels of these proteins in Bax-Bak deficient MEFs after glucose removal and we decided to check the status of these proteins in HeLa cells. As reported in figure 23, western blot analysis of HeLa subjected to glucose deprivation show a clear down-regulation of cIAP1 and cIAP2 (Figure 23A). To further investigate a role of the Ripoptosome in caspase-8 engagement in HeLa, we set up immunoprecipitation experiments of caspase-8 in normal conditions or under glucose deprivation. We could not co-precipitate RIPK1 with caspase-8 in none of the studied conditions (Figure 23B).

We next performed silencing experiment of RIPK1 using siRNA. We could not prevent cell death after glucose removal in RIPK1 silenced conditions (Figure 23C and 23D). Those data suggested that the Ripoptosome is not the cause of caspase-8 activation under glucose deprivation in the diverse studied cell lines.

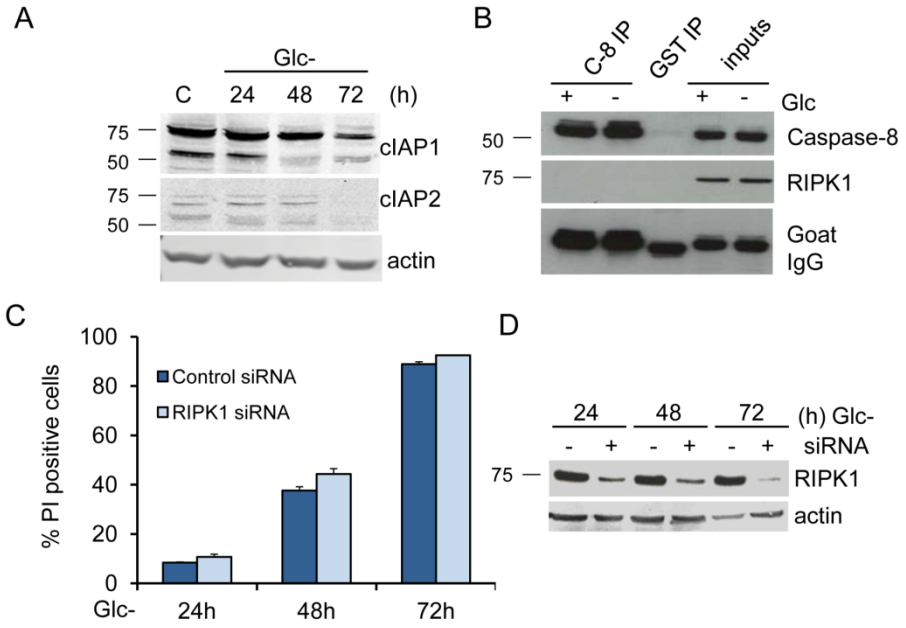

Figure 23: RIPK1 is not involved in cell death under glucose deprivation. **A**, HeLa cells were plated at 20% confluence in 60 mm dishes and 24 h later they were treated without glucose for 24, 48 and 72 h and collected for western blot analysis of cIAPs. **B**, HeLa were plated at 20% confluence in 100 mm dishes and 24 h later they were treated with Glc+ or Glc- for 24 h before performing immunoprecipitation of caspase-8. Anti-GST antibody was used as an isotype control. Western blot of caspase-8 and RIPK1 are reported. **C, D**, Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM RIPK1 siRNAs or the control siRNA. 24 h post transfection, cells treated without glucose for 24, 48 and 72 h were collected for PI analysis by FACS. The average and S.E.M. of three experiments are shown in (C). Western blot of Glc- treated cells, transfected with control and RIPK1 siRNAs are shown in (D).

4. The autophagosomal platform/iDISC is not involved in the activation of caspase-8 under glucose deprivation in different cell types.

A novel autophagosomal membrane platform has been described that seems to be important in recruiting and activating caspase-8 in different cell types, under proteasome inhibition (Laussmann et al., 2011; Pan et al., 2011; Young et al., 2012). Caspase-8 can form a complex with Atg5 maybe trough FADD interaction and has been shown to co-localize with LC3 and p62 on an autophagosomal membrane platform, called iDISC.

We previously showed that in Bax-Bak deficient MEFs and in HeLa cells subjected to glucose deprivation there is an accumulation of proteins involved in autophagy such as p62 and the lipidated form of LC3, LC3-II, due to an inhibition of the basal autophagy flux (Ramírez-Peinado et al., 2013). We wanted to test whether in HeLa cells subjected to glucose deprivation this autophagic platform could have a role in recruiting and activating caspase-8, resulting in cell death.

We first performed immunoprecipitation experiments of human caspase-8 in normal and in glucose deprived conditions for 24 hours in presence or absence of the pan-caspases inhibitor Q-VD and we could see a clear pull down of the lipidated form of LC3 and p62 only in glucose deprived conditions and not in the controls (Figure 24A).

On the contrary, in Bax-Bak deficient MEFs, we could not detect any interaction of mouse caspase-8 with those autophagic proteins (data not shown).

Then, to verify that a real interaction exists between human caspase-8, p62 and LC3, we performed immunoprecipitation of p62 in controls or glucose deprived conditions plus or minus the pan-caspase inhibitor Z-VAD. As shown in figure 24B, immunoprecipitation of p62 pulls down the

lipidated form of LC3 and the caspase-8, more precisely the cut and active form of caspase-8, which appears only after glucose withdrawal.

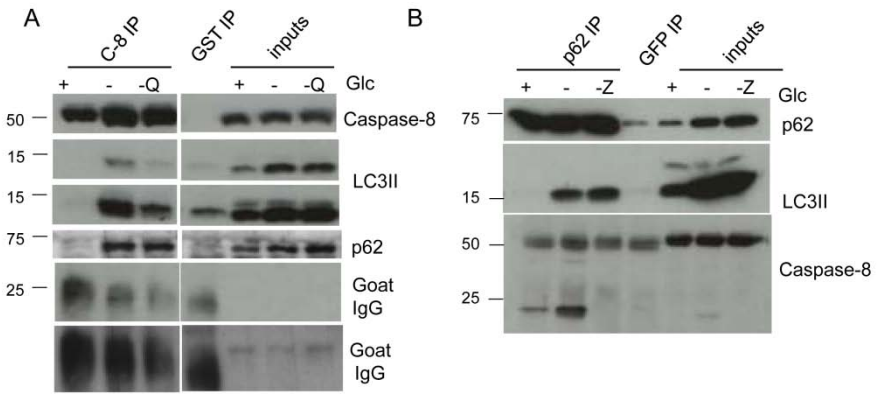

Figure 24: p62 and LC3-II are pulled down with caspase-8 immunoprecipitation in HeLa cells under glucose deprivation. A, B, Hela cells were plated at 20% confluence in 100 mm dishes and 24 h later they were treated with Glc+ (+), Glc- (-), Glc- plus 10μM Q-VD (-Q) or 20μM Z-VAD (-Z) for 24 h before performing immunoprecipitation of caspase-8 (A) or p62 (B). Anti-GST antibody (A) and anti-GFP antibody (B) were used as isotype controls. Western blot of caspase-8, p62 and LC3-II are shown.

As many groups have shown the presence of Atg5 in this platform, we next moved to check whether Atg5 could interact with caspase-8 in our conditions. Figure 25 shows an immunoprecipitation of caspase-8 in HeLa subjected to glucose deprivation for 24 hours. Atg5 is co-immunoprecipitated with the protease at higher levels under the treatment, although more caspase-8 is precipitated after glucose removal.

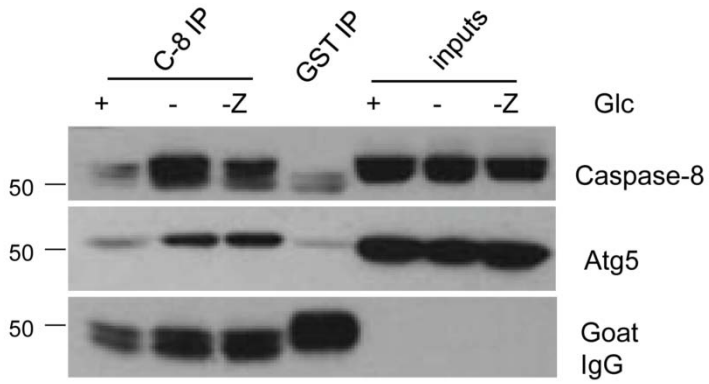

Figure 25: Caspase-8 and Atg5 are co-immunoprecipitated in HeLa cells under glucose deprivation. Hela cells were plated at 20% confluence in 100 mm dishes and 24 h later they were treated with Glc+ (+), Glc- (-), Glc- plus 20μM Z-VAD (-Z) for 24 h before performing immunoprecipitation of caspase-8. Anti-GST antibody was used as an isotype control. Western blot of caspase-8 and Atg5 are shown.

Recently, it has been shown that caspase-8 can be recruited on aggresomes or p62 foci to be fully activated, upon proteasome inhibition or ER stress (Tomar et al., 2013). We decided to perform immunofluorescence experiments to test first if there are some changes in the intracellular localization of caspase-8 under glucose deprivation and next if a possible co-localization of caspase-8 with p62 could exist.

In figure 26, pictures of confocal microscope of HeLa deprived or not of glucose for 24 hours, are shown (Figure 26A). Caspase-8 looks like a diffuse pattern of spots in the cytosol with no obvious changes after the treatment; in general it is more immune reactive after glucose deprivation. We could not observe a clear co-localization of caspase-8 with the spots of p62. When we quantify the rate of co-localization taking into account only the spots of p62 we obtain a minimum rate of co-localization that is maintained similar after glucose removal (Figure 26B).

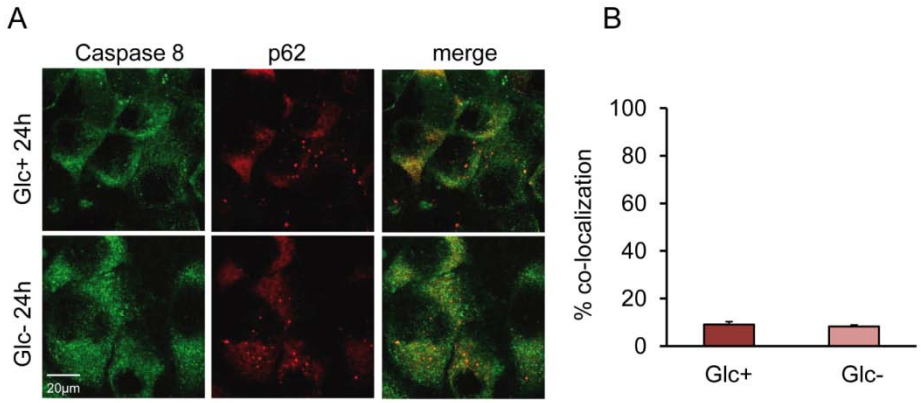

Figure 26: p62 does not colocalize with caspase-8 under glucose deprivation. A, B, Hela cells were plated at 20% confluence in 12 well plates on glasses coated with poly-L-lysine and 24 h later they were treated with Glc+ or Glc- for 24 h before performing immunofluorescence of caspase-8 and p62. Confocal microscope pictures of co-localization of caspase-8 with p62 spots are reported in A and the quantification with ImageJ software in B. Average and S.E.M. of three experiments are shown.

Afterwards, we verified if the caspase-8 could co-localize with ubiquitin spots, as p62 binds ubiquitinated proteins destined to autophagy degradation. For this reason, we performed immunofluorescence experiments of caspase-8 using an antibody against mono and poly-ubiquitinations and, as we can observe in figure 27A, caspase-8 spots do co-localize with ubiquitin spots, without obvious changes in the rate of co-localization after glucose removal (Figure 27B). The ubiquitination of caspase-8 on the residue K63 has been described to be essential for its full activation; so we checked by western blot if the immunoprecipitated fraction of caspase-8 could be ubiquitinated under glucose deprivation. We were not able to detect this ubiquitination (data not shown).

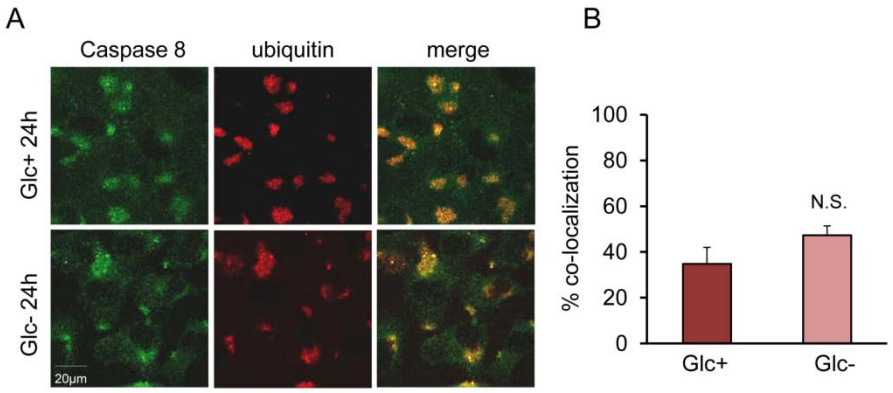

Figure 27: Caspase-8 is colocalizing with ubiquitin under glucose deprivation. A, B, Hela cells were plated at 20% confluence in 12 well plates on glasses coated with poly-L-lysine and 24 h later they were treated with Glc+ or Glc- for 24 h before performing immunofluorescence of caspase-8 and ubiquitin. Confocal microscope pictures of co-localization of caspase-8 with ubiquitin spots are reported in A and the quantification with ImageJ software in B. Average and S.E.M. of three experiments are shown.

