IGFBP-3 INDUCED BY RIBOTOXIC STRESS IS NOT SECRETED PRIOR TO NUCLEAR LOCALIZATION IN MAMMARY EPITHELIAL CELLS

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ABSTRACT OF THE THESIS

IGFBP-3 Induced by Ribotoxic Stress is Not Secreted

Prior to Nuclear Localization In Mammary Epithelial Cells

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Ribotoxic stressors such as anisomycin (ANS) and deoxynivalenol (DON) induce apoptosis in MAC-T cells. These agents also increase IGFBP-3 expression and knockdown of IGFBP-3 mitigates the apoptotic effects of these toxins. IGFBP-3 contains both a signal sequence and a nuclear localization sequence (NLS) and is thus both secreted and localized to the nucleus. Nuclear IGFBP-3 has been proposed to be important in its apoptotic effect. Following treatment with DON and ANS, nuclear IGFBP-3 is glycosylated, a hallmark of the secretory pathway. However, how it escapes the secretory pathway to traffic to the nucleus is unknown. Some studies have reported that extracellular IGFBP-3 is rapidly internalized and delivered to the nucleus, suggesting IGFBP-3 may require secretion and re-internalization prior to nuclear localization. To study trafficking of the endogenous protein, MAC-T cells were treated with ANS or DON. Fluorescent microscopy and Western immunoblot analysis demonstrated that ANS and DON induced nuclear localization of

IGFBP-3. Treatment of nuclear IGFBP-3 with the deglycosylation enzyme Endoglycosidase H (Endo H) resulted in a lower molecular weight band indicating nuclear IGFBP-3 contains a mannose or hybrid type glycan. In contrast, the sugar of secreted IGFBP-3 was not truncated using Endo H, but was deglycosylated using PNGase indicating complex-type glycosylation. Cells treated with Brefeldin A (BFA), an inhibitor of anterograde transport from the ER to the Golgi, still showed nuclear movement of IGFBP-3. Glycosylation and BFA data indicate that IGFBP-3 is not secreted and re-internalized prior to nuclear localization during ribotoxic stress.

Dedication

To my parents whose unwavering love and support has been the foundation for my achievement at Rutgers. Thank you for the sacrifices you've made to help me realize my dreams, and for the love and guidance along the way.

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List of Abbreviations

ALS	Acid-labile subunit
ANS	Anisomycin
Apaf-1	Apoptotic protease activating factor 1
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BFA	Brefeldin A
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2 interacting mediator of cell death
BiP	Immunoglobulin chain-building protein
DD	Death domain
DISC	Death inducing signaling complex
DON	Deoxynivalenol
Elk-1	ETS domain-containing protein
Endo H	Endoglycosidase H
ERAD	Endoplasmic-associated degradation
Erk	Extracellular signal-regulated kinase
ERManI	ER α 1,2 mannosidase I
FADD	Fas-associated death domain
FasL	Fas-ligand
GEF	GTPase-exchange factor

GlcNAc	N-acetylglycosamine
HBD	Heparin binding domain
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor -II
IGF1R	Insulin-like growth factor-I receptor
IGFBP	Insulin-like growth factor binding protein
IGFBP-3	Insulin-like growth factor binding protein-3
IP	Immunoprecipitation
M-6-P	Mannose-6-phosphate
MAC-T	Mammary alveolar cell
ManIA	ER mannosidase IA
MAPK	Mitogen-activated protein kinase
MCF-7	Michigan cancer foundation-7
MEC	Mammary epithelial cell
MOMP	Mitochondrial outer membrane permeability
NES	Nuclear export sequence
NFκB	Nuclear factor kappa B
NLS	Nuclear localization signal
NSCLC	Non-small cell lung cancer
Nur77	Nerve growth factor 1B
PAD	p97-associated deubiquitination process
PARP	Poly ADP ribose polymerase
PC-3	Prostate cancer cells
PDI	Protein disulfide isomerase

PI3K	Phosphatidylinositol-3-kinase
PNGase F	Peptide-N-glycosidase F
RARα	Retinoic acid receptor- α
Rpb3	Ribonucleic acid polymerase II binding subunit 3
RIP	Ribosome-inactivating protein
RPE	Retinal pigment epithelial cells
RTA	Ricin toxin chain A
RTB	Ricin toxin chain B
RXR α	retinoid-x-receptor-α
SAPK	Stress-activated protein kinase
STAT	Signal transducer and activator od transcription
TfR1	Transferrin receptor 1
TGN	Trans Golgi network
TM	Tunicamycin
TNF-α	Tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
UPR	Unfolded protein response
VDR	Vitamin D receptor
XBP1	X-box binding protein 1

CHAPTER 1

REVIEW OF THE LITERATURE

I. INTRODUCTION AND SIGNIFICANCE

Milk production and the role of apoptosis

Agriculture has been the sustenance of civilization and the support for an ever-growing population for the last 10,000 years. Today the scope of agriculture spans the cultivation of animals, plants and fungi for food. In the United States dairy farming is a major aspect of agricultural industry with milk production occurring in all 50 states and over 200 billion pounds of milk produced from dairy cattle each year. Commonly purchased dairy products include cheese, fluid milks, yogurt, butter, ice cream and whey. According to the USDA, consumption of dairy products is rising faster than the growth in population, creating a growing demand for higher milk yield efficiencies and driving innovations in efficient milk production.

Currently a dairy cow yields 23,042 pounds of milk per year, and this number has steadily risen over the last few years (22,545 pounds in 2014 and 22,655 pounds in 2015). Once milk production drops below a certain daily output, costs associated with animal maintenance outweigh profit from sale of the milk. In the dairy cow, lactation output is a function of the mammary epithelial cell number, with a peak in cell number and milk yield approximately 3-6 weeks after calving (Capuco, et al. 2001). After peak lactation there is a marked decline in milk yield attributed to a decrease in secretory cell number. The balance between survival and apoptotic signals determines lactation persistency and altering this balance in favor of cellular survival has the potential to delay the decrease in milk production. Therefore,

understanding the molecular mechanisms that regulate this process is fundamental to developing strategies to increase persistency in dairy cows. One promising area of investigation that may lead to technologies that promote cell survival is the role of the insulin-like growth factor (IGF)-axis, as IGF-I is a known mitogen and anti-apoptotic factor for mammary gland cells.

Role of IGFBP-3 and the IGF axis in growth

The IGF signaling system is crucial to the stimulation of growth and differentiation of multiple cell types in the majority of tissues. IGF-I is an integral component of the IGF signaling system and considerable data exist to support a role for IGF-I in the regulation of lactation in ruminants, though the precise mechanism is unknown (Cohick 1998). Both IGF-I and IGF-II are abundant in the circulation of lactating ruminants (Vicini, et al. 1991) and IGF-I mRNA is expressed in the bovine mammary gland (Sharma, et al. 1994). The physiological actions of the IGFs are mediated by a family of transmembrane receptors which includes the insulin, IGF-I and IGF-II/mannose-6-phosphate (M-6-P) receptors, with a majority of biological function carried out by activation of the IGF-I receptor (IGFIR) (Cohick and Clemmons 1993). IGF binding proteins (IGFBPs) are another major component of the IGF signaling system. The IGFBPs delicately balance signaling interactions by controlling the capacity of IGF-I and IGF-II to access cell surface receptors (Firth and Baxter 2002). IGFBPs have higher affinities for IGFs than the type-I IGF receptor, and therefore may prolong half-life of circulating IGF, sequester and transport IGF from vasculature or localize IGF to specific tissues. IGFBP-3 is the most abundant member of the IGFBP family circulating in serum, with levels more than 10-fold higher than the other IGFBPs (Baxter 1994). Classically, IGFBP-3 was believed to sequester IGF-I away

from its receptor, thereby preventing activation of IGF-stimulated signaling. In serum IGFBP-3 forms a ternary complex with IGF-I and acid-labile subunit (ALS) which greatly extends the circulating half-life and stability of IGFs (Baxter 1994).

The profuse circulating binding protein IGFBP-3 is known to interact with IGF-I by sequestering serum IGFs, effectively inhibiting IGF actions on the cell. IGF-I is a mitogen and acts on cells to stimulate proliferation by binding its receptor IGFIR. It differentially regulates IGFBP expression in primary bovine mammary fibroblasts and epithelial cells, suggesting distinct roles for IGFBPs in the coordination of mammary gland growth (Fleming, et al. 2005). In the MAC-T bovine mammary epithelial cell (MEC) line both IGF-I and insulin increase IGFBP-3 protein and mRNA levels (Cohick and Turner 1998). IGF-I regulates IGFBP-3 expression via phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (Sivaprasad, et al. 2004).

IGF-I independent actions of IGFBP-3

In addition to IGF-dependent actions, IGFBP-3 is also known to have IGF-independent actions. Historically, this stemmed from an experiment in which IGFBP-3 was added to IGF1R negative ($R^{(-)}$) fibroblast cells obtained from IGF1R knockout mice to test if IGFBP-3 induces apoptosis independently of IGFs and IGF receptors. Both exogenous IGFBP-3 protein and transfection of the IGFBP-3 gene significantly increased the apoptotic index and this effect was partially inhibited by IGFBP-3-specific antibodies (Rajah, et al. 1997). These results demonstrated a clear IGF-independent role of IGFBP-3 in pro-apoptotic effects. Studies from our laboratory have shown that IGFBP-3 mediates stress-induced apoptosis in MECs through a classic intrinsic apoptotic pathway via activation of caspase 3/7 (Grill and

Cohick 2000). In addition when IGFBP-3 is mutated to disable IGF binding (GGG-IGFBP-3) in MCF-7 breast cancer cells, there is an induction of apoptosis through the activation of caspases involved in a death receptor-mediated pathway independent of the IGF-IGF1R axis, specifically caspase-8 and -7 (Kim, et al. 2004). One report determined the first 95 residues of the amino-terminal region of IGFBP-3 were sufficient to induce apoptosis in MCF-7 cells (Bernard, et al. 2002). Further this proteolytic fragment of IGFBP-3, which fails to bind IGFs, inhibits mitogenic signals due to IGFIR activation or insulin (Lalou, et al. 1996). These experiments support an IGF-independent role for IGFBP-3 in the balance between cell death and proliferation which is further described in the sections below.

II. ROLE OF IGFBP-3 IN APOPTOSIS

Overview of apoptosis

Apoptosis is the natural process of programmed cell death whereby the balance between survival and death factors is skewed toward death. These apoptotic signals are mediated by two autonomous and interacting pathways associated with either cell surface death receptors or the mitochondria. The two primary pathways of apoptosis are extrinsic and intrinsic apoptosis, also referred to as ligand-mediated and ligand-independent apoptosis, respectively.

The extrinsic pathway of apoptosis is initiated when cell surface death receptors are activated by their ligands such as Fas-ligand (FasL), tumor necrosis factor (TNF)-α, and TNF-related apoptosis inducing ligand (TRAIL) (Thorburn 2004). Ligand-induced cross-link activation of intracellular death domains (DD) initiates recruitment of adaptor molecules such as Fas-associated death domain (FADD) (MacFarlane 2003). Subsequent recruitment and

aggregation of procaspase-8 to FADD forms the death inducing signaling complex (DISC), promoting auto-activation of caspase-8, which serves to activate downstream effector caspases such as caspase-3, -6 and -7 (Thorburn 2004). Caspases are cysteine-dependent aspartate-directed proteases known as the principle mediators of the apoptotic cell death response, as well as key players in autophagy (Tsapras and Nezis 2017). The effector or executioner caspases are named so for their protease activity and role in cellular destruction. Active caspases may affect nuclear membrane degradation and DNA fragmentation (Weinlich, et al. 2011).

Conversely, intrinsic apoptosis may be triggered by exposure of cells to stressors including growth factor withdrawal, UV radiation, heat shock, toxins (including anisomycin and deoxynivalenol), chemical stressors (such as chemotherapeutics), or oxidative stress. These stimuli lead to mitochondrial outer membrane permeability (MOMP) and the release of pro-apoptotic proteins (Parsons and Green 2010; Tait and Green 2010). Mitochondrial permeability and subsequent release of apoptogenic factors is controlled in part by the Bcl-2 family of proteins which are known to be critical checkpoints in apoptosis, as they reside immediately upstream of irreversible cellular damage (Danial 2007; Youle and Strasser 2008).

The Bcl-2 family consists of around 20 members that generally fall into two categories: promoters of apoptosis via increased MOMP (Bax, Bak, Bad, Bid, Bim and Bik) (Edlich 2017; Pena-Blanco and Garcia-Saez 2017) and protectors from apoptosis by prevention of cytochrome c release (Bcl-2, Bcl-XL, Bcl-XS). Cytochrome c functions by associating with apoptotic protease activating protein-1 (Apaf-1) and pro-caspase-9 to form the apoptosome, thereby triggering the conversion of pro-caspase-9 to active caspase 9 (Hill, et al. 2004).

