Altered nuclear import and function of p53 in calreticulin deficient cells

By

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A Thesis
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Altered Nuclear Import and Function of p53 in Calreticulin Deficient Cells

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

p53 (transcription factor) is a cell cycle regulatory protein, which is activated by DNA damage (e.g. UV irradiation, chemical treatment, etc.) and more specifically by DNA damage repair intermediate proteins [1-3]. Stimulation of p53 can induce cell cycle arrest or programmed cell death (apoptosis) [2]. Functions of p53 are achieved by the regulation of downstream targets of p53 including Bax, MDM2 and $p21^{WAF}$ which force the cell down one of these pathways. Loss of p53 function can result in an escape from proper cell cycle regulation and increased proliferation (cancer) [4]. Recently, calreticulin (CRT, an endoplasmic reticulum protein) has been shown to affect the rate of induction of apoptosis [5, 6]. Absence of CRT has also been shown to result in impaired nuclear import of nuclear factor of activated T cells (NF-AT3) [7] and myocyte enhancer factor C2 [8]. Therefore, the aim of this study was to investigate the role of CRT in the nuclear import of p53 and regulation of p53 function.

Mouse embryonic fibroblast cells expressing different levels of CRT (wt, *crt-/-* and CRT-*crt-/-*) were used to examine CRT's role in modulation of p53. Using these cell lines we have observed a significant decrease in p53 function. We also showed that this is mediated by a role of CRT in p53 protein stability/rapid degradation and nuclear localization of the p53 protein. Furthermore, we have shown the nuclear accumulation of MDM2 in *crt-/-* cells, suggesting increased ubiquitination and induced nuclear export of p53 protein. We also demonstrated the altered nuclear import of endogenous and over-expressed p53 in both non-treated and UV stressed conditions. These results in addition to a previous report on

decreased Akt/PKB activity in the CRT over-expressing cells, lead us to conclude that CRT regulates p53 function by affecting its stability and nuclear localization.

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I would be remiss if I didn't send a "shout out" to my lab-mates Tina, Lise, Melanie and Lori. Your friendship and assistance has been of monumental importance to me. To all the members within the Division of Stroke and Vascular Disease, your friendship and advice made my time at St. Boniface amazing.

To my friends and family, what can I say? I couldn't have done it without you! Your ability to divert my attention from the negatives and re-energize me with your words and actions meant the world to me. I thank you from the bottom of my heart.

LIST OF FIGURES

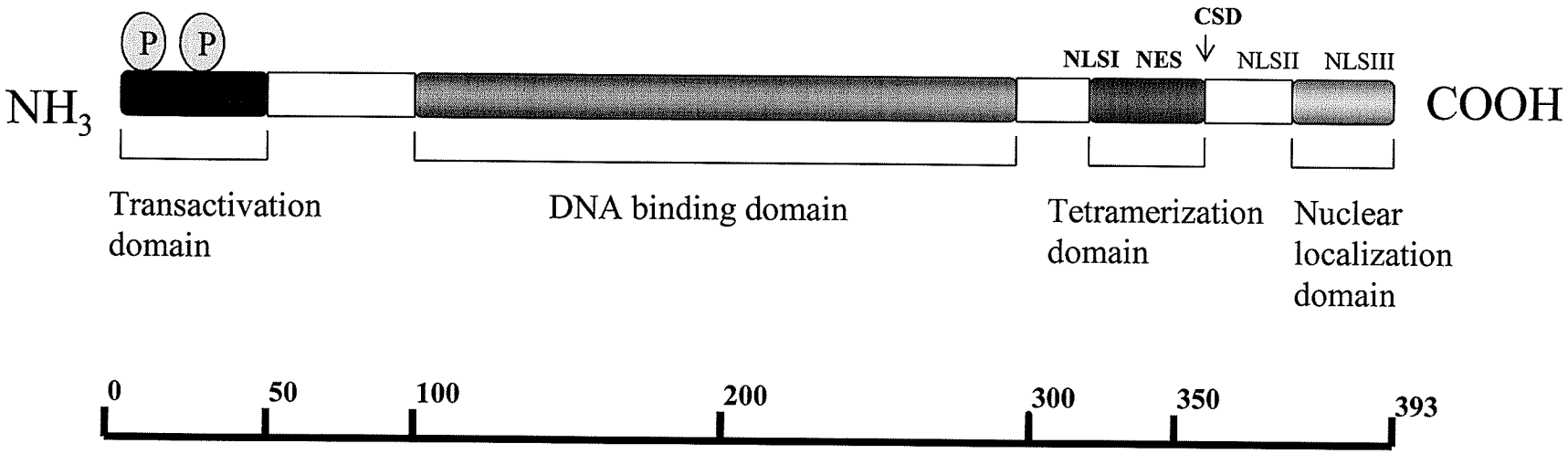
A. REVIEW OF LITERATURE

I. p53

1. p53: Protein structure and domains

p53 is a highly conserved phosphoprotein, which contains about 393 amino acids and can be subdivided into four domains **(Figure 1)**. The amino terminal domain is termed the transactivation domain and consists of a highly charged acidic region of about 50 residues [9]. Conservation within this domain, specifically amino acids 13-23 of p53, has been shown in a variety of diverse species (human, monkey, hamster, rat, chicken, mouse, trout and *Xenopus laevi*) [10, 11]. Amino acids within this conserved region (Phe19, Leu22, and Trp23), as shown by Lin *et al.* (1995) [12], are required for transcriptional activation of p53. The amino terminal region of p53 is known to be important in protein stability, dissociation from DNA and subcellular localization [13-15]. Several residues within this domain are potential targets for numerous cellular kinases such as ATM (ataxia telangiectasia), ATR (ataxia telangiectasia related), DNA-PK (DNA-activated protein kinase), Chk1 and Chk2/Cds1 (checkpoint kinase 1 and 2) [16, 17]. In fact, phosphorylation of amino acid residues 15 and 20 specifically is known to stabilize p53 and protect it from rapid proteolysis [13]. Addition of phosphate groups to these amino acids inhibit the negative regulation of p53 by blocking murine double minute clone 2 (MDM2) (HDM2 -human double minute clone 2). Binding of MDM2 to

Figure 1: Schematic diagram of p53 protein structure, showing different functional domains.



residues Phe 19, Try 23 and Leu 26 of p53 normally results in complex formation and eventual p53 degradation [18].

A sequence-specific, hydrophobic proline-rich region of p53 is important in DNA-binding of the transcription factor (positions 102-292) [19]. Point mutations within this domain are most frequently associated with p53-related tumor formation [19]. Specific residues within this domain directly interact with the nuclear chromatin. Interaction with DNA occurs through sequence specific contact within the major groove of the DNA coil while yet other regions interact with the DNA bases via hydrogen bonds [20]. Many groups have shown this domain to be a protease resistant, independently folded domain able to bind p53 consensus oligonucleotides [21-23]. The activity of the domain is dependent on Zn^{2+} binding but differs from conventional DNA-binding motifs including the well-known Zn-finger domain [23]. The structure of this domain was determined by Cho *et al.* (1994) [24]. Residues 94-312 within this domain interact to form a β-sandwich structure [24]. Maintenance of this structure is required for proper DNA interaction [25]. El-Deiry and colleagues [25] demonstrated that activated p53 homotetramer can bind to four DNA repeat sequences 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' [25]. A symmetrical p53-binding motifs are arranged in two pairs of inverted repeats [24], suggesting that p53 interacts with DNA as a tetrameric complex [25]. This hypothesis is consistent with studies showing p53 homotetramer assembly *in vivo* and *in vitro* [26, 27].

In its active form, p53 functions as a tetramer via binding through residues 323-356 [28]. This region of the protein is known as the tetramerization domain. The secondary structure of the tetramerization domain consists of approximately 1/3 β-strand (residues 326-334) and 2/3 α-helical (residues 337-355) separated by a sharp kink [23]. Tetramer formation and DNA-binding relies on the proper folding of this domain complex [25]. Inability of p53 to form a tetramer via this domain is consistently found in a variety of human tumors [25] (will be discussed in greater detail later). p53 monomers dimerize by forming an antiparallel beta sheet and through interactions between the helices; Leu 330 and Phe 338 in the beta strands drive dimerization [29]. Two of these dimers interact through their alpha helices to form the functional tetramer; hydrophobic residues Met 340, Phe 341, Leu 344, Leu 348, Ala 347, and Leu 350 are important in tetramer formation [29]. Folding of this domain is surprisingly stable despite its small size and lack of stabilizing elements such as disulfide bridges. A model proposed by Stommel *et al.* (1999) [30] suggests that tetramerization masks the primary nuclear export signal (NES) within p53, resulting in extended nuclear retention and DNA binding. Dismantling of the p53 tetramer into monomers or dimers is required to expose the protein's NES that mediates its nuclear export [30].

The C-terminal domain is termed the nuclear localization domain. This domain regulates the localization of the p53 protein. Conserved motifs, termed nuclear localization signals (NLS), are contained within this domain and enable p53 to be imported into the nucleus. Three putative NLS motifs reside in the C-terminus. A second region which can determine p53 localization is also found within this domain, the

cytoplasmic sequestration domain (CSD) (residues 326-355) [31], and can block the binding of p53 to nuclear import machinery, importin α, and inhibit its nuclear import. Nuclear translocation will be discussed in detail later in the introduction. The conversion of latent to active p53 was shown to be determined by modifications that occur in the C-terminal domain [32]. Acetylation of key lysine residues within the C-terminus signals this switch of activity [33]. Another function of the C-terminal domain is the ability to interact with damaged DNA and to stimulate the association of single-stranded DNA (as in after DNA excision repair) to double stranded [34].

2. Localization

i. Cytoplasm-Nucleus

In normal cells, p53 continually cycles between cytoplasm and nucleus throughout the cell replication cycle. p53 accumulates in the cytoplasm during G_1 phase and translocates into the nucleus during the G_1/S transition [35]. Inability to accomplish the proper cycling between nuclear import and export can result in cytoplasmic accumulation of p53 and various forms of human cancers (breast cancers, colon cancers, and neuroblastoma) [4]. Shuttling between nucleus and cytoplasm is controlled by both the nuclear localization sequence (NLS) and nuclear export sequence (NES). Classical NLSs consist of a single stretch of several basic amino acids. p53 contains three putative NLSs in the C-terminus [36]. NLSI (PQPKKP) (residues 316-322) has been shown to be the most active of the three in directing p53 nuclear import as well as the most extensively studied [31, 36]. Little data is available on NLSII or NLSIII of p53. Other regions of p53 can also play a role in import besides the NLSs. A basic motif containing Lys^{305}-Arg^{306} works together with NLSI to enhance nuclear import of p53 [31]. Mutation or absence of these amino acids resulted in decreased nuclear import of p53 [31]. These stretches are recognized by import machinery (importin α: docker, importin β: transporter to nuclear envelope). Loss of NLS recognition results in p53 cytoplasmic accumulation and potential cancer [37]. A C-terminal cytoplasmic sequestration domain (CSD, residues 326-355) can inhibit nuclear import of p53 [38]. Previously, CSD had been shown to interfere with importin α binding to p53 by masking access to NLSI [38].

Deletion of CSD has been shown to enhance p53 nuclear import due to increased importin α binding as determined by *in vitro* binding assay and suggests a role for CSD sequestering p53 to the cytosol [31].

Prior to activation and nuclear shuttling, latent p53 is thought to be associated with cytoskeletal microtubule networks via its N-terminus [15]. Giannakakou *et al.* (2000) [15] demonstrated the *in vitro* association of p53 with polymerized microtubules via N-terminus (amino acids 1-25) by p53-tubulin co-polymerization as well as *in vivo* in human keratinocytes (preliminary results). Removal of the N-terminus of p53 resulted in a loss of tubulin binding [15]. Furthermore, disruption of microtubule networks with paclitaxel or vincristine resulted in reduced p53 nuclear accumulation in A549 cells (human renal cancer line) as well as impaired p53 transactivation of MDM2 and p21 [15]. p53 association with microtubule motor dynein has also been suggested by immunocytochemistry and confocal microscopy. Giannakakou *et al.* (2000) [15] showed co-localization of anti-p53 as well as anti-dynein intermediate-chain antibodies. Also addition of anti-dynein antibody to cells resulted in inhibition of p53 nuclear import [15]. This 'intracellular highway' may assist p53 phosphorylation by bringing it into close proximity to protein regulators (Phosphoinositol 3 kinase (PI3) and BRC1) as well as nuclear import [15]. Latent nuclear p53, has also been observed tethered by RNA [39]. Rubbi *et al.* (2000) [39] have shown p53 preferentially linked to sites of actively transcribing genes. This observation provides evidence on how relatively few p53 proteins molecules can monitor DNA fidelity during cell replication.

ii. Nuclear Export

Nuclear export of p53 is mediated primarily by a highly conserved NES in its tetramerization domain. This NES consists of a leucine-rich stretch of amino acids. Mutation of the primary NES results in nuclear accumulation of p53 and absence of nuclear export [40]. Recently, Zhang, *et al.* (2001) [41] reported the identification of a second NES within the transactivation domain of p53 (residues 11 to 27). This N-terminal NES, in addition to the C-terminal NES, was necessary for p53 nuclear export [41]. The authors showed an inhibition of p53 export after DNA damage (UV irradiation) attributed to stress-induced phosphorylation of two lysine residues within the N-terminal NES [41] (described in more detail later). This evidence suggests another means of p53 nuclear retention post DNA damage, allowing for extended access to chromatin [41].

Exportin 1 (Crm1) mediates export of NES containing proteins. Tetramerization of p53 prevents Crm1 access to C-terminal NES, preventing nuclear export of the complex [30]. Degradation of p53 the oligomer into dimers or monomers is required for the unmasking of this NES [30]. Despite evidence presented by Zhang *et al.* (2001) [41], mutational disruption of the p53 C-terminal region has been shown to result in nuclear export inhibition [42]. Therefore, nuclear export of p53 must occur by means of another NES. MDM2 (negative regulator of p53) also contains an NES and interacts with p53 in the nucleus [43]. Nuclear export of p53 with a mutated C-terminal NES has been demonstrated to be due to the interaction between Crm1 and the NES on MDM2 [43].

3. p53: Role within the cell

p53 is a relatively short-lived protein with a half-life of approximately thirty to sixty minutes [44]. If left in the inactivate/latent form, p53 is quickly turned over via ubiquitin-dependent degradation. Cellular stresses resulting in DNA-damage have been shown to stimulate p53 activation, nuclear translocation and gene expression [3]. However, the posttranslational modifications of latent p53 satisfy the primary rapid response to stress. Maltzman and Cyzyzk in 1984 [1] were the first to report p53 accumulation following exposure to UV light. Work in the early 1990's determined a model for the function of p53 by which genotoxic damage induced transactivation of p53 leading to cellular growth arrest or apoptosis [2]. Many groups have shown p53 induction following exposure to a variety of stress conditions (UV/IR radiation, hypoxia, oncogenic signals, etc.), chemical agents (5-fluorouracil, etoposide, adriamycin, strauosporine, etc.), as well as oncogenes (Maf, etc.) [42, 45, 46]. DNA damage repair intermediates have also been shown to induce p53 response. Loss of p53 function has been shown to results in the absence of apoptosis and progression of tumors from benign to a malignant state [19]. Genetic instability associated with p53 loss of function (DNA binding, localization associated mutations) is known to be prevalent in many human cancers. Polyak *et al.* (1997) [47] suggested a model of p53-induced apoptosis in colorectal cancer. Evidence demonstrating a cascade in which p53 induces the production of reactive oxygen species (ROS) suggests the role oxidative damage has on mitochrondria and its stimulation of apoptosis [48].

i. Cell Cycle arrest

Protection of genomic material from errors is crucial in cell division. p53, in concert with other regulatory proteins (Myc, Rb, E2F), accomplishes this by binding to DNA (excision-repair) and negatively regulating mitosis machinery (i.e. p16 and cyclin D_1-Cdk4) [49]. $p21^{WAF1/CIP1}$, a downstream target of p53, also binds cyclins and Cdk complexes: cyclin-D_1-Cdk4, cyclin E-Cdk2, cyclin A-Cdk2, and cyclin A-Cdc2 [10]. Binding inhibits continuation of cellular division and allows the cell to make required DNA repairs before recommencing mitosis [10]. DNA replication by DNA polymerase PCNA is inhibited by p21 binding [10]. Formation of a p21-PCNA complex blocks PCNA DNA replication but not its role in DNA damage repair [10]. A rise in p53 protein levels (and downstream target genes) has been associated with G_1/S arrest [50]. However Cross *et al.* (1995) [50] demonstrated p53's role in G_2/M arrest. This group demonstrated p53-mediated G_2/M arrest in *p53-/-* mouse embryonic fibroblast (MEF) cells after treatment with mitotic spindle inhibitors, such as nocodazole. These cells were allowed to reinitiate M phase of mitosis thereby increasing the ploidy of the cells 4-8x [50]. Arrest at G_2/M checkpoint requires the involvement of p53 C-terminus as shown by Nakamura *et al.* (2001) [51]. Posttranscriptional modifications of key lysine residues within this domain appear to be necessary for proper cyclin inhibition and cycle arrest [52]. The fate of cell cycle arrest rather than cell death is determined by the amount of DNA damage [10]. DNA-strand breaks generated by genotoxic agents activate the nuclear/DNA binding protein poly (ADP-ribose) polymerase-1 (PARP-1), DNA polymerase β and

DNA ligase III [53]. PARP-1 has been described as the 'Cinderella of the genome', destined as a housekeeper that can signal DNA rupture as well as base excision repair [54]. The role of PARP-1 in genome repair is to act indirectly in DNA opening by acting on excision machinery. However, this benefit of PARP-1 action can also lead to cell death [54]. PARP-1 can induce NAD^+ depletion resulting in a strain on glycolysis and mitochondrial pathways and energy storage [55]. Controversy surrounding PARP-1 in DNA repair led to the conception of the 'suicide hypothesis' to explain its function [55].

ii. Apoptosis

Mutation of the p53 gene has been recognized in slightly more than 50% of human cancers [10]. The loss of p53 function gives rise to the rapid malignant progression of tumors [56]. Mouse urogenital sinus cell lines isolated from *p53+/-* and *p53-/-* mice have also displayed a high rate of cancer (100%) [57]. The p53-targeted genes Bax, Fas/Apo-1, IGF-BP3, Killer/DR5, PIGs, PAG608, PERP, Noxa, PIDD, DRAL and p53AIP1 are thought to play a role in p53-induced cell death [42]. Apoptosis induced by these protein pathways typically involve mitochondrial membrane disruption [58]. Perturbation of mitochondrial organelles leads to the release of apoptotic activators and effectors of cell death from its lumen [58]. Mitochondrial potential alteration by rapid p53 translocation has been shown to result in cytochrome c release and procaspase-3 activation [59]. Other pro-apoptotic proteins undergo translocation to the outer mitochondrial membrane upon *apoptotic-stimulus*, including Bcl family member Bax [58, 60]. These proteins induce cytochrome c leakage resulting from increased

mitochondrial membrane permeability and the onset of apoptosis. Transactivation of gene expression by p53 occurs under a variety of circumstances and targets multiple genes depending on the severity of DNA-damage.

iii. Target Genes of p53

Activation of p53 has a variety of gene targets. More then 150 genes are hypothesized to be induced or inhibited by p53 including $p21^{WAF/CIP1}$, GADD45, MDM2, IGF-BP3, and Bax. These proteins carry out the roles of p53 in stimulating cell cycle checkpoint arrest or apoptosis. In many cases, loss of these downstream targets of p53 result in a negative phenotype (poor cell cycle arrest - embryonic lethality) [10].

a. $p21^{WAF/CIP1}$

p53-mediated transactivation of p21 expression occurs in response to some forms of DNA-damage (depends on severity). Gene activation of p21 can be regulated by the binding of p53 within its promoter (p53-binding site) 2.4 kb upstream of a p21 coding sequence [61]. Induction of p21 has been shown to suppress the growth of human brain, lung, and colon tumor cells in culture [61]. p21 is a cyclin-dependent kinase enzyme which binds a variety of cyclin-Cdk complexes to prevent cell division progression [10]. p21 null mice develop normally though fibroblast cell lines derived from them are partially deficient in G_1/S phase cycle arrest [10]. Loss of both p21 alleles from cancerous cell lines containing *wtp53* are unable to enforce G_1 arrest [47].

b. GADD45

DNA damage has also been shown to stimulate p53-mediated transactivation of growth arrest and DNA damage 45 gene (GADD45) [62]. Xiao *et al.* (2000) [62] demonstrated a significant increase of GADD45 mRNA in human fibroblasts after DNA damage (CPT treatment). Interaction between p21 and PCNA is thought to be stabilized by a chaperone function of GADD45 [10]. This interaction seems key in some cell lines, since over-expression of GADD45 is able to arrest cells in G_1 [10]. The ultimate nature of GADD45 is still very unclear however [10].

c. MDM2

The presence of an auto-regulatory feedback loop between p53 and murine double minute clone 2 (MDM2) was first suggested by Barak *et al.* (1993) [63]. Evidence of a rapid stimulation of mRNA production suggested the direct involvement of p53 as a transcription factor in MDM2 gene activation [63]. MDM2 is regulated, just as p53, by post-transcriptional modification (phosphorylation and sumoylation) [64, 65]. Phosphorylation by deoxynucleoside protein kinase (DNK-PK) of two clusters within MDM2 (N terminal p53 binding domain and central acidic region) has been shown to reduce MDM2's interaction with p53 [66]. Sumoylation takes place at lysine residue 446 of MDM2 [65]. Loss of modification results in autoubiquitination of lys-446 and MDM2 degradation. Downregulation of MDM2 ultimately results in increased p53 stability. ARF

(tumor suppresser protein) and pRb (cell cycle regulatory protein) have also been shown to downregulate MDM2 via complex formation. ARF acts directly to inhibit the p53-directed ubiquitin ligase activity, while binding of pRb blocks MDM2 degradation of p53.

d. IGF-BP3/Bax

Insulin-like growth factor binding protein-3, is another gene activation target of the p53 transcription factor [67]. Activation of IGF-BP3 is associated with the enhanced secretion of IGF-BP3 which is able to inhibit the IGF mediated signal pathway important in mitogenic signaling [67]. Blocking of this signaling pathway enhances the promotion of apoptosis with activation of the p53-transactivation target BCL-2 family member Bax [10]. Miyashita and Reed (1995) [68] demonstrated that Bax is also a gene activation target of p53. Upon DNA damage, Bax can tip the cell's fate to apoptosis by interacting with the anti-apoptotic protein BCL-2. This interplay can lead to mitochondrial caspase cascades and p53-mediated apoptosis [42].

