Delineating the Molecular Mechanism Behind the Role of HuR in Cell Death and Drug Resistance

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ABSTRACT

Apoptosis plays an essential role in the development and maintenance of tissue hemostasis. However, failure to undergo apoptosis is thought to represent the key to the development of several human diseases including cancers. The RNA-binding protein HuR (human antigen R) plays an important role in apoptosis and in carcinogenesis, as well as other cellular processes, including proliferation, and cell differentiation. We previously showed that HuR is required for both pro-survival and pro-apoptotic pathways, where the caspase-mediated cleavage of HuR determines the fate of the cell that is favored. Other posttranslational modifications such as phosphorylation and methylation have been shown to regulate the function of HuR. Recently, PARylation of HuR by PAR polymerase enzyme-1 (PARP1) was also shown to regulate the function of HuR during inflammation. However, the regulatory mechanism(s) of the pro-apoptotic function of HuR and the involvement of posttranslational modifications such as PARylation in this process is still elusive. In the first part of this thesis, I have identified PARylation as a regulatory mechanism that modulates the function of HuR in determining cell fate. My results showed that PARP1/2-mediated PARylation prevents the accumulation of HuR in the cytoplasm, subsequently resulting in a decrease in its cleavage, thereby inhibiting the pro-apoptotic function of HuR. I demonstrated that the combined depletion of PARP1 and PARP2 increases the cytoplasmic accumulation of HuR and thus increases its cleavage. HuR cleavage, consequently, increases its pro-apoptotic function as evidenced by the significant increase in the level of caspase-3 cleavage and in the number of apoptotic cells. Furthermore, I showed that the polymers of ADP-ribose (PAR), which is the product from PARPs' catalytic activity, binds HuR non-covalently through a consensus motif and that this binding is required for the nuclear localization of HuR as well as its association with the import factor Transpotin-2 (TRN2). Indeed, mutating the HuR PAR-binding site (HuR-PBS)

prevented PAR from binding to HuR, resulting in the cytoplasmic accumulation of HuR, and therefore advancing apoptosis. Thus, this work provides evidence for the importance of the PARP-mediated PARylation and the resulting PAR binding to HuR in regulating the function of HuR during apoptosis.

For decades, chemotherapeutic drugs have been shown to induce apoptosis in several cancer cells and tumors, yet many cells conferred multidrug resistance (MDR) which represents a major obstacle in cancer treatment, usually associated with resistance to apoptosis. Several studies associated HuR with the development of chemotherapeutic resistance in a variety of tumors. However, the mechanism in which HuR affects drug resistance in cancer cells and mediates *MDR1* mRNA expression, in particular, is not fully understood. In the second part of this thesis, using KB human cervical adenocarcinoma cells, I established the importance of HuR in the regulation of *MDR1* mRNA expression. I showed that HuR knockdown decreased the expression of *MDR1* mRNA and protein in the drug resistant KB-V1 cells. This effect, interestingly, is not due to a change in HuR expression nor a change in HuR cellular localization. Additionally, I showed that HuR binds an ARE in *MDR1* mRNA in drug resistant KB-V1 cells. Together, this work reveals a new role for PARylation in cell fate determination and implicates the non-covalent interaction of PAR and HuR as an important regulatory process required for the regulation of the pro-apoptotic function of HuR. Additionally, this work provides further insight on the HuR-mediated regulation of the *MDR1* mRNA thus linking HuR to the resistance of cells to drug treatment.

RÉSUMÉ

L'apoptose joue un rôle essentiel dans le développement et le maintien de l'hémostase des tissus. Cependant, on pense que la défaillance de l'apoptose représente la cause principale du développement de plusieurs maladies humaines, y compris les cancers. La protéine liant l'ARN HuR (human antigen R) joue un rôle important dans l'apoptose et la cancérogenèse, ainsi que dans d'autres processus cellulaires, notamment la prolifération et la différenciation cellulaire. Nous avons montré précédemment que HuR est nécessaire pour les voies pro-survie et pro-apoptotiques, où le clivage de HuR par la caspase détermine le destin de la cellule qui est favorisée. D'autres modifications post-traductionnelles telles que la phosphorylation et la méthylation se sont avérées réguler la fonction de HuR. Récemment, il a également été démontré que la PARylation de HuR par PAR polymerase enzyme-1 (PAPR1) régule la fonction de HuR pendant l'inflammation. Cependant, le(s) mécanisme(s) de régulation de la fonction pro-apoptotique de HuR et l'implication des modifications post-traductionnelles telles que la PARylation dans ce processus restent encore à élucider. Dans la première partie de cette thèse, j'ai identifié la PARylation comme un mécanisme régulateur qui module la fonction de HuR dans la détermination du destin cellulaire. Mes résultats ont montré que la PARylation médiée par PARP1/2 empêche l'accumulation de HuR dans le cytoplasme, entraînant par la suite une diminution de son clivage, inhibant ainsi la fonction pro-apoptotique de HuR. J'ai démontré que la déplétion combinée de PARP1 et PARP2 augmente l'accumulation cytoplasmique de HuR et donc son clivage. Le clivage de HuR augmente donc sa fonction pro-apoptotique, comme le montre l'augmentation significative du niveau de clivage de la caspase-3 et du nombre de cellules apoptotiques. En outre, j'ai montré que les polymères d'ADP-ribose (PAR), qui sont le produit de l'activité catalytique des PARPs, lient HuR de manière non covalente par l'intermédiaire d'un motif consensus et que cette liaison est nécessaire à la

localisation nucléaire de HuR ainsi qu'à son association avec le facteur d'importation Transpotin-2 (TRN2). En effet, la mutation du site de liaison PAR de HuR (HuR-PBS) a empêché PAR de se lier à HuR, ce qui a entraîné l'accumulation cytoplasmique de HuR et, par conséquent, l'avancement de l'apoptose. Ces résultats prouvent donc l'importance de la PARylation médiée par PARP et de la liaison de PAR qui en résulte à HuR pour réguler la fonction de HuR pendant l'apoptose.

Depuis des décennies, il a été démontré que les médicaments chimiothérapeutiques induisent l'apoptose dans plusieurs cellules cancéreuses et tumeurs, mais de nombreuses cellules présentent une résistance à multiples médicaments, ce qui représente un obstacle majeur dans le traitement du cancer, généralement associé à une résistance à l'apoptose. Cette résistance est médiée par un facteur codé par l'ARNm de la résistance multidrogue (MDR) 1. Plusieurs études ont associé HuR au développement de la résistance aux chimiothérapies dans une variété de tumeurs. Cependant, le mécanisme par lequel HuR affecte la résistance aux médicaments dans les cellules cancéreuses, et en particulier l'expression de l'ARNm *MDR1*, n'est pas entièrement compris. Dans la deuxième partie de cette thèse, en utilisant des cellules d'adénocarcinome cervical humain KB, j'ai établi l'importance de HuR dans la régulation de l'expression de l'ARNm *MDR1*. J'ai montré que le knockdown de HuR diminue l'expression de l'ARNm et de la protéine MDR1 dans les cellules KB-V1 résistantes aux médicaments. Il est intéressant de noter que cet effet n'est pas dû à un changement de l'expression de HuR ni à un changement de la localisation cellulaire de HuR. En outre, j'ai montré que HuR se lie à un élément riche en AU (nommé ARE) dans l'ARNm *MDR1* dans les cellules KB-V1 résistantes aux médicaments. L'ensemble de ces travaux révèle un nouveau rôle pour la PARylation dans la détermination du destin cellulaire et implique l'interaction non covalente de PAR et HuR comme un processus de régulation nécessaire à la régulation de la

fonction pro-apoptotique de la HuR. De plus, ces travaux apportent un aperçu supplémentaire sur la régulation de l'ARNm *MDR1* médiée par HuR, ce qui permet d'établir un lien entre HuR et la résistance des cellules aux traitements médicamenteux.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

Chapter I: PARP-mediated PARylation is a key mediator of HuR function during apoptosis.

- PARP1 and PARP2 regulate the cytoplasmic localization, cleavage, and pro-apoptotic function of HuR.
- PAR binds HuR non-covalently through a consensus motif.
- PAR binding prevents the pro-apoptotic function of HuR by promoting its nuclear localization.
- This work reveals a new role for PARylation in cell fate and implicates the non-covalent interaction of PAR and HuR as an important regulatory process required for the regulation of the pro-apoptotic function of HuR.

Chapter II: HuR mediates the post-transcriptional regulation of Multidrug resistance (MDR).

- HuR mediates the expression of *MDR1* mRNA and protein in drug resistant KB-V1 cells.
- The effect of HuR on the expression of MDR1 mRNA in KB-V1 cells is not due to a change in HuR expression nor a change in HuR cellular localization.
- HuR binds MDR1 mRNA in drug resistant KB-V1 cells.
- This work represents the first key milestone in our understanding of the regulatory mechanism underlying the role of HuR in MDR.

CONTRIBUTION OF AUTHORS

In chapter I, KA contributed to conceptualization, conducted the investigation and validation of experimental findings, wrote the original draft, and performed the formal analysis and visualization of experimental findings. **DTH** and **SM** contributed to the conceptualization and conducted the preliminary investigation and validation of experimental findings. **XJL and SB** helped by performing some of the western blot and qPCR experiments. **SDM** assisted with conceptualization, data analysis, and helped edit and review the manuscript. **JPG** and **GGP** performed the *in vitro* PAR binding assays and helped with the analysis of the data. **DYT** provided the technical and experimental expertise with the in vitro peptide mapping experiment and helped with data analysis and interpretation. **IEG** conceptualized, established, and directed the execution of the project, interpreted the data, reviewed, and edited the manuscript.

In chapter II, Dr. Isabelle Menard performed the work shown in (Figure 9A) and (Figure 10B and C). Kholoud Ashour performed all other work. To be submitted shortly.

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LIST OF ABBREVIATIONS

ABC ATP Binding Cassette

ActD Actinomycin D

AIF Apoptosis-inducing factor

Apaf1 apoptotic protease activating factor

ARE A/U-rich element

ATP Adenosine Triphosphate

Bax Bcl-2 associated X

Bcl-2 B-cell lymphoma protein 2

CARM1 coactivator-associated arginine methyltransferase 1

Caspase Cystenyl aspartic acid-protease

CRM1 chromosome region maintenance 1

Cyt c Cytochrome c

Dapi 4',6-diamidino-2-phenylindole

DISC Death-inducing signaling complex

DNA deoxyribonucleic acid

ELAV-1 Embryonic-lethal abnormal vision 1

Epi Epirubicin

FADD Fas-associated protein with death domain

G3BP Ras-GTPase-activating protein (SH3)-binding protein

GFP Green fluorescent protein

GST Glutathione S-transferase

HeLa Henrietta Lacks

HnRNP Heterogeneous nuclear ribonucleoproteins

HNS HuR nucleocytoplasmic shuttling

HuB Human antigen B

HuC Human antigen C

HuD Human antigen D

HuR Human antigen R

HuR-CP HuR cleavage product

lngRNA long non-coding RNA

LPS lipopolysaccharide

MARylation mono (ADP-ribosyl) ation

miRNA microRNA

mRNA messenger RNA

NLS Nuclear localisation signal

pADPr poly (ADP-ribose)

PARP Poly (ADP-ribose) polymerase

PARG poly (ADP-ribose) glycohydrolase

PARylation poly (ADP-ribosyl) ation

PBM PAR-binding motif

PBmt PAR binding mutant

PBS PAR-binding site

P-gp P-glycoprotein

PHAPI Putative HLA-associated protein-I

PKR protein kinase RNA

ProT Prothymosin α

RBD RNA-binding domain

RBP RNA-binding protein

RNA ribonucleic acid

RRM RNA recognition motif

STS Staurosporine

TRN2 Transportin 2

UTR untranslated region

WT wild type

ZnFs zinc finger domaines

INTRODUCTION:

1. Cell Death

Cell death is a fundamental event in all the domains of the life of a multicellular organism. The balance between cell death and cell survival plays a crucial role in regulating embryonic development, maintaining tissue homeostasis, and eliminating damaged as well as potentially harmful cells [1-3]. Disruption of cell death mechanisms lead to a variety of diseases, such as cancer. Over the last two decades, there have been various cell death pathways delineated in the biomedical literature [4]. Among these, there are three major forms of cell death that are widely known and classified by their cellular appearance: apoptosis (also known as type I cell death), autophagic cell death (type II), and necrosis (type III) (Figure 1.1) [5, 6]. Apoptosis, often referred to as "cell suicide", is the most studied class of these various cell death pathways. It can be triggered both internally (though the mitochondrial pathway) or externally (due to the activation of cell death receptors), as described below, and both routes are intricately regulated [7]. Apoptosis is characterized by cell shrinkage, membrane blebbing with the formation of apoptotic bodies (small intact vesicles), nuclear fragmentation (karyorrhexis), chromatin condensation (pyknosis) and chromosomal DNA fragmentation [3, 6]. Autophagic cell death, however, is characterized by the activation of the autophagy machinery and the formation of the autophagosome which is a large, double membraned, intercellular vesicle. In fact, the autophagic activity itself has been argued to act mainly as a natural, conserved survival promotor where it degrades and removes unwanted components through a regulated lysosome-dependent mechanism [5, 6]. Necrosis, in the other hand, occurs when a cell is severely damaged due to an external factor including but not limited to trauma, toxins and infections. Necrosis is characterized by cell swelling, uncontrolled

membrane rupture with cell content being expelled which often damage nearby cells, subsequently triggering inflammation [5, 8].

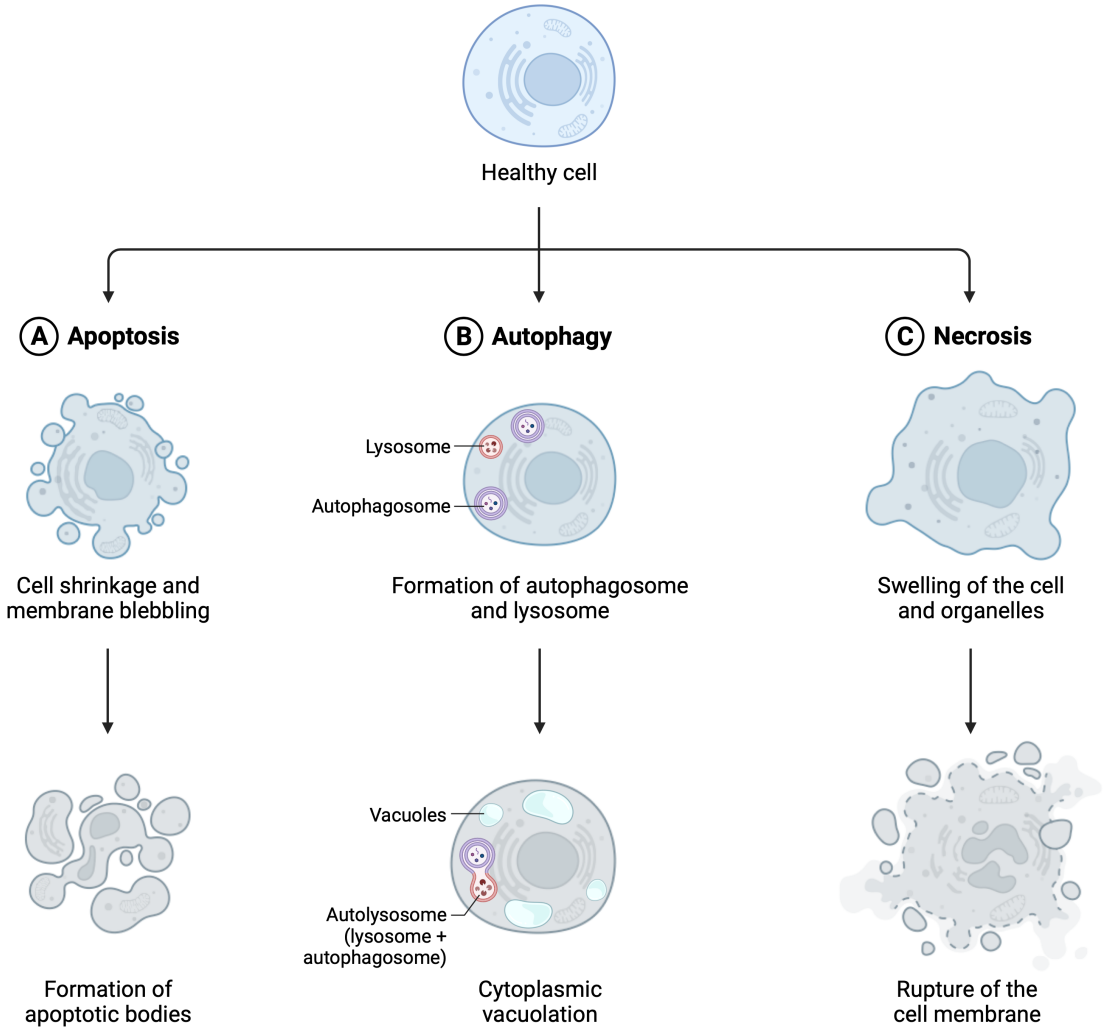

Figure 1.1: The three major forms of cell death.

A) Apoptosis is characterized by cell shrinkage, membrane blebbing with the formation of apoptotic bodies and others. B) Autophagy is characterized by the formation of the autophagosome which degrades and removes unwanted components through a regulated lysosome-dependent mechanism. C) Necrosis is characterized by cell swelling, uncontrolled membrane rupture [5, 8]. Created with BioRender.com (MD25S7G2L2)

1.1 Apoptosis

Apoptotic cell death or apoptosis is defined as a programmed cell death since it is not a spontaneous event. In fact, the process of apoptosis starts with a trigger that originates from a diversity of sources ranging from chemical activators, endocrine signalling from neighbouring cells, or internal sensors of cell damage [9]. The action and efficiency of the apoptotic mechanism is tightly regulated through highly complex, energy-dependent cascading events that involve the activation of a family of proteases known as caspases [9].

1.1.1 Caspases

Caspases are a family of cysteine-dependent, aspartate-specific proteases that cleave protein substrates after an aspartate residue, leading to their inactivation and, subsequently, resulting in cell death [10]. Generally, caspases exist in cells as inactive zymogens called pro-caspases [10]. In human, 12 members of the caspase family have been identified, all of which contain a structurally similar catalytic domain [5, 10]. To date, these enzymes have been found to play a key role in not only driving the cell to apoptosis but also in inflammation and cell differentiation. Members of caspases that have been known to play a role in apoptosis are subcategorized by their mechanism of action as initiator (Caspase-2, -8, -9, and -10), or executioner (Caspase-3, -6, and -7) caspases. Once a member of the initiator caspases is activated in response to an apoptotic stimulus, it can lead to the sequential activation of other caspases in a process commonly known as the “caspase cascade” [3, 10]. While initiator caspases exist as inactive monomers that are activated by their dimerization with adaptor protein, executioner caspases are produced as inactive dimers and they are activated through proteolytic cleavage by the active initiator enzymes, leading to the degradation of many protein substrates [5, 10-12]. Typically, the

activation of the caspase-dependent apoptosis cascade can be achieved through two main pathways: intrinsic and extrinsic (Figure 1.2) [10, 11].

