SCUOLA DI ALTA FORMAZIONE

DOTTORATO IN SCIENZE E BIOTECNOLOGIE MEDICHE

PHD PROGRAM IN SCIENCES AND MEDICAL BIOTECHNOLOGY

COORDINATOR: PROF EMANUELE ALBANO

STUDENT: **MIRIAM SAVINO** CYCLE: **XXIX** YEAR: **2014-2015**

TUTOR: PROF. CIRO ISIDORO

2nd YEAR ANNUAL REPORT

PREMISE

In February 17th, I moved from Prof. Graziani's Lab to Prof. Isidoro's Lab and consequently I have changed my PhD Project.

In these first six months of work in Prof. Isidoro's lab I had to learn (and still I am learning) new methods to study Autophagy and Lysosomal proteolysis and how these processes are regulated in certain diseases. In particular, the main objective of the studies performed in Prof. Isidoro's lab is to understand how autophagy is related to cell death and cell survival in 'in vitro' models of diseases.

EXPERIMENTAL RESULTS

1: Assessment of mitophagy in an 'in vitro' model of Cockayne syndrome.

From March to June, I have been involved in a project in collaboration with Dr Eugenia Dogliotti (of Istituto Superiore di Sanità in Rome) related to the Cockayne Syndrome. Our task was to better characterize the involvement of autophagy in the degradation of oxidized mitochondria (mitophagy) in relation to cell death, and to understand how Parkin could regulate this process.

I have contributed to this work with imaging in immunofluorescence of these processes. Below, I report the data that I have produced so far.

(I do not report the data produced in the laboratory of Dr. Dogliotti; the rationale for the experiments done in our lab is given in the legend to the figures).

The project is virtually ended. The manuscript titled "**Excessive fission of mitochondria leads to increased apopotosis in Cockayne Syndrome A cells**" has been submitted for publication and is presently under review.

2. PhD Thesis PROJECT

Next, in June, I have started to work on my PhD project, which is titled "**Mechanistic insights in the functional role of Cathepsin D in cell migration, vesicular traffic and in response to oxidative stress**". It is a new original project in which we wish to demonstrate the importance of Cathepsin D-mediated proteolysis in neuronal cell metabolism and behavior. I have to anticipate that the cells over-expressing Cathepsin D or genetically silenced (with a short-hairpin technology) for Cathepsin D were prepared in the laboratory of Prof. Isidoro by Dr Carlo Follo.

Below is a brief description of the project and results obtained until now.

1. **<u>Background</u>**

Human cathepsin D, hereafter CD, is a soluble aspartic endopeptidase ubiquitously expressed in endosomes and lysosomes, and is encoded by the CTSD gene located on chromosome 11p15.5 (Berg et al., 1995; Redecker et al., 1991). Its expression is highly regulated by growth factors and nutrient status (Roczniak-Ferguson et al., 2012; Settembre et al., 2011) and its activity is also highly regulated by cytokines, hormones and pH (Erickson et al., 2014, Springer Book).

Human CD is synthesized as an enzymatically inactive form of 52 kDa, that is co-translationally glycosylated in the endoplasmic reticulum and is subsequently proteolytically processed in post-Golgi compartments. First, pro-CD is processed into a 48-kDa single chain intermediate, that is partially active in late endosomes. The intermediate form of CD is further processed into the fully active mature double chain protease, composed of a N-terminal light chain of 14 kDa and C-terminal heavy chain of 34 kDa linked together by non-covalent interactions (Erickson et al., 2014, Springer Book)(Follo et al., 2007).

Similar to other aspartic protease, CD is synthesized into the lumen of rough endoplasmic reticulum as a pre-proenzyme that undergoes co-translational signal peptide cleavage and N-glycosylation with high-mannose sugars. After glycan processing the pro-enzyme is transported to the Golgi Complex, where the N-glycans are further modified to acquire the mannose-6-phosphate (M6P) group, and from here it is transported to downstream acidic compartments. This transport is primarily mediated by M6P receptors (MPR300 and MPR46) that recognize proCD tagged with the M6P (Hasilik and Neufeld, 1980). However, other pathways alternative to the MPR-mediated can operate the translocation of proCD from Golgi Complex to endosomes and lysosomes (reviewed in Castino and Isidoro, 2008).