4. Regulation of p53

i. Mutation

Missense mutations are the most common genetic alteration in human cancers. The majority of p53 missense mutations are located within the DNA-binding domain of the protein [19]. Hollstein *et al. (1994)* [19] created a database cataloging p53 mutations describing 2,500 mutations in the p53 gene in different human tumors and tumor cell lines. Residues Arg248 and Arg273 are the two most frequently altered amino acids. These mutations result in the loss of p53 transcriptional activity because of the loss of proper contact to the DNA [19]. Structural integrity of this domain is crucial for p53 target gene activation. Mutations resulting in the deletion or truncation of p53 also occur but are less frequent [69]. Studies have shown that individuals with these mutations are more predisposed to cancer due to a total loss of p53 function [69]. Impaired localization of p53 has also been shown to be an important factor in the development of p53-related cancer. Inhibited nuclear import of p53 results in cytoplasmic accumulation. This is a well-established characteristic of a variety of human tumors including colorectal cancer, breast cancer, and neuroblastoma [4]. The study by Jansson *et al.* (2001) [4] elucidated mutational hotspots within p53-induced colorectal cancer including amino acids: 175, 243, 244, 245, 248, 277 and 279. Within tumors examined, localization of p53 (nuclear or cytoplasmic accumulation) did not show a significant difference in the degree of cancer [4]. However colorectal tumors displaying both nuclear and cytoplasmic accumulation of p53 were shown to have a higher mutation rate [4].

ii. Degradation/Export

p53 protein level is tightly regulated via degradation by different proteases including proteasome activity and calpain.

a. MDM2

MDM2 is a RING-finger protein possessing E3 isopeptide ligase activity that serves as a bridge to allow transfer of ubiquitin molecules from E2 ubiquitin-conjugating enzyme to substrate [44]. MDM2-mediates ubiquitination of multiple Lys residues (370, 372, 373, 381, 382, and 386) on p53's C-terminus [70, 71]. The addition of ubiquitin molecules to the p53-MDM2 complex destines both for degradation. MDM2 interacts with the p53 amino-terminal domain across a stretch of approximately 115 amino acids [72]. This binding is sufficient to block p53's transcriptional activation and destines it for ubiquitin-mediated degradation [73]. Under normal conditions, p53 is expressed at low levels, which is under the control of rapid turnover through nuclear export and ubiquitin-mediated degradation by the 26S proteasome. There is some debate as to whether export of p53 is required for its proteasome degradation. Research by Moll and colleagues (1992) [40] demonstrated that MDA 231 human breast cancer cells treated with nuclear export inhibitor (Leptomycin B -- LMB) displayed **nuclear** p53 degradation.

Upon stress activation of p53 by DNA damage, Ser-15 and -20 become phosphorylated thereby inhibiting MDM:p53 complex formation and nuclear export [74, 75]. DNA damage also results in increased ARF levels, a tumor suppresser protein which can bind MDM2 and inhibit p53 degradation [76]. Downregulation of MDM2 production (mRNA and protein) after UV treatment has also been shown to be a mechanism in the regulation of p53 protein stability [52].

Loss of MDM2 function (MDM2 knockout mice) results in p53 nuclear accumulation and embryonic lethality [71]. Inhibition of nuclear export by the Crm1 inhibitor LMB (to be discussed later) has also shown to result in nuclear accumulation of p53 as well as increased expression. Experiments using LMB also demonstrate increased p53 half-life and transcriptional activity [73]. Binding of MDM2 to p53 can also inhibit other p53-protein interactions [73]. Since MDM2 binds the transactivation domain (N-termini of p53), it blocks p53's interaction with TBP-associated factors $TAF_{II}70$ and $TAF_{II}31$ inhibiting targeted gene transactivation [73].

b. Calpain

p53 degradation primarily occurs through the MDM2-mediated ubiquitination pathway, however p53 can also be a substrate for cleavage by the calcium-activated neutral protease, calpain [77, 78]. Cleavage of p53 was observed *in vitro*, generating an N-terminal truncated protein, however *in vivo* experimental results have been inconclusive [78]. Therefore the importance of calpain-mediated degradation of p53 is

still unclear. The inhibition of calpain by addition of calpastatin, calpain inhibitor I (ALLN - N-Acetyl-Leu-LeulNle-CHO) or Ca^{2+} chelating has been shown to cause stabilization of p53 in a variety of systems [77-79]. Pariat *et al.* (1997) [79] hypothesized that calpain-mediated degradation is dependent on the conformation of p53. Alterations in p53's tertiary structure increase its susceptibility to calpains [79].

iii. Postranslational Modifications

a. Phosphorylation:

Phosphorylation is important in stabilizing p53, a response to some stress signals. Stretches of serine residues clustered within the transactivation and nuclear localization domains of p53 are targets for phosphorylation [80]. Kinases, such as Chk1 and Chk2, appear to act on these residues to both stabilize and activate p53 in response to stress e.g. UV and ionizing radiation [81]. N-terminal modification is known to stabilize p53 by interfering with the ability of MDM2 to bind p53 (negative regulator) [75]. Seven serines (Ser-6, 9, 15, 20, 33, 37and 46) and two threonine residues (Thr-18, and 81) within the transactivation domain of p53 are phosphorylated in response to DNA-damage [16]. Specific roles for modifications of all nine residues are not clear. Interestingly Thr55 has been found phosphorylated in non-stressed conditions [82] and de-phosphorylated after DNA damage. Chk2 and Chk1 phosphorylate serine 20, which decreases protein turnover and thus leads to accumulation of p53 [83]. Post-transcriptional activation of p53 has been shown to be regulated by phosphorylation of serine 15 and 37 within the MDM2-binding site of p53 [13]. To examine the importance of these phosphorylation sites, Siliciano *et al.* (1997) [13] replaced Ser-9, 18 and 37 of p53 with asparitic acid. This alteration mimicks the charge characteristics of phosphorylated serine seen within stabilized wild-type p53 [13]. Absence of the proper charge by the loss of phospho-serine residues within the first 37 amino acids of p53 resulted in loss of proper transactivation [13]. Furthermore, C-terminal phosphorylation of p53 (Ser315 and Ser392) by cyclin-

dependent kinase or casein kinase II, respectively, also occurs, although these Ser residues appear to be important for p53 activation rather than p53 stability [9]. Modification by casein kinase II or PKR effectively activates p53's DNA binding and transcriptional ability [32]. Phosphorylation of p53 has also been shown to affect other forms of post-transcriptional modification such as acetylation [3]. Exposing cells to UV light or IR leading to DNA-damage has been shown to phosphorylate p53 at Ser 33 and 37 which resulted in an inhibition of acetylation of the C-terminal region of p53 [84].

b. Acetylation

Acetylation is a much more controversial form of p53 modification. Gu and Roeder (1997) [71] have shown *in* vitro acetylation of p53 after interaction with p300 HAT (histone acetyltransferase) and becoming acetylated *in vitro*. Further work with acetylation-specific antibodies for p53 showed that p300 as well as P/CAF HAT modifies multiple lysines within the C-terminal domain of p53 (p300: 372, 373, 381, 382 and P/CAF: 320) [3]. Sites Lys320 and Lys382 are acetylated in vivo in response to a variety of cellular stresses (i.e. IR and UV light) [3]. These alterations of p53 are thought to enhance sequence-specific DNA-binding. On the other hand Espinosa and Emerson *(2001)* [85] and Nakamura *et al.* (2000*)* [86] have shown acteylation does not assist p53 binding to large DNA molecules (i.e. p21 promoter) and in fact mutational deletion of lysine residues at positions 372, 373, 381, and 382 have no effect on transcriptional activation of p53. The topic is still very much up for debate.

c. Sumoylation

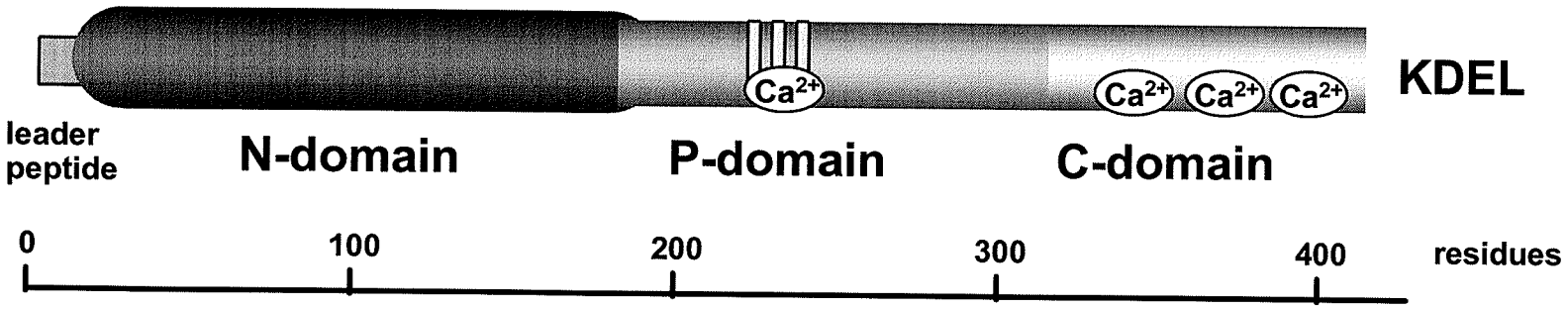
p53 was recently found to be covalently modified by SUMO-1 (Small Ubiquitin-related Modifier) [87]. Modification by SUMO-1 is thought to be very similar to ubiquitination in terms of enzyme machinery [17]. However, sumoylation, unlike ubiquitination targets only one molecule of SUMO-1 to the target residue [17]. With the use of the yeast-two hybrid system, SUMO-1 modification of Lys386 has been shown to enhance p53's transcriptional activity [17, 88]. An increase in Lys386 modification was noted in 293 cells after genotoxic stress [17]. Immunofluoresence data show co-localization of SUMO-1 and p53 within the nucleus [17]. However, identical results were obtained using a Lys386R p53 mutant [17]. Therefore, modification of p53 via sumoylation is not thought to be a major form of p53 regulation.

I. Calreticulin

1. Structure and domains of gene and protein

Calreticulin (CRT) is a 46 kDa protein (400 amino acids) first isolated by Ostwald and MacLennan (1974) [89] from rabbit skeletal muscle. CRT is an endo/sarcoplasmic reticulum (ER/SR) lumenal protein, which is important in Ca^{2+} homeostasis [90, 91], molecular chaperoning [92-94], and regulation of gene expression [95-98]. This multi-functional protein is sub-divided into three putative domains **(Figure 2)**. In addition to these defined domains, CRT also contains an N-terminal signal sequence and C-terminal KDEL ER retrieval signal. These terminal sequences allow localization of CRT to the ER/SR. The N-terminal domain is among the most conserved regions across species. This domain is highly folded, containing 8 anti-parallel β-stands of which three cysteine residues are known to form disulphide bridges (Cys^{120}-Cys^{146}). The N-terminal domain is able to bind Zn^{2+} with relatively high affinity [99]. Binding site location was determined using glutathione S-transferase fusion protein system and Zn^{2+}-IDA-agarose [99]. Several histidine residues within this domain are suggested to be involved in the binding of Zn^{2+} [99]. As an ER chaperone, CRT interacts with other ER resident chaperones involved in protein processing such as PDI (protein disulphode-isomerase) [100] and Erp57 (ER protein 57) [101] via N-terminal domain. This domain has also been shown to interact with the DNA-binding region of the glucocorticoid receptor [95] and α-integrin using *in vitro* assay methods [102]. Another

Figure 2: Schematic diagram of calreticulin protein and its functional domains.



putative function for this domain is as an inhibitor of angiogenesis (vasostatin, residues 1-180) [103]. Full length CRT has also been shown to inhibit angiogenesis [103]. Kuwabara *et al.* (1995) [104] demonstrated that addition of exogenous CRT resulted in its binding to endothelial cells *in vitro* and its localization to vascular endothelium *in vivo*. In this study CRT was able to stimulate nitric oxide release and suppress angiogenesis and endothelial cell migration [104]. Inoculation of athymic mice with vasostatin showed a significant reduction in tumor growth of human Burkitt lymphoma and human colon carcinoma [103]. CRT has been shown to modulate gene regulation mediated by the glucocorticoid receptor [95], androgen receptor [96], retinoic acid binding [96] and laminin binding [97, 98]. These functions might explain vasostatin's ability to specifically disrupt endothelial cell attachment and angiogenesis [98]. Research on the potential of vasostatin as a cancer therapeutic agent seems very promising. Lange-Asschenfeldt and colleagues (2001) [105] demonstrated vasostatin's potent inhibition of CA46 Burkitt lymphoma tumor growth in nude mice. Vasostatin-treated mice showed significantly reduced vascularity within cancer with no impairment of wound healing [98]. Vasostatin, as well as full-length CRT, has been shown to have anti-angiogenic and anti-tumor activities [106].

Residues 171-285 make up the P-domain of CRT which is named for its unique proline-rich regions. This region shows strong homology to calnexin (CNX), another ER chaperone protein [107]. Both CRT and CNX are known to act as chaperones for glycosylated proteins, but CRT may also function as a chaperone for nonglycosylated proteins [108]. The P-domain also contains one of CRTs Ca^{2+}-binding sites (high affinity,

low capacity). Nakamura *et al.* (2001) [109] demonstrated the role of the P-domain in Ca^{2+}-handling with calreticulin-deficient fibroblasts. Ca^{2+} release profiles (thapsigargin and ionmycin) showed transfection of either full-length CRT or P + C domain was able to restore ER Ca^{2+} levels to normal [109]. Recently the 3D structure of the P-domain of rat CRT was determined by nuclear magnetic resonance (NMR) spectroscopy [110]. This work has helped to elucidate the function of the P-domain as predicted by its structure including its protein-binding ability as an ER chaperone. Lectin-like activity of P-domain allows for strong interactions and the formation of complexes with a variety of ER proteins including PDI [100] and perforin [111].

Ca^{2+} binding also occurs within the C-domain (residues 286-400) of CRT. This domain is highly acidic and therefore is able to bind 25 mol Ca^{2+}/ mol protein, making CRT a major Ca^{2+} binding protein in the ER lumen [112]. Ca^{2+} binding within the C-domain of CRT has been shown to be important in regulating CRT interaction with PDI and ERp57 [101]. The C-domain also plays an antithrombotic role, binding blood-clotting factors [113] and inhibiting injury-induced restenosis [114].

2. Regulation of CRT gene expression

Calreticulin is an ubiquitously expressed, multi-functional ER lumenal protein. In the early embryonic phase of mouse development, CRT expression is high in the cardiovascular system [7]. CRT gene activity within the maturing cardiac system decreases further in gestation [7]. Antagonistic interplay between cardiac transcription factors Nkx2.5 and chicken ovalbumin upstream promoter-transcription factor 1 (COUP-1) regulate the expression of CRT during early heart development [115]. Loss of transcriptional activity of Nkx2.5 by targeted interruption of the Nkx2.5 gene results in embryonic-lethality at approximately 9-10 days postcoitum [116]. Reduced cardiac-specific expression of CRT in transgenic mice resulted in cardiac dysfunction and ventricular dilation causing sudden death due to complete heart block [117]. This CRT-dependent heart block is thought to be due to impaired L-type Ca^{2+} channel and gap junction connexin-40 [118] and connexin-43 [6, 119]. A significant decrease in the expression levels of these connexins results in severe arrhythmias and poor electrical signal conductance [120].

3. Localization of CRT

Calreticulin was first isolated from rabbit skeletal muscle sarcoplasmic reticulum (SR) [89] and was initially described as HACEP (high affinity calcium-binding protein). Controversy over the localization of CRT has spurred many studies. However repeated reports have shown CRT ER/SR lumen localization in many diverse species [121]. Opas *et al.* (1991) [122] showed ER localization of CRT in a variety of different cell lines by immunocytochemical methods. ER lumenal localization of CRT is maintained via a C-terminal KDEL sequence which causes the retention of the protein to the ER. Over-expression of CRT by Sonnichsen *et al.* (1994) [123] demonstrated that very little newly synthesized CRT can be found within the golgi after 5 minutes determined by pulse chase experiments labeled with [^{35}S] methionine. Additional immunodetection of over-expressed CRT with anti-CRT as well as anti-Erd2 (cytoplasmic tail of KDEL-receptor) indicated only a slight redistribution of Erd2 demonstrating continued ER retention [123]. If external pools of CRT exist, KDEL-mediated retention and retrieval would have to be inhibited by some means, such as retrieval machinery saturation or loss of KDEL retention sequence. Despite considerable evidence for ER localization, reports have localized CRT to cytoplasm [124], cell surface [93, 125], nuclear [126, 127], and even perhaps secreted [128]. Often these results are attributed to be an artifact of the antibody or the techniques used.

i. Endoplasmic Reticulum lumen localization

ER localization of the CRT protein is well established in a wide variety of cell types including human fibroblasts, human skeletal muscle cells, Madine-Darby canine kidney (MDCK) epithelial cells, rat bone cells, and L6 myoblasts [122]. Opas *et al.* (1991) [122] showed a strong degree of co-localization of anti-CRT antibodies and ER specific stain, TRITC-Con A demonstrated by fluorescence microscopy. Additional staining with acridine orange (RNA) and $DiOC_6$ (membranes) in quadruple label experiment showed CRT to be specifically localized within the rough ER lumen [122]. In addition to the ER lumen, CRT protein localization has also been detected within the NE lumen of MDCK epithelial cells as demonstrated by immunocytochemical localization with anti-CRT antibodies [122]. ER/NE localization is important in the regulation of the many functions of CRT. However, speculation of alternate locations for CRT have been postulated by many research groups due to largely *in vitro* experimental results (such as binding assays) as well as the shear variety of CRT's many known functions [93, 124, 127].

ii. Cytoplasmic localization

CRT is known to interact with several molecules which regulate cell adhesion, such as α3-integrins and vinculin [124]. Functions of CRT involving cytosolic proteins have lead to the model of cytoplasmic CRT. However, evidence for a cytoplasmic pool of CRT has never been substantiated. Coppolino *et al.* (1999) [129] suggested a direct

interaction of CRT-integrin by the inhibition of mitogen-activated protein kinase (MEK) by enhanced cell adhesion to collagen IV. Ito *et al.* (2001) [124] has also suggested a linkage between CRT-regulated cell adhesion and a possible cytoplasmic pool of CRT. This group demonstrated using immunoprecipitation and western blot analysis the direct interaction of CRT and α3 integrin over a 60-minute time span. Ito *et al.* [124] also showed a significant increase in CRT-integrin binding after anti-α3 integrin antibody stimulation. These researchers therefore determined this result to be evidence for the direct involvement of CRT in integrin function modulation. On the other hand, Fadel *et al.* (1999) [130] has suggested another mechanism by which CRT can affect cellular adhesion from the ER lumen. This group demonstrated the modulation of cell adhesion by CRT-regulated phosphotyrosine (Tyr-P) signaling rather than the direct interaction of CRT at cell adhesion points [130]. In cell lines under-expressing CRT, there was an increase in the total level of Tyr-P as determined by immunocytochemistry and western blot analysis [130]. This increase in Tyr-P coincided with a decrease in levels of both CRT and vinculin as determined by western blot analysis [130]. Opas *et al.* (1996) [131] has also demonstrated a modulation of vinculin expression and ER localization of CRT in mouse fibroblast cells over-expressing CRT. Interference reflection microscopic data by Opas *et al.* (1996) [131] showed focal contacts (cellular adhesion points) with ER localization of GFP-CRT. This data, therefore, can explain many of reported functions of CRT from the ER lumen.

iii. Cell Surface & secreted CRT

The cell surface incorporation/activity of CRT is very unclear. Functions such as modulation of cellular adhesion and migration are known to be influenced by CRT [124, 130-132]. However, the CRT protein lacks a transmembrane domain to interact with the cell membrane. Several immunorelated surface proteins (MHC I and HLA-A2 heavy chains for example) are known to be chaperoned by CRT via its lectin-like or protein-protein interacting domains as they are synthesized and transported in the ER [93, 94]. CRT has been shown to be associated with MHC I of activate of T lymphocytes by the use of primarily permeablized cell protein assays [93, 94]. However, these techniques (co-precipition, surface biotinylation and western, and immunofluorescence) involved cell permeablization and can result in a release of CRT from the ER. As a molecular chaperone, CRT is known to be bind a variety of proteins (i.e. MHC I), however this interaction does not always infer co-localization with full length (mature protein). Cell surface localization has also been noted in melanoma cells and virus-infected fibroblasts [93]. With the use of biotinylation and immunocytochemical methods, Robin Johnson's lab has showed small but significant amounts of CRT in large patches on the cell surface of the NG108-15 cell (a mouse neuroblastoma-rat glioma hybrid cell line) [125]. Xiao *et al.* (1999) [125] demonstrated the tight binding of CRT to surface molecules by immunoprecipitation of media showing an absence of CRT release. However, the existence of secreted CRT has been argued in the literature [127]. For this to be true, a model for ER retention escape would have to be established. Conditions, such as inflammatory response have shown protealic cleavage of CRTs KDEL sequence [133].