1.1.1.1 Intrinsic activation of caspases

In response to an internal stimulus such as DNA damage, the intrinsic pathway is initiated by the release of cytochrome c (Cyt c) from the mitochondria into the cytoplasm where it contributes to the formation of the apoptosome. The apoptosome is a large complex comprised of several proteins including Apaf1(apoptotic protease activating factor 1) and procaspase-9 as well as Cyt c [13]. As a consequence of its recruitment to the complex procaspase-9 dimerizes, leading to its activation. Once caspase-9 is activated, it proceeds to the cleavage and activation of other executioner caspases including procaspase-3 and -7, which in turn target and cleave important functional protein substrates such as Poly(ADP-ribose) polymerase (PARP), thus advancing apoptosis [14, 15].

1.1.1.2 Extrinsic activation of caspases

The extrinsic signaling pathway is triggered by the interaction of a transmembrane receptor with an extracellular ligand, also known as a death receptor and death ligand, respectively. These death receptors include members of the Tumour Necrosis Factor (TNF) receptor family such as Fas and TNF α receptors whose binding to their respective ligand is achieved through the death domain (DD) [9, 16]. For example, the binding of the Fas ligand to the Fas receptor results in the recruitment of the adaptor protein Fas-associated protein with death domain (FADD) to the receptor leading to its activation. FADD, in turn, is capable of binding monomeric procaspases such as procaspase-8, leading to the formation of the death-inducing signaling complex (DISC) and thereby the autocatalytic activation of caspase-8.

The Fas ligand, Fas receptor, FADD and procaspase-8 axis is the best-known model used to describe the mechanism of action of extrinsic-induced apoptosis [9, 16]. However, there are evidence showing that caspase 8, in particular, is capable of initiating apoptosis by not only targeting and cleaving executioner caspases such as caspase-3 but by also targeting pro-apoptotic proteins such as Bid. Bid is a member of the Bcl-2 family that is an important promoter of the release of Cyt c from the mitochondria, leading to apoptosome formation and subsequently the activation of the intrinsic pathway [17]. This demonstrates an interplay between the extrinsic and intrinsic pathway and that molecules in one pathway can influence the other [9].

1.1.1.3 Protein regulator of apoptosis

The regulation of apoptosis involves a number of proteins that can directly or indirectly inhibit or activate caspases and other pro- and anti-apoptotic players during the apoptotic process. Members of the Bcl-2 superfamily are well-known regulators of mitochondrial-induced apoptosis. They are subcategorized based on their function into (1) pro-apoptotic proteins (e.g., Bad, Bid, Bax) which mediate the release of Cyt c from mitochondria, and (2) anti-apoptotic proteins (e.g., Bcl-2, Bcl-x_L) which inhibit this release of Cyt c. All Bcl-2 family proteins contain a BH3 domain that is necessary for regulating the release of Cyt c through the mitochondrial outer membrane permeabilization (MOMP) [9, 18, 19]. Once Cyt c is in the cytoplasm and the apoptosome complex is formed, caspase-9 is activated, leading to the activation of downstream caspases such as caspase-3 and -7 [9, 18, 19]. Interestingly, the activity of these caspases is subjected to inhibition by members of Inhibitor of apoptosis proteins (IAPs) such as XIAP and Survivin [9, 20].

Several proteins have also been identified as regulators of the apoptotic process at the apoptosome level by either increasing or inhibiting its activity, such as PHAPI/PP32 and

Prothymocin α (ProTα), respectively [17, 21]. The role of these proteins in the apoptotic machinery will be further discussed in following sections.

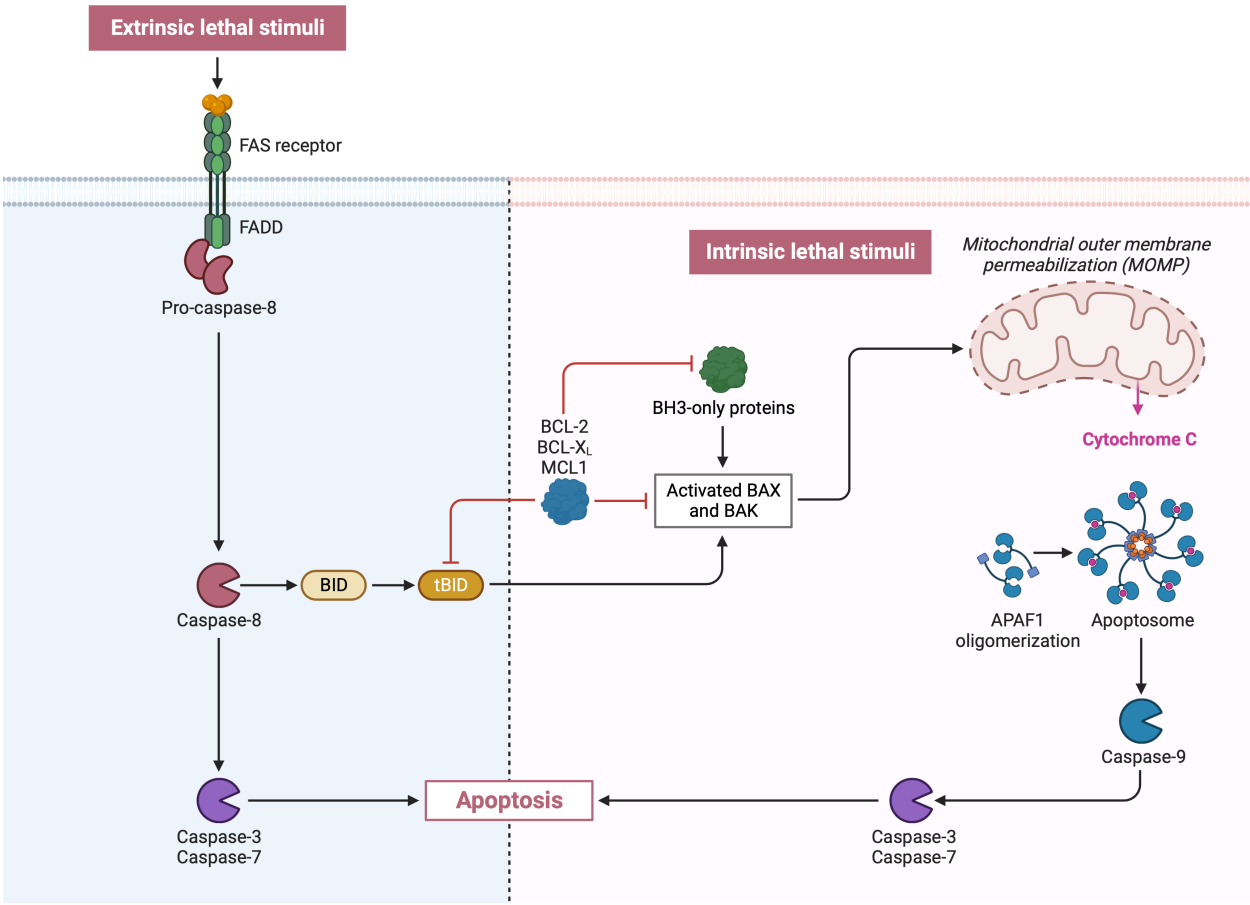

Figure 1.2: Extrinsic and intrinsic pathways of apoptosis.

As described above, apoptosis may be engaged extrinsically or intrinsically. In the intrinsic pathway, triggered by an internal stimulus, cytochrome c (Cyt c) is released from the mitochondria into the cytoplasm where it contributes to the formation of the apoptosome. The apoptosome complex composed of Apaf1, and pro-caspase-9 in addition to Cyt c. Consequently, caspase-9 is activated and proceeds to activate caspase-3 and -7, which target and cleaves many protein substrates, thus advancing apoptosis. The extrinsic pathway involves transmembrane receptor-mediated interaction. The binding of death ligand to death receptor (such as FAS) results in the recruitment of FADD (adaptor protein) which activates caspase-8. In turn, caspase-8 initiates apoptosis by targeting and activating caspase-3 and -7 or by targeting pro-apoptotic member of Bcl-2 family proteins. The latter are responsible for regulating the release of Cyt c from the mitochondria leading to the activation of the intrinsic pathway [9].

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1.1.2 Apoptosis in disease and cancer

While the apoptotic machinery is highly regulated by a variety of apoptotic players, an alteration in its activity contributes to a wide variety of human diseases ranging from neurodegenerative disorders to malignancies [22]. For example, an excess in the activity of caspases has been linked to Alzheimer's disease [23]. Additionally, the most common example of disease linked to an inactivation of apoptosis is cancer. For decades, chemotherapeutic drugs have been shown to induce apoptosis in several cancer cells and tumors, yet, many cells conferred a multidrug resistant (MDR) phenotype which is usually associated with resistance to apoptosis [24].

1.1.2.1 Multidrug resistance

MDR of cancer cells represent a major obstacle in cancer treatment. Resistance to drugs can be activated in response to both intrinsic and acquired mechanisms. While intrinsic mechanisms occur due to pre-existing factors, such as inherited genetic alterations or changes in the tumor microenvironment (TME), an acquired resistance can be induced by chronic exposure to these drugs [25]. Cancer cells treated with a single anticancer drug tend to develop cross-resistance to a wide variety of chemotherapeutic drugs to which they have never been exposed and with no obvious structural or functional similarities [25-27]. There are multiple mechanisms discussed in the literature that contribute to the development of clinical MDR.

1.1.2.1.1 Molecular mechanism of MDR

To date, the best studied mechanism of MDR is the reduction of drug accumulation that can be mainly achieved by increased cellular drug efflux or reduced cellular drug uptake. In general, drugs that are hydrophobic such as natural products vinblastine, vincristine, doxorubicin, daunorubicin, actinomycin D, etoposide, tenoposide, and paclitaxel enter cells through passive diffusion across the plasma membrane. However, water-soluble hydrophilic drugs such as

cisplatin, nucleoside analogues, and antifolates are unable to enter by passive diffusion and therefore require the help of specific transporters to enter through hydrophilic channels in the membrane [27].

Increased cellular drug efflux is mainly mediated by the presence of an efflux transporter pump known as P-glycoprotein (P-gp), a well characterized member of the ATP binding cassette (ABC) of transporters superfamily. The activity of this transporter reduces the efficacy of trans-cellular diffusion of drugs by promoting their efflux from the cells [25-27].

Another important mechanism that leads to MDR is the inhibition of the apoptosis signaling pathway by the induction of antiapoptotic molecules such as Bid and Bad, which are members of the Bcl-2 family of proteins that regulate the release of Cyt c from the mitochondria [28].

1.1.2.1.2 P-glycoprotein

P-glycoprotein is an adenosine triphosphate (ATP) binding cassette transporter (ABCB1) encoded by the multidrug resistance protein-1 (*MDR1*) gene. It is a membrane-bound protein of a molecular weight of approximately 170 kDa. P-gp is an efflux transporter pump, and similar to all ATP binding cassette transporter, it utilises ATP to actively transport substances out of the cell. It is expressed with basal ATPase activity in numerous normal cells such as the liver, kidneys, gastrointestinal track, testis, as well as the blood brain barrier (BBB), where it controls substances' absorption, distribution and elimination in the body, and performs protective and detoxifying activities [29-31]. Interestingly, it is found to be overexpressed in cancerous cells, where it functions as an efflux pump to a wide range of anticancer drugs and amphipathic compounds that are structurally and functionally varied. As such, P-gp reduces the efficacy of these compounds and confers therapeutic resistance by expelling them out of the cell and away from their target site [30, 32]. Structurally, P-gp consists of two homologous halves connected by a linker domain,

where each half consists of an N-terminal transmembrane domain (TMD) spanning six transmembrane (TM) α-helix segments, followed by a cytoplasmic nucleotide binding domain (NBD) (Figure 1.3). While the TMD is responsible for substrate binding and translocation, the NBD serves as the functional unit and is required for ATP binding and hydrolysis [26, 31]. Some studies have suggested that the NBD contains several conserved domains. However, further studies are needed to elucidate how P-gp could recognize a vast number of different compounds. There is also a controversial hypothesis surrounding the exact mechanism of action and the order of the steps leading to drug execution. The general mechanism of action accepted is that upon substrate binding, ATP hydrolysis by the cytosolic NBD derives a change in the conformation of P-gp structure from inward-facing (drug recognition and binding) to outward-facing (drug release) allowing substrate movement to the TMD and subsequently, its excretion out of the cell [33].

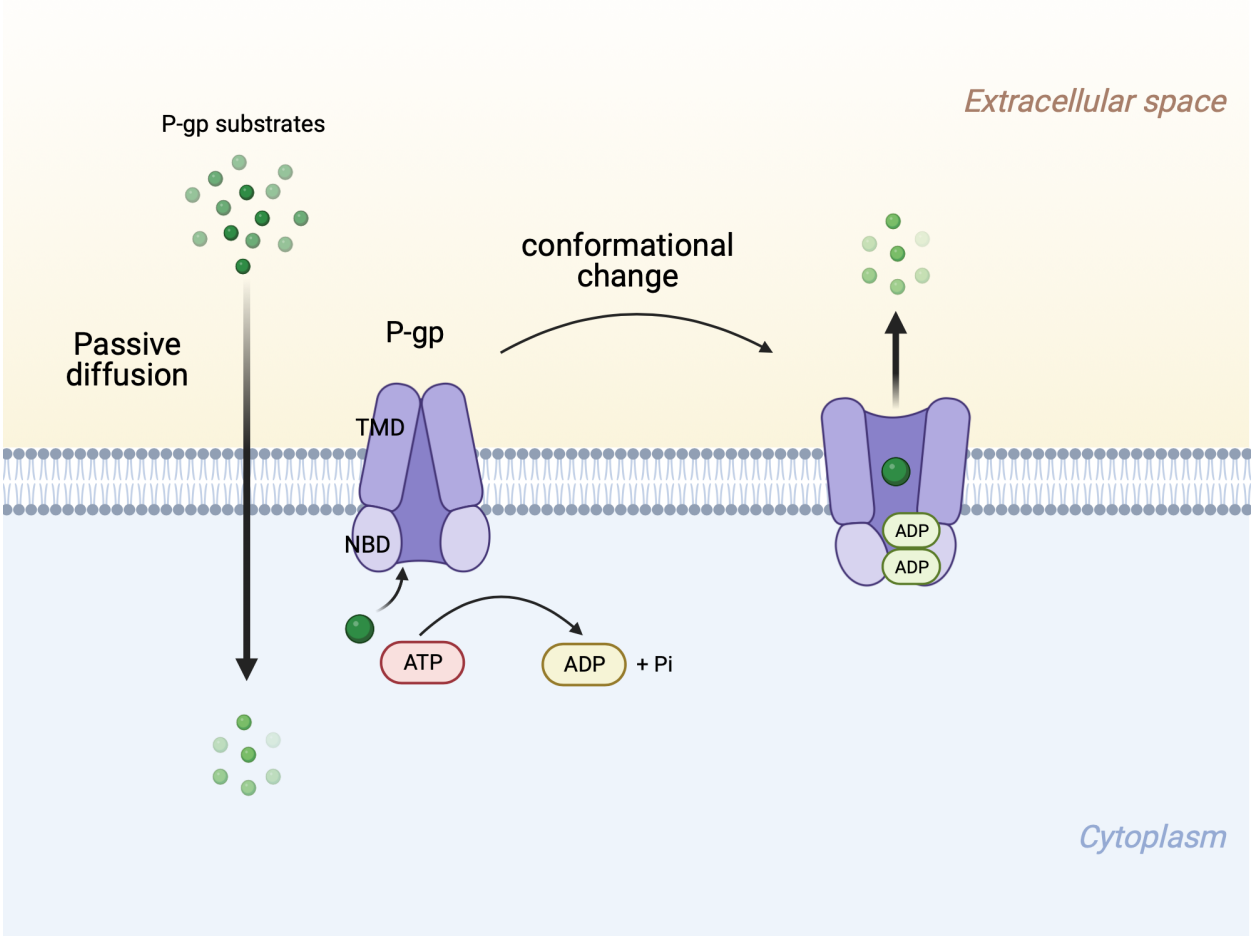

Figure 1.3: Model of P-glycoprotein transport.

P-glycoprotein (P-gp) is a membrane-bound protein and a member of ATP binding cassette transporter (ABCB1). It utilises ATP to actively transport substances out of the cell [29-31]. P-gp consists of two homologous halves connected by a linker domain. Each half consists of an N-terminal transmembrane domain (TMD) and a cytoplasmic nucleotide binding domain (NBD). Upon substrate binding, ATP hydrolysis by the cytosolic NBD derives a change in the conformation of P-gp structure from inward-facing (drug recognition and binding) to outward-facing (drug release) allowing substrate movement to the TMD and subsequently, its excretion out of the cell [33].

Adapted from [34].

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1.1.2.1.3 MDR regulation in cancer

P-gp has been reported to be highly expressed in patients with many blood malignancies, including lymphoblastic leukemia and acute myeloid leukemia, and in many solid tumors such as breast cancer, gastric cancer, small cell lung cancer, ovarian cancer, and many others [35]. Overexpression of P-gp in all cancer patients has been associated with poor clinical response, chemoresistance and impaired apoptosis. Several studies reported that downregulating the expression of MDR1 gene has been shown to lower P-gp expression and therefore serves as a successful tool to combat MDR. In colorectal carcinoma cells, for instance, silencing *MDR1* mRNA using siRNA has been shown to re-sensitize the cells to chemotherapeutic drugs and contribute to the apoptotic induction of tyrosine kinase inhibitor, increasing its intracellular accumulation, and subsequently reduces tumor metastasis [36].

While the inhibition of P-gp expression successfully increases the bioavailability of susceptible drugs and can re-sensitize MDR cells to chemotherapeutic drugs *in vitro*, little to no survival benefits were found in clinical trials due to toxicity [27]. Additionally, although the knockout of P-gp in mice does not lead to a lethal phenotype, it leads to a large difference in drug pharmacodynamics compared to the wild-type counterparts. Therefore, intense efforts have been invested on elucidating the role of of P-gp and the underlying regulatory mechanisms of MDR1 in different cancer cell lines to identify a variety of potential strategies to overcome MDR and improve the efficacy of cancer treatment.

Many studies have developed different treatment strategies to overcome MDR. These include strategies that target the expression of P-gp (such as the use of siRNA to target *MDR1* mRNA) or strategies that prevent drug efflux (such as drug encapsulation to increase delivery of drugs in the cells).

A number of genes, miRNAs, lncRNAs, and proteins were identified as a MDR biomarkers (modulator), and studies revealed that upregulating, downregulating or overexpress the expression of these biomarkers alone or in combination with the apoptosis-induced drugs increase the efficacy of the drugs against MDR cancers, making them promising therapeutic strategies to reverse MDR [25]. Additionally, these studies successfully used these biomarkers to distinguish between drug-resistant and drug-sensitive cancer cells, enhancing the efficacy of chemotherapy [25]. For example, certain miRNA such as miRNA-27a and miRNA-451 were found to be differentially expressed in the ovarian cancer cell line and its MDR counterparts [37]. Moreover, it was demonstrated that the expression of these miRNAs is associated with the activation of *MDR*1/P-gp expression and contributes to drug resistance in cancer cells [37].