Caspase activation is associated with the onset of apoptosis, as both intrinsic and extrinsic apoptosis pathways converge on what is called the execution pathway to execute the final stages of cell death (Elmore 2007). Downstream effector caspases (caspase-3, -6, -7) target a range of proteins for cleavage, including PARP, cytoskeletal proteins, and cytokeratins.

Ribotoxic stress in apoptosis

One pathway to induce apoptosis is via ribotoxic stress. Ribotoxic stress involves the activation of stress-activated protein kinases (SAPKs), members of the MAPK family, whose transduction cascades may lead to cell recovery after damage or apoptotic death (Iordanov, et al. 1997). Known potent inducers of ribotoxic stress are ribosome-inactivating proteins (RIPs) which enzymatically and irreversibly damage the cell ribosomes, effectively inhibiting protein synthesis (Stirpe 2004). Some RIPs impair the ribosome by acting as a rRNA *N*-glycosidase to cleave the glycosidic bond of a single adenine residue in the 3' end of the 28S rRNA subunit (Endo, et al. 1987; Narayanan, et al. 2005; Stirpe, et al. 1988). Trichothecenes such as deoxynivalenol (DON) function by binding to a specific adenine residue to prompt a conformational change of the ribosome followed by cleavage by internal RNases (He, et al. 2012; Pestka 2010). The antibiotic anisomycin (ANS) is another ribotoxic stressor which inhibits protein synthesis via association with the peptidyl transferase center of the ribosome without damage to the ribosomal subunit. Both ANS and DON are ribotoxins that have been used as a model in our lab to investigate the intrinsic apoptotic pathway in the MEC line MAC-T.

Published work from our lab shows ANS induces IGFBP-3 expression at both the mRNA and protein level, and that IGFBP-3 knockdown via siRNA attenuates ANS-induced

apoptosis in MAC-T cells (Leibowitz, et al. 2013). These findings implicate IGFBP-3 as a key mediator of intrinsic apoptosis. Unpublished data from our lab has further determined DON-induced apoptosis is attenuated with IGFBP-3 knockdown by siRNA in MAC-T cells, prompting the query of whether IGFBP-3 is part of a universal mechanism of ribotoxin-induced intrinsic apoptosis. The work in this thesis delves further into the question of the role of IGFBP-3 in ribotoxin-induced apoptosis.

The role of IGFBP-3 in cellular fate and apoptosis

IGFBP-3 is a multifunctional protein affecting both cell proliferation and survival (Firth and Baxter 2002) through both IGF-dependent and IGF-independent mechanisms (Mohan and Baylink 2002). Work in MEC has shown both TNF-α, a cytokine produced by immune cells to modulate inflammation, and IGF-I, which is a potent mitogen and cell survival factor for MECs, increase IGFBP-3 expression (Sivaprasad et al. 2004). This is an interesting observation considering TNFα and IGF-I exert opposing effects on MEC growth and survival (Cohick, et al. 2000; Leibowitz and Cohick 2009; Thorn, et al. 2008). Leibowitz and Cohick reported that endogenous IGFBP-3 is required for both proliferation and cytokine-induced apoptosis in MECs (Leibowitz and Cohick 2009) solidifying the pluripotent role for IGFBP-3 in cell fate and survival. However, a clear pro-apoptotic function of IGFBP-3 independent of IGF interaction has been demonstrated in addition to its role in modulating IGF function (Butt and Williams 2001; Oh, et al. 1995). Work in human breast cancer cells has supported an important role for IGFBP-3 in IGF-independent anti-proliferative actions (Oh et al. 1995). In non-small cell lung cancer (NSCLC) cell lines IGFBP-3 is a potent inducer of apoptosis *in vitro* and *in vivo* (Lee, et al. 2002).

How does IGFBP-3 mediate apoptosis?

IGFBP-3 is a secretory protein and as such may impart its apoptotic effects via death receptors on the outer membrane, as was reported for breast and prostate cancer cells (Ingermann, et al. 2010). However one study that mutated IGFBP-3 to diminish cell surface binding found IGFBP-3 was still growth inhibitory, could elicit cell cycle arrest, and induced apoptosis in human breast cancer cells (Butt, et al. 2002), suggesting substitute pathways. Alternatively, IGFBP-3 may enter into cell via endocytosis to activate various downstream signal transduction pathways (Baxter 2013; Shahjee and Bhattacharyya 2014). For example, recent investigations into the IGF-independent anti-angiogenic effects of IGFBP-3 found that the binding protein interacted with Erk1/2 and Elk-1 leading to inactivation and suppression of downstream transcriptional events necessary for growth (Kim, et al. 2011). Work in early differentiated chondrogenic cells showed IGFBP-3 specifically up-regulated gene expression of signal transducer and activator of transcription (STAT)-1 and that incubation with an antisense STAT-1 oligonucleotide abolished the IGF-independent apoptotic effect of IGFBP-3. Overexpression of IGFBP-3 in NSCLC cells inhibited PI3K and MAPK pathways, both of which are IGF-induced survival pathways (Lee et al. 2002).

Gene expression of IGFBP-3 is increased due to p53, suggesting p53 may impact the IGFBP-3 apoptotic effect. Brimberg *et. al.* demonstrated that IGFBP-3 mediates p53-induced apoptosis and is inhibited by treatment with IGF-I to antagonize IGFBP-3 action (Grimberg, et al. 2002). Conversely, treatment with TGF-β, a known apoptosis-inducing agent, results in induction of IGFBP-3 expression and a dose-dependent increase of apoptosis in PC-3 cells (Rajah et al. 1997). PC-3 cells are p53-negative therefore these results suggest that IGFBP-3 induces apoptosis through a pathway independent of p53. Butt *et. al.*

further supported a p53-independent mechanism of apoptosis using MCF-7 human breast cancer cells where transfection of IGFBP-3 modulated the ratio of pro-apoptotic proteins Bax and Bcl-2 to potentiate cell death (Butt, et al. 2000).

Additionally IGFBP-3 may impart its tumor suppressive actions via activation of caspase-dependent apoptosis (specifically caspase-8 and -3/-7) and cross-talk with the transcription factor NF-κB and its modulators (Han, et al. 2011). When IGFBP-3 is mutated to disable IGF binding (GGG-IGFBP-3) in MCF-7 breast cancer cells, there is an induction of apoptosis through the activation of caspases involved in a death receptor-mediated pathway independent of the IGF-IGF1R axis, specifically caspase-8 and -7 (Kim et al. 2004). Butt et al. also reported that caspase inhibition by Z-VAD-FMK treatment significantly blocked IGFBP-3 induced apoptosis in T47D human breast cancer cells (Butt et al. 2002).

Nuclear localization of IGFBP-3

IGFBP-3 contains a nuclear localization signal (NLS) and nuclear localization of IGFBP-3 has been recognized for nearly two decades. However, clarity on its role in the nucleus and mechanism of nuclear transport has been slow to emerge. The role of nuclear IGFBP-3 in the IGF-independent actions of IGFBP-3 is at present unclear and thus an important area of investigation.

The nuclear localization sequence

In 1994 Radulescu *et al.* decoded a five amino acid motif in the basic carboxy-terminal domain of IGFBP-3 that shared strong sequence homology with previously identified bipartite nuclear localization sequences (NLS) (Radulescu 1994; Spratt, et al. 1991). Follow-

up experiments by Schedlich *et. al.* determined that mutation within the putative NLS of IGFBP-3 prevented nuclear uptake in T47D cells (Schedlich, et al. 1998). The same group reported the NLS sequence motif directed nuclear translocation of IGFBP-3 by association with importin β, a nuclear transport protein (Gorlich, et al. 1995; Kubitscheck and Siebrasse 2017), and that it was specific and energy dependent, requiring both ATP and GTP hydrolysis (Schedlich, et al. 2000). Leibowitz *et al.* determined IGFBP-3 is directed to the nucleus in ANS-treated cells independent of caspase activation, but nuclear movement did not occur in IGF-I treated MAC-T cells (Leibowitz et al. 2013). Unpublished work from the Cohick laboratory with fluorescent microscopy has shown that nuclear localization of GFP-tagged IGFBP-3 is also a regulated event and association of IGFBP-3 with importin β is required for import in bovine MECs (Agostini-Dreyer 2014b). Interestingly IGFBP-5 contains a structurally similar bipartite motif and both IGFBP-3 and IGFBP-5 are found to translocate to the nucleus (Schedlich et al. 2000; Schedlich et al. 1998). IGFBP-2 (Azar, et al. 2014) and IGFBP-6 (Han, et al. 2009) have also been shown to localize to the nucleus, while the remaining members of the IGFBP family do not.

Actions of nuclear IGFBP-3

Role of nuclear IGFBP-3 in apoptosis

While nuclear localization of IGFBP-3 is well documented, it is still largely unknown if IGFBP-3 nuclear accumulation is a prerequisite for apoptosis. When the IGFBP-3 NLS was mutated in YFP-IGFBP-3 fusion proteins (KGRKR to MDGEA) prior to transfection into human prostate cancer cells, neither secretion nor concentration in the nucleus were required for the induction of caspase-dependent apoptosis (Bhattacharyya, et al. 2006). However another study found IGFBP-3 is able to translocate to the nucleus from the

extracellular compartment as a potent anti-proliferative agent where it functions by cell cycle blockade and induction of the intrinsic apoptotic pathway (Baxter 2001; Lee, et al. 2004).

In vitro work in osteosarcoma cells demonstrates a role for nuclear IGFBP-3 in inducing apoptosis in a caspase-dependent manner, evident by treatment with the broad-spectrum caspase inhibitor Z-VAD-FMK which abolishes nuclear IGFBP-3 induced apoptosis (Santer, et al. 2006). IGFBP-3 is known to interact with retinoid X receptor-α (RXRα) and retinoic acid receptor-α (RARα) to modulate RAR-signaling in the nucleus via residues in the N- and C-terminal domains of the binding protein (Schedlich, et al. 2007a). Other reports highlight the interaction between Nur77 and IGFBP-3 where IGFBP-3 acts as a trigger for Nur77 translocation as a possible mechanism for how IGFBP-3 may induce apoptosis (Wei and Li 2015). IGFBP-3 induces mitochondrial translocation of nuclear Nur77 that is dependent on RXRα and IGFBP-3 and Nur77 have additive effects on caspase 3/7 activation, indicating a role for mitochondrial Nur77 in IGFBP-3 induced apoptosis (Lee, et al. 2005). Additionally Agostini-Dreyer *et. al.* recently found that Nur77 plays a role in ANS-induced apoptosis in MAC-T cells possibly via association between endogenous IGFBP-3 and Nur77 in the nucleus and subsequent modulation of Nur77 phosphorylation and nuclear export (Agostini-Dreyer, et al. 2015).

Role of IGFBP-3 as a transcription factor and general mechanisms

A lingering question is whether IGFBP-3 interacts directly with DNA or chromatin, or complexes with transcription factors or other components of the genetic machinery. Reports indicate that nuclear IGFBP-3 may bind to transcription factors such as RXRα, RAR-α, VDR, Nur77 and ribonucleic acid polymerase II binding subunit 3 (Rpb3) to impart its

intracellular actions (Lee and Cohen 2002; Oufattole, et al. 2006; Schedlich, et al. 2004). One study determined a functional NLS is required to act as the binding domain for Rpb3, which recruits the polymerase complex to specific transcription factors for transactivation of genes in the nucleus (Oufattole et al. 2006). IGFBP-3 may also interact with histone-DNA complex directly or play a role in the DNA damage response via damage-induced apoptosis or as a transcriptional target of the tumor suppressor p53 (Baxter 2015). Additional support that IGFBP-3 may mediate its anti-proliferative effects via interaction with nuclear transcription factors comes from studies on osteoblast differentiation. IGFBP-5, which bares structural similarity to IGFBP-3 and is the only other IGFBP to contain a NLS, interacts with nuclear vitamin D receptor (VDR), to inhibit heterodimerization with (RXRα) and attenuate 1,25(OH)D3-induced expression of bone differentiation markers (Schedlich, et al. 2007b).

