FUNCTIONAL REMODELLING OF THE NUCLEOLUS BY LONG NONCODING RNA

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ABSTRACT

The nucleolus is a plurifunctional organelle in which structure and function are intimately linked. Though it is primarily known as the site of ribosomal biogenesis, the nucleolus is also capable of orchestrating the immobilization of a broad range of proteins under specific environmental conditions. This process, known as nucleolar sequestration, contributes to cell viability under stress. Despite the importance of this post-translational regulatory pathway, very little is known about the mechanisms that govern it. Here, we show that heat shock and acidosis, two physiological stimuli associated with nucleolar sequestration, induce the expression of long noncoding RNA (lncRNA) from stimulus-specific loci of the ribosomal intergenic spacer (IGS). These lncRNAs, in turn, immobilize proteins encoding a nucleolar detention sequence (NoDS) within a compartment of the nucleolus termed the detention centre (DC). The DC is a spatially and dynamically distinct region, characterized by an 8-anilino-1-naphthalenesulfonate (ANS)-positive hydrophobic signature. Its formation is accompanied by a redistribution of nucleolar factors and an arrest in ribosomal biogenesis. Silencing of regulatory IGS lncRNA prevents the creation of this structure and allows the nucleolus to retain its tripartite organization and transcriptional activity. Signal termination causes a decrease in IGS transcript levels and a return to the active nucleolar conformation. We propose that the induction of IGS lncRNA, by environmental signals, operates as a molecular switch that regulates the structure and function of the nucleolus.

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouridine
Ac.	Acidosis
ActD	Actinomycin D
ANS	8-anilino-1-naphthalenesulfonic acid
APC2	Anaphase-promoting complex subunit 2
ChIP	Chromatin immunoprecipitation
DC	Detention centre
DFC	Dense fibrillar component
DIC	Differential interference contrast
DMEM	Dulbecco's (modified) Minimum Essential Medium
DNA	Deoxyribonucleic acid
DNMT1	DNA (cytosine-5)-methyltransferase 1
DRB	5,6-dichloro-beta-D-ribofuranosylbenzimidazole
FBL	Fibrillarin
FC	Fibrillar centre
FISH	Fluorescence *in situ* hybridization
FLIP	Fluorescence loss in photobleaching
FRAP	Fluorescence recovery after photobleaching
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Granular component
GFP	Green fluorescent protein
HIF	Hypoxia inducible factor

HS	Heat shock
HSF1	Heat shock factor 1
Hsp70 (HspA1)	Heat shock protein of 70,000 mol. wt.
Hsc70 (HspA8)	Cognate of heat shock protein of 70,000 mol. wt.
IgG	Immunoglobulin G
IGS	Intergenic spacer
kb	kilobase
kD	kilodalton
lncRNA	Long noncoding RNA
MDM2	Murine double minute 2
MJ	Mathieu D. Jacob (author)
mRNA	Messenger RNA
NB	Northern blot
NEAT1	Nuclear paraspeckle assembly transcript 1
NoDS	Nucleolar detention sequence
nt	Nucleotide
PABP1	Polyadelynate binding protein 1
PCR	Polymerase chain reaction
POLD1	DNA polymerase catalytic subunit δ
PML	Promyelocytic leukemia protein
qPCR	Real-time quantitative polymerase chain reaction
rDNA	Ribosomal DNA
Rec.	Recovery

rRNA	Ribosomal RNA
RNA	Ribonucleic acid
RNF8	RING finger protein 8
RPA	Ribosomal protein A
RPL	Ribosomal protein L
RT-PCR	Reverse transcription polymerase chain reaction
shRNA	Short hairpin RNA
STAD	Subnuclear targeting arginine domain
STHD	Subnuclear targeting hydrophobic domain
TA	Timothy E. Audas (author)
TEM	Transmission electron microscopy
ThS	Thioflavin S
UT	Untreated
VHL	von Hippel Lindau

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CONTRIBUTIONS

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Jacob MD, Audas TE, Uniacke J, Trinkle-Mulcahy L, Lee S.
Mol Biol Cell. 2013. 24(18):2943-53.

2) Where no RNA polymerase has gone before: novel functional transcripts derived from the ribosomal intergenic spacer.
Jacob MD, Audas TE, Mullineux ST, Lee S.
Nucleus. 2012. 3(4):315-9.

3) An oxygen-regulated switch in the protein synthesis machinery.
Uniacke J, Holterman CE, Lachance G, Franovic A, **Jacob MD**, Fabian MR, Payette J, Holcik M, Pause A, Lee S.
Nature. 2012. 486(7401):126-9

4) The nucleolar detention pathway: A cellular strategy for regulating molecular networks.
Audas TE, **Jacob MD**, Lee S.
Cell Cycle. 2012. 11(11):2059-62.

5) Immobilization of proteins in the nucleolus by ribosomal intergenic spacer noncoding RNA.
Audas TE, **Jacob MD**, Lee S.
Mol Cell. 2012. 45(2):147-57.

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1. INTRODUCTION

1. INTRODUCTION

1.1. LONG NONCODING RNA

1.1.1. The central dogma of molecular biology

The central dogma of molecular biology, originally proposed by Francis Crick, depicts the relationship between information and function within biological systems (Crick, 1970). This model stipulates that information is passively contained within deoxyribonucleic acids (DNA), whereas function is the realm of proteins. Accordingly, ribonucleic acids (RNA) are mere intermediary transcripts that physically convey information from the genome to the sites of protein synthesis. This fundamental principle, considered as the pillar of cellular biology, has been challenged in recent years by a series of discoveries attributing various functions to noncoding transcripts. In fact, RNA is now emerging as an essential component of cellular biochemistry and organization. This thesis proposes functions for noncoding RNA in post-translational gene regulation and nuclear organization.

1.1.2. The RNA-world hypothesis

Proponents of the “RNA-world hypothesis” have anticipated the rise of functional noncoding RNAs for decades. RNA is capable of both storing genetic information and catalyzing enzymatic reactions, making it uniquely fitted to support the earliest stages of life on Earth. Eventually, this RNA-world would have been replaced by a more robust DNA-and-protein world, in which information and function are segregated (Benner et al., 1989; Gilbert, 1986; Joyce, 1989; Joyce, 1991; Waldrop,

1989). Interestingly, RNA plays a central role in this new order by providing the backbone of the molecular machinery that translates ribonucleic information into polypeptides. At the heart of this process, ribosomal RNA (rRNA) and transfer RNA (tRNA) work together to assemble amino acids into polypeptides, thus providing the first known examples of functional noncoding RNAs. It has been hypothesized that many additional noncoding RNAs would be identified, covering a wide range of cellular functions.