Additionally, a study on the RNA-binding protein HuR revealed that downregulating its expression impacted *MDR1* mRNA and consequently intensified the cytotoxic effect of epirubicin-induced drug resistance on colon cancer cells [38]. In this study, it was shown that siRNA-mediated HuR knockdown alone or in combination with Epirubicin (Epi) treatment cooperatively decreased the mRNA expressions of MDR transporter-related genes including *MDR1* mRNA and increased Epi-induced apoptotic. The efficacy of this effect was also increased by decreasing cellular mRNA levels of *Bcl-2*, and increasing *Bax* and *caspase-3 and -9* levels [38]. These findings and others provided insight into the importance of the role of HuR on regulating MDR1 expression, and subsequently, supressing MDR efflux transporter, apoptosis induction and circumventing MDR. The post-transcriptional role of HuR in regulating MDR1 expression will be further elaborated in the following sections.

2. The RNA binding protein HuR

The RNA-binding protein HuR (Human antigen R), also known as ELAV1, is a small, ubiquitously expressed protein. HuR, which was initially discovered in 1988 in *Drosophila melanogaster,* is one of four members of the Embryonic Lethal, Abnormal Vision (ELAV) family of RNA binding proteins (RBP). In contrast to HuR, the other three members, HuB, HuC, and HuD, are known to localize primarily in the brain and central nervous system. Structurally, HuR contains three highly conserved RNA binding domains (RBD) also known as RNA recognition motifs (RRM), and a hinge region between RRMs 2 and 3 that is termed the HuR Nucleocytoplasmic Shuttling (HNS) domain. While the HNS domain serves as a mediator for the shuttling of HuR between the nucleus and the cytoplasm, the RRM1 and RRM2 motifs are known to mediate the association of HuR with AU-rich elements (AREs) in the 3′-untranslated region (UTR) of its mRNA targets. RRM3, on the other hand, has been suggested to mediate the binding of HuR to U-rich sequences and to the poly(A) tail [39]. In addition, RRM3 has been shown to mediate protein-protein interactions and facilitate the oligomerization of HuR on target messages (Figure 1.4).

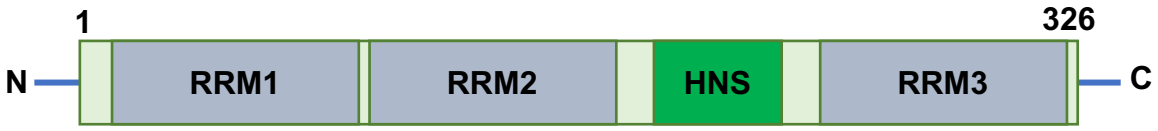

Figure 1.4: Schematic of the structure of HuR

HuR contains three RNA recognition motifs (RRMs) and a hing region (HNS) located between RRM1 and RRM2. RRM1 and RRM2 mediates binding to ARE-containing messages. RRM3 mediates binding to U-rich sequences, poly (A) tail, and facilitates protein-protein interaction and HuR oligomerization on target messages. HNS mediates HuR nucleocytoplasmic shuttling [39].

2.1 HuR functions

HuR is known to post-transcriptionally regulate gene expression at multiple levels. In the nucleus, HuR can bind to pre-mRNAs and influence the early processing events of these messages through alternative splicing as well as alternative polyadenylation mechanisms [40]. Whereas in the cytoplasm, HuR serves as a key regulator of other post-transcriptional events such as mRNA stabilization, nucleo-cytoplasmic localization, and translation [41]. One of the ways that HuR is able to mediate this array of functions is by competing or cooperating with other *trans*-acting factors. In fact, HuR was shown to complex with various RBPs such as KSRP, TTP, and YB-1 to promote the stability or the decay of various ARE-containing mRNAs [42-44]. As such, HuR can differentially influence the expression of many mRNA targets encoding proteins involved in several cellular pathways including cell proliferation, cell stress response, apoptosis, cell differentiation, senescence, as well as inflammation [44]. For example, HuR has been shown to compete with the KSRP-mediated decay and promote myogenesis in muscle cells by increasing the stability of many pro-myogenic messages such as *p21, MyoD*, and *myogenin* [42, 44]. Additionally, a recent study from our lab revealed that HuR regulates the stability of *c-Myc*, *MyoD,* and *myogenin* mRNAs by interacting with the RBP YB-1 [45].

HuR has been also shown to interact with a multitude of miRNAs to differentially regulate the expression of many ARE-containing mRNA targets. For example, a recent study from our lab demonstrated that HuR binds the *STAT3* mRNA 3'UTR and mediate its translation in muscle cells by preventing the recruitment of miR330-mediated STAT3 translation inhibition [46].

2.2 HuR role in cancer and Multidrug resistance

HuR is expressed in high abundance in a wide variety of cancers and is associated with high-grade malignancies and poor clinical prognosis in cancer patients [47]. Of particular interest,

several recent studies have shown that HuR plays a prominent role in mediating the development of chemotherapeutic resistance of tumors including pancreatic cancer [48, 49], colorectal cancer and many others [38, 50]. In fact, the silencing of HuR in human colorectal carcinoma cells led to their sensitization to epirubicin, which was associated with the inhibition of galectin-3/β-catenin signaling and the suppression of MDR transporters resulting in the activation of apoptosis [38]. Moreover, the same study showed that HuR silencing enhanced the epirubicin-induced apoptosis by decreasing the cellular level of *MDR1* mRNA indicating that HuR post-transcriptionally regulates MDR expression [38]. In addition, a recent study by Zhang *et al*. revealed that the lncRNA FENDRR countered Adriamycin resistance in chronic myelogenous leukemia cells by decreasing the expression of MDR1 through competitively binding to HuR and miR-184 [51]. This group also showed that HuR knockdown increased the stabilization of *MDR1* mRNA [51]. In spite of these observations, the mechanism through which HuR affects drug resistance in cancer cells and mediates *MDR1* mRNA stabilization, in particular, remains unexplored.

2.3 HuR role in cell fate

It is well known that HuR is required for both pro-survival and pro-apoptotic pathways [17, 18, 52]. We as well as others have demonstrated that, in response to stress, HuR initially binds and modulates the expression of various pro-survival messages such as *Bcl-2, Mcl-1, cyclin A, cyclin B1, cyclin D1, p21*, and *prothymosin α*. However, when the stress becomes severe, HuR shifts its function and modulates the expression of several pro-apoptotic factors including *c- myc, caspase-9, Bax, p53* and *p27* [18]. HuR's ability to differential shift from regulating pro-survival versus pro-apoptotic pathways is thus modulated by the severity of the stress. The mechanisms by which this occurs, however, remains to be elucidated.

2.3.1 Caspase mediated cleavage of HuR

Previous work from our lab showed that, in response to different apoptotic stimuli, HuR translocate to the cytoplasm, where it undergoes caspase-3 and -7 dependent cleavage at the aspartate (D)226 residue, thereby generating two HuR cleavage products (CPs), HuR-CP1 (24 kDa) and HuR-CP2 (8 kDa) [17, 18, 53, 54]. Importantly, although about 50% of the exported HuR is targeted for the cytoplasmic cleavage, our lab reported that this event represents the key element in HuR's functional switch from being an anti-apoptotic regulator during mild stress condition to a pro-apoptotic one under lethal conditions [18]. We also showed that HuR-CPs selectively binds and stabilizes the mRNA encoding the pro-apoptotic factor *caspase-9* but not the anti-apoptotic factor *prothymosin α* during the onset of apoptosis. The importance of HuR cleavage was demonstrated by the fact that, unlike wild-type HuR, a non-cleavable isoform of HuR (HuR_{D226A}) was not able to rescue apoptosis in a condition where the endogenous HuR was depleted [18]. Additionally, the fact that overexpression of HuR-CPs triggered cell death, in cells exposed to a mild stress, shed further light on the importance of HuR cleavage in promoting the pro-apoptotic function of HuR. Importantly, while several studies noted that HuR is overexpressed in many cancer cells, the fact that it is not cleaved in these cells further explains the preferential role of HuR in promoting cell survival in cancer patients [17, 55].

2.4 HuR regulation

2.4.1 Nuclear-cytoplasmic shuttling

HuR is a shuttling protein that translocates in and out the nucleus. Importantly, in addition to its ability to mediate the nuclear export of target messages, its functional impact on the post-transcriptional regulation of these transcripts is highly linked to the localization of HuR in the cytoplasm. Several studies have shown that the cellular movement of HuR is regulated through

the direct association of its HNS and RRM3 domains with adaptor proteins. While the nuclear export of HuR is mediated by its binding to export factors such as Putative HLA-associated protein-I (PHAPI/pp32), APRIL and CRM1; its nuclear import is mediated by its binding to transportin proteins such as Transportin-1 (TRN1) and Transportin-2 (TRN2) (Figure 1.5) [17, 18, 44, 53, 54, 56].

In addition, the localization of HuR was shown to be extensively regulated by post-translational modification, as will be discussed in the following sections [57-60].

2.4.2 Protein association

The pleiotropic and dichotomic function of HuR in many cellular processes including differentiation, apoptosis, and many others, was found to be regulated by protein-protein interactions. Previously published work from our lab identified HuR protein ligands, PHAPI and TRN2, as not only important regulators of HuR localization, but also modulators of its post-transcriptional function in response to various extracellular stimuli, including those able to trigger apoptosis. In 2008 we showed that, in response to lethal stress, PHAPI, a well-known activator of the apoptosome, binds to and colocalizes with HuR in the nucleus and further colocalize to the cytoplasm where both collaborate to activate the apoptotic pathway. This occurred due to the increased cleavage of HuR by caspase-3 and -7. We also showed that HuR-CP2 association with PHAPI is required for the PHAPI-mediated apoptosome formation and therefore, for promoting apoptosis. In fact, upon the induction of apoptosis, the non-cleavable isoform of HuR maintained its association with PHAPI and resulted in the decrease in HuR cleavage and subsequently, preventing PHAPI's pro-apoptotic activity [13, 17, 53].

Additionally, our lab showed that the TRN2-mediated import of HuR ensures the maintenance of HuR in the nucleus, while disrupting the association between HuR and TRN2

results in its cytoplasmic accumulation. Moreover, the disruption of this association in cells treated with STS blocks the nuclear import of full length HuR due the competitive binding of HuR-CP1 to TRN2 [17, 53, 54]. Together, these studies provided evidence of the direct correlation between the cytoplasmic accumulation of HuR that is mediated by protein partners, its cleavage and its ability to promote apoptosis.

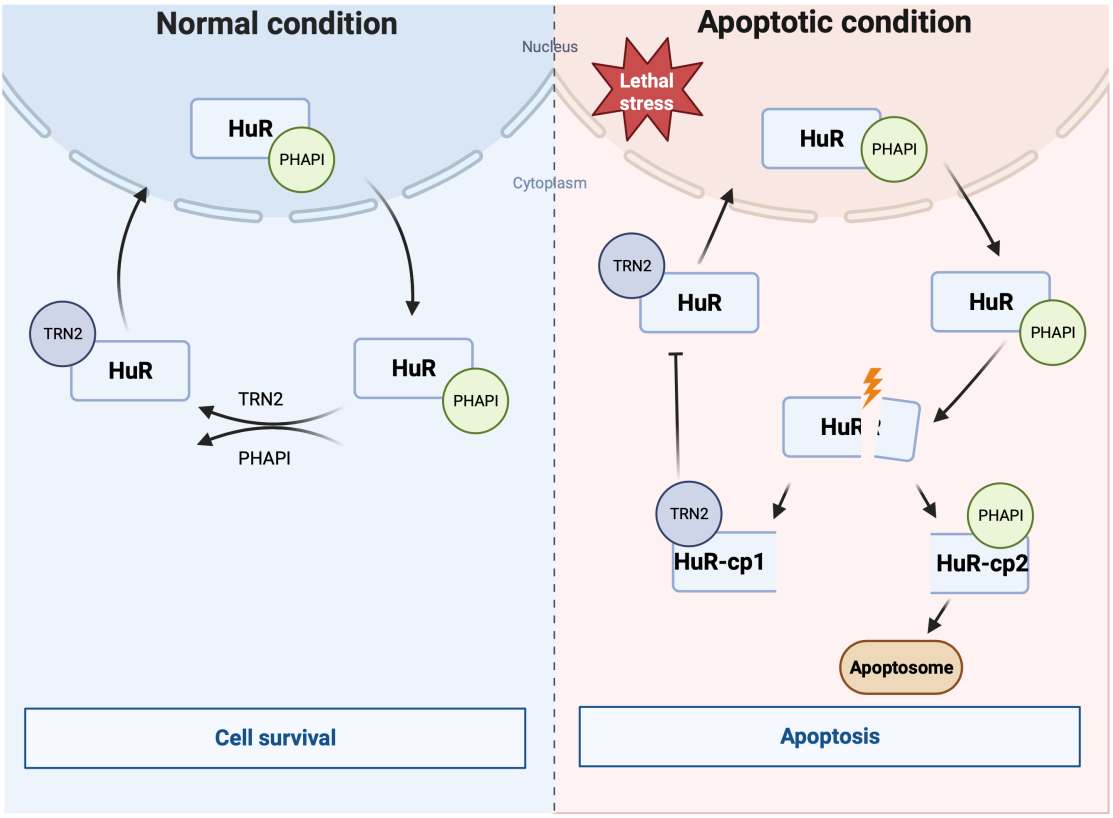

Figure 1.5: HuR cellular trafficking and protein association.

Model depicting the mechanism by which HuR association with protein partners regulates its trafficking and its apoptotic function. Under normal condition, HuR maintains its nuclear localization by promoting its interaction with the import factor TRN2. In response to a lethal assault, HuR and PHAPI translocate to the cytoplasm where HuR undergoes caspase mediated cleavage yielding HuR-CP1 and HuR-CP2. While HuR-CP2 interacts with PHAPI mediating the activation of apoptosome-formation, HuR-CP1 interacts with TRN2 preventing the reuptake of HuR back to the nucleus. HuR, therefore, accumulates in the cytoplasm, advancing apoptosis [16-18, 53].

Created with BioRender.com (GW25S7HL38)

2.4.3 Post-translational modification

The cellular localization of HuR as well as its RNA binding activity is known to be regulated by post-translational modification (PTMs) such as phosphorylation and methylation. Several studies reported that HuR is targeted for phosphorylation at different residues by several kinases such as cdk1, AMP-activated protein kinase (AMPK), PKC and p38; and for methylation by the methyltransferase CARM1. The PTM of residues within the RRMs influences the function of HuR in regulating RNA metabolism, while modification of residues within or near the HNS impacts HuR subcellular localization. For instance, phosphorylation of HuR by Chk2 at residues S88, S100 and T118 located within RRM1 and RRM2 modulates HuR binding to *SIRT1* mRNA and other mRNA targets [59]. On the other hand, phosphorylation by cdk1 at S202, situated within the HNS, facilitates HuR binding to the nuclear 14-3-3 triggering its nuclear retention [60, 61].

Recent studies showed that the newly discovered post-translational modification of HuR, poly (ADP-ribosyl) ation (PARylation), modulates its function under pro-inflammatory conditions [58, 62, 63]. It was reported that in response to inflammatory stimuli, poly (ADP-ribose) polymerase 1 (PARP1)-mediated PARylation targets the HNS-RRM3 region of HuR and modifies it at a conserved aspartate residue D226. Moreover, mutating this site (D226) or inhibiting PARP impacted HuR localization, its ability to associate with pro-inflammatory messages, as well its ability to oligomerize [58, 63]. More recently, unpublished study from our lab uncovered that PARylation of HuR by Tankyrase1 (TNKS1), also known as PARP5a, promotes the cytoplasmic accumulation and cleavage of HuR, as well as its ability to associate with promyogenic mRNAs to promote muscle fiber formation, underlining the physiological relevance of HuR PARylation (Mubaid *et al.* unpublished data). Although the role of HuR PARylation during inflammation [58, 63] and muscle cell differentiation (Mubaid *et al.* unpublished data) has been investigated, the

importance of this modification on the function of HuR in determining cell fate has never been delineated.

3. PARylation

3.1 Biochemistry of PARylation

Poly (ADP-ribosyl) ation (PARylation) is a post-translational modification process by which polymers of ADP-ribose (PAR) are catalysed by pADPr polymerase (PARP) enzymes. Using nicotinamide adenine dinucleotide (NAD^+) as a substrate, PARPs covalently modify target proteins by attaching the ADP-ribose units to acceptor amino acid residues via a unique ribose-ribose linkage. The attached PAR chains can vary in length and shapes. They can be linear or branched and their length may reach up to 10nm with more than 200 ADP-ribose residues [64, 65]. The catalytic domain of PARP is highly conserved in all members of the PARP family and is composed of a donor site, also known as the NAD+ binding pocket and an acceptor site for binding to the PARylation target molecule. The NAD+ binding pocket has a conserved Histidine-Tyrosine-Glutamate (H-Y-E) triad. While the Histidine and Tyrosine residues are responsible for NAD+ binding, the Glutamate residue is essential for the catalysis and elongation reaction of PAR chains that is characterised by the (*2'-1''*) ribose-ribose glycosidic bonds between ADP-ribose units. The branching reaction, on the other hand, is characterized by the formation of (*2''-1'''*) ribose-ribose glycosidic bonds [65-68].

PARylation is a highly dynamic, transient and reversible modification where PAR removal is catalysed by a number of hydrolyzing enzymes, including PAR glycohydrolase (PARG), terminal ADP-ribose glycohydrolase 1 (TARG1) and other mono- and poly-ADP-ribose hydrolases [65, 67-70]. PARG is the most studied PAR hydrolyzing enzyme and it is known to hydrolyze ribose-

ribose bonds to resolve PAR chains and leave behind the terminal ADP-ribose unit. The later ADP-ribose unit is removed by TARG and other amino acid-specific hydrolases [65, 68, 71].

3.2 Covalent and non-covalent PARylation

It is well-established that PARP-mediated PARylation of target proteins can occur via covalent modification or by non-covalent association to PAR. PARPs can mediate the covalent PARylation of target proteins at various residues, specifically at the aspartate (D), arginine (R), glutamate (E), lysine (K), serine (S), or tyrosine (Y) residues. In parallel, the catalyzed PAR chain can bind in a non-covalent manner with proteins that contain a conserved PAR-binding motif (PBM) [65]. Generally, this motif consists of a loosely conserved sequence of hydrophobic and basic amino acids which are often found to overlap with important functional domains such as DNA and/or RNA binding domains, exerting regulatory function within the cell [65].

Several recent studies are pointing to the importance of these two manners of PARylation on the function of their substrates and how there may be an interplay between the two modifications [61, 64, 72]. For instance, heterogeneous nuclear ribonucleoprotein A1(hnRNPA1), a well-known RBP, has been shown to be both PARylated covalently and can bind non-covalently to PAR or PARylated proteins. Recently, Duan *et al*. showed that hnRNPA1 is PARylated on Lysine 298 and mutating this site decreased its PARylation level and affected its localization [62]. They also showed that hnRNPA1 harbors a PAR binding motif, which when mutated, increased its covalent PARylation [62]. These observations led them to suggest that the non-covalent PAR binding reduces the hyper-PARylation of hnRNPA1 on the covalently PARylated Lysine residue.