IGFBP-3 as a multi-compartmental signaling molecule

In addition to playing a nuclear role in apoptotic signaling, there is work supporting the role of IGFBP-3 as a multi-compartmental signaling molecule. Paharkova and Lee determined there to be a highly conserved leucine-rich nuclear export sequence (NES) in the C-terminal region of IGFBP-3 similar to known NESs that fits established criteria (Bogerd, et al. 1996; Paharkova-Vatchkova and Lee 2010). Investigations into the role for IGFBP-3 in nucleo-mitochondrial translocation showed nuclear export to the mitochondria and endoplasmic reticulum is crucial to regulate the apoptotic properties of IGFBP-3, as impaired nuclear export retained RXR/Nur77 heterodimers in the nucleus and abolished apoptosis (Paharkova-Vatchkova and Lee 2010). Further work supporting nuclear export of IGFBP-3 demonstrated that IGFBP-3 and Nur77 associate in the cytoplasm in prostate cancer cell

apoptosis (Lee, et al. 2007). Co-immunoprecipitation and fluorescence immunocytochemistry analysis showed that IGFBP-3 and RXRα co-localize in both the cytoplasm and the nucleus, and uncovered an association between them. In addition, treatment of F9 RXRα knock-out cell lines with IGFBP-3 had no effects on apoptosis in comparison to F9 WT cells, indicating RXRα is required for apoptosis (Lee and Cohen 2002).

III. ROLE OF GLYCOSYLATION IN IGFBP-3 PHYSIOLOGY

IGFBP-3 contains a signal sequence as well as a NLS and is thus both secreted from the cell and found in the nucleus. Due to the heavy glycosylation status of serum IGFBP-3, the question of whether IGFBP-3 needs to be secreted prior to nuclear localization for apoptosis emerged. Interestingly, nuclear and secreted IGFBP-3 run at different molecular weights on SDS-PAGE gels in MAC-T cells (unpublished data). However to my knowledge there have been no attempts to classify the type of glycan present in nuclear IGFBP-3 versus secreted IGFBP-3 and the potential role glycosylation status plays in its apoptotic mechanism.

The secretory pathway

One-third of all proteins in eukaryotes enter the cellular secretory pathway. N-glycosylation begins in the ER with the addition of a core oligosaccharide sugar being placed onto a newly synthesized protein as it enters the ER membrane via co-translocation off the ribosome. After protein transfer, extensive covalent modifications in the form of trimmed and processed glycosylation and deglycosylation ensue by glycosyltransferases and glycosidases

(Kellokumpu, et al. 2016). In the ER partial trimming of the precursor oligosaccharide plays a role in glycoprotein folding and quality control (Leitman, et al. 2013; Ruddock and Molinari 2006). Glycans may serve as tags to control the fate of glycoproteins (Aebi, et al. 2010; Spiro 2004). Only when the protein is properly folded and tagged can it proceed to the Golgi for further processing and packaging. The trafficking of enzymes and proteins through the Golgi is acutely regulated to modulate remodeling of proteins and fine tune protein function. Enzyme distribution patterns show distinct concentration gradients within the Golgi stacks though there are no defined subcompartments (de Graffenried and Bertozzi 2004; Rabouille, et al. 1995; Schoberer, et al. 2010). Dysregulation of glycosylation in the Golgi due to disease has revealed the importance of glycosylation in tethering factors, protein sorting, maintaining Golgi pH homeostasis, and membrane fusion (Rivinoja, et al. 2009; Rosnoblet, et al. 2013). Further modifications may include demannosylation, galatosylation, fucosylation and sialylation. It is poorly understood how signaling cascades regulate glycan synthesis. One review focuses on how tight regulation of membrane trafficking through control of enzyme compartmentalization may be a regulatory mechanism of glycosylation, supported by the finding that glycosylation enzymes are usually distributed by their order of action (Bard and Chia 2016).

Different forms of glycosylation

There are two major types of glycosylation: O-linked where a sugar moiety is attached to an oxygen atom of an amino acid residue, and N-linked where the sugar moiety is attached to asparagine residues. The later often occurs with soluble, secreted proteins (Varki, et al. 2015). N-glycosylation occurs in all domains in life and while the core glycan being transferred may differ in prokaryotes, eukaryotes produce a conserved structure (Schwarz and Aebi 2011).

There are three types of N-linked glycans: polymannose (high-mannose), complex oligosaccharide (different sugars) and hybrid (of polymannose and complex). IGFBP-3 is an *N*-linked glycoprotein with three *N*-linked sites available for glycosylation at Asn89, Asn109, and Asn172 (Firth and Baxter 1999). The two glycoforms of IGFBP-3 commonly seen in protein analysis via polyacrylamide gel electrophoresis (PAGE) are from variable glycosylation at the N172 residue. It is believed these two occupied sites are important glycosylation sites for IGFBP-3 function (Masnikosa, et al. 2010).

N-linked glycosylation in the ER and the Golgi

As noted above, protein glycosylation begins in the ER with modifications occurring all along the endomembrane system, especially the Golgi apparatus which modifies proteins to produce mature glycoproteins. Investigations into the glycosylation status of proteins along the component organelles of the endomembrane system have utilized glycosidases and trafficking inhibitors to determine if there is differential glycosylation (Lam, et al. 2009; Shen, et al. 2017).

IGFBP-3 exists as two glycoforms which reduce to one 29 kDa form upon inhibition of N-linked glycosylation (Firth and Baxter 1999). Enzymatic deglycosylation is another method used to reduce a protein to its non-glycosylated form. Two enzymes used to discern glycosylation status are Endoglycosidase H (Endo H) and Peptide-N-Glycosidase F (PNGase F). Endo H is capable of deglycosylating only high-mannose glycan forms, while PNGase, which cleaves between the innermost GlcNAc and asparagine residues, is able to reduce all forms of glycans including the complex type (Schoberer and Strasser 2011; Shen et

al. 2017). Interestingly, Endo H sensitivity was found to be an indication that a protein had not trafficked to the Golgi yet for packaging or secretion (Tarentino and Maley 1974).

Brefeldin A (BFA) is a fungal macrocyclic lactone commonly used as an inhibitor of protein trafficking and secretion in mammalian and plant cells (Jackson and Casanova 2000). In the mammalian endomembrane system the target of BFA is a subset of Sec7-type GTPase-exchange factors (GEFs) that catalyze the activation of a small GTPase called Arf1p (Jackson and Casanova 2000). Arf1p is localized to the Golgi apparatus and is responsible for the formation of transport vesicles via recruitment of coat proteins such as COPI and clathrin. Treatment with BFA results in loss of COPI coats from the Golgi apparatus followed by the fusion of Golgi membranes with the ER creating hybrid ER-Golgi stacks (Helms and Rothman 1992; Nebenfuhr, et al. 2002), and a complete distribution of Golgi enzymes into the ER (Sciaky, et al. 1997). The newly formed BFA compartment inhibits ER to Golgi trafficking and consequentially results in an accumulation of high mannose proteins (Lam et al. 2009; Takatsuki and Tamura 1985) and a block in protein secretion, while protein synthesis is maintained (Misumi, et al. 1986). Proteins of cells treated with Brefeldin A may be treated with Endo H to probe for sensitivity, indicating the protein has not been able to reach cisternal stacks of the Golgi apparatus that edit sugars to contain complex-type glycans.

The role of glycosylation in IGFBP-3 physiology

Protein glycosylation is potentially involved in all aspects of human growth and development and has been shown to confer specific binding sites or modify the stability or function of carrier proteins (Kailemia, et al. 2017; Varki 2017).

IGFBP-3 is known to form a ternary complex of 150kD with IGF-I and acid-labile subunit (ALS) in serum (Baxter and Dai 1994), which is unable to cross into extracellular space due to its large size. Therefore the ternary complex must dissociate to enable IGF delivery to target tissues, suggesting a role for glycosylation in target protein delivery. One study found that IGF ternary complex formation depended on the glycosylation status of ALS, with complete deglycosylation abolishing the ability of IGFBP-3 to associate with ALS (Janosi, et al. 1999). To assess the role of IGFBP-3 glycosylation in ligand binding, Firth and Baxter created seven IGFBP-3 N-glycan mutants in various permutations and expressed the cDNAs. Ligand blotting using (^{125}I)IGF-I recovered all seven mutants indicating the ability to bind IGF-I was retained (Firth and Baxter 1995). There appeared to be no difference between the mutant forms and fully-glycosylated IGFBP-3 in ALS binding. However glycosylation status did alter cell binding characteristics, as the fully glycosylated form of IGFBP-3 had significantly less cell-surface association than the non-glycosylated mutant (Firth and Baxter 1999). While these findings suggest no role for IGFBP-3 glycosylation in ligand binding, other data propose glycosylation may change affinity for IGFs, though it is not essential for IGF binding (Nedic, et al. 2012). In addition, investigations of IGFBP-mediated endocytosis of microspheres in human retinal pigment epithelial cells (RPE) found a clear role of glycosylation status of IGFBP-3 and IGFBP-5, specifically in heparin binding-mediated internalization as treatment with heparin abolished phagocytic responses to microspheres coated with non-glycosylated IGFBP-3, but only partially inhibited response to glycosylated IGFBP-3 (Ainscough, et al. 2009).

One possible function of N-glycosylation is to aid in the *in vivo* folding and assembly of the nascent polypeptide chain, which is crucial in maintaining its conformation, directing its localization or ultimate secretion from the cell (Winterburn and Phelps 1972), and mediating protein-protein interactions. Both glycosylation and sialylation regulate ligand-binding equilibrium, though they have opposite effects. However glycosylation did not appear to affect IGFBP-3 ability to bind with its ligands IGF-I, IGF-II or ALS (Firth and Baxter 1995, 1999). IGFBP-3 from patients with diabetes possesses additional sialic acids and increased content of complex type N-glycans (Firth and Baxter 1999). These data suggest that decreased affinity of IGFBP-3 for its ligand due to sialylation may be a potential mechanism by which IGFBP-3 promotes tumor progression.

Differential glycosylation may regulate the half-life and clearance rate, as non-glycosylated IGFBP-3 is degraded more rapidly than the glycosylated form. Therefore alteration of glycosylation may be an adaptive mechanism to protect IGFBP-3 from degradation as IGFBP-3 is subject to cleavage from factors in serum. For example, matrix metalloproteinase-3 cleaves IGFBP-3 into six fragments, some containing heparin-binding domains. The fragments are capable of selectively binding to glycosaminoglycan moieties commonly attached to protein cores (proteoglycans), which may mediate the effects of IGFBP-3 at the cellular and extracellular interface (Fowlkes and Serra 1996). Additionally other enzymes such as plasmin, thrombin, cathepsin D, prostate-specific antigen, or nerve growth factor are also capable of cleaving IGFBP-3 (Nedic et al. 2012). Inhibiting N-linked glycosylation using tunicamycin (TM) causes cell death in malignant cells by down-regulating IGF-1 receptor at the cell surface (Dricu, et al. 1997).

Glycosylation of IGFBP-3 and cancer

Both *in vivo* and *in vitro* models have demonstrated a role for IGFBP-3 as a biological mediator of cancer cell apoptosis (see review (Baxter 2014)). In human prostate cancer cell lines, IGFBP-3 expression is typically diminished (Schwarze, et al. 2002), and in human and mouse models this decrease is associated with advanced disease (Hampel, et al. 1998; Kaplan, et al. 1999). The onset of tumorigenesis is dependent on the balance of survival and apoptotic factors. An overall shift in the balance toward anti-apoptotic factors leads to tumorigenesis. IGFBPs are known to interact with and affect these factors (Baxter 2014). As such there is a hazard significance associated with it in breast cancer risk assessment, as high levels and low levels have been associated with risk of breast cancer, suggesting IGFBP-3's actions may depend on the cellular environment (Burger, et al. 2005). IGFBP-3 may function as a tumor suppressor and is down regulated in some cancer tissues. In breast and prostate cancer cells IGFBP-3 mediates anti-tumor effects via cell death receptors (Ingermann et al. 2010). However many tumor types, including breast cancer, have an overexpression of IGFBP-3, implicating IGFBP-3 as a key player in breast cancer cell regulation.

In addition to a mechanistic role for IGFBP-3 in cancer and tumor progression, a pertinent role for glycosylation as a biomarker has also been implemented in cancer (Kailemia et al. 2017). In cancer cells a hallmark of malignancy is enhanced synthesis of highly branched and sialylated N-glycans. IGFBP-3 in human serum of breast cancer patients contains highly biantennary complex type N-glycans with a bisecting GlcNAc residue and high content of terminal sialylation, a common occurrence in metastatic cancer cells (Baricevic, et al. 2010). The extent of the changes increased with breast cancer severity. These findings suggest

breast cancer progression may cause alterations of IGFBP-3 glycosylation and that carcinogenesis may have dramatic transformation of the glycosylation mechanism.