In the early and late endosomes, single-chain CD plays a role in the proteolytic processing and activation of pro-enzymes and pro-hormones, whereas in the lysosomes, mature CD accomplishes a pivotal role in overall protein catabolism (Benes et al., 2008; Nicotra et al., 2010a). Under cellular stress circumstances leading to lysosomal membrane permeabilization, mature CD can relocate in the cytoplasm and trigger apoptotic cell death, whereas the massive rupture of the lysosomes would lead to necrotic cell death. All these functions have an impact on several pathophysiological process such as organ development (Follo et al., 2011, 2013;

Saftig et al., 1995); neurodegeneration (Mantle et al., 1995; Shacka et al., 2007), cancer (Gemoll et al., 2015; Nicotra et al., 2010a; Rodríguez et al., 2005), and oxidative stress (Hah et al., 2012). Studies on CD-deficient animal models, such as mouse and Drosophila knock-out, show severe neurodegeneration that resembles that of human infantile Neuronal Ceroid Lipofuscinosis type 10 (NCL10) (Koike et al., 2000; Myllykangas et al., 2005). Further, our lab has demonstrated that CD knock-down zebrafish larvae develops several phenotypic abnormalities, including microphthalmia and congenital myopathy (Follo et al., 2011, 2013). All together these data suggest that CD could play a key role in brain homeostasis, tissue development and cancer progression through its proteolytic action on a variety of substrates, most of which are still unknown.

AIMS OF THE PROJECT

The aim of this project is to understand the pathophysiological role of Cathepsin D in neuronal metabolism. To this end, in the laboratory of Prof. Isidoro have already been generated clones stably over-expressing CD or sh-mediated silenced for CD.
In addition, I will generate a stable cell line of neuroblastoma SH-SY5Y carrying human CD mutated at the processing region that leads to a stable, un-processable, intermediate single-chain CD, named hCD^m (Follo et al., 2007)). This new clone, together with shCD and overCD clones, will permit us to clarify and study in depth the possible different physiological roles of single and double-chain CD and to identify a list of candidate substrates of these two different molecular forms of the active enzyme.
In particular, we will study the phenotype of each clone in terms of:

- proliferation rate
- migration
- neuronal differentiation capability
- autophagy process
- resistance to oxidative stress

Finally, we will perform a transcriptome and microRNome analyses of the clones to search for the epigenetic alterations associated with the altered expression of CD forms, and will perform a proteomic analysis of the lysosomal content in order to identify possible CD substrates responsible for the phenotypes.

2. Experimental plan

In orther to clarify the phenotype and metabolism of shCD and overCD and hCD^m, the clones will be subjected to several investigations:

- A wound healing assay will be performed and then analysed through an appropriate software to observe the proliferation rate of cells expressing shRNA CD, hCD and hCD^m.

- The proliferation rate will be assessed through a growth curve and FACS analysis of the cell cycle (doubling time will be calculated). The contribution of cell death to the growth of the culture will be evaluated by assessing the presence of dead cells (necrotic = trypan blu-positive and propidium iodide-positive; apoptotic = annexin V-positive and SubG1)
- The Autophagy flux will be assessed through western blotting and immunofluorescence imaging of autophagy markers such as p62 and LC3 in co-staining with LAMP-1 and M6PR, and following the guidelines to which Prof. Isidoro has contributed (Klionsky et al., 2012).
- Treatment with Retinoic acid will be used to induce a mature neuronal differentiation of the neuroblastoma cells expressing sh-CD, hCD or hCD^{m}. We will measure the neurite outgrowth rate of each clone to clarify if CD is involved in this process.
- As we know from the literature that Thioredoxin (anti-oxidant enzyme) is a substrate of CD (Nicotra et al., 2010b) we will verify the response to oxidative stress treating our clones with hydrogen peroxide or dopamine (to mimics neurodegenerative conditions in which dopamine stress could be a determinant of these pathology) and observing differential response of our clones with regard to cell death (characterizing the type), to mitochondrial integrity/activity (MitoTracker Red Staining), to ROS production and which type is more represented in our clones (MitoSOX staining, Dichlorofluorescein staining in presence or absence of Deferoxamine/N-acetylcystein to distinguish among hydroxyl and anion superoxide radicals).

In parallel, we will start to characterize the potential epigenetic impact of CD absence and hCD^{Wt}/hCD^{m} overexpression. To this end, a transcriptomic and microRNAomic analysis will be performed in neuroblastoma cell line SH-SY5Y sham, shCD, overCD and hCD^{m} (collaboration with Dr. Chiorino of Edo Tempia Foundation). For this purpose, the cells will be exposed to treatments inducing the desired phenotype.
Finally, we will search for the potential substrate(s) of CD involved in the phenotypes as characterized above (i.e., cell proliferation, cell migration, neuronal differentiation and neurite outgrowth, autophagy, ROS response, cell death). To this end, we will analyse the lysosomal proteome in our clones (cultured in either control conditions or under appropriate treatments to induce the desired phenotype). This step implies a lysosomal fractionation and proteomic analysis to find differential spots and identify, with mass spectrometry, the protein/s differentially expressed (For the analysis we shall rely on the collaboration with Prof. Cavaletto, DISIT, Alessandria).
Together, the genomic/epigenomic and the proteomic analyses shall provide a complete picture of the metabolic pathway associated with the diverse phenotypes and a list of the potential CD substrates involved.