We previously showed that glucose deprivation induces a pull down of the lipidated form of LC3 with immunoprecipitated caspase-8. We next performed immunofluorescence experiment of caspase-8 and LC3 to check a possible co-localization of these two proteins. We could observe that caspase-8 do co-localize with LC3 at basal levels and after the treatment the rate of co-localization is significant increased (Figure 28A and 28B).

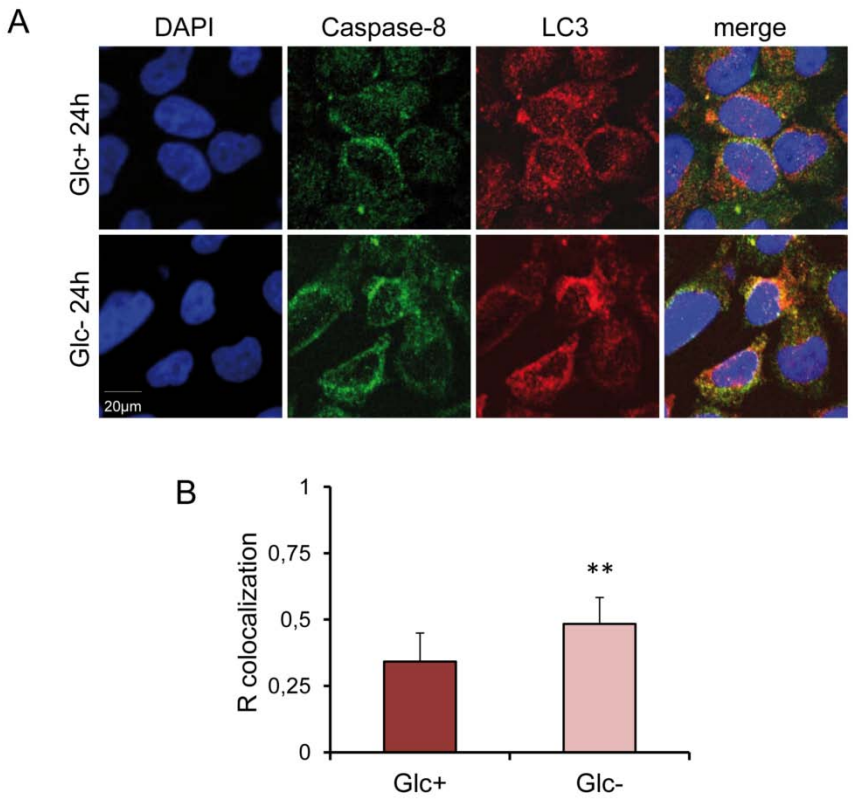

Figure 28: Caspase-8 is colocalizing with LC3 under glucose deprivation. A, B, Hela cells were plated at 20% confluence in 12 well plates on glasses coated with poly-L-lysine and 24 h later they were treated with Glc+ or Glc- for 24 h before performing immunofluorescence of caspase-8 and LC3. Confocal microscope pictures of co-localization of caspase-8 with LC3 are reported in A and the quantification with ImageJ software in B. Average and S.E.M. of three experiments are shown.

All these data suggest that p62, LC3 and possibly Atg5 are interacting with caspase-8 in some platform where they could be involved in its recruitment and activation under glucose deprivation.

In order to clarify the function of this platform in HeLa cells, we decided to perform silencing experiments of p62 and to check the effect of its transient silencing on the glucose deprivation-induced cell death. We have already shown that p62 siRNAs in Bax-Bak deficient MEFs did not prevent cell death by glucose removal suggesting that in that cell line this platform

is not essential for the induction of cell death (Ramírez-Peinado et al., 2013).

We used two different siRNA sequences against p62, one of those indicated as (2) in figure 29B, silenced very well the protein.

In both cases, the transient silencing of p62 did not prevent cell death observed under glucose deprivation (Figure 29A).

These results suggest that something else different from the autophagosomal platform is inducing cell death under glucose deprivation in HeLa cells.

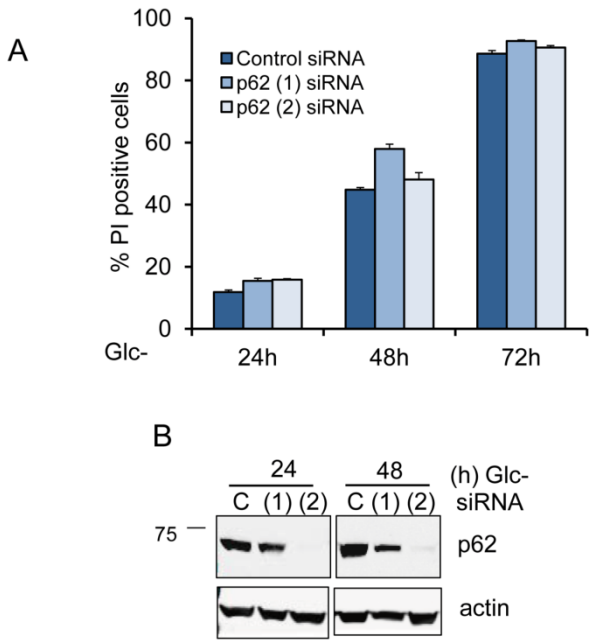

Figure 29: p62 is not involved in HeLa cell death under glucose deprivation. A, B, Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM p62 (1), p62 (2) siRNAs and the control siRNA. 24 h post transfection cells were treated without glucose for 24, 48 and 72 h and collected for PI analysis by FACS. The average and S.E.M. of three experiments are shown in (A). Western blots of cells treated without glucose for 24 h and 48 h, transfected with control, p62 (1) and p62 (2) siRNAs are shown in (B).

5. FADD, but not TRADD, is involved in death observed under glucose deprivation.

Michaeu and Tschopp described in 2003 that the signaling through TNF-receptor 1 (TNFR1) proceeds through the recruitment of the adaptor protein TRADD, the kinase RIPK1 and TRAF2 (the so-called complex I) which results in cell survival through the NF-KB pathway (Micheau and Tschopp, 2003). This signalization can also result in cell death if the adaptor protein FADD and the pro-caspase-8 are recruited to the first complex (forming the so-called complex II). In the absence of the inhibitor cFLIP, caspase-8 would be activated, initiating the apoptotic signaling.

We wanted to investigate if the adaptor molecules FADD and TRADD are essential for the assembly and recruitment of caspase-8 to the DISC under glucose deprivation.

For this reason we performed immunoprecipitation experiments of caspase-8 in normal and glucose deprived conditions in presence or absence of caspase inhibitors, looking for possible FADD and TRADD co-immunoprecipitation. While in Bax-Bak deficient MEFs we could not detect any interaction of caspase-8 with FADD (data not shown), we observed a clear interaction of the protease with FADD in HeLa cells, only after glucose removal (Figure 30A). We could not detect any interaction with TRADD (data not shown). Immunoprecipitation experiments of FADD still pulled down the lipidated form of LC3, but also the active form of caspase-8, suggesting a possible role of FADD in recruiting caspase-8 (Figure 30B).

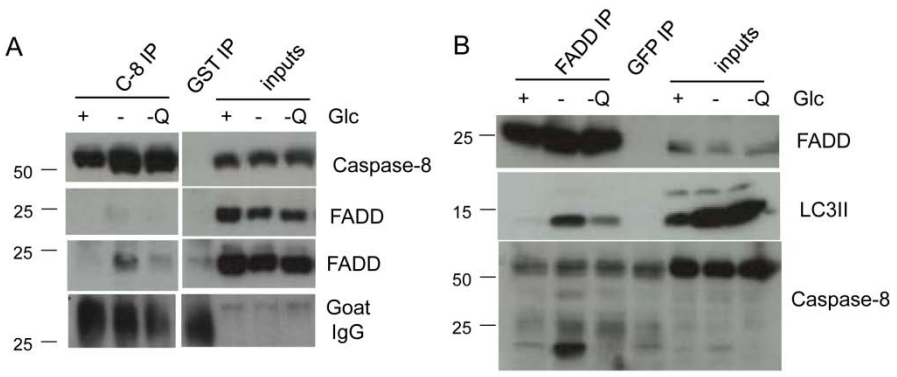

Figure 30: Caspase-8 and FADD are co-immunoprecipitated in HeLa cells under glucose deprivation. A, B, Hela cells were plated at 20% confluence in 100 mm dishes and 24 h later they were treated with Glc+ (+), Glc-(-), Glc- plus 10µM Q-VD (-Q) for 24 h before performing immunoprecipitation of caspase-8 (A) and FADD (B). Anti-GST antibody (A) and anti-GFP antibody (B) were used as isotype controls. Western blot of caspase-8, FADD and LC3-II are reported.

To further investigate the interaction of caspase-8 and the adaptor FADD, we performed immunofluorescence experiments in HeLa subjected or not to glucose deprivation for 24 hours (Figure 31). As shown by confocal microscope pictures and ImageJ co-localization analysis, we could detect a basal co-localization of caspase-8 with FADD which seems not to increase after the treatment.

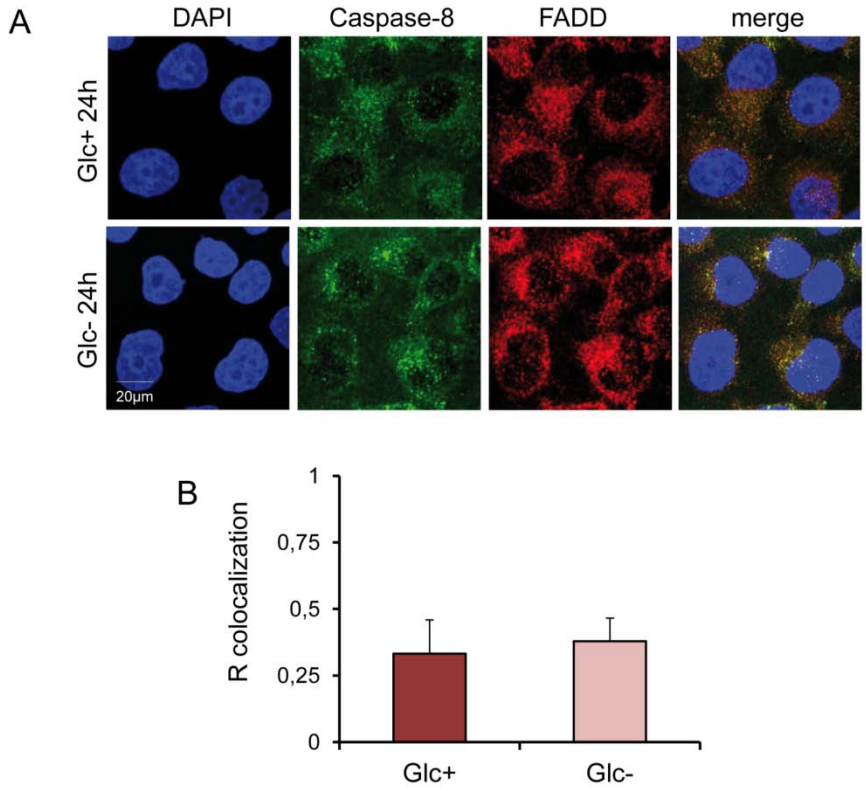

Figure 31: Caspase-8 is colocalizing with FADD under glucose deprivation. A, B, Hela cells were plated at 20% confluence in 12 well plates on glasses coated with poly-L-lysine and 24 h later they were treated with Glc+ or Glc- for 24 h before performing immunofluorescence of caspase-8 and FADD. Confocal microscope pictures of co-localization of caspase-8 with FADD are reported in A and the quantification with ImageJ software in B. Average and S.E.M. of three experiments are shown.

To clarify the role of FADD in recruiting caspase-8 in HeLa under glucose deprivation, we performed silencing experiments of FADD using two different sequences of siRNA. We could observe a clear protection from death with both sequences of FADD (Figure 32). A similar amount of protection with FADD siRNAs was obtained by a cross-linker antibody against Fas (CH11 Fas) which induces the canonical extrinsic pathway of apoptosis (Figure 32A).

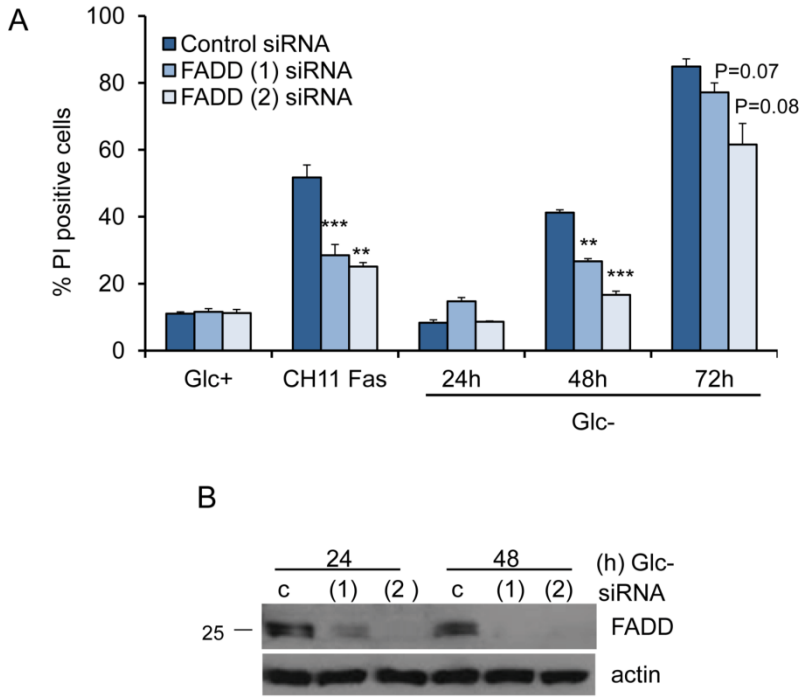

Figure 32: FADD siRNAs protect from death observed under glucose deprivation. A, B, Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM FADD (1) and (2) siRNAs and the control siRNA. 48 h post transfection cells were treated without glucose for 24, 48 and 72 h or with CH11 antibody for 48 h as control and collected for PI analysis by FACS. The average and S.E.M. of three experiments are reported in (A). Western blot of Glc- treated cells, transfected with control, FADD (1) and FADD (2) siRNAs are shown in (B).

