NUCLEOCYTOPLASMIC TRAFFICKING OF HUMAN GCN5 AND ITS ROLE

AS AN ACTIN-MODIFIER

NUCLEOCYTOPLASMIC TRAFFICKING OF THE HUMAN GCN5

ACETYLTRANSFERASE AND A NOVEL

ROLE FOR GCN5 IN THE NUCLEUS AS AN ACTIN-MODIFIER

By

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A Thesis Submitted to the School of Graduate Studies In Partial Fulfillment of the Requirements For the Degree Master of Science

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ABSTRACT

The first histone acetyltransferase to be described was GCN5, from the yeast species *Saccharomyces cerevisiae*. To date, the GCN5-related *N*-acetyltransferases (GNATs) comprise one of the largest enzyme superfamilies with over 10,000 identified members in sequenced genomes. This protein is known to acetylate specific lysine residues on the amino-terminal tails of nucleosomal histones, thereby loosening their contact with the tightly packed DNA and facilitating transcription.

In this study, I determined that GCN5 is able to shuttle between the nucleus and the cytoplasm using fluorescence recovery after photobleaching (FRAP). Mutational studies revealed that its nuclear import is regulated by a classical bipartite nuclear localization signal (NLS) that is dependent on the transporters importin α and β . In contrast, we found that GCN5 lacks a CRM1-dependent nuclear export signal (NES), as demonstrated by mutational and leptomycin B (LMB) studies; instead, IkB, a previouslydescribed transcription inhibitor with a CRM1-dependent NES, was found to modulate the export of GCN5 from the nucleus. This was initially discovered while performing the LMB assays, for which IkB served as a positive control, and was subsequently confirmed by mutational studies and protein complementation assays (PCAs). Furthermore, while the PCAs demonstrated a physical interaction between these two proteins *in vivo*, GST pull-down experiments were employed to confirm their interaction *in vitro*.

Furthermore, this study also revealed that over-expression of GCN5-eYFP in NIH 3T3 cells causes ~10% of the transfected cells to exhibit nuclear GCN5-eYFP-associated filaments; these structures were confirmed to be F-actin filaments comprised of β -actin through co-localization studies with both TRITC-phalloidin and a mRFP-\beta-actin construct. GCN5's acetyltransferase activity was shown to be responsible for the formation of these filaments through mutation of its catalytic residue. Moreover, a protein complementation assay (PCA) demonstrated an in vivo interaction between GCN5 and βactin, while FRAP analysis of a single filament showed that GCN5-eYFP molecules rapidly and randomly associate with these filaments along their entire length. Together these results suggest that GCN5's acetyltransferase activity is responsible for the structural maintenance of these filaments. Finally, GCN5-eYFP-associated filaments were found to be spatially separate from both lamin A (a nuclear envelope structural protein) and DNA; however, this does not exclude the possibility of an indirect interaction between these cellular constituents, as treatment of a live cell with Hoechst DNA stain, which disrupts the structure of DNA, was shown to disturb the structural integrity of these filaments.

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Finally, I would like to thank my friends and family, as both have been extremely emotionally and mentally supportive, despite a persistent ignorance for what I do in the lab!

LIST OF ABBREVIATIONS

αΜΕΜ	alpha Minimal Essential Medium
μg	microgram
μm	micrometre
ADCA	autosomal dominant cerebellar ataxia
ADP	adenosine diphosphate
ARD	ankyrin repeat domain
Asp	aspartic acid
ATP	adenosine triphosphate
BiFC	bimolecular fluorescence complementation
bp	base pairs
CAG-repeat	triplet code for glutamine residue (cytosine, adenosine, guanosine)
CFP	cyan fluorescent protein
CNS	central nervous system
CRM1	Chromosome region maintenance-1 protein
DIC	differential interference contrast
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRPLA	Dentatorubral-pallidoluysian atrophy
DsRed	Discosoma sp. red fluorescent protein
DTT	dithiothreitol
Е	glutamic acid
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced GFP
eYFP	enhanced YFP
F	forward oligo
F-actin	filamentous actin
FG motif	phenylalanine-glycine motif
FRAP	fluorescence recovery after photobleaching
G-actin	globular actin
GCN5	general control of amino-acid synthesis-5
GCN5L	long isoform of GCN5
GCN5S	short isoform of GCN5
GDP	guanosine diphosphate
GFP	green fluorescent protein
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GNAT	GCN5-related N-acetyltransferase

GPCR	G-protein coupled receptor
GST	glutathione-S-transferase
GTP	guanosine triphosphate
НАТ	histone acetyltransferase
HC1	hydrochloric acid
HDAC	histone deacetyltransferase
HEK 293 cells	human embryonic kidney 293 cell-line
hGCN5	human GCN5
hnRNP	heterogeneous nuclear ribonucleoprotein
Hz	Hertz
IR	importin B-hinding domain
	IrB kinase
IDTC	isopropyl R D 1 thiogalastopyraposide
	Isopropyi-p-D-1-titlogalactopyianoside
-dANK2/	IKB lacking its second ankyrin repeat
IκB	inhibitor of KB
KCl	potassium chloride
kDa	kilodalton
KH ₂ PO ₄	potassium phosphate monobasic
Laser	light amplification by stimulated emission of radiation
LB media	Luria-Bertani media
LMB	leptomycin B
LSM	laser scanning microscope
Μ	molarity (moles/litre)
MBE	mouse brain extract
mCer/mCerulean	monomeric cerulean fluorescent protein
MCF7 cells	human breast adenocarcinoma cell-line
Met	methionine
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mRFP	monomeric red fluorescent protein
mRNA	messenger RNA
MW	molecular weight
NA	numerical aperture
Na ₂ HPO ₄	sodium phosphate dibasic
NaCl	sodium chloride
NES	nuclear export signal
NESm	NES-mutant
NF-ĸB	nuclear factor-kB
ng	nanogram
NIH 3T3 cells	mouse embryonic fibroblast cell-line
	we chief only hore of which will have

NLS	nuclear localization signal
NLSm	NLS-mutant
nm	nanometre
NMI	nuclear myosin I
NPC	nuclear pore complex
PBS	phosphate-buffered saline
PCA	protein complementation assay
PEG	polyethylene glycol
Pre-mRNP	pre-messenger ribonucleoprotein
PSF	point spread function
PtdIns(4,5)P ₂	phosphatidylinositol-(4,5)-bisphosphate
PVDF	polyvinyl difluoride
Q	glutamine
R	reverse oligo
RGB	red green blue
RHD	Rel homology domain
RNA	ribonucleic acid
ROI	region of interest
rRNA	ribosomal RNA
S	serine
SAGA	yeast Spt-Ada-GCN5-acetyltransferase complex
SBMA	Spinal and bulbar muscular atrophy
SCA	spinocerebellar ataxia
SCA7	Spinocerebellar ataxia type 7
SCA7	SCA7 gene
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	serine
snRNP	small nuclear ribonucleoproteins
STAGA	SPT3-TAF _{II} 31-GCN5-L acetyltransferase complex
Т	threonine
TAF	TBP-associated factor
TAF _{II} 31	TBP-associated factor II 31
tGCN5	Tetrahymena thermophila homologue of GCN5
Thr	threonine
TIFF	tagged image file format
TNFa	tumour necrosis factor alpha
TRITC	tetramethyl rhodamine iso-thiocyanate
Tyr	tyrosine
UAS	upstream activation sequence
UV	ultraviolet
Y	tyrosine
yGCN5	yeast GCN5

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1. INTRODUCTION

The compartmentalization of genetic material within the nucleus of eukaryotic cells provides a powerful mechanism for the regulation of gene transcription and other nuclear processes. Specifically, it is within the nuclear compartment tht DNA replication and repair and RNA transcription occurs; in contrast, protein synthesis takes place in the cytoplasm of eukaryotic cells. Therefore, the cell requires mechanisms to regulate the transport of specific proteins, RNA, and ribonucleoproteins across the nuclear envelope in order to couple these two processes [1,2]. This thesis initially involved the study of the ataxin-7 protein, which in its disease state, is responsible for the polyglutamine disease Spinocerebellar ataxia type 7; our goal in this study was to determine potential ataxin-7binding partners using affinity chromatography and mass spectrometric analysis. However, we decided to change the focus of this thesis to involve the characterization of human GCN5 (hGCN5), which is a protein that has previously been shown to biochemically interact with ataxin-7. Human GCN5 is a well-described histone acetyltransferase, however its activity has never been examined at the cellular level. For this reason, we have focused on characterizing its ability to shuttle between the nucleus and the cytoplasm in vivo, and determining the mechanisms by which these events occur. Furthermore, we have found a novel role for hGCN5 as an actin-modifier, specifically increasing the polymerization of actin within the nucleus. We propose that this function of GCN5 may have a role either in the recruitment of actively-transcribed DNA to the nuclear periphery or in facilitating the rapid export of mRNA from the nucleus to the cytoplasmic compartment, where protein synthesis is known to occur.

1.1. GCN5 is a Histone Acetyltransferase

Nucleosomes are the structures that fold chromosomal DNA into chromatin. They are specifically made up of two molecules each of four core histones, namely H2A, H2B, H3 and H4. DNA is wrapped almost twice around this core octameric structure, while the nucleosomal cores are linked by short stretches of DNA, bound in part by "linker" histones H1 and H5 [3]. The nucleosomal structure is then stabilized by protein-protein interactions occurring between the histones of each octamer, and by electrostatic interactions between negatively-charged phosphates of the DNA backbone and the positively-charged lysine and arginine residues located on the histone tails [4]. These amino-terminal tails, which extend from each of the core histones, are subject to a variety of post-translational modifications, including acetylation, phosphorylation, and methylation; each of these modifications acts to uniquely modify the charge and function of the respective histone. Of these modifications, histone acetylation and deacetylation are the best characterized [5]; histone acetyltransferases (HATs) function in the addition of acetyl groups to specific lysine residues within the amino-terminal histone tails, while histone deacetyltransferases (HDACs) facilitate acetyl removal.

In eukaryotic cells, nucleosomal filaments that are 10 nm in diameter fold upon one another to form higher-order chromatin structures. A significant number of studies that

have shown that these nucleosomal and supranucleosomal chromatin arrangements are efficient at precluding the binding of a variety of proteins that are essential for transcription regulation [6]. Initially acknowledged through genetic studies involving the yeast species, *Saccharomyces cerevisiae*, analyses of the distributions of nucleosomes at active genes *in vivo* suggest that their positioning within promoter regions, in particular, represents a key mechanism of transcriptional regulation [7-10]. Acetylation of histone tails is thought to neutralize the positive charge of the lysine residues, thereby decreasing their affinity for the negatively-charged phosphate groups within DNA; this enables unfolding of the nucleosome such that transcription factors are able to access specific genes and activate their transcription [11].

In 1992, the first HAT enzyme was discovered in yeast and was named GCN5 (yGCN5; general control of amino-acid synthesis-5). This protein was found to have sequence homology to a family of bacterial enzymes that acetylate a set of related aminoglycosidic antibiotics [12], while shortly thereafter, a Tetrahymena thermophila homologue of GCN5 (tGCN5), was shown to catalyze histone acetylation [13]. To date, the GCN5-related N-acetyltransferases (GNATs) comprise one of the largest enzyme superfamilies with over 10,000 identified members in sequenced genomes [13]. Members of the GNAT superfamily have been found in all kingdoms of life, including archea, bacteria, insects, plants and mammals. Despite relatively little overall homology between some of the members, most consist of a structurally conserved core monomer fold that acts as the acetyl-CoA binding domain [14]. Although more than two dozen structures have been solved for various GNAT family members, these unfortunately have provided little indication of the type(s) of substrate(s) that each acetylates; substrate preferences have been shown to include antibiotics, metabolic intermediates, and proteins, where acetylation can occur either internally on the ε -amino group of lysine residues or at the α amino-terminus [14].

The catalytic mechanism of histone acetylation by yGCN5 has been known for some time: the reaction occurs through the formation of a ternary complex between the histone, an acetyl-CoA molecule and the HAT enzyme. The ε -amino group of the histone's lysine residue becomes deprotonated, thereby allowing it to attack the carbonyl carbon on the reactive thioester group of acetyl-CoA [15,16]. In a mutational study of yGCN5, an invariant glutamic acid residue (Glu173) within the catalytic domain was shown to be essential in that it serves as a general base catalyst that can deprotonate the histone substrate [17]; importantly, a recent study confirmed that the corresponding glutamic acid in the HAT region of the murine GCN5 protein (Glu568) is also responsible for its catalytic activity [15].

GCN5 is a member of a number of multi-subunit transcriptional complexes found in yeast, fly, and mammalian cells [18-20]. These include the yeast co-activator SAGA (Spt-Ada-GCN5-acetyltransferase) and the homologous mammalian STAGA (SPT3-TAF_{II}31-GCN5-L acetyltransferase) complexes, the yeast complex of which has been found to be required for normal transcription of ~10% of genes [21], including various stress-induced genes [22]. These complexes are recruited by activators to upstream activation sequences (UASs) of the respective promoters [23] whereby specific aminoterminal lysines on the histone H3 tail, including lysines 9 and 14 [24], are acetylated by the HAT component GCN5. Histone acetylation by this enzyme has been shown to cause displacement of promoter nucleosomes during transcription activation [25], while the complexes, in their entirety, have been implicated in aiding the recruitment of the TATAbinding protein (TBP), RNA polymerase II [26,27], and other coactivators [28,29] to the promoter regions to aid in transcription initiation [22]. Importantly, a number of studies have shown that GCN5's acetyltransferase activity is involved in the transcriptional regulation of a number of genes involved in cell-cycle progression [30-32], suggesting that there may be an important cellular mechanism in place for regulating this protein's transcriptional activities. This study has focused specifically on the processes of nucleocytoplasmic transport, with respect to protein compartmentalization, as a mechanism for regulating human GCN5's activity in the nucleus.

1.2. The Nuclear Pore Complex and Nucleocytoplasmic Transport

In eukaryotic cells, cellular functions are coordinated and maintained in an orderly manner through the continuous transport of a variety of molecules, including proteins, RNA, and ribonucleoproteins, between the nucleus and the cytoplasm. These two compartments are segregated by a double membrane called the nuclear envelope [33,34], through which structures known as nuclear pore complexes (NPCs) penetrate; it is through these complexes that cargo can enter and exit the nucleus. In a proliferating human cell, there can be between 3000-5000 NPCs [34,35]. These structures have been estimated to be ~125 MDa in vertebrates and ~60 MDa in yeast [36]. Each NPC consists of an eightfold rotational symmetry: extending outward from, and encircling the central core on each of the nuclear and cytoplasmic faces are eight spokes that make up the nuclear basket and cytoplasmic filaments, respectively [37]. The central pore itself is about 90 nm in length, while its diameter is approximated to be 30-40 nm at its narrowest point. Comprising the eukaryotic NPC is estimated to be ~30 proteins, termed nucleoporins, which are each present in eight copies to reflect the eightfold symmetry of the complex [38]. The nucleoporins that make up the inner channel of the pore contain phenylalanine-glycine (FG) repeats that may form a meshwork to restrict transport of large proteins, yet allow diffusion of small molecules and ions [39,40]; for active transport to occur, the NPC is able to open to approximately 25 nm, while passive diffusion of molecules smaller than 20-40 kDa is restricted by a 9 nm opening [41].

Macromolecules that rely on active transport into the nucleus generally require a nuclear localization signal (NLS) that can be recognized by an import receptor (karyopherin/importin) [42,43]. Certain NLS-containing cargoes interact with the import receptor (e.g. importin β), either directly, or via an adapter protein, such as importin α [44,45]. Importin α harbours a small amino-terminal importin β -binding (IBB) domain, through which it binds importin β , and a larger carboxy-terminal NLS-binding domain, which promotes its interaction with the NLS-bearing cargo [46]. In contrast, the importin β receptor physically mediates the translocation of the receptor/adaptor/cargo complex by interacting with nucleoporins within the NPC that contain FG-peptide repeats [47,48].

We proposed that hGCN5 may contain a type of classical NLS within its amino acid sequence that allows it to be imported into the nucleus via this transport mechanism.

Classical NLSs that are recognized by importin β /importin α receptors, which include some of the first NLSs to be defined, fall into two categories: 1) a monopartite signal, which has the consensus sequence of K-K/R-X-K/R, where X represents any amino acid and K and R represent a lysine or arginine, respectively; and 2) a bipartite signal [49]. The sequence of interest in hGCN5 is similar to the consensus sequence for the bipartite NLS, which involves two independent groups of basic residues that are separated by a non-conserved 10-12 amino acid linker region: this type of sequence was first described in the molecular chaperone, nucleoplasmin [50], which harbours the following NLS: ¹⁵⁵KRPAATKKAGQAKKKK¹⁷⁰ [51].

In contrast to nuclear import, which requires importins, specific proteins known as exportins are involved in regulating the nuclear export of specific proteins [52]. A number of cargoes contain nuclear export signals (NESs), first identified in the HIV-1 Rev protein [53,54], that are recognized by the CRM1/exportin1 receptor. We hypothesized that hGCN5 may contain a NES within its primary sequence that could be regulated by this factor.

CRM1/exportin1 is a member of the Karyopherin β family involved in a transport pathway that mediates the export of proteins containing a classic leucine-rich NES that is typified by the loose consensus sequence $\Phi X_{2\cdot3} \Phi X_{2\cdot3} \Phi x_{1\cdot2} \Phi$, where Φ most often represents residues such as leucine, isoleucine, phenylalanine, valine and/or methionine, and X implies a lack of strong selection for particular residues within these positions, although Bogerd *et al.* have shown that not just any amino acid is suitable [53-55]. The identification of proteins that are regulated by the CRM1 export factor has been greatly accelerated by the use of an antifungal agent known as leptomycin B (LMB): this compound is a highly specific and potent inhibitor of CRM1 function [56] in that it irreversibly alkylates a conserved cysteine residue (Cys529) within its cargo-binding domain [57,58]. This modification inhibits CRM1's ability to export any of its normal cargo; thus, in the event that a fluorescently-tagged protein of interest contains a CRM1dependent NES, one can quantify the percent change in nuclear localization following treatment with this drug. As such, LMB will be used to test the functionality of the putative NES in the carboxy-terminus of hGCN5.

