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EGF and Starvation Differentially Affect Neuroblastoma Cell Migration: Effects on HSC70 and Annexin A2

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ABSTRACT

Annexin A2 (Anxa2) is associated with a wide spectrum of solid tumors and hematological malignancies where its upregulated expression is correlated with migration, invasion, and progression. In neuroblastomas (NB), Anxa2 overexpression has been linked to multi-drug resistance. Additionally, the epidermal growth factor receptor (EGFR) has been shown to be overexpressed in the NB tissues, with a dysregulated signalling in multi-drug resistant NB cell lines. Whether EGFR stimulation regulates the Anxa2 protein levels and how this would impact the phenotype and behaviour of NB cells remain to be elucidated.

Our findings show that the EGFR stimulation enhanced the expression of Anxa2, while growth factors and amino acids starvation of NB cells elicited the opposite effect through induction of chaperone-mediated autophagy (CMA), a lysosome-mediated proteolytic pathway. Notably, short-term treatment of NB cells with EGF demonstrated increased activating phosphorylation of Annexin A2 and its co-localization with HSC70 and LAMP2A, two proteins involved in CMA. In addition, extended 24h EGFR stimulations increased the motility and migrating capabilities of the NB cells with high levels of Anxa2, indicating its vital role in the progression of NB. Parallel with our previous studies marking the crucial role of Cathepsin D (a lysosomal protease) in limiting the proliferative potential and improving the prognostic value of the NB patients, these findings further allow us to speculate on the underlying proteolytic cleavage mechanism of Anxa2 via lysosomal Cathepsin D.

In conclusion our study provides evidence for Anxa2 as a molecular marker for the highly motile and invasive NB cells, opening avenues for exploring the targeted disruptions of oncogenic proteins via CMA by caloric restriction mimetics (mimicking the starvation condition) in the therapeutic interventions of neuroblastomas.

1. INTRODUCTION

1.1 Neuroblastoma

Neuroblastoma (NB) is a highly aggressive solid malignancy that mainly affects children. It originates in the peripheral sympathetic nervous system, which is derived from neural crest cells (Louis and Shohet, 2015) and causes tumors in the sympathetic ganglia and/or adrenal glands. (65%) (Mlakar *et al.*, 2017). This type of cancer accounts for about 65% of all cases of tumors that develop in the nervous system. Neuroblastoma tumor cells are highly metastatic and frequently disseminate to other organs, such as bone marrow, lymph nodes, liver, intracranial and orbital tissues, lungs, and the central nervous system (Tolbert and Matthay, 2018).

The incidence rate of NB in children younger than 14 years old is about 11 to 13 cases per million, whereas in children aged less than 12 months is nearly 65 cases per million (Hsieh *et al.*, 2009). The overall incidence rate of NB is approximately 8.2 cases per million children annually without decrease, which could be correlated to the lack of specific clinical signs and early detection methods (Liu *et al.*, 2023). The etiology of NB is still unknown. However, it has been hypothesized that unlikely is related to exposure to environmental carcinogens, given its frequent onset in the infancy and childhood. NB is classified into three subtypes depending on a growth pattern: undifferentiated, poorly differentiated, and differentiated (Shimada and Ikegaki, 2019). Undifferentiated subtype tumors are characterized by the low expression of TrkA, therefore lacking the potential to differentiate, while differentiated NB cells display cellular differentiation, the appearance of differentiating neuroblasts, and predominantly neurite production. On the other hand, poorly differentiated subtypes show either cellular differentiation with tumor maturation or no tendency to differentiation or maturation (lower levels of TrkA expression) (Shimada and Ikegaki, 2019). In the clinical view, differentiating subtypes of neuroblastoma tumors in patients are associated with poor clinical outcomes.

NB is a type of cancer that can be classified into different risk categories based on biological and clinical factors (Sokol and Desai, 2019). To determine a patient's risk level, factors such as age at diagnosis, *MYCN* status, DNA ploidy index, stage of disease according to the International Neuroblastoma Staging System (INSS), and tumor histology using International Neuroblastoma Pathology Classification (INPC) criteria are commonly used by the Children's Oncology Group (COG) (Table 1) (Sokol and Desai, 2019). These factors are relevant for predicting the clinical outcome in neuroblastoma patients. In some cases, localized tumors such

as in L1 or L2 are classified as intermediate or high risk if the molecular profile shows *MYCN* amplification or chromosome 11q aberration (Monclair *et al.*, 2009).

Genetic risk factors in neuroblastoma range from larger chromosomal aberrations and activation of oncogenic pathways (Fransson *et al.*, 2020). Activation of molecular oncogenic pathways including phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, WNT/β-catenin signalling, p53-mouse double minute 2 homolog (MDM2) pathway, anaplastic lymphoma kinase (ALK) signalling pathway and rat sarcoma (RAS)-microtubule associated protein kinase (MAPK) signaling pathway and mesenchymal transition are involved in the pathogenesis of neuroblastoma (Zafar *et al.*, 2021). PI3K/Akt/mTOR pathway promotes chemoresistance in most NBs, and also contributes to *MYCN* stabilization. Located on chromosome 2p24.3, *MYCN* is a proto-oncogene that encodes a transcription factor. It plays a crucial role in regulating cell growth, differentiation, and apoptosis (Hogarty and Maris, 2012). A study has confirmed that inhibition of the PI3K/Akt/mTOR pathway leads to reduced levels of N-Myc protein in NB (Chesler *et al.*, 2006).

The currently available modalities of treatment include chemotherapy, local radiotherapy, surgery, immunotherapy, and stem cell transplantation (Brodeur, 2003; Maris *et al.*, 2007) (Figure 1). In low-risk patients, surgery alone may be sufficient whereas high-risk patients require to more intensive chemotherapy, surgery, irradiation, and high-dose chemotherapy with stem cells (Pearson *et al.*, 2008; Ladenstein *et al.*, 2010). Current therapies for NB have high toxicity and resistance, leading to poor survival rates. Therefore, an alternative approach is necessary to improve the quality of life for patients.

INSS Stage	**Description**
1	Localized tumor, grossly resected, no lymph node involvement
2A	Unilateral tumor, incomplete gross excision, negative lymph nodes
2B	Unilateral tumor with positive ipsilateral lymph nodes
3	Tumor infiltrating across midline or unilateral tumor with contralateral lymph nodes or midline tumor with bilateral lymph nodes
4	Distant metastatic disease
4S	Localized primary tumor as defined by stage 1 or 2 in patient under 12 months with dissemination limited to the liver, skin, and/or bone marrow (<10% involvement)
INRG Stage	**Description**

L1	Localized tumor with no image-defined risk factors (Monclair *et al.*, 2009)
L2	Localized tumor with one or more image-defined risk factors (Monclair *et al.*, 2009)
M	Distant metastatic disease
M2	Metastatic disease in children under 18 months with metastases limited to skin, liver, and/or bone marrow (<10% involvement)

Table 1. International Neuroblastoma Staging System (INSS) and International Neuroblastoma Risk Group Staging Sysem (INRGSS)

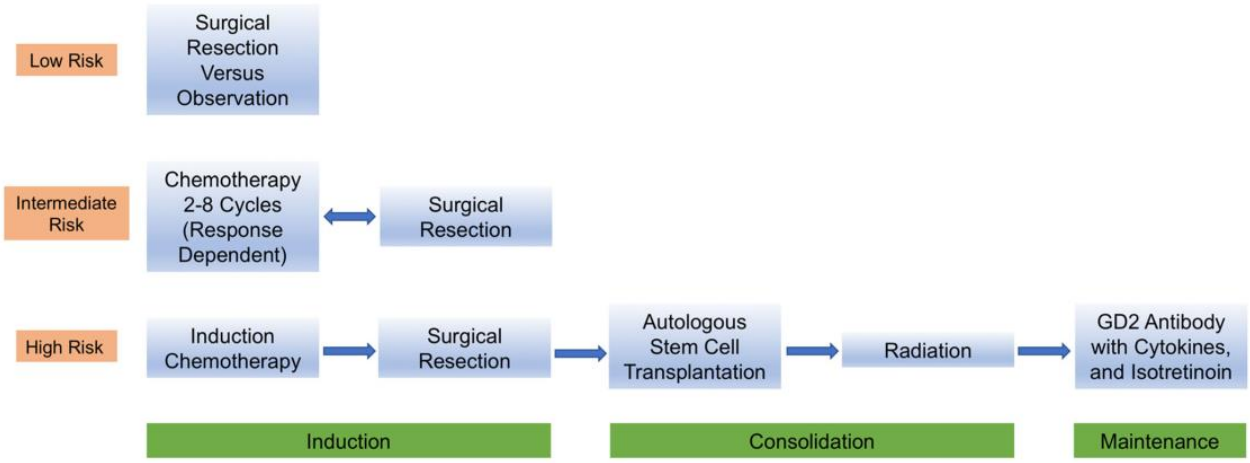


Figure 1. **Overview of treatment modalities for neuroblastoma patient by risk classification** (Adapted from (Tolbert and Matthay, 2018)).