Intriguingly, a high nuclear IGFBP-3 expression phenotype is strongly associated with a higher risk of prostate cancer recurrence and is a better predictor of tumor recurrence than well known indicators such as tumor margin status or capsular invasion, while cytoplasmic IGFBP-3 show no significance as a predictor (Seligson, et al. 2013). However the role of IGFBP-3 glycosylation was not examined in this study.

Role of glycosylation in IGFBP-3 nuclear localization and trafficking

Although there are many accounts of IGFBP-3 nuclear localization as mentioned above, little is known about the mechanism by which it localizes to the nucleus including if it first has to be secreted, or the role glycosylation plays in movement. Some reports indicate IGFBP-3 must be secreted prior to cellular uptake and re-internalization into the nucleus, as opposed to trafficking from the ER to the nucleus directly following protein folding as described below.

As mentioned above, IGFBP-3 glycosylation status may affect binding affinity. IGFBP-3 delivery may be accomplished by complex formation between IGFBP-3 with transferrin and transferrin receptor 1 (TfR1), as observed in colon cells from both non-cancer and cancer tissues (Miljus, et al. 2015). IGFBP-3 contains a transferrin-binding C terminal peptide region to allow transferrin binding, which in turn is able to bind the transferrin receptor (TfR1) for cellular uptake (Weinzimer, et al. 2001). IGFBP-3 also contains a binding sequence for caveolin, a well-known protein involved in vesicular formation for trafficking

and cellular uptake. Blocking TfR1-mediated endocytosis and inhibiting caveolae formation prevents both endogenous and exogenous IGFBP-3 re-uptake into the cell (Lee et al. 2004). Additionally inhibition of receptor-mediated endocytosis via clathrin-coated pits does not prevent nuclear uptake of Cy3-labeled IGFBP-3 in T47D cells, suggesting IGFBP-3 may re-internalize by a mechanism other than classical endocytosis (Schedlich et al. 1998). This same report also showed IGFBP-3 and -5 may share a common nuclear transport pathway, as saturation with 10-fold non-labeled plasma IGFBP-3 blocked detection of nuclear Cy3-labeled IGFBP-3 and IGFBP-5 suggesting possible competition for cell surface binding or internalization (Schedlich et al. 1998). Another study investigating IGFBP-3 mediated endocytosis in RPE cells found uptake of IGFBP-3 coated microspheres was unaffected by blockade of the transferrin receptor, relying more on the NLS and heparin binding domains (HBD) (Ainscough et al. 2009).

ERAD as an escape from the ER

As mentioned above, N-glycan processing occurs in the ER, where cellular machinery orchestrates quality control mechanisms and delegates proteins toward their proper fate (Lederkremer 2009). Misfolded or unassembled multi-subunit proteins are recognized by chaperones and associated factors with the help of N-linked glycosylation. Targeted proteins are then shuttled through the ER-associated degradation (ERAD) machinery for proteasomal degradation in the cytoplasm (Spiro 2004; Vembar and Brodsky 2008). This requires retrotranslocation through a Sec61 channel and deglycosylation by PNGase.

Some proteins are known to use ERAD machinery to escape the ER, most notably ricin. Ricin is a plant holotoxin that results in protein synthesis inhibition, stress-induced cell

signaling cascades and apoptosis. Ricin Toxin A (RTA), the catalytically active subunit of Ricin, is capable of inducing ribotoxic stress in mammalian cells even when mutated to have less effective enzymatic ribosome depurination (Jetzt, et al. 2012). In mammalian cells RTA showed enhanced toxicity due to inhibition of UPR (Wang, et al. 2011).

Ricin is of particular interest due to the manner in which it traffics through the cell (Spooner and Lord 2015). The plant toxin enters via endocytosis and passes through the Trans Golgi Network (TGN) by retrograde trafficking mechanisms toward the ER where it embeds into the ER membrane (Tsai, et al. 2002). The RTA and RTB subunits are reductively separated by interaction with protein disulfide isomerase (PDI) which remodels the holotoxin structure to allow cleavage. RTA then masquerades as a misfolded protein, effectively hijacking the ERAD machinery designed to chaperone proteins to the proteasome for degradation (Bellisola, et al. 2004). However RTA is not broken down by proteasomes due to lack of lysine residues (Deeks, et al. 2002; Lord, et al. 2003a); instead it refolds in the cytosol and inhibits protein synthesis at the ribosome and apoptosis ensues (Lord, et al. 2003b).

As IGFBP-3 is a glycosylated protein that is both secreted and found in the nucleus, we were interested in understanding how it escapes the secretory pathway for nuclear localization. Glycosylation status may play a role in deciding the fate of IGFBP-3 as a tag to direct compartmentalization, specifically toward ERAD machinery in the ER. Additionally, glycosylation is known to affect protein folding and may serve to reveal or conceal a NLS or secretion signal for IGFBP-3. ***The objective of this work was to investigate the role of glycosylation in nuclear localization of ribotoxin-induced IGFBP-3.*** Future work will

focus on determining whether IGFBP-3 utilizes ERAD machinery as a means of escaping the secretory pathway.

Figures

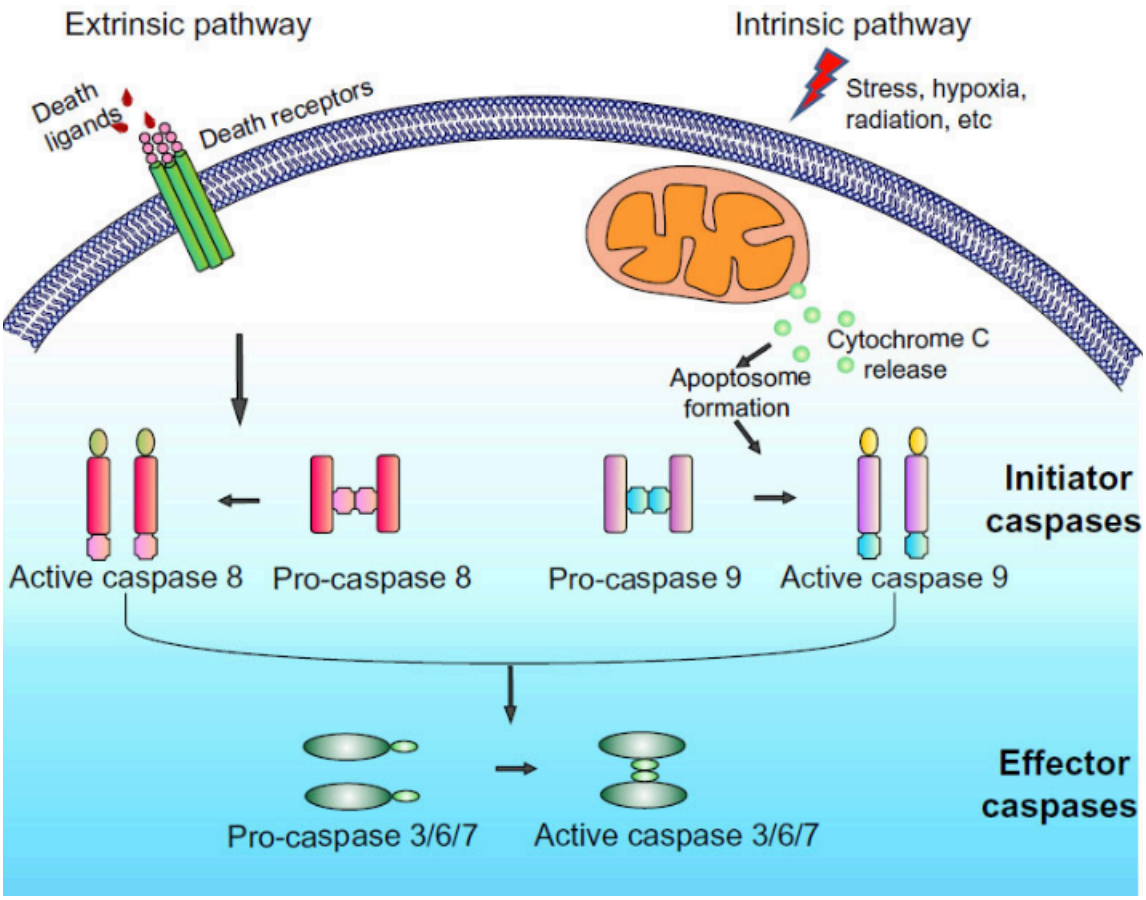

Fig. 1. Extrinsic vs. intrinsic apoptotic pathways. Ligand binding at cell surface death receptors induces downstream signaling, resulting in activation of caspase 8 and effector caspases 3/6/7. Intrinsic stressors such as hypoxia and radiation trigger mitochondrial membrane permeability, resulting in release of cytochrome c, followed by activation of initiator and effector caspases 3/6/7. Extrinsic and intrinsic pathways converge on the same effector caspases (Julian 2015).

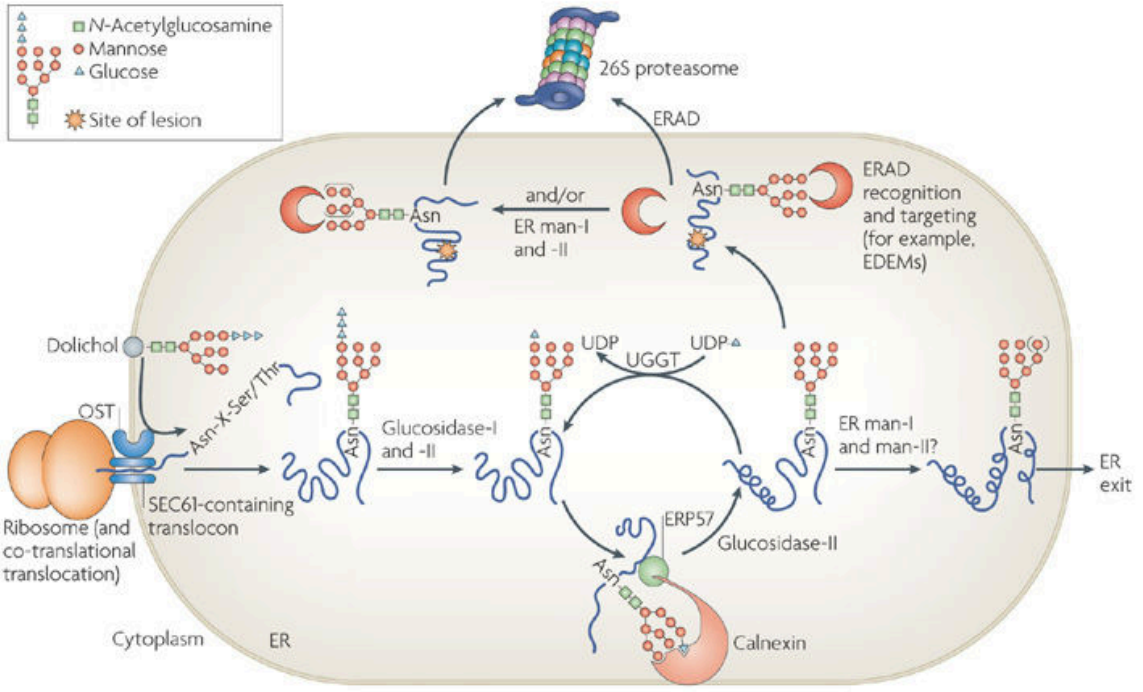

Fig. 2. Endoplasmic reticulum associated degradation (ERAD). Proteins translocate across the ER membrane and are shuttled to ERAD machinery by BiP chaperone proteins. Unfolded proteins are maintained in the ER by the addition of a mannose sugar until they are folded properly. If a protein fails to properly fold, ER manI removes a branch of the glycan, inhibiting further mannose addition. This allows the improperly folded protein to bind to EDEMs, which shuttle it through ERAD machinery for proteasomal degradation (Vembar and Brodsky 2008).

CHAPTER 2

IGFBP-3 Induced by Ribotoxic Stress is Not Secreted Prior to Nuclear Localization In Mammary Epithelial Cells

Introduction

IGF binding protein-3 (IGFBP-3) is proposed to play a role in apoptosis through IGF-independent mechanisms. In the MAC-T bovine mammary epithelial cell (MEC) line, the ribotoxic stressors anisomycin (ANS) and deoxynivalenol (DON) induce apoptosis and increase IGFBP-3 expression (Hanke 2017; Leibowitz et al. 2013). Knockdown of IGFBP-3 mitigates the apoptotic effects of these toxins, supporting a role for IGFBP-3 in the apoptotic response (Hanke 2017; Leibowitz et al. 2013). IGFBP-3 contains both a signal sequence and a nuclear localization sequence (NLS) and is thus both secreted and localized to the nucleus (Lee et al. 2004; Schedlich et al. 1998; Spratt et al. 1991; Xi, et al. 2007). Nuclear accumulation of IGFBP-3 has been proposed to be important in its apoptotic effect. IGFBP-3 was shown to interact with nuclear transcription factors RARα and RXRα, and modulates translocation of nuclear Nur77 to the mitochondria (Agostini-Dreyer et al. 2015; Schedlich et al. 2007a; Wei and Li 2015). Because nuclear localization of IGFBP-3 plays a role in intrinsic apoptosis, understanding how IGFBP-3 is trafficked to the nucleus is crucial to further delineating the apoptotic mechanism. However, how it escapes the secretory pathway to traffic to the nucleus is unknown.