A point that is not touched upon in the current study, but is important for understanding the process of nucleocytoplasmic transport, is the fact that the directionality of this transport system is largely influenced by an important Ran-GTP/GDP-binding protein. A number of proteins, including nucleotide exchange factors and GTPase-activating proteins, are maintained within the nuclear and cytoplasmic compartments, respectively; these factors are essential for maintaining a high RanGTP concentration in the nucleus, and conversely, a high RanGDP concentration in the cytoplasm [59-63]. Thus, it is the presence or lack of RanGTP in the particular cellular compartment that facilitates the assembly or the dissociation of the particular cargo complex, depending on whether it is an import or an export complex [63-66].

1.3. ΙκΒα, a Negative Regulator of NF-κB Signaling

During the evaluation of the putative NES in hGCN5 using LMB, our results suggested that the protein being used as a positive control for effective drug treatment, $I\kappa B$, may be involved in regulating the export of hGCN5. For this reason, the well-described inhibitory role of $I\kappa B$ will be discussed.

The coordinated import and export of transcriptional regulators to and from the nucleus is an important mechanism that the cell uses to regulate the expression of certain genes. The best known example of this phenomenon involves the transcriptional activators of the nuclear factor- κ B (NF- κ B)/Rel family, which are responsible for the inducible expression of a large number of genes involved in immune response, inflammation, cell survival, and proliferation [64,65,67,68]. The most abundant form of NF- κ B is the p65/p50 heterodimer, which is present in essentially all cells [65].

In the absence of an extracellular signal, $I\kappa B$ retains the NF- κB dimer in the cytoplasm [69-73] by means of a CRM1-dependent NES located within its aminoterminus [74]. Additionally, $I\kappa B$ is able to mask at least one of NF- κB 's two NLSs [75], thereby preventing its re-import into the nucleus. Conversely, upon appropriate signaling, the I κB kinase becomes activated and phosphorylates I κB on two critical residues, Ser-32 and Ser-36; this signals its polyubiquitination and subsequent degradation by the 26S proteasome [76]. This event liberates NF- κB and exposes its NLSs, allowing it to enter the nucleus and initiate transcription of specific genes. Upon signal termination, NF- κB induces the expression of its inhibitor, I κB [77]; this protein then enters the nucleus where it dissociates NF- κB from its promoter/enhancer regions and mediates its export from the nucleus. NF- κB remains sequestered in the cytoplasm by I κB until further signal-induced activation [78].

There are multiple members in the IKB family, including IKB α (previously referred to as MAD3 [65,79]), IKB β and IKB ϵ , which differ in their constitutive turnover and their signal-induced degradation [64]; however, IKB α (hereafter referred to simply as IKB) has been found to be the major player in the control of NF-KB signaling [80]. IKB is composed of a surface-exposed amino-terminal region, followed by a central, protease-resistant domain that consists of five tandemly-repeated ankyrin repeats. The ankyrin repeat, which is made up of 33 amino acids, is one of the most common protein-protein interaction motifs in nature. Finally, connected to the core through a flexible linker [81], is a compact, highly acidic carboxy-terminus that resembles the PEST motif that has been associated with rapid protein turnover [80,82,83]. Both the central ankyrin repeat domain and the linker region are required for the interaction of IKB with a number of the NF-KB subunits, including p65 [84].

1.4. Actin in the Nucleus

Following experiments with transiently-expressed fluorescently-tagged hGCN5, we noted the existence of nuclear filaments in a portion of the transfected cells. Due to a number of studies that have suggested the presence of actin in the nucleus, and specificially in its polymerized form, we proposed that these filaments may be polymerized nuclear actin.

Actin is a 43 kDa [85], globular ATPase that is separated into two lobes by a cleft that forms the ATP (adenosine triphosphate)-binding site. This protein cycles between a monomeric, ATP-bound state (globular or G-actin) and a polymerized, filamentous form termed F-actin, which results in ATP hydrolysis [85]. Actin filaments are composed of two strands that wrap around each other to form a double right-handed helix. There are three isoforms of actin in higher eukaryotes, namely α -, β -, and γ -actin; the former is muscle-specific, while the latter two are found in all cell types [86]. Since its discovery, actin has been shown to be an important cytoskeletal protein that is involved in processes such as mitosis, cytokinesis, cell motility, muscle contraction, maintenance of cell shape, and endocytosis [87]. As such, for the most part, it has been considered to be solely a cytoplasmic protein. However, a number of recent studies have shown that it also exists in the nucleus, even in the absence of physical disruption of the nuclear envelope. For instance, a cell-permeable cross-linker was used in one study and resulted in the covalent attachment of actin to DNA *in vivo* [88]. Furthermore, a number of studies have shown that actin is able to both import and export to and from the nucleus [56,89,90].

A well-characterized model system that has been routinely used to test for the presence of nuclear actin has been the *Xenopus* oocyte nucleus, which is large, and easily extracted without much fear of cytoplasmic contamination. Within this nucleus, it has been estimated that actin is at a level of 4-6 mg/mL, which is near the critical concentration for polymerization [88]. Specifically, using these nuclei, it has been estimated that roughly 75% of nuclear actin is freely soluble, while approximately 25% is associated with the nuclear matrix [91-93], a network of fibres, analogous to the cytoskeleton, that is thought to exist within the cell nucleus. This, in addition to other studies that have shown actin to be associated with a similar nucleoskeleton [94], suggests that nuclear actin may be an important structural component of this matrix.

Polymerization of actin involves the hydrolysis of an ATP molecule that is normally associated with monomeric actin; the energy of hydrolysis contributes to actin polymerization [95]. Furthermore, although its not necessary, actin is often processed by acetylation of its amino-terminus in order to increase the efficiency of filament formation [96]. Class I actin genes encode for Met-Asp(Glu)-actin, while class II actin genes encode for Met-X-Asp(Glu)-actin, where X is usually a cysteine [96]. Amino-termini of both are removed in an acetylation-dependent processing reaction yielding acetyl-Asp(Glu)-actin lacking the initiator methionine [97,98]; however, the specific acetyltransferase used in this processing has yet to be described.

Microscopic and ultrastructural analyses have confirmed that polymerized actin is present in the nucleus [99,100], while the use of F-actin filament-stabilizing or - disrupting agents has suggested that at least some of the nuclear actin is in its

polymerized state [101]. A study conducted by Clark *et al.* [101] employed electron microscopy to visualize the ultrastructural arrangement of the nuclear F-actin filaments, which they identified through decoration with rabbit skeletal muscle myosin subfragment-1 (S-1); they noted that the actin filaments appeared to be randomly distributed throughout the nucleoplasm, with occasional masses of oriented filaments in the periphery of the matrix [102]. Another study noted F-actin in the nuclei of neuronal cells by heavy meromyosin labeling [103]. Furthermore, using the technique known as fluorescent recovery after photobleaching (FRAP), McDonald *et al.* [104] have recently shown that nuclear actin is in dynamic equilibrium between its monomeric and polymeric forms within living cells.

The non-muscle form of actin, β -actin, is the type that has routinely been observed in the nucleus [105], while this finding has been further confirmed by mass spectrometry analysis [106]. One proposed role for nuclear actin, in the early years of its discovery, was that it may influence changes in chromatin organization, which occur during the cell cycle and/or during development [107-109]. Other studies, which reported a close association between actin filaments and the mitotic spindle [85,91,110], have raised the possibility that actin may have a role in chromosome movement during mitosis.

Recently, the repertoire of possible functions for nuclear actin has grown to include many closely related nuclear processes, some of which include those which were initially proposed. It has been shown to be involved in chromatin-remodeling, as a component of many SWI/SNF-related chromatin-remodeling complexes, from both humans and insects, as well as a variety of histone acetyltransferase complexes [85,91]. Additionally, extensive research has supported the role of actin in transcription by each of the three eukaryotic RNA polymerases, as well as in RNA processing and export; specifically, nuclear actin has been found to be associated with a variety of heterogeneous nuclear ribonucleoproteins (hnRNPs) [87]. These proteins are known to form complexes that play an essential role in the splicing of mRNA precursors.

1.5. Fluorescent Protein Technology

The majority of the following thesis involved the use of fluorescent protein technology, including variants of the original green fluorescent protein (GFP; 238 amino acids. GFP was first identified by Shimomura *et al.* [111] as a companion protein to aequorin, the famous chemiluminescent protein from *Aequorea victoria*. GFP was found to convert the blue emission from aequorin to the green fluorescence observed in intact cells and animals. Morin and Hastings [112] later found the same colour-shift in the related *Obelia* (a hydroid) and *Renilla* (sea pansy) species. In 1974, Morise *et al.* [113] successfully purified and crystallized GFP, in addition to determining its quantum yield and absorbance spectrum. In 1992 by Prasher *et al.* successfully cloned GFP [114], while it was more fully characterized and expressed in non-jellyfish organisms by Chalfie *et al.* [114] in 1994. Since this time, the use of fluorescent protein tags has remarkably advanced the field of cellular biology through the ability to monitor proteins within living cells. Due to its small size of 26 kDa, GFP, which is usually monomeric, can be suitably

used as a reporter through either amino- or carboxy-terminal fusion to numerous proteins of interest. It has a low toxicity in mammalian cells, as well as an intrinsic ability to fluoresce without the need for additional cofactors [115]. Moreover, it is highly resistant to denaturation, except under harsh environmental conditions [113].

The spectral characteristics of GFP are due to a covalently-bound chromophore, *p*-hydroxybenzylideneimidazolinone [116], which is formed by the internal cyclization of residues 65-67, which are Ser-Tyr-Gly in the native protein, and 1,2 dehydrogenation of the Tyr residue [117]. A single amino acid substitution, Ser⁶⁵ \rightarrow Thr⁶⁵ (S65T), was found to accelerate fluorophore formation from a time constant of 2 hours to 0.5 hour [118,119], while several additional mutations throughout the protein enabled it to fold properly at 37°C, to resist aggregation at high concentrations, and to diffuse more efficiently in living cells [118]. The expression of GFP was further improved by altering some of the codons and improving the translational initiation sequences to ones which are more efficiently expressed in the organisms in which the protein is routinely used [120,121]; a number of these mutations gave rise to an enhanced variant of GFP, termed eGFP. GFP is an 11-stranded β -barrel (referred to as a β -can) with an α -helix running up the axis of the cylinder. The chromophore is attached to the α -helix, and is buried almost completely within the cylinder [118], along with a number of polar groups and structured water molecules that are buried adjacent to the chromophore [122].

Other fluorescent proteins that have been derived from wild-type GFP include the enhanced yellow fluorescent protein (eYFP) [123]. A variant of eYFP, known as Venus, has specific mutations that allow it to mature faster and more efficiently at 37° C than traditional eYFP, and decrease its environmental sensitivity [124]. Additionally, for the purpose of multi-coloured imaging, a red fluorescent protein has also been developed. The original protein, termed DsRed, was obtained from the *Discosoma* species, however it forms tetramers that hinder its use as a fluorescent tag. It has since been replaced by a monomeric red fluorescent protein, mRFP1 [125]. This protein was constructed by the insertion of arginine residues between each subunit interface of DsRed, in addition to random and site-directed mutagenesis [125]. Although this DsRed variant has a slightly lower extinction coefficient, quantum yield, and photostability, it is monomeric, and matures 10 times faster, thus showing equivalent brightness [126]. Furthermore, it has recently been found that a Gln \rightarrow Thr mutation in mRFP1, termed mRFPQ66T, causes it to be substantially more blue-shifted [127], and two- to three-fold brighter [128] than traditional mRFP1.

A technique that has been employed in this study to examine possible proteinprotein interactions is bimolecular fluorescence complementation (BiFC); this technique was adapted from other protein complementation assays (PCAs) showing that nonfunctional fragments of a protein such as β -galactosidase could be brought together to restore enzymatic activity [129]. This finding was extended to the study of proteinprotein interactions, such that only proteins that interacted could restore this enzymatic activity when fused to these fragments [130,131]. Later studies showed that nonfluorescent fragments of eYFP could be brought together to restore fluorescence that varied with intracellular Ca²⁺ levels when fused to calmodulin and the M13 calmodulinbinding peptide [132]. Similarly, BiFC, which was first developed using fragments of eYFP [133], has subsequently been improved by the use of fragments of the eYFP derivative, Venus [134], which folds more efficiently at higher temperatures [133]. The positions of the fragment truncations have also been optimized by Shyu *et al.* [133], and were shown to be at residue 173 for the amino-terminal fragment and starting at residue 155 for the carboxy-terminal fragment [135]. Furthermore, it has recently been shown that these BiFC constructs can be additionally fused to functional fluorophores such as mRFP1 and CFP, in order to visualize their normal cellular distributions [136].

Fluorescence recovery after photobleaching (FRAP) is another technique that has been increasingly popularized due to the use of fluorescent protein tags such as GFP. This technique, which was developed over 30 years ago [137], can be used *in vivo* to study the dynamic lateral mobility and binding kinetics of a fluorescently-tagged protein of interest [137]. In these experiments, the fluorescent molecules are irreversibly photobleached in a region of interest (ROI) within the living cell through the use of a focused, high-intensity laser beam. Over time, the bleached ROI increases in fluorescence intensity as the bleached molecules are replaced by non-bleached recombinant proteins from the surrounding areas of the cell. Quantitative FRAP can be used to determine the mobile fraction of the protein of interest, as well as its rate of mobility [138]. Furthermore, an application of FRAP using bikaryon cells (cells with two nuclei), which was specifically used in this study, has been described by Howell *et al.* [139] to examine the ability of fluorescently-tagged proteins to dynamically shuttle between the nuclear and cytoplasmic compartments.

1.6. Project Overview

This project initially began with the study of the Spinocerebellar ataxia type 7 (SCA7) disease protein, ataxin-7. Using affinity chromatography and mass spectrometry, our initial goal was to determine which specific protein(s) interact with ataxin-7's previously-described phosphoprotein-binding domain [140]. This domain is interestingly situated between the recently-described NES [140] and an NLS, suggesting that understanding proteins that bind to this region might help us to better understand why ataxin-7 actively shuttles between the nucleus and the cytoplasm [141-144]. The background on ataxin-7 and the SCA7 disease, as well as preliminary results for this part of the project are appended at the end of the thesis (see Appendix).

Additionally, we set out to study whether hGCN5, a protein recently shown to directly interact with ataxin-7 within the human STAGA complex [138], might modulate or affect ataxin-7's ability to shuttle between the nucleus and the cytoplasm. However, during this study, which involved determining whether hGCN5 was able to independently shuttle between the two compartments, we made some serendipitous discoveries that redefined the goals of this study. These goals, which focused on the characterization of hGCN5, included testing its ability to actively shuttle between the nucleus and the cytoplasm, and determining the mechanisms that regulate this process, as well as explaining its relationship with filamentous structures observed in the nucleus upon its over-expression.

We hypothesized that hGCN5 was, at the very least, able to import into the nucleus, and possibly even export from this compartment. Using a bikaryon shuttling assay employing FRAP [73], we showed that hGCN5 is capable of shuttling both to and from the nucleus. Additionally, through motif-scanning and site-directed mutagenesis, we showed that hGCN5 harbours a classical bipartite NLS that is most likely recognized by the import factors, importins α and β . In contrast, using both site-directed mutagenesis and leptomycin B (LMB) assays, we concluded that hGCN5 does not contain an internal CRM1-dependent NES.

To assess the effectiveness of the LMB treatment, the I κ B protein was used as a positive internal control since it contains a well-defined CRM1-dependent NES [145]. In doing this, however, we noted that in its presence, GCN5 was consistently less nuclear than when it was either expressed alone or with a control fluorophore protein. This prompted us to study the ability of I κ B to modulate GCN5's nuclear export *in vivo* through mutational analysis of specific regions within I κ B. Furthermore, we conducted GST pull-down assays to confirm I κ B's ability to interact with GCN5 *in vitro*. These results were further supported using an *in vivo* PCA. Together, our results suggested that GCN5's histone acetyltransferase activity may be, at least partially, regulated by I κ B through an export mechanism similar to that which it uses to modulate the activity of the well-known transcription factor, NF- κ B.

Moreover, we found that hGCN5 can also be exported by the mRNA export factor, TAP/NXF1, suggesting that this protein may be regulated through more than one pathway. The interest in the ability of GCN5's nuclear export to be regulated by this mRNA export factor was enhanced by the observation that, upon over-expression of GCN5-eYFP, ~10% of the transfected cells show the presence of GCN5-eYFP-associated nuclear filaments. These filaments were hypothesized to be nuclear actin filaments, which was confirmed by co-expression with mRFP- β -actin, as well as co-imaging of these structures with TRITC-phalloidin-labelled nuclear actin filaments. The interaction between GCN5 and nuclear actin was also confirmed by BiFC. Using a catalyticallyinactive GCN5 mutant, we showed that GCN5's acetyltransferase activity is most-likely responsible for the formation of these filaments, while FRAP applied to a single filament demonstrated that GCN5's acetyltransferase activity is important in both the formation and maintenance of these filaments.

In visualizing the spatial arrangement of these GCN5-eYFP-associated filaments in comparison to the nuclear envelope (labeled using a fluorescently-tagged nuclear envelope protein, mRFP-lamin A [125]) or DNA, or both, we have found that these filaments do not appear to cross in 3D space with either of these other structures. However, we did note that these filaments are able to terminate in areas overlapping with lamin A locations, and that these filaments often appear to confine some of the stained DNA to the nuclear periphery. Furthermore, our results show that these filaments are transient and are dependent on the structural integrity of DNA, as they are disrupted overtime following the addition of an intercalating DNA stain.

Interestingly, nuclear actin has recently been shown, in several studies, to be involved in transcription by all three mammalian RNA polymerases. And furthermore,

recent studies have noted the recruitment of certain actively-transcribed genes to the nuclear periphery. Thus, we propose a model whereby GCN5 increases transcriptional activity and processing through both its previously-described histone acetyltransferase activity, as well as through acetylation, and hence formation, and structural maintenance of nuclear actin filaments. We propose that these filaments are involved in facilitating the transport of either actively-transcribed genes or the transcribed mRNA to the nuclear periphery, where it can be efficiently exported. This may occur through an acto-myosin motor, as there has recently been a myosin protein described to localize specifically to the nucleus. With such important roles in the regulation of transcription, it would make sense for hGCN5, like other transcription factors, to be regulated at the level of its nucleocytoplasmic transport ability; this includes its nuclear import, which was found to be controlled by the karyopherin alpha/beta pathway, as well as its export, which was found to be modulated by IkB and the mRNA export factor, TAP/NXF1. The importance of having multiple pathways regulating its export is evident when considering the high occurrence of transcription-related pathologies, including cancer and certain neurodegenerative diseases.