1.2 CATHEPSIN D

Cathepsin D (CD) is a ubiquitously expressed lysosomal aspartic endo-protease that is involved in proteolysis of polypeptides in lysosomes (Benes, Vetvicka and Fusek, 2008). Human CD is coded by the *CTSD* gene located in the short arm of chromosome 11, position (11p) 15.5, and it contains 9 exons with 16 transcripts (Augereau *et al.*, 1988). *CTSD* gene expression is regulated by a hybrid promoter that enables both TATA-independent (binding site for specificity protein (Sp) 1) and TATA-dependent transcription initiations (Cavaillès, Augereau and Rochefort, 1993). The promoter region contains high GC content and Sp1 binding sites (Cavaillès, Augereau and Rochefort, 1993) regulated by steroid hormones, growth factors IGF-1, TNF-α and EGF, and retinoic acid (Vidoni *et al.*, 2016).

In 1926, the term "Cathepsin" was introduced by Willstätter and Bamann, and cathepsin protease families are classified into three subgroups based on amino acids in their active site,

including cysteine (11) (B, C, F, H, K, L, O, S, V, X, and W), aspartate (2) (D and E) and serine (2) (A and G) (Patel *et al.*, 2018). Lysosomes are rich of more than 60 hydrolytic enzymes including proteases, phosphatases, nucleases, lipases, glycosidases, sulfatases, and phospholipases (Trivedi, Bartlett and Pulinilkunnil, 2020) to degrade the substrate delivered through endocytosis, phagocytosis, and autophagy (Gallwitz *et al.*, 2022; Isidoro, 2023).

CD plays a crucial role in various biological processes that occur in low pH conditions ranging from 3.8 to 5.0. CD is involved in multiple cellular biological processes like cell cycle progression, differentiation and migration, morphogenesis and tissue remodelling, immunological processes, ovulation, fertilization, neuronal outgrowth, angiogenesis, and apoptosis (Lkhider *et al.*, 2004; Castino *et al.*, 2008; Follo *et al.*, 2011, 2013; Khalkhali Ellis and Manne, 2014).

Mutations in the human gene that encodes CD result in a lysosomal storage disorder that can lead to various pathological conditions such as atherosclerosis, cancer, cardiovascular disease, and neurodegenerative disorders such as Alzheimer's, Huntington's, and Parkinson's diseases (Vidoni *et al.*, 2016). The production of active and mature form of CD involves several steps, including transcription, synthesis with post-translational modifications on the rough endoplasmic reticulum (RER) as a pre-pro-enzyme followed by several proteolytic cleavages and transport to lysosomal destination (Liaudet-Coopman *et al.*, 2006; Follo *et al.*, 2007, 2013).

CD is synthesized as a single chain pre-pro-enzyme with 412 amino acids and glycosylated at N-linked glycosylation sites on two asparagine residues 134 and 263. The formation of two active chain enzymes of CD is initially removed signal peptide (pre-peptide about 20 amino acids) and followed by pro-peptide of 44 amino acids. CD is further cleaved to remove about seven amino acids from NH2 terminus and certain sequences Ser-Ala-Ser-Ser-Ala-Ser-Ala-Leu at the position 97-105. The two CD glycosylated chains (Asn70, Asn199) are carrying mannose-6-phosphate (M6P) motifs that are recognized by the M6P receptors and involved in CD lysosomal routing and cellular uptake of secreted pro-cathD (Masson *et al.*, 2010). Finally, CD intermediate is processed by cysteine endo-peptidase (cathepsin B or L) and autocatalysis to form two active chains of light CD (14 kDa) and heavy (34 kDa) and catalytic site contains aspartic amino acid residues at 33 and 231 position respectively (Metcalf and Fusek, 1993) (Figure 2). Matured CD enzyme is then transported to the lysosomal compartments comprising of two lobes which is a cleft of the catalytic site of two aspartate residues. This double chain CD consists of 346 amino acid residues with three domains including an N-terminal domain

(1-188 amino acids), an inter-domain region (160-200 amino acids), and C-terminal domain (189-346 amino acids) (Baldwin *et al.*, 1993). CD enzyme is associated with cleavage of structural and functional proteins and peptides. This CD-mediated proteolysis of substrate is to be resulted activation and inactivation polypeptide hormones and growth factors, activation of enzymatic precursors, processing of enzyme activators and inhibitors, antigen processing and regulation of cell death (Castino *et al.*, 2002, 2007; Benes, Vetvicka and Fusek, 2008).

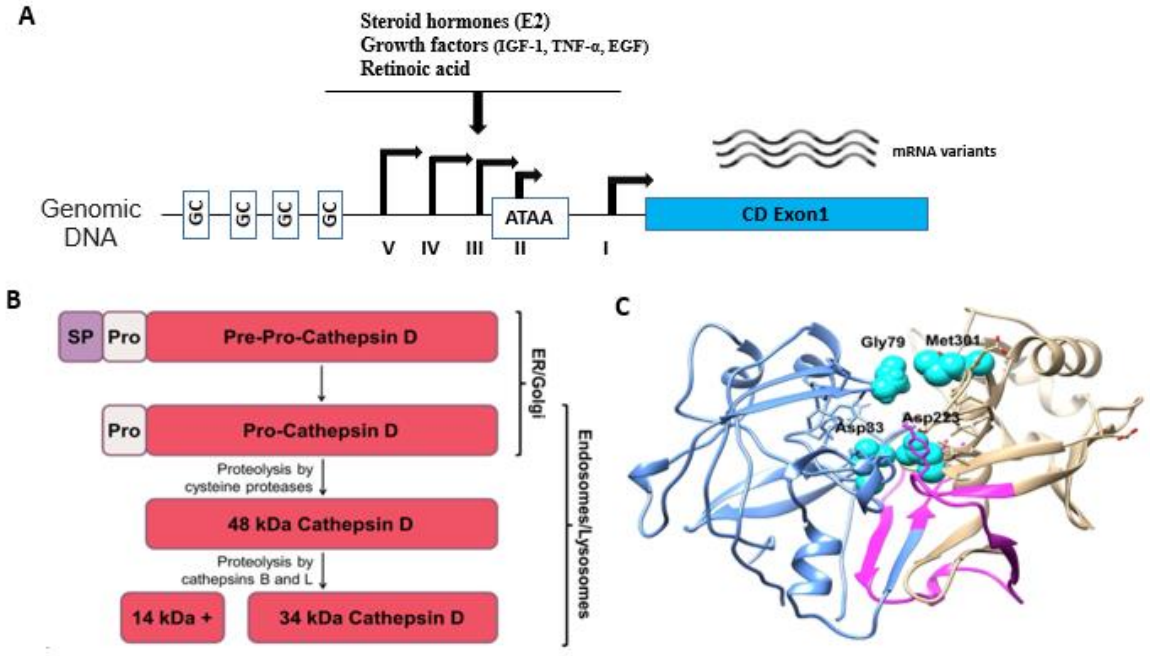


Figure 2. Cathepsin D transcription, maturation and structure. (A) Cathepsin D promoter region. Five major transcription start sites (TSS-I to -V) for CD gene are indicated by arrowheads. These five transcription initiation sites respectively map at 20, 44, 51, 60 and 72 from the first base of the initiation codon (adapted from (Zaidi *et al.*, 2008). (B) Major steps of cathepsin D maturation. SP-signal peptide; Pro-prodomain; kDa - kilodaltons; ER-endoplasmic reticulum (Adapted from (Mijanovic *et al.*, 2021). (C) Mature CD structure. Crystal structure of CD illustrating the N-terminal domain (1-188 amino acid residues) in blue color, C-terminal domain (189-346 amino acid residues) in gold color, and an inter-domain region (160-200 amino acid residues) in pink color as well as the important aspartic acids and flap tip residues (also known as hairpin loop). Catalytic aspartic amino acids are represented as sticks (Adapted from (Arodola and Soliman, 2016).