There are conflicting reports describing a mechanism for nuclear accumulation of IGFBP-3. Some studies have reported that extracellular IGFBP-3 is rapidly internalized and delivered to the nucleus, suggesting IGFBP-3 may require secretion and re-internalization prior to nuclear localization. In osteosarcoma cells IGFBP-3 added to growth medium is internalized by clathrin- and caveolin-mediated pathways (Micutkova, et al. 2012). Additionally, in prostate cancer cells exogenous IGFBP-3 is reported to bind transferrin, and then get internalized as a complex with transferrin (Tf) and transferrin receptor (TfR) (Lee et al.

2004). Both of these systems rely on addition of exogenous IGFBP-3, so whether they represent what occurs when endogenous IGFBP-3 is secreted is uncertain. Experiments investigating the role of endogenous or transfected IGFBP-3 are inconsistent. When prostate cancer cells are transfected with IGFBP-3 that lacks the signal peptide required for secretion, IGFBP-3 is found in the nucleus and can still induce apoptosis (Bhattacharyya et al. 2006). In contrast, TGF-β induces production and nuclear localization of IGFBP-3, however nuclear accumulation of IGFBP-3 is attenuated by the addition of an anti-IGFBP-3 antibody indicating that secretion is required (Lee et al. 2004).

Additionally, protein glycosylation may play a role in IGFBP-3 modes of action. Endogenous IGFBP-3 present in cell lysates runs as a doublet. The two isoforms are due to N-linked glycosylation, where sites N_{89} and N_{109} are glycosylated with sugars of 4 kDa and 4.5 kDa respectively, while N_{179} has variable occupancy (Firth and Baxter 1999). Previous work from the Cohick lab found that exogenous IGFBP-3 that had trafficked to the nucleus due to treatment with ANS was glycosylated (unpublished data). One possible function of N-linked glycosylation is to assist in folding the polypeptide chain to maintain conformation, thus aiding to direct its localization within or ultimate secretion from the cell (Winterburn and Phelps 1972).

The objectives of the present study were to extend work using ANS to investigate the role of IGFBP-3 in ribotoxic stress to DON, and to further determine how nuclear IGFBP-3 induced by ribotoxic stress traffics to the nucleus. To study this, we treated MAC-T cells with ANS and DON to examine IGFBP-3 expression and nuclear localization. We then used deglycosylation enzymes Endoglycosidase H (Endo H) and Peptide-N-glycosidase F

(PNGase F) to determine whether IGFBP-3 that has localized to different compartments is differentially glycosylated. We also treated with Brefeldin A, an inhibitor of anterograde trafficking to the Golgi, which results in blocked secretion (Helms and Rothman 1992). Our results demonstrate that IGFBP-3 can traffic to the nucleus independent of COPI-mediated retrograde transport. Moreover, nuclear IGFBP-3 contains ER-, but not Golgi-specific glycosylation indicating newly synthesized IGFBP-3 enters the ER prior to trafficking to the nucleus. Collectively the data support a model that suggests trafficking of ribotoxin-induced IGFBP-3 to the nucleus involves transport from the ER, possibly via retrograde translocation, in the MEC line.

Materials and Methods

Chemical reagents

Dulbecco's Modified Eagle Medium (DMEM-H, with high glucose 4.5 g/L D-glucose), penicillin, and streptomycin were purchased from Life Technologies (Carlsbad, CA). Phenol red-free (PRF) DMEM-low glucose media, gentamycin, bovine insulin, ANS, and fetal bovine serum (FBS) were purchased from Sigma (St. Louis MO). Recombinant human IGF-I (100% identical to bovine IGF-I) was obtained from Peprotech (Princeton, NJ). Antibodies against the following proteins were purchased as indicated: HSP60 and importin-β (Abcam, Cambridge, MA), His-tag (Genscript, Piscataway, NJ), PARP and cleaved caspase-3/-7 (Cell Signaling Technology, Danvers MA), lamin AC (Santa Cruz), and anti-rabbit IgG (GE, Pittsburgh, PA). The anti-bovine-IGFBP-3 antibody was produced in-house (Agostini-Dreyer 2014a). The enzymes Endo H and PNGase F were purchased from New England Biolabs (Ipswich, MA). The following inhibitors were purchased as indicated: Importazole (Millipore, Billerica, MA) and Brefeldin A (Sigma Aldrich, St Louis, MO). Hoechst 33342 was purchased from Invitrogen. Superfect transfection reagent was purchased from Qiagen (Valencia, CA) and deoxynivalenol was from Sigma Aldrich, St Louis, MO.

Cell culture

The bovine MEC line MAC-T (Huynh et al., 1991) was routinely maintained in complete media consisting of DMEM containing 4.5 g/liter D-glucose (i.e., DMEM-H), 20 U/ml penicillin, 20 μg/ml streptomycin, 50 μg/ml gentamicin, 10% FBS, and 5 μg/ml insulin. For experiments, cells were plated at a concentration of incomplete media at 1 x 10^4 cells/cm^2 and grown to confluence in phenol red-free DMEM-H containing 10% FBS and antibiotics

and without insulin. Except where otherwise noted, cells were washed with phosphate-buffered saline (PBS), and incubated in serum-free (SF) DMEM-H supplemented with 0.2% BSA and 30 nM sodium selenite prior to exposure to treatment in PRF SF DMEM-H with antibiotics and without insulin.

Construction of GFP-tagged IGFBP-3 (IGFBP-3-GFP)

A PCR reaction was used to add a 6x-His tag to the C terminus of bovine IGFBP-3. *Xho*I and *BamHI* restriction sites were added to the 5' and 3' ends of bovine IGFBP-3, respectively, producing a 900 bp fragment. The 50 µl PCR reaction contained 20 ng DNA template (pRc/RSV IGFBP3, Grill and Cohick, 2000), 20 pmol forward primer 5'-ATATTACTCGAGTAATGCTGCGGGCACGCCCCGCGCTC-3', 20 pmol reverse primer 5'-ACAAGTGGATCCACCTTGCTCTCCATGCTGTAGCAGTC-3', 2 mM Mg SO_4, 0.2 mM of each dNTP, 5% DMSO and 1 unit Platinum *Taq* High Fidelity DNA Polymerase (Life Technologies, Carlsbad, CA). The cycling parameters were 94˚C 2 min; 30 cycles of 94˚C 1 min, 50˚C 1 min, 68˚C 1 min; 68˚C 10 min. The IGFBP-3-GFP fragment was purified using Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel, Bethlehem, PA). IGFBP-3-GFP was digested with *Xho*I and *BamHI*. After vector dephosphorylation with Anarctic Phosphatase (New England Biolabs) the insert was purified as described above and ligated using T4 DNA Ligase (Invitrogen, Carlsbad, CA). The ligation reaction was used to transform One Shot TOP 10 competent cells (Invitrogen). After miniprep, colonies were screened for positive clones by restriction digestion. Bovine IGFBP3-GFP construction was confirmed by sequencing.

Transient transfection of IGFBP-3

MAC-T cells were plated in complete media at 3.5 x 10^4 cells/cm^2. The next day subconfluent cells were transfected with a plasmid encoding cDNA for IGFBP-3-GFP or eGFP as a control. Plasmids were prepared using the EndoFree plasmid Maxi Kit (Qiagen, Valencia CA). Cells were transfected using SuperFect combined with plasmid in a 1:5 ratio for 100 x 25 mm^2 dishes and in a 1:10 ratio for 8-well μslides (Ibidi, Martinsried, Germany). The transfection mixture was prepared in DMEM-H without additives, vortexed for 10 sec, and incubated at RT for 10 min. Spent media were removed from cells and replaced with fresh complete media and the transfection mixture. Cells were incubated with transfection mixture for 3 h then media were removed without PBS washes and replaced with fresh complete media. Following a 24 h recovery in serum-containing media, cells were rinsed twice in PBS and incubated with fresh SF DMEM-H for 1 h then treated as indicated in the figure legends.

Cell lysis and Western immunoblotting

Cytosolic and nuclear fractionations were obtained by lysing cells in hypertonic buffer (20 mM Hepes pH 7, 10 mM KCL, 0.1% Triton, 20% Glycerol) supplemented with protease inhibitors with 10 strokes of the Dounce homogenizer followed by centrifugation for 5 min at 1000 x g. Pellets were resuspended in buffer C (20 mM HEPES pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol) supplemented with protease inhibitors, incubated 4°C 30 min, then centrifuged at 13,250 RPM for 10 min to obtain the nuclear fraction. To collect whole cell lysates (WCL) cells were washed with ice cold 1X PBS and collected by scraping in Complete Lysis Buffer (10 μg/ml aprotinin, 80 mM β-glycerophosphate, 2 mM EDTA, 2 mM EDTA, 50mM HEPES, 10 μg/ml leupeptin, 1 mM

phenylmethylsulfonylfluoride, 0.1% SDS, 10 mM sodium fluoride, 2 mM sodium orthovanadate, 1% Triton X-100, 10 μg/ml trypsin inhibitor). Cell lysates were incubated on ice for 30-40 min and then pelleted at 4 °C in a centrifuge for 15 min at 13,000 x g. Lysates were assayed for protein content with the BioRad Protein Assay (BioRad, Hercules, CA). For cytoplasmic fractions and WCL 50μg of total protein was run, and 30μg for the nuclear fraction. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) on 12.5% or 15% gels and transferred to nitrocellulose (0.2 μm; Bio-Rad); (Millipore, Bedford, MA) membranes. Membranes were blocked for 1 h at room temperature in Tris buffered saline + 0.05% Tween-20 (v/v) (TBS-T) and 5% non-fat dried milk (w/v), then incubated with primary antisera at 4°C overnight with gentle agitation. Membranes were then washed in TBS-T and incubated for 1 h at room temperature with appropriate HRP-conjugated secondary antibodies. Peroxidase activity was detected with ECL Prime (GE, Pittsburgh, PA). Chemiluminescence was detected with the Fluorchem FC2 (Protein Simple, Santa Clara, CA).

Fluorescence microscopy

MAC-T cells were plated in complete media at 3.5 x 10^4 cells/cm^2 on 8-well Ibidi μ-slides, transfected and treated as described above. Cells were then fixed with 10% neutral buffered formalin (Fisher Scientific, Waltham, MA). Nuclei were stained with Hoechst (Invitrogen). Cells were stored in mounting media (Ibidi) and images were acquired with an Olympus FSX100 microscope.

Results

ANS and DON induce nuclear localization of IGFBP-3

Previous work from our lab has shown that the ribotoxin ANS induces nuclear localization of IGFBP-3 (Agostini-Dreyer 2014b). To test whether IGFBP-3 nuclear localization is part of a universal ribotoxic stress response, we treated MAC-T cells with ANS or DON, a trichothecene that also activates the ribotoxic stress response (Pestka 2010). Cells were transfected with IGFBP-3-GFP which carries a molecular weight of approximately 64 kDa making it too large for passive diffusion through the nuclear pore (Hoelz, et al. 2011) and treated ± ribotoxin. As shown in Fig. 1A, cells incubated in serum-free (SF) conditions exhibited punctate staining with IGFBP-3-GFP detected exclusively in the cytosol, indicating it was excluded from the nucleus in untreated cells. Treatment with ANS and DON both resulted in nuclear import of IGFBP-3-GFP, indicating nuclear localization of exogenous IGFBP-3 is specific and inducible with ribotoxins. Hoechst staining indicated the nuclei were intact and cells were viable after 2-hour treatment with the ribotoxins.

To test whether ribotoxin-induced endogenous IGFBP-3 localizes to the nucleus, MAC-T cells were treated ± ANS or DON and fractionated into nuclear and cytosolic components. Treatment with either ribotoxin induced nuclear localization in MAC-T cells, while nuclear localization was not observed under untreated SF conditions (Fig. 1B), confirming the results obtained with transfected IGFBP-3 and fluorescent microscopy.