1.1.3. The emergence of noncoding RNAs in modern biology

The number of genes in the human genome is currently estimated at 30,000 to 40,000 (Consortium et al., 2007; Ewing and Green, 2000; Lander et al., 2001; Venter et al., 2001). However, this number remains controversial due to the ambiguous definition of a gene as "protein-coding". Existing methods for the identification of genes are mainly centered on polyadenylated mRNA sequencing and on computational predictions based on coding exons (Aparicio, 2000; Roest Crollius et al., 2000). These approaches are therefore biased towards conserved and abundant protein-coding genes, and surely neglect many other types of transcripts (Claverie, 2005; Eddy, 1999; Eddy, 2001). In fact, there is mounting evidence that the human genome contains a surprisingly large number of noncoding transcripts, with as much as 90% of DNA being transcribed (Huttenhofer et al., 2001; Lagos-Quintana et al., 2001). Noncoding RNAs are traditionally classified as follows: tRNA and rRNA in protein synthesis, microRNA (miRNA) and small interfering RNA (siRNA) in translational repression and gene silencing, small nuclear RNA (snRNA) in RNA

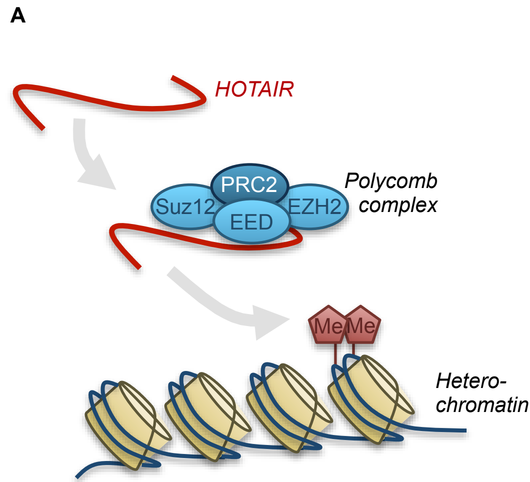
splicing, and small nucleolar RNA (snoRNA) in rRNA modification (Eddy, 2001; Matera et al., 2007). In recent years, an emerging field of study has been focusing on a new type of RNA: long non-coding RNA (lncRNA). lncRNAs are arbitrarily defined as being over ~200nt in size, on the basis of standard RNA purification protocols that exclude smaller transcripts. Due to their unexpected abundance and low sequence conservation, lncRNAs were initially considered to be non-functional transcriptional background (Struhl, 2007). However, new evidence suggests otherwise (Mattick, 2005). Despite their sequence divergence, lncRNAs contain conserved splicing patterns, secondary structures and subcellular localizations (Cawley et al., 2004; Ponjavic et al., 2007). Additionally, lncRNAs have been shown to be specifically expressed at various stages of embryonic development (Amaral and Mattick, 2008; Dinger et al., 2008) and to bind proteins (Mercer et al., 2008). They are involved in chromatin modification (Mayer et al., 2008; Mayer et al., 2006; Morris et al., 2008; Nagano et al., 2008; Pandey et al., 2008; Rinn et al., 2007), transcriptional regulation (Feng et al., 2006; Wang et al., 2008), post-transcriptional processing (Beltran et al., 2008; He et al., 2008; Willingham et al., 2005) and subcellular structural organization (Clemson et al., 2009; Sasaki et al., 2009; Sunwoo et al., 2009). Despite these functional insights, the biological role and physiological relevance of lncRNAs remain for the most part poorly understood. Of the >3000 lncRNAs that have been discovered, less than 1% have been characterized (Guttman et al., 2009; Tsai et al., 2011). The functional characterization of these transcripts has been a dynamic and challenging area of modern biology (Mercer et al., 2009).

1.1.4. Specific functions for long noncoding RNAs

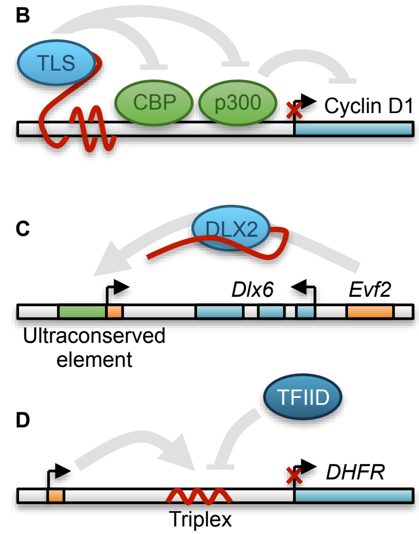
One of the best-characterized lncRNAs is the Hox antisense intergenic RNA (HOTAIR), a noncoding transcript commonly overexpressed in metastatic breast cancer and associated with poor clinical prognoses (Gupta et al., 2010). HOTAIR functions *in trans* to recruit the Polycomb chromatin remodelling complex PRC2 to the HOXD gene locus, thereby silencing it **(Figure 1A)** (Rinn et al., 2007). Other transcripts, such as Xist/RepA and Kcnqot1, function in a similar manner (Pandey et al., 2008; Zhao et al., 2008). The ability of lncRNAs to mediate interactions between protein complexes and DNA sequences allows a small number of chromatin remodelling complexes to target a wide array of genetic loci (Mercer et al., 2009). Another well-characterized example of lncRNA activity is the repression of cyclin D1 transcription in human cell lines. Stress-induced lncRNAs can modulate the activity of the RNA-binding protein TLS (translocated in liposarcoma), allowing it to inhibit the histone acetyltransferase activity of the CREB-binding protein (CBP) and p300 **(Figure 1B)** (Mercer et al., 2009; Wang et al., 2008). Conversely, lncRNAs can also operate as cofactors, as with the mouse Evf2 transcript. Expressed from an enhancer region, this RNA induces the expression of the *Dlx6* gene by recruiting the transcription factor DLX2 **(Figure 1C)** (Feng et al., 2006; Mercer et al., 2009). DNA-RNA triplex structures are another mechanism by which lncRNA regulate gene expression. By forming a triplex on the promoter sequence of the DHRF (dihydropholate reductate) locus, a lncRNA is capable is preventing transcription factor IID (TFIID) binding, thus modulating RNA pol II recruitment **(Figure 1D)** (Martianov et al., 2007; Mercer et al., 2009). The ability of RNA to anneal with

Figure 1. Mechanisms of action of known lncRNAs. (A) HOTAIR recruits the polycomb chromatin remodelling complex to the HoxD locus. The result is a tri-methylation of lysine 27 residues of histones and a silencing of chromatin. **(B)** RNA-binding protein TLS is recruited by a lncRNA to repress the histone acetyltransferase activity of CREB binding protein (CBP) and p300, and silence of Cyclin D1 gene. **(C)** The Evf2 lncRNA acts as a co-activator to the transcription factor DLX2, thus regulating the expression of the Dlx6 gene. **(D)** A lncRNA forms a triplex with the promoter region of the DHFR gene, thereby preventing the binding of general transcription factor TFIID. **(E)** An antisense lncRNA masks the 5'UTR of the Zeb2 mRNA to inhibit alternative splicing by the spliceosome. **(F)** NEAT1 is an architectural RNA that recruits DBHS-protein to its site of transcription to induce the nucleation of paraspeckles. Paraspeckles, in turn, retain several mRNAs, including A-to-I hyperedited transcripts. **(A-E)** Adapted from Mercer et al., 2009. **(F)** Adapted from Fox and Lamond, 2010.

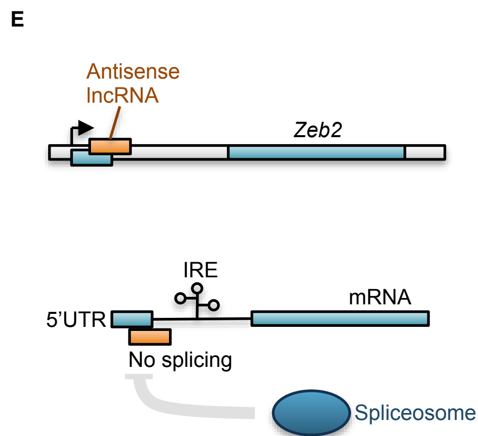
Chromatin remodeling



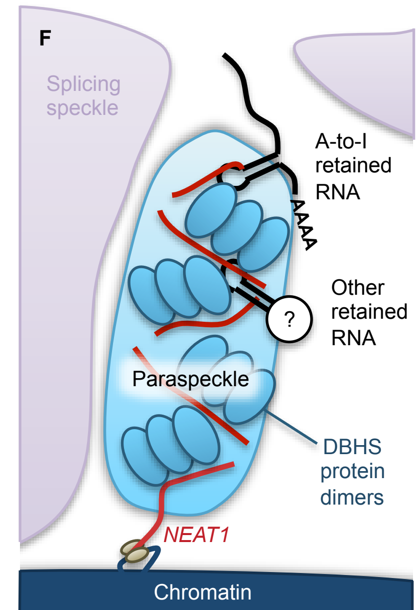
Transcriptional control



Post-transcriptional processing



Nuclear organization



complementary sequences allows for post-transcriptional control at the messenger RNA (mRNA) level. For instance, the Sip1 antisense RNA binds the 5'UTR of the Zeb2 mRNA to inhibit alternative splicing of this region **(Figure 1E)** (Beltran et al., 2008; Mercer et al., 2009). These findings highlight the importance of long noncoding transcripts in the transcriptional and post-transcriptional regulation of gene expression (Mattick, 2001).