CD plays an important role in cancer development and has dual biological functions, including proteolytic and non-proteolytic activities (Berchem *et al.*, 2002). CD is implicated in several critical steps of cancer progression, like the maintenance of balance between cell death and survival, intracellular matrix (ECM) degradation, invasion, and neo-angiogenesis (Briozzo *et al.*, 1991) (Figure 3). CD is overexpressed with hypersecretion in various solid tumors

including lung, ovarian, prostate, breast, endometrial, malignant glioma,melanoma and non-Hodgkin's lymphomas (Lösch *et al.*, 1996; Isidoro *et al.*, 1997; Konno *et al.*, 2001; Vetvicka, Vetvickova and Benes, 2004; Fukuda *et al.*, 2005; Trincheri *et al.*, 2007; Castino *et al.*, 2009; Nicotra *et al.*, 2010; Zhu *et al.*, 2013). The extracellular proCD activates the RAS/MAPK and PI3K/AKT pathways in fibroblasts and human endothelial cells. It acts as an autocrine and paracrine growth factor for an unidentified receptor (Pranjol *et al.*, 2018).

Based on current understanding, cathepsin S and B are involved in breaking down receptors and reducing EGFR signaling (Huang *et al.*, 2016). Neuroblastomas with low CD levels progress more quickly than those with higher levels, particularly in patients at INSS Stage 4. Additionally, high levels of CTSD mRNA are negatively correlated with a group of genes associated with cell cycle and proliferation. Furthermore, patients with neuroblastomas expressing high levels of the lysosomal protease CD have a better prognosis (Secomandi *et al.*, 2022).

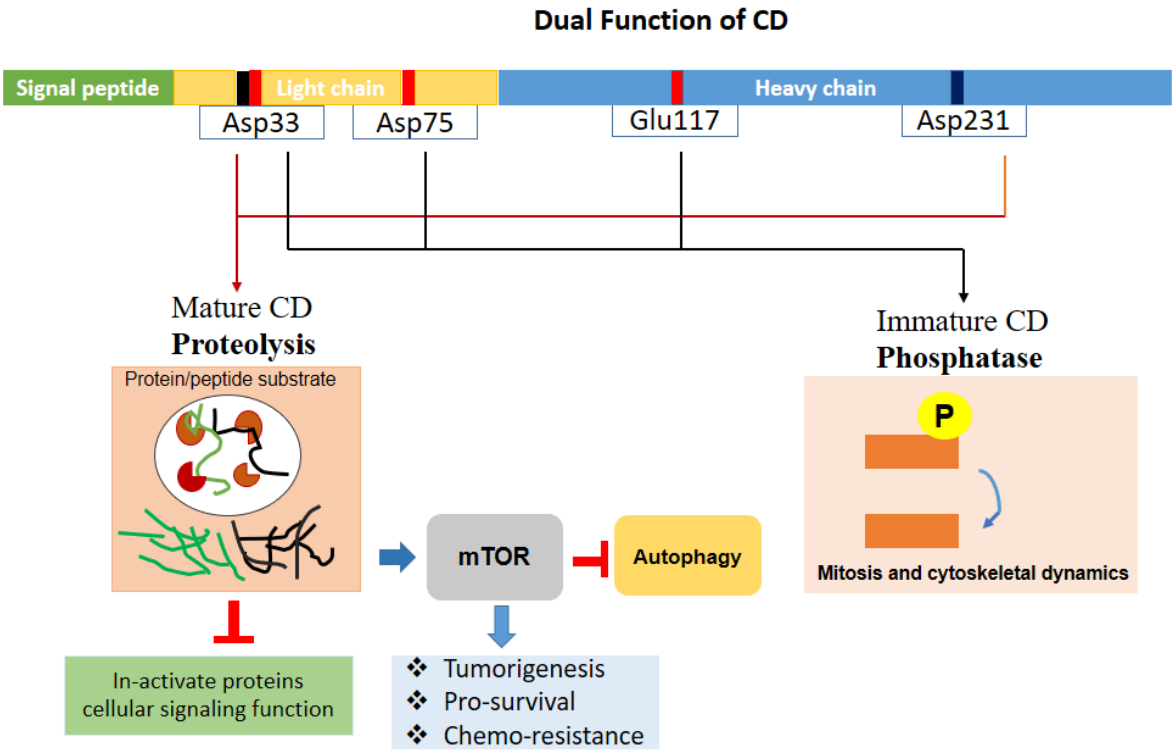


Figure 3. Overview of the dual cellular function of CD. In the lysosomes acidic compartment, matured CD hydrolyzes protein and peptide substrates yielding certain amino acids role in mTOR and reducing the activities of proteins. Whereas, in neutral pH in the nucleus, immature CD precursors are involved in de-phosphorylation and modulate cytoskeletal dynamics and cell mitosis process (Adapted from (Liu *et al.*, 2021).

1.3 ANNEXIN A2

Annexin A2 (ANXA2), a 36 kDa protein, is coded by the *ANXA2* gene located in the long arm of human chromosome 15, position (15q) 22.2 (Lei *et al.*, 2023). ANXA2 exists in four forms, membrane-bound, secretory, nuclear, and cytoplasmic (Wang and Lin, 2014), and it is associated with different processes including membrane trafficking (Matos *et al.*, 2020), vascular homeostasis (Xiu *et al.*, 2016), signal transduction (Mazaki *et al.*, 2019) such as cell proliferation, survival, invasion and metastasis and DNA integrity and synthesis ((Lei *et al.*, 2019) (Figure 4). Membrane-bound ANXA2 has the tendency to enhance calcium, phospholipid, and heparin binding, as well as F-actin binding, and plasminogen-binding sites in the C-terminal region (Kassam *et al.*, 1997; Lokman *et al.*, 2011). These enhancements lead to further molecular events. Furthermore, cytoplasmic ANXA2 can perform various subcellular functions such as actin remodelling, protein assembly, exocytosis, and endocytosis (Grindheim, Saraste and Vedeler, 2017) due to its ability to form aggregations.

ANXA2 is found overexpressed in several types of aggressive malignancies such as breast cancer, neuroblastoma (Wang *et al.*, 2017), prostate cancer, colorectal carcinoma, renal cell carcinoma, pancreatic cancer, multiple myeloma, ovarian cancer, and glioblastoma (Bao *et al.*, 2009; Lokman *et al.*, 2011, 2016; Chen *et al.*, 2018). ANXA2 expression levels are positively correlated with tumor size, histological morphology change, invasion and proliferation (Chen *et al.*, 2018), pathological tumor node metastasis stage (Li *et al.*, 2021) and chemo-resistance (Wang *et al.*, 2017). ANXA2 is also involved in cellular pathways by upregulating key epithelial to mesenchymal transition (EMT) regulator genes such as Snail, Slug, and Twist via EGF-STAT3-mediated pathways, promoting (Lo *et al.*, 2007; Sullivan *et al.*, 2009; Wendt *et al.*, 2014) angiogenesis. ANXA2 negatively affects p53 function through the JNK/c-Jun pathway, reducing the expression of apoptosis-promoting genes such as p21, GADD45 and BAX (Wang and Lin, 2014; Feng *et al.*, 2017). ANXA2, in complex with PGK initiates DNA replication (Vishwanatha, Jindalf and Davis, 1992), while as a monomer, it is involved in DNA synthesis, cell proliferation, cell cycle progression (Vishwanatha and Kumble, 1993) and protects DNA damage from oxidative stress (Madureira *et al.*, 2012).