EXPERIMENTAL WORK performed in the period June-September 2015

3. Materials and methods

Cell Lines and Vectors: Human neuroblastoma SH-SY5Y cells were used and cultivated under standard culture condition (37°C; 95 v/v% air: 5 v/v% CO_2) in 50% Minimum Essential Medium (MEM) (SIGMA, St. Louis), 50% Ham's F12 Nutrient Mixture (HAM) (SIGMA, St. Louis) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO BRL), 1w/v% of a penicillin-streptomycin solution (SIGMA, St. Louis), 2mM di L-glutamine (SIGMA, St. Louis). In the past years a member of our lab, Carlo Follo, had generated three clones of SH-SY5Y stably transfected with pcDNA4/TO carrying human CTSD gene under constitutive promoter (clone overCD), pENTR/H1/TO carrying shRNA for CD (clone shCD) and finally with empty vector pENTR/H1/TO (clone sham, used as control cell line).

Wound Healing Assay: sub-confluent cell monolayer were scratched with yellow tip to make a wound. Cells were let to migrate for at least 48 hours or until the wound is healed. Contrast phase images were taken every 24 hours and then analyzed through TScratch Software.

FACS subG1 analysis/Doubling Time: the three clones were seeded in a manner that after 24 hours were at 60% confluency. At 24 hours from the seeding, cells were counted to set the t0 of the Doubling Time. 48 hourse from seeding harvest the cell, count them for the t1 of the Doubling time, and prepare them for subG1 analysis fixing in Ethanol 70% o/n at 4°C and stain with 500ng/ml of Propidium Iodide (PI) for 15 min. PI stained cells were then subjected to FACS analysis and the resulting data analyzed with WinMDI Software.
Doubling time were calculated with on-line tool at the website http://www.doubling-time.com/

Western Blotting and Antibodies: cells were treated for 3-6 hours with several autophagy inducer (Rapamycin 100nM –mTOR inhibitor -, Lithium 1mM – mTOR-independent pathway - and Earl's Balanced Salt Solution – EBSS). Protein lysates were obtained and quantified with Bradford's Assay, then 25 µg of total protein were loaded and separated through SDS-PAGE, after transfer to PVDF, the filter was blotted with p62 1:500 (rabbit, Cell Signaling, Cod. 5114), LC3B 1:1000 (rabbit, Sigma, Cod. L7543) and α-Tubulin is used as loading control.

4. Results

Since we are moving in several research line, these results are a summary of preliminary data collected until now.

Cathepsin D affect the migration behaviour of SH-SY5Y cell line.
As first experiment we performed a Wound Healing Assay to test the migration capability of sham, shCD and overCD.
As shown in Figure 1, whilst the overexpression of CD seems to affect the migration behavior of SH-SY5Y cell line strongly inhibiting it, the CD knock down clone has a totally opposite effect. This result suggest us that the aspartic protease Cathepsin D can influence cell migration pathway and also rise up the question "How a protease within a lysosome can influence the migration?". Further study will be performed in the next future to clarify this aspect through transcriptomic and microRNAomic analysis in collaboration with Dr. Chiorino of Edo Tempia Foundation, and through analysis of vescicle trafficking/fusion using immunofluorescence and proteomic assays.

Cathepsin D could be involved in the control of cell cycle
SH-SY5Y sham, shCD and over CD clones were subjected to cell cycle analysis and their doubling time was calculated. As shown in Figure 2A, both clones shCD and overCD seems to increase S phase without altering G2/M phase but, in this single experiment, we could speculate that in overCD clone we saw a slight increase of subG1 population.
Doubling Time calculation (Figure 2B) instead, highlight a very slow cell cycle in overCD clone while in shCD cell cycle is accelerated.
All together these results lead us to think that CD could play an indirect role in cell cycle dynamics. To begin the next experiments, surely these data must be confirmed with other two experiments and then we could clarify the mechanistic role of CD in cell cycle through immunofluorescence imaging of p21 and ki67.

Analysis of Autophagy Flux
Firstly, to assess the autophagy flux in our clones, is required to know which autophagy inducer is the best for all three clones. To clarify this aspect we treated the cell for 3-6 hours with mTOR inhibitor (Rapamycin) for mTOR-dependent pathway of autophagy, Lithium Chloride for mTOR-independent pathway and EBSS for amino acids starvation.
As shown in Figure 3 Rapamycin is able to induce autophagy at 3 hours in all cell clones while LiCl and EBSS have a major effect at 6 hours. Since this is the first time course experiment we need to reproduce the data at least other two times and then, after the selection of the best inducer, we could verify autophagy flux in immunofluorescence imaging using antibody

combination LC3/LAMP1 (for Autolysosome formation) and M6PR/LAMP1 (for Amphisome formation.