2. MATERIALS & METHODS

2.1. Plasmids

All fluorescent fusion protein constructs were made using the same type of background vector, with either an amino-terminal or carboxy-terminal fluorescent protein tag, purchased from BD Clontech. All PCR reactions were performed using Deep Vent (New England Biosciences), except for the inverse-PCR and QuikChange mutagenesis reactions, which were performed using PfuUltra DNA polymerase (Stratagene). QuikChange mutagenesis reactions were performed as per the manufacturer's instructions. All restriction endonucleases were purchased from New England Biosciences, and all constructs were ligated using T4 DNA ligase (New England Biosciences). For each of the constructs, Table 1 (below) lists which oligos were used along with their corresponding sequences.

pGCN5-eYFP was constructed using the peYFP-N1 vector, while GCN5 was amplified by polymerase chain reaction (PCR) using the oligos RT0869 and RT0870 to introduce *XhoI* and *Eco*RI restrictions sites. The vector and insert were cut with the mentioned restriction endonucleases and ligated. The inactive mutant of GCN5, namely pGCN5-E575Q-eYFP, was constructed using the QuikChange II Site-Directed Mutagenesis kit (Stratagene), using oligos RT0946 and RT0947 to create the E575Q mutation. pGCN5-NLSm-eYFP was also created using the QuikChange Mutagenesis kit by employing oligos RT0925 and RT0926 to introduce the K446A and R447A mutations within the putative NLS (residues 433 - 450). Similarly, pGCN5-NESm2-eYFP was constructed using the mutagenesis kit with oligos RT0884 and RT0885 to introduce the F827A and L829A mutations in the putative carboxy-terminal NES (residues 821 - 829).

pmRFP-C1 was made by Joanna Graczyk by PCR-amplifying mRFP from cDNA [73], (kind gift of R.Y. Tsien, University of California, San Diego), using the oligos RT0355 and RT0337 to introduce NheI and BspEI restriction sites. The PCR product and peGFP-C1 were digested with the mentioned restriction endonucleases and ligated to produce a construct with the mRFP gene introduced into a peGFP-C1 background. pmRFP-IkB was constructed by Meghan Holmes by PCR-amplifying IkB with the oligos RT0794 and RT0795 that introduced EcoRI and XbaI sites. The insert and the pmRFP-C1 vector were digested with the mentioned restriction enzymes and ligated. pIkB-GFP was purchased from BD Clontech. pmRFP-IkB-AANK2 and plkB-AANK2-GFP, which deleted a 33 amino acid region, known as the second ankyrin repeat, beginning with residue 105 of IkB, were both made by inverse-PCR employing PfuUltra DNA polymerase (Stratagene) and the oligos RT0923 and RT0924, with the pmRFP-IkB and pIkB-GFP constructs as templates, respectively. Both phosphorylation of the blunt-ends and ligation of the constructs were performed in one step using T4 Polynucleotide kinase (T4 PNK, New England Biosciences) and T4 DNA ligase in the latter enzyme's buffer at 25°C. pmRFP-IkB-NESm1 was created using the QuikChange mutagenesis kit (Stratagene) and the oligos RT0919 and RT0920 to introduce the I52A and L54A mutations into I κ B's amino-terminal NES (residues 45-54) [146].

pmRFP-TAP/NXF1 was constructed by Harry Kim by PCR-amplification of the TAP/NXF1 gene from a Homo Sapien cDNA library using oligos RT0721 and RT0722 to introduce *Eco*RI and *Bam*HI restriction sites, respectively. This PCR product, along with the pmRFP-C1 vector, were then cut with the appropriate restriction enzymes and ligated.

pmRFP- β -actin and pmCer- β -actin were made directly from peYFP- β -actin (BD Clontech) by removing the β -actin sequences from the parental constructs using the *Bam*HI and *XhoI* restriction endonucleases. The following inserts were ligated into mRFP-C1, which had been digested with the same enzymes. pmRFP-lamin A was constructed by PCR-amplifying lamin A from cDNA purchased from ATCC: Cell Biology Collection, using the oligos RT1052 and RT1053, which introduced *Eco*RI and *KpnI* restriction sites. The mRFP-C1 and the insert were then digested with the appropriate enzymes and ligated.

For the GST pull-down experiments, using recombinant wild-type and $\Delta ANK2$ mutant GST-I κ B, pGEX-I κ B and pGEX-I κ B- $\Delta ANK2$ were created using the pGEX-5X-1 vector backbone. Oligos RT1030 and RT1031 were used to PCR-amplify both I κ B and I κ B- $\Delta ANK2$, which introduced *Bam*HI and *Eco*RI sites. The inserts and vector were digested with the mentioned restriction endonucleases and ligated.

For Bimolecular Fluorescence Complementation (BiFC), the following constructs were made. The pVenus-C1 vector was a kind gift from R. Y. Tsien, University of California [126]. pVenus-N172 was made by Jianrun Xia using inverse-PCR and the oligos RT1026 and RT1027, which deleted the latter portion of the Venus fluorescent protein, beginning with amino acid 173. The complementary vector, pVenus-C155 was also made by J. Xia by employing the same method, using oligos RT1028 and RT1029, which deleted the first 154 amino acids, in addition to introducing a methionine codon to enable translation of the latter portion of the Venus protein, beginning at residue 155. Both vectors were phosphorylated using T4 PNK and ligated with T4 DNA ligase, concurrently, in the latter enzyme's buffer. pmRFPQ66T, which is substantially more blue-shifted than traditional mRFP1 [127], two- to three-fold brighter [127], and which has been recently tested in BiFC experiments by Jach et al. [138], was constructed using the oligos RT0954 and RT0955 to introduce the Q66T mutation using the QuikChange mutagenesis kit. Following this, a pmRFPQ66T-Venus-N172 (i.e. pmRFPQ66T-NVenus) construct was made by J. Xia, which would allow for the visualization of the fused protein's localization, using oligos RT1042 and RT1043 to produce a mRFPQ66T PCR product with NheI and AgeI sites. The vector and insert were digested with the appropriate restriction enzymes and ligated. The complementary vector was made by introducing a mCerulean marker into the pVenus-C155 vector, creating pmCer-Venus-C155 (i.e. pmCer-CVenus), which was also made by J. Xia. To amplify and introduce NheI and AgeI sites into the mCerulean product, oligos RT1044 and RT1045 were employed. The insert and vector were then digested and ligated.

The experimental constructs for BiFC were made as follows: pmRFPQ66T-NVenus-GCN5 was created using the pmRFPQ66T-NVenus vector as a backbone, which had been purified from the ER2925 dam⁻ strain of bacteria. GCN5 was PCR-amplified with the oligos RT1036 and RT1037, introducing *Kpn*I and *Xba*I sites. The vector and insert were digested with the appropriate restriction enzymes and ligated. From herein, this construct will be referred to as mRFP-NVenus-GCN5. For the pmCer-CVenus-I κ B, -I κ B- Δ ANK2, and -I κ B-NESm1 constructs, each of the inserts was PCR-amplified using the oligos RT1038 and RT1039, which introduced *Sac*II and *Bam*HI restriction sites. The constructs were then ligated, following digestion of the inserts and the pmCer-CVenus backbone with the mentioned enzymes. Finally, pmCer-CVenus- β -actin was created by amplifying the β -actin sequence from the pmRFP- β -actin construct using the oligos RT1040 and RT1041, which similarly introduced *Sac*II and *Bam*HI sites. The insert and vector backbone were digested with the appropriate enzymes and ligated.

Each of the constructs was either PCR-screened to identify the presence of the insert, and subjected to diagnostic digestion, or PCR-sequenced by the McMaster Mobix facility (McMaster, University). Moreover, if possible, the constructs were examined by fluorescence imaging to further confirm their correct expression and their predicted cellular localization.

Construct	Oligo #	F/R	Oligo sequence (5'-3')
pGCN5-eYFP	RT0869	F	GATCCTCGAGGCCACCATGGCGGAACCTTCC
	RT0870	R	GATCGAATTCACTTGTCAATGAGGCCTCC
pGCN5-E575Q-eYFP	RT0946	F	CCACCCAGGGCTTCACGCAGATTGTCTTCTGTGCTGTC
	RT0947	R	GACAGCACAGAAGACAATCTGCGTGAAGCCCTGGGTGG
pGCN5-NLSm-eYFP	RT0925	F	GACCCTGGAGGATGCCGCGCGCGCCCCGTGTGATGGGTG
	RT0926	R	CACCCATCACACGGAGCGCCGCGGCATCCTCCAGGGTC
pGCN5-NESm2-eYFP	RT0884	F	GGAGAAGTTCTTCTACGCCAAGGCCAAGGAGGGAGGCC
			TC
	RT0885	R	GAGGCCTCCCTCCTTGGCCTTGGCGTAGAAGAACTTCT
			СС
pmRFP-C1 (made by	RT0355	F	GAGATCGCTAGCGCTACCGGTCGCCACCATGGCCTCCT
J. Graczyk)			CCGAGGACG
	RT0337	R	CATGTCCGGAGGCGCCGGTGGAGTG
pmRFP-IxB(made by	RT0794	F	GATCGAATTCTATGTTCCAGGCGGCCGAGCGC
M. Holmes)			
	RT0795	R	GATCTCTAGAGCAATCCTAGGTAGCGGTGG
pIxB-GFP	N/A	N/A	Purchased from BD Clontech
pmRFP-IKB-DANK2/	RT0923	F	GAGCTCCGAGACTTTCGAGG
pIKB-AANK2-GFP			
	RT0924	R	GAGGAAAGCCAGGTCTCCC
pmRFP-IKB-NESm1	RT0919	F	CAAGGAGCTGCAGGAGGCCCGCGCGCGAGCCGCAGGAGG
			TG
	RT0920	R	CACCTCCTGCGGCTCGGCGCGGGGCCTCCTGCAGCTCCT
			TG
pmRFP-TAP/NXF1	RT0721	F	GATCGAATTCAATGGCGGACGAGGGGAAG
(made by H. Kim			
	RT0722	R	GATCGGATCCTCATGAATGCCACTTCTGG

Table 1: Constructs made with their corresponding oligo sequences.F, forward oligo;R, reverse oligo.

peYFP-β-actin	N/A	N/A	Purchased from BD Clontech
$pmRFP-\beta-actin$	N/A	N/A	Cut directly from peYFP- β -actin
pmCer- β -actin	N/A	N/A	Cut directly from peYFP- β -actin
pmRFP-lamin A	RT1052	F	GATCGAATTCTATGGAGACCCCGTCACAGCG
	RT1053	R	GATCGGTACCTTACATGATGCTGCAGTTCTGGG
pGEX-IKB/	RT1030	F	GATCGGATCCCCATGTTCCAGGCGGCCGAGC
pGEX-IKB-AANK2			
	RT1031	R	GATCGAATTCTCATAACGTCAGACGCTGGCC
pVenus-C1	N/A	N/A	Kind gift from R.Y. Tsien (University of California)
pVenus-N172 (made	RT1026	F	TCCGGACTCAGATCTCGAG
by J. Xia)			
	RT1027	R	CTCGATGTTGTGGCGGATC
pVenus-C155 (made	RT1028	F	GACAAGCAGAAGAACGGCAT
by J. Xia)			
	RT1029	R	CATGGTGGCGACCGGTAG
pmRFPQ66T (made	RT0954	F	CTCAGTTCACGTACGGCTC
by J. Xia)			
	RT0955	R	GAGCCGTACGTGAACTGAG
pmRFPQ66T-Venus-	RT1042	F	GATCGCTAGCGATGGCCTCCTCCGAGGAC
N172 (i.e.			
pmRFPQ66T-NVenus)			
(made by J. Xia)			
	RT1043	R	GATCACCGGTAGGGCGCCGGTGGAGTGGC
pmCer-Venus-C155	RT1044	F	GATCGCTAGCGATGGTGAGCAAGGGCGAG
(i.e. pmCer-			
CVenus) (made by			
J. Xia)			
	RT1045	R	GATCACCGGTAGCTTGTACAGCTCGTCCATG
pmRFPQ66T-NVenus-	RT1036	F	GATCGGTACCATGGCGGAACCTTCCCAGGC
GCN5 (i.e. pmRFP-			
NVenus-GCN5)			
	RT1037	R	GATCTCTAGACTTGTCAATGAGGCCTCCCTCC
pmCer-CVenus-	RT1038	F	GATCCCGCGGGCATGTTCCAGGCGGCCGAGC
IKB/-IKB-AANK2/-			
IKB-NESm1)		<u> </u>	
	RT1039	R	GATCGGATCCTCATAACGTCAGACGCTGGCC
pmCer-CVenus-β-	RT1040	F	GATCCCGCGGGCATGGATGATGATATCGCCGCG
actin			
	RT1041	R	GATCGGATCCCTAGAAGCATTTGCG

2.2. Tissue Culture

The cell lines used in the following experiments were either NIH 3T3 (ATCC, CCL-2) or HEK 293 (kind gift from D. W. Andrews, McMaster University). NIH 3T3 cells were predominantly used for microscopic imaging, while HEK 293 cells were used to produce whole cell or nuclear extracts of endogenous NF- κ B or GCN5, respectively, for the GST pull-down assays.

NIH 3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen), while HEK 293 cells were cultured in Alpha Minimal Essential Medium (α MEM, Invitrogen), each of which was supplemented with 10% Fetal Bovine Serum (Invitrogen). Both cell types were incubated at 37°C with 5% CO₂.

Following growth to ~80% confluency, cells were passaged with either 10% trypsin-EDTA (Invitrogen), for NIH 3T3 cells, or phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄) plus α MEM, for HEK 293 cells. Frozen stocks were prepared in 5% DMSO and stored in a liquid nitrogen tank.

For live-cell imaging, 25 mm glass-bottomed dishes were prepared as described previously [138], and stored in 70% ethanol; the dishes were washed twice with PBS, prior to use. For live-cell imaging, approximately 70,000 to 150,000 NIH 3T3 cells were seeded 16 to 24 hours prior to transfection. For fixed-cell imaging, 70,000 to 100,000 cells were seeded on acid-washed coverslips placed in 25 mm dishes. Similar incubation times prior to transfection were followed, as described for live-cell imaging.

2.3. Transfections

All transfections were performed using polyethylenimine (ExGEN 500, Fermentas). For 25 mm dishes, 2.5 to 3 μ g total plasmid was transfected (either single or co-transfection), according to the manufacturer's instructions, except that 100 μ l of 150 mM NaCl was used for up to 3 μ g of DNA. All plasmids used for transfections were purified using the GenEluteTM Plasmid Miniprep Kit (Sigma-Aldrich) or the polyethylene glycol (PEG)/lithium chloride method.

2.4. Leptomycin B (LMB) Assay

NIH 3T3 cells expressing either GCN5-eYFP or GCN5-NESm2-eYFP alone, or in combination with mRFP-I κ B, were used for the following assays. For both single and co-transfections, a total of 2.5 µg DNA was used. Both untreated control and experimental cells had their serum-supplemented media replaced by serum-free media. For the experimental condition, the designated cells were treated with 10 ng/mL leptomycin B (LMB, Sigma) for 13 hours. Prior to imaging, the cells were treated with Hoechst dye as a nuclear marker. For each condition, a total of 90 cells were imaged and subjected to quantitative analysis to determine percent nuclear localization of each construct (see below for equation used for quantification).

2.5. Widefield Fluorescence Microscopy

Live-cell microscopy was performed on a Nikon TE200-inverted fluorescence microscope with a 175 W Xenon arc lamp (Sutter Instruments LB-LS/17) light source. A

Nikon $63 \times$ plan apochromat (NA 1.4) oil-immersion objective was used. The following band-pass excitation and emission filter sets were used for the specific fluorophores: Hoechst dye: 387 nm and 447 nm; mCerulean: 438 nm and 483 nm; eGFP: 472 nm and 520 nm; eYFP: 500 nm and 542 nm; mRFP: 562 nm and 624 nm (Semrock). Each channel for a particular image was captured separately using a monochrome camera (Hamamatsu model C4743-95 and controller). The software, Simple PCI version 5.3.1.081004 (Compix, Inc.) was used to control the microscope and capture the pseudo-coloured, merged images. Exposure time for each image ranged from 0.09 to 1.5 seconds at a resolution of 512×512 pixels, unless stated otherwise. Images obtained for the LMB assays were captured using this microscope, and saved as 24-bit RGB, tagged image file format (TIFF) uncompressed files. The TIFF files were then subject to pseudo-colouring, and brightness and contrast adjustments using the Laser Scanning Microscope (LSM) Image Browser version 4.0.0.157 (Carl Zeiss). Corel PhotoPaint and CorelDraw version 13 (Corel Corporation) were used to further enhance the brightness and contrast of the images to prepare them for publication.

Some micrographs were also captured using a Leica DMI 6000 B widefield fluorescence microscope with a Hamamatsu Orca ER-AG camera. A $100 \times$ plan apochromat (NA 1.4) oil-immersion objective was used, while the images were captured using Volocity 4 software (Improvision). Image z-series captured with this microscope were system-optimized with respect to step sizes and deconvolved using the *Iterative Restoration* algorithm of Volocity 4 software using theoretical widefield point spread functions (PSFs).

2.6. Laser Scanning Confocal Microscopy

Fluorescence recovery after photobleaching (FRAP) experiments, specifically, were performed on a Leica TCS SP5 MP confocal microscope (Leica Microsystems), using a $63 \times$ glycerol-immersion objective (NA 1.3). An Argon ion laser at 95% intensity was used for excitation and bleaching of the eYFP fluorophore, while the laser was reduced to 4-5% intensity for the time-lapsed recovery images. LAS AF confocal software (Leica Microsystems) was used to control the microscope, photobleach, and capture the images. Images were captured at either 400 or 1000 Hz laser-line rastering speed depending on the experiment. Corel PhotoPaint and CorelDraw version 13 (Corel Corporation) were used to brighten and enhance the contrast of the images to prepare them for publication.