1.1.5. Long noncoding RNAs in nuclear organization

The nuclear enriched abundant transcript 1 (NEAT1; also known as Men ε/ß) is a particularly relevant lncRNA in that its function differs from that of other known noncoding transcripts. Whereas the majority of lncRNAs operate by regulating the expression of protein-coding genes, NEAT1 is an architectural transcript that mediates the formation of paraspeckles, the most recently identified nuclear bodies (Bond and Fox, 2009; Clemson et al., 2009; Fox et al., 2002; Sasaki et al., 2009; Sunwoo et al., 2009). Current models stipulate that paraspeckles formation begins with the synthesis of NEAT1 by RNA polymerase (pol) II, immediately following cell division. Before NEAT1 has a chance to diffuse away from its site of site of transcription, it assembles into complexes with DBHS (Drosophila Behaviour Human Splicing) proteins (Mao et al., 2011; Sasaki and Hirose, 2009) (Figure 4B). DBHS proteins include Non-POU domain-containing octamer-binding protein (P54NRB/NonO), Splicing factor proline and glutamine rich (PSF/SFPQ) and paraspeckles component 1 (PSP1/PSPC1) (Fox et al., 2002). In turn, paraspeckles are believed to play a critical role in the control of gene expression through the

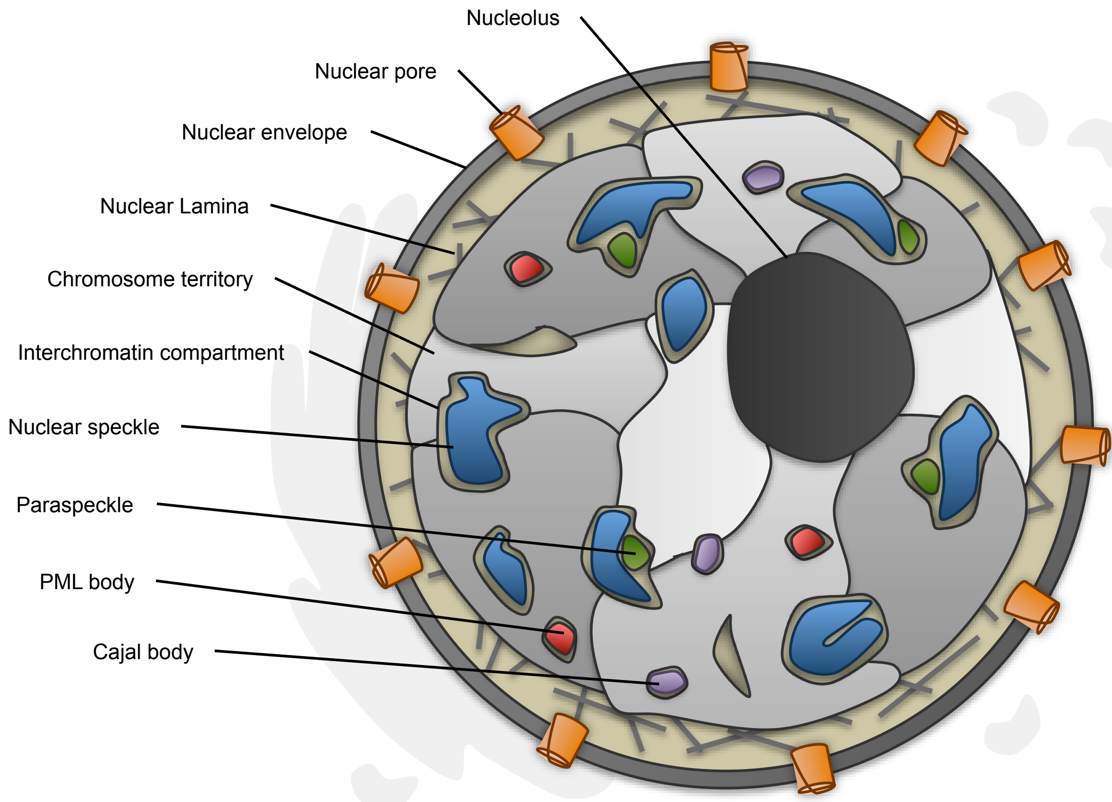
nuclear retention of A-to-I hyperedited mRNAs **(Figure 1F)** (Prasanth et al., 2005). NEAT1 thus provides evidence that the unique properties of noncoding RNAs can be harnessed to maintain proper nuclear organization and function.

1.2. NUCLEAR ORGANIZATION AND DYNAMICS

1.2.1. Compartmentalization and self-assembly within the nucleus

The cell nucleus is the site of genome maintenance and expression. As such, it displays a highly ordered organization that is regulated by interactions between chromatin, RNA and protein complexes (Fraser and Bickmore, 2007). Important research efforts are dedicated to understanding the influence of this architecture on gene expression, particularly in response to stress and physiological stimuli. Structural and functional compartments of the nucleus include the nucleolus, the nuclear lamina, Cajal bodies, PML bodies, splicing speckles and paraspeckles **(Figure 2)** (Fraser and Bickmore, 2007; Matera, 1999; Spector, 2001). Briefly, the nucleolus is primarily known as the site of ribosomal biogenesis, although it has been shown to coordinate stress responses (Boisvert et al., 2007; Pederson, 1998b). The nuclear lamina is a fibrillar network that regulates the structure of the nucleus; it plays a mechanistic role, tethers chromosomes to the nuclear periphery and regulates cell division (Aebi et al., 1986; Gerace and Blobel, 1982; Gerace et al., 1984; Gruenbaum et al., 2005; Guelen et al., 2008; Reddy et al., 2008). Cajal bodies are rich in splicing factors and may be involved in modifying newly transcribed RNA (Carmo-Fonseca, 2002b; Cioce et al., 2006; Dundr et al., 2004; Hebert and Matera, 2000; Ogg and Lamond, 2002; Sleeman and Lamond, 1999). PML bodies are often

Figure 2. Organization of the mammalian cell nucleus. The nucleus is characterized by an assortment of bodies and domains that are structurally and functionally distinct. These include the nucleolus, nuclear pores, the nuclear envelope, the nuclear lamina, chromosome territories, interchromatin compartments, nuclear speckles, paraspeckles, PML bodies and cajal bodies. Adapted from Lanclot et al., 2007.