5. References

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6. Elenco delle pubblicazioni

- "Excessive fission of mitochondria leads to increased apopotosis in Cockayne Syndrome A cells". Barbara Pascucci*, Mariarosaria D'Errico*, Alessandra Romagnoli, Chiara De Nuccio, Miriam Savino, Donatella Pietraforte, Manuela Lanzafame, Angelo Salvatore Calcagnile, Paola Fortini, Sara Baccarini, Donata Orioli, Paolo Degan, Sergio Visentin, Miria Stefanini, Ciro Isidoro, Gian Maria Fimia, Eugenia Dogliotti. (IN REVIEWING)
- "The role of Cathepsin D in the pathogenesis of human neurodegenerative disorders". Chiara Vidoni, Carlo Follo, Miriam Savino, Marina A. Melone and Ciro Isidoro. - Review - (IN SUBMISSION)

7. Poster sessions

- 51° Congresso dell'Associazione di Neuropatologia e Neurobiologia Clinica
  4-6 Giugno 2015 Verona
  "Toxicity and clearance of mutant Huntingtin in human neuroblastoma cells subjected to oxidative stress: role of the autophagy lysosomal system"

8. List of Seminars and Lessons

28/10/14	**Natural compounds as modulators of epigenetic events**	**Prof. March Diederich**
06/11/14	**Dysregulated antigen receptor signaling:molecular lessons from two congenital lymphoproliferative disorders**	**Dr. A. Snow**
14/11/14	**Tissue engeneering: the state of the art**	**Dott. F. Boccafoschi**
22/11/14	**Stem cell in the regeneration and repair of the tissues and organs**	**Prof. M. Prat**
27/11/14	**Nuove sfide ed opportunità dell'epidemiologia**	**Prof. Laura Baglietto**

	molecolare per lo studio dei tumori	
28/11/14	**Humoral responses to HCV infection and clinical outcomes**	**Prof. Arvind Patel**
04/12/14	**Uncovering the role of β-HPV in field cancerization: a collaboration in progress**	**Prof. Girish Patel**
05/12/14	**Focus on the liver: from basics of NAFLD to hot topics in HBV & HCV infections"**	**Prof. Rifaat Safadi**
16/12/14	**From the legend of Prometheus to regenerative medicine**	**Prof. Antonio Musarò**
17/12/14	**Microglia microvescicles: messengers from the diseased brain**	**Prof. Furlan**
16/01/15	**Pregi e difetti dei nuovi anticoagulanti orali nella pratica clinica**	**Prof. Giancarlo Agnelli**
19/01/15	**Anticancer strategy targeting cell metabolism in ovarian cancer**	**Dr. Yong-Sang Song**
20/01/15	**Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states**	**Dr. Tonino Alonzi**
21/01/15	**Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis**	**Prof. Valeria Poli**
27/01/15	**Myeloid cells as therapeutic target in cancer**	**Prof. Antonio Sica**

11/03/15	**Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells**	**Prof. Darko Bosnakovski**
09/04/15	**Signal control in iNKT cell development and function**	**Prof. Xiaoping Zhong,**
21/04/15	**Actin-based mechanisms in the control of gene expression and cell fate**	**Prof. Piergiorgio Percipalle**
07/05/15	**An integrated approach to the diagnosis and treatment of ovarian cancer**	**Prof. John McDonald**
25/05/15	**Ribosomopathies**	**Prof. Steve Ellis**
05/06/15	***Recent Developments in (cutaneous) Human Polyomavirus Research***	**Prof. Mariet C.W. Feltkamp,**
10/06/15	**Basic of scientific research**	**Prof. Nicoletta Filigheddu**
09/07/15	**Miniworkshop on "Biotechnology for Dermatology"**	**Dr Gwenaël ROLIN** **Thomas LIHOREAU – Ingénieur hospitalier**
28/07/15	**Le cellule staminali nel danno renale acuto e nel trapianto di rene**	**Dott. Vincenzo Cantaluppi**
03/09/15	**Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD)**	**Prof. Darko Bosnakovski**