The Leica TCS SP5 MP confocal microscope (Leica Microsystems) was also used to capture images that would be used for quantification of nuclear localization of GCN5eYFP and its mutants, as well as in combination with different mRFP-IkB variants.

Similarly, this microscope was used to capture z-series micrographs (with a system-optimized step-size) of GCN5-eYFP-associated filaments, either alone, or in combination with mRFP-lamin A, as were the results of bimolecular fluorescence complementation (BiFC) using mRFPQ66T-NVenus-GCN5 and mCer-CVenus- β -actin. Finally, focal plane images of these filaments in combination with mRFP- β -actin and/or

TRITC-phalloidin were captured using this microscope. Each of the aforementioned images was sequentially scanned to prevent cross-talk between the eYFP and mRFP channels, while the image z-series were deconvolved using the *Iterative Restoration* algorithm of the Volocity 4 software (Improvision) with theoretical confocal PSFs.

2.7. Fluorescence Recovery After Photobleaching (FRAP)

FRAP experiments demonstrating shuttling of GCN5-eYFP were based on a protocol described by Howell *et al.* [138], with some modifications. Thirty-five millimeter glass-bottomed dishes were seeded with NIH 3T3 cells and transiently transfected with 2.5 μ g of the pGCN5-eYFP construct. Following 24 hours transfection, the serum-supplemented media was replaced with serum-free media for 12 hours in order to induce the formation of natural bikaryons. Prior to bleaching, three images were taken of the bikaryon and were designated as pre-bleach images. Bleaching of the donor nucleus required 12 passes of the laser at full power every 1.314 seconds. Fluorescence recovery from the acceptor to the donor nucleus was monitored and captured over a 20 minute period (1 min/scan) at a 400 Hz laser-line rastering speed, until the fluorescence intensity in the bleached and non-bleached regions equilibrated. The FRAP assays were conducted and imaged using the Leica TCS SP5 MP confocal microscope. Eight bikaryons were tested for GCN5's ability to shuttle between the nucleus and the cytoplasm.

For FRAP analysis of a single GCN5-eYFP-associated nuclear actin filament, 2.5 μ g of pGCN5-eYFP was transfected into NIH 3T3 cells, followed by 36 hours of expression. These images were captured at a 1000 Hz laser-line rastering speed and a resolution of 256×256. Prior to bleaching the filament, three pre-scans were obtained, followed by 5 passes of the Argon laser at 98% intensity to bleach the filament; each of these scans took 150 milliseconds. Time-lapsed recovery was obtained at 30 second intervals for a total of 15 minutes.

2.8. Co-expression of GCN5-eYFP and mRFP-IkB Permutations

Various pGCN5-eYFP constructs, including the wild-type pGCN5-eYFP construct, pGCN5-NLSm-eYFP and pGCN5-NESm2-eYFP, in addition to the empty vector, peYFP, were co-transfected with various permutations of the pmRFP-I κ B construct. These included the wild-type pmRFP-I κ B construct, pmRFP-I κ B- Δ ANK2, and pmRFP-I κ B-NESm1. Co-transfections were performed in NIH 3T3 cells using 1.5 µg of each of the GCN5 and I κ B constructs and incubated for 24 hours prior to imaging. Triple-channel imaging, including DIC images, was performed using the Leica TCS SP5 MP confocal microscope. Ninety cells per condition were captured and subjected to quantitative analysis of percent nuclear localization of each of the various GCN5 permutations in the presence of the variant forms of I κ B (see below for equation used in quantification).

2.9. Image Analysis

Image analysis was conducted using ImageJ version 1.34s (National Institute of Health, USA). In order to measure the percent nuclear localization (as a function of percent nuclear fluorescence intensity), three region of interests (ROIs) were manually defined for each image: the nucleus, the total cell, and a region in the background. An output of area (in number of pixels) and mean fluorescence intensity were provided for each ROI defined. The following formula was used for the quantification of percent nuclear localization:

% nuclear localization = [(mean_{nuclear fluorescence} - mean_{background fluorescence}) x nuclear area] x 100 [(mean_{total cell fluorescence} - mean_{background fluorescence}) x total cell area]

2.10. Statistical Analysis

All data was analyzed in Microsoft Excel 2003 (Microsoft Corporation). Error bars in the histograms represent the standard errors of the mean intensities for each condition (over three independent experiments), both of which were calculated using Microsoft Excel's 'Descriptive Statistics' function. Statistical analysis was done in Excel using the 'Student's t-test, assuming unequal variances', with a confidence level (α) of 0.01. P-values were also calculated in Excel.

2.11. GST Pull-down Assays

For the GST pull-down assays, recombinant GST-I κ B and GST-I κ B- Δ ANK2 proteins, as well as the GST control, were used. Following overnight expression of each of the respective pGEX constructs in BL21-AI *E. coli* at 37°C in antibiotic-treated 2xLB media, 4 mL of each culture was inoculated into separate 400 mL volumes of media and further grown at 37°C to OD 0.5-0.6. Each culture was subsequently induced by the addition of 0.1 mM IPTG and 0.2% arabinose, and grown for another 2 hours. The GST-fusion proteins were purified using Glutathione Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's instructions, with the following modifications: the proteins were incubated with the beads for 1 hr at 4°C, solubilization of the recombinant proteins with Triton X-100 was omitted, and 20 mM glutathione (reduced) in PBS was used to elute the GST proteins. Each of the recombinant proteins was subsequently dialyzed overnight (with two buffer changes) in dialysis buffer (0.1 M NaCl, 10 mM Hepes pH 7.4, 10% glycerol) at 4°C.

For the control pull-down experiment, we tested the ability of recombinant GST-I κ B to precipitate endogenous NF- κ B using HEK 293 whole cell lysate that had been prepared using NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Igepal, protease inhibitors). For the experimental pull-down of endogenous GCN5, HEK 293 nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction

kit (Pierce) and the HaltTM Protease Inhibitor Cocktail, EDTA-free (Pierce) according to the manufacturer's instructions. For both experiments, the lysates (whole cell or nuclear) were rotated for 3 hours at 4°C in GST pull-down buffer (20 mM Tris-Cl pH 8.0, 200 mM NaCl, 1mM EDTA pH 8.0, 0.5% Igepal, protease inhibitors) that contained equimolar concentrations of GST and GST-IKB, as well as GST-IKB- Δ ANK2, in the case of the GCN5 pull-down assay. Subsequently, 100 µl of 50:50 slurry Glutathione Sepharose 4B beads (Amersham Biosciences) were added and incubated for an additional 2 hours (a beads alone control was also used). Afterwards, the beads were spun down at low speed, washed twice with PBS, followed by the addition of SDS-loading buffer and DTT. All the samples for the above mentioned experiments were electrophoresed on 10% polyacrylamide gels for 1.5 hours at 20 mA.

2.12. Western Blot Analysis

Following SDS-PAGE, the proteins were transferred to 0.45 μ m PVDF membrane (Pall) overnight at 20 V. To blot for NF-KB, rabbit anti-NF-KB p65 primary antibody (3034, Cell Signaling) was used at 1:1000. In contrast, blotting for GCN5 was performed using rabbit anti-GCN5 primary antibody (ab18381, Abcam) at 1:1000. The secondary antibody used for both blots was goat anti-rabbit IgG peroxidase conjugate (A4914, Sigma) at 1:3000. To blot for GST, and the associated fusion proteins, the blots were incubated in mouse anti-GST-tag primary antibody (A00014, GenScript Corporation) at 0.05 μ g/mL, followed by goat anti-mouse HRP-conjugated secondary antibody (NEF822, Perkin Elmer Life Sciences Inc.) at a 1:5000 dilution. Protein bands in all cases were visualized using chemiluminescent Western Lighting reagent (Amersham Biosciences).

2.13. Bimolecular Fluorescence Complementation (BiFC) Assays

For the protein complementation assay (PCA) between GCN5 and IKB and its mutants (Δ ANK2 and NESm1), 1.5 µg of each of the appropriate plasmids was transiently-transfected into NIH 3T3 cells in live-cell culture dishes, followed by 36 hours of protein expression. A number of images (>50 cells) were captured of the GCN5:IKB-wild-type-expressing cells. For each of these images, the eYFP channel was set to appropriately see eYFP expression (at approximately the same intensity for each image). The exposure times for each image in the eYFP channel were recorded and averaged, equaling a 0.04 second exposure time. To test for BiFC, each subsequent image, including controls, was then imaged at this exposure time for the eYFP channel, in order to keep the experiments consistent. Of importance is the fact that a background level of BiFC between GCN5 and the Htt 81-588 protein, which served as the negative control, was only observed when the exposure time in the eYFP channel was increased almost 10-fold, to between 0.2 and 0.3 seconds. Additionally, each protein was expressed alone and imaged in each of the mRFP, mCerulean and eYFP channels to serve as

controls. All images were captured using a Nikon TE200-inverted fluorescent microscope.

To test for complementation between mRFP-NVenus-GCN5 and mCer-CVenus- β -actin, 2.5 µg and 0.5 µg of plasmid were transfected, respectively, followed by 36 hours of expression. Representative z-series of images (with system-optimized step-sizes) were captured using the Leica TCS SP5 MP confocal microscope with sequential imaging for each of the mRFP, mCerulean, and eYFP channels. The z-series images were then deconvolved using Volocity 4 (Improvision) as previously stated, followed by the use of Imaris version 5.5.1 (Bitplane Scientific Solutions) to obtain a cross-sectional slice of the 3D image. CorelDraw version 13 was subsequently used to brighten and enhance the contrast of each subset of images.

2.14. Imaging of GCN5-eYFP-associated Nuclear Actin Filaments

For image analysis of GCN5-eYFP-associated nuclear actin filaments, 2.5 μ g of pGCN5-eYFP was transiently-transfected into NIH 3T3 cells, either seeded in 25 mm glass-bottomed dishes for live-cell imaging or on acid-washed coverslips for fixed cells, followed by 36 hours of protein expression. The representative images were collected as a z-series using the Leica TCS SP5 MP confocal microscope (Leica Microsystems) with low laser power at 200 Hz laser-line rastering speed. Representative images were subjected to digital decovolution using Volocity 4 (Improvision) as previously stated, in addition to 3D-rendering through voxel visualization using Imaris version 5.5.1 (Bitplane Scientific Solutions).

For the micrographs confirming the nature of the GCN5-eYFP-associated filaments, two types of images were collected. For the first, 2.5 μ g of pGCN5-eYFP was co-transfected with 0.5 μ g of pmRFP- β -actin into NIH 3T3 cells that were seeded in 25 mm glass-bottomed dishes. After 36 hours of expression, live-cell imaging was performed. The second experiment involved transfecting the same cell-line, seeded on acid-washed coverslips, with 2.5 μ g of pGCN5-eYFP. The protein was expressed for a similar time-frame, followed by fixation of the cells with 4% paraformaldehyde. These cells were then washed extensively with PBS, permeabilized with 0.1% Triton X-100 in PBS, and further washed. Staining of F-actin filaments was performed using 50 μ g/mL of TRITC-phalloidin for 40 minutes at room temperature, followed by washing in PBS, and mounting the coverslips with 90% glycerol/PBS mounting media. These image sets were also captured using the Leica TCS SP5 MP confocal microscope (Leica Microsystems) with sequential scanning of the two channels.

2.15. Analyses of GCN5-eYFP-associated Nuclear Actin Filaments in the Presence of mRFP-lamin A and/or Hoechst DNA Dye

For the images showing GCN5-eYFP-associated filaments with respect to the nuclear envelope structural protein, lamin A, 2.5 μ g of pGCN5-eYFP was co-transfected with 0.5 μ g of pmRFP-lamin A, followed by 36 hours of expression in NIH 3T3 cells. Z-series images were captured with sequential scanning and system-optimized step-sizes using the Leica TCS SP5 MP confocal microscope.

Representative images showing GCN5-eYFP-associated filaments with respect to DNA was accomplished by transiently-transfecting NIH 3T3 cells with 2.5 μ g of pGCN5-eYFP for 36 hours, followed by fixing of the cells as stated previously. The cells were then incubated with Hoechst dye 33258 (4 μ g/mL) for 15 minutes at 37°C, followed by washing. The coverslips were fixed and images were captured using a Leica DMI 6000 B widefield fluorescence microscope.

Similarly, for the representative micrographs showing the spatial arrangement of GCN5-eYFP-associated filaments with respect to both mRFP-lamin A and DNA, the cells were transfected with similar quantities of plasmid DNA as described above, fixed, then stained with Hoechst dye 33258 (4 μ g/mL). These image sets were also captured as system-optimized z-series using the Leica DMI 6000 B widefield fluorescence microscope.

Images representing the temporal effect of Hoechst dye on GCN5-eYFPassociated nuclear filaments were captured using the Leica DMI 6000 B widefield fluorescence microscope, immediately following the exchange of normal media with media containing Hoechst dye 33258 (4 μ g/mL). The eYFP and DAPI channels were captured sequentially as a temporal, system-optimized z-series, with 5 minutes per timepoint. Following image-series capture, each of the micrographs described in this section was deconvolved using Volocity 4 (Improvision), and 3D voxel-rendered using Imaris version 5.5.1 (Bitplane Scientific Solutions). All movies from these studies (shown as Supplementary Material) were also created using Imaris 5.5.1.
3. RESULTS & DISCUSSION

3.1. GCN5 is a Shuttling Acetyltransferase

Using a fluorescence recovery after photobleaching (FRAP) assay designed specifically to assess the ability of a protein of interest to shuttle to and from the nucleus, as first described by Howell *et al.* [69], Figure 1 shows the ability of GCN5-eYFP to dynamically shuttle between the nucleus and the cytoplasm of a natural mouse NIH 3T3 bikaryon (a cell with two nuclei). Panel A shows an image of the cell following two prebleach scans, while panel C is a post-bleach image (t=0) immediately following successive scans of a high intensity laser that was used to bleach the fluorescent fusion proteins in the acceptor nucleus and the cytoplasm. Over the time-course of 15 minutes following the bleaching event, unbleached GCN5-eYFP molecules are observed to recover into the acceptor nucleus, as seen in panels E, F and G. This indicates that recombinant GCN5 is able to import and export to and from the nuclear compartment. Corresponding differential interference contrast (DIC) images are also shown in this figure to illustrate the health of the cell during the time-course of the experiment (panels B, D, F, H, and J).

3.2. GCN5 has a Bipartite NLS and a Putative NES

Figure 2A shows the amino acid sequence of human GCN5 (hGCN5). The putative bipartite nuclear localization signal (NLS), $_{433}$ KRTLPENLTLEDAKRLRV₄₅₀, is highlighted in red. Additionally, a potential CRM1-dependent nuclear export signal (NES) was identified in the carboxy-terminus of the protein, and has the sequence $_{821}$ LEKFFYFKL₈₂₉ (highlighted in blue). The black, bolded amino acids represent those which were mutated to test the validity of these sequences. Figure 2B compares the percent nuclear localization for each of the labeled constructs (GCN5-eYFP versus the NLS- and NES-mutants), using the eYFP vector as a control (n=3, N=30). The results confirm the presence of a NLS in GCN5, where wild-type GCN5-eYFP is approximately 45% more nuclear than the NLS-mutant (p= 2.33×10^{-6}). Additionally, the results suggest the presence of a NES in the carboxy-terminus of GCN5, since the difference between percent nuclear localization of the wild-type and NES-mutant is statistically significant (p= 5.0×10^{-4}). Figure 2C shows representative images of each construct.

3.3. GCN5 Lacks an Internal NES, but its Export from the Nucleus is Regulated by IκB

Figure 3 shows the results of four separate leptomycin B (LMB) experiments that

were performed to further assess the functionality of the putative NES in GCN5; each assay consisted of three independent experiments where N=30. These assays focused either on the wild-type GCN5-eYFP or the NES-mutant, GCN5-NESm2-eYFP, proteins. In each case, mRFP-IkB was used as either an internal or an external positive control, while the percent nuclear localization of each protein was quantified as described in "Materials & Methods". IKB is used to show the efficacy of the LMB treatment since it has a well-defined CRM1-dependent NES. Figures 3A and 3B use LMB to test the putative NES in wild-type GCN5-eYFP. In Figure 3A, where IkB serves as an external control, there is a statistically significant difference between the non-treatment and treatment conditions for GCN5-eYFP (p=0.032). This suggests that GCN5 may have a functional NES, although the results are not conclusive when one compares this difference to that obtained with the control protein, IkB. There is a highly significant difference between the two conditions for this positive control ($p=1.8 \times 10^{-4}$), as would be expected for a protein containing a functional NES of this type. Figure 3B shows the results obtained when $I\kappa B$ was used as an *internal* control. In the absence of treatment, GCN5-eYFP's nuclear signal decreases considerably when co-expressed with IkB (compare Figures 3A and 3B; 61.7% versus 43% nuclear localization of GCN5-eYFP, respectively). Thus, although there is a highly significant difference seen in this case between the non-treatment and treatment conditions ($p=9.18 \times 10^{-6}$), this is proposed to be due to the export of GCN5-eYFP from the nucleus by mRFP-IkB, using its known NES, in the absence of treatment. IkB is known to regulate the export of another transcription factor, NF-kB. In contrast, LMB treatment is presumed to sequester this complex in the nucleus; this would be due to CRM1's inability to mediate the export of IkB because of its inhibition by LMB.

Using site-directed mutagenesis, the last two hydrophobic residues in the putative NES of GCN5 were mutated to alanines to create GCN5-NESm2-eYFP (see Figure 2A for the GCN5 amino acid sequence); if this sequence is a functional CRM1-dependent NES, these mutations would be expected to inactivate its function. Figure 3C shows the results of treating mutant-expressing cells with LMB, with mRFP-IKB serving as an external control. This figure shows a lack of a statistically significant difference between the non-treatment and treatment conditions for GCN5-eYFP (p=0.195). In contrast, there was a highly significant difference between the two conditions for the mRFP-IkB control protein $(p=1.11\times10^{-5})$, demonstrating that the LMB treatment was effective. Although these results appear to suggest that GCN5 *does* have a functional CRM1-dependent NES, Figure 3D demonstrates otherwise. In this experiment, where mRFP-IkB was used as an internal control, the GCN5-NESm2-eYFP protein was capable of nuclear export, similar to wild-type GCN5-eYFP (compare Figures 3C and 3D; 65.9% versus 45.5% nuclear localization). Furthermore, in the presence of the mRFP-IkB control protein, this mutant showed similar nuclear localization in the non-treatment condition to that seen for wildtype GCN5-eYFP (compare 43% versus 45.5% nuclear localization for the wild-type and mutant proteins in Figures 3B and 3D, respectively). These figures demonstrate that this mutant is able to export from the nucleus with a comparable efficiency to the wild-type construct in the presence of IkB. Moreover, in Figure 3D, the results show that upon treatment with LMB, like wild-type GCN5-eYFP, there is a highly significant difference

between the non-treatment and treatment conditions (p=0.0001). Together, these results further support the idea that GCN5 lacks an internal CRM1-dependent NES, but that its nuclear export is regulated by IkB's NES.