3.3 The importance of PARylation in regulating protein function

Various proteomic analyses identifying PARylated and PAR-binding proteins revealed that RBPs constitute a major subset of the targeted proteins. RBPs are known to play important roles

in many of the RNA regulatory pathways through multiple mechanisms. Further studies revealed that PARP-mediated PARylation or PAR binding to RBPs can affect their localization, activity, or their binding to RNA, and therefore can alter their function [71, 73, 74]. For example, several studies on hnRNPs showed that their modification by PARP1 inhibited their ability to bind RNA and influenced their function in regulating alternative splicing and translation [73-75]. Additionally, as mentioned above, previous studies have reported that the PARP1-mediated PARylation of HuR in LPS-induced cells, affected its localization and function [58, 63].

It is also well known that the generated PAR polymers, as a consequence of PARP activation in response to DNA damage, modulate the recruitment of RBPs to DNA-damaged sites, which plays a critical role in facilitating the DNA repair process. For instance, the RBP NONO is recruited to the DNA-damaged site in a PARP1-dependent manner and therefore guides the DNA-damage repair machinery to repair the damage [71, 76].

PARP has also been shown to modulate the function of these RBPs during stress response by regulating their recruitment to cytoplasmic entities named stress granules (SGs). These stress granules serve as an important reservoir for the translationally stalled mRNAs and their associated RNA-binding proteins. It has been shown in various studies that the association of the RBP G3BP1 with PAR is necessary for the assembly of SGs under stress condition. Furthermore, PARylation of hnRNP-A1 has been shown to modulate its recruitments to SGs and therefore affects the translation of its mRNA targets [62, 74, 77].

Numerous evidence shows that when acceptor proteins are being targeted for PARylation or association with the bulky and negatively charged PAR polymers, a change in their folding occurs and consequently disrupts or uncovers motifs that are important for preventing or promoting interactions. For example, the E3 ligase RNF146 has been shown to undergo a conformational

change of the RING domain as a consequence of PAR binding to its PAR conserved domain which leads to increased E3 ligase activity [78].

3.4 PARPs Family

The PARP family of proteins consist of seventeen members that are known to be involved in various cellular processes. Among the PARPs, PARP1, PARP2, PARP5a (TNKS1), and PARP5b (TNKS2) are designated as the ‘’*bona fide* PARPs’’ since they harbour the PARylation activity and are characterised with the presence of the conserved glutamate residue (Glu988).[66, 70] Other PARPs are putative mono(ADP-ribose) polymerases that mainly induces MARylation except for PARP-9 and PARP-13. The latter are considered inactive PARPs due to the fact that they do not have PARP signature motif that binds NAD+ nor do they have Glu988 [66, 79].

The most characterised and well-studied enzyme of the PARP family is PARP1, and it is the one that is known to synthesizes the most PAR in cells [77, 79, 80]. Structurally, PARP1 is a 116-kDa protein, consisting of three major functional domains: (1) DNA-binding domain (N terminal) containing three zinc finger (ZnFs) structure responsible for PARP1’s recognizing and binding to DNA strand breaks, a nuclear localization sequence (NLS), and a caspase-cleavage site (2) auto-modification domain (central), containing a BRCT motif and (3) catalytic domain (C terminal), containing the PARylation active site also known as the (PARP signature) [65-68, 77, 79-81]. The catalytic domain is highly conserved in all PARP members. PARP2 has the closest homology to PARP1 in the C-terminal catalytic domain with 69% similarities, but it lacks the N-terminal ZnFs and the BRCT domain found in PARP1 (Figure 1.6) [66, 68, 77, 79, 80, 82].

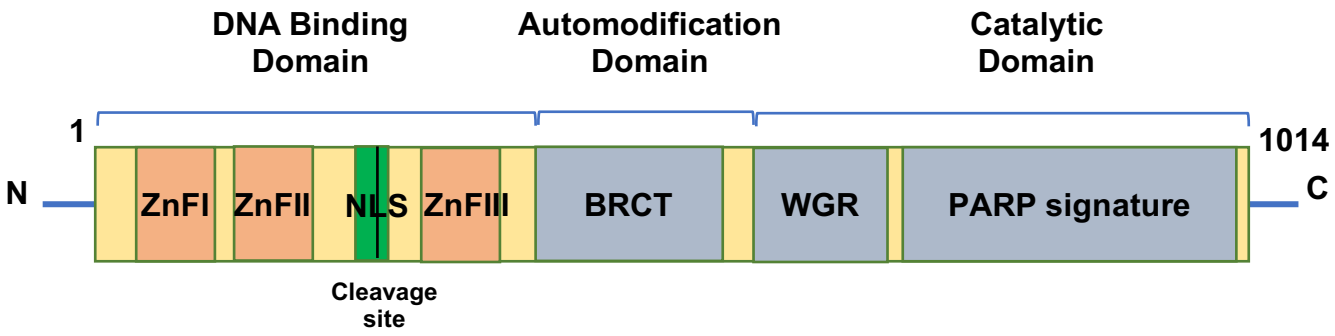

Figure 1.6: Schematic representation of PARP1 structure

PARP1 consists of three major motifs: (1) DNA-binding domain (N terminal) containing three zinc finger (ZnFs) structure, a nuclear localization sequence (NLS), and a caspase-cleavage site (2) auto-modification domain (central), containing a BRCT motif and (3) catalytic domain (C terminal), containing the PARylation active site also known as the (PARP signature) [65-68, 77, 79-81].

3.5 PARP1 and PARP2 role in apoptosis

The catalytic activity of PARP1 is normally initiated in response to a break in the DNA strand. When the DNA damage is mild and manageable, PARP1 detects and recruits DNA damage response factors to repair the damage and hereby acts as a cell survival factor. However, when the damage is irreparable, PARP1 is cleaved in the nucleus in a caspase-dependent manner by caspase 3 and 7, thereby leading to apoptosis [83, 84]. In fact, PARP1 cleavage represents an important step to revoke the overactivation of PARP1 in response to severe DNA damage and inhibits the unnecessary depletion of NAD+ and ATP levels which could lead to necrosis instead of apoptosis. Once PARP1 is cleaved, two inactive cleavage products are generated, the 24-kDa and 89-kDa fragments. The smaller 24kDa fragment, which contains the DNA-binding motif and the NLS, remains irreversibly bound to the DNA break and therefore inhibits the activity of full length PARP1 by competition. In parallel, the 89kDa fragment which contains the auto-modification and catalytic domains relocalizes to the cytoplasm and facilitates caspase-mediated DNA fragmentation leading to apoptosis [14, 66, 68, 79, 84, 85].

Recent studies also showed that PAR translocation from the nucleus to the cytoplasm represents a crucial step in the parthanatos pathway, which is a programmed cell death that is initiated by PARP1 overactivation in response to DNA damage. PAR polymers produced, mainly from the auto-modification of PARP1 itself due to its overactivation, are believed to remain bound to the 89kDA fragment which serves as a carrier for PAR translocation to the cytoplasm where PAR triggers the release of the apoptosis-inducing factor (AIF) from the mitochondria. Consequently, AIF is imported to the nucleus and associates with DNAase, resulting in DNA fragmentation in a caspase-independent manner [84].

In addition to the fundamental role of PARP1 in DNA damage repair and apoptosis, PARP1 is also known to be involved in other cellular process including cell differentiation, immune response, and transcriptional regulation.

Similar to PARP1, the activity of PARP2 is initiated in response to DNA damage and both play an important role in the DNA damage repair process, nevertheless, PARP2 preferentially targets DNA gaps but not nicks. In fact, PARP2 is ranked the second major PAR-producing PARP after PARP1 and it is found to compensate for most of the residual DNA-dependent PARP1 activity in PARP1-deficient mice [66, 68, 78, 79]. Additionally, the function of PARPs is essential since the loss of both PARP1 and PARP2 in mice is embryonically lethal, suggesting an important functional overlap between PARP1 and PARP2. Furthermore, PARP2 null mice display defects in T cell development, erythropoiesis, and spermatogenesis that are not found in PARP1 null mice, suggesting that PARP2 has a unique function although its function is found to be redundant to that of PARP1 [86].

RATIONAL

Apoptosis is an evolutionary form of programmed cell death that plays a crucial role in regulating embryonic development, maintaining tissue homeostasis, and eliminating damaged and potentially harmful cells [1-3]. The activity and efficiency of the apoptotic mechanism is tightly regulated through highly complex, energy-dependent cascading events that involve the activation of a set of proteases known as caspases which promote apoptosis through the cleavage of protein substrates [9]. Interestingly, previous work from our lab revealed that during caspase-mediated apoptosis, the RNA-binding protein HuR is cleaved at the aspartate (D)226 residue, thereby generates two cleavage products (CPs). This proteolytic event occurs upon the migration of HuR to the cytoplasm in response to different apoptotic stimuli [17, 18, 53, 54]. It is well-known that during the stress response, HuR initially modulates the expression of various pro-survival messages such as prothymosin α. However, when the stress is severe, HuR shifts its function, and via its CPs, modulates the expression of many pro-apoptotic factors such as caspase-9 [18]. Importantly, the caspase-mediated cleavage is required for the pro-apoptotic function of HuR [18]. In various cancers, HuR is found to be overexpressed, however it does not get cleaved and consequently its pro-apoptotic function is impaired. Therefore, delineating mechanisms involved in determining whether HuR will favor a pro-survival or pro-apoptotic cell fate might provide a therapeutic option where the function of HuR may be targeted for intervention. Post-translational modifications such as phosphorylation and methylation have been shown to regulate the function of HuR. Recently, PARylation of HuR by PAR polymerase enzyme-1 (PARP1) was also shown to regulate the function of HuR during inflammation [58]. However, the regulatory mechanism(s) mediating the pro-apoptotic function of HuR and the involvement of post-translational modifications such as PARylation in this process has never been investigated. Therefore, the main

objective of my study in the first part of this thesis was to identify a regulatory mechanism through which the pro-apoptotic function of HuR is regulated. During these studies, we identified PARylation as a key post-translational modification that modulates the function of HuR in determining cell fate.

The loss of apoptotic control is thought to responsible for the onset of several human diseases including cancers [22]. For decades, chemotherapeutic drugs have been shown to induce apoptosis in several cancer cells and tumors. In numerous cases, however, these cells/tumors develop a multidrug resistant (MDR) which represent a major obstacle in cancer treatment [24]. The MDR of cancer cells is usually associated with resistance to apoptosis. Overexpression of P-glycoprotein (P-gp), a membrane-bound protein encoded by the MDR1 gene, is one of the major players responsible for the drug resistance in cancer cells [32]. Of particular interest, several recent studies have shown that HuR plays a prominent role in mediating the development of chemotherapeutic resistance of tumors including pancreatic cancer [48, 49], colorectal cancer and many others [38, 50]. Indeed, although it has been reported that HuR is able to modulate the expression of the MDR1 mRNA and P-gp protein in many cancer cells, the mechanism in which HuR affects drug resistance and mediates *MDR1* mRNA expression, particularly in the KB human cervical adenocarcinoma cells, is not fully understood [38, 48, 49, 51]. Therefore, the objective of the second part of my research was to provide further insight on the HuR-mediated regulation of the *MDR1* mRNA thus linking HuR to the resistance of cells to drug treatment and bringing a new hope for the researcher to overcome MDR.

By determining the regulatory mechanisms of the function of HuR in cell fate, we would be able to further gain insight into potential strategies to target this function of HuR, especially in cancer cells, where the apoptotic mechanism is known to be dysregulated. Additionally,

understanding how HuR contributes to the development of drug resistance in a variety of cancer cells and tumors allows a better understanding of the role of HuR in cancer, and the importance to target its activity therapeutically. Further studies offer an interesting opportunity to demonstrate whether regulatory mechanisms of HuR such as PARylation could play a potential role in modulating the function of HuR in regulating the expression of MDR. Therefore, this would open a new avenue to utilise PARylation related drugs as beneficial therapeutics to drive the pro-apoptotic function of HuR thus inducing apoptosis in these cells and subsequently overcoming MDR.

RESULTS

1. PARP1 and PARP2 regulate the cytoplasmic localization, cleavage, and pro-apoptotic function of HuR

In order to establish the role of PARylation on the function of HuR during apoptosis we assessed, as a first step, whether HuR is associated with PAR polymers in HeLa cells treated, over a three-hour period, with staurosporine (STS), a well-known apoptotic inducer. We have previously demonstrated, as described in [17] and shown in (Figure 1A) that the treatment of these cells with STS for 3h hours induces both the cleavage of HuR and PARP1. We show, under these conditions, by performing co-immunoprecipitation experiments, that although PAR associates to HuR in untreated cells, this interaction decreases when the cells were treated with STS for up to 3h (Figure 1B). The decreased interaction of HuR with PAR, interestingly, appears to coincide with the cleavage of PARP1 under these conditions suggesting that PARP1 is involved in mediating the interaction of HuR with PAR in untreated cells.

As mentioned earlier, we have previously shown that in response to lethal stress the accumulation of HuR in the cytoplasm is required for its cleavage and pro-apoptotic function (Figure 2A) [17]. Since the interaction of HuR with PAR only occurs in untreated cells where HuR is localized in the nucleus, we decided to assess if PARP1-mediated PARylation prevents the accumulation of HuR to the cytoplasm. PARP2 has highly redundant function to PARP1, and both are cleaved in a caspase-dependent manner during apoptosis [82, 87]. Thus, we sought to assess if depleting both PARPs individually, and in combination, using siRNAs specifically targeting each PARP would affect the localization of HuR (Figure 1C). We observed that these siRNAs efficiently depleted the expression of both PARPs by more than 90% in these cells (Figure 2B). By performing immunofluorescence experiments, we further observed that although knocking

down PARP1 or PARP2 increased the cytoplasmic localization of HuR in untreated conditions, this effect was more prominent when cells were simultaneously treated with siRNAs targeting both proteins (Figure 1C). Additionally, this observation was reproduced using Talazoparib, a well-known PARP1/2 inhibitor (Figure 1D). Our results, therefore, suggest that the interaction of HuR with PAR in the nucleus regulates its nucleocytoplasmic shuttling function under normal conditions in Hela cells.

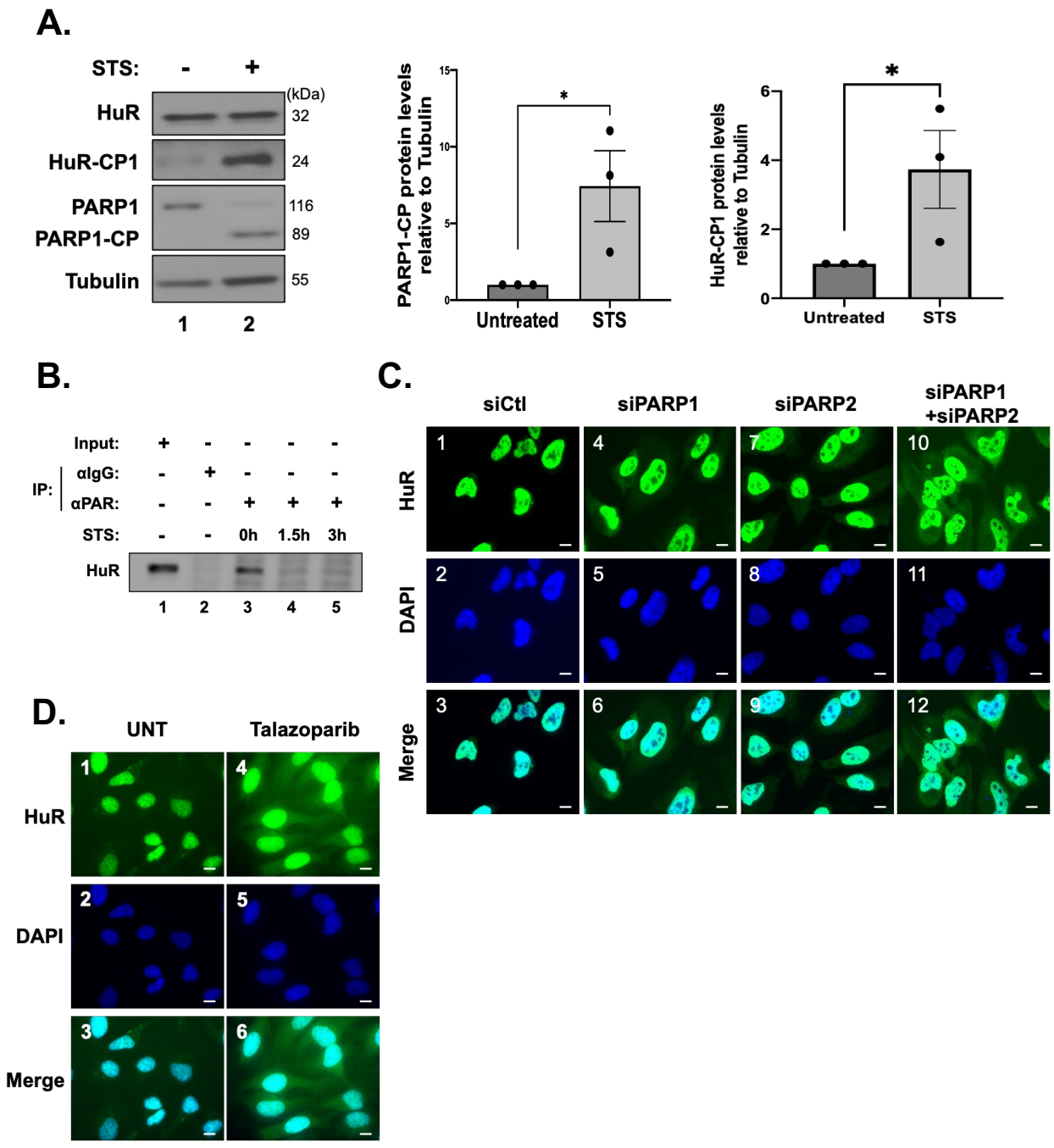

Figure 1: The cytoplasmic translocation and cleavage of HuR in response to an apoptotic stimulus correlates with the cleavage of PARP1/2

A) (Left) Hela cells treated with or without 1 μM STS for 1.5 h were collected, lysed, and used for western blot analysis with antibodies against HuR, PARP1 or α-tubulin (loading

control). **(Right)** Densitometric quantification of HuR-CP1 and PARP1-CP signals in the western blot relative to α-tubulin signal. Values were quantified using ImageJ.

B) Lysates obtained from Hela cells treated with 1 μM STS for (0, 1.5, 3 h) were used for immunoprecipitation experiments using antibodies against PAR or IgG as a negative control. The binding of PAR to HuR was then assessed by western blot using an anti-HuR antibody (3A2). All blots shown in the figure are representative of 3 independent experiments.

C) HeLa cells were transfected with siRNA targeting PARP1 and/or PARP2 or a control siRNA. These cells were then fixed, permeabilized, and stained with antibodies against HuR. DAPI was used to stain nuclei. Images are representative of three independent experiments. (Scale bars, 10 μm).

D) Immunofluorescence experiments demonstrating HeLa cells treated with and without 1μm-Talaxzoparib. After 24 hours of treatment, cells were fixed, stained, permeabilized and stained with antibodies against HuR. DAPI was used to stain nuclei. Images of a single representative field are shown and are representation of three independent experiments. (Scale bars, 10 μm).

Data presented in Figure 1 are +/- the S.E.M. of three independent experiments with *P<0.05, by unpaired *t*-test.