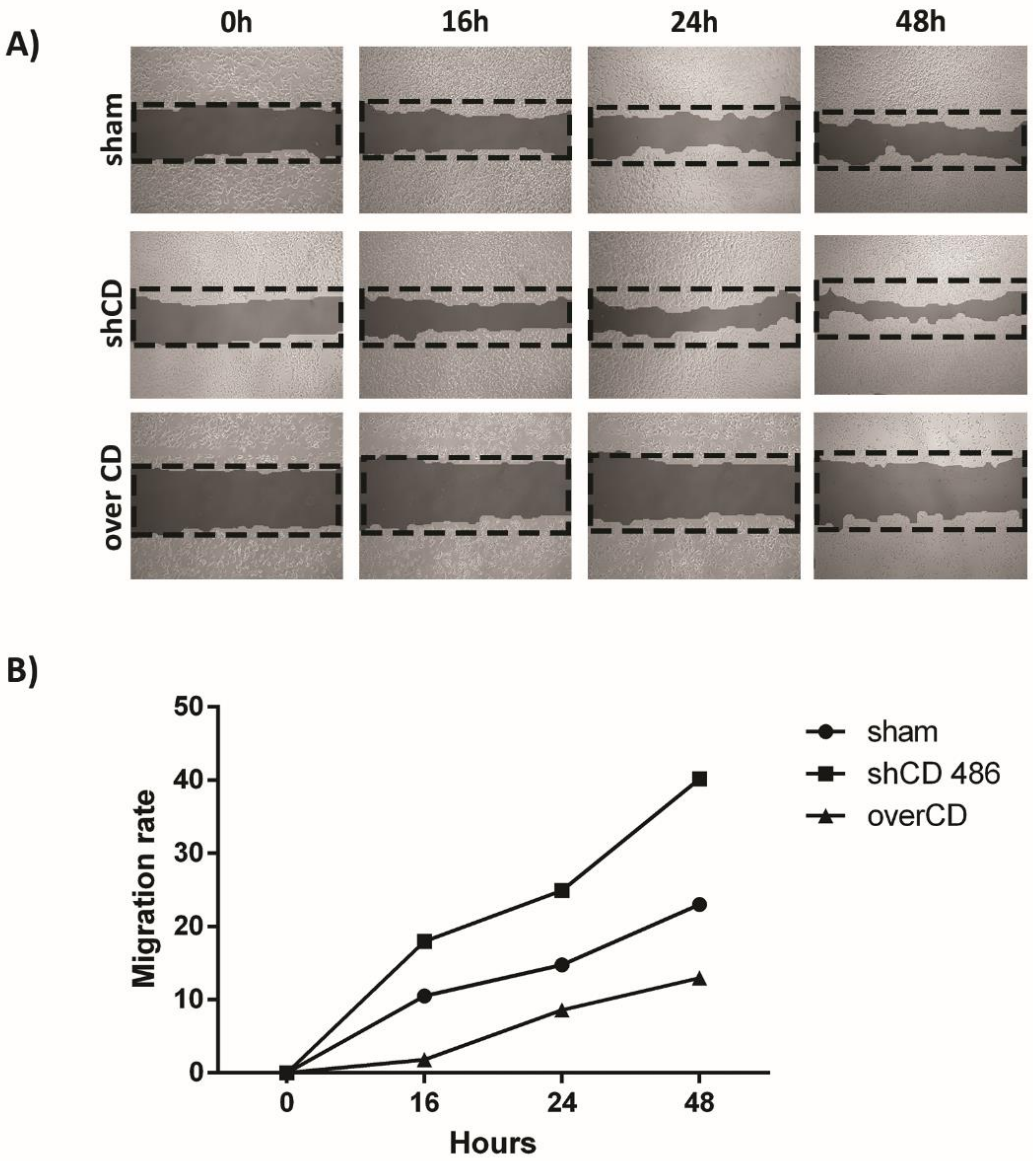
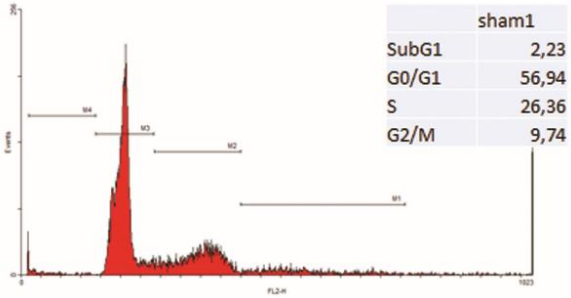
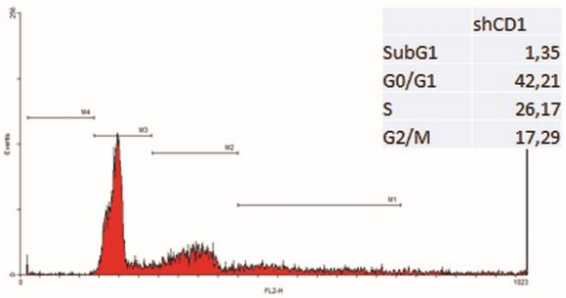


Figure 1: Lack of Cathepsin D notably increase the migration rate of SH-SY5Y cells. A) Wound Healing Assays were performed in stable transfected human neuroblastoma SH-SY5Y cell line carrying empty vector pCDNA H1/TO (sham), shRNA (shCD) and human CTSD gene (overCD). Rapresentative images of three independent experiments were shown. B) Graph of migration rate of SH-SY5Y clone sham, shCD, overCD (GraphPad, Prism). Migration analysis and image optimization was performed with T-scratch software (http://www.cse-lab.ethz.ch).