Figure 4 compares the nuclear localization of each of the wild-type, NLS-mutant and NES-mutant forms of GCN5-eYFP in the presence of different permutations of mRFP-IkB. In its wild-type form, upon co-expression with the mRFP vector, GCN5eYFP exhibits 72% nuclear fluorescence. However, upon co-expression of the wild-type proteins, GCN5-eYFP and mRFP-IKB, GCN5-eYFP's nuclear fluorescence is reduced to 39%, further suggesting that IKB is mediating GCN5's export from the nucleus. IKB has several ankyrin repeats which have been shown to mediate its interaction with NF-KB; specifically, the first ankyrin repeat has been demonstrated to confer the strong inhibitory property of IKB on this transcription factor [147]. Additionally, Sachdev et al. [73] have suggested that the second ankyrin repeat mediates Ran-independent nuclear import of IKB. We proposed that this second ankyrin repeat may be responsible for mediating the interaction between IKB and GCN5. In support of this hypothesis, Figure 4 shows that upon co-expression of wild-type GCN5-eYFP and a mutant mRFP-IKB construct lacking the second ankyrin repeat (mRFP-IkB-dANK2), GCN5-eYFP's percent nuclear fluorescence is restored to 69%, which is comparable to GCN5-eYFP's nuclear fluorescence when co-expressed with the mRFP vector alone, at 72% (p=0.081). Moreover, when wild-type GCN5-eYFP is co-expressed with a NES-mutant form of IkB [148,149], mRFP-IkB-NESm1, GCN5's nuclear fluorescence returns to 71%, further implicating IKB's NES in the ability to regulate GCN5's export. However, when the NLS-mutant form of GCN5, GCN5-NLSm-eYFP, is co-expressed with mRFP-IkB-NESm1, the former protein's percent nuclear localization is only 14% compared to 11.6% when GCN5-NLSm-eYFP is co-expressed with the control mRFP vector. Although the difference has a high statistical significance (p=0.007), the biological effect is relatively minor in comparison to the other results using the NES-mutant of IKB. This is likely because the NLS in GCN5 is more efficient than the NES in IKB. In addition, once GCN5 is in the nucleus, it has an affinity for DNA-wrapped histones where it is most likely recruited into the STAGA complex. This supposition could also explain the reason why wild-type GCN5-eYFP remains considerably nuclear (39%) in the presence of wild-type mRFP-IKB, although it is still drastically lower than when GCN5-eYFP is co-expressed with a control protein. Substantiating the fact that GCN5 lacks an internal NES, GCN5-NESm2-eYFP's co-expression with mRFP gives a similar nuclear fluorescence (72%) to when wild-type GCN5-eYFP is expressed with mRFP (73%) (p=0.14). Interestingly, the difference in the percent nuclear localization between the conditions where wild-type versus the NES-mutant of GCN5 was co-expressed with mRFP-IkB (38.4% versus 45.9%, respectively) was highly significant (p=0.001). While the results displayed statistical significance, the effect observed was weak relative to other studies of NES mutations in proteins, or in our studies of LMB treatment of IKB. The mutations in the putative NES of GCN5 may have affected the interaction between GCN5 and IKB, such that IKB was not as efficient at mediating its export.

3.4. Endogenous GCN5 Interacts with Recombinant IKB in a GST Pulldown Assay

We first performed a control GST pull-down assay to confirm that our recombinant I κ B-GST protein was able to successfully pull-down endogenous NF- κ B, of which I κ B is the predominant inhibitor. Figure 5A panel a shows the GST and I κ B-GST proteins, in addition to the beads alone control. Figure 5A panel b shows that, as expected, only the I κ B-GST recombinant protein is able to pull down endogenous NF- κ B (compare with panel a). This indicates that the recombinant I κ B protein is in the proper conformation to bind to a known protein partner. The slightly visible band below the NF- κ B protein band in panel b is most likely cross-reactivity between the anti-NF- κ B antibody and the I κ B portion of the fusion protein, since there is no cross-reactivity seen in the lane with GST alone (compare with panel a). Since the NF- κ B antibody is polyclonal and I κ B is known to interact with NF- κ B, it is possible that the antigen protein was not generated properly, or else NF- κ B and I κ B may share a similar epitope.

Following the determination that recombinant I κ B-GST is able to bind a known partner, we tested its ability to bind our protein of interest, GCN5. The first blot of Figure 5B panel a shows 10% input of the nuclear HEK 293 extract; the two bands observed are consistent with the predicted molecular weight of ~95 kDa, and are thought to be different isoforms of GCN5. In accordance with this proposal, there have been multiple differentially-spliced forms of *GCN5* transcripts found in both human and mouse cells, which are thought to result in two different isoforms of GCN5[150]. The short form (GCN5S) is similar in length to yGCN5, while the long form (GCN5L) is thought to be the predominantly-expressed isoform in human and mouse cells, and appears to be the form most often found within the mammalian HAT complexes [148,149].

The second blot in Figure 5B panel a was treated with anti-GCN5 antibody. As expected, there was no interaction between GCN5 and the beads alone control (compare with panel b). The protein band seen in the lane with the GST control (compare with panel b) suggests cross-reactivity between the GCN5 antibody and the GST moiety, perhaps due to the high molarity of GST protein. Importantly, it can be seen that endogenous GCN5 is able to bind to IKB-GST (compare with panel b), and interestingly, it shows a stronger interaction with the short form of GCN5 (GCN5S). This result may further explain the lack of total nuclear export of GCN5-eYFP in the presence of mRFP-IKB, as this study has focused solely on the long variant of GCN5 (GCN5L), which has an extended amino-terminal domain [151]; it is possible that this domain may somehow partially occlude the region in which IKB normally binds to GCN5, thereby decreasing the export efficiency of the long GCN5 variant. Moreover, in contrast to the previous conclusion that GCN5-eYFP was incapable of binding to the Δ ANK2-mutant of IKB (see Figure 4), this figure shows that GCN5 is capable of interacting with the recombinant IKB-AANK2-GST protein. Furthermore, the strength of the interaction between the AANK2-mutant and GCN5 qualitatively appears to be similar to that observed for wildtype IKB-GST. An explanation for this occurrence is provided following an important observation noted in the subsequent figure. The blot in panel b indicates the presence of the GST proteins, each at their expected molecular weights of 25, 63 and 62 kDa, respectively.

3.5. GCN5 Interacts with IkB in vivo, as Shown by BiFC

The results in Figure 6 were obtained using a protein complementation assay (PCA), specifically bimolecular fluorescence complementation (BiFC). Each of the individual constructs used in this experiment were expressed in Figure 6A to act as controls; these images demonstrate both the proteins' normal localization, as well as the lack of signal in the eYFP channel (indicating a lack of a protein-protein interaction) in the absence of potential protein partners. Likewise, Figure 6B shows representative images of mRFP-NVenus-GCN5 co-expressed with the control protein, mCer-CVenus-Htt 81-588; this protein has been shown to shuttle, but does not interact with the GCN5 protein, as only background levels of protein complementation are observed (eYFP channel) (see Materials & Methods regarding the estimation of background levels for this assay). Furthermore, it can be seen that mRFP-NVenus-GCN5 maintains a significant amount of nuclear signal in the presence of this huntingtin construct, demonstrating that this huntingtin fragment is not mediating the export of GCN5-eYFP.

Figure 6C shows representative images of the co-expression of mRFP-NVenus-GCN5 with mCer-CVenus-I κ B. While mRFP-NVenus-GCN5 is slightly less nuclear than in control images (compare with Figure 6A panel d, and Figure 6B panels a, d, g, and j), we see that mCer-CVenus-I κ B is mostly observed in the cytoplasm (mCer channel), as is the BiFC signal (eYFP channel). Interestingly, panels d-f show a case where mCer-CVenus-I κ B is in both the nucleus and the cytoplasm (mCer channel), consistent with mRFP-NVenus-GCN5's cellular localization (mRFP channel). Furthermore, as evidenced by the signal in the eYFP channel, these proteins are interacting within both of these compartments. Each of these micrographs, therefore, adds support to the proposal that I κ B can mediate GCN5's export from the nucleus, while retaining the exported protein in the cytoplasm.

Figure 6D is a collection of representative images of mRFP-NVenus-GCN5 coexpressed with mCer-CVenus-I κ B- Δ ANK2. Recall that results from Figure 4 suggested that this mutant was incapable of interacting with GCN5, due to the restoration of its normal percent nuclear localization. In contrast, the results of Figure 5 demonstrated that a recombinant delta-ankyrin repeat mutant of I κ B is, in fact, capable of binding to GCN5, and qualitatively to a similar extent as wild-type recombinant I κ B. While Figure 6D panels a-c show that mCer-CVenus-I κ B- Δ ANK2 can have a similar protein distribution to the wild-type I κ B construct, these panels further indicate that this mutant is able to interact with GCN5, and importantly, it can occur within the cytoplasm, similar to that which is observed with wild-type I κ B. However, more often than this occurrence, and very interesting, was the finding that the I κ B- Δ ANK2 protein was able to not only enter the nucleus but appeared to be trapped there, where it significantly interacted with GCN5, as is shown in Figure 6D, panels d-1. Thus, it would seem that by deleting the second ankyrin repeat in I κ B, this mutant may be somehow retained in the nucleus with GCN5, perhaps due to an inability to bind an unknown factor involved in mediating the export of the GCN5:I κ B complex. Interestingly, the CRM1-dependent NES in I κ B (residues 45 – 54) is relatively close in the primary sequence to the second ankyrin repeat (residues 105 – 127). Thus, efficient export of the GCN5:I κ B complex may require a steric interaction between the NES of I κ B and its second ankyrin repeat that gives the complex a higher affinity for the CRM1 export factor. Furthermore, the fact that these two complexes appear to be sequestered in the nucleus could explain the initial results, where GCN5-eYFP's percent nuclear localization was significantly restored following co-expression with the Δ ANK2-mutant of I κ B (see Figure 4), and corroborates the results shown in Figure 5B, which demonstrated that this mutant is still capable of interacting with GCN5.

Finally, Figure 6E shows representative images of the results of co-localization between mRFP-NVenus-GCN5 and mCer-CVenus-I κ B-NESm1. Consistent with the results shown in Figure 4, it was found that in the majority of transfected cells the GCN5 protein was predominantly retained in the nucleus, where it showed significant interaction with the NES-mutant of I κ B. Thus, we conclude that GCN5 does, in fact, bind I κ B, while the short variant of GCN5 may bind to this transcription regulator more efficiently, although the function of this variant is not yet known. Our results show that I κ B is capable of mediating GCN5's export through its amino-terminal, CRM1-dependent NES, which may potentially involve the second ankyrin repeat of I κ B for efficient export.

3.6. GCN5 can be Exported by the mRNA Export Factor, TAP/NXF1

As an unexpected result, the mRNA export factor, TAP/NXF1, was similarly found to facilitate the export of GCN5 from the nucleus, and to an even greater extent than IkB as shown in Figure 7. Figure 7A shows the percent nuclear localization of both proteins with the control vectors (eYFP or mRFP, respectively). As can be observed, there is a significant difference between the percent nuclear localization of GCN5-eYFP in the presence of the control protein (mRFP; 72%), versus 24% upon co-expression with mRFP-TAP/NXF1 (p<0.001). Correspondingly, the mRFP-TAP/NXF1 protein is significantly less nuclear when co-expressed with GCN5-eYFP versus the eYFP protein alone. Figure 7B shows representative images of each of the co-expressed proteins, which compliments the quantitative results. Thus, these results show that TAP/NXF1 can export GCN5 from the nucleus, and may act alongside IkB in the regulation of its activity by its compartmentalization in the cytoplasm. This link between GCN5 and TAP/NXF1 is very interesting: while GCN5 is known to facilitate transcription of certain genes, hnRNPs are involved in the processing of the nascent mRNA transcripts. These processed transcripts are then exported from the nucleus by the TAP/NXF1 receptor. Thus, there may be an indirect or direct association between GCN5 and the processed transcript.

3.7. Control Proteins Do Not Mediate the Export of GCN5-eYFP from the Nucleus

To validate the capacity of both $I\kappa B$ and TAP/NXF1 to mediate or facilitate the export of GCN5, we tested the ability of two other proteins that are known to shuttle between the nucleus and the cytoplasm, to relocate GCN5-eYFP to the cytoplasm. Figure 8A shows representative images of GCN5-eYFP co-expressed with ataxin-3-Q28-mRFP. Despite the ability of ataxin-3 to shuttle, GCN5-eYFP remained predominantly in the nucleus (panels b, e, and h), while the merged images (panels c, f and i) show co-localization of these two proteins only within the nuclear compartment. Likewise, a huntingtin fragment, Htt-Ex1-wt-mRFP, that also shuttles between the two compartments was unable to qualitatively alter the nuclear localization of GCN5-eYFP. This is shown in Figure 8B, where the GCN5-eYFP signal is predominantly in the nucleus (panels b, e, and h) and co-localization between these proteins only occurs within this compartment (panels c, f, and i). Thus, our results showing that GCN5's export can be regulated by IkB and TAP/NXF1 appear to be valid, as two other proteins that are known to shuttle were unable to alter GCN5's nuclear localization.

3.8. GCN5-eYFP is Associated with F-actin Filaments in the Nucleus

As previously stated, actin has recently been acknowledged to exist in the nucleus, where it has been demonstrated to be involved in transcription regulation and mRNA export. Interestingly, upon over-expression of GCN5-eYFP, ~10% of the transfected cells consistently show nuclear filaments that are highlighted by the GCN5-eYFP protein, as observed in Figure 9A. These filaments, shown as both 3D volume-rendered and isosurfaced images (panels a and b, respectively), span across the entire nucleus in various directions, while they are often seen to wrap around the periphery of the nucleus; however, they appear to routinely exclude the nucleoli. These GCN5-eYFP-associated nuclear filaments in the nucleus, as shown in Figure 9B, and as well, are labelled by the specific F-actin-binding toxin phalloidin as shown in Figure 9C. The merged images in both cases show the overlap in signal between the mRFP/TRITC and eYFP fluorophores and dye.

With these results, we believe that it is the higher expression levels of GCN5eYFP that enable the F-actin filaments to be appropriately visualized. In the case of endogenous levels of GCN5, these polymerized filaments are not readily seen, particularly by the use of the specific F-actin-binding toxin, phalloidin; however, a number of studies have suggested the existence of these actin filaments in the nucleus. As such, we have proposed that it is GCN5's acetyltransferase activity that, at least partially, accounts for the polymerization of actin in the nucleus, and that as a result of overexpression of this enzyme we are able to efficiently visualize these filaments in the nucleus. Importantly, these filaments appear to be enhanced by the over-expression of a nuclear protein with a known enzymatic activity that affects actin polymerization in the cytoplasm in other protein contexts.

3.9. GCN5 Interacts with Nuclear β -actin in vivo, as Shown by BiFC

BiFC was used to confirm the physical interaction between transiently-expressed GCN5 and β-actin. Figure 10 panels A-D are control images showing each of the channels when mCer-CVenus-B-actin was expressed alone. Note that in the absence of the GCN5 protein (mRFP channel), there is very little fluorescent signal in the cell nucleus (mCer channel). Furthermore, as expected, in the absence of a potential interaction partner (mRFP channel), there is a lack of BiFC signal (eYFP channel). The set of control images for the expression of mRFP-NVenus-GCN5 alone was shown in Figure 6A panels d-f; despite the fact that these particular images were captured using a widefield fluorescence microscope, the important point to consider is that there is no signal in the eYFP channel in the absence of an interaction partner. Finally, Figure 10, panels E-H show three NIH 3T3 cells co-expressing mRFP-NVenus-GCN5 and mCer-CVenus- β -actin: panel E shows the expression of mRFP-NVenus-GCN5 in these three cells (mRFP channel) (note the difference in protein expression levels between the middle and the two outer cell nuclei). Panel F shows the localization of the mCer-CVenus- β -actin fusion-protein (mCer channel) in the presence of the GCN5 construct. As can be seen, there is β -actin present in the nuclei expressing higher levels of the GCN5 fusion-protein, while there is very little observable signal in the nucleus expressing lower levels of GCN5. Correspondingly, the BiFC signal (panel G; eYFP channel) is predominantly present in the nuclei of the two outer cells, while there is very minimal BiFC in the centre nucleus, and the cytoplasmic compartments. Panel H shows the merged image of each of the channels, where it is evident that GCN5 is, in fact, interacting with β-actin, and specifically in the nucleus, which coincides with its normal residence and function in this compartment.

Noteworthy is the fact that with these constructs, we were unable to find cells containing the nuclear filaments that had been previously observed. There are a number of possible explanations for this finding: firstly, the presence of the additional fluorophores on each of the proteins may sterically hinder GCN5's ability to catalyze the acetylation, and hence, the polymerization of nuclear actin filaments (evidence for an enzymatically-dependent relationship between GCN5 and nuclear β -actin are shown below). Secondly, in contrast to the previous constructs, for this experiment GCN5 was tagged on is amino-terminus, which may have also affected its catalytic activity. Moreover, actin polymerization has been shown to be more efficient when the monomers are amino-terminally acetylated; thus, the addition of a second exogenous protein on the amino-terminal end of this protein could have resulted in less actin polymerization, despite the presence of GCN5. Lastly, an important advantage of this PCA, in comparison to other types, is the ability to visualize transient interactions; this is due to the fact that the complex made by joining the two fluorescent-protein fragments is stable

and resists dissociation [15]. Thus, this exact characteristic could be one of the principle reasons for the lack of nuclear-filament formation, as later results suggest a correlation between the presence of these filaments and the ability of GCN5-eYFP to form transient associations with them.