Sonnichsen *et al.* (1994) [123] however has shown that removing the KDEL sequence from CRT resulted in very minimal secretion of CRT from the cells. Increased vesicular transport may also play a role by saturating ER-retention machinery. The cell has a limited amount of KDEL receptors, and under this theory, non-retained ER proteins might be able to escape from the ER and reach the cell surface [125]. Without KDEL retention, CRT could potentially be released into the circulation. Secreted CRT has been documented in the progression of pathological diseases including systemic lupus erythematosus (SLE) [133], rheumatoid arthritis [134], celiac disease [135], complete congenital heart failure [136], halothane hepatitis [137] and Sjorens syndrome [133]. Rokeach *et al.* (1991) [138] demonstrated the existence of autoantibodies in an SLE patient's sera against CRT which also tested negative for Ro/SS-A. The Ro/SS-A human antigen is thought to be comprised of four protein components and CRT and has also been shown to be associated with disease [139]. Ro/SS-A shares 92% amino acid sequence identity with CRT from rabbit skeletal muscle and also stimulates an immune response in Sjorens syndrome and some subsets of systemic lupus erythematosus [133]. Extensive evidence contests the ER localization of CRT suggesting alternate locations might be due to cross-reactivity, cell permeablization or technical artifact.

iv. Nuclear localization

Nuclear export of the glucocorticoid receptor (GR) is thought to be among the functions of CRT [127]. CRT is known to modulate gene regulation by a variety of steroid receptors including GR [95]. Holaska *et al.* (2001) [127] attempted to show an

interaction of CRT and the DNA-binding domain of GR as well as regulation of GR nuclear export by CRT. The link between CRT and GR was the presence of complementing stretches of amino acids (KLGFFKR) [95, 102] which Holaska *et al.* (2001) [127] suggested might act as a nuclear export recognition. Burns *et al.* (1994) [95] showed the *in vitro* interaction of CRT and GR through this complementing domain. However, the regulation of GR transcriptional activity by CRT has been shown to be mediated from within the ER [140]. Localization of CRT to the nucleus has never been conclusively proven though it has been reported by multiple groups [126, 127]. Holaska *et al.* (2001) [127] first suggested the existence of nuclear CRT after mass spectrometry determined that a 60kDa polypeptide with high nuclear export activity was CRT. This group went further to suggest CRT as a nuclear exporter of PKI by demonstrating their direct interaction in permeablized cell fractions. However, the suggestion of contamination of sub-cellular fractionation as well as staining and fixation artifacts were later blamed for these experimental results [127].

4. Major functions of Calreticulin

i. Chaperone/protein-folding

The precise tertiary structure of molecules including ion channels, surface receptors, integrins and transporters is key to their function. The newly synthesized membrane and secreted proteins are processed in the ER for proper folding. The consequence of the misfolding of proteins within the ER often results in decreased or loss of activity. Quality control performed during protein processing within the ER involves the interaction and modification by chaperones. Jorgensen *et al.* (2000) [141] demonstrated this quality control by showing the preferential binding of CRT to heat-denatured ovalbumin indicating CRTs recognition of partially unfolded, monoglucosylated glycoproteins in the ER. CRT and calnexin (CXN) are both well-characterized homologous ER chaperones [107]. The monoglycosylated proteins are chaperoned by the lectin-like domains of CRT and CXN resulting in the addition of specific carbohydrate moieties or chains [142]. Studies utilizing immobilized CRT and radiolabeled oligosaccharides demonstrated that CRT's interaction was limited to monoglycosylated polymannose components [143]. Optimal binding of glycoproteins by CRT was shown via $Glc_1Man_9GlcNAc$ oligosaccharide [143]. Presence and arrangement of N-linked oligosaccarides (including monoglucosylated polymannose moieties) are known to affect CRT affinity [143] as well as signal proper folding of ER-processed glycoprotein [144]. Processing of glycoproteins undergo ATP- and Ca^{2+}- dependent cycling within the ER that requires the recruitment of other ER chaperones [100, 145,

146]. One of the CRT chaperone substrates is the major histocompatibility complex (MHC) class I molecule which are composites of polymorphic heavy chains, integral membrane glycoproteins (α) and light chains ($\beta_2$microglobulin) [147]. *In vitro* data with MHC I [94] has resulted in multiple groups suggesting a cytoplasmic localization of CRT [93, 147] (discussed earlier). Other CRT chaperone substrates include thioredoxin [145], myeloperoxidase [148], viral hemagglutinin [149], transferrin [150] and HIV virus type I envelope glycoprotein [107].

ii. Calcium homeostasis

Regulation of calcium within ER/SR is accomplished by a battery of membrane bound and lumenal proteins. Interplay between these Ca^{2+} sequestering molecules (calreticulin, calsequestrin, etc.) and ion channels ($InsP_3$/RyR receptors, SERCA pump, and cell membrane Ca^{2+} channels) modulate Ca^{2+} homeostasis. CRT has been hypothesized to interact with intraluminal loops of SERCA and perhaps the $InsP_3$-receptor [151]. This interaction may allow CRT to further influence $[Ca^{2+}]_{ER}$ [151, 152]. Consequences from loss of regulation by CRT results in accumulation of misfolded proteins, activation of ER chaperones and ER to nucleus and ER to plasma membrane signaling (reviewed by Michalak, 1999) [153]. In fact, CRT knockout mice are embryonical lethal due poor cardiac development perhaps brought about by defects in the regulation of Ca^{2+} homeostasis (to be discussed later) [7].

Agonist stimulated calcium release via InsP_3-mediated pathways is inhibited in CRT-deficient MEF cells [5, 7]. Mesaeli *et al.* (1999) [7] suggested that this reduced response to bradykinin (which activates the InsP_3-mediated pathway) might be due to the loss of the CRT Ca^{2+} sequestering function and reduced IP_3-sensitive Ca^{2+} pool or that the subcellular compartments containing CRT are directly related to the IP_3-sensitive Ca^{2+} pool. In fact, research by Roderick *et al.* (1998) [154] has shown an increase in the Ca^{2+} store in HeLa cells over-expressing CRT suggesting CRT's role in Ca^{2+} storage within the ER lumen. This poor Ca^{2+} homeostasis has been demonstrated by impaired contraction of *crt -/-* cardiomyocytes derived from differentiated ES cells [8]. These authors suggested that the mechanism of inhibition is due to decreased phosphorylation of ventricular myosin light chain 2 (MLC2) in *crt-/-* cells due to impaired organization of myofibrils [8]. However Mesaeli *et al.* (1999) [7] have reported spontaneous contraction in cardiomyocytes cultured from 14.5-day-old *crt-/-* embryos. These conflicting results suggest that cell type as well as CRT level plays a role in cell phenotype and its response to stimulus. Decreased cytosolic Ca^{2+} in ES-derived cardiac cells also influences nuclear transport of transcription factor myocyte enhancer factor C2 [8]. However, this nuclear exclusion has been shown to be reversible upon ionomycin-triggered Ca^{2+} release [8]. Impaired NF-AT3 (nuclear factor of activated T cells) nuclear import has also been demonstrated in *crt-/-* MEF cells [7].

iii. Cell Adhesion

CRT has also been shown to affect cell adhesion [130-132, 155, 156]. However, the mechanism by which CRT modulates cell adhesions is not completely understood. Early experiments demonstrated *in vitro* binding of CRT-integrin α3 resulting in modulation of its function [124, 156]. Direct interaction at points of adhesions would require cytoplasmic CRT. However, Opas *et al.* (1996) [131] demonstrated the indirect modulation of cell adhesion via the phosphotyrosine pathway. Their results showed CRT can regulate vinculin from the ER lumen. Vinculin is an adhesion-specific cytoskeletal protein involved in the formation and stabilization of focal contacts as well as zonulae adherens [156]. Fadel *et al.* (1999) [130] demonstrated an inverse relationship between CRT and phosphotyrosine (Tyr-P) levels, such that cells under-expressing CRT show increased total Tyr-P. Results from Opas *et al.* (1996) [131] and Fadel *et al.* (1999) [130] suggest CRT modulation of cell adhesion is accomplished by the regulation of gene expression of adhesion molecules (vinculin) or modulation of Tyr-P signaling pathways.

iv. Apoptosis

Recent studies have shown targeted deletion of CRT in mice resulted in the embryonic lethality of *crt* -/- embryos 14.5-16.5 days during gestation [7, 157]. Decreased ventricular cell mass was associated with increased apoptosis of cardiac myocytes [157]. Death of CRT-knockout mice is likely due to this cardiac lesion. Over-expression of CRT in HeLa cells has been demonstrated to cause drug-dependent

apoptosis sensitivity [5]. In contrast, calreticulin-deficient cells showed a resistance to apoptosis [5]. Evidence strongly suggests that cross talk between the ER and mitochondria, involving the second messenger Ca^{2+} could determine cell fate upon apoptotic-stimulus [5].

Over-expression of CRT in H9c2 cardiomyoblast cells have also shown a significant decrease in protein kinase B/Akt activity [6]. This decrease in Akt signaling is caused by increased phosphatase activity (protein phosphatase 2A) which results in the dephosphorylation of Akt [6]. Suppression of Akt signaling promotes the differentiation-dependent apoptosis of H9c2 cardiomyoblasts [6]. Recent research involving the reduction of $[Ca^{2+}]_{ER}$ has demonstrated the inhibition of apoptosis upon ceramide-induced Ca^{2+} release [158]. This mechanism might explain the observed regulation of apoptosis by CRT as shown by Nakamura *et al.* (2000) [5]. Treatment with ceramide has been shown to cause an increase in cytoplasmic Ca^{2+} concentration resulting in prolonged mitochondrial Ca^{2+} accumulation and alterations in morphology resulting in membrane permeability of the organelle [159]. Mitochondrial damage is known to cause the release of cytochrome c and various pro-/caspases, which are major pro-apoptotic intermediates [42]. Regulation of ER Ca^{2+} stores via the over-expression of Bcl-2, as shown by Pinton *et al. (2001)* [158], demonstrated how lowered $[Ca^{2+}]_{ER}$ (similar to that seen in *crt-/-* cells) can protect the cell from mitochondrial leakage and apoptosis.

III. Nuclear Transport

1. Nuclear envelope and pores

Eukaryotic genetic material is separated from the cytoplasm by the nuclear envelope (NE). This barrier consists of a double membrane structure and nuclear pore complexes (NPC) [160]. The outer layer of the NE is morphologically continuous with the ER and has attached ribosomes. The inner membrane interacts with the nuclear lamina and has multiple chromatin attachment points. Lumenal contents of the peripheral ER and NE are continuous [161]. Therefore, calcium handling associated with the ER also occurs within the NE [162]. The level of lumenal diffusion within ER/NE is determined by the level of calcium store [163]. In fact, an increase in cytosolic calcium longer than ten minutes has been shown to disrupt ER tubules and prevent lumenal diffusion of large molecules [163]. Structural changes of the NE/ER network due to persistent Ca^{2+} increases have been shown to be functionally important in induction of gene expression [164], mitosis [165], and apoptosis [158, 166]. NE Ca^{2+} stores are regulated by ion channels on the cytoplasmic and possibly nuclear face of the membrane like the ones found on the ER [167]. The nuclear envelope's Ca^{2+} stores have been shown to be important in the regulation of transport through the NPC [162, 168-170].

The NPC is a macromolecular structure, which spans both the inner and outer membrane of the NE. Bi-directional trafficking between the two compartments must pass through this pore. The NPC is comprised of a complex of 100-200 different polypeptides

and has a mass of 124,000 kDa [171]. Proteins that make up the NPC are termed nucleoporins. Some nucleoporins contain O-linked N-acetylglucosamine carbohydrate modifications, which may allow for proper segregation during mitosis [172]. Density of NPCs is dependent on the metabolic activity of the cell [173]. Metabolic levels [174], protein manipulation (such as p53) [175], and NE Ca^{2+} stores [170] have been shown to have an effect on levels and direction of transport through the NPC. Overall diameter of the NPC is 130 nm with a dynamic central aqueous channel opening of 9 nm in resting cells (reviewed in Lee, 1998) [8]. High resolution scanning electron microscopy (SEM) of the NPC has allowed visual evidence of the pore's superstructure. Hinshaw *et al.* (1992) [176] utilized SEM to discover the NPC's three ring nature: cytoplasmic, nucleoplasmic and distal ring. Eight cytoplasmic filaments extend outward from the cytoplasmic ring and are hypothesized to be involved in docking and delivery of the cargo complex to the pore [177]. The nucleoplasmic face also houses filaments, which extend 20-40nm into the nucleus terminating at a coaxial ring structure forming a "nuclear basket" [178]. The function of this "nuclear basket" is unclear, though it may also be involved in docking and trafficking of material across the channel. Within the central pore of the NPC resides a central body named a nuclear plug [170]. The depth of the plug within the channel has been shown to be dynamic and able to be affected by a variety of agents (ionomycin, Ca^{2+} chelators, thapsigarin, InsP_3). Raising of the nuclear body results in inhibited nuclear translocation [170]. Therefore, NE/ER Ca^{2+} handling is very important in the proper workings of the NPC (discussed in further detail later).

2. Import/Export signals and machinery

Nuclear transport can occur in one of two ways: active (requiring energy) or passive. Size of the molecule, and not their structure, largely determines the method of translocation. Small molecules (< 60 kDa) and ions are able to passively diffuse through the nuclear pore [179]. Formation of gradients between nucleus and cytosol results in passive diffusion through the NPC until equilibrium is met. However, retention of small nuclear proteins via binding, or tethering, to non-diffusible structures within the nucleoplasm can mediate nuclear accumulation [180]. Active transport of macromolecules (>60kDa) containing a nuclear localization signal (NLS) through the NPC requires additional machinery involved in recognition and docking as well as the expenditure of energy for the translocation [181].

The NLS often consists of a stretch of basic residues (PKKKRKV), or two small clusters of basic amino acids (KRPAAIKKAGQAKKKK) 5-14 amino acids apart [182]. Alteration or absence of the NLS results in a loss of transport ability [183]. Protein machinery such as importin α, importin β, and RanGTPase are of key importance in facilitating nuclear import. Importin α, recognizes and binds to proteins with an NLS [178]. This complex is bound by a second importin species, β, and directed to the cytoplasmic face of the NPC [178]. Importin α therefore acts as an adaptor to bridge the interaction between the NLS and importin β via its importin β-binding domain [178]. The importin heterodimer and substrate protein bind to the nucleoporin NUP358p/RanBP2 situated on the cytoplasmic fibrils extending from the outer ring of the NPC. This

docking to the NPC is an energy-independent step [178]. The docked complex then migrates to the center pore of the NPC where it interacts with transporter components [184]. Once through the channel the cargo is released into the nucleoplasm in a process appearing to involve the molecule Ran GTPase [185].

Formation of the importin α/β-NLS-RanGDP complex plus GTP mediates nuclear pore crossing [186]. Dissociation of importin α, β, at the nucleoplasmic face of NPC is promoted by the presence of RanGTP allowing the release of cargo. A gradient of RanGDP and RanGTP is formed between the nucleus and cytosol. Gradient formation is due to the continuous cycling of Ran GTPase between compartments largely due to localization of GDP-GTP exchange factor (RCC1) (nucleus) [187] and RanGAP1 (hydrolysis stimulator) (cytosol) [188]. RanGDP (from import complex) is exchanged with RanGTP, which then facilitates nuclear export and is shuttled back to the cytosol [189]. RCC1 stimulates nucleotide exchange rate by five orders of magnitude [189]. Hydrolysis of RanGTP to RanGDP stimulated by RanGAP1, results in the release of cargo after export. Bischoff *et al.* (1994) [190] purified RanGAP1 from HeLa cell lysate and demonstrated its ability to induce GTPase activity.

Nuclear export also requires a specific conserved sequence of amino acids to be present on cargo. The first identification of such a sequence was in the HIV-1 transactivating protein Rev [191]. This *nuclear export signal* (NES) is well conserved and high in leucine residues (LPPLERLTL) [191]. Disruption of leucine residues in this sequence results in an inability to export and subsequent nuclear accumulation [192].

Similar to nuclear import, nuclear export also requires machinery to recognize NES. The discovery of the antifungal antibiotic leptomycin B (LMB) was the first major tool used in uncovering molecules required for nuclear export. LMB treatment has been shown to block Rev protein export [193]. LMB targets Crm1 (chromosome region maintenance 1), which has been localized to nucleoplasmic face of the NPC and is a member of the importin β family of proteins [194]. Ossareh-Nazari *et al.* (1997) [195] demonstrated Crm1's ability to bind to an NES. These authors' data obtained from co-immunoprecipitation and NES affinity column binding provide strong evidence for the involvement of Crm1 in nuclear export [195]. Demonstration of LMB's inhibitory ability over the formation of NES/Crm1/Ran complex by Fornerod *et al.* (1997) [194] was a major advancement in understanding the role of Crm1 in nuclear export. Formation of this tripartite complex is necessary for their removal from the nucleus. These findings resulted in the renaming of Crm1 to exportin 1 [194]. Docking of exportin1/cargo is hypothesized to involve nucleoporins Rip (Rev interacting protein) and Rab (Rev activation domain binding protein). Formation of the Rip/exportin1/Rev-NES complex serves as a docking mechanism to the NPC [196].

3. Regulation of nuclear translocation

The NE/ER [Ca^{2+}] has been shown to be important in the conformational regulation of the NPC [162, 163, 170, 197-199]. Atomic force microscopy (AFM) has allowed researchers to examine the topology of the NPC both in control and depleted conditions. InsP_3-mediated calcium release from NE lumenal stores has been shown to cause a raising of the plug (11.6nm depth to2.1nm, as compared with NPC outer surface) as well as a decrease in nuclear pore diameter (67.9nm in width to 34.2nm)[170]. Addition of BAPTA-AM (Ca^{2+} chelator) demonstrated similar results to InsP_3-induced NPC plugging [162]. Both treatments resulted in the inhibition of active nuclear import but not the passive diffusion of smaller molecules and ions through the NPC [162]. However, Greber *et al.* (1995) [197] reported a decrease in passive diffusion after ER Ca^{2+} was depleted by the Ca^{2+} ionophore, ionomycin. Models to explain this Ca^{2+}-sensitive conformation change have implicated nucleoporin gp210 [200]. Topological mapping has shown that a large domain of gp210 resides within NE lumenal space, with only the C-terminal tail interacting directly with NPC [197]. gp210 possesses a putative calcium binding EF-hand domain which is thought to mediate the regulation of the NPC by monitoring lumenal Ca^{2+} [184]. Also, inhibition of nuclear passage through the NPC has been demonstrated by addition of antibodies recognizing the gp210 lumenal domain [200]. Incubation of isolated nuclei in a solution containing ATP and Ca^{2+} allows for the replenishing of nuclear envelope Ca^{2+} stores resulting in lowering of the central plug [170]. Nuclear export however is not influenced by treatment with InsP_3 or BAPTA-AM [198]. Strubing *et al.* (1999) [198] demonstrated directionality of passage through NPC

can be affected. Inhibition of nuclear import of GFP tagged glucocorticoid receptor (GR) was demonstrated by the addition of BAPTA-AM in human embryonic kidney cell line. However, nuclear export of GFP tagged MAP kinase activated protein kinase 2 (MK2) after the same BAPTA-AM treatment was not effected [198]. These authors suggested the possibility of a depletion-independent mechanism to account for the interference of the nuclear import pathway [198].