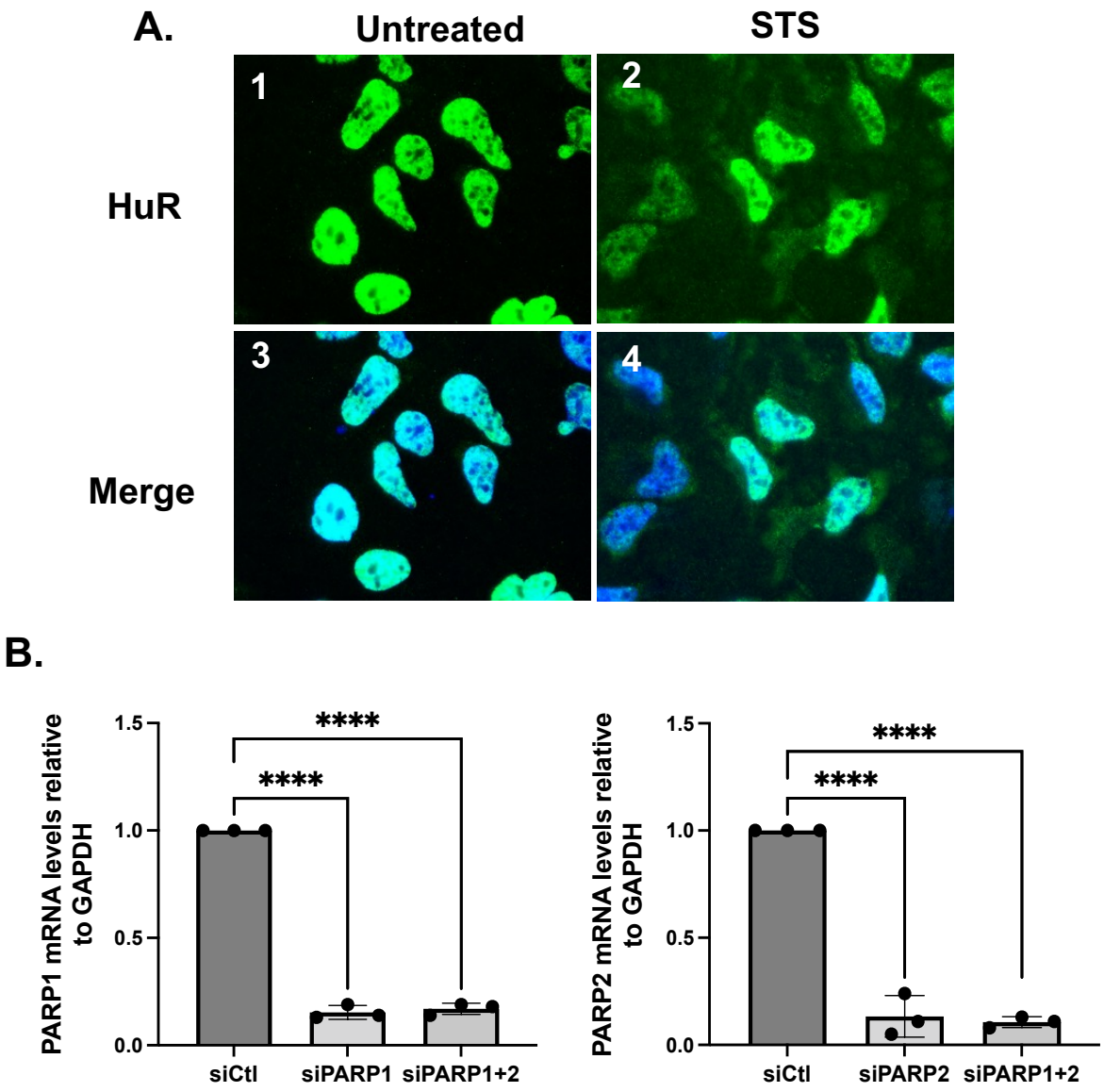

Figure 2:

A) Immunofluorescent experiments demonstrating the localization of HuR in Hela cells treated with or without 1 µM STS for 1.5 h.

B) Total RNA was isolated from HeLa cells transfected with siRNA targeting PARP1 and/or PARP2 or a control siRNA and RT-qPCR analysis was performed using primers for PARP1 and PARP2 to determine mRNA level for the validity of knockdown efficiency.

Next, to determine the impact of PARP1 and PARP2 on the apoptotic function of HuR, we assessed if knocking down these PARPs affects its cleavage. We noticed that HuR cleavage is significantly increased in PARP1 depleted cells under normal conditions (Figure 3A lane 2). We observed, similarly, a trend in HuR cleavage in cells depleted of PARP2 (Figure 3A lane 3). This cleavage, however, was further significantly increased with the double knockdown of PARP1 and PARP2 compared to cells treated with control siRNAs (Figure 3A lane 4). These results indicate that the PARylation of HuR could play a potential role in modulating its pro-apoptotic function. Since depleting these PARPs resulted in the cleavage of HuR in untreated conditions, mimicking what we observed in the apoptotic conditions, we next questioned the impact of depleting these PARPs on caspase-3 cleavage, another well-established event in apoptosis. As expected, silencing PARP1 and PARP2 together showed a significant increase in the cleavage of caspase-3 (Figure 3A, lane 4). This result was further confirmed by performing flow cytometry experiments which demonstrated a significant increase in the number of Annexin V-positive cells in siPARP1 and siPARP2 treated cells (Figure 3B). Together, these findings highlighted the importance of PARP1/2-mediated PARylation on HuR's pro-apoptotic function.

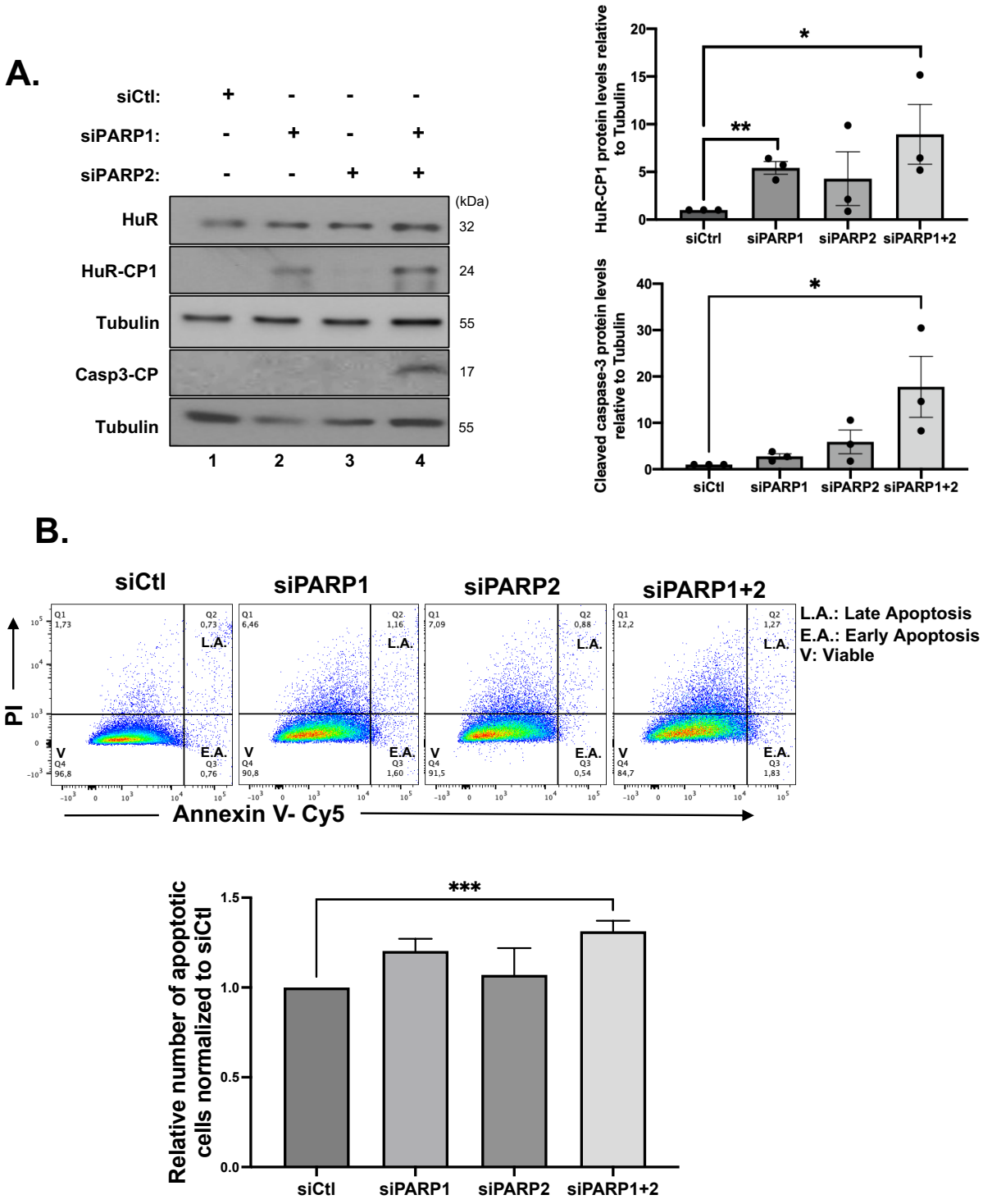

Figure 3: PARP1/2 knockdown increases HuR cleavage and triggers apoptosis

A) HeLa cells were transfected with siRNA targeting PARP1 and/or PARP2 or a non-specific control siRNA (siCtl). Lysates were used for western blot analysis (left panel) with

antibodies against HuR, Caspase3 cleavage product (CP) and α-tubulin. Densitometric quantification (Right panel) of HuR-CP1 and Caspase3-CP levels. Values were quantified using ImageJ, normalized to tubulin and shown relative to siCtl.

B) HeLa cells treated with siRNA as described in A were analyzed by staining with annexin V–Cy5 and PI (Propidium Iodide) and by flow cytometry. The relative number of apoptotic cells was determined for siPARP1 and/or siPARP2. The values are relative to control siRNA treated cells.

Data presented in Figure 2 are +/- the S.E.M. of three independent experiments with *P<0.05, ***P<0.001 by unpaired *t*-test.

2. PAR binds HuR non-covalently through a consensus motif.

It is well-established that PARP-mediated PARylation of target proteins can occur via either covalent modifications or by non-covalent association to PAR [80]. Both mechanisms were shown to entail different functional consequences on the affected proteins [62, 71]. Thus, as a first step, we decided to investigate if the non-covalent association of PAR to HuR could mediate its pro-apoptotic function. By performing an *in-vitro* dot blot assay we demonstrated that HuR, unlike BSA and GST (used as negative controls), non-covalently binds to PAR (Figure 4A). Next, we wanted to determine the exact PAR binding site on HuR. To this end, we performed an *in-vitro* peptide mapping experiment where we generated small peptide fragments spanning the complete HuR sequence. Each fragment is about 20 amino acids in length. We found that several fragments (B6, B7, E1) of HuR exhibited binding to PAR with various strength (Figure 4B). However, only one of these (E1) harbours a region (amino acids 201-208 of HuR) that exhibits 76% similarity to a well-known consensus PAR binding site ($[HKR]_1$-X_2-X_3-$[AIQVY]_4$-$[KR]_5$-$[KR]_6$-$[AILV]_7$-$[FILPV]_{8)}$ [80, 88]. Therefore, we dubbed this element as the HuR PAR Binding Site (HuR-PBS).

To better understand the importance of this site on the function of HuR, we generated a mutant isoform of HuR (HuR^{pbmt}) whereby the (+) charged arginines (R) and histidines (H) residues within the HuR-PBS were converted into alanines (A) (Figure 4C top). Using the dot-blot approach mentioned above we demonstrated that unlike the wild type HuR (HuR^{wt}), the HuR PAR binding mutant (HuR^{pbmt}) lost its ability to bind PAR (Figure 4C bottom). We next assessed the importance of this site for PAR binding in HeLa cells by transfecting the cells with GFP, GFP-HuR^{wt} and GFP-HuR^{pbmt} followed by a co-IP experiment where we immunoprecipitated PAR and immunoblotted with anti-HuR (Figure 4D). We demonstrated that although PAR binds to GFP-HuR^{wt} this interaction is completely abrogated due to mutation of the PAR-binding site (Figure

4D). Together, these results reveal that HuR non-covalently interacts with PAR through the harbored PAR-binding motif.

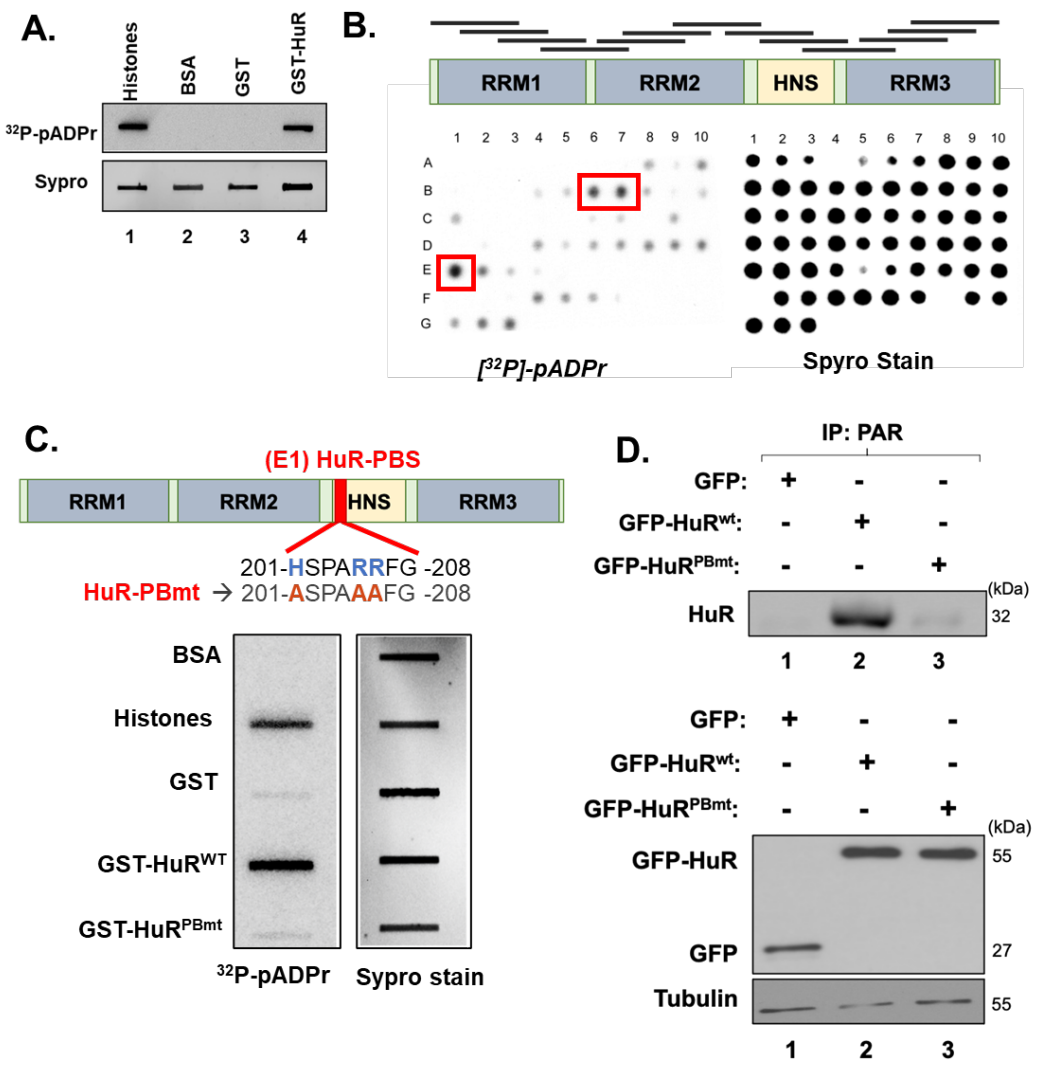

Figure 4: PAR binds HuR non-covalently through a consensus motif

A) Recombinant GST and GST-HuR proteins as well as recombinant Histone (positive control) and BSA (negative control) were blotted directly onto nitrocellulose membrane, rinsed, incubated with a radiolabeled ^{23}P-pADPr and analyzed by autoradiography. Sypro Ruby stain was used to demonstrate the integrity and quantity of the proteins.

B) HuR protein was fragmented into 63 small peptides (each fragment is 20 amino acids in length with 5 staggered amino acids) used for peptide mapping experiment. All fragments were blotted onto nitrocellulose membrane and processed as in A.

C) (**Top**) Schematic showing location of PAR binding site of HuR. Mutation of this site was generated by substituting the positively charged amino acid Histidine and Arginine by

hydrophobic Alanines residues. (**Bottom**) Slot blot assay was performed using recombinant GST-HuR^{wt}, GST-HuR^{PBmt} protein and GST/BSA as a negative control, while Histone as a positive control. Sypro Ruby stain was used to demonstrate the integrity and quantity of the proteins.

D) (**Top**) Total cell extracts obtained from HeLa cells transfected with GFP, GFP-HuR^{wt} or GFP-HuR^{PBmt} were used for immunoprecipitation experiments using antibodies against PAR. The binding of PAR to HuR was then assessed by western blot using anti-HuR antibody. (**Bottom**) Transfection efficiency was assessed by determining the levels of these proteins in the input using anti-GFP and α-tubulin as a loading control. Immunoprecipitation results are representative of three independent experiments.

3. PAR binding prevents the pro-apoptotic function of HuR by promoting its nuclear localization

Our results described above show that the depletion of PARP1/2 decreased the nuclear localization of HuR. This is likely due to the decreased interaction of HuR to PAR. To assess if this is the case, we next assessed the impact of mutating the HuR PAR-binding site on its cellular localization. Immunofluorescence assays revealed that HuR^{Pbmt} but not HuR^{wt} accumulates in the cytoplasm of untreated HeLa cells, mimicking the observations obtained due to the knockdown of PARP1/2 (Figure 5A). Together our results therefore suggest that the non-covalent association of PAR with HuR plays an important role in modulating its cellular localization in normal HeLa cells.

We and others have shown that the nucleocytoplasmic translocation of HuR, during apoptosis, is mediated by its association with adaptor proteins for nuclear export such as PHAP-I and with the import factors such as transportin-2 (TRN2), [17, 21, 89]. To determine whether mutating the PAR binding site would have an impact on the differential association of HuR with these proteins, we immunoprecipitated PHAPI and TRN2 individually and assessed their association with GFP-HuR^{wt} or GFP-HuR^{Pbmt} (Figure 5B). We observed that, unlike HuR^{wt}, the HuR^{Pbmt} isoform loses its association with TRN2 (Figure 5B right) but not with PHAPI (Figure 5B left). This finding suggests that an intact HuR-PBS is required for the association of HuR with TRN2 and its retention in the nucleus.