Nuclear import of IGFBP-3 is dependent on importin- β

ANS and DON both induced nuclear localization of transfected IGFBP-3-GFP (Fig. 1A) and endogenous IGFBP-3 (Fig. 1B) suggesting that nuclear import of IGFBP-3 is a

regulated event. Leibowitz *et al.* determined that IGFBP-3 is directed to the nucleus in ANS-treated cells independent of caspase activation, but nuclear movement did not occur in IGF-I treated MAC-T cells (Leibowitz et al. 2013). Co-immunoprecipitation data from the Cohick lab has shown ANS promotes association between transfected IGFBP-3-his and importin-β. Additional fluorescent microscopy data showed ANS-induced nuclear localization of IGFBP-3 is dependent on importin-β. (Agostini-Dreyer 2014b).

To investigate if nuclear import of DON-induced IGFBP-3 is also facilitated by importin-β we treated cells ± DON to promote nuclear movement. We then treated with or without importazole, a small molecule inhibitor of importin-β. As shown in Fig. 2, under SF conditions cells exhibited punctate staining while ANS and DON resulted in nuclear accumulation of IGFBP-3-GFP. In the presence of importazole this transport was attenuated by importazole, indicating IGFBP-3 does utilize importin-β for nuclear import.

Nuclear and secreted IGFBP-3 are different molecular weights

IGFBP-3 often is detected as a doublet which has been shown to be due to different glycosylation forms (Firth and Baxter 1999). Interestingly, we made the observation that when samples of conditioned media containing secreted IGFBP-3 and cell lysates containing intracellular IGFBP-3 were run side by side on a gel, the forms of IGFBP-3 clearly migrated differently (Fig. 3). To investigate this further, MAC-T cells were treated with the ribotoxin ANS or DON for 6 h to induce IGFBP-3. Whole cell lysates were either collected in CLB or fractionated to obtain cytoplasmic and nuclear extracts. Conditioned media was also collected after 18 h of toxin treatment from duplicate dishes. As shown, IGFBP-3 collected from conditioned media of cells treated with ANS or DON migrated as a wide band of

approximately 38 to 45 kDa, while cellular IGFBP-3 ran at a doublet of 31-38 kDa. There was no difference in molecular weight of IGFBP-3 localized within the cell (whole cell lysate, cytoplasmic or nuclear).

Intracellular and secreted IGFBP-3 are differentially glycosylated

Since IGFBP-3 is glycosylated, we presumed that the differences in molecular weight described above were due to differences in glycosylation status. Glycosylation is a hallmark of the secretory pathway and may be characterized by both size and type of sugar moiety. To determine the types of N-glycans present on IGFBP-3, cells were treated with toxins to induce endogenous IGFBP-3 then conditioned media (Fig. 4A) and whole cell lysates (Fig. 4B) were subjected to enzymatic digestion. PNGase F deglycosylates all glycosylation types including complex, while Endo H can only reduce mannose or hybrid glycans. Digestion of secreted IGFBP-3 with Endo H did not result in a reduced band, but treatment with PNGase F decreased IGFBP-3 to a lesser glycosylated form of approximately 31 kDa. Conversely, intracellular IGFBP-3 was reduced with both deglycosylation enzymes to 29 kDa and 32 kDa respectively. The ability of Endo H to deglycosylate intracellular IGFBP-3 (nuclear and cytoplasmic) but not IGFBP-3 from conditioned media suggests intracellular and secreted IGFBP-3 are differentially glycosylated. Specifically, intracellular IGFBP-3 contains mannose or hybrid glycans, while secreted IGFBP-3 carries complex glycosylation.

Non-secreted IGFBP-3 localizes to the nucleus

Previous work in the Cohick lab has determined that preventing re-internalization via treatment with Pitstop2, an inhibitor of clathrin-mediated endocytosis (CME), did not

reduce nuclear accumulation of ANS-induced IGFBP-3. This suggested nuclear IGFBP-3 is not derived from secreted protein (Agostini-Dreyer 2014b).

BFA is an inhibitor of anterograde protein transport from the ER to the Golgi complex and thus is used as an inhibitor of protein secretion. Therefore it was used to examine whether IGFBP-3 needs to be secreted prior to nuclear localization. MAC-T cells were treated with a ribotoxin ± Brefeldin A (BFA), fractionated and immunoblotted. As shown in Fig 6, analysis of conditioned media from BFA-treated cells confirmed that BFA successfully inhibited IGFBP-3 secretion. As expected, the ribotoxins ANS and DON both stimulated nuclear accumulation of endogenous IGFBP-3. Hindering IGFBP-3 secretion with BFA did not inhibit ribotoxin-induced nuclear trafficking. These findings suggest non-secreted IGFBP-3 is able to traffic to the nucleus.

To corroborate these findings cells were transfected with IGFBP-3-GFP and treated with a ribotoxin ± BFA. Fluorescent microscopy images (Fig. 7A and 7B) show successful transfection of IGFBP-3-GFP with no nuclear accumulation in SF conditions. ANS and DON treatments induced nuclear localization of IGFBP-3-GFP. Treatment with secretion blocker BFA did not attenuate IGFBP-3 nuclear trafficking suggesting non-secreted IGFBP-3 localizes to the nucleus.

Discussion

ANS and DON are ribotoxins with similar modes of action (Schmeits, et al. 2014). Both ribotoxins are low molecular weight molecules capable of passive diffusion through cell membranes, interaction with the ribosome at the peptidyl transfer center of the 28S subunit of rRNA, and activation of SAPK signaling pathways that are evolutionarily conserved in all eukaryotic cells (Gray, et al. 2008; He et al. 2012; Weber, et al. 1999). Knockdown experiments using siRNA to inhibit IGFBP-3 expression determined IGFBP-3 mediates both ANS and DON induced apoptosis in MAC-T cells (Hanke 2017).

IGFBP-3 is both secreted and contains a NLS, allowing for multi-compartmental localization across the cell. Nuclear localization of IGFBP-3 is associated with its role in apoptosis (Lee et al. 2004; Leibowitz et al. 2013; Xi et al. 2007). The Cohick lab previously established that ANS induces nuclear localization of IGFBP-3 in MAC-T cells (Leibowitz et al. 2013). MAC-T cells were treated with 0.1 μM ANS for 3 h or 8 h. While nuclear accumulation of IGFBP-3 was detected at both 3 h and 8 h treatment with ANS, treatment for 8 h resulted in greater caspase 3/7 activation indicating apoptosis. Additionally IGFBP-3 mRNA expression followed a concentration and time-dependent pattern, with the most significant increases following 6 h treatment with 0.1 μM ANS. Therefore this work used a similar treatment of 0.1 μM ANS for 6 h to induce IGFBP-3 expression. The work in this thesis shows that 6 h treatment with DON also resulted in IGFBP-3 nuclear localization in MAC-T cells. Previous work from the Cohick lab also demonstrated that nuclear IGFBP-3 mediates ANS-induced apoptosis through activation and nuclear export of Nur77 in MAC-T cells (Agostini-Dreyer et al. 2015). These data establish a nuclear function for IGFBP-3 in

intrinsic apoptosis. In light of these findings, we investigated whether ribotoxic stress induces a universal effect of IGFBP-3 nuclear localization.

IGFBP-3 has a five amino acid NLS motif in the basic carboxy-terminal domain, which allows nuclear uptake in cells (Radulescu 1994) The NLS sequence motif was shown to direct nuclear translocation of IGFBP-3 by association with importin-β, a nuclear transport protein. This was shown to be a specific and energy dependent reaction, requiring both ATP and GTP hydrolysis (Schedlich et al. 2000). Additionally IGFBP-3 has been reported to use the importin-β subunit for nuclear import in breast cancer cells (Schedlich et al. 2000). To determine if nuclear import of ribotoxin-induced IGFBP-3 uses similar mechanisms, IGFBP-3-GFP transfected MAC-T cells were treated with or without ribotoxin, and with or without importazole, then monitored for cellular distribution of IGFBP-3. GFP-tagged IGFBP-3 is 64 kDa, which surpasses the threshold of 45 kDa for passive diffusion through nuclear pores and therefore limits nuclear uptake to regulated translocation using nuclear pore complexes (Hoelz et al. 2011).

Fluorescent microscopy data showed that IGFBP-3 shuttled to the nucleus following ANS or DON treatment. Additional treatment with importazole inhibited ribotoxin-induced nuclear accumulation of IGFBP-3. These data supports that nuclear uptake of IGFBP-3 during ribotoxic stress is a regulated event mediated by NLS association with importin-β, rather than passive diffusion. However, the physiological switch that triggers nuclear movement over secretion and the mechanism by which IGFBP-3 traffics from the ER to the nucleus remains unclear.

An interesting observation in the present results was that IGFBP-3 collected from cultured media after ribotoxin treatment consistently migrated at a higher molecular weight band than intercellular IGFBP-3. Similar direct comparisons are not present in the literature and suggest several variations of glycosylation. Work using the deglycosylation enzymes Endo H and PNGase F has distinguished N-linked glycosylation types (Shen et al. 2017). Using these endoglycosidases, we found that secreted IGFBP-3 in the CM contains complex-type glycans, while nuclear and WCL IGFBP-3 contains only mannose or hybrid glycans. Unpublished work from the Cohick lab also showed exogenous nuclear IGFBP-3 is glycosylated. Digestion of the fractions with Endo H resulted in a single band of approximately 29 kDa, indicating that nuclear IGFBP-3 is glycosylated with non-complex glycans (high mannose or hybrid). While both enzymes de-glycosylated intracellular IGFBP-3, the resulting bands varied in size due to the enzyme cleavage site. This is because digestion with PNGase F deaminates the asparagine residue to aspartic acid, leaving the oligosaccharide intact. However, Endo H cleaves between the N-acetylglucosamine residues of the core oligosaccharide, leaving one N-acetylglucosamine residue attached to the asparagine of N-linked glycans.

The differential glycosylation exhibited by nuclear and secreted IGFBP-3 may imply alternate routes of IGFBP-3 trafficking. Proteins edited with glycosyltransferases in the ER are Endo H-sensitive, meaning they can be reduced to a non-glycosylated form with the enzyme Endo H because they carry a high-mannose glycan or a hybrid glycan. In contrast, proteins edited in the cisternae of the Golgi are unable to be cleaved with Endo H due to their complex type glycans. This is because in the Golgi lumen, mannosidase II acts to cleave two mannose sugars, rendering the glycoprotein resistant to attack by the highly specific

Endo H. All later structures in the pathway are also resistant to Endo H enzymatic activity, therefore treatment with this enzyme is used to distinguish complex from high-mannose oligosaccharides. This explains how secreted IGFBP-3, which must first pass through the Golgi, contains a complex-type glycan that can only be de-glycosylated using PNGase F.

Having established that IGFBP-3 glycoforms differ depending on localization, specifically that nuclear and secreted IGFBP-3 contains non-complex and complex sugar moieties respectively, we set out to determine if non-secreted IGFBP-3 can localize to the nucleus. MAC-T cells treated with ribotoxin and Brefeldin A, which inhibits protein transport from the ER to the Golgi apparatus thereby inhibiting secretion, had similar amounts of nuclear IGFBP-3 as cells treated with ribotoxin alone. These findings indicate that COPI mediated translocation from the ER to the Golgi is not a required step for direction of IGFBP-3 to the nucleus. This indicates secretion is not necessary for nuclear localization of IGFBP-3. These findings are novel in implying IGFBP-3 exits the secretory pathway prior to reaching the Golgi, as anterograde transport from the ER was inhibited by BFA.

The question then emerged of how IGFBP-3 escapes the secretory pathway to traffic to the nucleus. It is possible that endogenous IGFBP-3 produced in response to ribotoxins gets sent to the ER where its secretion signal gets modified or turned off via glycosylation while the NLS is activated, resulting in nuclear accumulation. Future experiments will use CRISPR-cas9 genome editing technology to mutate the N-linked glycosylation sites of IGFBP-3 from asparagine to alanine. This will determine how inhibition of IGFBP-3 N-linked glycosylation affects the ability of ribotoxins to induce IGFBP-3 nuclear localization in MAC-T cells. However, this does not address why some IGFBP-3 is sent to the nucleus

while the rest gets secreted into the cultured media. Our goal for further elucidating the mechanism of IGFBP-3 nuclear localization is to determine how ribotoxin induced IGFBP-3 escapes from the ER.