RATIONALE

Following cell stress such as UV irradiation, chemotherapeutic drug treatment or hypoxia, p53 is key in determining cell fate between apoptosis and cell cycle arrest. The proper functioning of p53 depends on its accumulation in the nucleus following cell stress leading to activation of downstream target genes. The nuclear translocation of macromolecules (including p53) through the nuclear pore complex (NPC) has been shown to be dependent on proper Ca^{2+} homeostasis within the nuclear envelope. A decrease in the Ca^{2+} concentration in the lumen of the nuclear envelope leads to a conformational change of the NPC that inhibits the passage of molecules between the cytosol and the nucleus [170]. Calreticulin (CRT) is a major Ca^{2+} sequestering protein within the lumen of the ER and the nuclear envelope. In CRT deficient cells, the absence of CRT has been shown to result in an altered Ca^{2+} homeostasis [7] [5]. Impaired nuclear import of NF-AT3 (nuclear factor of activated T cells) [7] and myocyte enhancer factor C2 [8] has also been shown in CRT deficient cells, suggesting a possible role for CRT in the regulation of nuclear import. However, no data is available on nuclear translocation of p53. Recently, CRT has been demonstrated to play a role in drug and UV induced apoptosis. These studies showed that over expression of CRT increased the sensitivity of the cells to an apoptotic signal. Alternatively, deletion of CRT was shown to increase the cell's resistance to these stimuli [117]. Over-expression of CRT has also been associated with a decreased PKB/Akt kinase signaling activity [6]. The Akt serine-threonine kinase pathway is known to result in the phosphorylation of MDM2 causing its nuclear translocation [201]. Active MDM2 binds to p53 resulting in its inhibition and degradation [202]. Therefore, the aim of this study was to investigate the role of CRT in p53-

mediated apoptosis. We tested the hypothesis that CRT regulates p53 induced apoptosis by modulating its localization and expression. Our results demonstrate that the loss of CRT impairs p53 localization and expression. In addition, the nuclear localization of MDM2 in cells deficient in CRT suggests a direct role for ubiquitination and protein degradation in the modulation of p53 function. These results demonstrate a possible mechanism by which CRT can affect cell sensitivity to apoptotic stimulation.

B. MATERIALS AND METHODS

1. Materials

Prestained SDS-PAGE Protein Standards, Low range, was purchased from Bio-Rad Laboratories (Hercules, CA). ALLN (N-Acetyl-Leu-Leu-Nle-CHO) calpain inhibitor I and FluoroSave Reagent were purchased from Calbiochem (La Jolla, CA). LipofectAMINE reagent was from Invitrogen (Burlington, ON). Hoechst 33258 and DAPI nuclear stains were purchased from Molecular Probes, Inc. (Eugene, OR). Supersignal West Dura Extended Duration Substrate for detection of chemiluminescence was from Pierce (Rockford, IL). Blocking reagent for western blotting and immunocytochemistry was purchased from Roche Diagnostics (Montreal, PQ). Luciferin, adriamycin (doxorubin) and coenzyme A were from Sigma-Aldrich (Oakville, ON). All other reagents were of Molecular Biology grade and were purchased from Sigma-Aldrich or Fisher.

2. Plasmids

The pcDNA-CRT-HA expression vector (gift from Dr. M. Michalak, University of Alberta) was used to transfect *crt-/-* cells (will be referred to as CRT-*crt-/-*). To study nuclear import of p53 in *wt*, *crt-/-*, and CRT-*crt-/-* cells, a plasmid encoding EGFP tagged human p53 was purchased from Clonetech (pp53-EGFP). This plasmid contains a CMV promoter and results in continuous expression of EGFP-p53 in cultured cells. To examine

changes in the transcriptional activity of p53 by examining its downstream target genes, a plasmid containing a *cis*-acting enhancer element specific to p53 (pp53-TA-LUC) was used purchased from Clonetech. This plasmid contains p53 response elements, as found in p53 target genes, upstream of a luciferase reporter gene. To determine the effects of different levels of CRT expression on the regulation of p53 gene activity we used a plasmid containing the human p53 promoter upstream of a luciferase reporter gene (hp53-Luc). This plasmid was a gift from Dr. D. Reisman (University of South Carolina).

3. Cell culture and transfection

Wt, *crt-/-*, and CRT-*crt-/-* MEF cells were cultured in DMEM containing 10% FBS. Equal numbers (10^5 or $2x10^6$ cells/plate) of cells were plated at equal numbers, depending on the experiment. To achieve this, cells were trypsinsized and counted using a hemocytometer (Fisher). For immunocytochemistry or confocal microscopy, cells were plated on glass coverslips (thickness #1), otherwise cells were plated in regular petri dishes. After an overnight (ON) incubation at 37°C, cells were treated or transfected. Cells were transfected with 5μg total DNA using LipofectAMINE™ (Invitrogen) following the manufacturer's protocol. 16 hours after transfection, the media was changed to DMEM+10% FBS. Cells were incubated for an additional 24 hours to allow for the expression of the transfected DNA, then processed for confocal microscopy or reporter gene assay as described below.

4. Treatments

i. ALLN

As described in the literature review, the p53 protein has a short half-life and undergoes rapid degradation. Therefore, to be able to detect p53 in our experiments cells were pre-treated with 20μM ALLN (calpain inhibitor) for 2-6 hours, depending on the experiment, at 37°C. The cells were then divided into three groups; control untreated, UV treated or adriamycin treated. All these of treatments were done in the presence of ALLN.

ii. UV

Media was aspirated from each plate, prior to UV treatment, and cells were washed with PBS. Cells were then irradiated with 25 J/m^2 UV light in a microprocessor-controlled UV crosslinker (Spectrolinker: XL-1000, Spectronics Corp.). Following this treatment media (DMEM with 10% FBS) containing ALLN was added to the plates, and cells were allowed to recover by incubating them for 2 or 6 hours, depending on the experiment, at 37°C.

iii. Adriamycin (Doxorubicin)

To chemically activate p53, cells were treated with adriamycin. In these experiments, cells were incubated in media containing 400 ng/ml adriamycin and ALLN for two hours at 37°C.

5. Western blotting

For western blot analysis, cells were plated at a density of $2x10^6$ cells/plate. After the different treatments, cells were lysed using RIPA lysis buffer (250 mM NaCl, 1% NP-40, 20 mM Tris, pH 7.8, 1 mM EDTA, 1 mM EGTA, 1 mM Sodium Vanadate, 30 mM NaF, 10 mM $Na_4P_2O_7$, 1% Tx100, 0.5% NaDOC, 0.1% SDS) containing a protease inhibitor cocktail (Sigma-Aldrich). Cells were scraped off and nuclei and membrane fragments were removed by centrifugation for five minutes at 7,000 rpm at 4°C in the Eppendorf centrifuge 5804 R. The protein concentration in the supernatant was determined using the Dc protein assay kit (Bio-Rad). Lysates containing 50μg of protein were separated on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred to nitrocellulose membrane. The membrane was incubated in the blocking reagent for 15 minutes, followed by incubation with the primary antibody [mouse anti-p53 (1:10,000 Ab-1, Oncogene), goat anti-CRT (1:300 a gift from Dr. M Michalak, University of Alberta), or mouse anti-actin (1:300 Sigma-Aldrich)], followed by HRP-secondary antibody (1:10,000, Jackson Laboratories). The protein bands were visualized by chemiluminescence (SuperSignal West Dura extended duration substrate) and quantified on a Fluoro-S MultiImager (Bio-Rad).

6. Tissue isolation and preparation

Tissue samples were obtained from 14-days-old embryonic mouse hind-limb of *wt*, heterozygous (*crt*+/-) and homozygous CRT deficient mice (*crt*-/-). Tissue was

homogenized in RIPA lysis buffer containing protease inhibitor cocktail as described above. Particulate matter was removed by centrifugation at 7,000 rpm at 4°C for 10 minutes. The protein concentration was determined in the supernatant. 50 μg of protein was separated by 10% SDS-PAGE and p53 was detected by western blot analysis as described above.

7. Immunocytochemistry and fluorescent microscopy

Cells were plated on glass cover slips and incubated overnight. Following treatment with ALLN, cells were fixed for 15 minutes in 4% paraformaldehyde. Cover slips were incubated with blocking reagent for 20 minutes and then with primary antibody (monoclonal anti-p53 or anti-MDM2) for one hour at room temperature in a humidified chamber. Antibodies were diluted in blocking reagent (MDM2 (Clonetech) - 2μg/ml, p53 (Clonetech) - 5μg/ml final concentrations). FITC labeled anti-mouse antibody (Jackson Laboratories, Inc.) was used at 1:100 dilution in blocking reagent. Cover slips were incubated in secondary antibody for one hour at room temperature. Each cover slip was mounted on a microscope slide using Vectashield mounting media containing DAPI (Vector Laboratories, Inc.). The fluorescent signal from FITC and DAPI was captured using a Ziess epi-fluorescent microscope. Cells transfected with EGFP-p53 were washed and then fixed in 4% formaldehyde for 15 minutes at room temperature. Cold methanol (80%) was used to permeablize the cells. Nuclei were stained with 0.12 μg/ml Hoechst 33258 (Molecular Probes) for 20 minutes at room temperature. Each cover slip was mounted on a microscope slide using FluoroSave™ Reagent (Calbiochem). Fluorescence

was visualized using BioRad MRC-600 confocal microscope (GFP: excitation-emission 489/508 and Hoechst 33258: excitation-emission 346/460). Fields were captured at both wavelengths and images were processed using a Silicon Graphics (SGI) computer.

8. Reporter gene assay

Cells were co-transfected with a luciferase reporter plasmid (hp53-Luc of pp53-TA-Luc) and β-Gal plasmid. 48 hours following transfection, cells were lysed by addition of NP40 lysis buffer (100 mM Tris, pH 7.8, 0.5% NP-40). Aliquots of each cell lysate were assayed for luciferase activity as described in Waser *et al* (1997) [203]. Briefly, aliquots of cell lystate were mixed with luciferase assay buffer (500 mM Tricine, 100 mM Mg-carbonate, 1 M $MgSO_4$, 50 mM EDTA, 1 M dithiothreitol (DTT), 10 mM coenzyme A, 100 mM luciferin, 100 mM ATP) in a luminometer (Lumat LB 9507, Berthold Tech.) and breakdown of luciferin was measured after a 20 second incubation. β-galactosidase activity in the same cell lysate was measured using a final concentration of 20 mM o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate (as described in Waser *et al.* (1997) [203]). This is a colorimetric assay and was quantified using OD_{420} (MRX Revelation 4.22, Dynex technologies). Experiments were done in triplicate and were repeated 5-6 times. To normalize for transfection efficiency the ratio of luciferase to β-galactoside activity was collected and results were plotted as a mean +/- SE.

9. Statistics

Graphs were tabulated by establishing a mean and standard error for individual experimental results. In addition, student t-test was used to determine degree of significance of specific results.

RESULTS

p53 function

p53 acts as a transcription factor able to induce the expression of several genes by binding to a specific DNA motif (response-elements). To examine the role of CRT on p53 mediated gene activation we used a luciferase based reporter assay as described in Materials and Methods. Figure 3 shows a significant decrease (approximately 17.6% that of *wt*) in the p53 dependent expression of luciferase in CRT deficient cells as compared to *wt* cells. Reduced p53 tetramer binding to these response-elements may translate to decreased p53-mediated transcriptional activity. This result suggests a role for CRT in the regulation of p53 function and programmed cell death. This modulation of p53 function by CRT may occur through regulatory mechanisms such as impaired p53 protein expression (protein stability) or changes in sub-cellular localization of p53. Therefore we examined the effect of CRT expression levels on p53 protein levels and gene transcriptional regulation.

Expression of p53

Alteration of p53 protein expression levels is known to result in a change in its function [19]. Therefore we examined p53 expression in the presence or absence of CRT protein expression using western blot analysis. Figure 4A shows the level of CRT expression in whole cell lysates of *wt, crt-/-*, CRT-*crt-/-* cell lines. As seen in Figure 4A, the level of

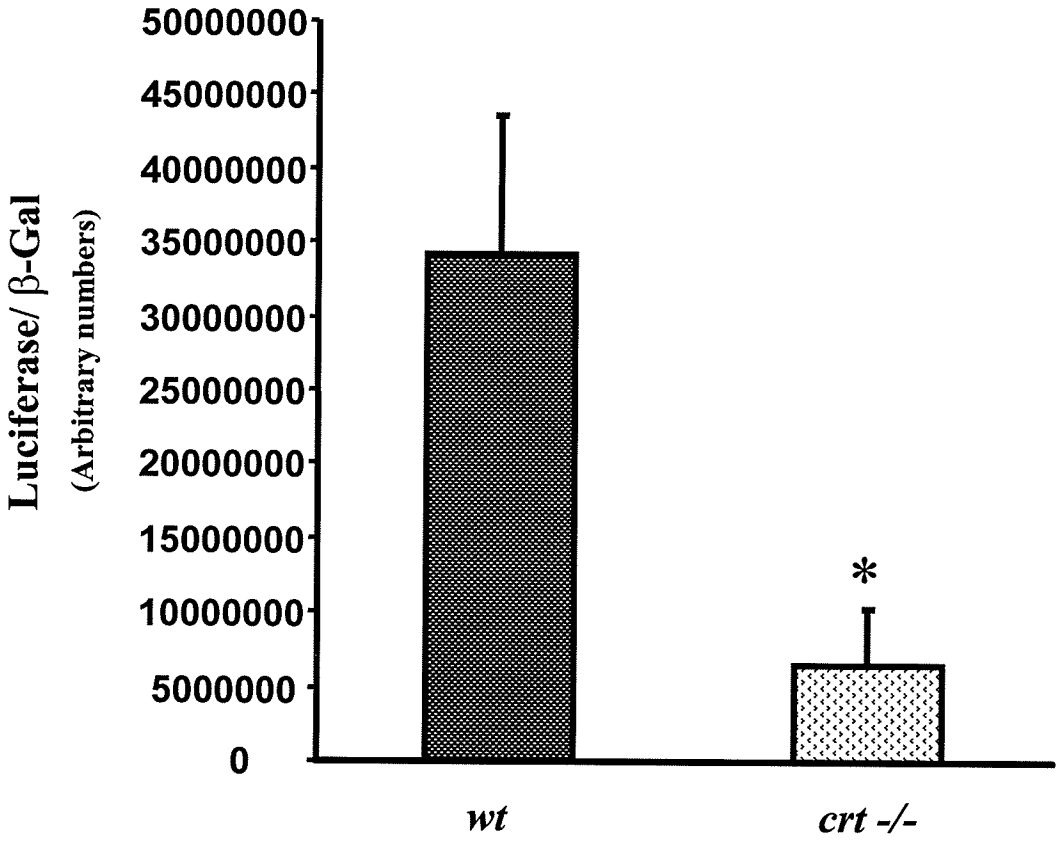


Figure 3. Alteration of p53 function with changes in CRT expression. *Wt* and *crt-/-* MEF cells were co-transfected with a plasmid containing a p53-response element upstream of a luciferase reporter gene and a plasmid encoding β-Galactosidase as described in Materials and Methods. Data represents normalized values of the luciferase activity to β-Gal activity. Bars represent the mean of five different experiments ±SE; each experiment was done in triplicates.

* $P < 0.001$ as determined by student t-test.

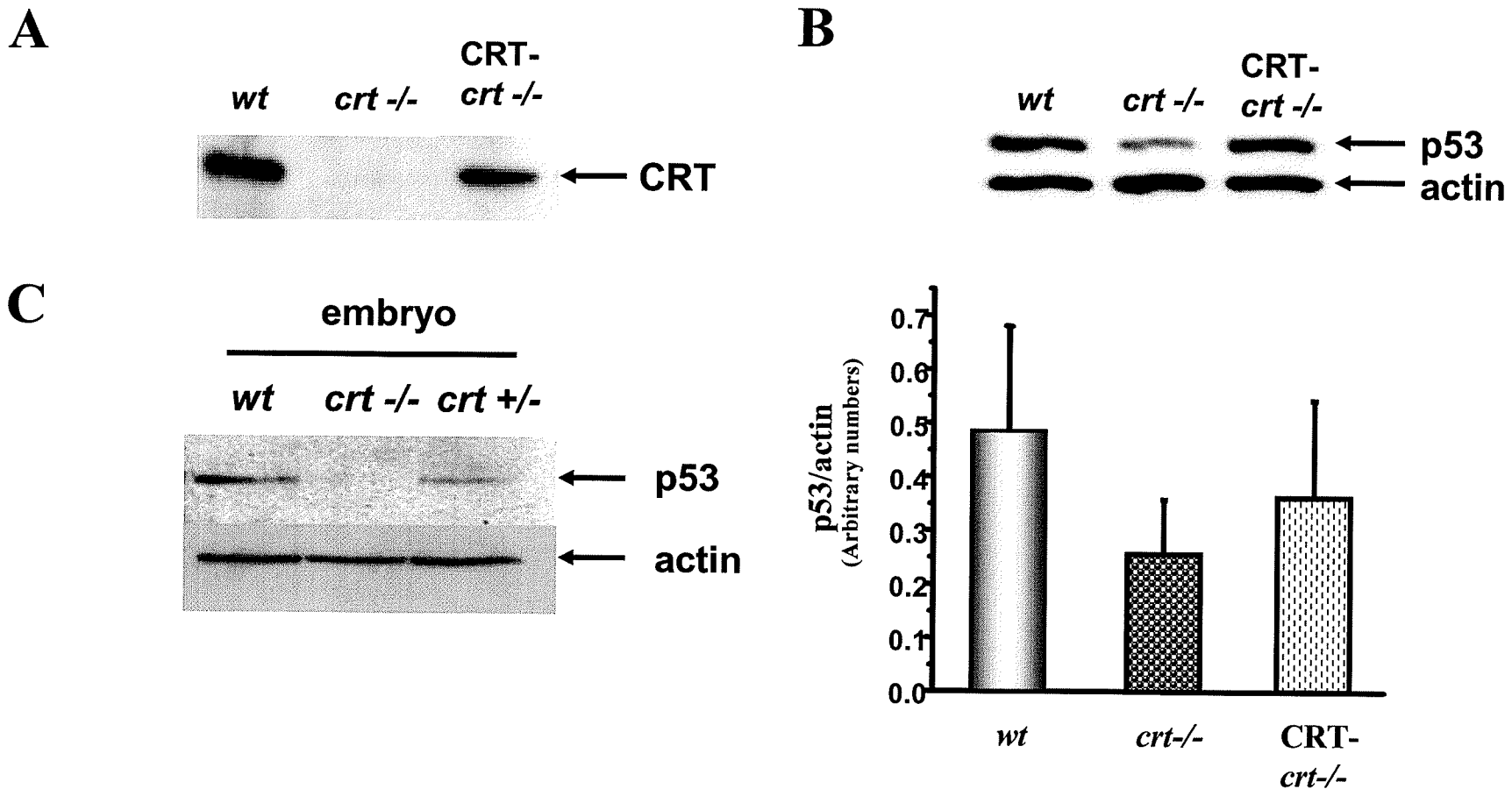


Figure 4. Analysis of CRT and p53 protein levels in *wt, crt-/-* and CRT-*crt-/-* MEF cells. (A) Western blot of MEF cultured cell lines probed with anti-CRT antibody as described in Materials and Methods. (B) Western blot analysis of p53 and actin expression in *wt, crt-/-* and CRT-*crt-/-* cell lines. Bands were quantified as discribed in Materials and Methods. The p53 values were normalized to actin values and plotted as Bar graph. (C) Western blot of lysate from tissue sample of *wt, crt-/-* and *crt+/-* mice embryos were probed with anti-p53 and anti-actin antibodies.

CRT expression in CRT transfected *crt-/-* (CRT-*crt-/-*) cells, is only 60% of *wt*. Figure 4B shows a significant decrease in p53 protein in *crt-/-* cells as compared to *wt* and CRT-*crt-/-* cells. To control for equal loading the same blots were also probed with anti-actin antibody as described in Materials and Methods (Figure 4B). A bar graph (Figure 4B) summarizes the average of densitometric quantification of four western blots and shows a 50% reduction in the p53 protein level in *crt-/-* cells as compared to *wt*. This decrease was reversed in CRT-*crt-/-* cells, but this recovery was not complete (Figure 4B). A similar decrease in the p53 protein level was observed in homogenates prepared from embryonic tissue (Figure 4C) and rules out changes in p53 expression due to cell culture conditions. Figure 4C shows that the p53 protein level in the heterozygous CRT null (*crt*+/-) mouse embryonic tissue is approximately half of that of *wt* embryos. Furthermore, p53 protein was not detectable in the homozygous CRT null (*crt-/-*) embryonic homogenate (Figure 4C). This suggests a direct relationship between CRT expression and p53 protein level. These observed changes could be attributed to altered transcriptional regulation of the p53 gene or reduced protein stability and rapid degradation of p53.