Moreover, we wanted to check if TRADD could be participating in the formation of the DISC recruiting caspase-8 after glucose deprivation.

Consequently, we transiently silenced TRADD in HeLa cells that were then deprived of glucose. As we can see by figure 33A, both siRNAs of TRADD decrease very well the levels of the protein, but none of them prevents death induced by glucose deprivation (Figure 33B).

These results confirmed that FADD, but not TRADD, is essential for caspase-8 recruitment and activation in HeLa cells subjected to glucose deprivation.

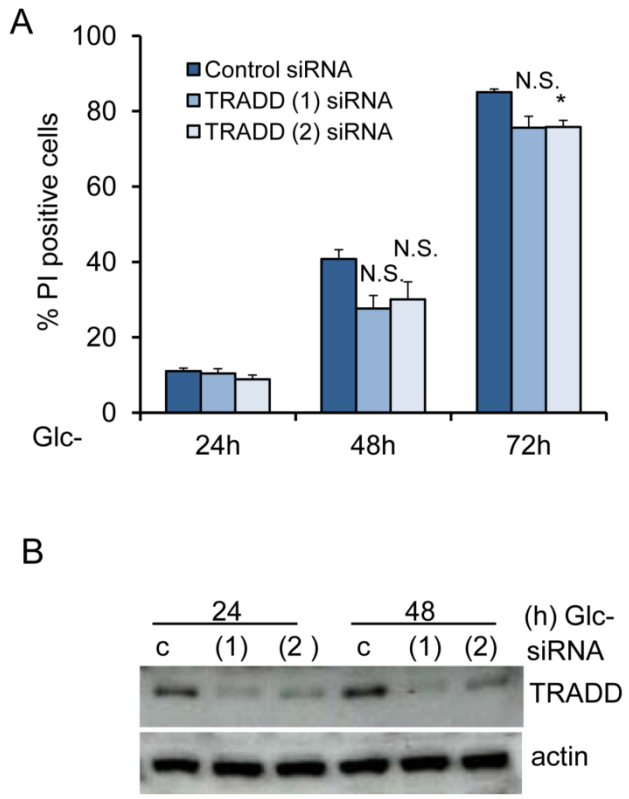

Figure 33: TRADD siRNAs do not protect from death observed under glucose deprivation. A, B, Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM TRADD (1) and (2) siRNAs and the control siRNA. 24 h post transfection cells were treated without glucose for 24, 48 and 72 h and collected for PI analysis by FACS (A). The average and S.E.M. of three experiments are reported. Western blot of Glc- treated cells, transfected with control, TRADD (1) and TRADD (2) siRNAs are shown in (B).

6. Glucose deprivation induces ER stress and death receptors expression.

Our group have previously described that glucose deprivation induces ER stress in different cell lines due maybe to an accumulation of misfolded proteins in the ER (León-Annicchiarico et al., 2015). If ER stress is persistent or the cell cannot deal with it, it can result in cell death. We decided to check whether glucose deprivation induces ER stress in our model.

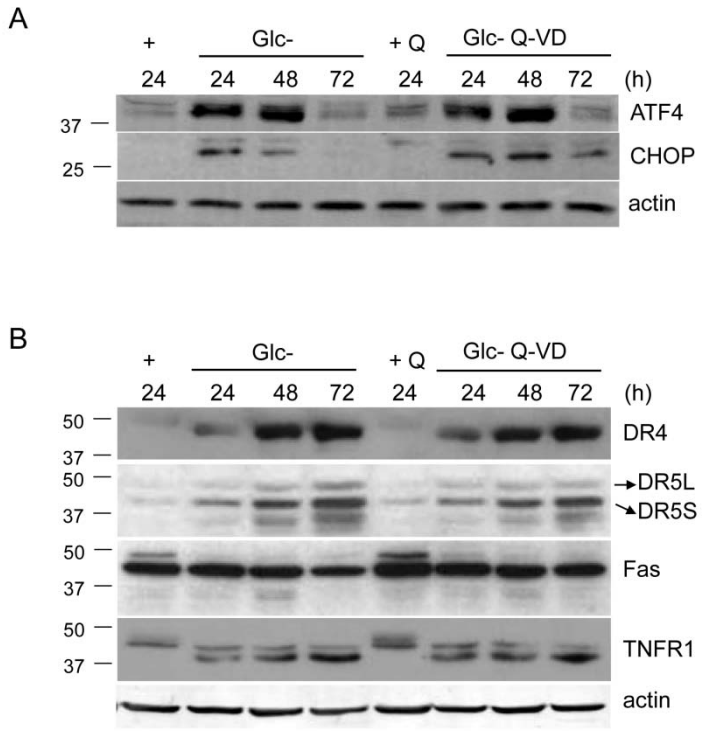

Figure 34: Glucose deprivation induces ER stress and death receptors. A, B, Hela cells were plated at 20% confluence in 60 mm dishes and 24 h later they were treated with Glc+ or Glc- plus or minus 10µM Q-VD (Q) for 24, 48 and 72 h and then collected for western blot of ER stress markers: ATF4 and CHOP (A) and death receptors: DR4 (TRAIL-R1), DR5 (TRAIL-R2), Fas and TNFR1 (B).

As shown in figure 34A, glucose removal induces in HeLa cells an increase not only of ATF4 but also of CHOP, observed already at 24 hours of glucose deprivation and then decreasing at later time points.

Recently, different groups have shown that ER stress inducers can trigger cell death through the up-regulation of DR5 and DR4 and both were found regulated directly by CHOP on their promoters (Li et al., 2015; Lu et al., 2014; Yamaguchi and Wang, 2004).
We wondered if also in our model of glucose deprivation it could exist an induction of death receptors due to the persistent ER stress.
We performed western blot analysis of the death receptors DR4, DR5, Fas and TNFR1 in HeLa cells and we observed that DR4 and DR5 were clearly up-regulated at protein levels after glucose deprivation, already at the 24 hours of treatment (Figure 34B). We could also observe the appearance of a third band of DR5 after glucose removal that could be due maybe to a less glycosylation of the receptor.

Fas and TNFR1 receptors were not clearly induced by the treatment but we could see a shift in size of the bands relative to those receptors due maybe to a differential state of glycosylation (Figure 34B).

We also perfomed q-PCR analysis to define if also the mRNA levels of the death receptors were increased under glucose deprivation. We can detect an increase of DR5 mRNA levels at short time points of glucose deprivation, 8 hours, which was then reduced at longer time points (Figure 35).

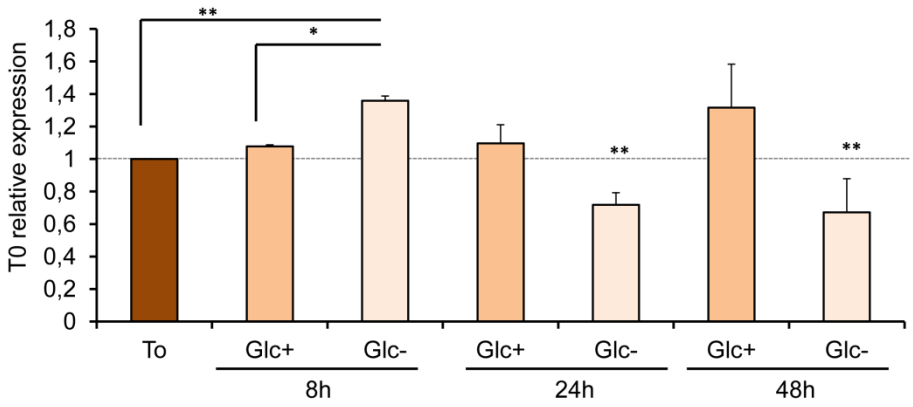

Figure 35: Glucose deprivation induces DR5 expression at a short time point of glucose deprivation. Hela cells were plated at 20% confluence in 60 mm dishes and 24 h later they were treated with Glc+ or Glc- for 8, 24 and 48 h and collected for q-PCR analysis. DR5 mRNA levels relative to housekeeping gene and time 0 (T0) are reported. The average and S.E.M. of three experiments are reported.

No significant changes were detected for the mRNA levels of DR4 receptor, while TNFR1 and Fas were downregulated at 24 hours of glucose removal (Figure 36).

We also wondered what happens to TRAIL under glucose deprivation and if it could be responsible for DR4 and DR5 induction. qPCR analysis show that TRAIL is not induced after glucose withdrawal; on the contrary, it is considerably down-regulated at 24 hours of treatment (Figure 36).

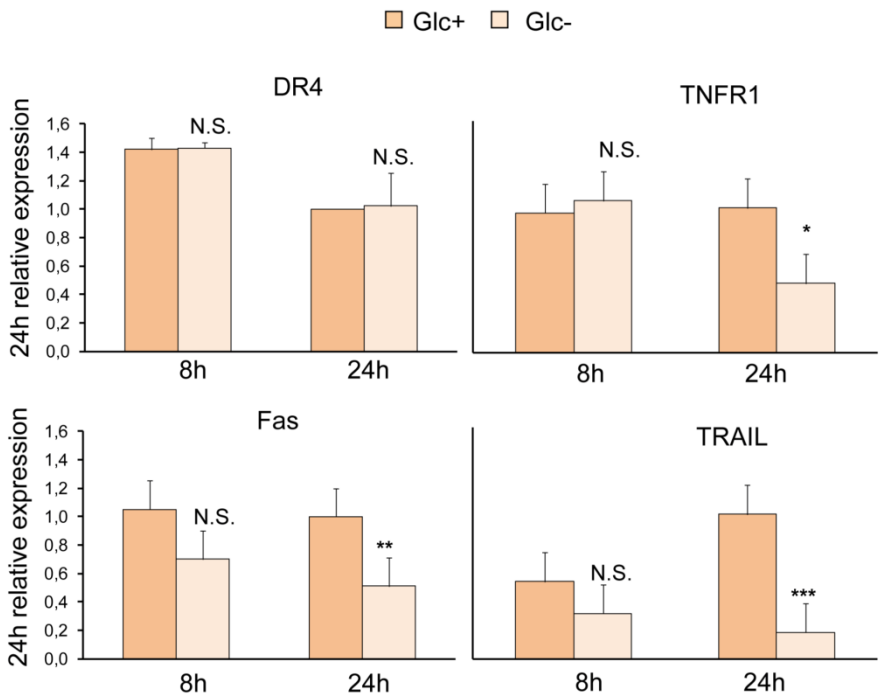

Figure 36: Glucose deprivation does not induce DR4, TNFR1, Fas and TRAIL mRNAs. Hela cells were plated at 20% confluence in 60 mm dishes and 24 h later they were treated with Glc+ or Glc- for 8, 24 and 48 h and collected for q-PCR analysis. DR4, TNFR1, Fas, TRAIL mRNA levels relative to housekeeping gene and time 24 h are reported. The average and S.E.M. of three experiments are reported.

7. Glucose deprivation induces apoptosis through ATF4-dependent DR5 up-regulation.

We already described that caspase-8-dependent apoptosis in Bax/Bak deficient MEFs subjected to glucose deprivation occurs independently of death ligands (Caro-Maldonado et al., 2010). To confirm that death observed under glucose deprivation is not dependent on death ligands

interaction with their receptors in HeLa cells, we silenced TRAIL using two different sequences of siRNA (Figure 37). Both siRNAs work very well, in fact they decrease considerably the mRNA levels of TRAIL as shown in figure 37B.

We could not prevent cell death with none of the siRNA, suggesting that the induction of the receptors under glucose deprivation occurs independently of upregulation of their ligand.

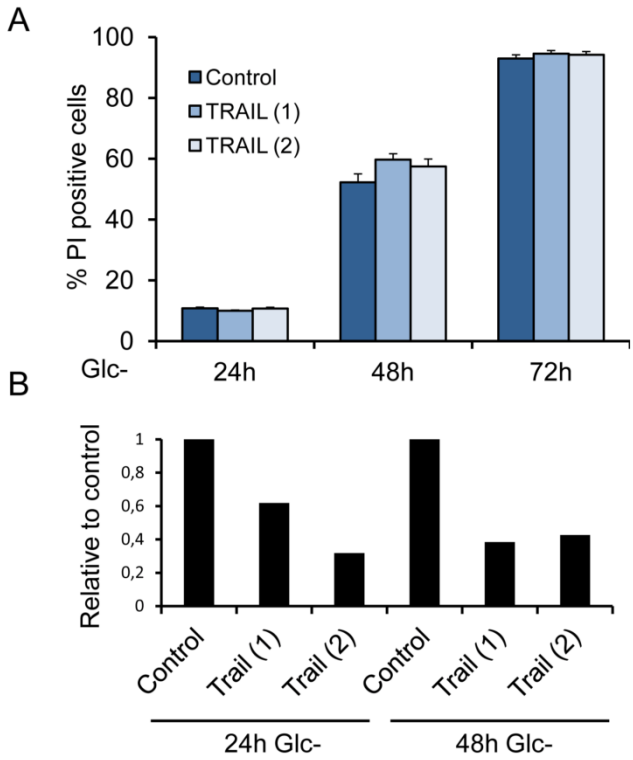

Figure 37: TRAIL is not involved in cell death under glucose deprivation.