Ricin is a protein known to escape the ER by hijacking the endoplasmic reticulum associated degradation (ERAD) machinery (Spooner and Lord 2015). ERAD is a highly conserved process, by which terminally misfolded glycoproteins are targeted and chaperoned by ER degradation-enhancing alpha mannosidase-like proteins (EDEM) across the ER membrane and into the cytosol for proteasomal degradation (Hosokawa, et al. 2001). This tightly regulated pathway is initiated by the oligomerization and autophosphorylation of the ER stress-sensor IRE1, which splices an intron from the X-box binding protein 1 (XBP1) mRNA (Yoshida, et al. 2001). The spliced XBP1 mRNA is translated into an efficient transcription factor that triggers the expression of EDEMs. ERAD also requires the removal of certain mannose residues from the misfolded N-linked glycoprotein. Mannosidase enzymes in the ER such as mannosidase IA (ManIA) and ER α 1,2 mannosidase I (ERManI) trim and target misfolded glycoproteins for ERAD (Ogen-Shtern, et al. 2016).

Studies are presently underway using Kifunensine, a potent inhibitor of endoplasmic reticulum mannosidase 1 (ERM1). This blocks processing of glycoproteins in the ER by obstructing EDEM association with targeted proteins and subsequent protein shuttling through Sec61 to the cytoplasm. Treatment with Eeyarestatin 1 (ES1), which interferes with the p97-associated deubiquitination process (PAD) at Sec61, could also determine whether IGFBP-3 utilizes ERAD machinery to exit the ER (Wang, et al. 2008). An identified

pathway of ER escape could explain how nuclear and cytoplasmic IGFBP-3 is glycosylated similarly, and how nuclear IGFBP-3 is glycosylated without first being secreted.

An understanding of the mechanism for nuclear import of IGFBP-3 is important in establishing how IGFBP-3 exerts its apoptotic effects (Bach 2017). The glycosylation of nuclear IGFBP-3 prompted the theory that IGFBP-3 is trafficked through the secretory pathway, and expelled from the cell prior to re-internalization and nuclear localization. However, in light of the results presented here, this pathway seems unlikely. We determined that nuclear and secreted IGFBP-3 carry different types of N-linked glycans, with intracellular IGFBP-3 containing simple (high-mannose or hybrid types), and secreted IGFBP-3 containing complex glycosylation. Thus it would require energy expenditure for removal of the complex glycan prior to nuclear entry. We also established that non-secreted IGFBP-3 is capable of nuclear localization in MAC-T cells, and that IGFBP-3 likely escapes the secretory pathway from the ER. Work using BFA showed that inhibition of vesicular transport between the ER and the Golgi did not hinder nuclear localization, and suggested IGFBP-3 escapes the secretory pathway prior to reaching the Golgi. Future work will focus on the mechanism of how ribotoxin-induced IGFBP-3 escapes the ER.

Figures

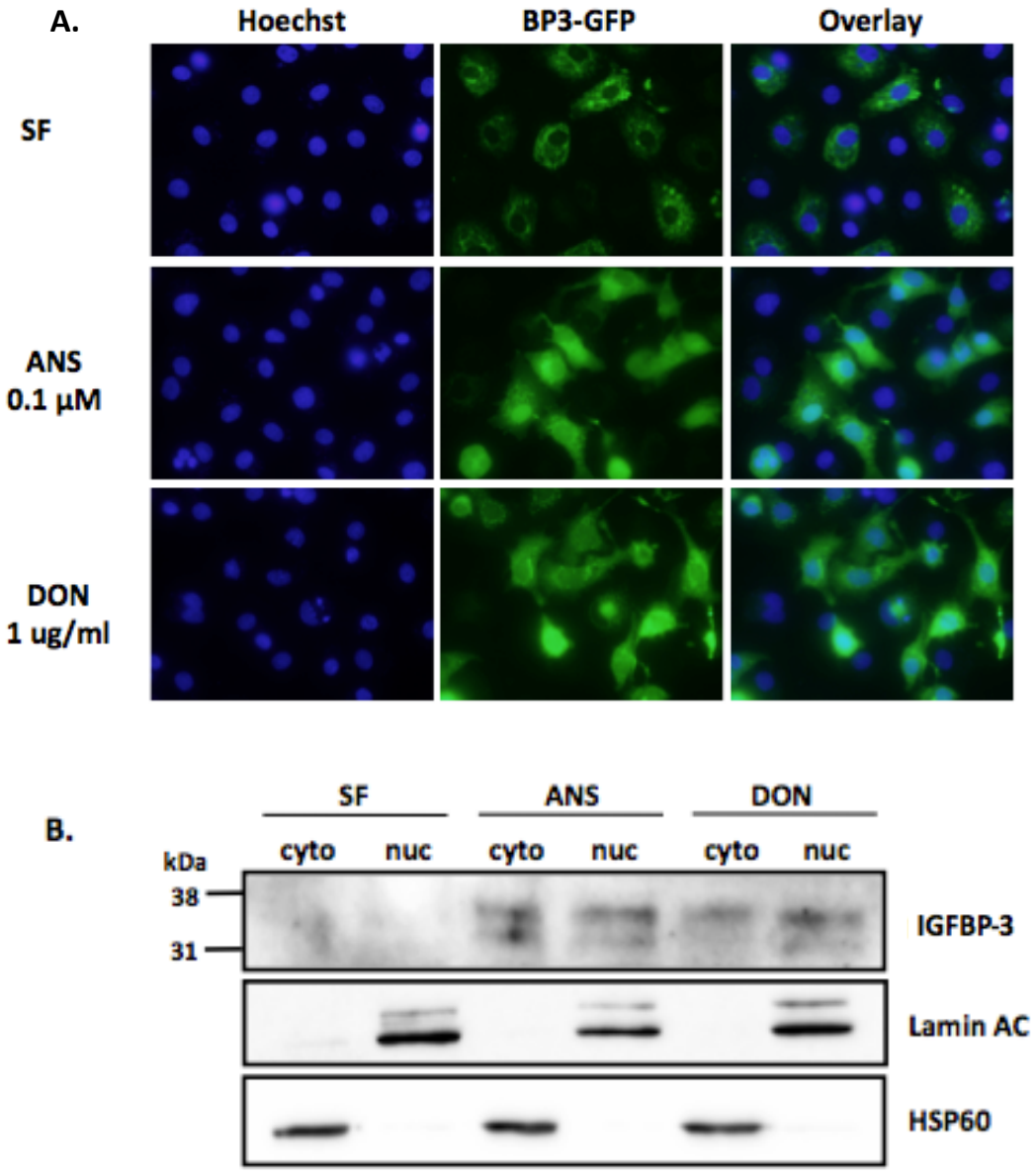

Fig 1. ANS and DON induce nuclear localization of IGFBP-3. (A) MAC-T cells transfected with IGFBP-3-GFP were treated 2 h ± 0.1 µM ANS or 1 µg/ml DON. Cells were fixed in formalin then nuclei were stained with Hoechst. Images were acquired with an Olympus FSX100 microscope. Images are representative of two independent experiments. (B) MAC-T cells were treated 6 h ± 0.1 µM ANS or 1 µg/ml DON, then fractionated into nuclear and cytoplasmic components and Western immunoblotted for IGFBP-3. Lamin AC and HSP60 served as controls for nuclear and cytoplasmic loading, respectively. Results are representative of three independent experiments.

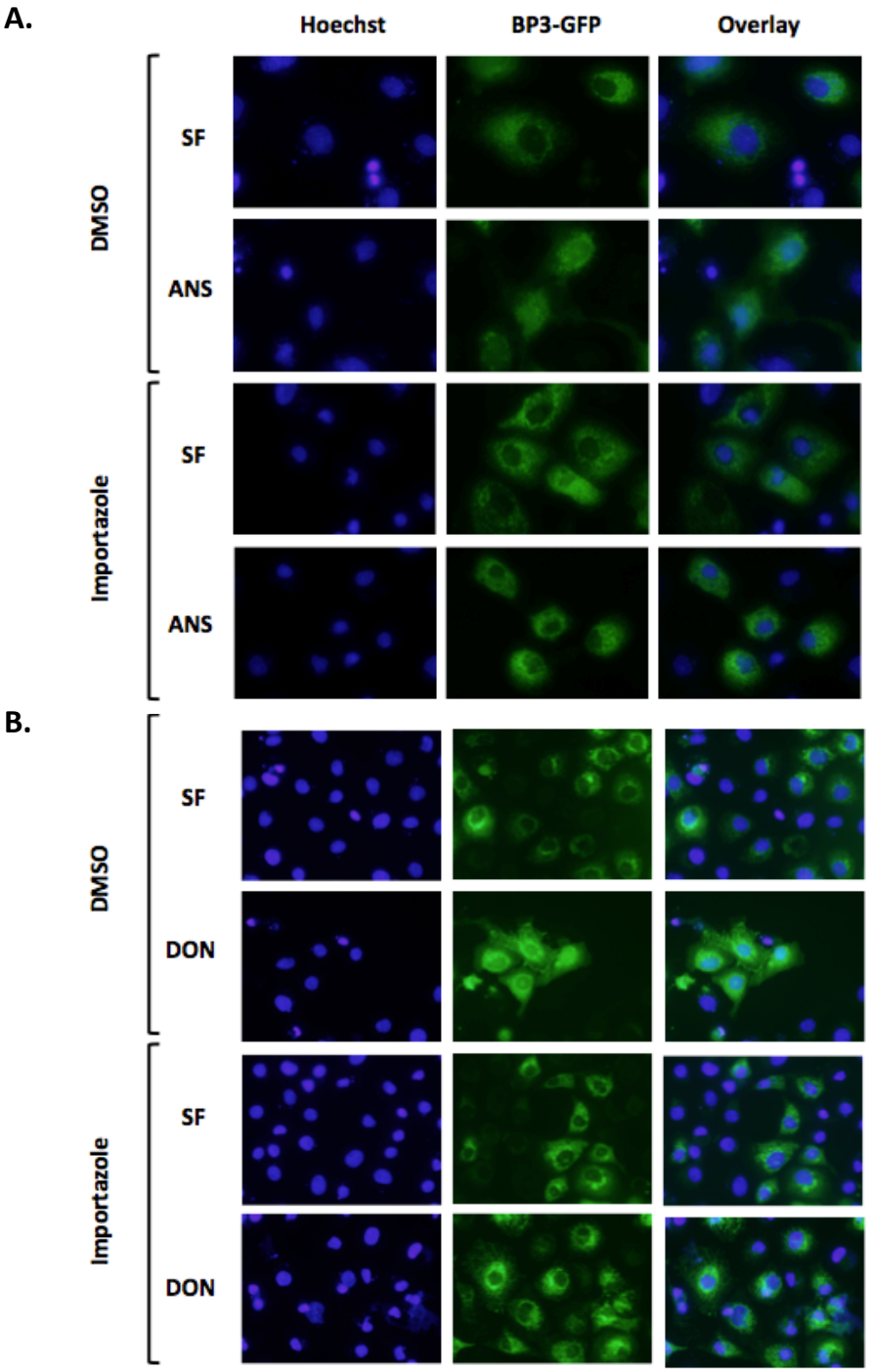

Fig 2. Importazole reduces nuclear import of IGFBP-3-GFP. Cells transfected with IGFBP-3-GFP were treated 4 h ± 0.1 μM ANS (A) or 1 μg/ml DON (B) ± 40 μM importazole. Cells were fixed in formalin then nuclei were stained with Hoechst. Images were acquired with an Olympus FSX100 microscope. Images are representative of two independent experiments. **(**Fig. 2A from Agostini-Dreyer, unpublished data, 2014)

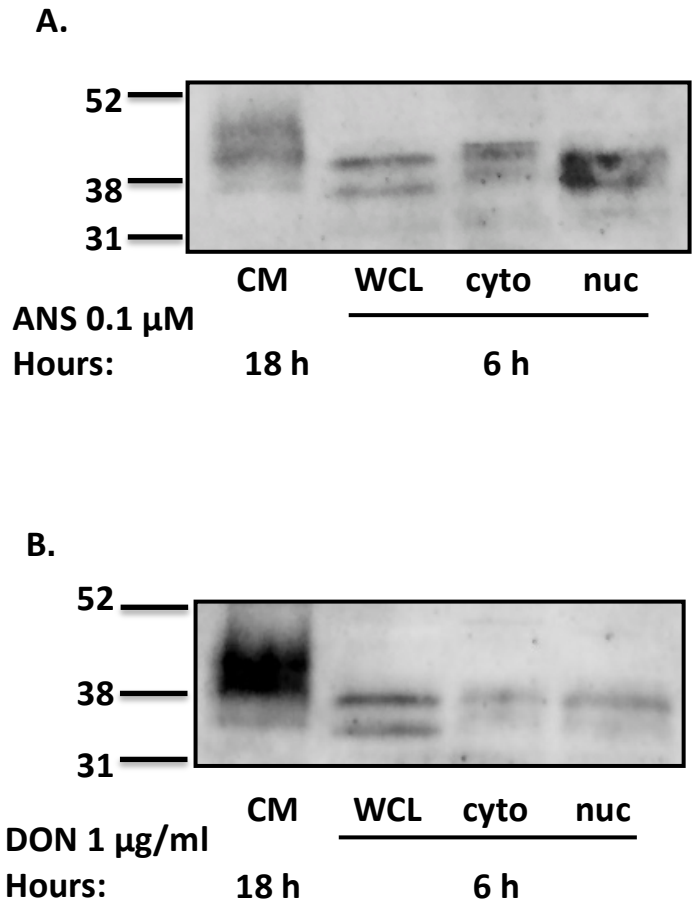

Fig 3. Secreted IGFBP-3 is differentially glycosylated compared to intracellular IGFBP-3. MAC-T cells were treated with (A) 0.1 µM ANS or (B) 1 µg/ml DON for 6 h or 18 h. After 6 h cells were fractionated or whole cell lysates (WCL) were collected then Western immunoblotted for IGFBP-3. Conditioned media (CM) were collected following 18 h incubation with treatments and immunoblotted for IGFBP-3. Gels are representative of at least 3 experiments.