Transcriptional regulation of p53 gene

To investigate changes in the p53 gene expression we studied the activity of the p53 promoter using a luciferase reporter gene assay. Figure 5 shows that in *crt-/-* cells the p53 promoter activity was increased by approximately 150% as compared to *wt*. In addition re-introducing CRT to *crt-/-* cells decreased p53 promoter activity (Figure 5). However

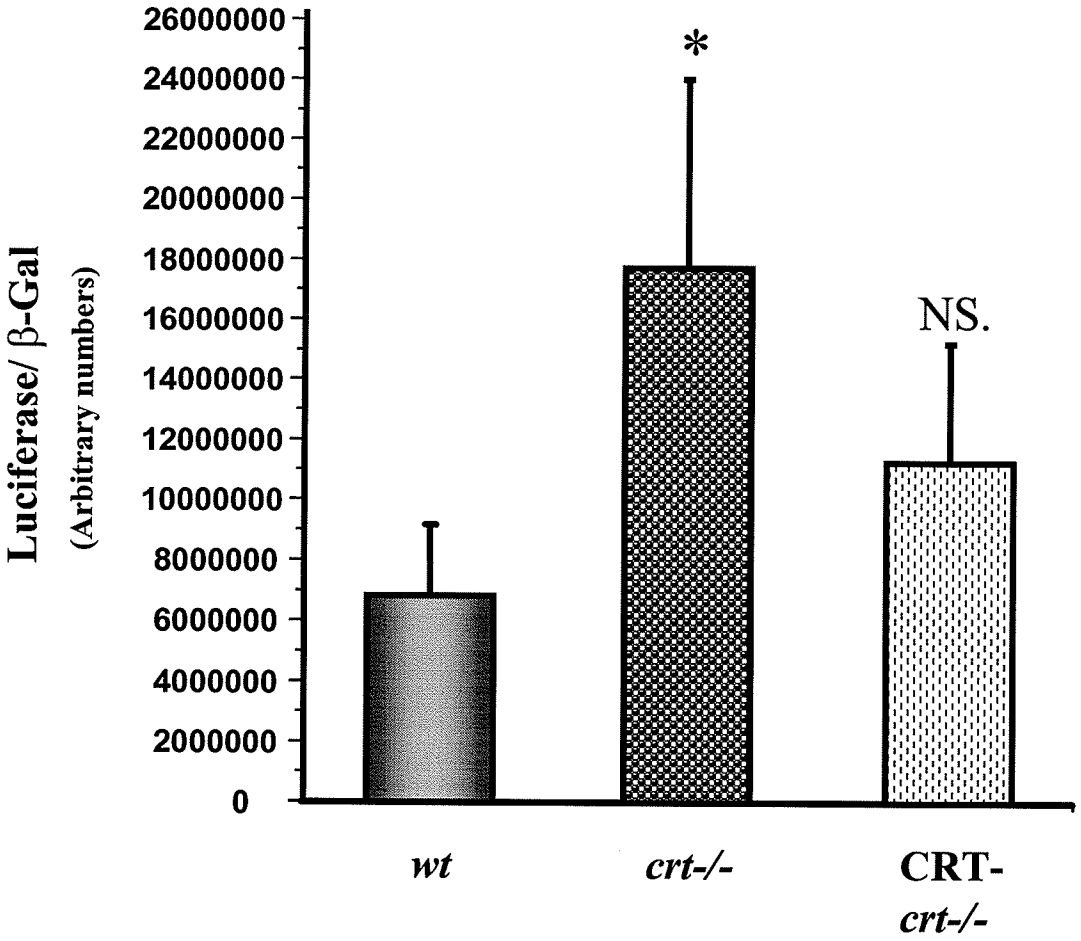


Figure 5. Transcriptional activity of the p53 gene in *wt*, *crt-/-* and CRT-*crt-/-* cells. p53 promoter activity was measured using a luciferase reporter plasmid containing human p53 promoter. Luciferase values were normalized to β-Gal acitivity as described in Materials and Methods. Bars represent the mean of four experiments ± SE; each experiment was done in triplicate.

* $P < 0.05$ vs. *wt*

NS. Indicates not significantly different from the *wt*.

this activity was not reduced to the *wt* level (approximately 60% greater than *wt*) (Figure 5). The observed difference in the *wt* and CRT-*crt*-/- could be attributed to lower level of CRT expression in CRT-*crt*-/- (Figure 4A). These results suggest that regulation of p53 by CRT is not due to changes in gene transcription but rather it might be due to rapid degradation of p53 protein by ubiquitination and proteasome activity.

Localization of p53

Altered p53 cellular localization can also affect the function of p53. Proper nuclear import of p53 is key in the regulation of its ability to regulate activate gene transcription [2]. Impaired nuclear import results in the cytoplasmic accumulation of p53 and is known to be a marker for breast cancer [204], colorectal cancer [205], and neuroblastoma [206] in humans. To investigate the relationship between changes in nuclear import of p53 and altered CRT expression we used immunocytochemistry to localize endogenous p53 protein (Figure 6). Prior to localization, cells were treated with the calpain inhibitor ALLN to limit rapid degradation of p53. Figure 6A shows that in *wt* cells p53 is predominantly localized to the nucleus. Superimposing of DAPI stain with p53 fluorescence allows for the co-localization of the two signals and confirmation of nuclear localization (Figure 6A"). However, in *crt*-/- cells the p53 signal was cytosolic as shown in Figure 6 B, B". The cytoplasmic accumulation of p53 was seen upon superimposing of FITC fluorescence (p53) and DAPI (Figure 6B"). This altered nuclear import was reversible as seen in CRT-*crt*-/- cells (Figure 6 C, C"). There was no difference in p53

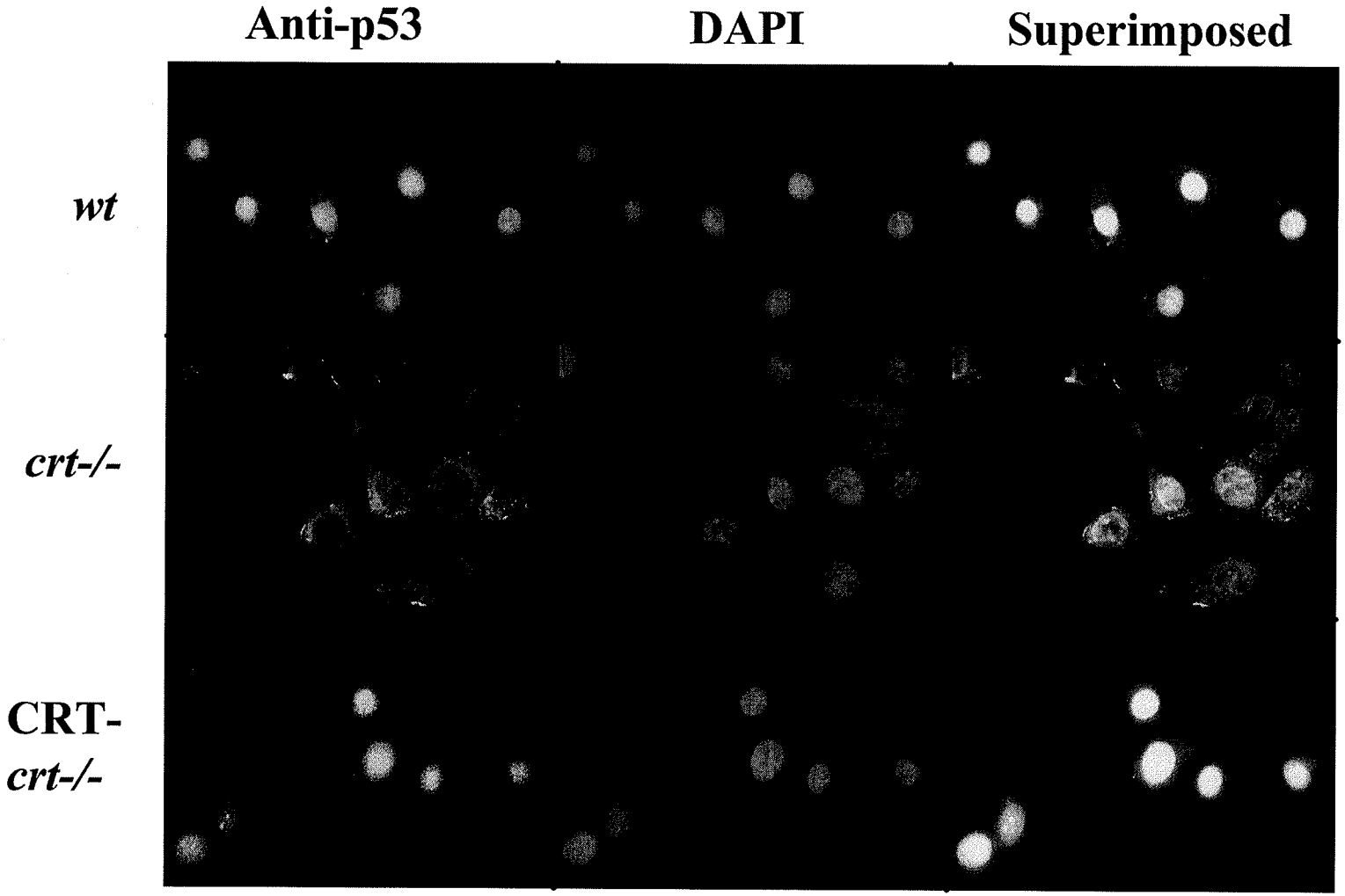


Figure 6. Endogenous p53 protein localization. Immunocytochemical localization with anti-p53 antibody via fluorescent microscopy (A, B, C). MEF cells (*wt, crt-/-*, and CRT *crt-/-*) were stained with DAPI nuclear marker (A', B', C') for cellular localization. Superimposed p53/DAPI fluorescence (A'', B'', C'') allows for co-localization of p53 to nucleus.

localization in *wt* and CRT-*crt-/-* cells (Figure 6 A, A" and 6 C, C"). To test if the defect in nuclear import of p53 could be stimulated by activation of p53, cells were treated with UV treatment and allowed to recover for 6 hours before processing for immunocytochemistry (Figure 7). DNA damage via UV irradiation is known to stimulate p53 activation and its nuclear accumulation [1]. As shown in Figure 7 (A, C), following UV treatment p53 is completely localized to the nuclei in the *wt* and CRT-*crt-/-* cells. Figure 7 (A", C") shows the co-localization of p53 and DAPI in the nucleas confirming its location. However in the *crt-/-* cells the p53 signal is present in the cytosol and the nuclear p53 has a punctate pattern (Figure 7B). This punctate pattern appears to be associated with the nucleoli. Previous reports have shown accumulation of p53 and MDM2 to the nucleoli in the absence of cellular stresses [39].

MDM2 localization

MDM2 is a strong negative regulator of p53 function through the binding to the N-terminus of the p53 protein [207]. Ubiquitination of p53, by E3-ligase activity of MDM2, stimulates the nuclear export of p53 and its degradation via the proteasome pathway [73]. Figure 8 shows that in the *wt* and CRT-*crt-/-* cells the MDM2 signal is largely cytoplasmic (Figure 8 A, C). The position of the nuclear signal was confirmed by DAPI staining (Figure 8 A", C"). However, in *crt-/-* cells the MDM2 signal is almost entirely nuclear (Figure 8B) and co-localizes with the nuclear specific stain DAPI (Figure 8B"). These results suggest that in absence of CRT, MDM2 is activated and binds to p53 to mediate its ubiquitination and degradation. Changes in the localization of MDM2 after

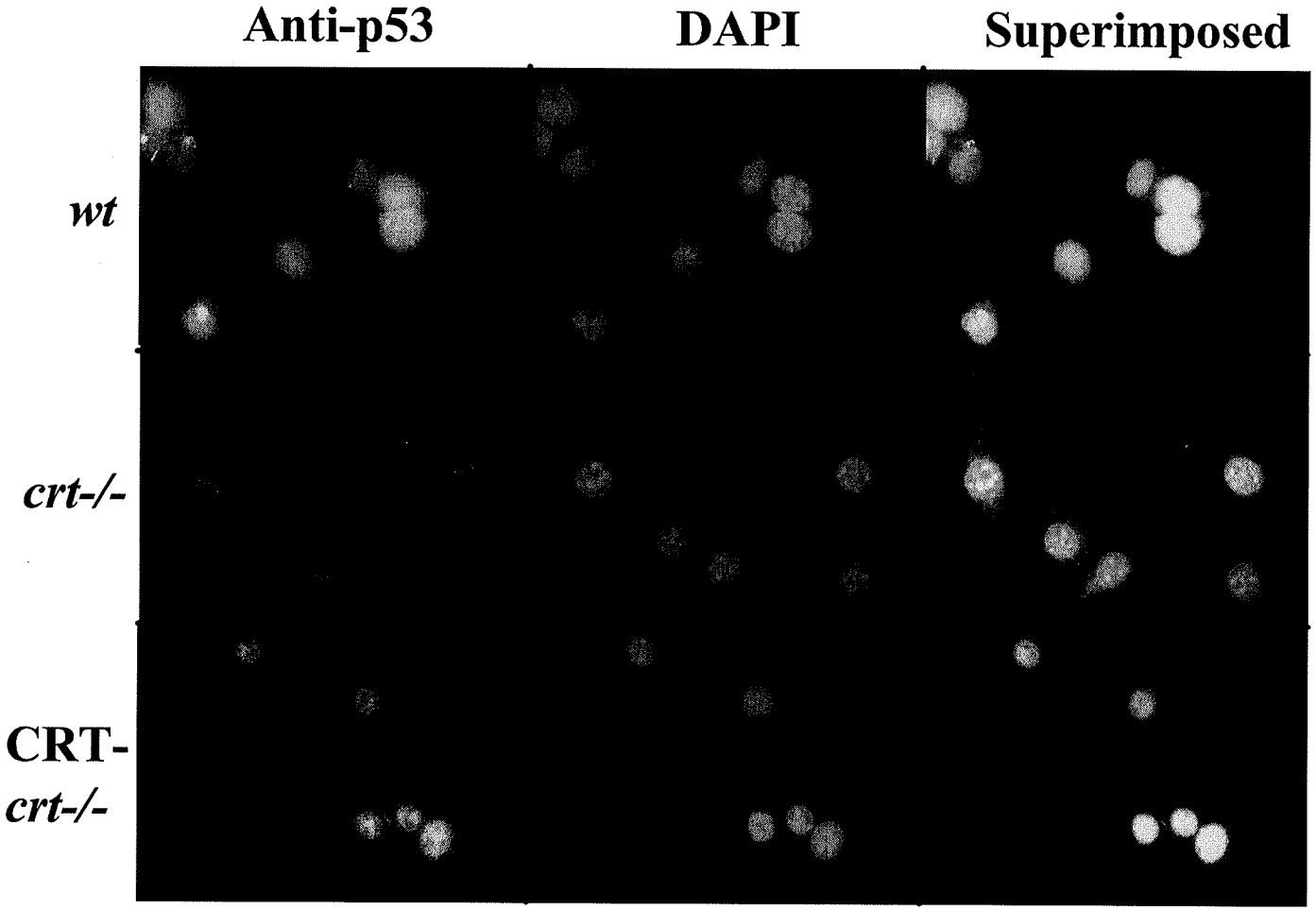


Figure 7. Localization of p53 in MEF cells following UV treatment and a 6 hour recovery. Cells (*wt, crt-/-*, CRT-*crt-/-*) were treated with UV as described in Materials and Methods followed by immunocytochemical detection of p53 (A, B, C). Cells are stained with DAPI nuclear marker (A', B', C') and signals were captured using a Zeiss epi-fluorescent microscope. A'', B'', C'' are superimposed images of FITC and DAPI.

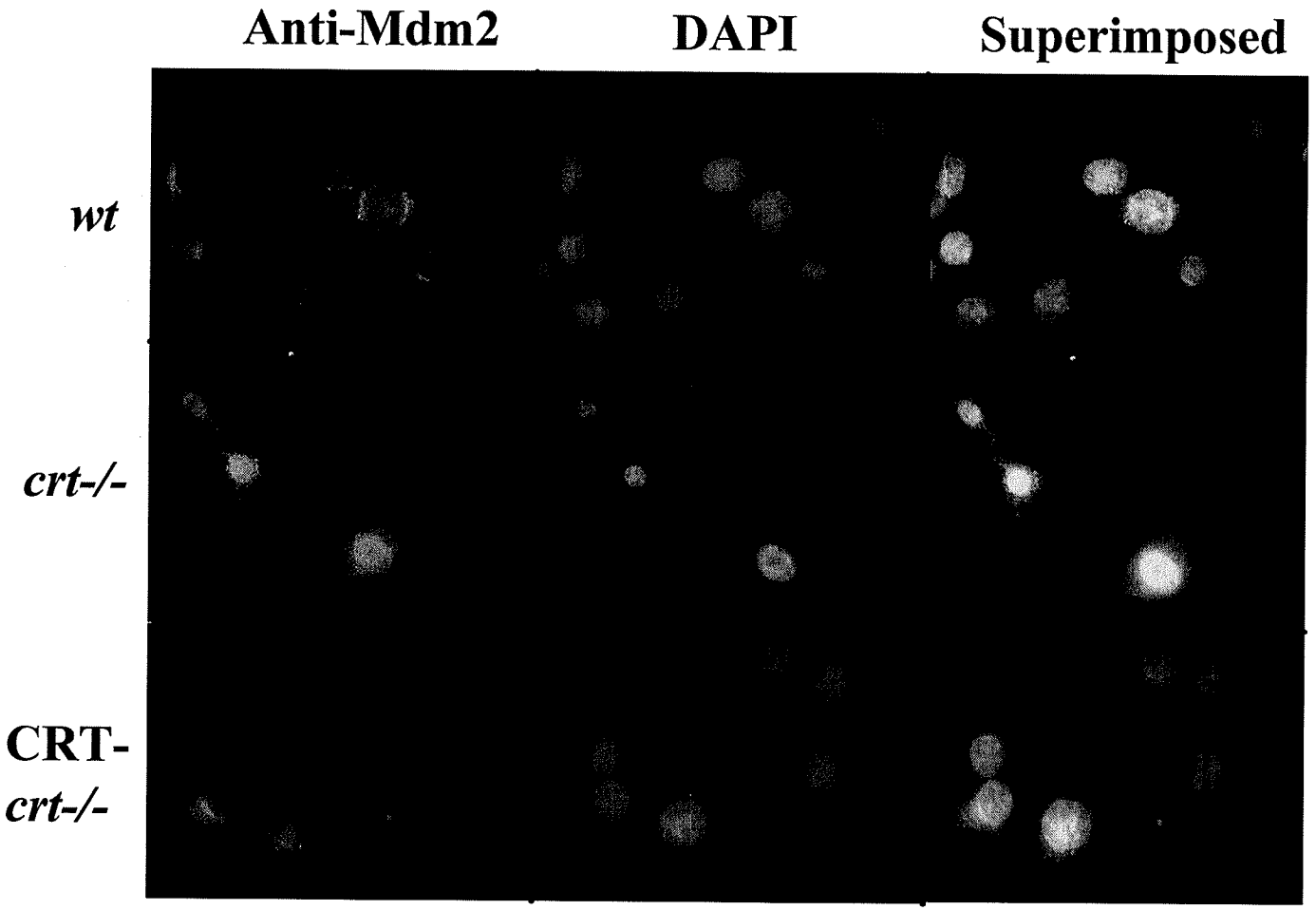


Figure 8. Localization of endogenous MDM2 protein. Anti-MDM2 antibodies were used to detect protein by immunocytochemistry (A, B, C). Nuclear localization was confirmed with DAPI nuclear marker (A', B', C'). Overlay of MDM2/DAPI signals (A'', B'', C'') show the cellular localization of MDM2 in *wt, crt-/-* and CRT-*crt-/-* cells.

UV treatment were also studied in these cells (Figure 9). As in non-treated cells, w*t* and CRT-*crt-/-* cells displayed cytoplasmic localization of MDM2 (Figure 9 A, C). Co-localization with DAPI (Figure 9 A', C') confirms the cytoplasmic localization after UV induction (Figure 9 A", C"). On the other hand, CRT deficient cells showed a decrease in nuclear MDM2 protein signal (Figure 9B) after UV treatment as compared to control non-treated *crt-/-* cells (Figure 8B). An inhibition of MDM2 nuclear import after UV stress might provide a mechanism by which p53 is detected in the nucleus. This data suggests a possible role for CRT in MDM2-mediated modulation of p53 function in CRT deficient cells.

Localization of over-expressed p53

Transfection of EGFP-p53 fusion protein in MEF cells (*wt, crt-/-*, and CRT-*crt-/-*) was used to examine whether over-expression of p53 could stimulate its nuclear import in CRT deficient cells (Figure 10). Over-expression has been shown to induce its nuclear translocation and activity [19]. Figure 10 shows the nuclear accumulation of EGFP-p53 fluorescence within w*t* and CRT-*crt-/-* cells (Figure 10 A, C) while this signal is cytosolic in *crt-/-* MEF cells under control conditions (Figure 10B). Superimposed images confirm nuclear and cytoplasmic localization respectively (Figure 10 A", B", C"). Upon nuclear import, p53 can mediate controlled cellular breakdown (apoptosis) often shown by DNA fragmentation and nuclear fragmentation [2]. Figure 10 A, C arrows show the nuclear fragmentation in the *wt* and CRT-*crt-/-* cells overexpressing EGFP-p53, indicating induction of apoptosis in these cells.