A, B, Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM TRAIL (1) and (2) siRNAs and the control siRNA. 24 h post transfection cells were treated without glucose for 24, 48 and 72 h and collected for PI analysis by FACS (A). The average and S.E.M. of three experiments are reported. q-PCR analysis of HeLa transfected with TRAIL (1) and TRAIL (2) siRNAs and the control siRNA is shown (B).

Recently, different groups have shown that ER stressors such as tunicamycin or thapsigargin induce cell death through CHOP dependent up-regulation of DR5 (Lu et al., 2014; Martín-Pérez et al., 2014).

We wanted to study if the ER stress induced under glucose deprivation could be the cause of death and of induction of DR5 and DR4.

For that reason, we performed silencing of ATF4 and CHOP in HeLa cells using different siRNA sequences to check if those proteins were involved in the induction of death receptors and the resulting cell death. The siRNAs of ATF4 prevented very well cell death observed under glucose deprivation (Figure 38A). The induction of DR5 but not of DR4 was also impaired under this condition (Figure 38B).

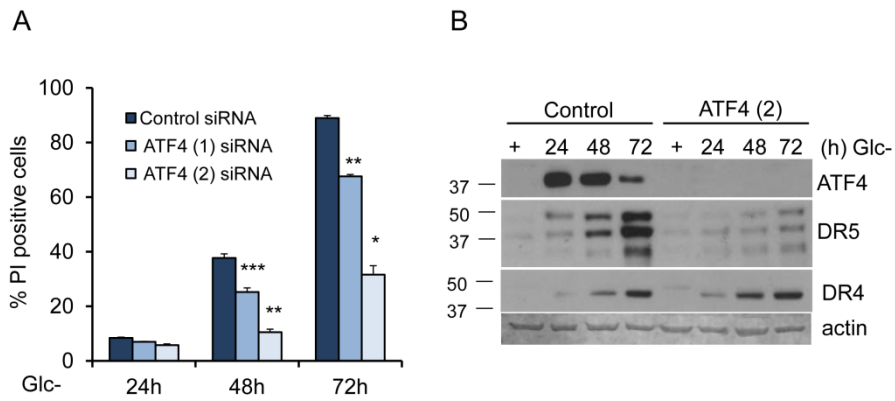

Figure 38: ATF4 induces cell death and DR5 induction under glucose deprivation. A, B, Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM ATF4 (1) and (2) siRNAs and the control siRNA. 24 h post transfection cells were treated without glucose for 24, 48 and 72 h and collected for PI analysis by FACS (A). The average and S.E.M. of three experiments are reported. Western blot of ATF4, DR5 and DR4 of cells transfected with control and ATF4 (2) siRNAs are shown in (B).

The siRNA against CHOP did not prevent cell death due to glucose withdrawal (Figure 39A), neither the induction of DR5 or DR4 was prevented when CHOP was silenced (Figure 39B).

All these data indicated that DR5 but not DR4 expression is regulated by ATF4 under glucose removal in HeLa cells.

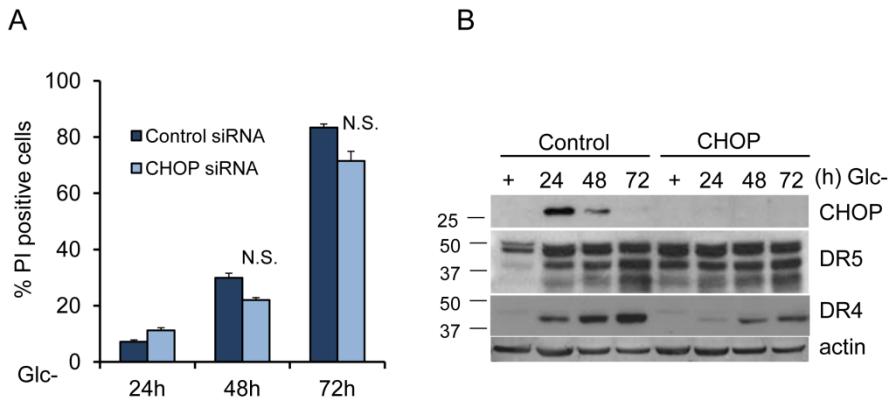

Figure 39: CHOP does not participate in cell death or DR5 induction under glucose deprivation. A, B, Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM CHOP siRNA and the control siRNA. 24 h post transfection cells were treated without glucose for 24, 48 and 72 h and collected for PI analysis by FACS. The average and S.E.M. of three experiments are reported (A). Western blot of CHOP, DR5 and DR4 of cells transfected with control and CHOP siRNAs are shown (B).

The group of Dr. A. Ashkenazi showed that DR5 is up-regulated under ER stress and it localizes, among other localizations, at the Golgi apparatus, where it is maybe recruiting the DISC platform for its activation (Lu et al., 2014). We performed immunofluorescence experiments in HeLa cells treated with plus or minus glucose for 24 hours and we studied co-localization of DR5 with GM130, a Golgi Marker, or calnexin, an ER marker.

We showed that DR5 localizes at the Golgi and it looks in more compact structures after glucose removal, where maybe it is being accumulated and activated (Figure 40). We could not detect co-localizaton of DR5 with calnexin in this cell line, as shown by the co-localization analysis in figure 41.

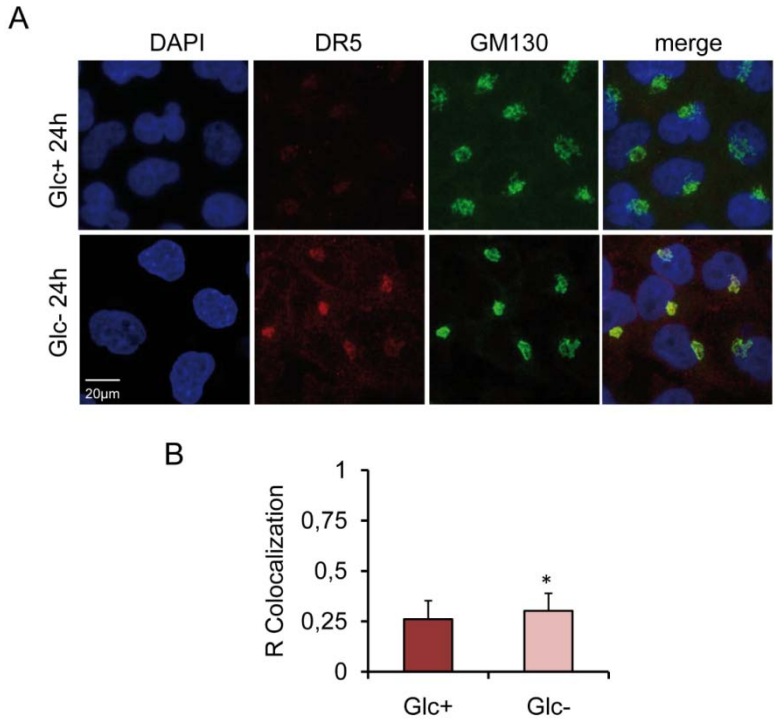

Figure 40: DR5 localizes at Golgi apparatus. A, B, Hela cells were plated at 20% confluence in 12 well plates on glasses coated with poly-L-lysine and 24 h later they were treated with Glc+ or Glc- for 24 h before performing immunofluorescence of GM130 and DR5. Confocal microscopy pictures of co-localization of GM130 with DR5 are reported (A). The quantification of the colocalization analysis with ImageJ software is reported in B.

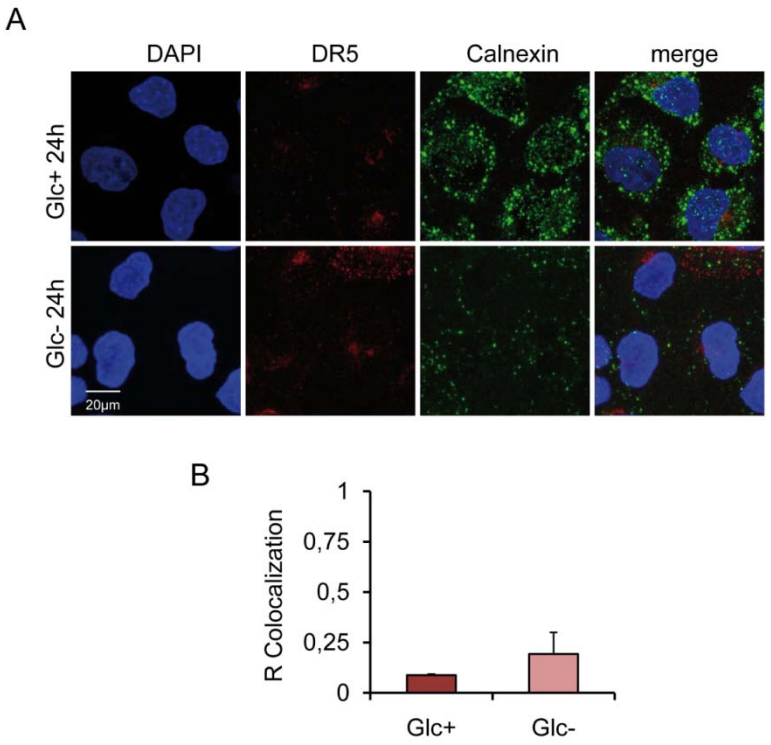

Figure 41: DR5 localizes partially with calnexin in ER. A, B, Hela cells were plated at 20% confluence in 12 well plates on glasses coated with poly-L-lysine and 24 h later they were treated with Glc+ or Glc- for 24 h before performing immunofluorescence of Calnexin and DR5. Confocal microscopy pictures of co-localization of Calnexin with DR5 are reported (A). The quantification of the colocalization analysis with ImageJ software is reported in B.

Then, we wanted to verify if actually a complex between caspase-8 and DR5 was formed upon glucose removal in HeLa. We performed immunoprecipitation analysis of caspase-8 in HeLa subjected to 24 hours of glucose deprivation.

We could observe that DR5 is pulled down with caspase-8, more precisely; the short isoform of the receptor is co-immunoprecipitated with the enzyme, after glucose removal (Figure 42).

All these data suggest that DR5 is induced under glucose deprivation in a ligand-independent way but dependent on ATF4. The receptor is accumulated at the Golgi apparatus where maybe it is oligomerizing and recruiting caspase-8 to form the iDISC.

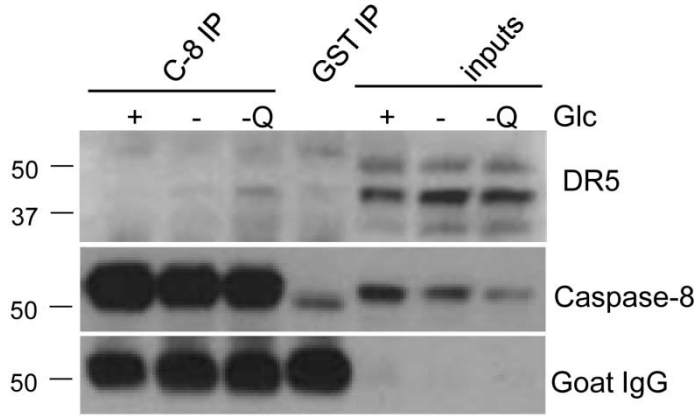

Figure 42: Caspase-8 and DR5 are co-immunoprecipitated in HeLa cells under glucose deprivation. Hela cells were plated at 20% confluence in 100 mm dishes and 24 h later they were treated with Glc+ (+), Glc- (-), Glc- plus 10μM Q-VD (-Q) for 24 h before performing immunoprecipitation of caspase-8. Anti-GST antibody is used as isotype controls. Western blot of caspase-8 and DR5 are reported.

8. Human tumor cells are dying under glucose deprivation in a TRAIL-R2/DR5-dependent manner.

To verify the role of DR5 in death due to glucose deprivation in human tumor cells, we decided to silence the receptor using different siRNA sequences against the two different isoforms of the receptor, the long and the short one, as reported in figure 43.

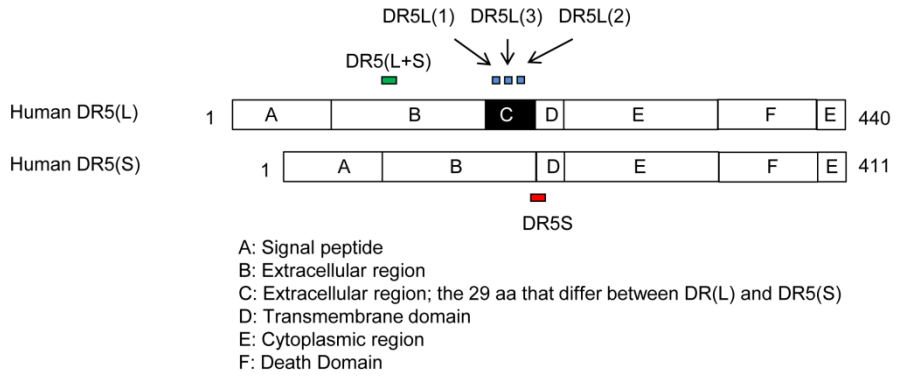

Figure 43: Scheme of siRNA sequences against human DR5 receptor. The regions of human DR5 receptor where siRNA sequences targeted its mRNA are reported. In green it is shown the sequence against both isoforms, the long and short one, DR5 (L+S); the sequences against the long isoform, DR5L (1), DR5L (2), DR5L (3) are indicated in blue and in red it is shown the one against the short isoform, DR5S.