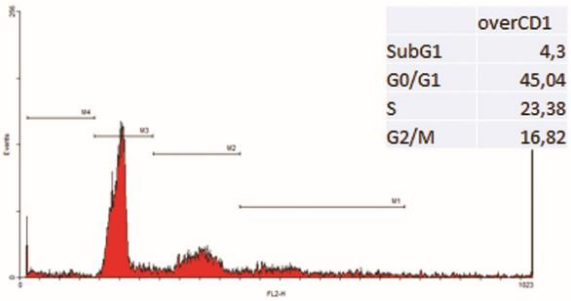
A)



	sham1
SubG1	2,23
G0/G1	56,94
S	26,36
G2/M	9,74

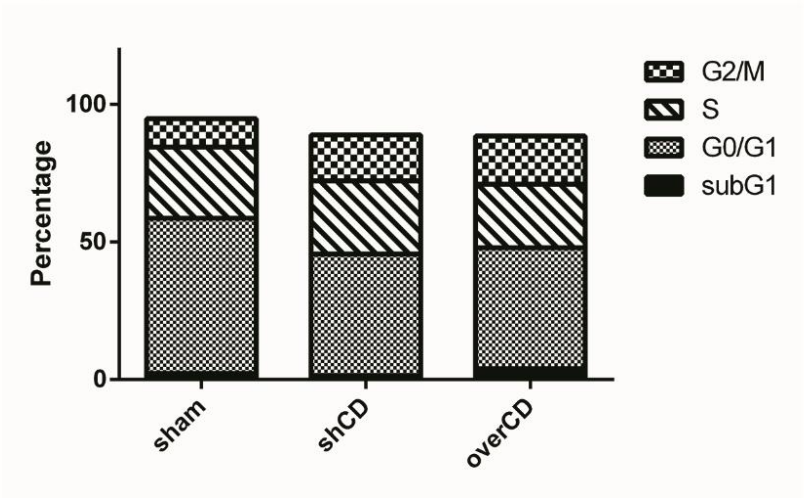


	shCD1
SubG1	1,35
G0/G1	42,21
S	26,17
G2/M	17,29



	overCD1
SubG1	4,3
G0/G1	45,04
S	23,38
G2/M	16,82

B)



	Doubl Time (Hrs)
Sham	41,09
shCD	22,3
overCD	169,2

Figure 2: Cathepsin D seems to affect the normal cell cycle. A) SubG1 analysis of SH-SY5Y sham, shCD and overCD was assayed in quadruplicate in basal conditions. Analysis were performed with WinMDI software (http:// facs.scripps.edu.html). B) Graphical image of subG1 analysis (GraphPad Prism) and table of Doubling time of every clone were calculated with on-line tool http://www.doubling-time.com/compute.php