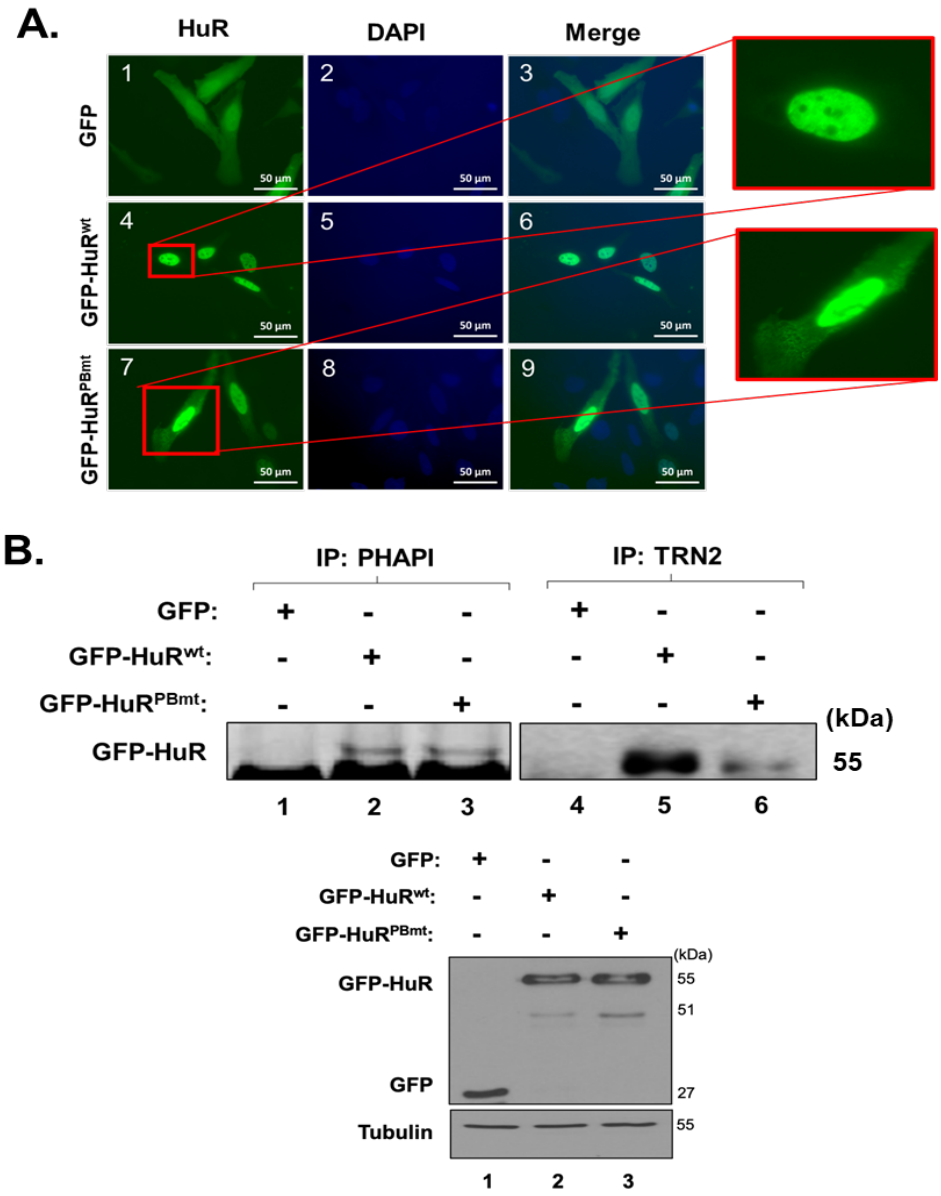

Figure 5: HuR binding to PAR modulates its cellular localization in Hela cells

A) HeLa cells transfected with GFP, GFP-HuR^{wt} and GFP-HuR^{PBmt} were fixed, permeabilized, and stained with antibodies against HuR and DAPI. Images are representative of 3 independent experiments. (Scale bars, 50 µm).

B) Total cell extracts obtained from HeLa cells transfected as in A were used for immunoprecipitation experiments using antibodies against PP32/PHAPI (left panel) or TRN2 (right panel). Immunoprecipitated complex was then assessed by western blot using HuR antibodies. The blots are representative of 3 independent experiments.

We have previously shown that the cleavage of HuR is tightly related to its cytoplasmic accumulation, due to the competition of HuR-CP1 with full length HuR for the binding to TRN2, leading to the accumulation of full length HuR in the cytoplasm. Therefore, we next determined whether mutating the HuR-PBS would affect the cleavage of HuR. We observed that the GFP-HuR^{Pbmt} is cleaved to a greater extent than GFP-HuR^{wt} (Figure 6A). Interestingly, we observed that the expression of HuR^{Pbmt} increased the cleavage of caspase-3 to a greater extent than cells expressing HuR^{wt} (Figure 6A). To determine the physiological importance of PAR binding, we performed flow cytometry analysis to assess the cell fate of HuR^{Pbmt} expressing cells compared to cells expressing HuR^{wt}. These results further supported our findings described above and showed an increase in annexin V positive cells expressing HuR^{pbmt} (Figure 6B), providing evidence for the anti-apoptotic role conferred to HuR by the binding to PAR. In conclusion, we demonstrate that HuR can non-covalently bind to PAR, and that this binding alters its pro-apoptotic function through the regulation of its localization, further underlining the importance of HuR localization in its pro-apoptotic function.

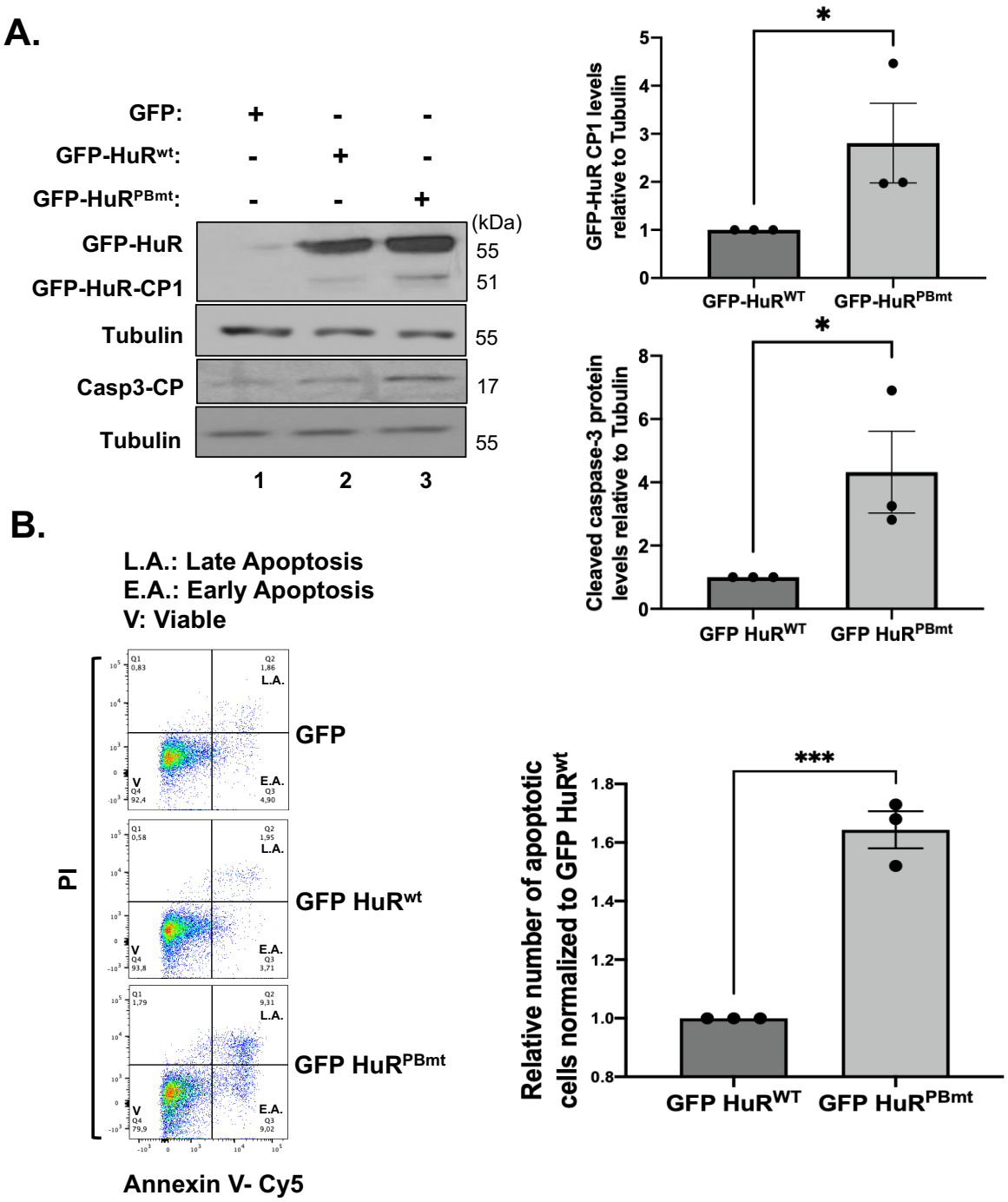

Figure 6: PAR binding to HuR negatively affect its pro-apoptotic function

A) HeLa cells were transfected with GFP, GFP-HuR^{wt} and GFP-HuR^{PBmt}. (**Left**) Lysates were used for western blot analysis with antibodies against HuR, Caspase3-CP and α-tubulin.

(**Right**) Densitometric quantification of HuR-CP1 and Caspase3-CP levels were normalized to α-tubulin level and shown relative to GFP-HuR^{wt}.

B) HeLa cells transfected as described in A were analyzed by staining with annexin V–Cy5 and PI and analyzed by flow cytometry. The relative number of apoptotic cells was determined for GFP-HuR^{wt} and GFP-HuR^{PBmt} transfected HeLa cells. The values were normalized to GFP.

Data presented in Figure 5 are +/- the S.E.M. of three independent experiments with *P<0.05, ***P<0.001 by unpaired *t*-test.

4. HuR promotes P-gp and *MDR1* mRNA expression in drug resistant KB-V1 cells

As P-gp is the key player for the emergence of the drug resistant phenotype in many cell types, we started our investigation by assessing the expression levels of P-gp and *MDR1* mRNA in both KB and KB-V1 cells, drug sensitive and drug resistant cells, respectively. We showed, by western blot, that P-gp is expressed only in KB-V1 cells but not in the parental (drug sensitive) cell line (Figure 7A). Consistently, RNA isolated from both cell lines, followed by RT-qPCR analysis, showed that *MDR1* mRNA is expressed in KB-V1 cells but not in KB cells (Figure 7B). As described earlier, HuR has been recently shown to mediate the regulation of MDR1 mRNA and protein level and contributes to the stability of MDR1 mRNA in CML cells, however, the mechanism by which HuR mediates the regulation of MDR1 mRNA in KB-V1 is still unknown [51]. Therefore, we next assessed whether HuR was involved in regulating the expression of P-gp/*MDR1* in these drug resistant KB-V1 cells. Western blot analysis demonstrated that the depletion of HuR resulted in decreased P-gp protein levels as well as *MDR1* mRNA level compared to the control siRNA treated conditions (Figure 8A-B). To verify that the difference in P-gp and *MDR1* mRNA expression observed in resistant versus sensitive cells is not due to a variation in the levels of HuR, we assessed whether the expression of HuR differs between the parental KB-3-1 and their resistant KB-V1 counterparts. Western blot analysis showed that HuR protein levels were similar in both cell types, indicating that the increased expression of the *MDR1* mRNA in KB-V1 cells is not due to a change in the expression of HuR (Figure 9A). Since the localization of HuR is tightly related to its function [90, 91], it is possible that a difference in the localization of HuR between both cell lines could be the cause of the differential effect on the expression of P-gp and *MDR1* mRNA. Towards this end, we performed immunofluorescence experiments to assess the localization of HuR in both cell lines. We showed, by performing these

experiments, that HuR is localized in the nucleus of both cell lines suggesting that the effect described above on *MDR1* mRNA is not due to a change in HuR cellular localization (Figure 9B).

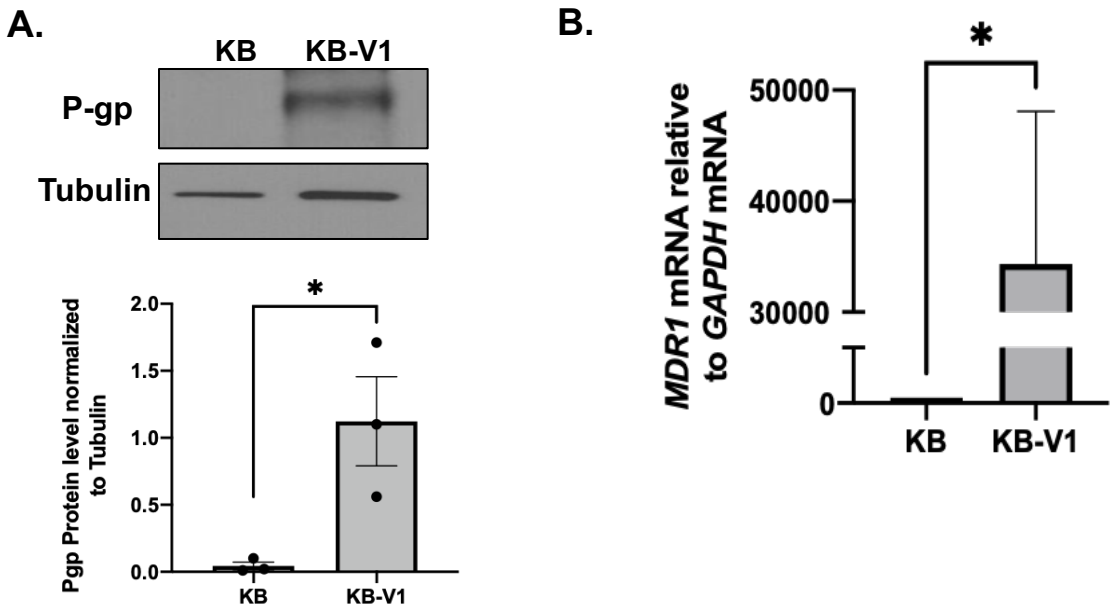

Figure 7: P-gp protein and *MDR1* mRNA expression is increased in drug resistant KB-V1 cells.

A) (**Top**) Lysates from multidrug resistant KB-V1 cells and parental non-resistant KB cells were used for western blot analysis using antibodies against P-gp and α-tubulin. (**Bottom**) Densitometric quantification of P-gp signals in the western blot relative to α-tubulin signal. Values were quantified using ImageJ. Blot is representative of 3 independent experiments.

B) Total RNA from KB-V1 and KB cells were isolated and subjected to RT-qPCR analysis using primers for MDR1 and GAPDH. Data presented are +/- the S.E.M. of three independent experiments with *P<0.05, by unpaired *t*-test. (See also [92, 93])

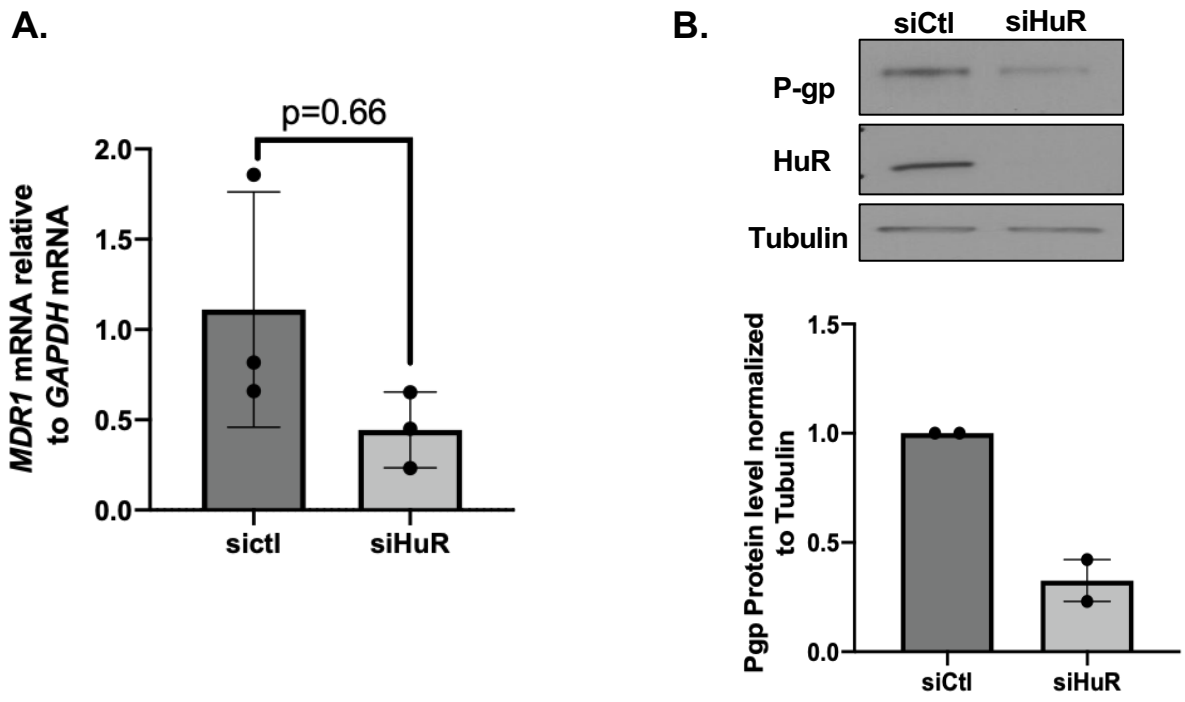

Figure 8: HuR promotes P-gp and *MDR1* mRNA expression in drug resistant KB-V1 cells.

A) Total RNA was isolated from KB-V1 cells transfected with siRNA targeting HuR or a control siRNA and RT-qPCR analysis was performed using primers for MDR1 and GAPDH. Data presented are +/- the S.E.M. of three independent experiments with *P<0.05, by unpaired *t*-test.

B) (**Top**) KB-V1 cells were transfected with siRNA targeting HuR or a non-specific control siRNA (siCtl). Lysates were used for western blot analysis using antibodies against P-gp, HuR, and α-tubulin. (**Bottom**) Densitometric quantification of P-gp level are representative of two independent experiments. Values were quantified using ImageJ, normalized to tubulin and shown relative to siCtl.