3.10. GCN5's Acetyltransferase Activity is Responsible for the Formation of Nuclear F-actin Filaments

The catalytic residue of yeast GCN5 (yGCN5), an invariant glutamic acid (E173), has been known for years [17], while the equivalent residue in mouse GCN5 was recently confirmed to similarly be responsible for this homologue's catalytic activity [152]. The sequence alignment for yeast, mouse and human GCN5 is shown in Figure 11A. Thus, we decided to use the knowledge of this catalytic residue to test if it was, in fact, GCN5's acetyltransferase activity that was responsible for the formation of the nuclear F-actin filaments. In Figure 11B, an assay was performed in which, following 36 hours of protein expression, the number of transfected cells harbouring visual nuclear filaments was counted for each of the wild-type and mutant-GCN5 (GCN5-E575Q-eYFP) constructs. Three and ten independent experiments were performed for each of the wild-type and mutant constructs, respectively, where in each case N=100. The histogram in Figure 11B shows the percentages of transfected cells with filaments for the individual constructs, where the wild-type GCN5-eYFP construct was significantly more correlated with the production of visual nuclear filaments than the mutant protein (12.3% versus 0.33%, respectively; p<0.001). Representative images are shown in Figure 11C; panels a-d show the filaments typically seen in a GCN5-eYFP-expressing cell, while panels e-h show various cells expressing the mutant, GCN5-E575Q-eYFP, where there was either a lack of visual filaments or, unlike that seen with the wild-type GCN5-eYFP construct, the filaments were very poorly defined. Thus, these results suggest that it is, in fact, GCN5's catalytic activity that is responsible for the formation of these nuclear F-actin filaments, which is consistent with the previously-noted increase in the efficiency of actin polymerization upon its amino-terminal acetylation.

3.11. The GCN5-eYFP-associated F-actin Filaments are Spatially-Separate from the Nuclear Envelope and DNA, but are Transient and Dependent on the Structural Integrity of DNA

Figure 12A shows the results of the co-expression of GCN5-eYFP and mRFPlamin A. Lamin A is an intermediate filament protein that weaves together with similar proteins to form a shell that lines the inner surface of the nuclear envelope, and is thus, a useful protein for highlighting the edge of the nucleus. Using both 3D volume-rendered (panels a-c) and isosurfaced (panels d-f) images, it can be noted that, for the most part, the GCN5-eYFP-associated filaments are spatially separate from the nuclear envelope (see panels c and f). Importantly, however, in some cases it was found that these filaments could terminate in areas overlapping with lamin A locations.

Similarly, using Hoechst 33258 DNA stain, which preferentially binds A:T-rich heterochromatic regions, Figure 12B suggests that these filaments are also spatially uncoupled from DNA. A representative cell is shown as both volume-rendered (panels a-c) and isosurfaced (panels d-f) 3D images. Figure 12B panels c and f are the merged images showing a lack of spatial interaction between these two components.

Furthermore, co-expression of GCN5-eYFP with mRFP-lamin A, and subsequent fixing and staining of the DNA with Hoechst dye suggested a lack of a spatial interaction between each of the three components, as shown in Figure 12C. Again, these nuclei are represented as 3D-volume (panels a-d) and isosurfaced images (panels e-h), where panels d and h show the merged images of these three components.

Despite these results, it is important to note that the ability to observe a physical interaction between two or more components within a cell is a function of the resolutionlimit of the microscopic technology being utilized. As well, the visualization of DNA is limited by the properties of the particular DNA stain used; for instance, Hoechst dye 33258 was used in this experiment, and has been shown to preferentially bind to A:T-rich regions in the minor grooves of open chromatin [153]; thus, only specific regions of DNA are highlighted. However, despite these limitations, it is interesting to note from these figures that some of the stained DNA appears to be confined between the nuclear envelope and the filaments; in agreement with a number of studies showing the recruitment of actively-transcribed DNA to the nuclear periphery, perhaps this polymerized actin acts as a structural matrix that retains these genes within the region closest to the NPCs.

In opposition to the speculation that these nuclear filaments are an overexpression artifact, Figure 13 demonstrates that their existence is transient and dependent on the structural integrity of DNA. Using volume-rendered (Figure 13A) and isosurfaced image sequences (Figure 13B), this figure shows that over a period of 35 minutes following the addition of Hoechst dye the filamentous structures eventually lose their structural integrity, as evidenced by the dispersion of the GCN5-eYFP signal (Figure 13A panel f). A time-lapsed, rotational 3D image sequence can also be seen as a supplementary video (Video 4). This ablation of GCN5-eYFP-assoicated nuclear filaments is significant due to the fact that staining of live cells with DNA-intercalating compounds can be toxic through the alteration of DNA structure. Furthermore, these dyes are capable of inhibiting DNA replication and transcription, for instance, by the prevention of TATA protein-binding [154]. Thus, despite the lack of an observable 3D spatial interaction between DNA and these filaments, this result suggests that these structures may be indirectly associated with DNA; this would most likely be through the transcription of various genes, as actin and actin filaments have previously been implicated in processes of mRNA transcription and nuclear export of nascent transcripts.

Moreover, a number of recent studies in yeast have demonstrated the specific translocation of actively-transcribed genes to the nuclear periphery. For example, one study using *Saccharomyces cerevisiae* has shown that the protein Mlp1, a component of the inner nuclear basket of the NPC, specifically interacts with components of the SAGA

histone acetyltransferase complex, including GCN5, Ada2, and Spt7, within the promoter regions of the *GAL* genes [155]. These findings suggest a physical interaction between SAGA and the NPC, and support previous microscopic results that demonstrated the translocation of *GAL* genes to the nuclear periphery upon activation [155]. Furthermore, using RNA-fluorescence in-situ hybridization, this study demonstrated that *GAL* loci that were constrained to the nuclear periphery had higher transcriptional activity than those remaining in the intranuclear space [155], while these specific genes were found to be tethered to the NPC protein Nup1 by the Sus1 and Ada2 proteins of the SAGA complex, as well as Sac3, a mRNA export factor [145]. These results, which have been proposed to enable the prompt export of the nascent transcripts through the NPCs, are even more intriguing given the fact that some of the GCN5-eYFP-associated actin filaments appear to confine a portion of the stained DNA to the nuclear periphery. Thus, we propose that these filaments may have a structural role in the recruitment and positioning of transcriptionally-active DNA to the periphery of the nucleus, particularly by acting as a nuclear matrix that limits the nucleoplasmic space in which the DNA can reside.

3.12. GCN5-eYFP Molecules Have a Strong and Randomized-affinity for the Nuclear F-actin Filaments

Finally, FRAP analysis was used to monitor the recovery of GCN5-eYFP proteins over the length of a single nuclear filament. Figure 14A shows the ROI prior to bleaching, while panels B-H illustrate the recovery of GCN5-eYFP proteins over the entire length of this filament during a 15 minute time-period. This rapid recovery, which begins within seconds following the bleaching period, lends support to the idea that GCN5 is involved in maintaining the structural integrity of these filaments, presumably through its acetyltransferase activity, rather than being an inert or immobile component of these filaments. Importantly, these filaments observed with GCN5-eYFP overexpression are unlikely to be over-expression artifacts, as this result shows that they are not static aggregates of over-expressed protein, but that they have a directed affinity for these filaments.

Figure 14 panels I and J show the positions and respective fluorescence recovery curves of three distinct ROIs along the length of the bleached region of the filament; as can be noted, despite differences in the initial average intensities of the fluorescent protein populations at each of these ROIs, the slopes of the recovery curves are qualitatively similar. Thus, these results demonstrate that the rapid association of GCN5-eYFP with these filaments is due to a randomized-affinity between GCN5 and β -actin, most likely through a diffusive process, as opposed to interacting with the ends of the filaments and tracking along their length. This observation further substantiates the theory that GCN5 is involved in preserving a polymerized state of nuclear actin.

4. CONCLUDING REMARKS

Human GCN5 (hGCN5) was traditionally believed to only reside within the nuclear compartment, where it is known to be involved in the process of histone acetylation that facilitates transcription activation. However, in this study we have shown, using FRAP analysis, that this protein is not only able to enter the nucleus, specifically by means of a classical bipartite NLS via the karyopherin alpha/beta pathway, but is also capable of exporting from the nucleus. Rather than containing an internal CRM1-dependent NES, we have shown that the nuclear export of GCN5 is regulated by IkB and the mRNA export factor, TAP/NXF1. In contrast, its subcellular localization was unchanged upon co-expression with the control proteins ataxin-3-Q28-mRFP and Htt-Ex1-wt-mRFP.

By co-expressing specific mutants of $I\kappa B$ with GCN5, we further demonstrated that the localization of GCN5 is, at least partially, dependent on a functional NES within I κB . Moreover, GST pull-down assays confirmed an *in vitro* interaction between these two proteins, while recombinant I κB preferentially interacted with the short isoform of GCN5 (GCN5S); as it was the long isoform (GCN5L) that was used in these studies, this finding might partially explain the inability of I κB to completely export GCN5 from the nucleus. Importantly, PCAs using fluorescent protein fragments further validated the GCN5:I κB interaction *in vivo*. While the wild-type I κB construct was found to preferentially interact with GCN5 in the cytoplasm, where it is expected to inhibit its activity, the I κB -NESm1 construct was unable to redistribute GCN5 from the nucleus to the cytoplasm, despite an interaction between the two proteins as shown by significant BiFC signal.

Furthermore, it was initially concluded that the I κ B-mutant lacking its second ankyrin repeat was unable to interact with GCN5, due to its inability to alter the nuclear localization of GCN5; however, the results of the GST pull-down assay and BiFC experiments suggested otherwise. Together the results suggest that this I κ B-mutant is capable of interacting with GCN5, but that deletion of this specific repeat may interfere with the ability of this complex to be properly exported from the nucleus. For instance, this repeat may be involved in binding to another protein that facilitates the nuclear export of the GCN5:I κ B complex; specifically, the long variant of GCN5 that was used in this study may require an external factor to enhance the efficiency of its nuclear export. Interestingly, the CRM1-dependent NES in I κ B (residues 45 – 54) is relatively close in the primary sequence to the second ankyrin repeat (residues 105 – 127), such that there may be a structural conformation that involves an interaction between these two sequences; this interaction could, in turn, improve the affinity of the complex for its export factor, CRM1.

Prior to this study, the only known function for hGCN5 involved histone acetylation, an activity of which has been tightly correlated with its presence within co-activator complexes such as STAGA. However, our results indicate that GCN5 is also involved in the acetylation, and hence polymerization, of nuclear β -actin.

Upon over-expression of GCN5-eYFP, we routinely observed nuclear filaments in ~10% of the transfected cells. Our co-localization studies demonstrated that these filaments are made up of nuclear β -actin and are of the polymerized form, as evidenced by TRITC-phalloidin-staining within the nucleus. Furthermore, a specific PCA was used to demonstrate an *in vivo* interaction between these two proteins. Importantly, using a catalytically-inactive GCN5-mutant, GCN5-E575Q-eYFP, we showed that there was a significant decrease in the number of transfected cells harbouring nuclear filaments; moreover, FRAP analysis of a single filament showed that GCN5-eYFP has a strong, disorganized binding-affinity for these structures, as it does not specifically track along their lengths. Together these results strongly suggest that GCN5 actively sustains the polymerized form of actin within the nucleus by perpetually maintaining this protein in its acetylated form. Importantly, despite over-expression of the recombinant GCN5-eYFP protein, our results show that these filaments are transient and are highly dependent on the structural integrity of DNA, as they are readily disrupted by the addition of an intercalating DNA stain such as Hoechst dye, which is toxic to live cells.

Additionally, we found that the GCN5-eYFP-associated nuclear filaments are spatially separate from both the nuclear envelope and DNA, as they do not appear to cross in 3D space. We did note, however, that in some instances these filaments terminate in areas overlapping with lamin A locations, and that these structures often appear to confine heterochromatic DNA to the nuclear periphery. These results are important in refuting the argument that the filaments are due to an over-expression artifact, since the recombinant protein does not form random protein aggregates, but appears to be somehow organized at the cellular level.

Interestingly, nuclear actin has been shown, in several studies, to be involved in transcription by all three mammalian RNA polymerases. And furthermore, recent studies have noted the recruitment of certain actively-transcribed genes to the nuclear periphery. Thus, we hypothesize that GCN5 increases and facilitates transcriptional activity and processing in two ways: 1) through its previously-described histone acetyltransferase activity; and 2) through acetylation, and hence formation, and structural maintenance of nuclear actin filaments. These filaments are proposed to be involved in facilitating the transport of either actively-transcribed genes or the transcribed mRNA to the nuclear periphery, where it can be efficiently exported. This may occur through an acto-myosin motor, as there has recently been a myosin protein found to exist specifically within the nucleus.

In conclusion, in addition to its previously-described role as a histone acetyltransferase, this study has shown that the catalytic activity of hGCN5 allows it to also function as an actin-modifier, specifically by increasing the efficiency of actin polymerization within the nucleus. These important functions, which have both been associated with transcriptional processes, are expected to require appropriate levels of regulation: similar to the type of regulation that is seen with a variety of other transcription factors, this study has demonstrated that the function of hGCN5 is, at least partly, dependent on its cellular localization. While its nuclear import is dependent on the karyopherin alpha/beta pathway, its export appears to be regulated by both $I\kappa B$ and TAP/NXF1. This overlap in the regulation of hGCN5's nuclear export seems reasonable,

as many diseases are the result of transcription dysregulation, including cancers and certain neurodegenerative diseases, such as the polyglutamine disease Spinocerebellar ataxia type 7 (SCA7). Interestingly, hGCN5 has been shown to physically interact with the protein, ataxin-7, that in its mutated form, is responsible for SCA7 [156] (see Appendix for additional information).

Future directions for this research are two-fold, involving both $I\kappa B$ and the nuclear actin in relation to the activity of GCN5. Firstly, it would be extremely valuable to be able to show a direct interaction between $I\kappa B$ and GCN5 *in vivo* using either FRET-AB or FRET-FLIM, the latter of which is not dependent on excitation intensity or fluorophore concentration [157,158].

Furthermore, future experiments should involve determining which extracellular factor(s) are involved in stimulating the I κ B-mediated export of GCN5. TNF α is a potent stimulator of NF- κ B-export by I κ B; thus, this factor was tested for its ability to similarly induce the I κ B-dependent redistribution of GCN5. Despite using MCF7 cells, which are more adherent and hardy than NIH 3T3 cells, we had difficulties with drug-induced cell death without observing a noticeable change in the distribution of the two proteins. However, this experiment was performed using over-expressed fluorescently-tagged proteins: in such a case, without similar over-expression of the proteins involved in mediating the TNF α -stimulated signaling pathway, it would be unlikely that there would be a significant change in the localization of recombinant GCN5 before the occurrence of cell death. Thus, this experiment could be significantly improved by using endogenous levels of GCN5 and I κ B with immunofluorescence staining following different timepoints of drug treatment. Moreover, in this case, the levels of TNF α that would be required to observe an effect would presumably be much lower.

Another important experiment that should be performed to further validate the role of GCN5 in nuclear actin polymerization is to use a general HDAC inhibitor, such as Trichostatin A, which acts to inhibit histone deacetylation by binding to and chelating the zinc ion within the catalytic site of the HDAC through its hydroxamic acid group [138]. Following drug-level optimization, we propose to be able to visualize nuclear F-actin filaments resulting from the activity of endogenous GCN5, due to a decrease in the rates of deacetylation compared to control conditions. The existence of polymerized actin within the nucleus will be evaluated using TRITC-phalloidin, which if observed within this compartment, can be attributed to acetyltransferase activity, and specifically to that of GCN5 considering our previous findings. This result would also suggest that under normal cellular conditions, there is a highly regulated equilibrium involving the formation and disassembly of these actin filaments that may be maintained at concentrations that are below the resolution-limit of the microscopes employed in this study.

Furthermore, an *in vitro* assay to test the polymerization of actin by hGCN5 could be performed. This assay is done in a test tube with a limiting concentration of salts; the effect on actin polymerization would be compared using recombinant wild-type hGCN5 and its catalytically-inactive mutant. The polymerization process can then be monitored in a variety of ways: 1) since when the filaments become long enough to entangle, the solution becomes more viscous, the efficiency of polymerization could be measured as a decrease in flow rate by a viscometer; 2) it could be tested by ultracentrifugation, since Factin will pellet while G-actin will not; or 3) one can label the G-actin monomers with a fluorescent dye, the fluorescence spectrum of which will change upon actin polymerization.

Finally, in order to determine if these actin filaments are members of a nuclear acto-myosin transport process, one should co-express a fluorescently-tagged nuclear myosin I protein (NMI-eYFP-NLS) with GCN5-mCer. If these filaments are acting as tracts, then one would expect to see co-localization between the myosin proteins and the filaments highlighted by GCN5-mCer. In the event of co-localization, one could go on to test for direct interaction between the two proteins using FRET-AB and/or FRET-FLIM analysis.

5. FIGURES

Figure 1: GCN5-eYFP dynamically shuttles between the nucleus and the cytoplasm. A FRAP assay using the technique described by Howell *et al.* [159-161] illustrates GCN5-eYFP's nucleocytoplasmic shuttling ability in a NIH 3T3 bikaryon cell. Panel A shows GCN5-eYFP protein levels prior to bleaching with an Argon laser at 95% power. Panel C represents the post-bleach image at t = 0 sec. GCN5-eYFP is observed to progressively shuttle from the un-bleached 'donor nucleus' to the bleached 'acceptor nucleus' during a 15 minute post-bleach recovery period (panels E, G, and I). The corresponding DIC images, shown in panels B, D, F, H and J, demonstrate the health of the bikaryon cell over the time-period of the experiment. This experiment was performed and imaged using a Leica TCS SP5 MP confocal microscope. Scale bar represents 10 μ m.



Figure 1

Figure 2: GCN5 has a classical bipartite nuclear localization signal (NLS), and a putative CRM1-dependent nuclear export signal (NES).