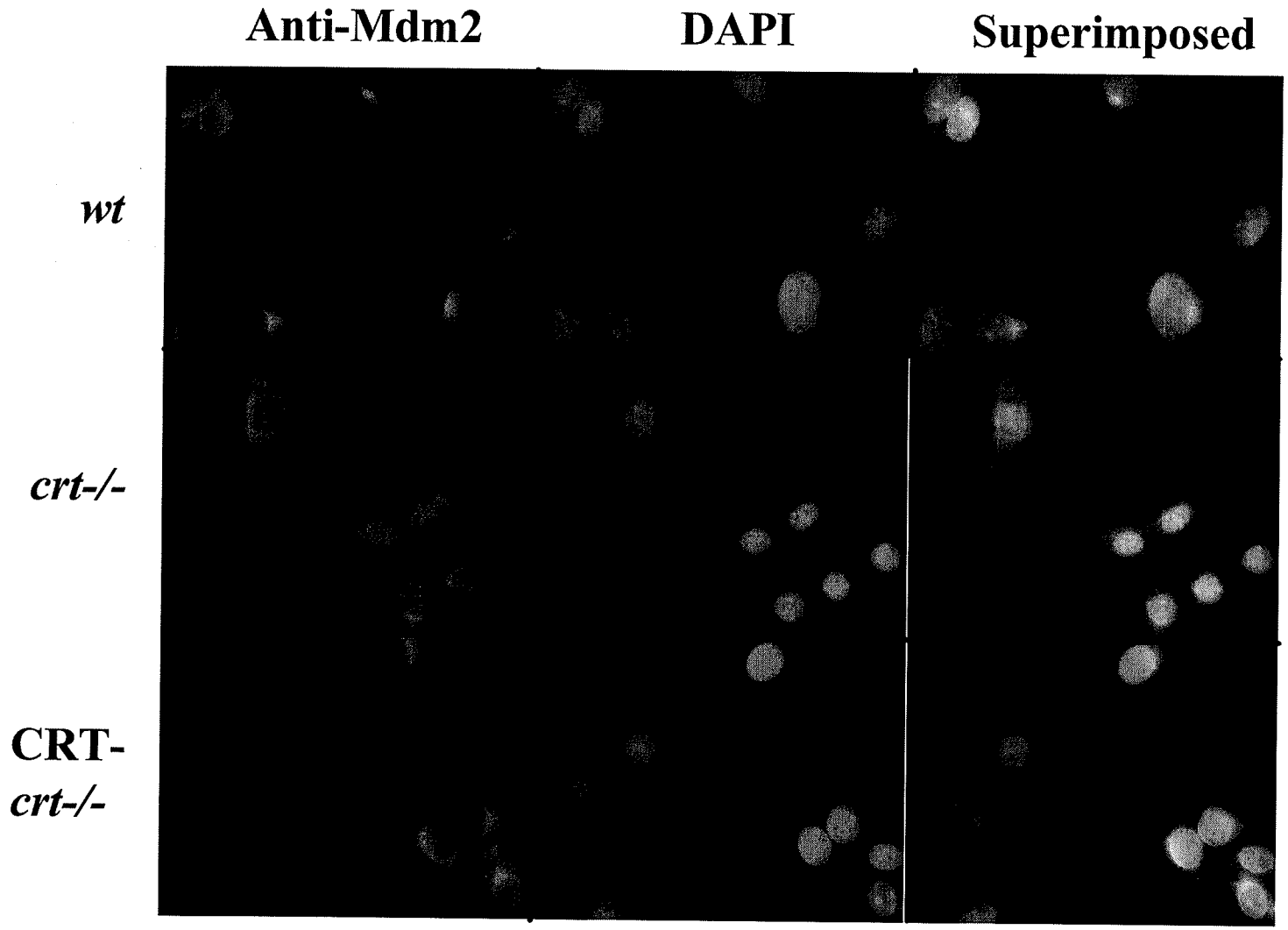


Figure 9. MDM2 cellular localization after UV-induced stress. Detection of endogenous MDM2 protein localization was detected by immunocytochemistry and fluorescent microscopy. Images represent MDM2 (A, B, C), DAPI fluorescence (A', B', C') and superimposed signal (A'', B'', C'').

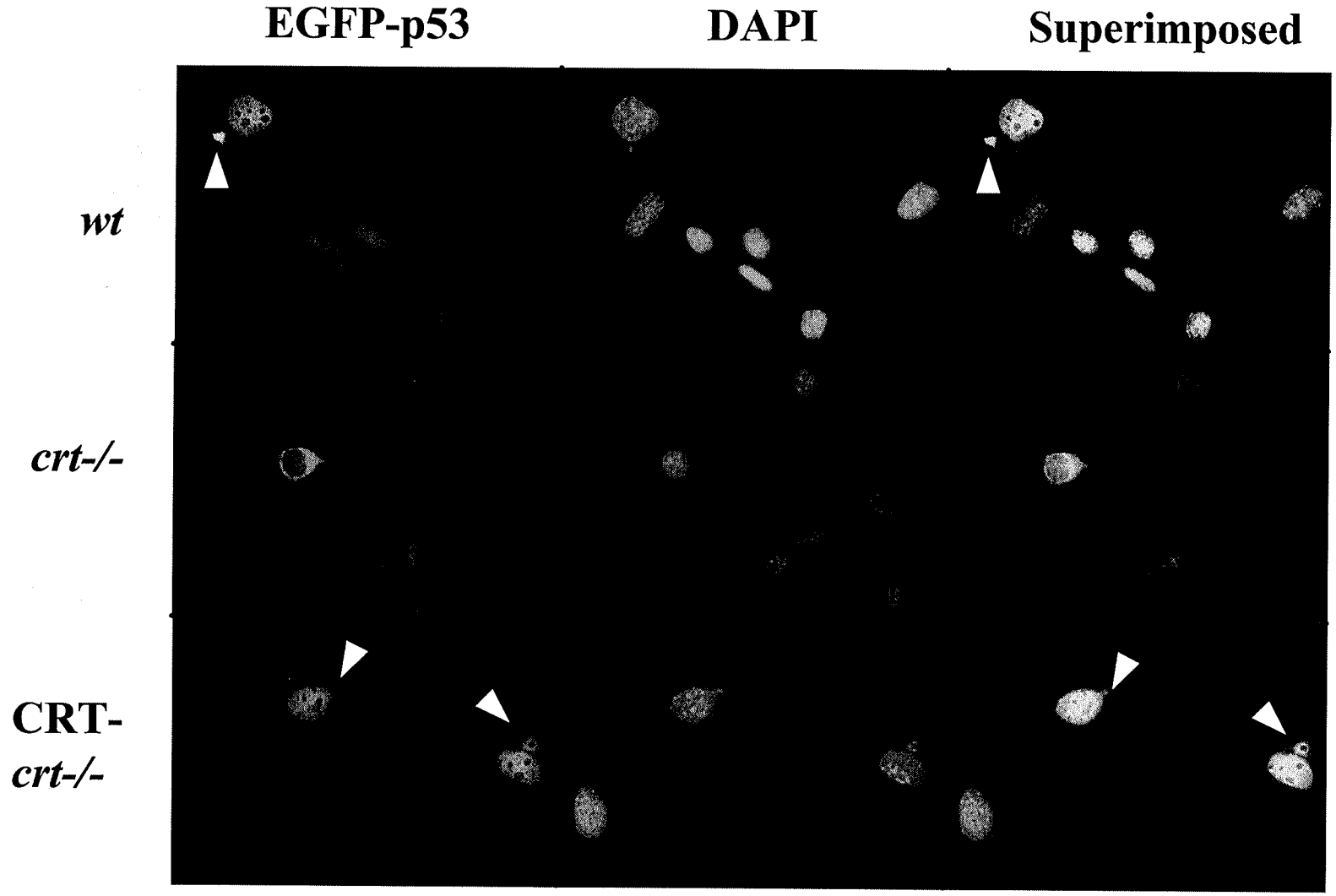


Figure 10. Nuclear translocation of p53 with different CRT expression was detected by EGFP-p53. The plasmid encoding for EGFP-p53 fusion protein was transiently transfected in MEF cells (*wt, crt-/-* and CRT-*crt-/-*) as described in Materials and Methods. EGFP signal was captured using Zeiss epi-fluorescent microscope (A, B, C). A', B', and C' show the location of the nuclei in MEF cells. A'', B'', and C'' are superimposed images of EGFP and DAPI confirming the nuclear localization or exclusion of the EGFP-p53 protein. Arrowhead points to the apoptotic bodies in *wt* and CRT-*crt-/-* cells.

Cell stress leading to DNA damage is known to stimulate p53 activity as well as nuclear import [40, 45]. To examine whether DNA damage might induce the nuclear translocation of EGFP-p53 in *crt-/-* cells, we treated the cells with UV treatment or adriamycin (Figure 11, 12) as described in Materials and Methods. As seen in Figure 11B UV treatment results in a partial nuclear translocation of EGFP-p53 with the majority of the signal remaining in the cytosol in *crt-/-* cells as confirmed by Hoechst 33258 nuclear specific staining (Figure 11B') and overlay (Figure 11B"). Nuclear signal was maintained in both *wt* and CRT-*crt-/-* cells (Figure 11 A", C") as observed in non-treated control images (Figure 10, A", C"). Treatment with adriamycin (Figure 12*)* resulted in a similar EGFP-p53 localization as was seen with UV treatment (Figure 11). CRT deficient cells showed only a very slight nuclear fluorescence (Figure 12B), but the majority of the signal remains in the cytoplasm as confirmed by Hoechst 33258 staining (Figure 12B"). In the *wt* and CRT-*crt-/-*, the EGFP-p53 signal remains nuclear (Figure 12 A, C), similar to the untreated cells (Figure 10).

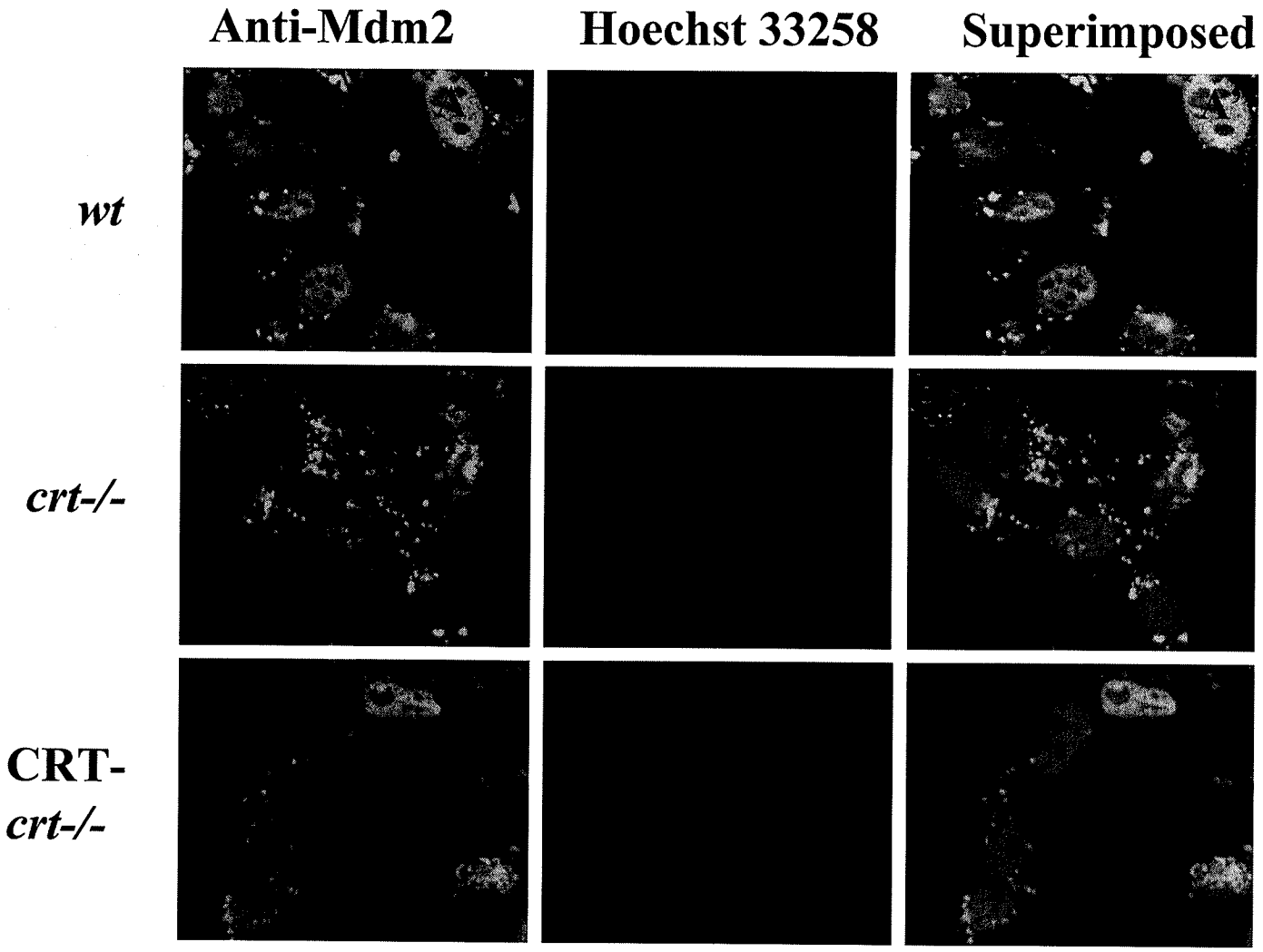


Figure 11. Nuclear localization of EGFP-p53 following UV treatment. Confocal microscopy of EGFP-p53 transfected cells (*wt, crt-/-*, and CRT-*crt-/-*) to detect p53 localization after treatment with UV light. EGFP signal was captured using Bio-Rad confocal microscope (A, B, C). Hoechst 33258 nuclear stain was used to determine the location of the nuclei in MEF cells (A', B', C'). A'',B'' and C'' are overlays of EGFP and Hoechst 33258 images confirming nuclear localization of EGFP-p53 fusion protein.

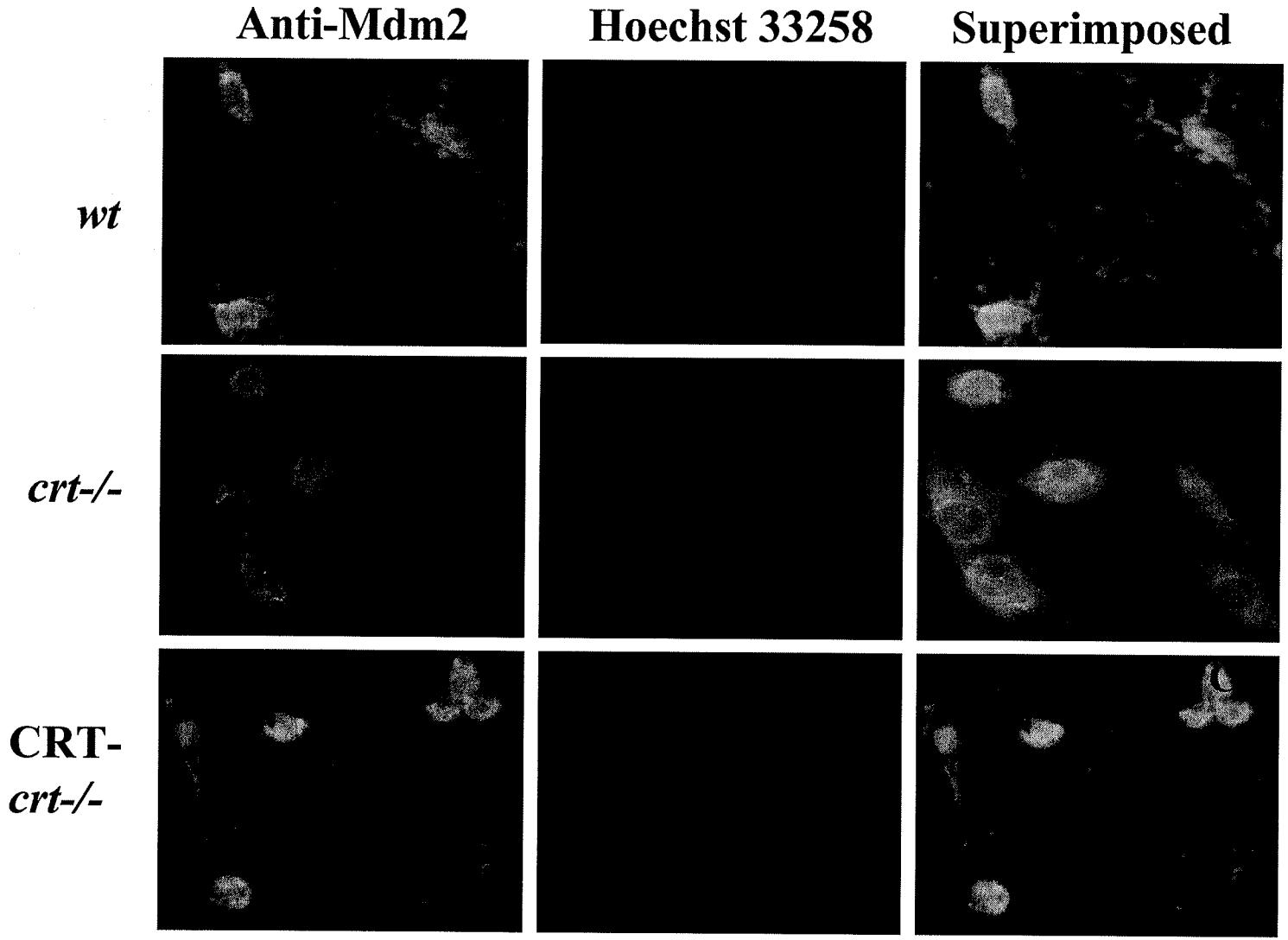


Figure 12. Nuclear localization of EGFP-p53 following adriamycin treatment. Confocal microscopy was used to determine EGFP-p53 localization with different CRT expression (*wt, crt-/-* and CRT-*crt-/-*). A, B, and C show the EGFP signal in MEF cells. A', B' and C' images show the location of the nuclei within each panel as determined with Hoechst 33258 nuclear stain. Superimposing of EGFP-p53 and Hoechst 33258 signals (A'', B'', C'') confirms the nuclear localization of EGFP-p53 protein.

D. DISCUSSION

In this study we showed modulation of tumor suppressor p53 function by CRT. Furthermore, we provided evidence that this regulation is mediated by a decrease in the p53 protein level and altered p53 localization. Cellular stresses resulting in DNA damage is known to stimulate p53 [80] which increases transactivation of downstream genes [2]. Upon activation, p53 tetramerizes and binds to the p53 response element region within the promoter of target genes regulating the transcription of these genes [47]. Using a reporter plasmid containing the p53 response-element we showed a significant decrease in the p53 function in *crt-/-* cells. The decreased p53 function would result in impaired cell cycle regulation and escape from apoptosis. Indeed, the *crt-/-* cells have been shown to be more resistant to apoptotic stimuli such as UV treatment (Mesaeli and Phillipson, submitted) and staurosporine [5]. These findings suggest a possible role for CRT in modulating p53-mediated apoptosis through the reduction of p53 mediated gene transactivation. The inability of p53 to recognize and activate p53-response elements within target genes exists in a high percentage of human cancers [19]. Modulation of p53 function can be regulated by several mechanisms including change in protein expression, rapid protein degradation/protein stability and regulation of nuclear localization of p53.

Regulation of p53 expression levels is important for modulation of the cell cycle. Indeed, loss of p53 (gene targeted deletion) has been demonstrated to give rise to a rapid malignant progression of tumors [56]. Our results are the first to show a relationship between p53 and CRT protein levels as demonstrated by the significant decrease of p53

protein in both cells and embryonic tissues lacking CRT. The re-introduction of CRT in *crt-/-* cells resulted in the recovery of the p53 protein level to that similar to the *wt.* However, the level of p53 in CRT-*crt-/-* cells did not reach that of the *wt.* This is due to lower CRT expression (only 60% of *wt*) in these cells. This observed decrease in p53 protein could explain the increased resistance of *crt-/-* cells to UV treatment induced apoptosis (Mesaeli and Phillipson, submitted). Furthermore, we showed that this decrease in p53 protein was not due to a defect in p53 gene transcription. In contrast, we showed a marked increase in the p53 promoter activity in *crt-/-* cells as compared to *wt* or CRT-*crt-/-* cells. This data suggests a post-translational regulation of p53 protein in the absence of CRT.

p53 is a relatively short-lived protein with a half-life of between thirty and sixty minutes [44]. Under normal conditions, p53 protein level is regulated by rapid degradation mediated by calpain and MDM2 [73, 77, 78]. In our experiments, we used a calpain inhibitor to inhibit its proteolytic activity, leaving the MDM2-proteasome pathway intact. MDM2 is a RING finger protein with an E3 ligase activity and has been shown to bind and ubiquitinate p53 [44]. The MDM2-mediated ubiquitination of p53 alters its nuclear localization by interaction with Crm1 and nuclear export [43, 70, 71]. Once in the cytosol the ubiquitinated p53 is degraded by the proteasome pathway [73]. Our results demonstrate nuclear accumulation of MDM2 in CRT deficient cells, which would suggest an increased p53 degradation and subsequent reduction in apoptosis. This increase in MDM2 in the nucleus was accompanied by a decreased nuclear p53 signal and decreased p53 protein level. These observations led us to conclude that in the *crt-/-*

cells the activated MDM2 induce nuclear export and degradation of p53. Recently Kageyama *et al.* (2002) [6] have shown that over-expression of CRT leads to a decrease in Akt activity and increase in the susceptibility of these cells to apoptosis. Therefore, one can assume that in the absence of CRT, Akt activity would perhaps increase. Figure 13 is a schematic diagram showing the mechanism by which CRT might influence p53 protein function. Here we propose that in absence of CRT the Akt/PKB activity is up regulated resulting in activation of MDM2. Previous reports have shown that activation of Akt results in the phosphorylation of MDM2 [201]. The activated MDM2 is then translocated into the nucleus where it binds to p53 protein inducing its nuclear export and subsequent degradation [202]. In fact we have shown an increase in the nuclear accumulation of MDM2 in *crt-/-* cells and reduced nuclear p53 in these cells. These findings can elucidate the decreased sensitivity of CRT deficient cells to UV treatment-induced apoptosis (Mesaeli and Phillipson, submitted) [5].