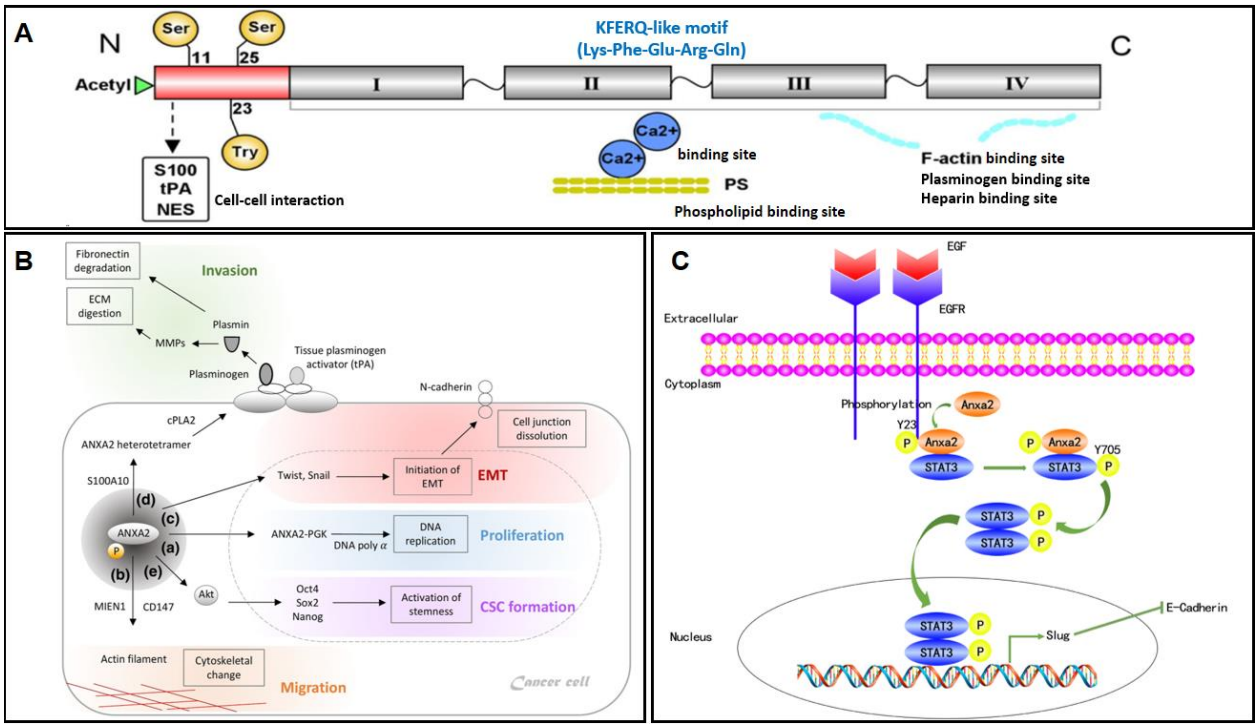


Figure 4. Structure of the ANXA2 protein domains and in cancer progression. (A) ANXA2 consists of commonly variable N-terminus and conserved core domain of C-terminus. tPA = tissue plasminogen activator, NES = nuclear export signal (Adapted from (Lei *et al.*, 2023) (B) ANXA2 in cancer progression, (a) ANXA2-3-phosphoglycerate kinase (ANXA2-PGK) complex serves. (b) MIEN1 phosphorylates ANXA2. (c) ANXA2 promotes the endothelial-mesenchymal transition (EMT) through the Twist/Snail molecular cellular pathway. (d) ANXA2 heterotetramer complex links to the plasminogen and tissue plasminogen activator (tPA), plasminogen to be cleaved into plasmin. (e) ANXA2 also plays a role in stemness-related transcription factors (Adapted from (Chen *et al.*, 2018). (C) ANXA2 mediates EGF-induced EMT in breast cancer by interacting with STAT3. Phosphorylated STAT3 translocates from the cytosol to the nucleus, and upregulates the expression of EMT associated transcription factor Slug, which finally induces EMT (Adapted from (Wang *et al.*, 2015).

1.5 AUTOPHAGY

The term ‘autophagy’ was introduced by Christian de Duve in 1963, and it is a crucial molecular pathway that plays a major role in maintaining cellular homeostasis in dopaminergic neurons by acting as a quality control mechanism in the cell (Isidoro *et al.*, 2009). Cells can survive under stressful conditions, such as nutrient starvation, oxidative stress, hypoxia, ER stress, metabolic stress, and others, due to the ability to recycle macromolecules (Kroemer and Piacentini, 2015). Till date, there are three major forms of autophagy based on the pathway to deliver the cargo including macro-autophagy (referred to as autophagy), chaperone-mediated

autophagy (CMA), and micro-autophagy (non-selective lysosomal degradative process) (Li, Li and Bao, 2012; Levy, Towers and Thorburn, 2017).

CMA is a lysosomal proteolysis pathway that selectively degrades cytosolic proteins directly crossing the lysosomal membrane without vesicle formation in response to stress conditions, ensuring cellular quality control (Kaushik and Cuervo, 2018; Tekirdag and Cuervo, 2018). CMA is operated through direct recognition of protein substrates bearing KFERQ-like motifs by the cytosolic chaperone heat-shock cognate 70 kDa (HSC70) for translocation into lysosomes for degradation, upon binding lysosomal-associated membrane protein 2A (LAMP2A) (Kaushik and Cuervo, 2018) (Figure 5). The motifs-like domain of proteins contains mainly a penta-peptide of amino acids sequence (Lys-Phe-Glu-Arg-Gln). CMA plays a role in protecting against oncogenic pathways via actively involving degradation of pro-oncogenic proteins including MYC and bHLH transcription factor (Gomes, Menck and Cuervo, 2017), translationally controlled tumor proteins 1 (TPT1/TCTP) (Bonhoure *et al.*, 2017), MDM2 (Lu *et al.*, 2010), and epidermal growth factor receptor pathway substrate 8 (EPS8), hexokinase 2 (HK2), galactose lectin-3 (Li *et al.*, 2018) reducing the amounts of transcriptional factors in cells.

Furthermore, some studies have shown that key glycolytic enzymes are identified as substrates of CMA such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase muscle isozyme (PKM), aldolase, and HIF1A (Ferreira *et al.*, 2013; Tasset and Cuervo, 2016) to reduce the amounts of enzymes in cells. Up-regulation expression of cytoplasmic proteins of HSC70 and LAMP2A in breast, colorectal, and brain glioma cancer patients' tissue samples are correlated with the progression and survival of cancer cells (Rios *et al.*, 2021).