A) Protein sequence for human GCN5 (hGCN5) with putative bipartite NLS (red) and putative CRM1-dependent NES (blue) (bolded, black letters represent residues mutated to alanines). B) NIH 3T3 cells were transiently-transfected with each of the GCN5-eYFP constructs, including the wild-type, mutant NLS (NLSm) and mutant NES (NESm2) variants of GCN5-eYFP. The histogram compares the percent nuclear localization (calculated as described in "Materials & Methods") each of these constructs using the eYFP vector as a negative control. *, Φ represent p<0.01; error bars represent ±1 standard error calculated from three individual experiments, where N=30. Two-tailed Student's ttest was used for statistical analysis. C) Representative images of the corresponding proteins expressed in NIH 3T3 cells (panels a-d). Images were captured on a widefield Nikon TE200-inverted fluorescent microscope. Scale bar represents 10 μ m.

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MAEPSQAPTPAPAAQPRPLQSPAPAPTPTPAPSPASAPIPTPTPAPAPAP	50
AAAPAGSTGTGGPGVGSGGAGSGGDPARPGLSQQQRASQRKAQVRGLPRA	100
KKLEKLGVFSACKANGTCKCNGWKNPKPPTAPRIDLQQPAANLSELCRSC	150
EHPLADHVSHLENVSEDEINRLLGMVVDVENLFMSVHKEEDTDTKQVYFY	200
LFKLLRKCILQMTRPVVEGSLGSPPFEKPNIEQGVLNFVQYKFSHLAPRE	250
RQTMFELSKMFLLCLNYWELETPAQFRQRSQAEDVATYKVNYTRWLCYCH	300
VPQSCDSLPRYETTHVFGRSLLRSIFTVTRRQLLEKFRVEKDKLVPEKRT	350
LILTHFPKFLSMLEEEIYGANSPIWESGFTMPPSEGTQLVPRPASVSAAV	400
VPSTPIFSPSMGGGSNSSLSLDSAGAEPMPGEKRTLPENLTLEDAKRLRV	450
MGDIPMELVNEVMLTITDPAAMLGPETSLLSANAARDETARLEERRGIIE	500
FHVIGNSLTPKANRRVLLWLVGLQNVFSHQLPRMPKEYIARLVFDPKHKT	550
LALIKDGRVIGGICFRMFPTQGFTEIVFCAVTSNEQVKGYGTHLMNHLKE	600
YHIKHNILYFLTYADEYAIGYFKKQGFSKDIKVPKSRYLGYIKDYEGATL	650
MECELNPRIPYTELSHIIKKQKEIIKKLIERKQAQIRKVYPGLSCFKEGV	700
RQIPVESVPGIRETGWKPLGKEKGKELKDPDQLYTTLKNLLAQIKSHPSA	750
WPFMEPVKKSEAPDYYEVIRFPIDLKTMTERLRSRYYVTRKLFVADLQRV	800
IANCREYNPPDSEYCRCASALEKFFYFKLKEGGLIDK	







Figure 2

Figure 3: GCN5 does not have an internal NES, but its nuclear export may be regulated by $I\kappa B$.

Leptomycin B (LMB) assays were employed to test the functionality of the putative NES in the carboxy-terminus of GCN5, with mRFP-IKB serving as either as an internal or external positive control for the effectiveness of the LMB treatment. NIH 3T3 cells were transiently-transfected with either pGCN5-eYFP (or pGCN5-NESm2-eYFP) alone, or in combination with pmRFP-IKB, and allowed to express for 24 hours. Each of the control and experimental dishes had their serum-supplemented media replaced by serum-free media, while the experimental dishes were additionally treated with 10 ng/mL of LMB for 13 hours. All histograms show the percent nuclear localization of the designated proteins in the presence or absence of LMB, with mRFP-IKB being used as either an internal or external control: A) GCN5-eYFP - mRFP-IKB used as an external control: B) GCN5-eYFP - mRFP-IKB used as an internal control; C) GCN5-NESm2-eYFP - mRFP-IKB used as an external control; D) GCN5-NESm2-eYFP - mRFP-IKB used as an internal control. * represents p<0.01, ** represents p<0.05; error bars represent ± 1 standard error calculated from three individual experiments, where N=30. Two-tailed Student's t-test was used for statistical analysis. Images were captured using a widefield Nikon TE200 fluorescent microscope.







Figure 4: GCN5-eYFP's localization can be manipulated by co-expression with permutations of mRFP-I κ B.

NIH 3T3 cells were transiently-transfected with the appropriate constructs and allowed to express for 24 hours. Following this period, live-cell imaging was performed using a Leica TCS SP5 MP confocal microscope. This histogram shows that the percent nuclear localization of GCN5-eYFP (and its NLS- and NES-mutants) is largely determined by the cellular distribution of mRFP-IKB and its corresponding NES- and Δ ANK2 (i.e. dANK2)-mutants. *, Φ represent p<0.01; error bars represent ±1 standard error calculated from three individual experiments, where N=30. Two-tailed Student's t-test was used for statistical analysis.



Figure 4

Figure 5: Recombinant wild-type IKB and its Δ ANK2-mutant both interact with GCN5 *in vitro*.

A) GST pull-down of NF- κ B p65 by GST-I κ B as a positive control showing the ability of the recombinant I κ B protein to interact with a known partner. Panel a: blotting was performed using rabbit anti-NF- κ B p65 primary antibody, followed by goat anti-rabbit IgG peroxidase conjugate; panel b: GST proteins were blotted for using mouse anti-GST-tag primary antibody, followed by goat anti-mouse HRP-conjugated secondary antibody. B) GST-I κ B and the GST-I κ B- Δ ANK2 mutant protein both interact with GCN5, while the the GST control protein does not. Panel a: blotting of GCN5 was performed using rabbit anti-GCN5 primary antibody, followed by goat anti-rabbit IgG peroxidase

conjugate. Panel b: GST proteins were blotted for in a similar manner as described above.







Figure 6: IKB and its Δ ANK2- and NESm1-mutants each interact with GCN5 in vivo.

Representative images are shown for each of the control and experimental BiFC assays; all images captured in the eYFP channel were subjected to a 0.04 second exposure level (as described in "Materials & Methods"). Parts A and B are controls: A) The individual proteins were expressed alone, with images taken of each of the mRFP, mCerulean and eYFP channels (panels a-o); B) Representative images of BiFC assays using a huntingtin fragment (Htt 81-588) that does not interact with GCN5 (panels a-1). Parts C, D and E are experimental PCAs: C) Representative images of mRFP-NVenus-GCN5 expressed with wild-type mCer-CVenus-IkB (panels a-i); D) mRFP-NVenus-GCN5 co-expressed with the mCer-CVenus-IkB- Δ ANK2 mutant (panels a-1); E) mRFP-NVenus-GCN5 co-expressed with a 512x512 resolution using a widefield Nikon TE200 fluorescent microscope. Scale bar represents 10 μ m.

A)	mRFP channel	mCer channel	eYFP channel
	a	b mCer-CVenus-Htt 81-588	c
	d mRFP-NVenus-GCN5	e	f
	g	h mCer-CVenus-IkB	Ť
	j.	K mCer-CVenus-IkB-ΔANK2	
	m	n mCer-CVenus-IkB-NESm 1	0

B)



Figure 6

C)	Not film and that the		
	mRFP channel	mCer channel	eYFP channel
	a	b	с
	8	0	0
	mRFP-NVenus-GCN5	mCer-CVenus-lkB	BIFC - Venus
	d	e	r Aller Aller
	mRFP-NVenus-GCN5	mCer-CVenus-IkB	BIFC - Venus
	9	h	0
	mRFP-NVenus-GCN5	mCer-CVenus-IkB	BIFC - Venus



E)

mRFP channel mCer channel eYFP channel





Figure 7: The mRNA export factor, TAP/NXF1, can export GCN5 from the nucleus. NIH 3T3 cells were transiently-transfected with the appropriate constructs, followed by protein expression for 24 hours. Live-cell imaging was performed using a Leica TCS SP5 MP confocal microscope. A) Histogram showing differences in percent nuclear localization of GCN5-eYFP when co-expressed with mRFP-TAP/NXF1 versus the control protein, mRFP. Error bars represent ±1 standard error calculated from three individual experiments, where N=30. A two-tailed Student's t-test was used for statistical analysis. B) Representative images of GCN5-eYFP co-expressed with either an mRFP control protein or mRFP-TAP/NXF1 (panels a, b), and of mRFP-TAP/NXF1 in the presence of GCN5-eYFP or eYFP alone (panels c, d). Scale bar represents 10 μ m.

A)





Figure 7

Figure 8: In contrast to mRFP-IKB and mRFP-TAP/NXF1, the control proteins ataxin-3-Q28-mRFP and Htt-Ex1-wt-mRFP do not export GCN5 from the nucleus. NIH 3T3 cells were transiently-transfected with the appropriate constructs, followed by 24 hours of protein expression. Live-cell imaging was performed using a widefield Nikon TE200 fluorescent microscope. A) Representative images of ataxin-3-Q28-mRFP (panels a,d,g), a known shuttling protein, co-expressed with GCN5-eYFP (panels b,e,h). Merged images are shown in panels c, f and i. B) Representative images of Htt-Ex1-wt-mRFP (panels a,d,g), another protein known to shuttle between the nucleus and the cytoplasm, co-expressed with GCN5-eYFP (panels b,e,h). Merged images are shown in panels c, f and i. Scale bar represents 10 µm.



B)

a	b	с
4	Ø	8
Htt-Ex1-wt-mRFP	GCN5-eYFP	Merge
d	e	f
Htt-Ex1-wt-mRFP	GCN5-eYFP	Merge
g	h	
Htt-Ex1-wt-mRFP	GCN5-eYFP	Merge

Figure 8

Figure 9: Over-expression of GCN5-eYFP induces the formation of nuclear filaments that co-localize with nuclear β -actin filaments.

A) Over-expression of GCN5-eYFP induces the formation of nuclear filaments, with which it is associated in ~10% of the total number of transfected cells. Each image was captured as a system-optimized z-series. Panel a is a deconvolved 3D volume-rendered image of a representative nucleus harbouring these filaments, while panel b is the same nucleus, but rotated, and has been isosurfaced and given a 3D appearance (when red-cyan glasses are used); B) GCN5-eYFP-associated nuclear filaments co-localize with mRFP- β -actin filaments in the nucleus of live cells and B) with TRITC-phalloidin-labelled actin in the nucleus of fixed cells. Each figure is representative of six images captured, following 36 hours of protein expression, on a Leica TCS SP5 MP confocal microscope. Furthermore, each channel for (B) and (C) was sequentially scanned to prevent signal bleed-through between the eYFP and the mRFP/TRITC channels. Scale bar represents 10 μ m.



Volume

3D Isosurface

B)







Figure 9

Figure 10: GCN5 and β -actin interact *in vivo*, as shown by BiFC.

Panels A-D are control micrographs of mCer-CVenus- β -actin showing a lack of BiFC signal in the absence of a potential binding-partner. The corresponding control images for mRFP-NVenus-GCN5 were previously shown in Figure 14A, panels d-f (these particular images were captured with a widefield fluorescence microscope). Panels A-D are control images of a cell expressing only mCer-CVenus- β -actin. Panels E-H demonstrate an *in vivo* interaction between the aforementioned proteins; as can be noted, there is only significant BiFC signal (eYFP channel) in the nuclei where each of the proteins are sufficiently expressed (compare the outer two nuclei with the middle nucleus). Scale bars represent 10 μ m.



Figure 10
Figure 11: A conserved glutamic acid residue in the catalytic domain of human GCN5 is responsible for the formation of the actin filaments.

A) Protein sequence alignment of part of the HAT domain for human GCN5 and its yeast and mouse homologues. The conserved glutamic acid responsible for GCN5's catalytic activity is outlined. B) Histogram of the number of cells showing filaments in cells expressing either GCN5-eYFP or the catalytically-inactive mutant, GCN5-E575Q-eYFP. For wild-type GCN5-eYFP, three independent experiments were performed, while for GCN5-E575Q-eYFP, ten independent experiments were performed, each with N=100. * represents p<0.01; error bars represent ±1 standard error of the means of the independent experiments. A two-tailed Student's t-test was used for statistical anaylsis. C) Representative nuclei expressing each of the proteins: panel a shows typical filaments observed in the nuclei of cells expressing wild-type GCN5-eYFP, while panels b-e show representative nuclei of cells expressing the GCN5-E575Q-eYFP mutant. Representative images were captured on a Leica TCS SP5 MP confocal microscope. Scale bars represent 10 μ m. B)

A)

S. Ce	erevisiae	159	VVGGITYRPFDKREF#EIVFCAISSTEQVRGYGAHLMNHLK	198
Mus I	musculus	553	VIGGICFRMFPTQGFTEIVFCAVTSNEQVKGYGTHLMNHLK	593
Ното	sapiens	560	VIGGICFRMFPTQGFTEIVFCAVTSNEQVKGYGTHLMNHLK	600



C)



GCN5-E575Q-eYFP





Figure 12: GCN5-eYFP-associated actin filaments do not co-localize with mRFPlamin A or DNA.

A) GCN5-eYFP was co-expressed with mRFP-lamin A in NIH 3T3 cells for 36 hours. Cells were fixed with 4% paraformaldehyde, mounted, and subjected to z-series imaging using a Leica TCS SP5 MP confocal microscope. B) GCN5-eYFP was expressed for a similar time-frame as noted above, followed by fixing. Cells were then treated with 4 μ g/mL Hoechst dye 33258 for 15 minutes at 37°C, followed by extensive washing. C) GCN5-eYFP was co-expressed with mRFP-lamin A for 36 hours, proceeded by fixing and incubation with Hoechst dye as stated above. Images shown in B and C were captured as system-optimized z-series using a Leica DMI 6000 B widefield fluorescence microscope. Each micrograph was subjected to 3D deconvolution using Volocity 4, and volume-rendered and isosurfaced using Imaris 5.5.1. Scale bars represent 5 μ m.





Figure 12

Figure 13: GCN5-eYFP-associated nuclear filaments are transient and dependent on the integrity of DNA structure.

Micrographs represent the effect of Hoechst dye on GCN5-eYFP actin filaments over time in the 3D (from 10 - 35 minutes post-application of the dye). A) volume-rendered images over time (panels a-f); B) Isosurfaced images over time (panels a-f). All images were captured on the Leica DMI 6000 B widefield fluorescence microscope at 5 minute timepoints, with a system-opitimized 0.2 μ m step-size, and deconvolved using Volocity 4 software and theoretical PSFs. 3D images were then manipulated on Imaris 5.5.1. Scale bar represents 10 μ m.



Figure 13

Figure 14: GCN5-eYFP recovers quickly and evenly over actin filaments.

Panels A-H represent a FRAP experiment: A) pre-bleach, filament was subjected to bleaching in ROI outlined in red; B-H) post-bleach recovery, showing 2.5 minute increments; I) Image following the final recovery time-point, with bleached filament outlined in green, and three regions of interest (ROIs) outlined in cyan, yellow, and magenta, from the bottom to the top of the filament, respectively; J) Line graph showing the recovery of each of the corresponding ROIs. Note that the pixel intensities initially differed, giving rise to separate lines; however, the slope of each line is qualitatively similar to the others, as well as to that of the filament as a whole, indicating that GCN5-eYFP is not tracking along the filament, but rather binding to it in a randomized manner. Scale bar represents 5 μ m.



Figure 14

6. APPENDIX

6.1. INTRODUCTION: Spinocerebellar Ataxia Type 7 (SCA7)

6.1.1. SCA7 and Human GCN5

Spinocerebellar ataxia type 7 (SCA7) is a neurodegenerative disease characterized by cerebellar ataxia and visual impairment due to progressive and selective loss of neurons within the cerebellum, brainstem and retina [162]. The disease is caused by the expansion of a CAG repeat in the first coding exon of the *SCA7* gene, resulting in an expanded polyglutamine tract in the amino-terminal region of the disease protein, ataxin-7 [163-165]. The same type of unstable mutation is known to cause eight other neurodegenerative disorders, including Huntington's disease, Spinal and bulbar muscular atrophy (SBMA), Dentatorubral-pallidoluysian atrophy (DRPLA), SCA1-3, 6 and SCA17/TBP disease [144].

Despite important differences between the polyglutamine diseases, a number of the polyglutamine disease proteins have been found to be involved in transcriptional regulation, including the androgen receptor (the protein responsible for SBMA), the TATA-binding protein (TBP) (which is responsible for SCA17), and recently, ataxin-7. Ataxin-7's role in transcriptional regulation is partly based on its interaction with the histone acetyltransferase, GCN5, in the mammalian STAGA (SPT3-TAF9-ADA-GCN5 acetyltransferase) and TFTC (TBP-free-TAF-containing) complexes [166,167], which function to connect transcriptional activators, and their associated activation domains, with the basal transcription machinery [143].

In retinas of SCA7 transgenic mice, Helmlinger et al. [143] noted a downregulation of various rod-photoreceptor-specific genes, which they found to be correlated with an increase in STAGA complex recruitment and subsequent histone H3 hyperacetylation by GCN5 [143]. This is in striking contrast to what is normally observed, where hyperacetylation causes chromatin de-condensation that is typically correlated with an increase in transcription of the specific gene(s). These researchers propose that their observations are, indeed, due to the presence of mutant ataxin-7 in this complex; however, their results suggest that it is not due to a disruption in STAGA complex assembly, or even a lack of GCN5's HAT activity. Instead, they suggest a number of alternative possibilities that may be responsible for the down-regulated transcription of rod-specific genes that is characteristic of SCA7 pathogenesis: these include, for instance, that the expanded polyglutamine tract in mutant ataxin-7 may act as a non-discriminating chromatin-interacting domain that can cause indiscriminate recruitment of the STAGA complex to various sites, thereby resulting in histone hyperacetylation and chromatin de-condensation at inappropriate positions within the genome. They hypothesize that this could lead to a decrease in the number of appropriately-positioned transcriptional co-activators, leading to a down-regulation of genes that are normally highly transcribed. Alternatively, they suggest that mutant ataxin7 may affect proper formation of the pre-initiation complex, thereby preventing efficient transcription.