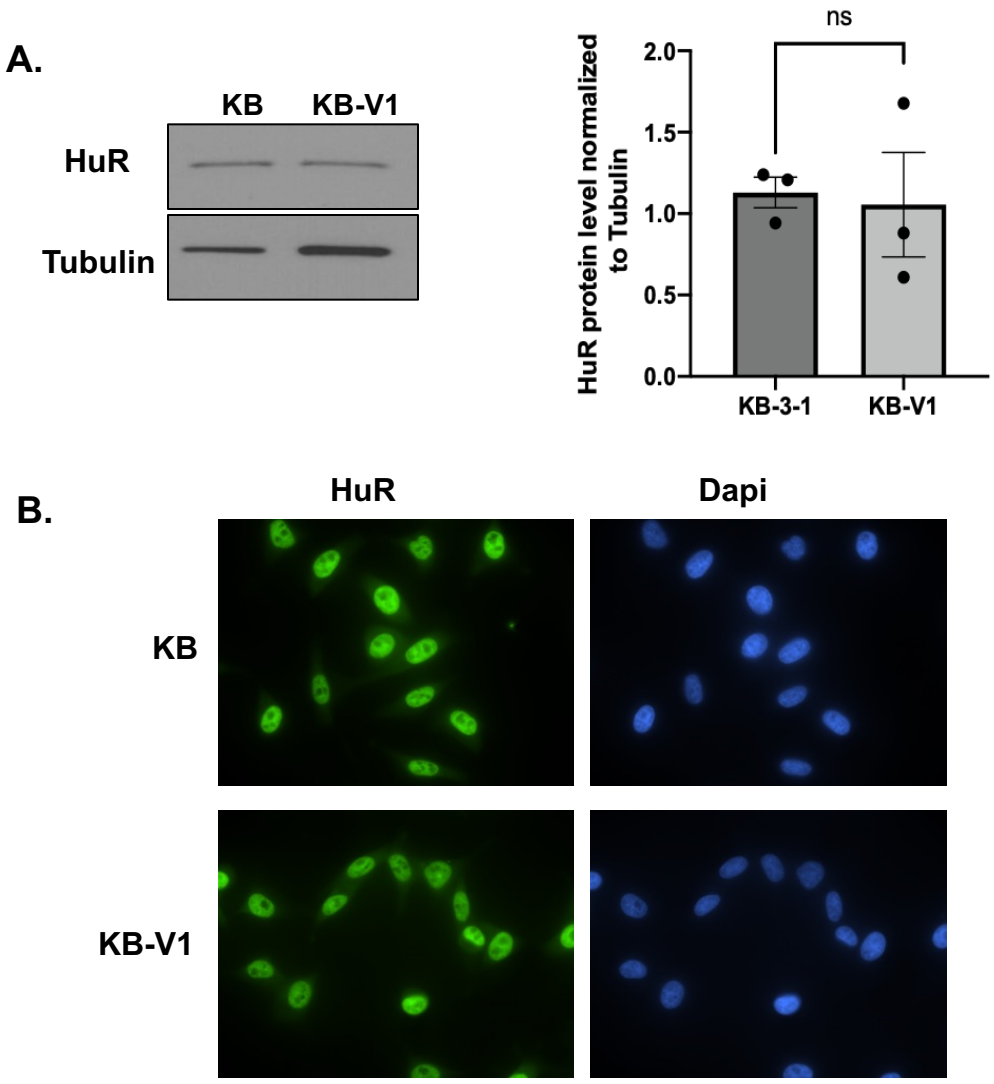

Figure 9: HuR protein levels and cellular localization are similar in multidrug resistant KB-V1 cells and parental non-resistant KB cells.

A) (**Left**) Lysates from both KB-V1 and KB-3-1 cell lines were used for western blot analysis using antibodies against HuR and α-tubulin. (**Right**) Densitometric quantification of HuR signals in the western blot relative to α-tubulin signal. Values were quantified using ImageJ. Blots shown in the figure are representative of 3 independent experiments.

B) KB-V1 and KB cells were fixed, permeabilized, and stained with antibodies against HuR and DAPI and subjected for immunofluorescence microscopy. Images are representative of 3 independent experiments.

5. HuR associates with the *MDR1* mRNA in drug-resistant KB-V1 cells

Previously published studies showed that the 3'UTR sequence of *MDR1* mRNA, which is seventy percent AU- rich, is important for its expression. Moreover, the 3'UTR of *MDR1* mRNA was shown to be bound by HuR, which resulted in mediating its stability in CML cells [13]. However, the exact HuR binding site in the *MDR1* 3'UTR has not yet been identified. To identify whether HuR binds to *MDR1* mRNA in our KB-V1 cell system, we performed RNA immunoprecipitation (RIP)-coupled to RT-qPCR experiments using anti-HuR and anti-IgG antibodies (used as a negative control). Our results showed that HuR binds *MDR1* mRNA in KB-V1 cells but not the parental cell line (Figure 10A). Subsequently, we sought to determine whether HuR interacts with the AU-rich region of the *MDR1*-3'UTR. To this end, an RNA electromobility shift assay was performed, using the recombinant GST-HuR and GST proteins and radiolabeled cRNA probe spanning the MDR1 AU-rich element. Results demonstrated, by performing these experiments, that HuR, but not the GST negative control, binds to the AU-rich region in the 3'UTR of *MDR1* mRNA.

In conclusion, our results demonstrate that the differential expression of *MDR1* mRNA and its protein product, P-gp, in KB cells versus their multidrug resistant counterparts, KB-V1 cells, is due to a differential regulation by HuR, which is mediated by the binding of HuR to an AU-rich element in the 3'UTR of the *MDR1* message.

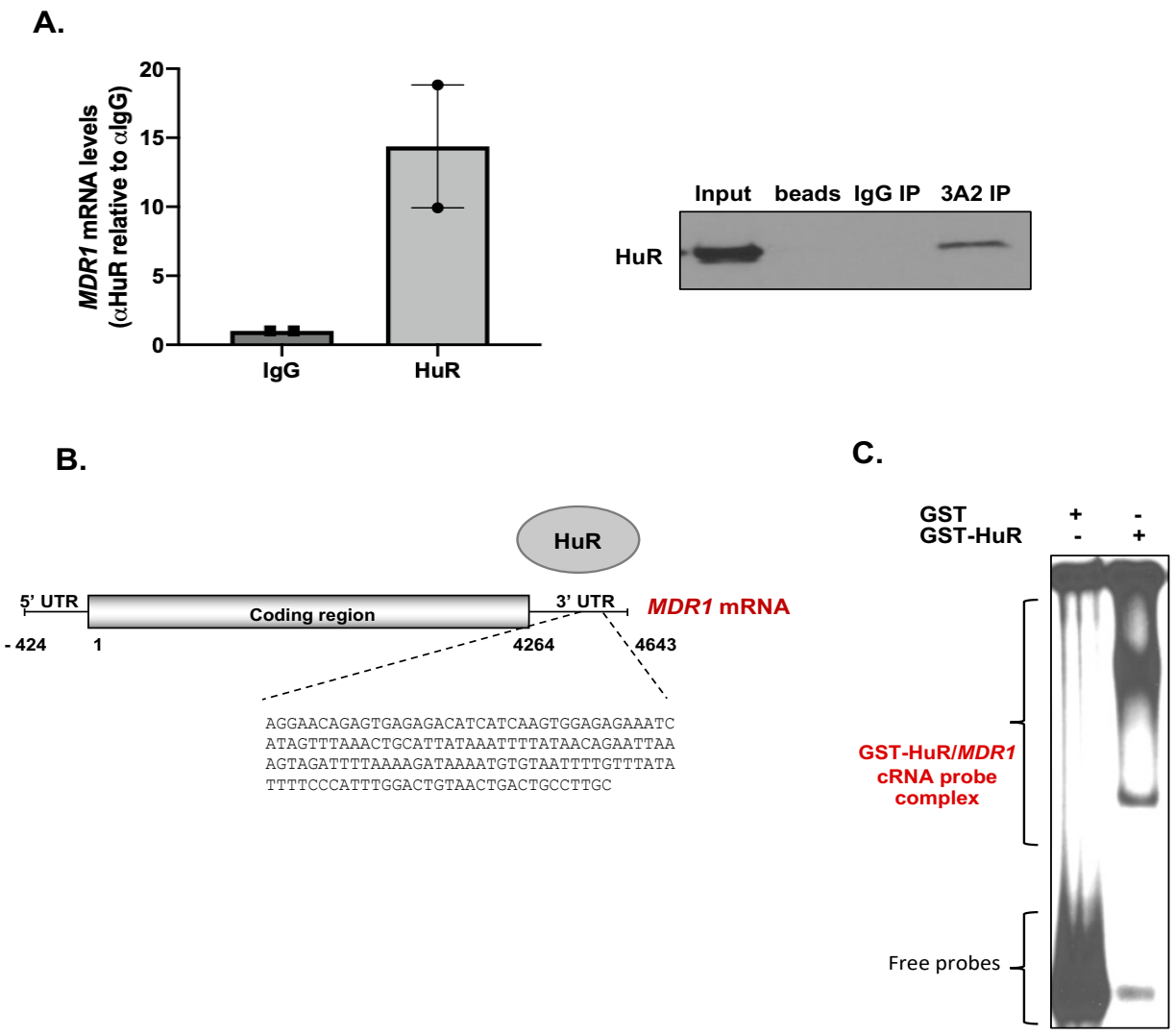

Figure 10: HuR associate with MDR1 mRNA through an AU-rich region located in its 3'UTR.

A) (**Left**) RNA-Immunoprecipitation coupled to RT-qPCR experiments were performed using anti-HuR (3A2) or anti-IgG antibodies on total extract from KB-V1 cells. (**Right**) western blot assessing the immunoprecipitation of HuR. Data shown are representative of two independent experiments.

B) Schematic representation of the human MDR1 cDNA and location of the region within its 3'UTR that was used to generate a radiolabeled RNA probe for RNA electromobility shift assay (REMSA).

C) Gel-shift binding assay performed by incubating 500 ng of GST-HuR with the radiolabeled MDR1 mRNA probe. The image is representative of three independent experiments.

MATERIAL AND METHODS

Cell culture, transfection, and treatment

HeLa CCL-2 cells (American Type Culture Collection), the human cervical carcinoma-derived KB-3-1 cell line and the vinblastine (VBL) resistant variant KB-V1 (DSMZ) were grown and maintained in DME (Dulbecco's modified Eagle) media (Invitrogen) containing 10% FBS (Sigma) and 1% penicillin/streptomycin (Sigma). The media for KB-V1 cells was additionally supplemented with 1ug/ml vinblastine (VBL) to maintain the full resistance phenotype. Plasmid and siRNA were transfected as described [90] using the Polyplus jetPRIME transfection reagent and 0.5ug/mL and 50nM/mL of plasmid and siRNA, respectively. Plasmid transfection was done on 80% confluent HeLa cells, whereas siRNA transfection was done on 60% confluent HeLa and KB resistant cells. siRNAs were purchased from Ambion: siPARP1 (ID: s1097), siPAPR2 (ID: 111561) and siHuR (ID: s67964). For the STS treatment (Sigma Aldrich), HeLa cells were exposed to 1uM STS for 1.5 or 3 hours. Treatments were done 24 hours post transfection. For PARP inhibitor experiments, cells were treated with 1uM Talazoparib (Selleckchem) for 24 hours.

Plasmid construction and protein purification

The GFP-HuR^{wt} and GST-HuR plasmids were generated as described [17]. The GFP-HuR^{pbmt} and GST- HuR^{pbmt} plasmids were generated (by mutating the Histidine and Arginine amino acids to Alanines) by Norclone Biotech Laboratories (London, ON, Canada). The GST, GST-HuR^{wt} and GST-HuR^{pbmt} recombinant proteins were generated by transforming BL21 with the respective plasmids. The expression of the proteins was induced by IPTG (0.5mM for 4hours at 37°C) in a 1-liter culture. The bacteria were collected and lysed. The GST proteins were pulled down using Glutathione Sepharose beads and processed as previously described [21].

Protein extraction and immunoblotting

Total cell extract from HeLa cells and KB parental and resistant cells were prepared as described previously [94, 95]. Briefly, cell extracts were lysed with mammalian lysis buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton, 10 mM pyrophosphate sodium, 100 mM NaF, 1 mM EGTA, 1.5mM $MgCl_2$, 1 X protease inhibitor (Roche) and 0.1 M orthovanadate), then lysates were collected after centrifugation for 15 minutes at 12000rpm. Western blotting was performed as described [17] using the following antibodies: HuR (3A2 [96], 1:1000), α-tubulin (Developmental studies Hybridoma Bank, 1:10000), Cleaved Caspase-3 (Cell signaling, 1:1000), full length PARP (Cell signaling, 1:1000), GFP (JL-8, Living colors, 1:1000) and P-glycoprotein (Invitrogen, 1:1000). Quantification of bands on western blot was done using ImageJ (Fiji) software and normalized to α-tubulin. Statistical analysis for significance was performed using GraphPad software with a one-tailed unpaired *t*-test.

Binding (Dot/Slot blot) assay and peptides mapping experiments

These experiments were performed as described [88]. Briefly GST, GST-HuR^{wt}, GST-HuR^{pbmt}, Histone (positive control) and BSA (negative control) or peptides spanning the HuR protein (fragmented into 63 peptides; each is 20 amino acids in length) were dot-blotted directly onto nitrocellulose membrane. The blot was then rinsed three times with TBST (Tris-buffered saline with 0.1% Tween 20 detergent) and incubated with radioactive pADPr (^{32}P-pADPr) generated by auto-modified PARP1, washed, and probed for retention of the pADPr. After incubation for 1 hour at room temperature with gentle agitation, the membrane was washed, dried, and subjected to autoradiography. Peptides/ full length proteins were incubated with Sypro Ruby stain in order to demonstrate their integrity and event distribution.

Immunoprecipitation

Immunoprecipitation experiments were performed as previously described [97, 98]. Briefly, antibodies against anti-PAR 10H clone (Tulips) , anti-TRN2 [98], and anti-PP32/PHAPI (Santa Cruz), anti-HuR (3A2) [96] and/or IgG (Jackson ImmunoResearch Laboratories) were incubated with 60 µl of protein A-Sepharose slurry beads (GE Healthcare) (washed and equilibrated in cell lysis buffer) for 4h at 4 °C. Beads were washed three times with cell lysis buffer (25mM Tris-HCl pH 8.0, 650mM NaCl, 0.05% Tween-20, 100mM NaF, 1X protease inhibitors) and then incubated with 800 µg of total cell extracts (TCE) overnight at 4 °C. Beads were subsequently washed three times with cell lysis buffer and Lamelli dye was added to the immunoprecipitated proteins for analysis by western blot.

Immunofluorescence

Immunofluorescence was performed as previously described [54]. Cells were fixed in 3% Paraformaldehyde (Sigma) for 20min and then permeabilized with 0.1% Triton-X 100 in PBS-goat serum for 20 min. After permeabilization, cells were incubated with primary antibodies against HuR/3A2 (1:1500) in 1% normal goat serum/PBS at room temperature for 1 hr. The cells were then incubated with the secondary antibody (Alexa Fluor® 488) and 4',6-diamidino-2-phenylindole (DAPI) (for nuclear staining). Images were taken at room temperature with a 63X oil objective on an inverted Axiovert 200M microscope with an Axiocam MRm digital camera (Zeiss).

Annexin V–Cy5/PI assay

Cells in the tissue culture dishes were collected 24h post-transfection using trypsin to detach them from the plates. Cell pellets were then processed as described by the apoptosis detection reagent kit protocol (Abcam; ab14147). Apoptotic and necrotic cells were identified by

annexin V–Cy5 and Propidium Iodide (PI) staining, respectively, using the flow cytometry analyzer (FACSCanto II). The flow cytometry work was performed in the Flow Cytometry Core Facility for flow cytometry and single cell analysis of the Life Science Complex. The data analysis was done using FlowJo software.

Quantitative RT-qPCR

RNA was extracted from cell extracts using Trizol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using the 5X iScript reagent (Bio-Rad) according to the manufacturer's protocol. Each cDNA sample was diluted 20 folds and used to detect the mRNA levels of *PARP1, PARP2, MDR1* and *GAPDH (*used as a loading control) using the SsoFast EvaGreen reagent (Bio-Rad Laboratories). The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt is the difference in Ct values between the target and reference genes (GAPDH). Primers used for qPCR are as follows. PARP1 (F: 5'-CCC AGG GTC TTC GAA TAG-3', R: 5'-AGC GTG CTT CAG TTC ATA C-3'), PARP2 (F: 5'-GGA AGG CGA GTG CTA AAT GAA-3',R: 5'-AAG GTC TTC ACA GAG TCT CGA TTG-3'), GAPDH (F: 5' - AAG GTC ATC CCA GAG CTG AA - 3', R: 5' - AGG AGA CAA CCT GGT CCT CA – 3'), MDR1 [99] (F: 5'-GGGAGCTTAACACCCGACTTA-3', R: 5'-GCCAAAATCACAAGGGTTAGCTT-3').

RNA electrophoretic mobility-shift assay (REMSA)

500ng of GST or GST-HuR were incubated with 50 000 cpm of ^{32}P-labelled cRNA in a total volume of 20 ul EBMK buffer (25 mM HEPES pH 7.6, 1.5 mM KCl, 5mM $MgCl_2$, 75 mM NaCl, 6% sucrose, and protease inhibitors) at RT for 15 minutes. Two microliters of a 50 mg/ml heparin sulfate stock solution were then added to the reaction mixture for an additional 15 min at

RT to prevent nonspecific protein- RNA binding. Finally, 1ul of loading dye was added and samples were loaded on a 4% polyacrylamide gel containing 0.05% NP-40 and ran for 2 hr. at 180 V. Gels were then fixed in 7% acetic acid/10% ethanol, dried, and exposed O/N at -80°C.

DISCUSSION

In this study we identify PARylation as a regulatory mechanism that modulates the function of HuR in determining cell fate. Our results show that PARP1/2-mediated PARylation prevents the accumulation of HuR to the cytoplasm resulting in a decrease in its cleavage, and inhibition of HuR's pro-apoptotic function. We demonstrated that the combined depletion of PARP1 and PARP2 increases the cytoplasmic accumulation of HuR and thus increases its cleavage. HuR cleavage, consequently, increases its pro-apoptotic function as evidenced by the significant increase in the level of caspase-3 cleavage and in the number of apoptotic cells. Furthermore, we showed that PAR binds HuR non-covalently through a consensus motif and that this binding is required for the nuclear localization of HuR as well as its association with the import factor TRN2. Indeed, we found that mutating HuR-PBS prevented PAR from binding to HuR resulting in the cytoplasmic accumulation of HuR and therefore advancing apoptosis. Thus, our work provides evidence for the importance of the PARP-mediated PARylation and the resulting PAR binding to HuR in regulating the function of HuR during apoptosis (Figure 11).