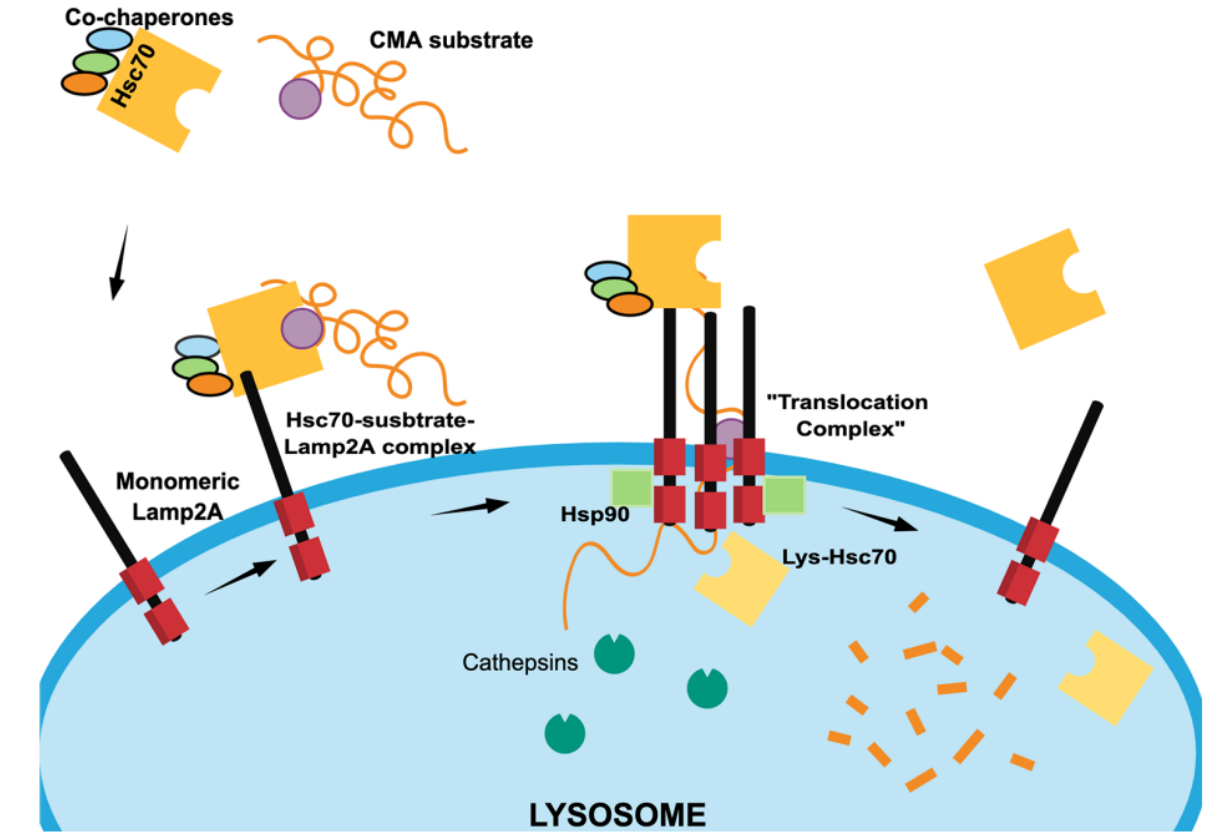


Figure 5. Scheme of different chaperone-mediated autophagy (CMA) steps. In the cytosol, heat shock cognate 70 (Hsc70) protein recognizes CMA protein substrates containing KFERQ-like motifs (purple circle) with the assistance of co-chaperones Hip, Hop and Hsp40 (light blue, green and orange ovals) to form a "Hsc70 substrate complex". The Hsc70-substrate complex interacts with monomeric lysosome associated membrane protein type 2A (Lamp2A) at the lysosomal membrane to induce the formation of a "translocation complex" through Lamp2A oligomerization, which is regulated at the outer lysosome membrane by the Glial Fibrillary Acidic Protein (GFAP), Protein Phosphatase 1 (PHLPP1), and Elongation Factor 1a (EF1a) proteins (not shown). Once the "translocation complex" is formed, the CMA substrate protein unfolds to enters at the lysosomal lumen thanks to the assistance luminal Hsc70 (lys-Hsc70) and Hsp90 (green squares). Once, the CMA substrate protein is degraded by cathepsins and other proteases, the "translocation complex" dissociates, Hsc70 is recycled and Lamp2A returns to a monomeric state (Adapted from (Rios *et al.*, 2021).

2. THE AIM OF THIS STUDY

Increased EGFR expression has been associated with more aggressive neuroblastomas. Our recent studies confirm that stimulating EGFR enhances the proliferative potential of neuroblastoma cells by downregulating the lysosomal protease Cathepsin D. This study sought to assess the migratory response of the neuroblastoma cells under EGFR activation or deprivation. Particularly, we investigate the impact of EGFR and starvation stimuli on the chaperone-mediated cytosolic regulation of Annexin A2, a known protein that regulates the migration and invasion of various carcinomas but still remains uncharacterized for neuroblastomas (Figure 6).

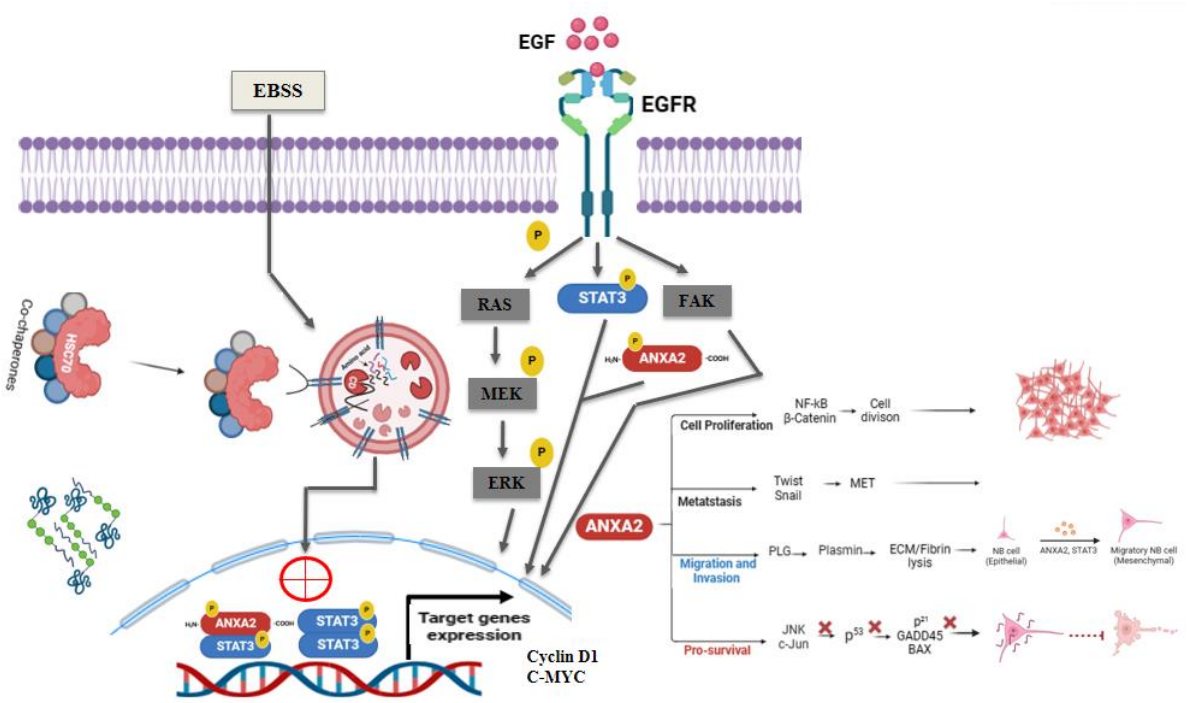


Figure 6. EBSS modulation of HSC70 expression and induce Chaperon-Mediated Autophagy whereas EGF promotes cell-migration through Annexin A2.

3. MATERIALS AND METHODS

3.1 CELL CULTURE AND REAGENTS

Human neuroblastoma cell lines, IMR-32 and SK-N-BE2 were obtained from the American Type Culture Collection (cod. CCL-127, cod. CRL-2271, ATCC, Rockville, MD, USA, respectively). Human neuroblastoma cells LAN-5 were obtained from German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) (DSMZ, cod. ACC673, Braunschweig, Germany). SK-N-BE2, LAN-5 and IMR-32 cell lines were grown with Roswell Park Memorial Institute (RPMI)-1640 (cod. R8758; Sigma-Aldrich Corp., St. Louis, MO, USA) with supplemented of 10% heat-inactivated fetal bovine serum (FBS, cod. ECS0180L; Euroclone, Milan, Italy), 1% penicillin/streptomycin (PES) (PES, cod. P0781; Sigma-Aldrich Corp.), 1% L-glutamine (cod. G7513; Sigma-Aldrich Corp.), 1% sodium pyruvate (cod. S8636; Sigma-Aldrich Corp.), under standard conditions (37 ^{o}C, 95 v/v% air: 5 v/v% CO2) and humidified incubator (Secomandi *et al.*, 2024). Treatments included 20 ng/mL epidermal growth factor (EGF, cod. E5036; Sigma-Aldrich Corp.), dissolved in 10 mM acetic acid and Earle's Balanced Salt Solution (EBSS) with sodium bicarbonate and phenol red (EBSS, cod. E2888; Sigma-Aldrich Corp.).

Cell line	TP53*	MYCN +
IMR-32	wt	amp
LAN-5	wt	amp
SK-N-BE(2)	404G>T (C135F)	amp

Table 2. Genetic profiles of neuroblastoma cells, wt, wild-type; amp, amplified; *from direct DNA sequencing of the entire coding region of TP53. Mutation is shown by nucleotide change followed by amino acid change. + Genomic amplification status (Adapted from (Van Maerken *et al.*, 2006)).

3.2 ANTIBODIES

Expressed protein levels were analyzed by using immunoblot. Antibodies included: anti-β-tubulin (mouse 1:1000, (55 kDa); Sigma-Aldrich), anti-HSC70 (mouse (1:500); Santa Cruz Biotechnology)). For immunofluorescence antibodies included: anti-annexin A2 (1:100); PAS-88522, Invitrogen ThermoFisher Scientific), anti-HSC70 (mouse (1:50), Santa Cruz Biotechnology), anti-LAMP2A (rabbit, (1:50); Santa Cruz Biotechnology), anti p-annexin A2 (mouse (1:50); Santa Cruze Biotechnology). Detection was via goat anti-mouse secondary

antibody (For WB, goat anti-rabbit (1:10,000); for IF, goat anti-rabbit (1:1000) XA339480, A32731, Alexa FluorTM Plus 488 IgG (H+L), Invitrogen, USA, and For WB, goat anti-mouse (1:10,000); for IF, goat anti-mouse (1:1000) XC344348, A32727, Alexa FluorTM Plus 555 Invitrogen, USA and IF, DAPI (1:1000)) with chemiluminescence detection. All proteins band densitometry were semi-quantified by Image Lab Software (Bio-Rad, software).