We transfected HeLa cells with different siRNA sequences against DR5, and 24 hours post transfection we deprived them of glucose and we detected PI incorporation at cytometer at 24 and 48 hours of treatment.

As reported in figure 44A, we obtained a significant protection from death due to glucose deprivation only with the siRNA sequence DR5L (2) but not with the other sequences. In figure 44B, we can observe that all siRNA sequences silenced very well their specific targeted isoform as reported by western blot analysis.

Moreover, we can observe that DR5L (2) is the only sequence which decreases the levels of the long isoform without altering the levels of the short one. In spite of decreasing very well the levels of the large isoform, DR5L (1) and DR5L (3) induce an accumulation of DR5S and the appearance of a third band of DR5 which could be an unknown isoform contributing to cell death.

DR5L (3) is also inducing a change in the shape of the cells, which became larger than the controls, but they are still dying after the treatment (Figure 45).

The DR5S is silencing very well the short isoform but it is inducing a third band of DR5 which is the same observed in the control siRNA-transfected cells after glucose removal; it could be a less glycosylated isoform still able to induce cell death (Figure 34B).

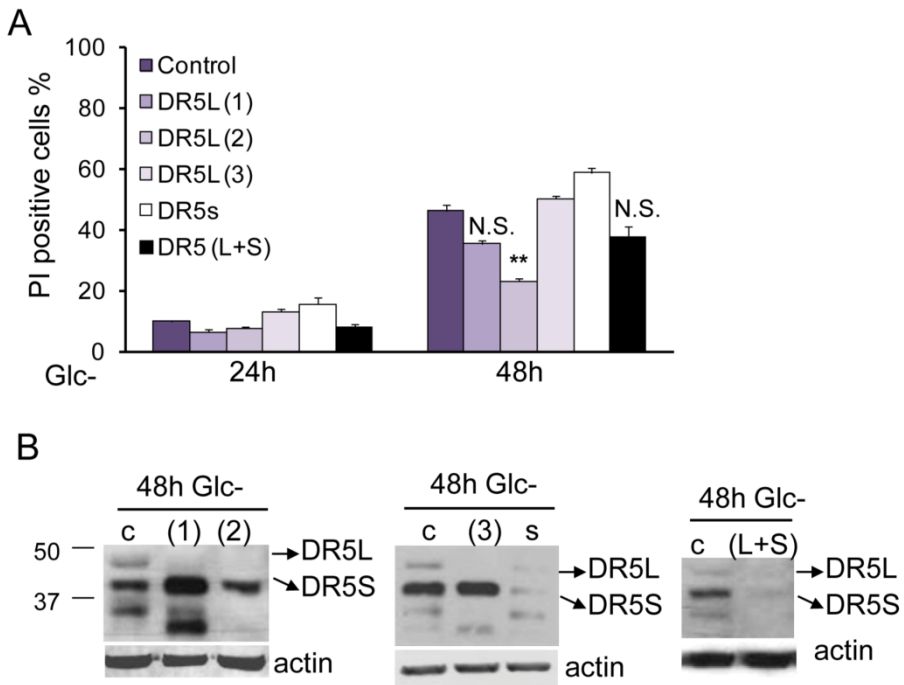

Figure 44: DR5L siRNA prevents cell death under glucose deprivation in HeLa. A, B, Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM DR5L (1), DR5L (2), DR5L (3), DR5S, DR5 (L+S) siRNAs and the control siRNA. 24 h post transfection cells were treated without glucose for 24 and 48 h and collected for PI analysis by FACS. (A) The average and S.E.M. of three experiments are shown. (B) Western blot of DR5 of cells transfected with control and DR5 siRNAs.

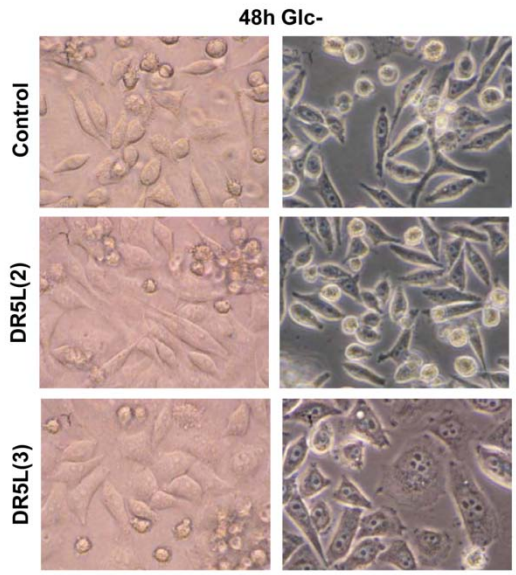

Figure 45: DR5L (2) siRNA protects from death due to glucose deprivation in HeLa. Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM DR5L (2), DR5L (3) and the control siRNA. 24 h post transfection cells were treated without glucose and some pictures were taken at 48 h of treatment at the OLYMPUS inverted microscope.

As we observed a strong induction of DR5 receptor under glucose deprivation and we could prevent death only with one sequence of siRNA against it, we wondered if a percentage of the observed death could still be due to the mitochondrial pathway of apoptosis that is functional in HeLa cells.

As shown in figure 46, silencing DR5 receptor in HeLa stably overexpressing Bcl-xL prevented cell death in a more pronounced way. All three siRNA sequences against the large isoform of DR5 protected from death while DR5S did not. In HeLa-Bcl-xL we can still detect a change in the shape of the cells due to the transfection with DR5L (3) sequence, but, contrary to what observed in HeLa, also this sequence prevents cell death.

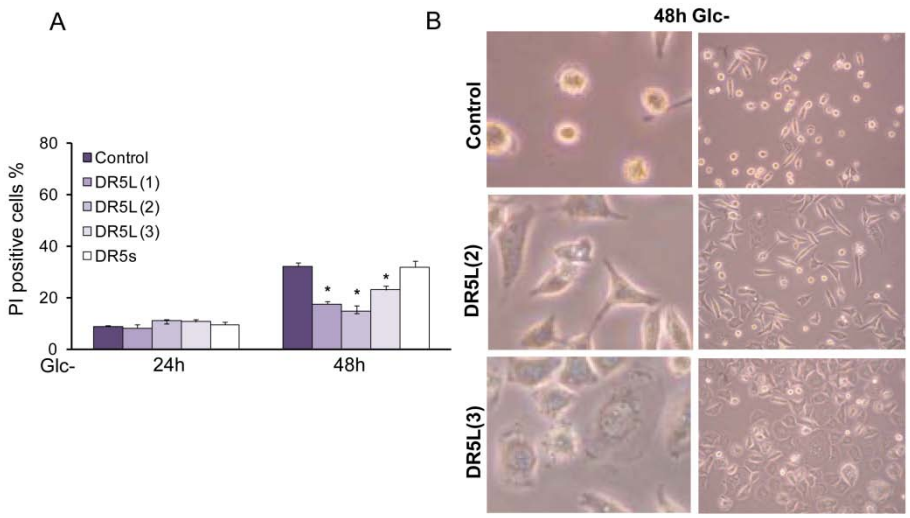

Figure 46: DR5L siRNAs prevent cell death under glucose deprivation in HeLa-Bcl-xL. A, B, Hela-Bcl-xL were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM DR5L (1), DR5L (2), DR5L (3), DR5S siRNAs and the control siRNA. 24 h post transfection cells were treated without glucose for 24 and 48 h and collected for PI analysis by FACS. The average and S.E.M. of three experiments are reported. (A). Pictures of cells transfected with control or DR5L (2) and DR5L (3) siRNAs and treated for 48 h without glucose are shown (B).

We then checked if Bax/Bak deficient HCT116 coul be protected by DR5 siRNAs under glucose deprivation. We decided to perform LDH test to quantify cell death in this cell line, because we were not able to reproduce the protection from cell death observed by eye with cytometer analysis, suggesting that these cells are too fragile to collect them for FACS.

As reported in figure 47 the DR5L (2) sequence and also the DR5S prevents death under glucose deprivation in this cell line.

These data suggest that different isoforms of DR5 receptor could have a major role in triggering apoptosis due to glucose deprivation, depending on cell type.

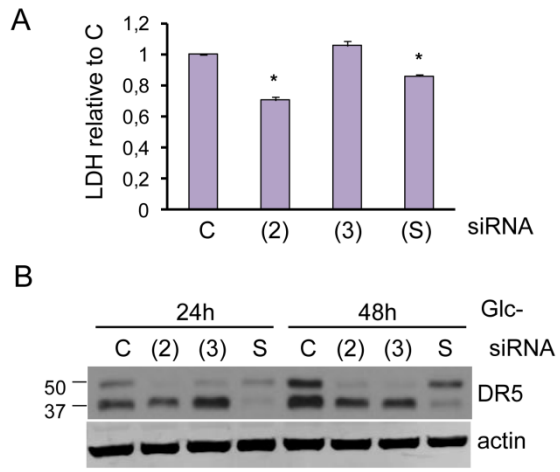

Figure 47: DR5L siRNA prevents cell death under glucose deprivation in Bax/Bak deficient HCT 116. A, B, Bax/Bak deficient HCT116 were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM DR5L (2), DR5L (3), DR5S and the control siRNA (C). 24 h post transfection cells were treated without glucose for 72 h and collected for LDH test. The average and S.E.M. of three experiments are reported (A). Western blot of DR5 of treated cells transfected with control and DR5 siRNAs are shown (B).

Moreover, we wanted to investigate if DR4, similarly to DR5, could have a role in regulating apoptosis due to glucose deprivation. We performed silencing experiments in HeLa cells using two different siRNA sequences against DR4, DR4 (1) and DR4 (2), and we deprived them of glucose, 24 hours after transfection.

The analysis of PI incorporation at cytometer is reported in figure 48A. DR4 siRNAs protect from cell death but not in a significant way (n=3). As observed in figure 48B the silencing of DR4 induces an increase in DR5 levels, compared to the controls. However, the combination of DR4 siRNAs with the best sequence against DR5L, DR5L (2) does not improve the protection.

These data suggest that DR5 could have a major role in inducing cell death after glucose withdrawal in HeLa cells.

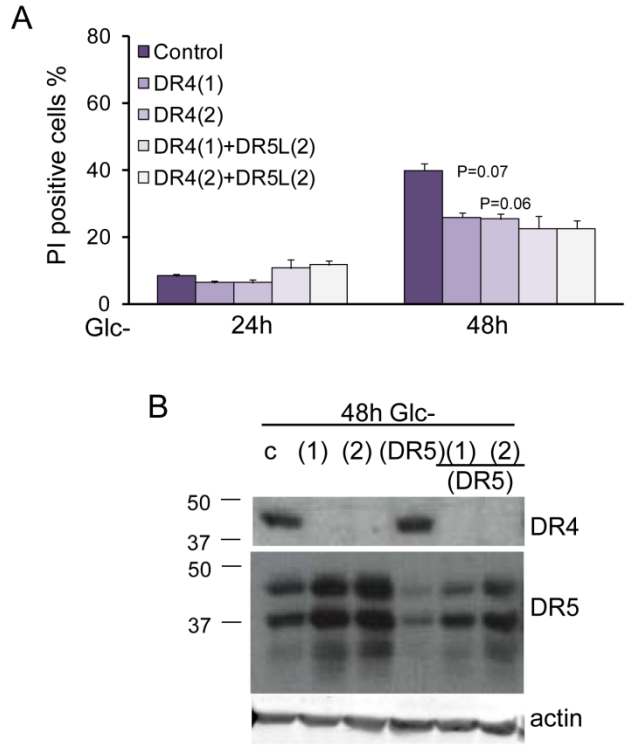

Figure 48: DR4 siRNAs do not prevent cell death under glucose deprivation in HeLa. A, B, Hela cells were plated at 50% confluence in 6 well plates and 5 h later they were transfected with 50nM DR4 (1), DR4 (2), alone or in combination with DR5L (2) and the control. 24 h post transfection cells were treated without glucose for 24 and 48 h and collected for PI analysis by FACS. The average and S.E.M. of three experiments are reported (A). Western blot of DR4 and DR5 of cells transfected with control and DR4 siRNAs alone or in combination with DR5L (2) are shown (B).

9. A cytoskeleton platform works as scaffold for caspase-8 activation in Bax/Bak deficient MEFs.

It has been described by many groups that TRAIL and Fas ligand treatment induce, in different cell types, a caspase-8 dependent apoptosis where the caspase-8 forms aggregates through its DED-domains; a condition which was essential for its activation (Dickens et al., 2012; Schleich et al., 2012).
In HeLa we observed that caspase-8 is diffuse in all cytoplasm of the cells with a punctate pattern, without significant changes after glucose deprivation. However, we detect that caspase-8 in Bax-Bak deficient MEFs changed its signal pattern after the treatment (Figure 49)

As reported in figure 49, immunofluorescence experiments of caspase-8 in Bax/Bak deficient MEFs show that there is an increase in the intensity of the signal of caspase-8 after glucose removal.
Moreover, we observed that, under glucose deprivation, caspase-8 forms filaments that are similar to the filaments already described in literature by Siegel and colleagues in 1998. Those filaments could be aggregates of caspase-8 which is oligomerizing to be activated.