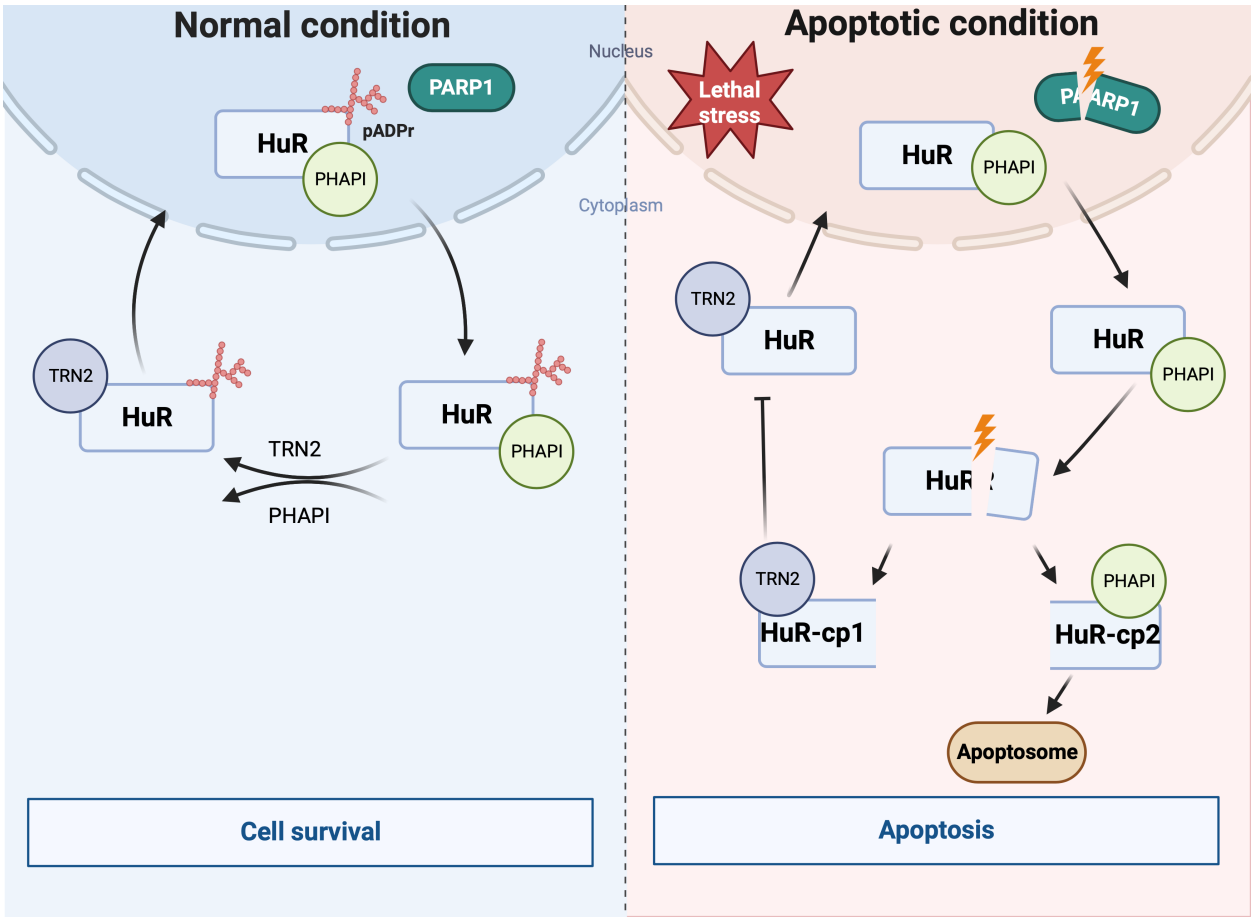

Figure 11: Proposed model

Model depicting the mechanism by which HuR association with PAR polymers regulates its apoptotic function. Under normal condition, HuR interacts with PAR polymers through its PAR Binding Site (HuR-PBS) maintaining its nuclear localization by promoting its interaction with the import factor TRN2. In response to a lethal assault, HuR loses its binding to PAR concurrently with the cleavage of PARP1. HuR/PHAPI translocate to the cytoplasm where HuR undergoes caspase mediated cleavage yielding HuR-CP1 and HuR-CP2. While HuR-CP2 interact with PHAPI mediating the activation of apoptosome-formation, HuR-CP1 interacts with TRN2 preventing the reuptake of HuR back to the nucleus. HuR, therefore, accumulates in the cytoplasm, advancing apoptosis.

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Although PARylation of HuR has been previously shown to regulate the function of HuR during inflammation [58, 63] as well as muscle cell differentiation (Mubaid *et al.* unpublished data), the importance of this modification on HuR function in cell fate was not assessed. Indeed, recent studies by Ke *et. al* revealed that, in response to inflammatory stimuli, PARP1- mediated PARylation of HuR binds to its HNS-RRM3 region and modifies it at a conserved aspartate residue D226. They demonstrated that mutating this site (D226) or inhibiting PARP impacted HuR localization, its ability to associate with pro-inflammatory messages as well its oligomerization [58, 63]. More recently, our lab uncovered that PARylation of HuR by Tankyrase1 (TNKS1), also known as PARP5a, promoted HuR cytoplasmic accumulation and cleavage as well as its ability to associate with promyogenic mRNA during myogenesis (Mubaid *et al.* unpublished data). In this study, however, we identified a consensus PAR binding motif located within the HNS of HuR, and we showed that PARP1/2-mediated PARylation and PAR binding to HuR through this identified motif mediates its subcellular localization and function during apoptosis.

Although HuR is predominantly a nuclear protein under normal conditions, its HNS domain encompasses a nucleocytoplasmic shuttling sequence allowing it to shuttle between the nucleus and the cytoplasm in response to various stimuli, such as stress signals [18, 61, 69, 98]. This translocation is important for the HuR-mediated post-transcriptional regulation of many mRNA targets including mRNA localization, stabilization, and translation, and has been shown to have physiological relevance by affecting cell fate and muscle cell differentiation [17, 54, 98, 100]. Several studies have reported that post-translational modification (PTMs) of residues within the RRMs influenced the function of HuR in regulating RNA metabolism, while modification of residues within or near the HNS impacted HuR subcellular localization [55, 56, 61]. For example, phosphorylation of HuR by Chk2 at HuR residues S88, S100 and T118 located within RRM1 and

RRM2 modulates HuR binding to SIRT1 mRNA and other mRNA targets [59]. On the other hand, phosphorylation by cdk1 at S202 facilitated HuR binding to the nuclear 14-3-3 triggering its nuclear retention [60, 61]. Previously, our lab and others have shown that the localization of HuR is dependant of its HNS, which mediates the differential association of HuR with protein partners for nuclear export, such as PHAPI and APRIL, and with the import factors TRN1-2, and importin α [17, 21, 101]. It has been shown that, under normal condition, HuR is localized mainly to the nucleus [17]. However, in response to lethal stress, HuR and PHAPI translocate to the cytoplasm where it is cleaved in a PKR- dependent pathway by caspase-3 and -7 yielding two cleavage products (HuR-CP1 and HuR-CP2) [16-18, 53]. Moreover, our lab showed that HuR-CP1 associate with TRN2 preventing the nuclear reuptake of HuR, thus causing HuR to accumulate in the cytoplasm [54]. In this present study, we identified the non-covalent binding of PAR to HuR as a regulatory mechanism mediating its association with these partners and therefore, its pro-apoptotic function. Given that the HuR-PBS is located in the HNS, it is not surprising that it regulates the localization of HuR. Our results demonstrate that an intact PAR binding to HuR is required for its binding with TRN2 in particular, and that mutating this site resulted in the loss of this binding. As HuR binding to different protein partners seems to influence the function of HuR during apoptosis, it would be valuable to determine if mutating the PBS on HuR would have an impact on HuR association with different protein ligands in other cell systems. Also, as previous observations highlighted the importance of HuR cleavage products during the onset of apoptosis, it would be interesting to investigate the role of PARP mediated modification on HuR and/or binding to PAR on the role of HuR-CPs as pro-apoptotic players.

In addition to the ability of PARylation to covalently modify acceptor protein at specific residues, a number of proteins, also known as PAR readers, can be modified by the non-covalent

attachment of PAR polymers to consensus PAR binding motifs [16, 28]. In fact, many RBPs have been shown to be bound by PAR covalently and non-covalently, both of which lead to the alteration of their functions [16]. Previous studies have reported that HuR is covalently PARylated by PARP1 at the D226 residue in LPS-induced cells, which affected its localization and function [58, 63]. Moreover, HuR also has been shown, in a proteome-wide analysis of PAR-associated proteins, to bind PAR non-covalently [81]. In our study we confirmed that PAR binds HuR non-covalently and we identified the consensus PAR binding site and showed its physiological importance in the anti-apoptotic function of HuR. Although the covalent PARylation of HuR at the D226 residue is critical in regulating its function and localization in macrophages [58], our results indicate that this modification is likely not involved in regulating HuR function in normal HeLa cells. Indeed, we observed that mutating the PAR-binding site on its own was sufficient to completely inhibit the pulldown of HuR due to the immunoprecipitation of PAR from normal Hela cells (Figure 3D) and that, furthermore, the HuR-PAR binding mutant is localized to the cytoplasm in these cells where it is cleaved (Figure 4 and 5).

Many recent studies are now pointing to the importance of these two manners of PARylation on the function of their substrates and how there may be an interplay between the two modifications [61, 64, 72]. For instance, heterogeneous nuclear ribonucleoprotein A1(hnRNPA1), a well-known RBP, has been shown to be PARylated covalently and it can also bind to PAR or PARylated proteins non-covalently. Recently, Duan *et al*. showed that hnRNPA1 is PARylated on Lysine 298 and mutating this site decreased its PARylation level and affected its localization[62]. They also showed that when the PAR binding motif is mutated, it increased its covalent PARylation [62]. These observations led them to suggest that the non-covalent PAR binding reduces the hyper-PARylation of hnRNPA1. Therefore, it would not be surprising that such an

interplay exists between the covalent and non-covalent PARylation of HuR during apoptosis and potentially other systems. Importantly, this study shows that mutating the non-covalent PARylation binding site prevented oligomerization of hnRNPA1 and prevented the formation of stress granule [62]. This impact is not surprising, since PARylation is suggested to nucleate membranelles organelles, including stress granules [64, 72, 77]. HuR has been shown to locate in membranelles organelles and is well known to form oligomers, which might be potentially regulated by PARylation, similar to hnRNPA1 [77]. It is thus possible that the non-covalent binding of HuR can prevent, as was shown for hnRNP A1, its covalent modification by PARPs under normal conditions. In doing so, this event may explain the differential role of HuR in modulating the survival or death of cells under normal or stress-induced conditions.

Our work, thus, has furthered our understanding of the role of HuR in apoptosis, showing that it is regulated by PARylation. Moreover, understanding the regulatory mechanism underling the pivotal role of HuR in cell fate will bring a new hope to find therapies to overcome many diseases, such as numerous cancers, that are associated with the increased cytoplasmic localization of HuR.

In the second part of this thesis, we identified the importance of HuR in the regulation of the expression of *MDR1* mRNA in KB human cervical adenocarcinoma cells. We showed that *MDR1* mRNA and P-gp protein are expressed differentially in KB and KB-V1 cells, drug sensitive and drug resistant cells, respectively. Moreover, we demonstrated, by western blot and RT-qPCR analysis, that the expression of both *MDR1* mRNA and P-gp protein is only present in the KB-V1 cells but not in the parental (drug sensitive) cell line. Moreover, we showed that HuR knockdown decreased the expression of *MDR1* mRNA and P-gp protein in the drug resistant KB-V1 cells. We elucidated that the effect seen is not due to a variation in HuR expression nor in HuR cellular localization. Additionally, using RNA-IP and REMSA, we showed that HuR binds specifically to an ARE in *MDR1* mRNA in drug resistant KB-V1 cells. Together, this work provides insight on the HuR-mediated regulation of the *MDR1* mRNA thus linking HuR to the resistance of cells to drug treatment.

Previously published studies showed that the 3'UTR sequence of *MDR1* mRNA, which is 70% AU- rich, is important for its expression. In parallel, HuR is known to bind the 3'UTR of various ARE-containing mRNAs and regulate their gene expression at multiple levels. Indeed, a recent study in the human ovarian cancer (OC) cells showed that HuR specifically binding to the *MDR1* mRNA 3′UTR, but not its 5′UTR or coding area, enhances its stability and therefore increases its expression. Additionally, HuR was shown to interact with *MDR1* mRNA 3'UTR thereby mediating its stability in CML cells [51]. However, the exact HuR binding site in the *MDR1* 3'UTR has not yet been determined. In our study we identified, by performing an RNA electromobility shift assays using the recombinant GST-HuR protein, that HuR interacts specifically with an AU-rich region in the MDR1-3'UTR. However, the functional relevance of this AU-rich motif bound by HuR on the expression of the *MDR1* mRNA is yet to be identified.

The next steps of the project thus would involve identifying the minimum binding site within the ARE mediating the binding to HuR and, furthermore, validate the functionality of the potential HuR binding site (HuR BS) in regulating the expression of *MDR1* mRNA. To do so, we will generate Renilla-luciferase (R-*Luc*) constructs that express wild-type MDR1-3′UTR or the MDR1-3′UTR in which the HuR BS is mutated. Then, using these reporter constructs we will determine the impact of mutating this site on the luciferase activity of the constructs, and in the ability of HuR to associate with their mRNAs. Next, we will assess the impact of the identified HuR BS on the expression of *MDR1* mRNA in the drug resistant KB-V1 cells, by assessing the mRNA levels of the mutant construct compared to the wildtype construct. We will also perform rescue experiment whereby MDR1 depleted cells are transfected with constructs expressing full length *MDR1* mRNA with and without mutation of the HuR BS identified above. We hypothesize that the full-length MDR1 3'UTR isoform that will be used in these experiments will rescue the depletion of *MDR1* mRNA, while the expression of the HuR BS mutant construct will not rescue the expression of *MDR1* mRNA, which would confirm that HuR promotes the MDR phenotype of these cells.

It is well established by our laboratory and others that the association of HuR with various protein partners modulates the function of HuR in various processes [42]. In fact, HuR was shown to complex with various RBPs such as KSRP, TTP, and YB-1 to promote the stability or the decay of various ARE-containing mRNAs [42-44]. As such, HuR can differentially influence the expression of many mRNA targets encoding proteins involved in several cellular pathways including cell proliferation, cell stress response, apoptosis, cell differentiation, senescence, as well as inflammation [44]. However, it is not yet known if HuR associates with protein partner to mediate the regulation of *MDR1* mRNA expression in drug resistant cells. Hence, as a next step

we will perform a pull-down experiments, followed by mass-spectrometry analysis to identify HuR protein ligands in both KB-3-1 and KB-V1 cells. Since HuR is known to collaborate with other RBPs to stabilize target messages, we will focus on determining the impact of the main RBPs known to interact with HuR to promote the stability of target messages on the function of HuR during multidrug resistance of cancer cells. We will perform RNP-immunoprecipitation experiments using lysates depleted of these RBP ligands or HuR and assess the ability of HuR and these RBPs, respectively, to bind to *MDR1* mRNA. As such, we will identify the network of RBPs that regulate the expression of MDR1.

As mentioned earlier, one of the ways that HuR is able to mediate its cellular trafficking and functions in various systems is modulated by PTMs [59, 60, 102, 103]. Up to date, little is known about the involvement of PTM in mediating the role of HuR in drug resistance. In fact, HuR has been shown to be stabilized by NEDDylation mediated by the murine double minute 2 (Mdm2) protein in ovarian cancer cells resistant to carboplatin [102]. This study showed that the treatment of ovarian cancer cells with the NEDDylation inhibitor MLN4924 inhibited the NEDDylation process of HuR and overcame the drug resistance of ovarian cancer cells to carboplatin [102]. Additionally, it was shown in another study by Latorre *et al.* that the doxorubicin-induced apoptosis effect in breast cancer (MCF-7) cells is dependent on the translocation of HuR to the cytoplasm and on its phosphorylation upon doxorubicin exposure [103]. Moreover, treating MCF-7 cells with rottlerin, known for its ability to block HuR phosphorylation, inhibited the translocation of HuR, consequently maintaining HuR in the nucleus, and decreasing the doxorubicin-induced apoptosis. Although other PTMs, such as PARylation have been shown recently to play an important regulatory role in HuR functions in various processes, including apoptosis in the first part of this present study, the potential role of

PARylation in drug resistance has never been investigated. Thus, it would be of great interest to identify the impact of such modifications on the HuR-mediated multidrug resistance of cancer cells, in general, and on the expression of MDR1 in KB-V1 cells, in particular.

Although several previous studies revealed that HuR is necessary to evoke the apoptotic effect mediated in response to chemotherapeutic treatment in many cancer cells, only a few mechanisms were reported on how HuR regulates apoptosis in drug resistant cancer cells. For instance, a study by Lin *et al.* revealed that the downregulation of HuR intensified the cytotoxic effect of epirubicin (Epi)-induced drug resistance in colorectal cancer cells [38]. Moreover, they showed that treating cells with an siRNA targeting HuR alone or in combination with Epi decreased *Bcl-2* mRNA levels while increasing *Bax* and *caspase-3 and -9* mRNA levels thereby, enhancing the Epi-induced apoptosis [38]. On the contrary, downregulating HuR expression in breast cancer cells decreased doxorubicin-induced apoptosis due to the decrease in the doxorubicin-mediated cytoplasmic translocation of HuR [103]. Importantly, although HuR in the latter study shows an increase in its cytoplasmic localization upon doxorubicin treatment, it did not appear to undergo caspase-mediated cleavage. This abnormal behaviour of HuR might be the foundation of the anti-apoptotic response of the cells, since it is opposing to our previous reports demonstrating that the cytoplasmic concentration of HuR in other cell system correlates with its ability to be cleaved and therefore its ability to bind and stabilize the pro-apoptotic mRNAs such as *caspase-9* thus inducing apoptosis. Additionally, these observations advocate the need of further studies investigating the importance of HuR cleavage in various cancer cell and imply whether HuR cleaved product (HuR-CP1 and -CP2) contributes to the drug resistance in these cells. Together, these findings and others provide insight into the importance of the role of HuR in regulating apoptosis induction and circumventing MDR. Moreover, these studies suggest that

targeting HuR could be a useful therapeutic strategy to induce apoptosis in many cancer cells exhibiting resistance to many chemotherapeutic drugs.

All in all, this work provides evidence that PARylation acts as a regulatory mechanism controlling the apoptotic function of HuR which can be potentially targeting therapeutically. It provides further insight on the activity of HuR in cancer, as well as its role in promoting MDR, urging for the need of further studies to discover therapeutic strategies to target the function of HuR in cancer. Thus far, HuR inhibitors have been shown to be cytotoxic, due to the pleotropic function of HuR. Therefore, discovering regulatory mechanisms, such as PTMs, could prove beneficial in targeting specific functions of HuR, including the function of HuR in MDR. For instance, if PARylation proves to regulate the function of HuR in MDR, PARP inhibitors may be useful, especially that PARP inhibitors are FDA-approved and already used in cancer therapy.

APPENDICES

i. Extended List of Publications

Journal Publications

1. Sánchez, B.J., Busque, S., de los Santos, Y.L., **Ashour**, **K.**, Sadek, J., Lian, X.J., Khattak, S., Di Marco, S., Gallouzi, I.E. The formation of HuR/YB1 complex is required for the stabilization of target mRNA to promote myogenesis. Nucleic Acids Research, Volume 51, Issue 3, 22 February 2023, Pages 1375–1392, https://doi.org/10.1093/nar/gkac1245
2. Mubaid S., Ma J.F., Omer A., **Ashour K.**, Lian X.J., Sanchez B.J., Robinson S., Cammas A., Dormoy-Raclet V., Di Marco S., Chittur S.V., Tenenbaum S.A., Gallouzi I.E. HuR counteracts miR-330 to promote STAT3 translation during inflammation-induced muscle wasting. Proc Natl Acad Sci U S A. 2019 Aug 27;116(35):17261-17270. doi: 10.1073/pnas.1905172116. Epub 2019 Aug 12. PMID: 31405989; PMCID: PMC6717253.
3. Sadek J., Omer A., Hall D., **Ashour K.**, Gallouzi I.E.. Alternative polyadenylation and the stress response. Wiley Interdiscip Rev RNA. 2019 Sep;10(5):e1540. doi: 10.1002/wrna.1540. Epub 2019 May 2. PMID: 31050180.

Papers in Preparation or under Revision

1. **Ashour**, **K.,** Hall, D., Mubaid, S., Lian, X.J., Gagné, J.P., Di Marco, S., Poirier, G.G., and Gallouzi, I.E. (To be submitted shortly, 2023).
2. Mubaid S., Adjibade P., Hall D., Busque S., Lian X.J., **Ashour K.**, Tremblay A.M., Carlile G., Gagné J.P., Di Marco S., Thomas D., Poirier G., Gallouzi I.E. Tankyrase-1 regulates RBP-mediated mRNA turnover to promote muscle fiber formation. (In Revision, Nucleic Acid Research, 2023)

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