3.3 TREATMENT ASSAY

50,000 cells density /cm^2 were seeded in 35mm petri dishes (P35) and treated after 48 h in order to make sure all cells adhered and grow. Adhered cells of IMR-32, LAN-5 and SK-N-BE2 were incubated in RPMI with supplement of 1% PES, 1% L-glutamine and 1% sodium pyruvate without FBS for 4 h. After that, cells were treated in RPMI completed media with EGF and only EBSS for 45 min.

3.4 PROTEIN EXTRACTION

For the protein extraction, cells were treated with EGF and EBSS and then harvested at specific time-points. To prepare cell lysates, treated cells in P35 were harvested with aspirated the media. Adhered cells were washed once with PBS and dissolved with lysis buffer 250 µL per P35 (lysis buffer composed of, sodium deoxycholate (NaDOC) 0.2%, sodium fluoride (NaF) 50 mM, sodium orthovanadate (Na_3VO_4) 1 mM, protease inhibitor cocktail (PI) (1:300) and frozen at -20 ^oC for overnight to disrupt the cells.

3.5 PROTEIN DOSAGE

For loading lysates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), each lysates were homogenized using a Microson ^{TM} ultrasonic cell disruptor XL (Misonix, Farmingdale, NY, USA) for about 40 seconds. The protein concentration of each lysates were determined using the Bradford assay (Bradford, 1976) with a bovine serum albumin (BSA) standard. Sample lysates were prepared with a final protein concentration of 25 µg/µL to be loaded with a SDS sample buffer and afterwards stored at -20 ^{o} C.

3.6 WESTERN BLOTTING

Sample lysates were prepared with a final protein concentration of 25 µg/µL in SDS sample buffer (5X Leammli sample buffer, loading buffer). Each sample lysates were heated at 95 ^{0} C on the heat blocker (Multi-block heater) for 10 min. Total cell lysates were loaded with pre-stained protein ladder as protein size control and separated by 12.5 % SDS-PAGE at 80 V for 30 min and followed at 120 V for hours. Blotting was performed using polyvinylidene fluoride

(PVDF) membranes with activated in methanol for 30 minutes (Bio RAD #1620177); for 2:45 h at 100 constant voltage. Membranes were blocked with 5% non-fat dry milk (cod. sc-2325; Santa Cruz Biotechnology), 0.2 % Tween-20 in PBS for 1 h at room temperature (RT), then incubated with primary antibodies at cold room 4 °C for overnight. The next day, after washing with 0.05 % TritonX-100 three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. After membrane washed three times with 0.05 % TritonX-100, the bands were detected using Enhanced Chemiluminescence reagents (ECL, cod. NEL105001EA; Perkin Elmer, Waltham, MA, USA) and developed with a ChemiDoc XRS instrument (BioRad, Hercules, CA, USA). Intensity of the bands were estimated by densitometry using Quantity One Software.

3.7 IMMUNOFLUORESCENCE IMAGING

To analyses protein expression, IMR-32, LAN-5 and SK-N-BE 2 cell lines were seeded on the coverslips with a density of 20,000 cells per cm^2 and allowed to attached and grow before treatment. Adhered cells of IMR-32, LAN-5, SK-N-BE2 were incubated in RPMI with supplement of 1% PES, 1% L-glutamine and 1% sodium pyruvate without FBS for 4 h. After that, cells were treated with RPMI completed media and with EGF and EBSS for 45 min. At the end of each experiment, the coverslips were collected and fixed in cold methanol, permeabilized with 0.2% Triton-PBS and then re-fixed with cold methanol. The coverslips were incubated with the specific primary antibodies dissolved in 0.1% Triton-PBS with 10% FBS. The following day, the coverslips were incubated with Goat-Anti-Rabbit IgG Alexa FluorTM Plus 488 IgG or Goat-Anti-Mouse IgG Alexa FluorTM Plus 555 secondary antibodies for 1 h at room temperature. Nuclei were stained with the UV fluorescent dye DAPI (4, 6-diamidino-2-phenylindole). DAPI and secondary antibodies were dissolved in 0.1% Triton-PBS with 10% FBS. Afterward, coverslips were mounted onto slide glasses using SlowFade antifade reagent (cod. S36936; Life Technologies, Paisley, UK). Images were acquired by fluorescence microscopy (Leica DMI6000, Leica Microsystems, Wetzlar, Germany) with supported by Leica Application Suite (LAS) X software (X version, 3.3.3.16958).

3.8 WOUND HEALING ASSAY

70,000 cells density/cm^2 of IMR-32, LAN-5 and SK-N-BE2 were seeded in P35 culture plates using the completed media. Once adhered, the cell monolayer was scratched with a 200-µL pipette tip. After that, cells were treated with EGF and photographed under a microscope

(ZEISS, Germany) at zero and each 24h up to 96h. The data was quantified using ImageJ software (version 1.54; NIH). The migration rate (healing rate) was calculated as follows: migration rate (%) = (original wound width-wound width at 24 h)/original wound width×100%.

4. RESULTS

4.1 EGF promotes Annexin A2 and HSC70 colocalization

To investigate the impact of starvation and EGFR stimulations on the cytosolic levels of Annexin A2 (Anxa2) and the chaperone involved in CMA, we performed an immunofluorescence double staining for Annexin A2 (green) and HSC70 (red) (panels in Figure 1A). The integrated densities of the green signal in Figure 1C showed that LAN-5 presented low levels of Annexin A2 in comparison to IMR-32 and SKNBE2 cells, which presented the highest level. Notably, the short-term stimulation with 20ng EGF (45 minutes) evidently increased the co-localization between Annexin A2 and HSC70 (yellow signal) compared to the control that suggests their fusion (figure 1B). Also, the neuroblastoma cells treated with EBSS showed a higher accumulation of red signal corresponding to the HSC70 while the Anxa2 levels have significantly reduced compared to the control.

CMA is constitutively expressed at basal levels in mammalian cells and can be upregulated in response to starvation conditions (Cuervo *et al.*, 1995). Upon induction of CMA, certain genes that are associated with recognition of substrate, co-chaperons and stabilizer and translocator of substrates could be upregulated to promote CMA-mediated proteolysis of proteins. As confirmed by the fluorescence staining, expression of HSC70 protein was also found upregulated in the western blot analysis (Figure 1D) for all the three neuroblastoma cell models, in response to the starvation induced via EBSS treatment. All together these data suggest that starvation and growth factors are differently modulating genes associated with migration and CMA.