Nucleolus
Nuclear pore
Nuclear envelope
Nuclear Lamina
Chromosome territory
Interchromatin compartment
Nuclear speckle
Paraspeckle
PML body
Cajal body

associated with Cajal bodies and are suspected to regulate transcription (Dellaire and Bazett-Jones, 2004; Hodges et al., 1998; Hofmann and Will, 2003; Muratani et al., 2002; Negorev and Maul, 2001; Takahashi et al., 2004). Nuclear speckles are dynamic structures that assemble around transcription sites to process newly-synthesized mRNA (Lamond and Spector, 2003; Mintz and Spector, 2000; Spector and Lamond, 2011; Spector et al., 1993; Spector et al., 1983; Zhang et al., 1994). Paraspeckles are small bodies associated with nuclear speckles and are believed to regulate the nuclear export of hyperedited mRNAs (Bond and Fox, 2009; Chen and Carmichael, 2009; Clemson et al., 2009; Fox et al., 2005; Fox et al., 2002; Fox and Lamond, 2010; Sasaki and Hirose, 2009; Sasaki et al., 2009; Scadden, 2009; Sunwoo et al., 2009). Disruption in the internal organization of the nucleus is often associated with pathologies (Bouteille et al., 1967; Handwerger and Gall, 2006; Zimber et al., 2004). For instance, loss of PML bodies has been linked promyelocytic leukemia (Bernardi and Pandolfi, 2007; Borden, 2002). Abnormal Cajal bodies have been reported in some cancers and inherited neurodegenerative diseases (Cioce and Lamond, 2005; Hebert et al., 2001). Similarly, the nucleolus is central to cancer and also plays a role in aging (Guarente, 1997; Marciniak et al., 1998; Montanaro et al., 2008; Yankiwski et al., 2000; Zimber et al., 2004; Zink et al., 2004). Genetic information itself is segregated into chromosome territories, with decondensed, transcriptionally active euchromatin separated from condensed, transcriptionally repressed heterochromatin **(Figure 2)**. Chromatin is tethered to the nuclear lamina (Mekhail and Moazed, 2010), and gene-rich regions tend to localize to the nuclear core, whereas gene-poor regions remain at the periphery (Foster and Bridger, 2005;

Lanctot et al., 2007). Throughout the cell cycle, or when required by cellular demand, chromatin undergoes directed movement throughout the nuclear space in order to activate or repress relevant gene clusters (Levi and Gratton, 2008; Levi et al., 2005; Spector, 2001; Thomson et al., 2004). Protein complexes, such as the ones involved in DNA replication, repair, transcription or editing, assemble and reassemble as needed into distinct domains throughout the nuclear space. Even large bodies, such as paraspeckles, can nucleate *de novo* upon expression of their associated lncRNA (Chen and Carmichael, 2009). This plasticity, which is at the heart of nuclear function, is one of the emerging themes of the past decade (Akhtar and Matera, 2006). The nucleus as a whole is continuously sensing internal and environmental cues in order to fine-tune gene expression and provide the most optimal adaptation to evolving conditions.

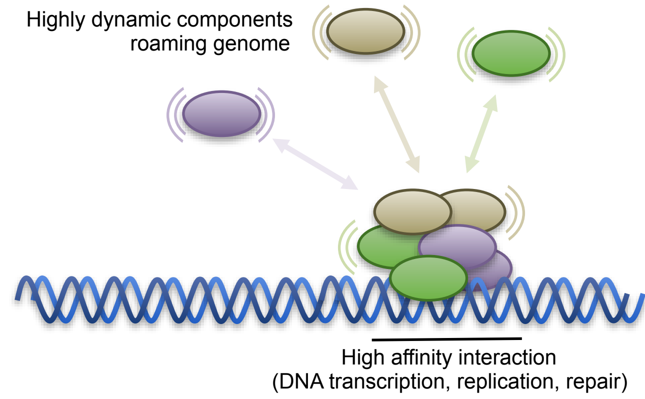
1.2.2. Macromolecular crowding and protein mobility

The ability of the nucleus to self-organize and remodel on cue relies on the extraordinary dynamic properties of its constituents. Up to 30% of the its volume is composed of proteins (400 mg/mL), a scramble of highly dynamic and reactive molecules that continuously collide and bounce off one another (Ellis, 2001a; Ellis, 2001b; Ellis and Minton, 2003; Hancock, 2004; Minton, 2000; Misteli, 2007; Misteli et al., 1997). Proteins diffuse throughout the cell at high speeds and interact in a stochastic (i.e. probabilistic) fashion. Other macromolecules, such as oligonucleotides, display similar dynamic properties (Politz et al., 1998; Politz et al., 1999). This highly chaotic and dynamic environment is paradoxically indispensable

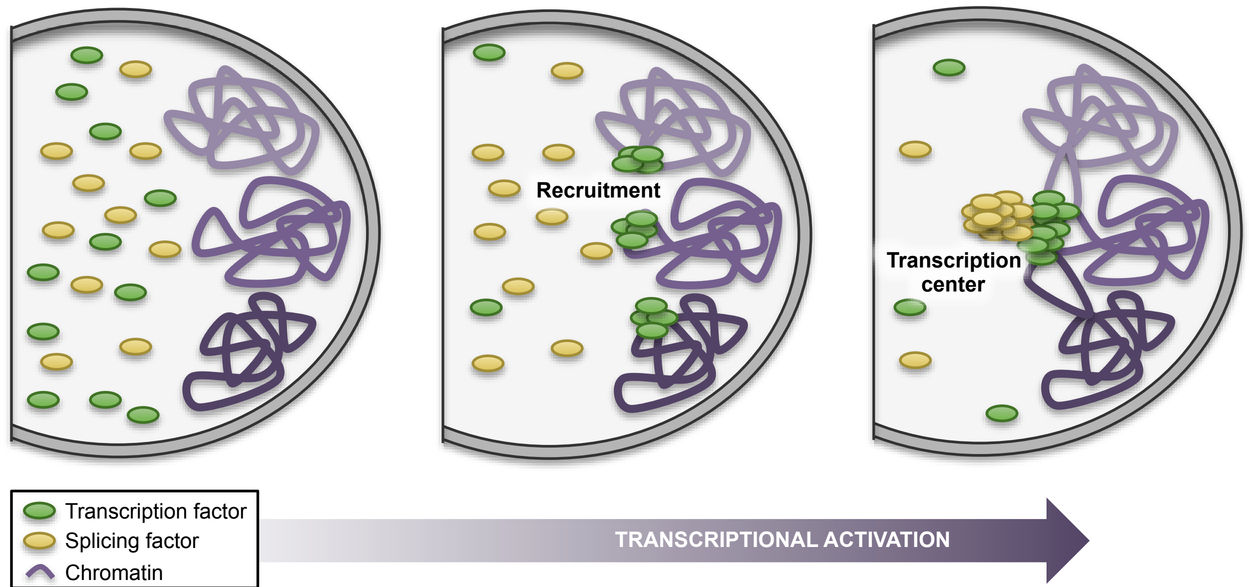
to generate order and to maintain proper cellular function. Protein mobility ensures their availability throughout the cell and generates an ever-changing yet stable framework for specific interactions and self-assembly to occur (Matera et al., 2009; Misteli, 2001; Phair and Misteli, 2000; Phair and Misteli, 2001). Without this plasticity, signals would not be readily transmitted and complexes would not assemble. The various components of DNA replication, transcription and repair machineries, for instance, roam the nuclear space until they are temporarily recruited by specific high-affinity interactions **(Figure 3A)**. This sometimes leads to the formation of large, specialized structures in which relevant factors are consolidated **(Figure 3B)**. Although they appear superficially static, protein complexes are in fact inherently dynamic and subjected to a continuous exchange of mobile factors. Individual proteins undergo rapid cycles of binding/unbinding, with dwell periods of only a few seconds (Carmo-Fonseca, 2002a; Dundr et al., 2000; Lander et al., 2001; Misteli, 2007; Phair and Misteli, 2000; Phair and Misteli, 2001; Phair et al., 2004; Tsai and McKay, 2005). Released proteins are free to scan the nucleoplasm in search of other interactions. The transient and probabilistic accumulation of dynamic proteins at high-affinity loci is referred to as "steady-state". This wide-scale model of self-assembly results in an emerging system in which structure and function are intimately linked. For that reason, interfering with nuclear processes results in substantial alterations to the architecture of the nucleus (Lamond and Spector, 2003).