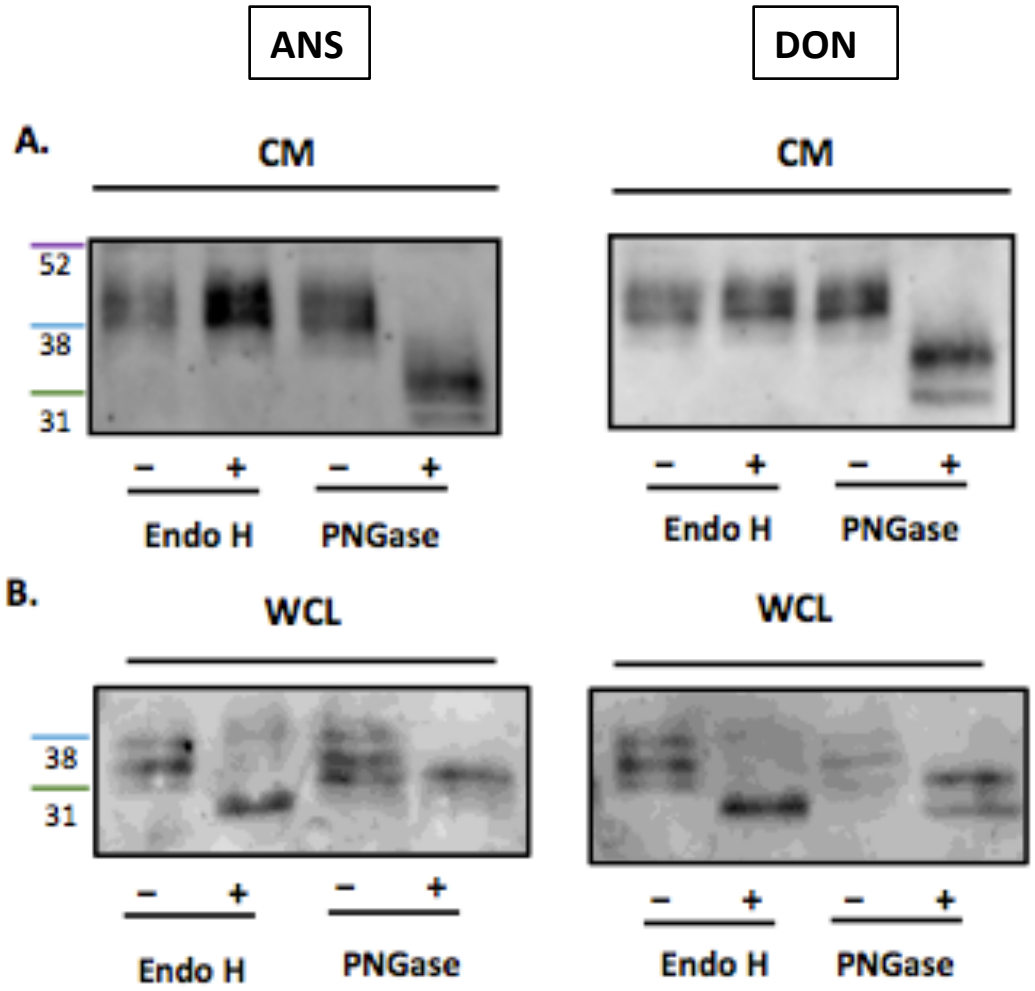

Fig 4. IGFBP-3 in the conditioned media is not sensitive to Endo H digestion. MAC-T cells were treated (A) 18 h + 0.1μM ANS or 1 μg/ml DON and conditioned media (CM) was collected or (B) 6 h + 0.1μM ANS or 1 μg/ml DON and whole cell lysates (WCL) were collected. CM and WCL were treated ± Endoglycosidase H or PNGase F to deglycosylate proteins, separated by SDS-PAGE and immunoblotted for IGFBP-3.

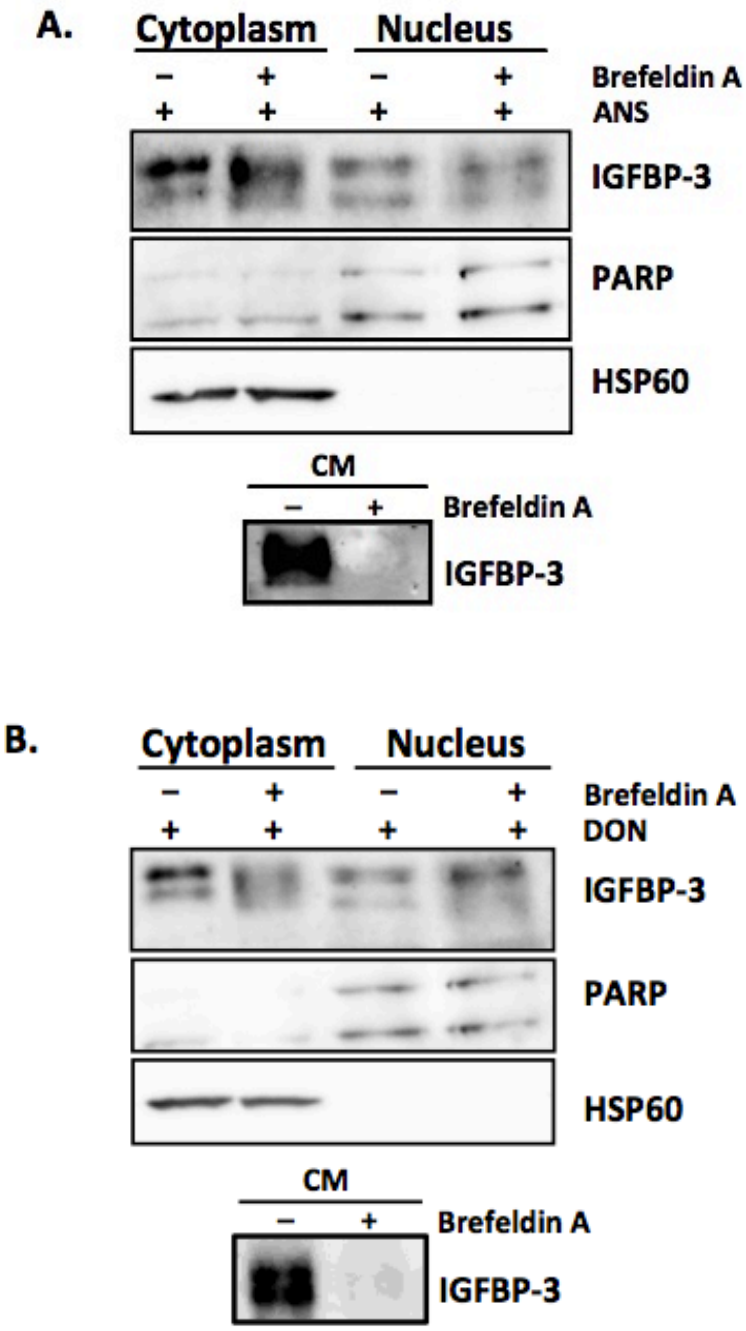

Fig 5. Non-secreted endogenous IGFBP-3 localizes to the nucleus. Cells were treated with either (A) ANS or (B) DON ± Brefeldin A. Conditioned media were collected and cells were fractionated. Samples were Western immunoblotted for IGFBP-3. Lamin AC and HSP60 served as nuclear and cytosolic loading controls, respectively. Results are representative of three independent experiments.

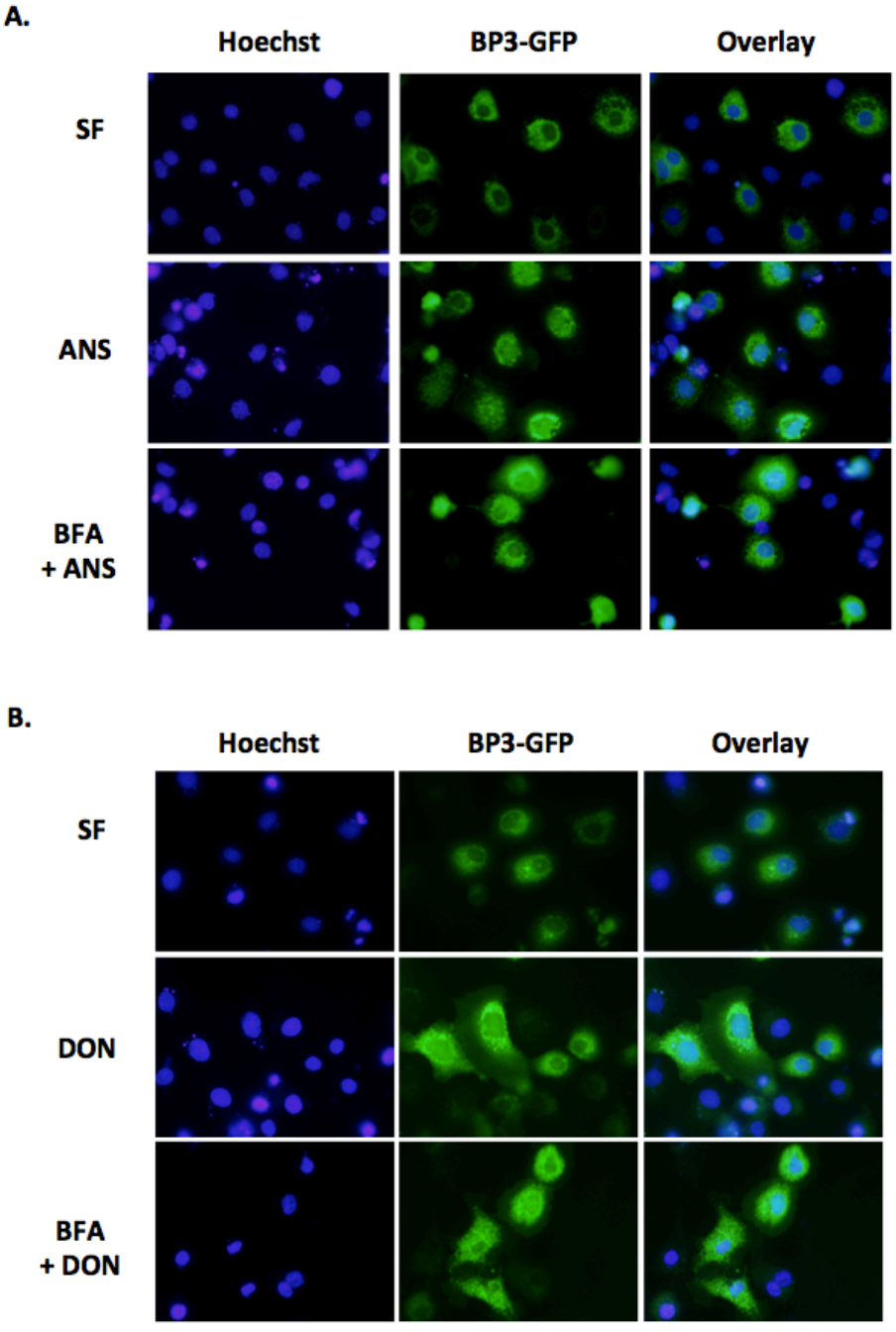

Fig 6. Non-secreted exogenous IGFBP-3 localizes to the nucleus. (A) Cells were co-treated for 1.5 h with 0.1 μM ANS and 10 μg/ml Brefeldin A. (B) Cells were co-treated for 2 h with 1 μg/ml DON and 10 μg/ml Brefeldin A. Images are representative of two independent experiments.

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