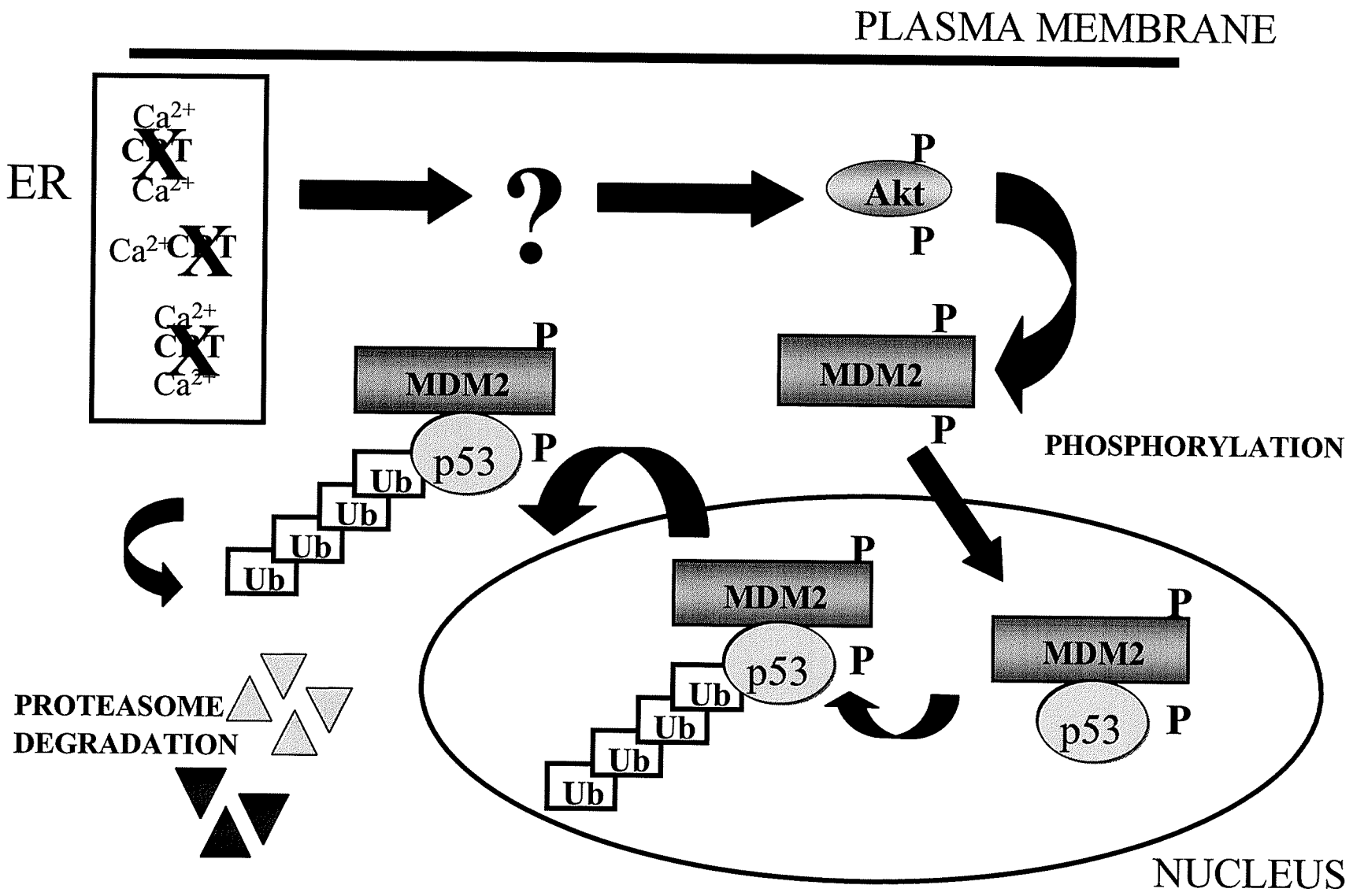
UV-stimulation of p53 localization has been known for some time and is well established in the literature [1]. Here we showed that in contrast to non-treated cells, the *crt-/-* cells resulted in accumulation of MDM2 in the cytosol. The diminished level of nuclear MDM2 protein could explain the presence of p53 in the nuclei of *crt-/-* cells as seen in Figure 7. The appearance of a punctuate pattern of nuclear p53 has been described previously for inactive p53 in HepG2 cell line [39]. Because the *crt-/-* cells are resistant to UV treatment-induced apoptosis, one can assume that the nuclear p53 accumulation following UV treatment is inactive.

Nuclear shuttling of tumor suppresser protein p53 is crucial for its function as a transcription factor. Impaired nuclear import has been shown to result in cytoplasmic accumulation of p53 and various forms of human cancers (breast cancers, colon cancers, and neuroblastoma) [4]. As described in the literature review, nuclear import of p53 can be stimulated by DNA insult by a variety of cellular stresses (UV, IR, chemotheraputic agents, oncogenes, hypoxia etc.) [1, 42, 45, 46]. The nuclear import of p53 is accomplished primarily by a nuclear localization signal (NLS) located at the protein's C-terminus [36]. Nuclear import occurs through the nuclear pore complex (NPC) which is the only channel spanning the nuclear envelope [160]. The outer leaflet of the NE is morphologically continuous with the ER and has attached ribosomes. The lumenal contents of the peripheral ER and NE are also continuous [161]. Therefore, calcium handling associated with the ER also occurs within the NE [162]. As described in the literature review, nuclear envelope Ca^{2+} stores are known to be important in the regulation of transport through the NPC [162, 168-170]. Loss of proper Ca^{2+} homeostasis results in inhibition of nuclear import due to a conformational change or plugging of the NPC [162, 170]. Two reports have shown a defect in Ca^{2+} homeostasis seen in *crt-/-* cells resulting in impaired nuclear import of nuclear factor of activated T cells (NF-AT3) [7] and myocyte enhancer factor C2 [8]. Similar to these studies we also observed an impaired nuclear translocation and cytoplasmic accumulation of p53 in CRT deficient cells.

Over-expression of p53 is known to induce its nuclear accumulation [19]. Here we showed that expression of EGFP-p53 fusion protein in both *wt* and CRT-*crt-/-* cells

resulted in nuclear localization of p53. In these cells, the p53 pro-apoptotic nature could also be noted by the nuclear fragmentation. However, in *crt-/-* cells the p53 signal was completely cytoplasmic and there was no nuclear fragmentation. Our results indicate a defect in appearance of nuclear import of p53 in *crt-/-* cells even when this protein was over-expressed. Furthermore, we showed that UV treatment or adriamycin treatment was not able to completely localize p53 to the nucleus. Here we noted only marginal nuclear import of EGFP-p53 in *crt-/-* cells strengthening the hypothesis that nuclear import of p53 is altered within CRT deficient cells. Our results in addition to the finding of Kageyama *et al.* (2002) [6] demonstrating decreased Akt/PKB activity in CRT over-expressing cells, lead us to conclude that CRT regulates p53 function by affecting its stability and nuclear localization.

Figure 13: Schematic diagram showing the mechanism of regulation of p53 protein by calreticulin.



REFERENCES

1. Maltzman, W. and L. Czyzyk, *UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells.* Mole. and Cell. Biology, 1984. **4**: p. 1689-1694.
2. Kastan, M.B., O. Onyekere, and D. Sidransky, *Participation of p53 protein in cellular response to DNA damage.* Cancer Res., 1991. **51**: p. 6304-6311.
3. Sakaguchi, K., et al., *DNA damage activates p53 through a phosphorylation-acetylation cascade.* Genes and Dev., 1998. **12**: p. 2831-2841.
4. Jansson, A., M. Gentile, and X. Sun, *p53 mutations are present in colorectal cancer with cytoplasmic p53 accumulation.* Int. J. Cancer, 2001. **92**: p. 338-341.
5. Nakamura, K., et al., *Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis.* J. B. C., 2000. **150**(4): p. 731-740.
6. Kageyama, K., et al., *Overexpression of calreticulin modulates protein kinase B/Akt signalling to promote apoptosis during cardiac differentiation of cardiomyoblasts H9c2 Cells.* J. B. C., 2002. **277**(22): p. 19255-19264.
7. Mesaeli, N., et al., *Calreticulin is essential for cardiac development.* J. B. C., 1999. **144**(5): p. 857-868.
8. Li, J., M. Puceat, and C. Perez-teric, *Calreticulin reveals a critical Ca2+ checkpoint in cardiac myofibrillogenesis.* J. B. C., 2002. **158**(1): p. 103-113.
9. Woods, D.B. and K.H. Vousden, *Regulation of p53 function.* Exper. Cell. Res., 2001. **264**: p. 56-66.

10. Levine, A.J., *p53, the Cellular Gatekeeper for Growth and Division.* Cell, 1997. **88**: p. 323-331.

11. Rebhan, M., *HotMolecBase: p53 tumor suppressor.* http://bioinformatics.weizmann.ac.il/hotmolecbase/entries/p53.htm, 1997.

12. Lin, J., Teresky, A. K., and Levine, A. J., *Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants.* Oncogene, 1995. **10**(12): p. 2387-2390.

13. Siliciano, J.D., C.E. Canman, and Y. Taya, *DNA damage induces phosphorylation of the amino terminus of p53.* Genes and Dev., 1997. **11**: p. 3471-3481.

14. Cain, C., *et al.*, *The N-terminus of p53 regulates its dissociation from DNA.* J. B. C., 2000. **275**: p. 39944-39953.

15. Giannakakou, P., D.L. Sackett, and Y. Ward, *p53 is associated with cellular microtubules and is transported to the nucleus by dynein.* Nature Cell Bio., 2000. **2**: p. 709-717.

16. Meek, D.W., *Multisite phosphorylation and the integration of stress signals at p53.* Cell Signalling, 1998. **10**: p. 159-166.

17. Kwek, S.S., et al., *Functional analysis and intracellular localization of p53 modified by SUMO-1.* Oncogene, 2001. **20**: p. 2587-2599.

18. Kussie, P.H., et al., *Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain.* Science, 1996. **274**(5289): p. 948-953.

19. Hollstein, M., et al., *Database of p53 gene somatic mutations in human tumors and cell lines.* Nucleic Acid Res., 1994. **22**(17): p. 3551-3555.

20. Bray, S.E., C. Schorl, and P.A. Hall, *The Challenge of p53: Linking Biochemistry, Biology, and Patient Management.* Stem Cells, 1998. **16**: p. 248-260.

21. Pavletich, N.P., K.A. Chambers, and C.O. Pabo, *The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots.* Genes Dev., 1993. **7**(12B): p. 2556-64.

22. Bargonetti, J., et al., *A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein.* Genes and Dev., 1993. **7**(12B): p. 2565-74.

23. Arrowsmith, C.H. and P. Morin, *New Insights into p53 function from structural studies.* Oncogene, 1995. **12**(7): p. 1379-1385.

24. Cho, Y., et al., *Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations.* Science, 1994. **265**: p. 346-355.

25. El-Deiry, W.S., et al., *Definition of a consenus binding site for p53.* Nature Genet., 1992. **1**: p. 45-49.

26. Kraiss, S., et al., *Oligomerization of oncoprotein p53.* J Virol, 1988. **62**(12): p. 4737-4744.

27. Weinberg, R.A., *Tumor suppressor genes.* Science, 1991. **254**(5035): p. 1138-1146.

28. Sturzbecher, H.W., et al., *A C-terminal alpha-helix plus basic region motif is the major structural determinant of p53 tetramerization.* Oncogene, 1992. **7**(8): p. 1513-1523.

29. Jeffrey, P.D., S. Gorina, and N.P. Pavletich, *Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms.* Science, 1995. **267**(5203): p. 1498-1502.

30. Stommel, J.M., N.D. Marchenko, and G.S. Jimenez, *A leucine-rich nuclear export signal in the p53 tetramerization domain: regualtion of subcellular localization and p53 activity by NES masking.* EMBO journal, 1999. **18**(6): p. 1660-1672.

31. Liang, S. and M.F. Clarke, *A Bipartite Nuclear localization signal is required for p53 nuclear import regulated by a carboxyl-terminal domain.* J. B. C., 1999. **274**(46): p. 32699-32703.

32. Hupp, T.R., D.W. Meek, and C.A. Midgley, *Regulation of the specific DNA binding function of p53.* Cell, 1992. **71**: p. 875-886.

33. Prives, C. and J.L. Manley, *Why is p53 acetylated?* Cell, 2001. **107**: p. 815-818.

34. Zhou, J., et al., *A role for p53 in base excision repair.* EMBO journal, 2001. **20**(4): p. 914-923.

35. Liang, S. and M.F. Clarke, *Regulation of p53 localization.* Eur. J. Biochem., 2001. **268**: p. 2779-2783.

36. Shaulsky, G., et al., *Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigensis.* Mol. Cell Biol., 1990. **10**: p. 6565-6577.

37. Kim, I., et al., *Truncated form of importin alpha identified in breast cancer cell inhibits nuclear import of p53.* J. B. C., 2000. **275**(30): p. 23139-23145.

38. Liang, S., D. Hong, and M.F. Clarke, *Cooperation of a single lysine mutation and a C-terminal domain in the cytoplasmic sequestration of the p53 protein.* J. B. C., 1998. **273**(31): p. 19817-19821.

39. Rubbi, C.P. and J. Milner, *Non-activated p53 co-localizes with sites of transcription within both the nuclioplasm and the nucleolus.* Oncogene, 2000. **19**: p. 85-96.

40. Moll, U.M., G. Riou, and A.J. Levine, *Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion.* PNAS, 1992. **89**: p. 7262-7266.

41. Zhang, Y. and Y. Xiong, *A p53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation.* Science, 2001. **292**(5523): p. 1910-1915.

42. Moll, U.M. and A. Zaika, *Nuclear and mitochondrial apoptotic pathway of p53.* FEBS Letters, 2001. **493**: p. 65-69.

43. Roth, J., M. Dobbelstein, and D.A. Freedman, *Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein.* EMBO journal, 1998. **17**: p. 554-564.

44. Ashcroft, M. and K.H. Vousden, *Regulation of p53 stability.* Oncogene, 1999. **18**: p. 7637-7643.

45. Lowe, S.W., et al., *p53-dependent apoptosis modulates the cytotoxity of anitcancer agents.* Cell, 1993. **74**: p. 957-967.

46. Hale, T.K., et al., *Maf Transciptionally Activates the Mouse p53 Promoter and causes a p53-dependent Cell Death.* J. B. C., 2000. **275**(24): p. 17991-17999.

47. Polyak, K., et al., *Genetic determinants of p53-induced apoptosis and growth arrest.* Genes and Dev., 1996. **10**: p. 1945-1952.

48. Polyak, K., et al., *A model for p53-induced apoptosis.* Nature, 1997. **389**: p. 300-305.

49. Dotto, G.P., *p21 WAF1/Cip1: more than a break to the cell cycle?* Biochim Biophys Acta, 2000. **1471**(1): p. M43-56.

50. Cross, S.M., C.A. Sanchez, and C.A. Morgan, *A p53-dependent mouse spindle checkpoint.* Science, 1995. **267**: p. 1353-1359.

51. Nakamura, S., et al., *C-terminus of p53 is required for G2 arrest.* Oncogene, 2001. **21**: p. 2102-2107.

52. Inoue, T., et al., *MDM2 can promote the ubiquitination, nuclear export, and degradation of p53 in the absence of Direct binding.* J. Bio. Chem., 2001. **276**(48): p. 45255-45260.

53. Valenzuele, M.T., R. Guerrero, and M.I. Nuniz, *PARP-1 modifies the effectiveness of p53-mediated DNA damage response.* Oncogene, 2001. **21**: p. 1108-1116.

54. Gaal, J.C., *et al., Cellular euthanasia mediated by a nuclear enzyme: a central role for nuclear ADP-ribosylation in cellular metabolism.* Trends Biochem. Sci., 1987. **12**: p. 129-130.

55. Chiarugi, A., *Poly(ADP-ribose) polymerase: killer or conspirator? The 'suicide hypothesis' revisited.* Trends in Pharma., 2002. **23**(3): p. 122-129.

56. Kemp, C.J., et al., *Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors.* Cell, 1993. **74**(5): p. 813-822.

57. Thompson, T.C., et al., *Loss of p53 function leads to metastasis in ras+myc-initiated mouse prostate cancer.* Oncogene, 1995. **10**(5): p. 869-879.

58. Hsu, Y.T., K.G. Wolter, and R.J. Youle, *Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 3668-3672.

59. Marchenko, N.D., A.I. Zaika, and U.M. Moll, *Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling.* J. B. C., 2000. **275**: p. 16202-16212.

60. Gross, A., et al., *Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis.* EMBO journal, 1998. **17**(14): p. 3878-3885.

61. El-Deiry, W.S., et al., *WAF1, a potential mediator of p53 tumor suppression.* Cell, 1993. **75**: p. 817-825.

62. Xiao, G., et al., *A DNA damage signal is required for p53 to activate GADD45.* Cancer Res., 2000. **60**: p. 1711-1719.

63. Barak, Y., et al., *MDM2 expression is induced by wild type p53 activity.* EMBO journal, 1993. **12**(2): p. 461-468.

64. Hay, T.J. and M.D. W., *Multiple sites of in vivo phosphorylation in the MDM2 oncoprotein cluster within two important functional domains.* FEBS Letters, 2000. **478**: p. 183-186.

65. Buschmann, T., S.Y. Fuchs, and C.-G. Lee, *SUMO-1 modifications of MDM2 prevents its self-ubiquitination and increases MDM2 ability to ubiquitinate p53.* Cell, 2000. **101**: p. 753-762.

66. Mayo, L.D., J. Turchi, and S.J. Berberich, *MDM2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53.* Cancer Res., 1997. **57**: p. 5013-5016.

67. Buckbinder, L., R. Talbott, and S. Valesco-Miguel, *Induction of the growth inhibitor IGF-binding protein 3 by p53.* Nature, 1995. **377**: p. 646-649.

68. Miyashita, T. and J.C. Reed, *Tumor suppressor p53 is a direct transcriptional activator of human bax gene.* Cell, 1995. **80**: p. 293-299.

69. Donehower, L.A., et al., *Deficiency of p53 accelerates mamary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability.* Genes and Dev., 1995. **9**: p. 882-895.

70. Geyer, R.K., Z. Yu, and C.G. Maki, *The MDM2 RING-finger domain is required to promote p53 nuclear export.* Nat. Cell Biol., 2000. **2**: p. 569-573.

71. Gu, J., et al., *Identification of p53 sequence elements that are required for MDM2-mediated nuclear export.* Mole. and Cell. Biology, 2001. **21**(24): p. 8533-8546.

72. Momand, J., G.P. Zambetti, and D.C. Olson, *The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation.* Cell, 1992. **69**: p. 1237-1245.

73. Freedman, D.A. and A.J. Levine, *Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6.* Mole. and Cell. Biology, 1998. **18**(12): p. 7288-7293.

74. Chehab, N.H., et al., *Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage.* PNAS, 1999. **96**: p. 13777-13782.

75. Shieh, S.Y., et al., *DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2.* Cell, 1997. **91**: p. 325-334.

76. Honda, R. and H. Yasuda, *Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53.* EMBO journal, 1999. **18**: p. 22-27.

77. Gonen, H., et al., *On the involvement of calpain in the degradation of the tumor suppressor proetin p53.* FEBS Letters, 1997. **406**(1-2): p. 17-22.

78. Kubbutat, M. and K. Vousden, *Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability.* Mol. and Cell BIo., 1997. **17**(1): p. 460-468.

79. Pariat, M., et al., *Proteolysis by calpains: a possible contribution to degradation of p53.* Mol. and Cell BIo., 1997. **17**(5): p. 2806-2815.

80. Giaccia, A.J. and M.B. Kastan, *The complexity of p53 modulation: emerging patterns from divergent signals.* Genes and Dev., 1998. **12**(19): p. 2973-2983.

81. Lakin, N.D. and S.P. Jackson, *Regulation of p53 in response to DNA Damage.* Oncogene, 1999. **18**: p. 7644-7655.

82. Gatti, A., et al., *Phosphorylation of human p53 on Thr-55.* Biochemistry, 2000. **39**: p. 9837-9842.

83. Chehab, N.H., et al., *Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53.* Genes and Dev., 2000. **14**(3): p. 278-288.

84. Appella, E. and C.W. Anderson, *Post-translational modifications and activation of p53 by genotoxic stresses.* Eur. J. Biochem., 2001. **268**: p. 2764-2772.

85. Espinosa, J.M. and B.M. Emerson, *Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment.* Mol. Cell, 2001. **8**: p. 57-69.

86. Nakamura, S., R.J. A., and M. T., *Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination.* Mole. and Cell. Biology, 2000. **20**: p. 9391-9398.

87. Rodriguez, M.S., et al., *SUMO-1 modification activates the transcriptional response of p53.* EMBO journal, 1999. **18**(22): p. 6455-6461.

88. Gostissa, M., A. Hengstermann, and V. Fogal, *Activation of p53 by conjugatetion to the ubiquitin-like protein SUMO-1.* EMBO journal, 1999. **18**(6462-6471).

89. Ostwald, T.J. and D.H. MacLennan, *Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum.* J. B. C., 1974. **249**: p. 974-979.

90. Liu, N., et al., *Decreasing calretiulin expression lowers the Ca2+ to bradykinin and increases sensitivity to ionmycin in NG-108-15 cells.* J. B. C., 1994. **269**(28635-28639).

91. Bastianutto, C.E., F. Clementi, and P. Codazzi, *Overexpression of calreticulin increase the Ca2+ capacity of rapid exchanging Ca2+ stores and reveals aspects of their lumenal microenvironmant and function.* J. C. B., 1995. **130**: p. 847-855.