Figure 3. Protein mobility and self-assembly in the nucleus. (A) Individual proteins are highly dynamic molecules that passively diffuse throughout the nuclear space, screening for binding sites. Transient high-affinity interactions lead to the formation of functional complexes at sites of action. Components of these complexes undergo rapid cycles of binding/unbinding, with very short dwell periods. **(B)** Self-assembly of dynamic macromolecules into large complexes leads to the formation of functional bodies within the nucleus, such as transcription centres. **(A, B)** Adapted from Misteli, 2007.

A



B

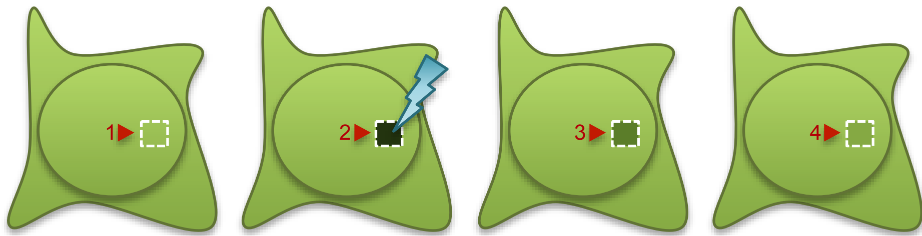


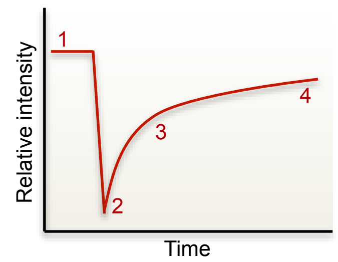
1.2.3. Assessing molecular dynamics using photobleaching

Although nuclear bodies seem stable over time, due to the absence of visible structural changes by time-lapse microscopy, they undergo constant remodelling at the molecular level. This molecular movement can be observed and quantified using photobleaching technologies: Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss In Photobleaching (FLIP) (Liebman and Entine, 1974; Liebman et al., 1974; Lippincott-Schwartz et al., 2003; Lippincott-Schwartz and Patterson, 2003; Patterson et al., 1997; Poo and Cone, 1974; Reits and Neefjes, 2001; White and Stelzer, 1999). In both cases, Green Fluorescent Protein (GFP)-tagged proteins are introduced to cells and allowed to assume their proper spatio-dynamic profile. A high-intensity laser is then used to "photobleach" a small area of the cell, photochemically destroying the fluorophores within it. With FRAP, the fluorescent signal of a small region is quenched by a single bleaching event, and recovery of fluorescence within that region is measured by time-lapse microscopy. Recovery of signal suggests a dynamic exchange between bleached and unbleached regions **(Figure 4A)**. On the other hand, with FLIP, a small area in the cell is continuously bleached and diminution of total cellular fluorescence outside that region indicates a dynamic behaviour, since mobile proteins will eventually cross the bleaching area **(Figure 4B)**. Mobility, as measured by photobleaching, is highest in freely diffusing proteins (e.g. GFP) **(Figure 4C-1)**. Complex-associated proteins spend more time bound to static structures, and therefore display reduced mobility **(Figure 4C-2)**. Histones, which are wrapped in DNA within nucleosomes, appear virtually immobile during interphase **(Figure 4C-3)** (Kimura, 2005; Lever et al., 2000).

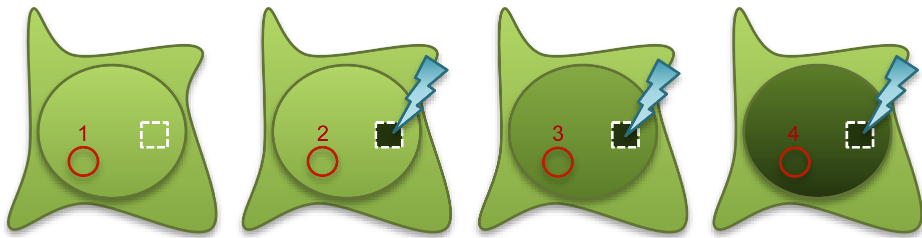
Figure 4. Empirical assessment of protein mobility by photobleaching. (A) In fluorescence recovery after photobleaching (FRAP), the fluorescent signal of a small region is quenched by a single bleaching event, and recovery of fluorescence within that region is measured by time-lapse microscopy. **(B)** in fluorescence loss in photobleaching (FLIP), a small area in the cell is continuously bleached and diminution of total cellular fluorescence outside that region indicates a dynamic behavior. **(C)** Freely-diffusing proteins are the most dynamic, whereas proteins that associate with complexes (e.g. chromatin-associated proteins) display reduced mobility. Histones appear immobile.

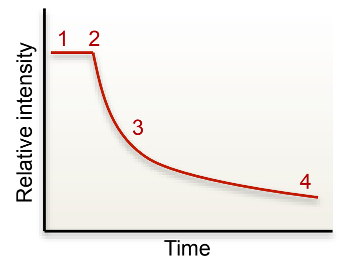
A. Fluorescence Recovery After Photobleaching (FRAP)



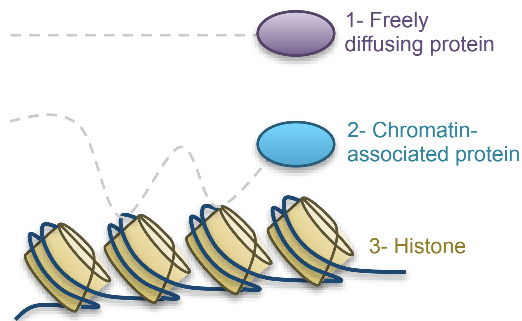


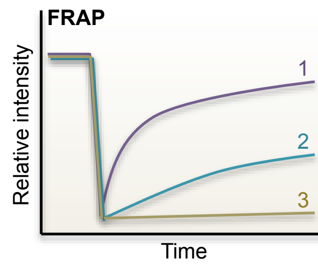
B. Fluorescence Loss In Photobleaching (FLIP)