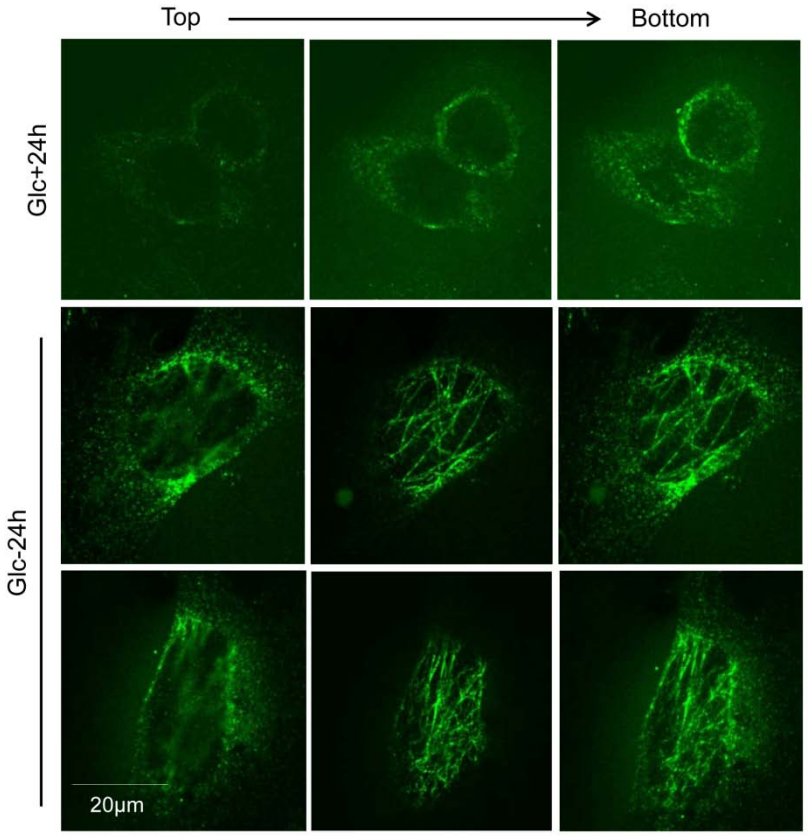

Figure 49: Caspase-8 forms aggregates like filaments in Bax/Bak deficient MEFs under glucose deprivation. Bax/Bak deficient MEFs were plated at 20% confluence in 12 well plates on Poly-L-Lysine pre-coated glasses. 24 h later they were treated with glucose deprivation for 24 h more. Caspase-8 immunofluorescence was performed and some confocal microscope pictures of staining from the top to the bottom of the cells are reported.

Using immunofluorescence we observed that caspase-8 aggregates do not co-localize with mitochondria, neither with actin filaments as we cannot detect any co-localization with Red mitotracker or phalloidin staining (Figure 50). However, we were not able to detect mitochondria after glucose deprivation in these cells; another marker of mitochondria should be used.

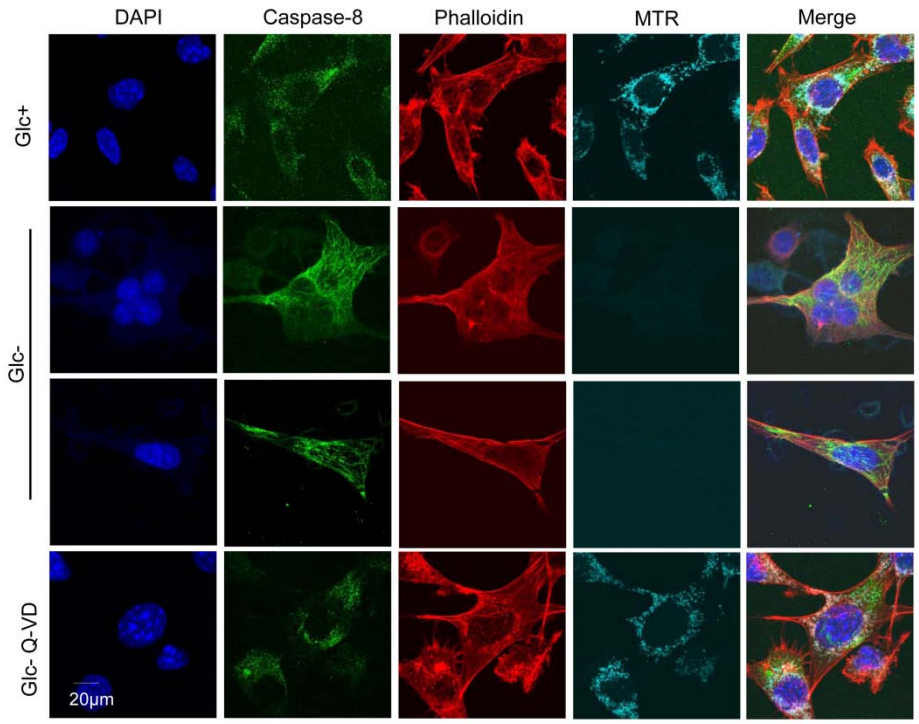

Figure 50: Caspase-8 does not colocalize with F-actin. Bax/Bak deficient MEFs were plated at 20% confluence in 12 well plates on Poly-L-Lysine pre-coated glasses and 24 h later treated with glucose deprivation plus or minus 10 µM Q-VD for 24 h. Caspase-8 immunofluorescence was performed and co-localization analysis with Phalloidin and Red Mitotracker (MTR) at confocal microscopy are reported.

We could detect co-localization of these death filaments with β-tubulin, as reported in figure 51. Quantifying the percentage of co-localization with an Image J software analysis, we concluded that the rate of co-localization between caspase-8 and β-tubulin filaments was significantly higher in the treated samples compared to the controls (Figure 51a).

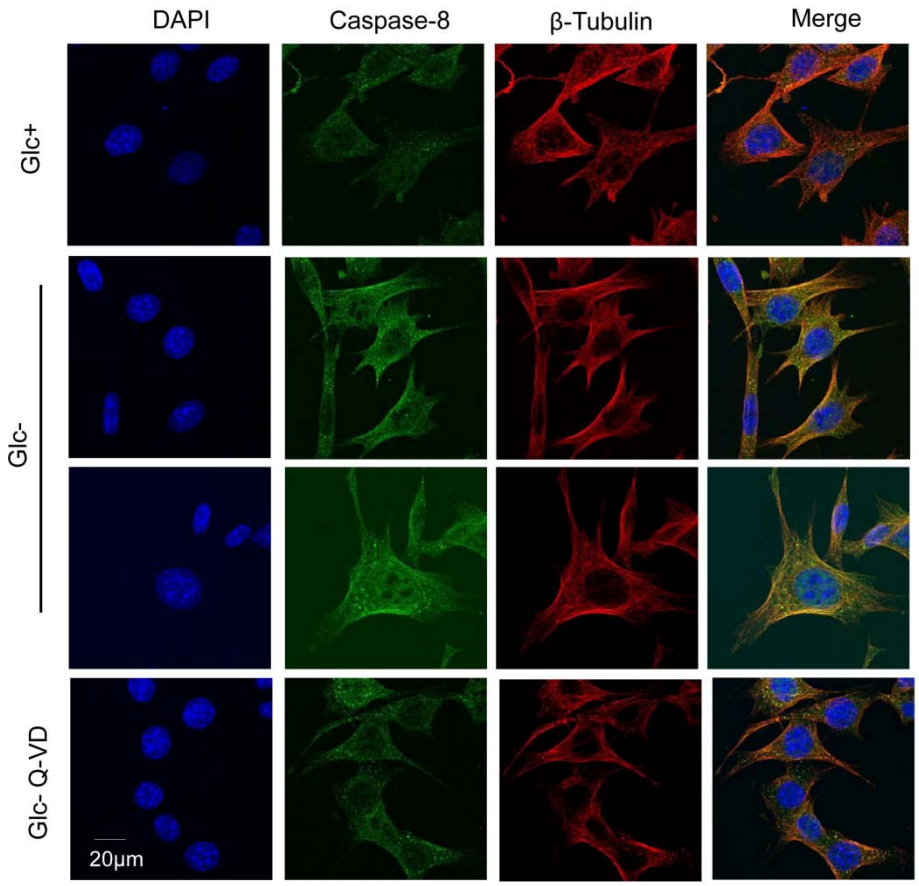

Figure 51: Caspase-8 colocalizes with β-tubulin filaments. Bax/Bak deficient MEFs were plated at 20% confluence in 12 well plates on Poly-L-Lysine pre-coated glasses and 24 h later treated with glucose deprivation plus or minus 10μM Q-VD for 24 h. Caspase-8 immunofluorescence was performed and co-localization analysis with β-tubulin at confocal microscopy are reported.

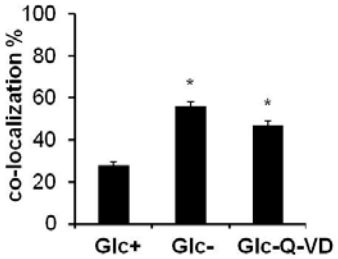

Figure 51a: Caspase-8 colocalizes with β-tubulin filaments. Co-localization analysis of caspase-8 and β -tubulin with Image J software analysis is shown.

Immunoprecipitation of caspase-8 in this cell line also reveal a weak pull-down of tubulin that was higher in the glucose deprived samples than in the controls (Figure 52).

These data, as a whole, showed that in Bax/Bak deficient MEFs, caspase-8 could be recruited on a microtubule scaffold which could be important for its oligomerization and full activation under glucose deprivation.

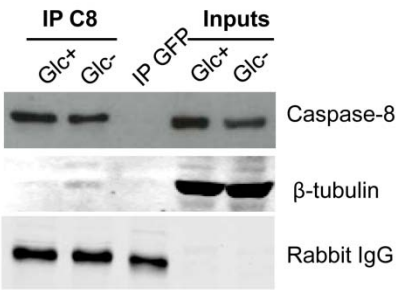

Figure 52: Caspase-8 co-precipitates with β-tubulin filaments. Bax/Bak deficient MEFs were plated at 20% confluence in 100 mm dishes and 24 h later were treated with Glc+ or Glc- for 24 h before performing immunoprecipitation of caspase-8. Anti-GFP antibody is used as isotype control. Western blot of caspase-8 and β-tubulin are reported.

10. A mass spectrometry analysis detects many cytoskeleton proteins under glucose deprivation upon caspase-8 and FADD immunoprecipitation in HeLa cells.

Finally, we decided to perform a mass spectrometry analysis to detect in a semi-quantitative way which were the proteins interacting with immunoprecipitated caspase-8 and FADD under glucose deprivation, trying to define exactly the components of the platform recruiting and activating caspase-8 in human cells.

We set up the best conditions to immunoprecipitate caspase-8 and FADD in HeLa cells in normal and in glucose deprived conditions and the samples were subjected to a mass spectrometry analysis at the IDIBELL proteomics platform.

The higher scores obtained with this analysis, for both immunoprecipitated proteins, were components of cytoskeleton, such as spectrin, myosin, actin, which seem to be increasingly bound to FADD and caspase-8 after glucose removal (Table 14).

These data indicated that also in HeLa cells a cytoskeleton platform could have an important role in recruitment and activation of caspase-8.

Another important finding in the mass spectrometry analysis is the presence of an important effector of the ER stress, GRP78, in both immunoprecipitated samples, that is increased after glucose deprivation (Table 15).

More analysis have to be performed to confirm these observation and verify that the interaction of these proteins with caspase-8 and FADD are real or are only the results of an artifact due to a general increase in the abundance of these proteins after glucose removal.

Number	Protein name	Glc+ IP FADD	Glc- IP FADD	Glc+ IP C-8	Glc- IP C-8
SPTB2_HUMAN	Spectrin beta chain, non-erythrocytic 1	251,4	689,7	261,5	1208,6
SPTN1_HUMAN	Spectrin alpha chain, non-erythrocytic 1	241,2	1131,1	438,8	1893
MYH9_HUMAN	Myosin-9	309,6	1249,5	411,3	1700,8
MYL6_HUMAN	Myosin light polypeptide 6	108,6	323,5	122,2	324,7
ACTN1_HUMAN	Alpha-actinin-1	130,7	350,7	267,4	691,8

Table 14: FADD and caspase-8 IP scores with a 2 fold increase or higher after glucose removal. Cytoskeleton proteins that are common at the two analyses (FADD and caspase-8 IPs).

Number	Protein name	Glc+ IP FADD	Glc- IP FADD	Glc+ IP C-8	Glc- IP C-8
IMB1_HUMAN	Importin subunit beta-1	45,2	132,3	81,4	161,7
PHB2_HUMAN	Prohibitin-2	51,7	220,9	46,3	131
GRP78_HUMAN	78 kDa glucose-regulated protein	236,5	616,5	274	1111,7

Table 15: FADD and caspase-8 IP scores with a 2 fold increase or higher after glucose removal. Not cytoskeleton proteins that are common at the two analyses (FADD and caspase-8 IPs).

VII. DISCUSSION

VII. DISCUSSION

1. Glucose deprivation induces caspase-8 dependent apoptosis in human cell lines: are we on the right DISC?

The principal goal of this thesis was to understand the molecular mechanism of cell death under glucose deprivation in different cell types. Previously, our group has shown that Bax/Bak deficient MEFs, with an impaired mitochondrial apoptotic pathway, die in a caspase-8 dependent manner under glucose deprivation. We also described that silencing of caspase-8 in HeLa cells prevents significantly death induced by glucose withdrawal, depicting an important role of caspase-8 as apical caspase in this process (Caro-Maldonado et al., 2010).