These findings by Helmlinger et al. [168] are in contrast to those found using yeast and stably-transfected HEK 293 cell models. Employing these model systems, both McMahon et al. [141] and Palhan et al. [169-171] found that mutant ataxin-7 inhibits nucleosomal histone acetylation by interfering with the recruitment of SPT3, ADA2b and TAF12 into the STAGA complex, as the latter two subunits have been previously shown to act as modulators of GCN5's HAT activity within the SAGA complex [168]. The discrepancies between the aforementioned in vivo results and these in vitro results are likely due to the differences in the model systems used. For instance, McMahon et al. [144] studied the incorporation of human ataxin-7 into the yeast SAGA complex; despite homologies between the various subunits that were studied, there are significant differences, including the presence of the polyglutamine tract and a third domain in mammalian ataxin-7 that are absent in the yeast homologue, Sgf73 [172,173]. Thus, incorporation of polyglutamine-expanded ataxin-7 into the yeast SAGA complex, rather than the human STAGA complex, may result in unpredicted changes in structure, composition, and/or function of the complex being studied. Furthermore, over-expression of other polyglutamine-expanded proteins in yeast has been shown to result in more rapid nuclear aggregate formation than that which is seen in mouse models [141]; thus, these aggregates could be responsible for decreasing the availability of SAGA complex subunits, such as SPT3, ADA2b and TAF12, thereby affecting the complex's HAT activity. Furthermore, Palhan et al. [141] analyzed STAGA complexes from transfected cells using a single antibody against recombinant ataxin-7: this could have resulted in the partial purification of other complexes containing over-expressed ataxin-7. Lastly, no transcriptional and/or chromatin alterations were reported in this study, making it difficult to correlate impaired HAT activity with SCA7 pathology [174,175].

6.1.2. Clinical Manifestations and Neuropathology of SCA7

SCA7, previously known as OPCAIII or ADCA type II, shows an autosomaldominant pattern of inheritance. Globally, families afflicted with this disease are relatively rare, with a reported prevalence of less than 1/100,000 [176]; however, this disease has been found to be the major type of autosomal-dominant cerebellar ataxia (ADCA) diagnosed in Sweden and Finland [142,177]. SCA7 is characterized by strong genetic anticipation presenting as a younger age of onset and a more severe progression of the disease in successive generations [160,161,178,179]. The age of onset can range from a few months to >75 years and the duration (i.e. time of onset until death) can be decades or as short as 3-4 months [161,179]. The range of symptoms also varies dramatically between SCA7 patients, although cerebellar ataxia, manifesting itself as disturbed coordination of movements, gait ataxia (wide-based gait) and dysarthria (speech impairments) are observed in most patients [161,179,180]. Another hallmark of SCA7 is loss of vision, which distinguishes this disease from other cerebellar ataxias [161,180]. The visual problems are usually initiated by defects in blue/yellow colour discrimination, followed by progressive macular degeneration that results in decreased visual acuity and eventual blindness [178,181,182].

6.1.3. The SCA7 Gene and its Polymorphic Mutation

The gene causing SCA7 was mapped to chromosome 3p12-21.1 by three independent groups in 1995 [162], and was cloned in 1997 [162,183]. The mutation responsible for SCA7 was found to be the expansion of a CAG-triplet repeat in the first coding-exon of the gene, resulting in an expanded polyglutamine tract in the disease protein, ataxin-7 [162,184]. Thus, SCA7 became the eighth neurodegenerative disorder shown to be caused by an expanded polyglutamine domain.

Genetic analysis showed that normal SCA7 alleles give rise to 4-35 CAG repeats, while pathological lengths vary from 37 to 306 [161,179]. Like all other polyglutamine disorders, SCA7 is characterized by an inverse correlation between the repeat length and the age of onset of the disease. Moreover, repeat length has been correlated with symptom-severity, such that patients with moderate repeat lengths often present with various forms of ataxia, while patients with longer repeats are additionally more susceptible to visual problems [185,186]. Studies of parent-to-child transmissions of the CAG repeat revealed that there is instability of the repeat during germ-line transmission, most often giving rise to an *expansion* in the SCA7 gene [161,162,179,180]. Gender has been demonstrated to influence the stability of the repeat in many polyglutamine disorders, including SCA7, where it is especially unstable and prone to expansion during spermatogenesis. In agreement with this, most of the infantile cases of SCA7, having extreme repeats, are the result of paternal transmissions [162].

6.1.4. The Ataxin-7 Protein

The SCA7 gene encodes an 892 amino acid protein, ataxin-7, with a predicted molecular weight of 95 kDa and unknown function [187,188]. Ataxin-7 exhibits a ubiquitous expression pattern with highest levels expressed in the heart, skeletal muscle, and pancreas, and moderate levels of expression in the brain, liver and kidney [188]. Within the retina, ataxin-7 has been localized to the optic nerve fibres, the cytoplasm and processes of the ganglion cells, the cell bodies of the inner nuclear layer, and the inner segments of the rod and cone photoreceptors [188-190]. Within the central nervous system (CNS), ataxin-7 has been observed in both affected and unaffected regions of the brain [191].

Besides the polyglutamine domain, ataxin-7 has a defined central [192] and two carboxy-terminal nuclear localization signals (NLSs) [140]. In addition, Taylor *et al.* [140] have recently discovered a nuclear export signal (NES) (see Figure 23), and have confirmed ataxin-7's ability to shuttle between the nucleus and the cytoplasm. In support of this, both mutant and wild-type ataxin-7 have been observed within both the nuclear and cytoplasmic compartments in cell culture models [188,189,193,194], normal and

SCA7-affected brains [195], and transgenic mice models [139]. This suggests that ataxin-7 may have functional roles in both cellular compartments.

Additionally, overlapped by the central NLS and NES, ataxin-7 harbours a phosphoprotein-binding motif homologous to that which is found in arrestins [196,197]. as shown in Figure 15. The arrestin family is made up of four members, two of which are found in the cone and rod photoreceptor cells of the retina [198-200]. Originally discovered for their role in the desensitization of phosphorylated G-protein coupled receptors (GPCRs) [201,202], these proteins have recently been found to mediate GPCR internalization through the formation of clathrin-coated pits [203,204], and to act as scaffolds that link activated GPCRs to members of various signaling pathways [205,206]. Interestingly, several of the arrestins have been found to have nucleocytoplasmic shuttling ability [207], suggesting that ataxin-7 may have a cytoplasmic role analogous to a nuclear signaling arrestin. Furthermore, the fact that the NLS and NES overlap the phosphoprotein-binding domain is intriguingly similar to the organization of the ligandbinding domain and nucleocytoplasmic shuttling signals of the androgen receptor protein, the disease protein involved in SBMA. Normally, this protein is able to traffic freely between the nucleus and the cytoplasm, while binding of androgen to the receptor sterically occludes the NES, thereby preventing export from the nucleus [140]. The same type of mechanism is hypothesized to affect ataxin-7's localization through regulation by an unknown phosphoprotein. In the disease state, nuclear retention may become permanent, thereby contributing to neurodegeneration, as mutation of the NES has been shown to increase the toxicity of polyglutamine-expanded ataxin-7 [139].



Figure 15: Schematic diagram of ataxin-7 and its phosphoprotein-binding domain, which is overlapped by its NES and its central NLS. Figure adapted from [208]. $(A)_n$, $(Q)_n$, $(P)_n$, and $(S)_n$ represent poly-alanine, -glutamine, -proline, and -serine regions; NLS, nuclear localization signal; NES, nuclear export signal.

6.2. MATERIALS & METHODS

6.2.1. Immunofluorescence

In these experiments, the MCF7 breast carcinoma cell-line was used due to more efficient transient transfections of the ataxin-7 construct. The property of this cell-line that allows cytotoxic proteins to be more easily expressed is a lack of caspase-3 protein expression; this is due to a 47-base pair deletion within exon 3 of the *CASP*-3 gene, that abrogates translation of *CASP*-3 mRNA [209]. Caspase-3 is known to be a converging member of many pathways involving programmed cell death, or apoptosis [210].

For each of the co-transfections, 2.5 μ g of total plasmid DNA was transfected, including 1.25 μ g of ataxin-7 (Q10)-GFP and 1.25 μ g of the specific HA-tagged G-protein coupled receptor (GPCR). The proteins were expressed for 18-24 hours, followed by fixing with 4% paraformaldehyde. The cells were immuno-stained using rabbit anti-HA primary antibody (H6908, Sigma Aldrich) at 1:50 dilution and goat anti-rabbit Alexa Fluor 594 secondary antibody (A31631, Invitrogen, Molecular Probes) at 1:5000 dilution. Coverslips were mounted on slides using 90% glycerol/PBS. Representative images were captured using a Nikon TE200-inverted fluorescence microscope with a 63x (NA 1.3) objective.

6.2.2. Plasmid Construction

The phosphoprotein-binding domain of ataxin-7, along with the central NLS and NES, were PCR-amplified using oligos RT0844 and RT0845 (shown in Table 1), which introduced *Bam*HI and *XhoI* sites, respectively. The PCR-product and the pET41a vector were then digested with the appropriate enzymes, followed by ligation; this resulted in the creation of the atx7PBD-GST construct, which was used in subsequent affinity chromatography experiments.

6.2.3. Protein Induction and Purification

A freshly-transformed BL21-AI colony harbouring the atx7PBD-GST construct was inoculated overnight in 100 mL of Kanamycin-treated 2x Luria-Bertani (LB) media. The next morning, 1/10 of the culture was added to 1 L of media and grown to OD 0.5-0.6, followed by induction with 0.2% arabinose and 1 mM IPTG for ~2 hours at 37°C. Subsequent to confirming induction, the recombinant protein was purified essentially as described by the Amersham Glutathione Sepharose 4B protocol, except that 50 mM Hepes buffer pH 7.4 was substituted for Tris buffer and lysis of the cells with Triton-X 100 detergent was omitted. The recombinant protein was then cleaved from the GST-tag and eluted, giving rise to the atx7PBD peptide. The protein was further purified by employing BD Talon Metal Affinity Resin, as described by BD Biosciences, using the incorporated His-tag.

6.2.4. Affinity Chromatography

Initially, increasing concentrations of atx7PBD peptide (1, 2, and 4 mg/mL) were each incubated with 100 mg Affigel 10 beads and the appropriate amount of coupling buffer (0.1 M NaCl, 10 mM Hepes pH 7.4, 10% glycerol, 0.1 mM DTT) to make up a total volume of 800 µl for 4 hours at 4°C. Affinity chromatography was carried out using P200 tips, with glass wool placed in the tip, and 20 µl bed-volumes of ligandcoupled beads. Coupling buffer was washed over the beads, followed by mouse brain extract (MBE), and additional coupling buffer. Finally, two column volumes of 0.5 M NaCl solution were used to elute the associated proteins, which were then run on a 12% SDS-PAGE gel, followed by silver staining using the Pierce SilverSNAP II Staining kit (Pierce Biotechnology), according to the manufacturer's instructions. Afterwards, specific protein bands were excised and sent for in-gel trypsinization and mass spectrometry at a facility in Washington, DC.

6.3. RESULTS & DISCUSSION

In order to test if ataxin-7 was functioning as a shuttling β -arrestin, ataxin-7 (Q10)-GFP was independently co-transfected with a number of hemagglutinin (HA)tagged GPCRs that are known to be expressed in various retinal cell-types (see Table 2). As detailed in Table 2, in contrast to our initial hypothesis, none of the retinal-specific GPCRs examined either altered or disrupted the normal nuclear localization of ataxin-7, as demonstrated with three representative GPCRs in Figure 23A-C. Importantly however, there are a number of reasonable explanations for this: first of all, the cell-line in which the experiments were performed, the MCF7 cell-line (due to its ease in the transfection of the cytotoxic ataxin-7 construct), lacks retinal-specific GPCR-kinases that are normally responsible for facilitating the GPCR: arrestin interaction [211,212]. Furthermore, it is very likely that, in general, GPCRs are not natural substrates of ataxin-7, since the crystal structure of visual-arrestin indicates that arrestins contain two major domains, on the amino- and carboxy-termini, that are principally responsible for their affinity for and ability to associate with GPCRs [139]. Ataxin-7, in contrast, only contains a phosphoprotein-binding domain similar to that found in arrestins [210], which, within an arrestin, resides in the linker region between the two terminal domains [213-215]. Thus, rather than acting as an arrestin-like protein, it is more likely that this motif is a generic phosphoprotein-binding domain that could be important to ataxin-7 function or regulation by interacting with one of a myriad of phosphorylated proteins.

For this reason, we decided to take a broader approach in the search of the protein(s) that could potentially interact with this ataxin-7 domain. To do this, we created a construct containing the phosphoprotein-binding domain and the overlapping NES and NLS (shown in Figure 23), and expressed it as a GST-fusion protein (atx7PBD-GST) in BL21-AI *E. coli* cells. Following induction and purification using Glutathione Sepharose

4B beads, the recombinant peptide was cleaved from the GST-tag using thrombin protease. The protein was further purified by employing BD Talon Metal Affinity Resin that bound to the HIS-tag within the construct, and finally quantified by SDS-PAGE gel and Coomassie blue staining. Figure 24A shows the results of titrating the purified product, atx7PBD, which was estimated to be at a concentration of $\sim 3\mu g/\mu l$.

Affigel 10 resin, which has an affinity for $-NH_2$ groups, was employed to precipitate increasing concentrations (1, 2 and 4 mg/mL) of the atx7PBD peptide for affinity chromatography. Mouse brain extract (MBE) was passed over each of the columns, followed by eventual elution of the associated proteins with 0.5 M NaCl. The proteins obtained from each fraction were separated by SDS-PAGE, as shown in Figure 24B, while seven protein bands, each of which was absent from the beads-alone control lane and showed increasing concentration over the increasing ligand concentrations, were sent for mass spectrometry analysis. Three of these protein bands are visible in Figure 24B (as highlighted by arrows).

The results of the mass spectrometric analysis are appended as a data file termed "Mass spec results obtained from affinity chromatography with an atx7PBD ligand". Although the interactions between atx7PBD and some of the protein hits obtained were not further validated, it is interesting to note that this domain was found to interact significantly with both a dynamin-1 isoform and a number of tubulin protein isoforms, both of which are transport proteins that have been shown to be expressed in the brain (and retina, in the case of dynamin-1), and to be phosphorylated at specific residues [213-215]. This is intriguing due to the fact that our lab has found that in \sim 10% of the transfected cells, ataxin-7 exhibits an array of cytoplasmic filaments, which is in contrast to its normal nuclear localization. As well, as previously mentioned, ataxin-7 has recently been found to contain a CRM1-dependent NES and to efficiently shuttle between the nucleus and the cytoplasm, suggesting that ataxin-7 may play a specific role(s) in the cytoplasm, perhaps with respect to protein transport, in addition to the nucleus.

Furthermore, this domain was found to interact significantly with nucleolin, which is an acidic phosphoprotein that is located mainly in the dense fibrillar regions of the nucleolus, and is involved in the control of transcription of ribosomal RNA (rRNA) genes, and the nucleocytoplasmic transport of ribosomal components [216]. Importantly, this protein is expressed in the brain and is actively phosphorylated at two serine residues [215]. The fact that this protein is a potential interaction partner of ataxin-7 would correspond with its previously-described roles in transcription, and ability to shuttle in and out of the nucleus.

Other findings further suggest that ataxin-7 may also play a role in translation, including the fact that it was found to interact substantially with the translation elongation factor 2 (EF-2), the coatomer protein complex, which processes and transports proteins and vesicles, and exportin-t, which exports tRNAs from the nucleus.

Importantly, as controls, we also found hits for an interaction of this domain, which included both the NES and a NLS, with transport proteins such as importin- β and the importin- α re-exporter.

Thus, although these protein hits provide a starting point for finding potential protein partners of ataxin-7, more detailed analyses of these proteins need to be

performed to conclusively demonstrate their interaction and their biological significance, particularly as it relates to SCA7 pathology.

6.4. FIGURES

Table 2: GPCRs do not induce nucleocytoplasmic shuttling of ataxin-7. Ataxin-7 was co-expressed with each of ten GPCRs known to be expressed in the retina, none of which had the ability to redistribute ataxin-7 from the nucleus to the cytoplasm.

G-Protein Coupled Receptor	Acronym	Redistribution of Ataxin-7 (Q10)-GFP?
Arginine vasopressin receptor 1A	AVPR1A	No
Neurotensin receptor 1	NTSR1	No
Neurotensin receptor 2	NTSR2	No
Tachykinin receptor 1	TACR1	No
Tachykinin receptor 2	TACR2	No
Tachykinin receptor 3	TACR3	No
Somatostatin receptor 1	SSTR1	No
Vasoactive intestinal peptide receptor 2	VIPR2	No
Oxytocin receptor	OXTR	No
Bradykinin receptor B2	BDKRB2	No

Figure 16: AVPR1A, NTSR1, and NTSR2 are not binding-partners of ataxin-7. A-C) Representative micrographs showing the lack of interaction between ataxin-7 (Q10)-GFP and three specific GPCRs that are known to be expressed in the retina, including AVPR1A (A, panels a-d), NTSR1 (B, panels a-d), and NTSR2 (C, panels a-d). In each case, MCF7 cells were transiently-transfected with 1.25 μ g each of the ataxin-7 (Q10)-GFP and HA-tagged GPCR constructs and were allowed to express for 18-24 hours. Cells were fixed with 4% paraformaldehyde and immuno-stained using rabbit anti-HA primary and goat anti-rabbit Alexa Fluor 594 secondary antibodies. Images were captured on a Nikon TE200-inverted fluorescence microscope.



Figure 16

Figure 17: The recombinant atx7PBD peptide can potentially interact with a variety of proteins. A) Glutathione Sepharose 4B and Talon Metal Affinity resin were used sequentially to purify the recombinant atx7PBD construct from BL21-AI cells. The ~20 kDa peptide was run on a 12% SDS-PAGE gel and stained with Coomassie Blue stain. The concentration of the protein was estimated to be ~3 $\mu g/\mu l$. [Lane 1: Broad range protein marker; Lane 2: 1 μl of atx7PBD; Lane 3: 2 μl of atx7PBD; Lane 4: 5 μl of atx7PBD]. B) Results of affinity chromatography carried out with increasing concentrations (1, 2 and 4 mg/ml) of the atx7PBD peptide and mouse brain extract, where two column volumes of 0.5 M NaCl solution were to elute the associated proteins. The elution volumes were run on a 12% SDS-PAGE gel, followed by silver staining. Six potential protein bands, two of which are highlighted, were sent out for mass spectrometry to elucidate potential atx7PBD binding partners. MW = molecular weight. Results of mass spectrometry can be viewed as supplementary material on the CD, termed "Mass spec results obtained from affinity chromatography with an atx7PBD ligand".

A)



B)



Figure 17

7. REFERENCES

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