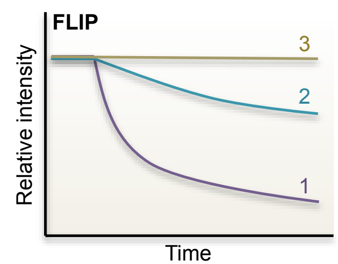




C







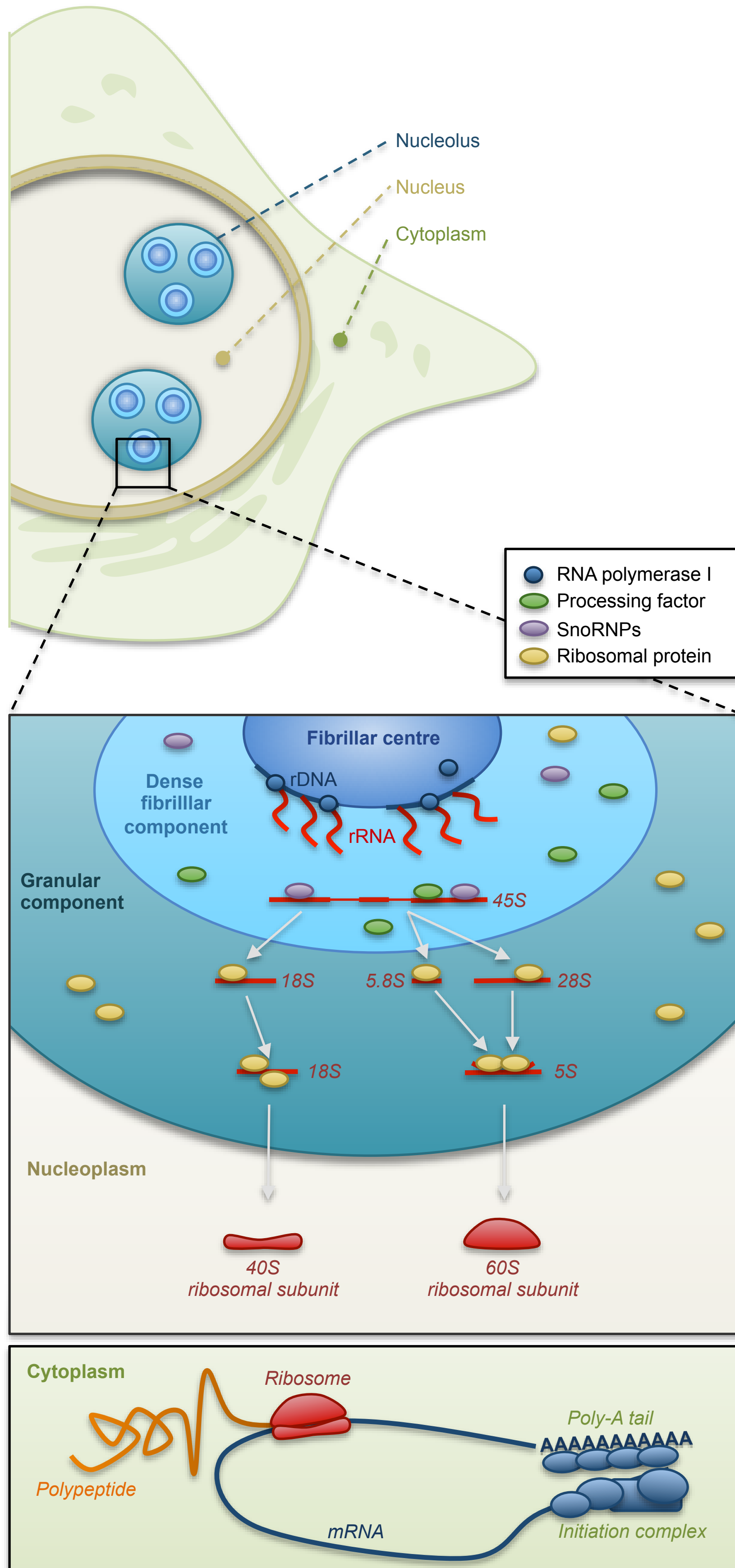
1.3. THE NUCLEOLUS

1.3.1. The nucleolus as the site of ribosomal biogenesis

The nucleolus is a large subnuclear structure that appears as a dark region in the nucleus of eukaryotic cells. Using optical microscopy, cytologists in the 19th century were able describe it in great detail without understanding its function (Montgomery, 1898). In the 1960s, advances in molecular biology allowed for the detection of RNA in the nucleolus, for the localization of ribosomal genes in the nucleolar region, and for the biochemical characterization of many of its components (Brown and Gurdon, 1964; Edstrom and Beermann, 1962; Edstrom et al., 1961; Miller and Beatty, 1969; Penman et al., 1966; Perry, 1960; Perry, 1962). These findings led to the rapid establishment of the nucleolus as the site of ribosomal biogenesis. We know today that nucleoli are structured around ~400 copies of ribosomal genes found in tandem on the short arm of all five acrocentric chromosomes (13, 14, 15, 21 and 22). Actively transcribed repeats cluster within chromosomal regions known as nucleolar organizing regions (NORs) (Lamond and Earnshaw, 1998; Melese and Xue, 1995; Thiry and Lafontaine, 2005). Nucleoli disassemble and reassemble around each mitotic cycle, recruiting during interphase all of the factors necessary for their basic structure and functional organization (Dundr et al., 2000; Hernandez-Verdun, 2011; Muro et al., 2010). Nucleoli are composed of three distinct regions that correspond to the different steps in the processing of ribosomes: the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC) (Kressler et al., 2010; Scheer and Hock,

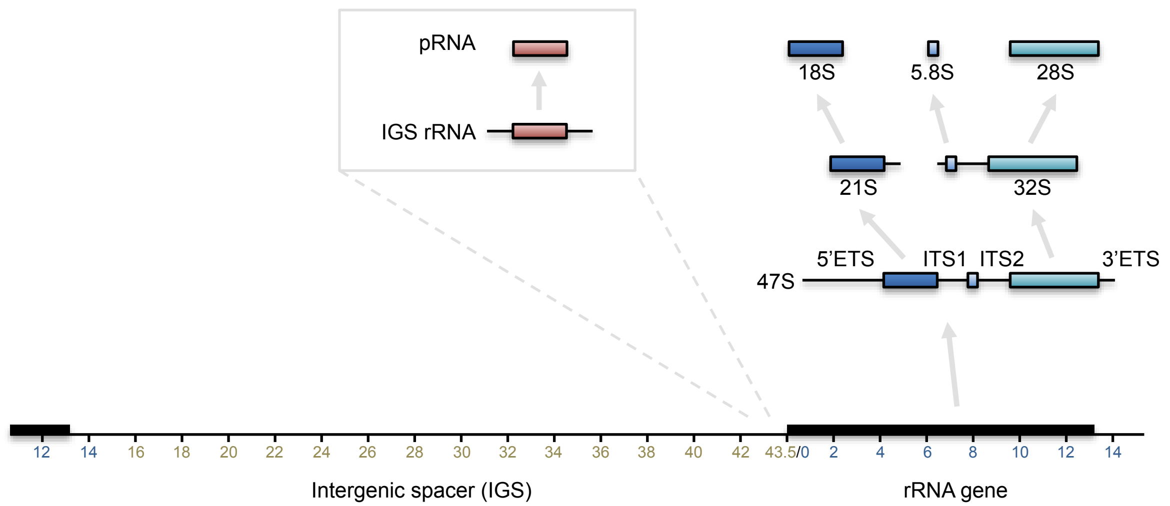
1999; Thiry and Lafontaine, 2005). Ribosomal DNAs (rDNA) is transcribed by a special RNA polymerase - RNA pol I - at the intersection between the FC and the DFC. The resulting product, known as the 47S pre-ribosomal RNA (rRNA), is a 13.3 kb polycistronic transcript comprised sequentially of the 5' external transcribed spacer (ETS1), 18S rRNA, internal transcribed spacer (ITS1), 5.8S rRNA, ITS2, 28S rRNA and 3'ETS **(Figure 5 and Figure 6)** (Fatica and Tollervey, 2002; Mullineux and Lafontaine, 2012). Pre-rRNA transcripts are then matured in the Dense Fibrillar Component through a series of processing and chemical modification steps that including cleavage, pseudouridylation and methylation (Matera et al., 2007). Ultimately, three distinct RNAs are produced (18S, 5.8S and 28S) and assembled with ribosomal proteins in the GC. 5.8S and 28S subunits assemble to form the large 60S ribosomal subunit, while 18S matures into the small 40S subunit. Both are then exported to the cytoplasm to become fully functional ribosomal subunits involved in mRNA translation **(Figure 5)** (Boisvert et al., 2007; Fatica and Tollervey, 2002; Fromont-Racine et al., 2003; Lafontaine and Tollervey, 2001; Sirri et al., 2008; Tschochner and Hurt, 2003). Therefore, the nucleolus provides the cell with the protein synthesis capabilities needed to sustain growth and proliferation (Lempiainen and Shore, 2009; Pederson and Kumar, 1971; Thomas, 2000). The process of ribosomal biogenesis accounts for the majority of RNA synthesized in the cell. Given the high energetic demand of this process, transcription of rDNA can be modulated in response to a variety of cellular stimuli, including aging, viral infection, amino acid starvation and toxic lesions (Banerjee et al., 2005; Belin et al., 2010; Chedin et al., 2007; Grummt, 1999; Grummt and Voit, 2010; Kao et al., 2004;