We tried here to clarify the molecular mechanism of this death in two different human cell lines: HeLa and Bax/Bak deficient HCT116 cells. HeLa cells are protected very well from death induced by glucose deprivation when the pan-caspase inhibitor Q-VD is added to the treatment, while Y-VAD, inhibitor of inflammatory caspases, does not prevent cell death. As shown in figure 18 and 19, Q-VD treatment in Bax/ Bak deficient HCT116 partially protects from cell death due to glucose removal, as well as the silencing of caspase-8. First of all, we observed that Bax/ Bak deficient HCT116 die very slowly when subjected to glucose deprivation, suggesting that perhaps a part of cell death in Bax, Bak wild-type cells could be due to the mitochondrial pathway. However, the fact that Q-VD treatment and caspase-8 siRNA cannot protect from all death induced by glucose deprivation in this cell line, indicates that there is a necrotic component. More experiments should be done to clarify this aspect. Necrostatin-1 treatment or RIPK1 silencing could help us to

understand if this cell line undergoes necroptosis when subjected to glucose deprivation.

Caspase-8 is generally activated in a DISC formed upon death ligand interaction with their receptors. However, it has been described that caspase-8 dependent apoptosis can occur independently of death receptors, suggesting that alternative platforms can be involved in its activation.

In order to define what platform is activating caspase-8 under glucose deprivation in HeLa cells, we first checked if some components of the innate immune system could be involved. Recently, it has been shown that caspase-8 dependent apoptosis can occur under virus infection independently of mitochondrial pathway (El Maadidi et al., 2014). This group showed that Bax/Bak deficient mouse or human cells, or Bcl-2 or Bcl-xL overexpressing cells were protected from Semliki Forest virus (SFV) infection when MAVS and caspase-8 were knocked-down. We focus on MAVS to see if it could be essential for caspase-8 recruitment and activation under glucose deprivation. We could not find any evidences of that in our model, because siRNAs of MAVS did not prevent death due to glucose withdrawal in HeLa (Figure 21). The two receptors of intracellular viral dsRNA, RIG-I and MDA5, as MAVS, contain two N-terminal CARDs which could potentially interact with caspase-8. Knocked-down experiments of these two proteins in HeLa cells could not protect from death under glucose deprivation, confirming that this pathway is not involved in the observed apoptosis (data not shown).

On the other hand, TRIF, an adaptor molecule involved in viral signalization particularly through TLR3 and TLR4, could also bind caspase-8, connecting innate immunity with our system (Marsh et al., 2009). Preliminary results in our group show that TRIF protects from death induced by glucose deprivation in HeLa cells. TRIF could be sequestering caspase-8 to the plasmatic membrane where there is TLR4 or to endosomes where there is TLR3, blocking its activation. It could also

prevent cell death activating the NF-KB pathway and maybe maintaining the levels of c-IAPs, FLIP or others molecules involved in the inhibition of caspase-8. Further experiments should be done to verify the role of TRIF in caspase-8 inhibition under glucose withdrawal in human models.

Recently, it has been shown by Tenev et al. and Feoktistova et al. that genotoxic stress induced by etoposide or TLR3 stimulation induces caspase-8 dependent apoptosis, independently of death ligands and the mitochondrial pathway (Feoktistova et al., 2011; Tenev et al., 2011). They described a new platform called Ripoptosome formed of caspase-8, FADD and RIPK1 which can result in caspase-8 dependent apoptosis or RIPK1-RIPK3 dependent necroptosis. Essential requirements for Ripoptosome formation and cell death initiation are cIAPs and FLIP inhibition and RIPK1 kinase activity. We wanted to define the possible role of Ripoptosome platform in death observed under glucose deprivation in Bax/Bak deficient MEFs and in HeLa cells. We already published that knock-down of RIPK1, the major player of this platform, or the treatment with Necrostatin-1, a specific inhibitor of this kinase, did not prevent cell death in Bax/Bak deficient MEFs (Caro-Maldonado et al., 2010).

Since we observed a downregulation of cIAP1 and cIAP2 in HeLa cells after glucose removal, we decided to silence RIPK1 also in this cell line. The knockdown of RIPK1 in HeLa cells did not protect from cell death due to glucose removal (Figure 23). However, the silencing of the protein is not complete, so a knockout mouse for RIPK1 could be used in the future to completely discard this pathway. A treatment with necrostatin-1 in HeLa could also help us to understand if RIPK1 is not essential in inducing cell death under glucose deprivation. Immunoprecipitation experiments of caspase-8 in glucose deprived conditions in both Bax/Bak deficient MEFs and HeLa cells did not show any interactions of caspase-8 with RIPK1. However, the reciprocal immunoprecipitation of RIPK1 in those conditions should be performed to confirm our results.

There is still the possibility that classical components of the DISC, such as FADD or FLIP, can be essential in caspase-8 activation or inhibition. We investigated if FADD or TRADD could have an active role in the recruitment of caspase-8 in HeLa under glucose deprivation. We observed a clear pull down of FADD in immunoprecipitation experiments of caspase-8 in HeLa cells in glucose removal conditions, but immunofluorescence experiments show that a basal co-localization of FADD with caspase-8 exists in normal conditions. When we performed silencing experiment of FADD in HeLa cells we could restore cell viability in glucose deprived conditions similar to the protection obtained from Fas-induced cell death (Figure 30). Knock-down of TRADD did not prevent cell death due to glucose withdrawal. All these data suggest that FADD, but not TRADD, is essential in inducing caspase-8 dependent cell death in HeLa cells subjected to glucose deprivation. However, we are not sure that the downregulation of TRADD is sufficient to achieve a biological effect because its silencing could not protect form death due to TNF treatment (not shown).

We tried to clarify what is the role of FLIP, the specific caspase-8 inhibitor, in cell death under glucose deprivation. Previously, we showed that FLIP overexpression, which inhibits death receptor-induced apoptosis, did not inhibit apoptosis induced by glucose removal in Bax/Bak deficient MEFs, suggesting that FLIP has no role in the inhibition of apoptosis in our system (Caro-Maldonado et al., 2010). Here, we over-expressed constitutively the murine FLIPL in HeLa cells and we could not observe any reduction of cell death under glucose deprivation. However, according to Prof. I. Lavrik, we were not able to detect endogenous FLIP in HeLa cells, but we observed that glucose removal is inducing a down-regulation of FLIP when it is ectopically over-expressed (Figure 20). This result suggests that a possible down-regulation of FLIP could be contributing in the activation of caspase-8 and the initiation of cell death in our model, leading us to carefully reconsider our first conclusions.

Recently, many groups have described a new death platform called iDISC, intracellular DISC, that is formed upon proteasome inhibition in different cell lines and is characterized by the presence of caspase-8 that is recruited on autophagosomal membranes by autophagic proteins such as Atg5, p62 and LC3 (Pan et al., 2011; Young et al., 2012). Pan et al showed that p62 and LC3 are essential for death induced by proteasome inhibition because shRNAs of p62 and LC3 prevented caspase-8 activation and the consequent cell death (Pan et al., 2011). Yong et al and Laussmann et al also showed that loss of FADD suppresses cell death due to the iDISC formed upon proteasome inhibition (Laussmann et al., 2011; Young et al., 2012).

Glucose deprivation could maybe induce the formation of an iDISC of FADD, caspase-8 and some autophagic proteins essential in the induction of apoptosis. We performed different experiments of immunofluorescence and immunoprecipitation of caspase-8 mainly in HeLa cells and we could observe some co-localization of caspase-8 with LC3 and some interaction of the protease with p62, LC3 and Atg5 in immunoprecipitation experiments under glucose deprivation.

We previously showed that glucose deprivation inhibits rather than inducing autophagy, resulting on accumulation of autophagic proteins such as p62 and LC3 in both Bax/Bak deficient MEFs and HeLa cells (Ramírez-Peinado et al., 2013). However, silencing experiments of p62 in Bax/Bak deficient MEFs did not prevent cell death due to glucose removal (Ramírez-Peinado et al., 2013).

Here, we show that also HeLa cells are never protected from cell death under glucose deprivation when p62 is knocked-down (Figure 29). It is known that caspase-8 is ubiquitinated when activated inside the DISC and it is bound by p62, a ubiquitin binding protein on the autophagosomal platform (Jin et al., 2009). We were not able to detect ubiquitinated caspase-8 by western blot. In our condition, p62 is binding directly or

indirectly caspase-8 and maybe it is sequestering it in the autophagosomal membrane for its autophagic degradation but not for its activation and initiation of apoptosis.

It still remains to clarify the role of Atg5 in the caspase-8 activation under glucose deprivation. Recently, it has been shown that tunicamycin and thapsigargin, well known ER stressor, can induce caspase-8 dependent apoptosis in Bax/ Bak or caspase-9 deficient cells (Deegan et al., 2014). They described that ER stress induce the formation of an autophagosomal platform where Atg5 and FADD are essential for caspase-8 recruitment and activation. We previously showed that knockdown of Atg5 in HeLa cells did not prevent cell death under glucose deprivation and immunoprecipitation of caspase-8 showed not clear interaction of this protein with Atg5 in our model (Figure 25).
It is possible that glucose deprivation induces an impairment of protein glycosylation inside the cell, resulting in ER stress. The consequent ER stress, if persistent, could trigger the formation of the stressosome, this new autophagosomal platform of FADD, Atg5 and caspase-8. However, our data suggest that this platform is not essential to activate caspase-8 and to initiate the apoptotic process under glucose deprivation.

2. Glucose deprivation induces ER stress and ATF4-dependent DR5 up-regulation independently of TRAIL

Persistent ER stress results in cell death generally through the mitochondrial pathway of apoptosis but different groups have shown that it can also induce apical caspase-8 induction on a iDISC platform, the same already described upon proteasome inhibition (Deegan et al., 2014; Tomar et al., 2013). Recently, Dr. A. Ashkenazi and colleagues described that ER stress inducers can trigger in different cell types a caspase-8

dependent cell death, through CHOP direct up-regulation of DR5 at mRNA levels (Lu et al., 2014).

We have already described that glucose deprivation induces ER stress, possibly due to an accumulation of misfolded proteins in the ER in different cell lines and if the ER stress is persistent can result in cell death. Performing western blot analysis in HeLa cells and in Bax/Bak deficient HCT116 we could detect a clear induction of one of the pathways induced by ER stress under glucose deprivation, showed by the increased levels of ATF4 and CHOP, the two main players of the PERK/eIF2α pathway of the UPR response.

It has been reported that in mammals other three kinases, apart from PERK, can phosphorylate eIF2α: the general control nonderepressible 2 (GCN2), which is activated in response to nutrient deprivation, in particular of essential amino acids; the double-stranded RNA-activated protein kinase (PKR), activated by viral infection and the heme-controlled inhibitor (HRI), induced by heme limitation. In order to clarify if glucose removal induces ATF4 and CHOP in a PERK dependent way, we should perform Knock-down experiments of PERK or use specific inhibitor of this kinase. But does the glucose deprivation induce also an increase in the other pathways induced by ER stress? We were not able to detect the spliced form of XBP1 by western blot in HeLa cells, maybe due to its low level of expression.

Western blot analysis also showed in HeLa cells a clear induction of TRAIL death receptors DR4 and DR5, while the TNFR1 and Fas are not clearly induced but they show a change in the size of the band due maybe to a differential glycosylation of the receptors. Glucose deprivation induces the appearance of a third band of DR5, lower than DR5L and DR5S that could be a less glycosylated form of the receptor or a novel not described spliced form which could trigger glucose deprivation dependent cell death (Figure 34). We have already described in the introduction the importance of post translational modifications of death receptors that regulate their

expression and function. It has been shown that O-glycosylation of both TRAIL receptors DR4 and DR5 and N-glycosylation of DR4 are essential for their activation in response to TRAIL. In our model, the lack of glycosylation seems to be not so critical for death receptors activation; the spatial accumulation of the receptors could be sufficient for their aggregation and initiation of apoptosis.

We also performed q-PCR analysis to study the effect of glucose withdrawal on the mRNAs levels of death receptors. We show that DR5, but not DR4 and the other receptors, is up-regulated also at mRNA levels at a short time point of glucose removal. The mRNA levels of DR4, TNFR1 and Fas are unchanged at 8 hours of treatment and down-regulated at 24 hours of glucose deprivation, suggesting a prevalent regulation of DR5 rather than the other death receptors by the treatment. However, DR4 does accumulate after glucose removal as detected by western blot. This could perhaps be due to the inhibition of autophagy induced by glucose deprivation which could block its degradation.

To further clarify the role of ER stress in the induction of death receptors we performed silencing experiment of the ER stress response proteins ATF4 and CHOP. As reported in figures 38 and 39, we can observe that the siRNAs of ATF4 but not CHOP protects considerably HeLa cells from cell death and impede the induction of the DR5 receptor in glucose deprived conditions. It is known in the literature that CHOP directly regulates the promoter of DR5, inducing its increase under ER stress condition (Yamaguchi and Wang, 2004). A recent report shows that ATF3 can also regulate DR5 promoter (Edagawa et al., 2014), but there are no evidences for direct ATF4 control of DR5 transcription. Our data confirm that glucose deprivation is inducing an ATF4-dependent DR5 up-regulation which could be involved in recruitment and initiation of caspase-8.

We found by immunoprecipitation experiments of caspase-8 in HeLa cells that glucose removal makes it interact with what seems to be the short

isoform of DR5; this is the one more induced by the treatment. The group of Dr. A. Ashkenazi showed that once induced upon ER stress, DR5 localizes at the Golgi apparatus or at ER depending on cell type (Lu et al., 2014). Our immunofluorescence experiments show that DR5 localizes prevalently at the plasma membrane and at the Golgi apparatus rather than at ER in HeLa cells deprived of glucose (Figure 40). In our model, the expression of DR5 is increased due to glucose deprivation-dependent ER stress and it is accumulated at the Golgi apparatus where it could recruit caspase-8 to initiate the apoptotic process.