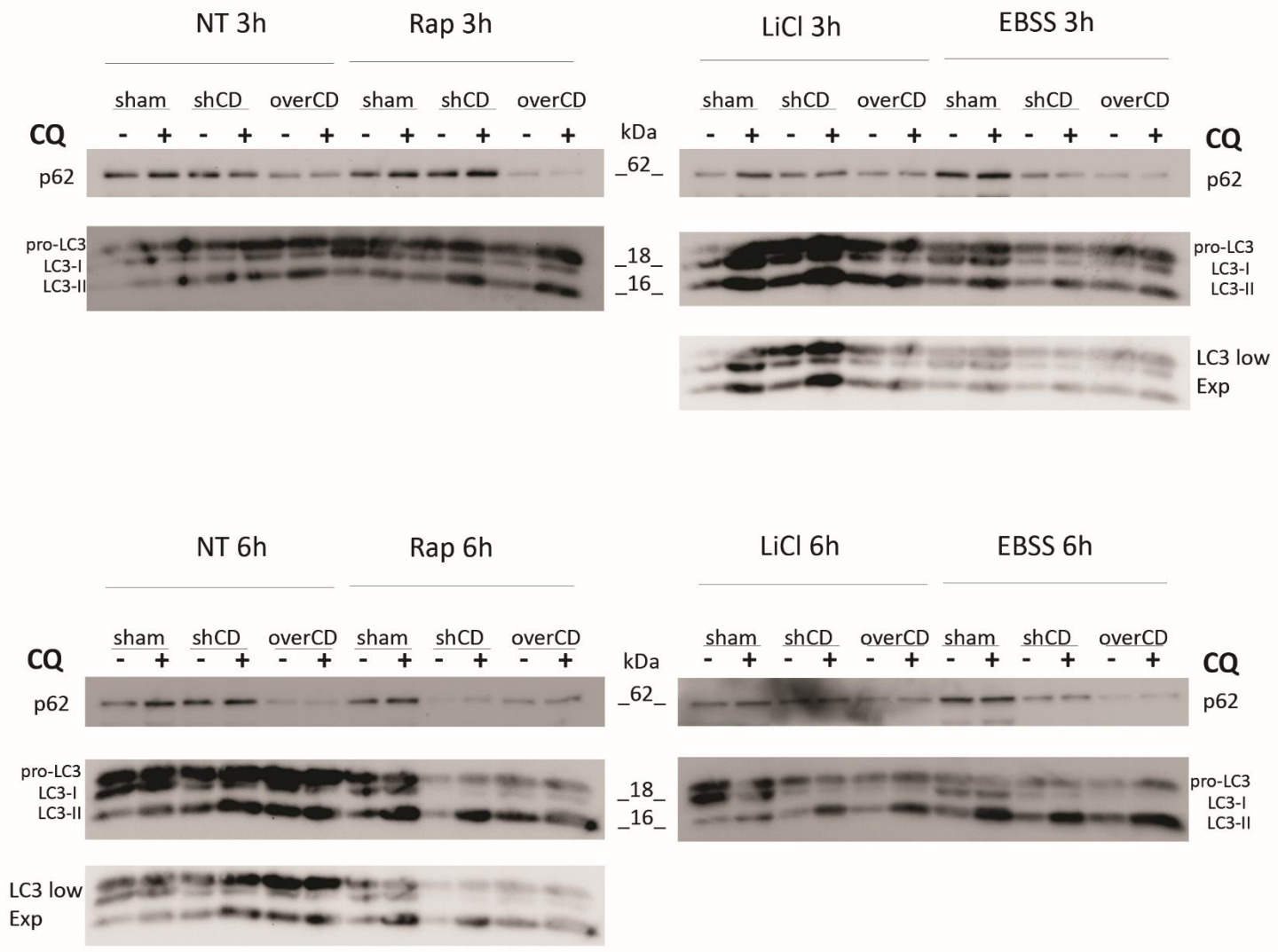


Figure 1: Selection of autophagy inducer for SH-SY5Y clones. Human neuroblastoma cell line sham, shCD and overCD were treated for 3-6 hours with several autophagy inducer in presence or absence of chloroquine to clarify the best time point and treatment to perform immunofluorescence analysis with autophagy markers. Worth to note that overCD clone has basal autophagyat very high levels in no-treatment condition.

Results from “Excessive fission of mitochondria leads to increased apopotosis in Cockayne Syndrome A cells”