Figure 5. Functional organization of the nucleolus. Nucleoli are composed of three distinct compartments that correspond to the different steps of ribosomal biogenesis: the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GV). Transcription of rRNA by RNA pol I takes places at the intersection between the FC and the DFC. Newly-synthesized pre-rRNA is processed within the DFC, and assembled with ribosomal proteins within the GC. Mature ribosomal subunits are then exported to the cytoplasm to take part in protein synthesis. Capped and poly-adenylated mRNAs are captured by ribosomes through their translation initiation complex and translated into a polypeptide sequence. Adapted from Boivert *et al.*, 2007



Nucleolus
Nucleus
Cytoplasm
RNA polymerase I
Processing factor
SnoRNPs
Ribosomal protein
Fibrillar centre
Dense fibrilllar component
rDNA
rRNA
Granular component
45S
18S
5.8S
28S
18S
5S
Nucleoplasm
40S ribosomal subunit
60S ribosomal subunit
Cytoplasm
Ribosome
Poly-A tail
AAAAAAAAAAA
Polypeptide
mRNA
Initiation complex

Figure 6. Synthesis of RNA from the ribosomal cassette. The human ribosomal cassette is composed of a ~13 kb transcribed region followed by a ~30 kb intergenic spacer. RNA polymerase I is recruited to a promoter region, upstream of the ribosomal gene and transcribes a single polycistronic transcript that is then processed by either of two pathways into the 18S, 5.8S and 28S rRNAs. pRNA is noncoding RNA emerging from the promoter region. Adapted from Jacob *et al*. 2012.


pRNA
IGS rRNA
18S
5.8S
28S
21S
32S
47S
5'ETS
ITS1
ITS2
3'ETS
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
43.5/0
2
4
6
8
10
12
14
Intergenic spacer (IGS)
rRNA gene

Shaw and Doonan, 2005). This regulation is mostly performed by transcription factors and chromatin-remodelling complexes, with additional mechanisms reported at the initiation, elongation and termination steps (Leary and Huang, 2001; Lempiainen and Shore, 2009; Santoro and Grummt, 2001). (Paragraph adapted from first author publication; Jacob *et al.*, 2012)

1.3.2. Structural flexibility of the nucleolus

The remarkable plasticity of the nucleolus is demonstrated during each mammalian cell cycle, with the structure disassembling at the onset of mitosis and reassembling at mitotic exit (Dimario, 2004; Dundr et al., 2000; Hernandez-Verdun, 2006; Leung et al., 2004; Roussel et al., 1996; Savino et al., 2001). It is also evident under conditions of cellular stress (Al-Baker et al., 2005; Boulon et al., 2010; Govoni et al., 1994; Mayer and Grummt, 2005; Rubbi and Milner, 2003; Welch and Suhan, 1985; Welch and Suhan, 1986). Actinomycin D (ActD)-mediated inhibition of transcription leads to an irreversible redistribution of both nucleolar and nucleoplasmic proteins into cap structures at the periphery of the nucleolus (Journey and Goldstein, 1961; Reynolds et al., 1964; Shav-Tal et al., 2005) and a correspondingly dramatic alteration in the nucleolar proteome (Andersen et al., 2005). Changes in the nucleolar proteome are also observed in response to DNA damage and viral infection (Boisvert et al., 2010; Boisvert and Lamond, 2010; Emmott et al., 2010; Hiscox, 2007; Lam et al., 2010; Montgomery et al., 1966; Moore et al., 2011). Structural alterations are noticeable in response to heat shock, viral infection and senescence (Bemiller and Lee, 1978; Dove et al., 2006; Pelham, 1984; Puvion-

Dutilleul and Macieira-Coelho, 1983; Simard and Bernhard, 1967; Welch and Suhan, 1985). A reversible disorganization of nucleolar structure can be induced by treatment with casein kinase 2 (CK2) inhibitor 5,6-dichloro-beta-D-ribofuranosylbenzimidazole (DRB) (Panse et al., 1999; Scheer et al., 1984). Under these conditions, RNA pol I remains active while nucleoli dissociate into substructures and disperse throughout the nucleoplasm as a necklace of transcribing “beads”. Nucleolar reformation upon removal of the drug is CK2-driven and ATP/GTP-dependent (Louvet et al., 2006). Although the functional significance of some of these responses remains unclear, they demonstrate that the structure of the nucleolus is highly responsive to a variety of stimuli (Olson and Dundr, 2005). Furthermore, the nucleolus harbours many proteins that are unrelated to ribosomal biogenesis, supporting the notion that it is a plurifunctional organelle (Andersen et al., 2005; Andersen et al., 2002; Boisvert et al., 2007; Coute et al., 2006; Leung et al., 2003; Pederson and Tsai, 2009; Pendle et al., 2005). In fact, the nucleolus has been reported to associate with additional structural elements, including perinuclear compartments in cancer cells (Ghetti et al., 1992; Huang et al., 1997; Huang et al., 1998; Matera et al., 1995; Pollock et al., 2011; Pollock and Huang, 2009) and intranucleolar bodies (Hutten et al., 2011), amongst other suspected subcompartments (Politz et al., 2000). (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

1.3.3. The ribosomal intergenic spacer

Human rRNA coding sequences are separated by a ~31kb region formerly referred to as the non-transcribed spacer (Gonzalez and Sylvester, 1995; Hillis and Dixon, 1991; Shaw and Jordan, 1995), which has not been attributed any significant function. Early work posited that this region either played a structural role within the nucleolus, allowing for the formation of tightly packed nucleolar organizing regions (Kaplan et al., 1993), or provided some form of genomic stabilization through the attachment of specific sequences to the nuclear matrix (Wachtler et al., 1991). Evolutionarily, the IGS appears to have increased in size over time, measuring only 2.5 kb in yeasts, 5.1 kb in *Drosophilia melanogaster,* 5.7 kb in *Xenopus laevis*, while the chickens, mouse and primate sequences are all approximately 30kb in length(Sylvester et al., 1986). At the sequence level the IGS differs considerably from the rRNA coding sequences, notably through its high level of variability both in length and nucleotide composition (Gonzalez et al., 1985; Gonzalez and Sylvester, 1995; Hillis and Dixon, 1991; Sasaki et al., 1987; Sylvester et al., 1989). Disparities in length have been attributed to unequal homologous exchanges (Erickson and Schmickel, 1985), while sequence divergences are caused by the incorporation of a large number of retrotransposons, or Alu elements (Sylvester et al., 1986) and microsatellite variations thought to be caused by slipped-strand mispairing during DNA replication (Levinson and Gutman, 1987). Despite the seemingly disorganized composition and a historical lack of evidence suggesting transcriptional activity, the ribosomal IGS appears to be more functionally relevant than previously believed. Promoter mapping studies of the

Xenopus laevi IGS identified spacer promoters that share 90% homologies with those of rRNA genes (Moss and Birnstiel, 1979; Moss et al., 1980). These promoters were capable of activating PolI-mediated transcription, though the resulting nascent transcripts were rapidly terminated well upstream of the rRNA sequences by T3 termination sites (Morgan et al., 1983). While similar promoters were also found in mice (Kuhn and Grummt, 1987), no known function was conclusively attributed to these transcripts, though a potential role in rRNA transcription activation was speculated (Caudy and Pikaard, 2002; Paalman et al., 1995). (Paragraph adapted from first author publication; Jacob *et al.*, 2012)