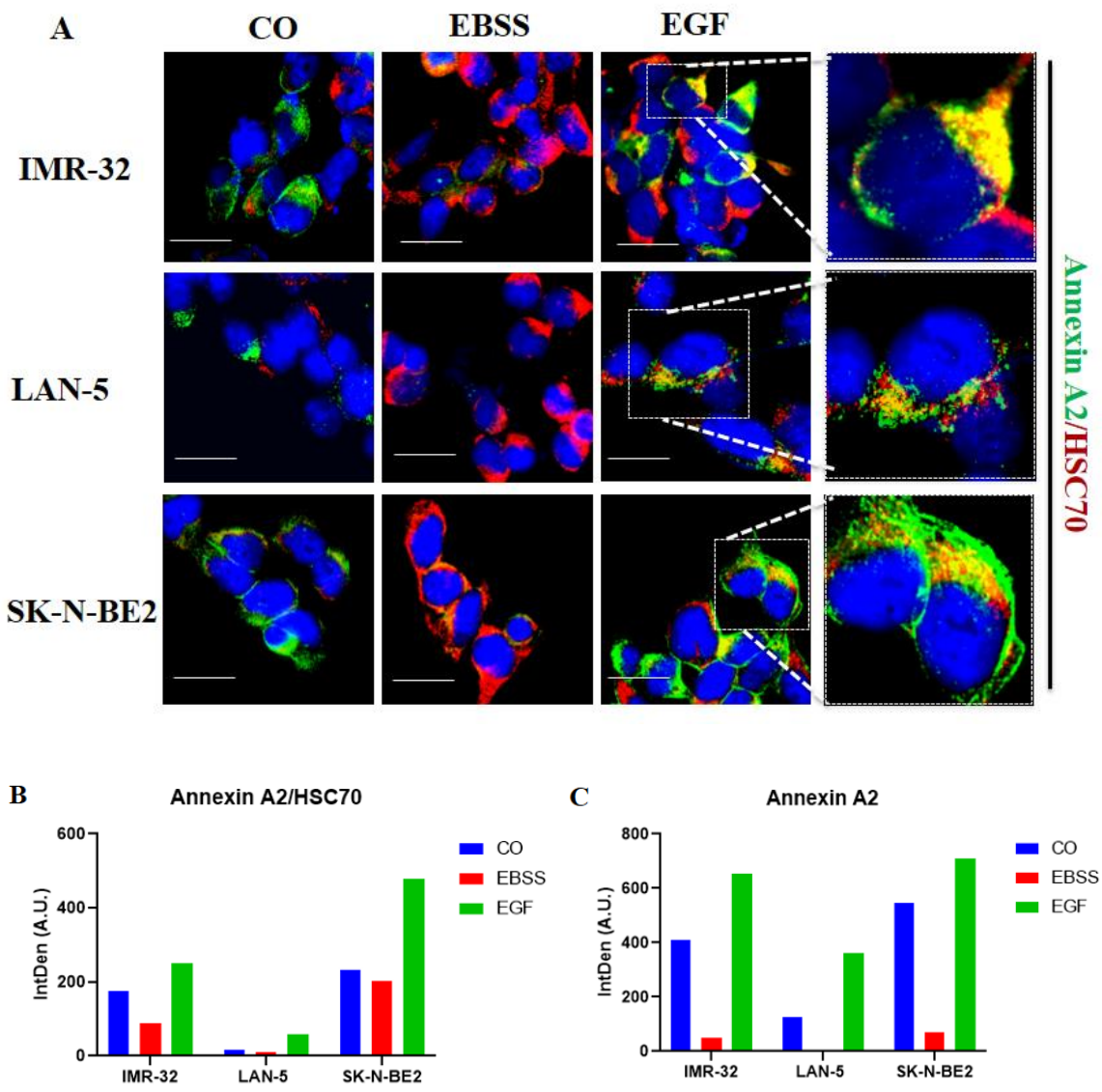


Figure 1. (A) **Immunofluorescence double stained for total annexin A2 (green) and HSC70 (red) after 45 min treatment**. (B) Graph represents the co-localization of Annexin A2/HSC70. (C) Graph shows the expression levels of Annexin A2 each condition. Scale bar = 25 µm; magnification = 63X. Representative images of different fields for each experimental condition were shown.

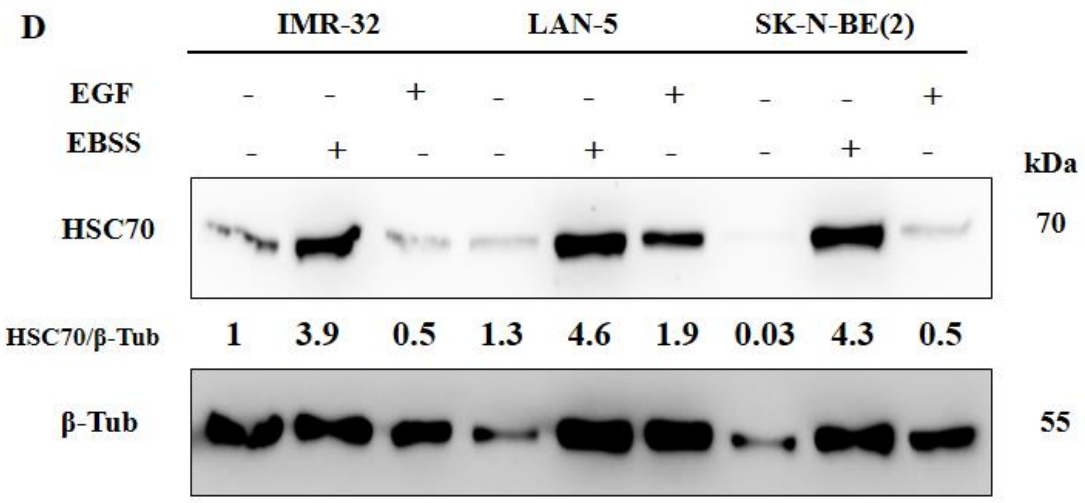


Figure 1D. Western blotting to assess the expression of HSC70 in the three neuroblastoma cell lines after short-term stimulation with EGF and EBSS.

4.2 EGF stimulation promotes Annexin A2 uptake into lysosomes through CMA

To further complete the circle and investigate the late stages of chaperone mediated autophagy, we also performed a fluorescence co-staining with CMA-specific lysosomal marker LAMP2A (green) and also visualized the activated or the phosphorylated Anxa2 (red) in the neuroblastoma cells in the presence of the aforementioned treatments. The treatment with EGF promotes the Annexin A2 phosphorylation and its further translocation into the lysosomes, suggested by the enhanced co-localization of p-Anxa2 and LAMP2A (yellow signal) in the immunofluorescence double staining (Figure 2A). This data suggests that Anxa2 is a substrate for the proteolytic degradation via CMA.

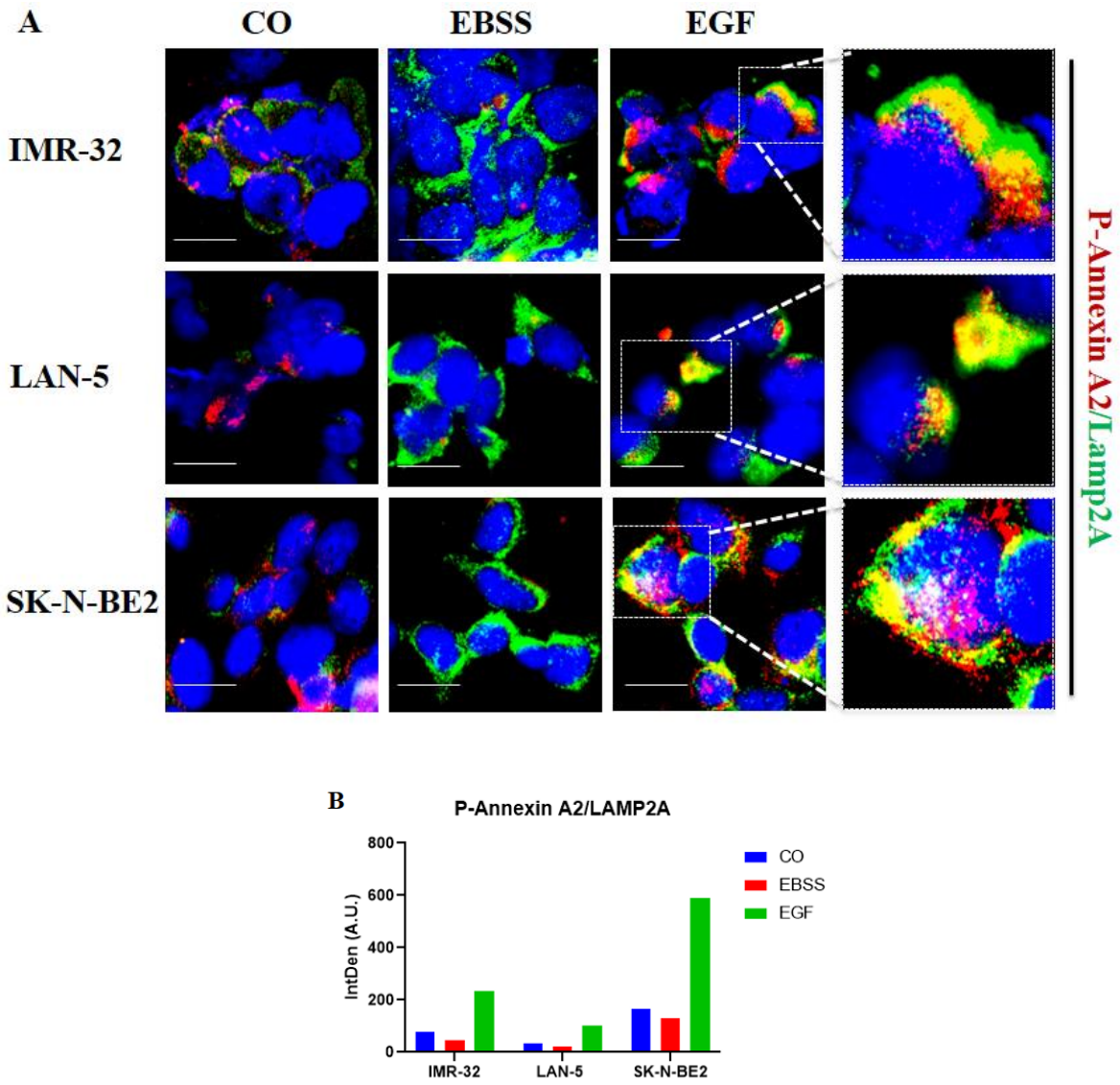
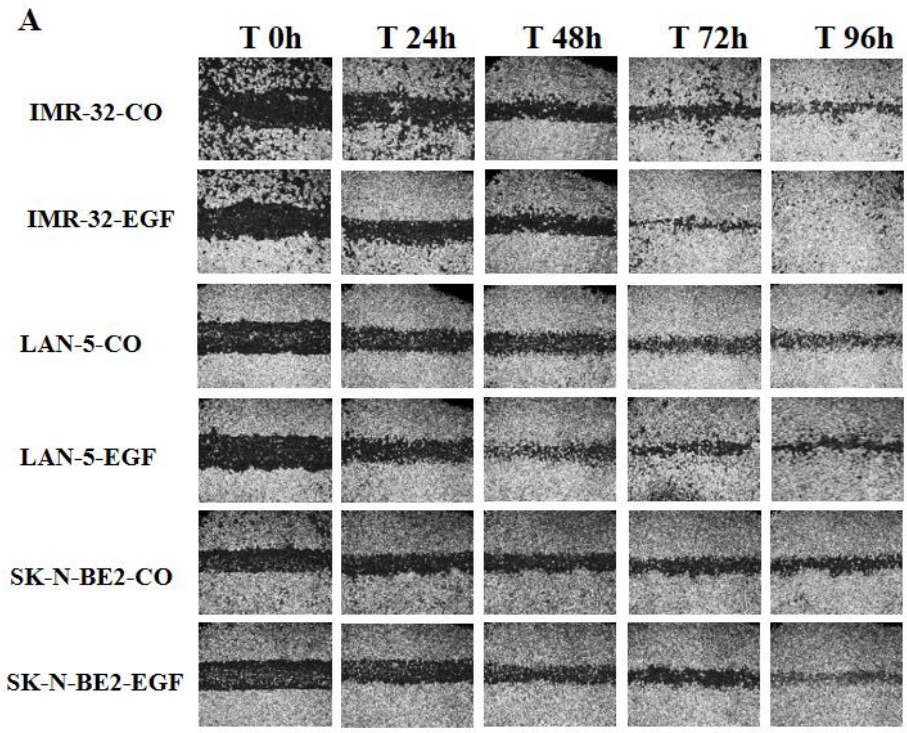