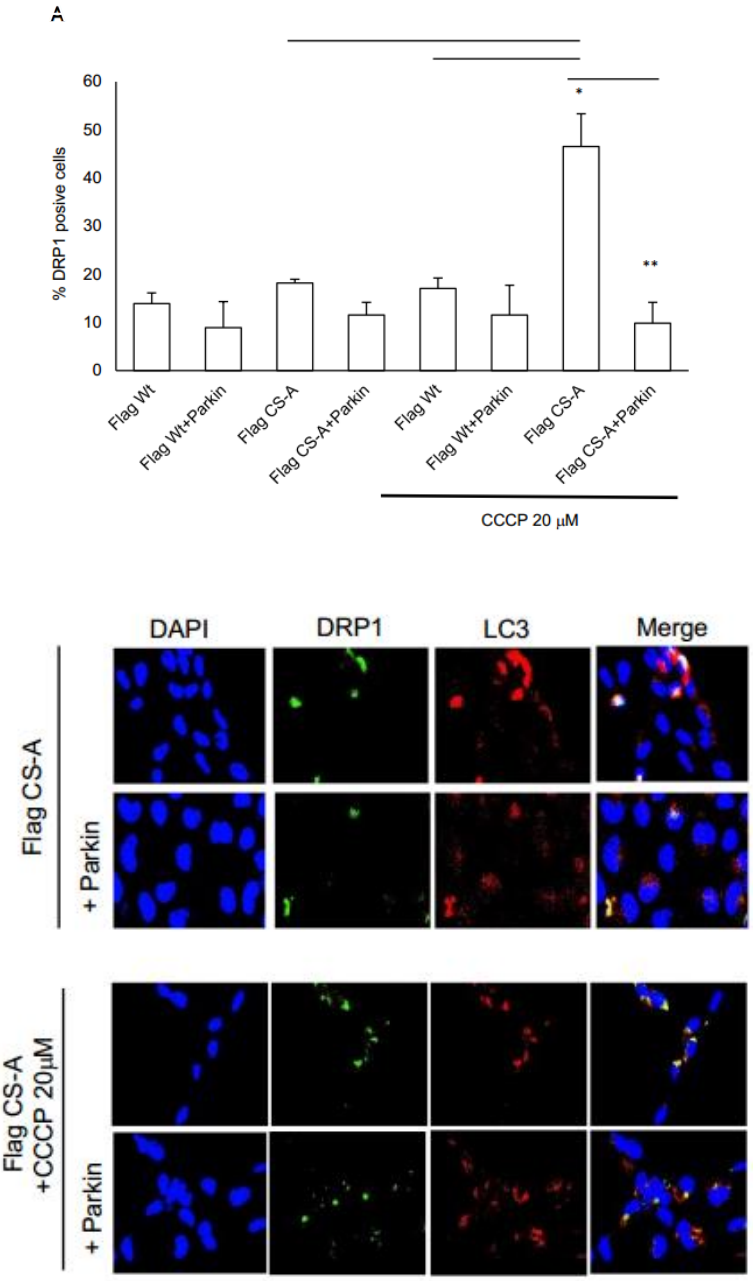


Figure CS-1 : Parkin overexpression rescues the CSA mitochondrial phenotype and decreases mitochondrial apoptotic Bax. (A) Top, mitochondrial DRP1 quantification in Flag WT and Flag CS-A cells w/o overexpression of Parkin, in basal and after CCCP (20 microM) treatment. (* p <0.01 **p<0.001). Bottom, immunofluorescence analysis of DRP1 and LC3 colocalization in Flag CS-A cells w/o overexpression of Parkin, in basal and after CCCP (20 microM) treatment.

B

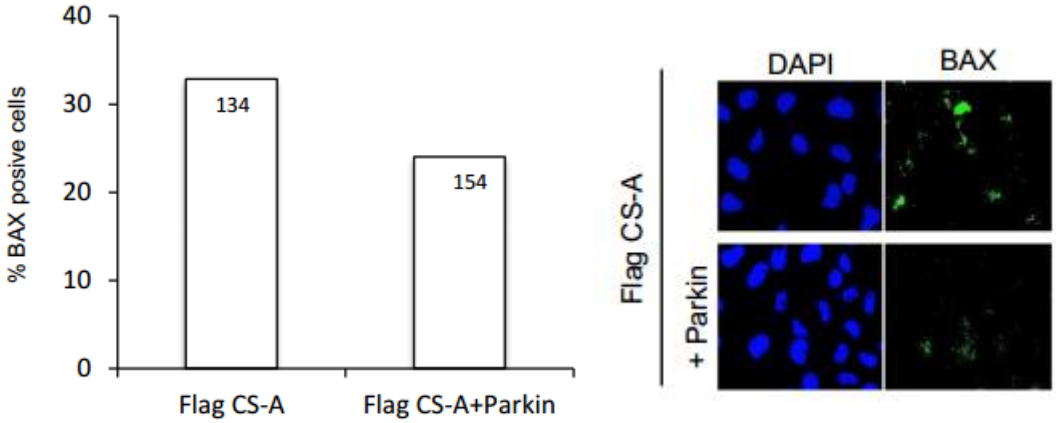


Figure CS-2 : Parkin overexpression rescues the CSA mitochondrial phenotype and decreases mitochondrial apoptotic Bax(. B) Left and right, Bax quantification and immunofluorescence analysis in Flag CS-A w/o overexpression of Parkin.

A

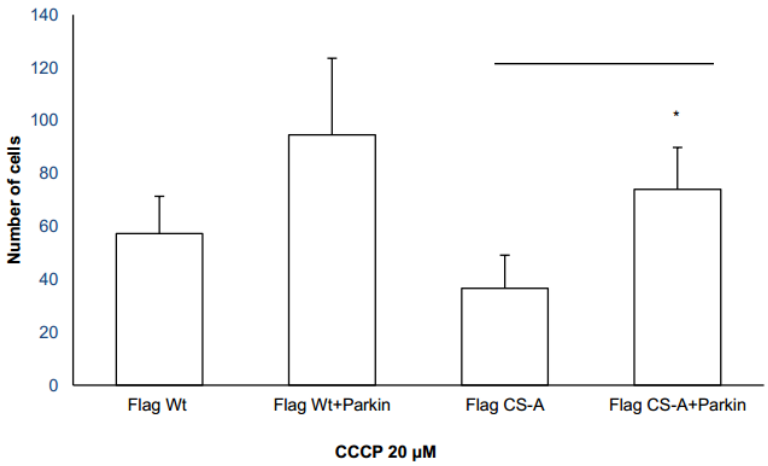


Figure CS-3: Parkin exerts a protective effect on cell survival. Quantification of DAPI positive cells in in Flag WT and Flag CS-A cells with or without overexpression of Parkin after CCCP treatment (20 microM for 16 h).