92. Krause, K.H. and M. Michalak, *Calreticulin.* Cell, 1997. **88**: p. 439-443.

93. Arosa, F.A., O. Jesus, and G. Porto, *Calreticulin is expressed on the cell surface of activated human peripheral blood t lymphocytes is association with major histocompatibility complex class I molecules.* J. B. C., 1999. **274**(24): p. 16917-16922.

94. Mancino, L., S.M. Rizvi, and P.E. Lapinski, *Calreticulin recognizers misfolded HLA-A2 heavy chains.* PNAS, 2002. **99**(9): p. 5931-5936.

95. Burns, K., et al., *Modulation of gene expression by calreticulin binding to the glucocorticoid receptor.* Nature, 1994. **367**(6462): p. 476-480.

96. Dedhar, S., et al., *Inhibition of nuclear hormone receptor activity by calreticulin.* Nature, 1994. **367**(6462): p. 480-483.

97. White, T.K., Q. Zhu, and M.L. Tanzer, *Cell surface calreticulin is a putative mannoside lectin which triggers mouse melanoma cell spreading.* J. B. C., 1995. **270**(27): p. 15926-15929.

98. Yao, L., S. Pike, and G. Tosato, *Laminin binding to the calreticulin fragment vasostatin regulates endothelial cell function.* J. Leuk. Biol., 2002. **71**: p. 47-53.

99. Baksh, S., et al., *Identification of the Zn2+ binding region in calreticulin.* FEBS Letters, 1995. **376**(1-2): p. 53-57.

100. Baksh, S., et al., *Interaction of calreticulin with protein disulphide isomerase.* J. B. C., 1995. **270**(52): p. 31338-31344.

101. Corbett, E.F., et al., *Ca2+ regulation of interactions between endoplasmic reticulum chaperones.* J. B. C., 1999. **274**(10): p. 6203-6211.

102. Rojiani, M.V., et al., *In vitro interaction of a polypeptide homologous to human Ro/SS-A antigen (calreticulin) with a highly conserved amino acid sequence in*

the cytoplasmic domain of integrin alpha subunit. Biochemistry, 1991. **30**(41): p. 9859-9866.

103. Pike, S., et al., *Vasostatin, a calreticulin fragment, inhibits angiogenesis and supresses tumor growth.* J. Exp. Med., 1998. **188**(12): p. 2349-2356.

104. Kuwabara, K., A.M. Pinsky, and A.M. Schmidt, *Calreticulin an antithrombotic agent which binds vitamin K-dependent coagululation factors, stimulates endothelial nitric oxide production, and limits thrombosis in canine cornary arteries.* J. B. C., 1995. **270**: p. 8179-8187.

105. Lange-Asschenfeldt, B., et al., *The angiogenesis inhibitor vasostatin does not impair wound healing at tumor-inhibiting doses.* J. Invest. Dermatol., 2001. **117**(5): p. 1036-1041.

106. Pike, S., et al., *Calreticulin and calreticulin fragments are endothelial cell inhibitors that suppress tumor growth.* Blood, 1999. **94**(7): p. 2461-2461.

107. Otteken, A. and B. Moss, *Calreticulin interacts with newly synthesized human immunodeficiency virus type 1 envelope glycoprotein, suggesting a chaperone function similar to that of calnexin.* J. B. C., 1996. **271**(1): p. 97-103.

108. Saito, Y., et al., *Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins.* EMBO journal, 1999. **18**: p. 6718-6729.

109. Nakamura, K., et al., *Functional specialization of calreticulin domains.* J. C. B., 2001. **154**(5): p. 961-972.

110. Ellgaard, L., R. Riek, and D. Braun, *Three-dimensional structure topology of the calreticulin P-domain based on NMR assignment.* FEBS Letters, 2001. **488**: p. 69-73.

111. Andrin, C., et al., *Interaction between a Ca2+-binding protein calreticulin and perforin, a component of the cytotoxic T-cell granules.* Biochemistry, 1998. **37**: p. 10386-10394.

112. Baksh, S. and M. Michalak, *Expression of calreticulin in Escherichia coli and identification of its Ca2+ binding domains.* J. B. C., 1991. **266**: p. 21458-21465.

113. Kuwabara, K., et al., *Calreticulin, an antithrombotic agent which binds to vitamin K-dependent coagulation factors, stimulates endothelial nitric oxide production, and limits thrombosis in canine coronary arteries.* J. B. C., 1995. **270**(14): p. 8179-8187.

114. Dai, E., et al., *Calreticulin, a potential vascular regulatory protein, reduces intimal hyperplasia after arterial injury.* Arterioscler Thromb Vasc Biol., 1997. **11**: p. 2359-2368.

115. Guo, L., et al., *COUP-TF1 antagonizes Nkx2.5-mediated activation of the calreticulin gene during cardiac development.* J. B. C., 2001. **276**(4): p. 2797-2801.

116. Lyons, I., et al., *Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5.* Genes and Dev., 1995. **9**: p. 1654-1666.

117. Nakamura, K., et al., *Complete heart block and sudden death in mice overexpressing calreticulin.* J. of Clin. Invest., 2001. **107**(10): p. 1245-1253.

118. Hagendoff, A., et al., *Conduction disturbances and increased atrial vulnerability in connexin 40-deficient mice analyzed by transesophageal stimulation.* Cirulation, 1999. **99**: p. 1508-1515.

119. Gutstein, D., *et al.*, *Conduction slowing and sudden arrhythmic death in mice with caridac-restricted inactivation of connexin 43.* Circ. Res., 2001. **88**: p. 333-339.

120. Maass, A. and L.A. Leinwand, *A role for calreticulin in the adult heart?* J. of Clin. Invest., 2001. **107**(10): p. 1223-1225.

121. Michalak, M., *Calreticulin.* R. D. Landes Co., Autsin, Tx, 1996.

122. Opas, M., et al., *Regulation of expression and intracellular distribution of calreticulin, a mjor calcium binding protein of nonmuscle cells.* J. Cell. Phys., 1991. **149**: p. 160-171.

123. Sonnichsen, B., et al., *Retention and retrieval: both mechanisms cooperate to maintain calreticulin in the endoplasmic reticulum.* J. Cell Science, 1994. **107**: p. 2705-2717.

124. Ito, H., Y. Seyama, and S. Kubota, *Calreticulin is directly involved in anti-alpha3 integrin antibody-mediated secretion and activation of matrix metelloprotease-2.* Biochem. and Biophys. Res. Comm., 2001. **283**: p. 297-302.

125. Xiao, G., et al., *Calrecticulin is transported to the surface of NG108-15 cells where it forms surface patches and is partially degraded in an acidic compartment.* J.Neuro. Res., 1999. **58**: p. 652-662.

126. Roderick, H., A. Campbell, and D. Llewellyn, *Nuclear localisation of calreticulin in vivo is enhanced by its interaction with glucocorticoid receptors.* FEBS Letters, 1997. **405**: p. 181-185.

127. Holaska, J.M., B.E. Black, and D.C. Love, *Calreticulin is a receptor for nuclear export.* J. B. C., 2001. **152**(1): p. 127-140.

128. Llewellym, D.H., H.L. Roderick, and S. Rose, *KDEL receptor expression is not coodinatedly up-regulated with ER stress-induced reticuloplasmin expression in HeLa cells.* Biochem. and Biophys. Res. Comm., 1997. **240**: p. 36-40.

129. Coppolino, M.G. and S. Dedhar, *Ligand-specific, transient interaction between integrins and calreticulin during cell adhesion to extracellular matrix proteins is dependent upon phosphorylation/dephosphorylation events.* Biochem J., 1999. **340**: p. 41-50.

130. Fadel, M.P., et al., *Calreticulin affects focal contact-dependent but not close contact-dependent cell-substratum adhesion.* J. B. C., 1999. **274**(21): p. 15085-15094.

131. Opas, M., et al., *Calreticulin modulates cell adhesiveness via regualtion of vinculin expression.* J. C. B., 1996. **135**(6): p. 1913-1923.

132. Fadel, M.P., M. Szewezenko-Pawlikowski, and P. Leclerc, *Calreticulin affects beta-catenin-associated pathways.* J. B. C., 2001. **276**(29): p. 27083-27089.

133. Eggleton, P., *et al.*, *Fine specificity of autoantibodies to calreticulin: epitope mapping and characterization.* Clin. Exp. Immunol., 2000. **120**: p. 384-391.

134. Verrick, F.A., *et al.*, *DR4Dw4/DR53 moelcules contain a peptide from the autoantigen calreticulin.* Tissue antigens, 1995. **45**: p. 270-275.

135. Tuckova, L., *et al.*, *Anti-giadin antibodies in patients with celiac disease cross-react with enterocytes and human calreticulin.* Clin. Immunol. Immunopathol., 1997. **85**(289-296).

136. Orth, T., *et al.*, *Complete congenital heart block is associated with increased autoantibody titres against calreticulin.* Eur. J. Clin. Invest., 1996. **26**(205-215).

137. Gut, J., *et al.*, *Mechanisms of halothane toxicity: novel insights.* Pharmcol. Ther., 1993. **58**: p. 133-155.

138. Rokeach, L.A., J.A. Haselby, and J.F. Meilof, *Characterization of the autoantigen calreticulin.* J. Imminol., 1991. **147**: p. 3031-3039.

139. Eggleton, P. and D.H. Llewellyn, *Pathophysiological roles of calreticulin in autoimmune disease.* Scand. J. Immunol., 1999. **49**: p. 466-473.

140. Michalak, M., et al., *Endoplasmic reticulum form of calreticulin modulates glucocorticoid-sensitive gene expression.* J. B. C., 1996. **271**(46): p. 29436-29445.

141. Jorgensen, C.S., et al., *Polypeptide binding properties of chaperone calreticulin.* Eur. J. Biochem., 2000. **267**: p. 2945-2954.

142. Helenius, A., *et al.*, *Calnexin, calreticulin and the folding of glycoproteins.* TRENDS in Cell Bio., 1997. **7**: p. 193-200.

143. Spiro, R., et al., *Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver golgi.* J. B. C., 1996. **271**(19): p. 11588-11594.

144. Sousa, M. and A.J. Parodi, *The molecular basis for the recognition of misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase.* EMBO journal, 1995. **14**(17): p. 4196-4203.

145. Nigam, S.K., et al., *A set of endoplasmic reticulum proteins possessing properties of molecular chaperones includes Ca(2+)-binding proteins and members of the thioredoxin superfamily.* J. B. C., 1994. **269**(3): p. 1744-1749.

146. Molinari, M. and A. Helenius, *Chaperone selection during glycoprotein translocation into the endoplasmic reticulum.* Science, 2000. **288**: p. 331-333.

147. van Leeuwen, J.E. and K.P. Kearse, *Deglucosylation of N-linked glycans is an important step in the dissociation of calreticulin-class I-TAP complexes.* PNAS, 1996. **93**(24): p. 13997-14001.

148. Nauseef, W.M., S.J. McCormick, and R.A. Clark, *Calreticulin functions as a molecular chaperone in the biosynthesis of myeloperoxidase.* J. B. C., 1995. **270**(9): p. 4741-4747.

149. Peterson, J.R., et al., *Transient, lectin-like association of calreticulin with folding intermediates of cellular and viral glycoproteins.* Mol Biol Cell, 1995. **6**(9): p. 1173-1784.

150. Wada, I., et al., *Chaperone function of calreticulin when expressed in the endoplasmic reticulum as the membrane-anchored and soluble forms.* J. B. C., 1995. **270**(35): p. 20298-20304.

151. John, L.M., J.D. Lechleiter, and P. Camacho, *Differential modulation of SERCA2 isoforms by calreticulin.* J. C. B., 1998. **142**(4): p. 963-973.

152. Xu, W., F.J. Longo, and M.R. Wintermantel, *Calreticulin modulates capacitative Ca2+ influx by controlling the extent of inositol 1,4,5-triphosphate-induced Ca2+ store depletion.* J. B. C., 2000. **275**(47): p. 36676-36682.

153. Michalak, M., E.F. Corbett, and N. Mesaeli, *Calreticulin: one protein, one gene, many functions.* Biochem. J., 1999. **344**: p. 281-292.

154. Roderick, H.L., et al., *Role of calretiulin in regulating intracellular Ca2+ storage and capacitative Ca2+ entry in HeLa cells.* Cell Calcium, 1998. **24**(4): p. 253-262.

155. Leung-Hagesteijin, C., et al., *Cell attachment to extracellular matrix substrates is inhibited upon downregulation of calreticulin, an intracellular integrin alpha-subunit-binding protein.* J. Cell Science, 1994. **107**: p. 589-600.

156. Coppolino, M., et al., *Inducible interaction of integrin alpha2beta1 with calreticulin-dependence on the activation state of the integrin.* J. B. C., 1995. **270**: p. 23132-23138.

157. Rauch, F., et al., *Heart, Brain, and Body Wall Defects in Mice Lacking Calreticulin.* Exper. Cell. Res., 2000. **256**: p. 105-111.

158. Pinton, P., D. Ferrari, and E. Rapizzi, *The Ca2+ concentration of the endoplasmic reticulum is a key determinant of cerimide-induced apoptosis: significance of the molecular mechanism of Bcl-2 action.* EMBO reports, 2001. **20**(11): p. 2690-2701.

159. Duchen, M.R., *Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death.* J. Physiol., 1999. **16**: p. 1-17.

160. Csermely, C., T. Schnaider, and I. Szanto, *Signalling and transport through the nuclear membrane.* Biochem. Biophys. Acta, 1995. **1241**: p. 425-452.

161. Dingwall, C. and R. Laskey, *The nuclear membrane.* Science, 1992. **258**(5084): p. 942-947.

162. Stehno-Bittel, L., C. Perez-Terzic, and D.E. Clapham, *Diffusion across the nuclear envelope inhibted by depletion of the nucleus Ca2+ store.* Science, 1995. **270**(1835-1838).

163. Subramanian, K. and T. Meyer, *Calcium-induced restructuring of nuclear envelope and endoplasmic reticulum calcium stores.* Cell, 1997. **89**: p. 963-971.

164. Sheng, M., M.A. Thompson, and M.E. Greenberg, *CREB: a Ca(2+)-regulated transcription factor phophorylated by calmodulin-dependent kinase.* Science, 1991. **252**: p. 1427-1430.

165. Helper, P.K., *The role of calcium in cell division.* Cell Calcium, 1994. **16**: p. 322-330.

166. Nicotera, P., B. Zhivotovsky, and S. Orrenius, *Nuclear calcium transport and the role of calcium in apoptosis.* Cell Calcium, 1994. **16**: p. 279-288.

167. Lee, M.A., et al., *Calcium regulation of nuclear pore permeability.* Cell Calcium, 1998. **23**(2/3): p. 91-101.

168. Perez-Terzic, C., M. Jaconi, and D.E. Clapham, *Nuclear calcium and the regulation of the nuclear pore complex.* BioEssays, 1997. **19**(9): p. 787-792.

169. Perez-Terzic, C., A.M. Gacy, and R. Bortolon, *Structural plasticity of the cardiac nuclear pore complex in response to regulators of nuclear import.* Circ. Res., 1999: p. 1292-1301.

170. Perez-Terzic, C., J. Pyle, and M. Jaconi, *Conformational states of the nuclear pore complex induced by depletion of nuclear Ca2+ stores.* Science, 1996. **273**: p. 1875-1877.

171. Fabre, E. and E.C. Hurt, *Nuclear Transport.* Curr. Opin. Cell Biol., 1994. **6**: p. 335-342.

172. Lubas, W.A., et al., *O-Linked GlcNAc transferase is a conserved nucleocytoplasmic protein containing tetratricopeptide repeats.* J. B. C., 1997. **272**(14): p. 9316-24.

173. Pante, N. and U. Aebi, *The Nuclear pore complex.* J. C. B., 1993. **122**: p. 977-984.

174. Dworetzky, S.I. and C.M. Feldherr, *Translation of RNA-coated gold particles through the nuclear pore of oocytes.* J. C. B., 1988. **106**: p. 575-584.

175. Feldherr, C.M., D. Akin, and R.J. Cohen, *Regualtion of functional nuclear pore size in fibroblasts.* J. of Cell. Sci., 2001. **114**(24): p. 4621-4627.

176. Hinshaw, J., B. Carragher, and R. Milligan, *Architecture and design of the nuclear pore complex.* Cell, 1992. **69**: p. 1133-1141.

177. Pante, N. and U. Aebi, *Sequential binding of import ligand to distinct nucleopore regions during nuclear transport.* Science, 1996. **273**: p. 1729-1732.

178. Nigg, E., *Nucleoplasmic transport: signals, mechanisms and regulation.* Nature, 1997. **386**(6627): p. 779-787.

179. Peters, R., *Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility.* Biochem. Biophys. Acta, 1986. **864**: p. 305-359.

180. Bonner, W.M., *Protein migration into nuclei. 1. Frog oocyte nuclei accumulate a class of microinjected oocyte nuclear proteins and exclude a class of microinjected oocyte cytoplasmic proteins.* J. C. B., 1975. **64**: p. 431-437.

181. Gerace, L., *Molecular trafficking across the nuclear pore complex.* Curr. Opin. Cell Biol., 1992. **4**: p. 637-645.

182. Dingwall, C., S.V. Sharnick, and R. Laskey, *A polypeptide domain that specifies migration of nucleoplasmin into the nucleus.* Cell, 1982. **2**: p. 449-458.

183. Robbins, J., et al., *Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence.* Cell, 1991. **3**: p. 615-623.

184. Sweitzer, T.D., D.C. Love, and J.A. Hanover, *Regulation of nuclear import and export.* Curr. Topics in Cell. Reg., 2000. **36**: p. 77-94.

185. Gorlich, D., N. Pante, and U. Kutay, *Identification of different roles of RanGDP and RanGTP in nuclear protein import.* EMBO reports, 1996. **15**(20): p. 5584-5594.

186. Moore, M.S. and G. Blobel, *The GTP-binding protein RAN/TC4 is required for protein import into the nucleus.* Nature, 1993. **365**: p. 661-663.

187. Ohtsubo, M., H. Okazaki, and T. Nishimoto, *The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA.* J. C. B., 1989. **109**: p. 1389-1397.

188. Melchior, F., et al., *Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase RAN/TC4 as an essential transport factor.* J. C. B., 1993. **123**: p. 1649-1659.

189. Klebe, C., et al., *The kinetic mechanism of RAN -- nucleotide exchange catalyzed by RCC1.* Biochemistry, 1995. **34**(39): p. 12543-12552.

190. Bischoff, F.R., et al., *RanGAP1 induces GTPase activity of nuclear Ras-related Ran.* Proc Natl Acad Sci U S A, 1994. **91**(7): p. 2587-2591.

191. Fischer, U., et al., *The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs.* Cell, 1995. **82**(3): p. 475-483.

192. Malim, M.H., et al., *Functional comparison of the Rev trans-activators encoded by different primate immunodeficiency virus species.* Proc Natl Acad Sci U S A, 1989. **86**(21): p. 8222-8226.

193. Wolff, B., J.J. Sanglier, and Y. Wang, *Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA.* Chem Biol., 1997. **4**(2): p. 139-147.

194. Fornerod, M., et al., *CRM1 is an export receptor for leucine-rich nuclear export signals.* Cell, 1997. **90**(6): p. 1051-1060.

195. Ossareh-Nazari, B., F. Bacheleria, and C. Dargemont, *Evidence for a role of CRM1 in signal-mediated nuclear protein export.* Science, 1997. **278**: p. 141-144.

196. Neville, M., et al., *The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export.* Curr Biol., 1997. **7**(10): p. 767-775.

197. Greber, U.F. and L. Gerace, *Depletion of calium from the lumen of endoplasmic reticulum reversibly inhibits passive diffusion and signal-mediated transport into the nucleus.* J. C. B., 1995. **128**(1/2): p. 5-14.

198. Strubing, C. and D.E. Clapham, *Active nuclear imprto and export is independent of lumenal Ca2+ stores in intact mammalian cells.* J. Gen. Physiol., 1999. **113**: p. 239-248.

199. Bustamante, J.O., *et al.*, *Calcium, ATP and nuclear pore channel gating.* Eur. J. Physiol., 2000. **439**: p. 433-444.

200. Greber, U.F. and L. Gerace, *Nuclear protein import is inhibited by an antibody to a lumenal epitope of a nuclear pore complex glycoprotein.* J. C. B., 1992. **116**: p. 15-30.

201. Mayo, L.D. and D.B. Donner, *A phospatidylinositol 3-kinase/Akt pathway promotes translocation of MDM2 from the cytoplasm to the nucleus.* PNAS, 2002. **98**(20): p. 11598-11603.

202. Ogawara, Y., et al., *Akt enhances MDM2-mediated ubiquitination and degradation of p53.* J. B. C., 2002. **277**(24): p. 21843-21850.

203. Waser, M., *et al.*, *Regulation of calreticulin gene expression by calcium.* J. B. C., 1997. **138**(3): p. 547-557.

204. Soong, R., et al., *Concordance betweem p53 protein overexpression and gene mutation in a large series of common human carinomas.* Hum Pathol, 1996. **27**: p. 1050-1055.

205. Bosari, S., et al., *Cytoplasmic accumulation of p53 protein: an independent prognostic indicator in colorectal adenocarcinomas.* J Natl Cancer Inst, 1994. **86**: p. 681-687.

206. Moll, U.M., et al., *Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors.* PNAS, 1995. **92**: p. 4407-4411.

207. Wu, X., et al., *The p53 mdm2 autoregulatory feedback loop.* Genes and Dev., 1993. **7**: p. 1126-1132.