In the extrinsic pathway of apoptosis, caspase-8 is activated upon death ligand interaction with their receptors and DISC formation. We previously showed that glucose deprivation induces caspase-8 dependent apoptosis in Bax/Bak deficient MEFs independently of death ligands (Caro-Maldonado et al., 2010). Cells were incubated with neutralizing antibodies against TNF, TNFR1, TRAIL, Fas-ligand or with TNF-R-FC or Fas-Fc and subjected to glucose deprivation. Inhibiting the interaction of death ligands with their receptors could not prevent cell death. As we observed a strong induction of DR5 in HeLa cells upon glucose withdrawal, we wondered if the treatment could induce the synthesis and secretion of TRAIL, which might kill cells in an autocrine way. On the contrary, q-PCR analysis of TRAIL mRNA in HeLa cells subjected to glucose deprivation show a significant reduction of TRAIL mRNA at 24 hours of treatment, leading us to discard this hypothesis. To better corroborate that the induction of DR5 and the resulting cell death is not dependent on the ligand, we performed silencing experiments of TRAIL under glucose deprivation in HeLa cells. The knock-down of TRAIL did not prevent cell death, showing that also in HeLa cells the activation of caspase-8 is independent of death ligands (Figure 37). However, we should treat HeLa cells also with neutralizing antibodies against the receptor, to be sure that the observed apoptosis is not dependent on interaction of a residual amount of TRAIL with its receptor.

Finally, we wanted to check if DR5 is really the cause of apoptosis observed under glucose deprivation in human cells. We performed a series of DR5 silencing experiments using different siRNA sequences against the long or the short isoform of DR5. We could observe a significant protection from death only with DR5L (2) siRNA in HeLa cells (Figure 44). The comprehension of this result is a bit difficult because when we silenced DR5L with DR5L (1) and DR5L (3) siRNAs we observed an induction of the short isoform of the receptor as well as the appearance of a third band that is absent in the controls which could be contributing in cell death under glucose deprivation. Moreover, the silencing of the short isoform of the receptor induces another band of the DR5 receptor that is the same size as the one observed in untransfected cells subjected to glucose deprivation. All these observations make more complicated the finding of the best protecting siRNA sequence.

When we performed the same experiments in HeLa overexpressing Bcl-xL, three siRNA sequences against DR5L prevented cell death in a significant manner (Figure 46). These results indicate that a component of cell death observed upon glucose withdrawal in HeLa cells could be due to the mitochondrial pathway of apoptosis because siRNAs of DR5 can better prevent death when the intrinsic pathway is blocked. Another possibility could be also that glucose deprivation induces not only caspase-8 activation in HeLa cells but also an amplification of the mitochondrial pathway of apoptosis. Silencing experiments of BID could help us in clarifying this aspect. Bax/Bak deficient HCT116, which cannot die by the mitochondrial pathway of apoptosis, were protected from cell death due to glucose removal when transfected with a siRNA sequence against DR5L and one against DR5S. This result indicates that the prevalence of one isoform or the other of DR5 to determine cell death under glucose deprivation might be dependent on cell type.

While DR5 is essential to induce cell death under glucose deprivation, it is still not so clear whether DR4, Fas and TNFR1 have an important role in

this death. Preliminary results show that knock-down of these death receptors does not prevent cell death after glucose removal in HeLa cells. Moreover, silencing of DR4 receptor was triggering an accumulation of DR5 receptor, suggesting that a sort of compensation between the two TRAIL receptors could exist (Figure 48).

3. A cytoskeleton platform could be involved in caspase-8 initiation under glucose deprivation

Recently, different groups have shown that the formation of death effector filaments due to the aggregation of caspase-8-DED domains is essential for its activation, upon TRAIL and FAS receptors stimulation (Dickens et al., 2012; Schleich et al., 2012).

Immunofluorescence experiments of caspase-8 in Bax/Bak deficient MEFs show a filament like staining of the protease after glucose deprivation which resemble the caspase-8-DED aggregates described after TRAIL and Fas treatment. These caspase-8 filaments co-localize with β-tubulin microtubules and the degree of co-localization is significantly increased after glucose withdrawal (Figure 51 and 51a).

The group of Stupack have shown in 2009 that Paclitaxel induces a caspase-8 dependent apoptosis in human endothelial and carcinoma cells and caspase-8 was found associated in a stronger manner with microtubules after paclitaxel treatment (Mielgo et al., 2009). They have also shown that expression of caspase-8-DEDs or paclitaxel treatment sensitizes to Fas induced apoptosis, suggesting that accumulation of DEDs on a microtubule scaffold could act to amplify death receptor-dependent caspase-8 activation. Preliminary results in our laboratory show that vincristine, a destabilizing microtubule agent, sensitizes Bax/Bak deficient MEFs to glucose deprivation. This data indicate that

caspase-8 activation by glucose deprivation could increase cell death after a microtubule perturbation. This is very important from a clinical point of view. Glucose deprivation as well as caspase-8 activating drugs such as TRAIL or proteasome inhibitors could contribute to the microtubule amplification of caspase-8 activation after taxanes treatment.

Preliminary results of a mass spectrometry analysis reveal that also in HeLa cells subjected to glucose deprivation the caspase-8 and the adaptor protein FADD are linked to a cytoskeleton platform that could be essential for its complete activation and the execution of apoptosis. Further experiments should be performed to validate the importance of this cytoskeleton platform for caspase-8 activation under glucose deprivation.

4. Molecular mechanisms of apoptosis induced by glucose deprivation

We have shown that glucose deprivation induces caspase-8 dependent apoptosis in different human cell lines. We have described that glucose deprivation induces ER stress due maybe to the accumulation of not glycosylated proteins and ATF4 up-regulates the expression of DR5. A platform of FADD, caspase-8 and DR5 is formed upon glucose withdrawal, independently of TRAIL. Whether caspase-8 is recruited on an iDISC in autophagosomal membranes by p62, LC3 and Atg5 interactions and if this translocation is essential for its activation under glucose deprivation, it is still not clear (Figure 53).

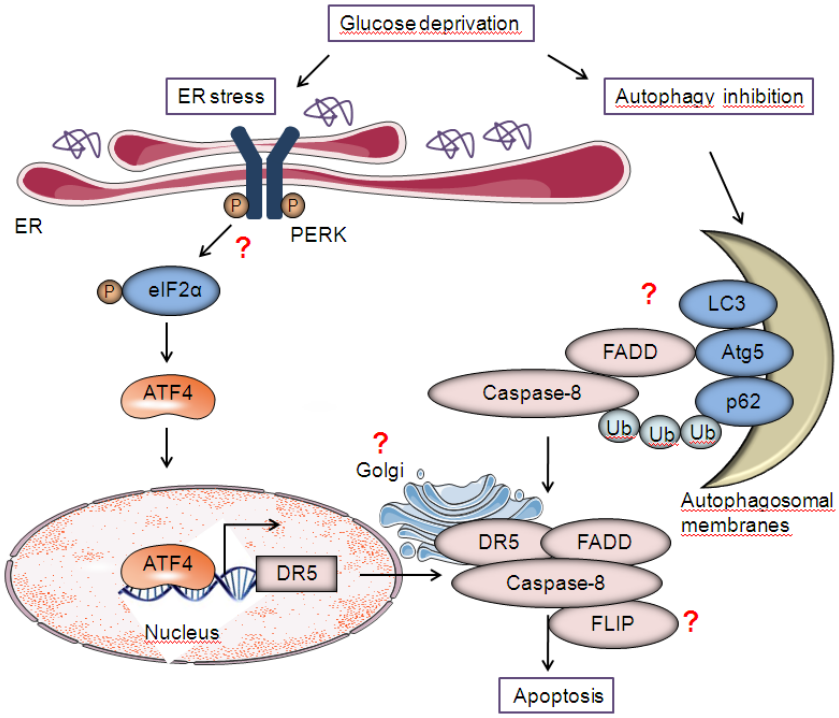

Figure 53: Model of cell death induced by glucose deprivation.
(Adapted from: Iurlaro R. and Munoz-Pinedo C., In press, FEBS Journal, 2015)

Anyway, some questions remain still open in this model. Is the ER stress induced by glucose deprivation really due to an impaired glycosylation of proteins? It has been proved that 2-DG, the analog of glucose currently used in clinical trials, induces autophagy in different cell lines not by depleting cells of ATP, but by interfering with N-linked glycosylation of proteins. Mannose could revert the resulting ER stress but not the ATP loss (Xi et al., 2011). Recently we have shown that 2-DG induces apoptosis while glucose deprivation induces necrosis in rhabdomyosarcoma cell lines. However, both treatments trigger ER stress as shown by an ATF4 accumulation (León-Annicchiarico et al., 2015). Several hexoses prevented glucose deprivation-induced necrosis, suggesting that ER stress induced by glucose deprivation is caused by an impairment in glycosylation of proteins. An experiment of glucose

deprivation in presence of mannose or N-acetylglucosamine should be performed in HeLa cells to clarify if also in our model glucose deprivation is triggering an impairment of protein glycosylation, which could be the cause of ER stress induction. Another possibility is that glucose withdrawal could result in amino acids starvation which in turn could induce the ER stress. Further analysis should be done to clarify this aspect.

We previously showed that 2-DG induces autophagy and a decrease of ATP levels in rhabdomyosarcoma that was reverted by mannose. On the contrary, glucose deprivation does not change the ATP levels and instead of inducing autophagy, inhibits it, even in HeLa cells (Ramírez-Peinado et al., 2013). The inhibition of autophagy could explain the observed accumulation of autophagic proteins such as p62 and LC3 in autophagosomes but if they are essential for recruitment of caspase-8 aggregates, contributing to its activation, it remains to be clarified.

Another important aspect that we did not mention is the role of the reactive oxygen species (ROS) in glucose deprivation-dependent apoptosis. ER stress may induce ROS production contributing to cell death, through ATF4 and CHOP, which are accumulated upon glucose withdrawal. CHOP induces the transcription of the endoplasmic reticulum oxidoreductin 1 (ERO1α) which results in a hyperoxidizing environment in the ER. Knock-down of ERO1α has been shown to protect C. *elegans* from tunicamycin treatment (Marciniak et al., 2004), suggesting a key role of ER in mediating ROS-dependent cell death. Treatment with N-acetylcysteine or other scavengers could help us to understand the importance of ROS in apoptosis due to glucose deprivation.

Moreover, other proteins could be involved in regulating caspase-8 activation under glucose deprivation. For instance, Bap31 is a protein resident in the ER which contains a death effector domain–like (DED-L) region that can bind some isoforms of procaspase-8 (Ng et al., 1997). The full lenght of Bap31 has been shown to inhibit Fas induced apoptosis

while the truncated p20Bap31 induces apoptosis. Recently, it has been shown that the mitochondrial fission protein Fis1 (Fission 1 homologue) can bind Bap31 to connect mitochondrial signals to the ER, forming a procaspase-8, Fis1, Bap31 platform which initiates apoptosis in different cell lines (Iwasawa et al., 2011). If some of these proteins are present in the caspase-8 activating platform induced by glucose deprivation is still a possibility. Gel filtration analysis would be useful to determine what platform is involved in capsase-8 activation under glucose removal and if one or more platforms could co-operate at the same time.

PEA-15/PED (phosphoprotein enriched in astrocytes 15kDa/phosphoprotein enriched in diabetes) is a phosphoprotein which contains a DED in its N-terminal region and can modulate death receptor-mediated apoptosis, binding FADD or caspase-8 at the DISC (Estellés et al., 1999). The status of its phosphorylation on Ser 116 determines its anti-apoptotic or apoptotic activity and it has also been shown to modulate MAP/ERK-dependent pathways, contributing in diverse biological functions. It has been shown that high levels of Ser 116 phosphorylated PEA-15 can protect glioblastoma cells from glucose deprivation-induced cell death through ERK1/2 signaling pathway and trough the up-regulation of the glucose transporter 3 (GLUT3) (Eckert et al., 2007). It would be interesting to study the phophorylation status of this protein and clarify if could exist some correlation with the apoptosis induced by glucose deprivation in our model.

The comprehension of the molecular mechanisms of apoptosis under glucose deprivation is really important to understand other pathological conditions such as ischemia, characterized by oxygen and glucose deprivation (OGD). Recently, it has been shown that stress signaling in response to OGD induces Bmf instead of Noxa, activating the mitochondrial pathway of apoptosis thus contributing to the injury in cortical neurons (Pfeiffer et al., 2014). Recently it has also been shown that ER stress is induced under OGD and CHOP knockout protects

neurons from ischemia. ER stress could be involved in signalization upon ischemia where a possible up-regulation of death receptors and consequent activation of caspase-8 might amplify the mitochondrial apoptotic pathway, contributing to cell death. All together these hypotheses make our findings really interesting.

VIII. CONCLUSIONS

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1. Glucose deprivation induces caspase-8 dependent apoptosis in HeLa and in Bax/Bak deficient HCT116 cells.

2. The apoptosis induced by glucose deprivation is not dependent on the Ripoptosome nor on the iDISC or autophagosomal platform.

3. FADD is essential for caspase-8 activation and cell death under glucose deprivation.

4. Glucose deprivation induces endoplasmic reticulum stress and induction of TRAIL death receptors.

5. ATF4 but not CHOP induces the up-regulation of DR5 and it is responsible for cell death upon glucose withdrawal.

6. DR5 induces apoptosis by recruiting caspase-8 maybe at the Golgi apparatus independently of TRAIL upregulation.

7. A cytoskeleton component could be essential for caspase-8 recruitment at the DISC in Bax/Bak deficient MEFs and in HeLa cells.

IX. BIBLIOGRAPHY

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X. ANNEX