Figure 2. Immunofluorescence double stained for phosphorylated annexin A2 (red) and LAMP2A (green) interaction after 45 min treatment. (B) Graph represents the co-localization of P-Annexin A2/LAMP2A (yellow signal). Scale bar = 25 µm; magnification = 63X. Representative images of different fields for each experimental condition were shown.

4.3 Low levels of Annexin A2 contrasts EGF-induced NB cell migration

Recently, our works have elucidated the proliferative potential of neuroblastoma cells in relation to the lysosomal proteolytic enzyme Cathepsin D (CD) (Secomandi *et al.*, 2022, 2024). To investigate the motility and migration of NB cell lines with different levels of CD and Annexin A2 we performed a wound healing assay. It revealed that IMR-32 with high levels of Anxa2 and low levels of cathepsin D migrated more by representing fastest closure of the

wound as compared to elevated CD and lower Anxa2 expressing LAN-5 cells (Figure 3A). This indicates that Anxa2 expression with low CD shows a positive correlation with NB cells migratory ability. Moreover, IMR-32 cells with low CD profile when treated with EGF exhibited higher migration rate as compared to LAN-5 and SK-N-BE2 cells with high CD profiles (Figure 3B). In line with our previous studies, EGF stimulation had the least impact on LAN-5 cells migration which possess high levels of CD in comparison to IMR-32 and SK-N-BE2.



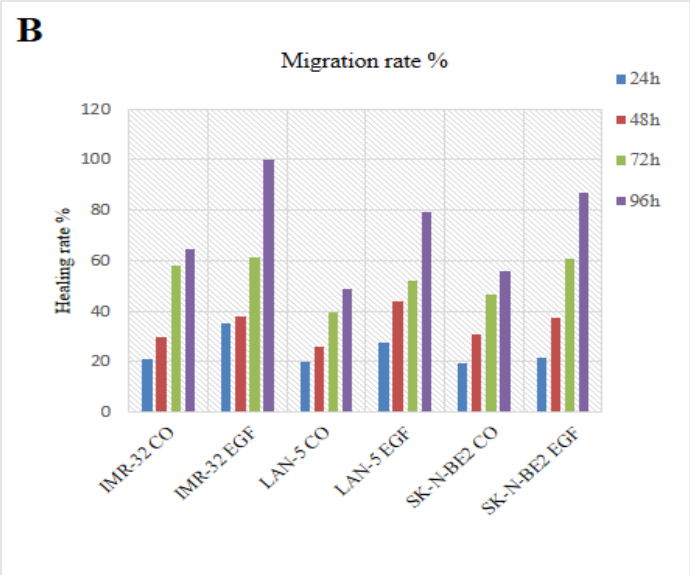


Figure 3. EGF induced the migration of IMR-32, LAN-5 and SK-N-BE2 cells. Assessment of wound healing rate was performed with 20 ng/mL EGF treatment. (A) The figure shows a graphical representation of cell migration rate. The treatment was repeated every 24h, until the end point of 96h. Time zero refers to the first day of scratching and treatment. Magnification = 5X. Representative images of wound healing for each experimental condition were shown.

5. DISCUSSION

Annexin A2 (Anxa2) is associated with a wide spectrum of solid tumors and hematological malignancies where its upregulated expression is correlated with migration, invasion, and progression. Particularly, in neuroblastomas Anxa2 overexpression has been liked to multi-drug resistance (Wang et al., 2017). Neuroblastoma (NB) is an embryonal tumor that develops from the sympathetic central nervous system. Also, the epidermal growth factor receptor (EGFR) has been shown to overexpress in the NB tissues with a dysregulated signalling in multi-drug resistant NB cell lines (Meyers *et al.*, 1988; Tamura *et al.*, 2007). However, the role of EGFR stimulation or deprivation is not well understood in regulating the Anxa2 protein levels and the chaperone-mediated autophagy (CMA) in the NB cells.

Our current study elucidates that in response to the EGF stimulation, Anxa2 levels increase as depicted by the fluorescence staining in figure 1A, whereas the starvation stimuli enhance the chaperone HSC70 in the neuroblastoma cells. Particularly, we also found that LAN-5 has the lowest levels of Anxa2 amongst the NB cell models. Since autophagy is a lysosomal recycling pathway for the cytosolic content which takes charge under the stress situations such as

starvation to maintain homeostasis, our data confirmed a significant increase in the cellular expression of vital HSC70 protein indicating towards the autophagic induction.

CMA is the selective removal of the damaged proteins through the identification of the KFERQ-motifs present in their amino acid sequences (Kaushik and Cuervo, 2018). Notably, our data further confirms that the cytosolic activated Anxa2, particularly under EGF stimulus, interacts with the CMA-specific lysosomal marker LAMP2A, highlighting the translocation of recognized pentapeptide substrate HSC70-Anxa2 to the lysosomes.

Recent works from our group further confirmed through an in-silico transcriptome study that neuroblastoma patients with high EGFR and high proteolytic enzyme Cathepsin D (CD) levels had a better prognosis than patients with high EGFR and low CD (Secomandi *et al.*, 2022). Moreover, preclinical in-vitro and in-vivo studies consistently reveal that diet modifiers that lower calorie intake have an influence on the tumor microenvironment and cancer metabolism, resulting in reduced cancer development and progression (Vidoni *et al.*, 2021). Taken all under consideration, our current data suggest that CD may be involved proteolytic process of annexin A2 in the neuroblastoma cells, caloric restriction mimetics such as resveratrol may present a therapeutic intervention that could accelerate the CD-mediated targeting of the oncogenic Anxa2, thereby playing a key role in improvising the neuroblastoma prognosis and holding back its progression. Study has been reported that calorie restriction improves chaperone-mediated autophagy through stabilize lysosomal membrane LAMP2A receptor (Jafari *et al.*, 2024).

Overall, our finding suggests that presence of KFERQ-like motif within target protein and binding with HSC70 and LAMP-2A could be applied to disrupt the cellular and molecular function of oncogenic proteins. This could be achieved through enhance the transcription of substrate recognizer (HSC70) and LAMP-2A proteins and initiation of CMA through caloric restriction.

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In the spirit of hope and freedom, I am reminded of a powerful quote by Nelson Mandela: ‘‘May your choices reflect your hopes, not your fears.’’

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