1.3.4. Promoter-associated ribosomal noncoding RNA

The best evidence of functional transcriptional activity within the IGS involves the promoter-associated RNA (pRNA) **(Figure 6)**. Analysis of the IGS region 2 kb upstream of the rRNA start site identified a 150-250 nucleotide PolI-mediated transcript, known as the promoter-associated RNA (pRNA). This molecule was shown to be involved in targeting TIP5, the large subunit of the nucleolar remodelling complex (NoRC), to the ribosomal cassettes. Recruitment of the NoRC results in the transcriptionally-repressive histone modifications H3K9me1 and H4K20me3 and in HP1 binding, ultimately inhibiting rRNA synthesis (Mayer et al., 2006; Schmitz et al., 2010). The secondary structure of the pRNA is apparently more conserved than its sequence. Though mouse and human pRNA transcripts share less than 50% sequence identity, both possess a highly conserved stem-loop structure. In fact, expression of the human variant can rescue the phenotype of pRNA depleted

mouse cells, demonstrating the significance of the structure (Mayer et al., 2008). The importance of secondary structure within the IGS is particularly striking considering the overall divergence in sequence similarity between mice and humans, as the IGS of these related species share only ~40% sequence similarity. Transcription of pRNA is driven by a spacer promoter that is 90% homologous to the rRNA promoter yet produces pRNA at a rate 1,000-fold lower (Santoro et al., 2010). The resulting transcript is present at very low levels throughout most of the cell cycle, as it is rapidly degraded by the exosome. However, during mid-S phase pRNA levels transiently increase 2-fold, allowing it to repress rRNA synthesis in late replication(Santoro et al., 2010). Although pRNA originates from a regions closely associated with rRNA, expression of these transcripts appears to be regulated independently (Santoro et al., 2010), establishing pRNA as the first example of a functional ribosomal IGS-derived transcript. (Paragraph adapted from first author publication; Jacob *et al.*, 2012)

1.3.5. The plurifunctional nucleolus

The view of the nucleolus as a mere factory for ribosomes remained until the late 1990s and early 2000s, when advances in nucleolar purification and mass spectrometry allowed for a characterization of the nucleolar proteome, revealing over 700 human proteins, 90 percent of which have yeast homologues (Andersen et al., 2005; Andersen et al., 2002; Coute et al., 2006; Leung et al., 2003; Ospina and Matera, 2002; Scherl et al., 2002). This high degree of conservation suggests an essential and plurifunctional role for the nucleolus (Boisvert et al., 2007; Olson et al.,

2000; Olson et al., 2002; Pederson, 1998b; Pederson, 2002). In fact, there is mounting evidence that the nucleolus is involved in many cellular processes, including the processing and/or export of mRNA, tRNA and SRP RNA (Bertrand et al., 1998; Boyne and Whitehouse, 2006; Jacobson and Pederson, 1998; Kadowaki et al., 1994; Ko et al., 2000; Paushkin et al., 2004; Pederson and Politz, 2000; Politz et al., 2000; Schneiter et al., 1995; Thompson et al., 2003; Wang et al., 1991), senescence (Guarente, 1997; Johnson et al., 1998; Marciniak et al., 1998), cellular stress (Mayer et al., 2005; Olson, 2004) and cell cycle regulation (Azzam et al., 2004; Cockell and Gasser, 1999; Pederson, 1998a; Sherr and Weber, 2000; Shou et al., 2001; Shou et al., 1999; Visintin et al., 1999). Most of these functions are related to the dynamic association of proteins with the nucleolar architecture. In 2005, a study published in *Nature* characterized the flux of close to 500 nucleolar proteins in response to different metabolic inhibitors, revealing significant changes in the nucleolar proteome over time (Andersen et al., 2005). In fact, despite the presence of a set of resident proteins, most nucleolar proteins shuttle rapidly between the nucleolus and the nucleoplasm (Carmo-Fonseca, 2002a; Dundr et al., 2000; Lander et al., 2001; Phair and Misteli, 2001; Tsai and McKay, 2005). This incredible ability of the nucleolus to retain and release various proteins throughout the life of the cell is particularly intriguing in that, unlike most organelles, it is not enclosed by a membrane. Uncovering the exact role of the nucleolus and the mechanism by which is selectively modifies the proteome would help understand some of the cell's most basic adaptive mechanisms.

1.4. NUCLEOLAR SEQUESTRATION OF PROTEINS

1.4.1. Definition

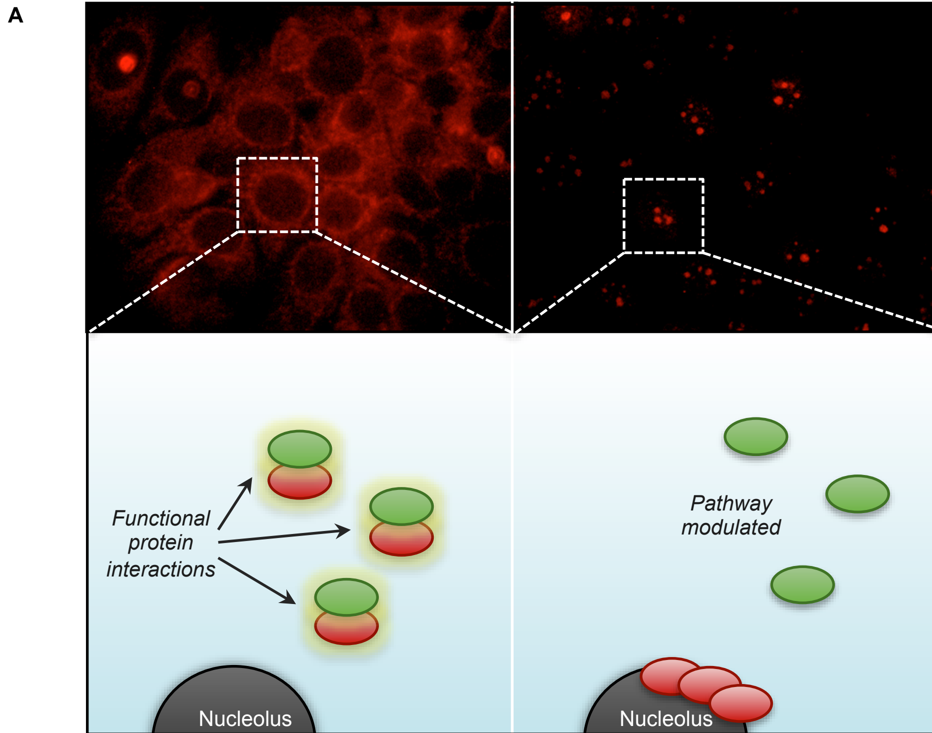
Nucleolar sequestration is a post-translational regulatory mechanism in which the nucleolus captures selected proteins in response to specific physiological or stress conditions. Capture by the nucleolus inactivates proteins by preventing their free diffusion, and therefore their interaction with substrates. Depending on the function of the targeted proteins, nucleolar sequestration can be inhibitory or activatory for the associated pathways **(Figure 7A)**.

1.4.2. Examples in the literature

Several examples of functional nucleolar sequestration have been reported over the past decade and a half **(Figure 7B)**. Cell division cycle 14 (CDC14), a yeast tyrosine-phosphatase involved in mitosis regulation, represents one of the first examples of nucleolar sequestration. In late anaphase, it is released from the nucleolar RENT complex, allowing it to triggers mitotic exit (Shou et al., 1999; Visintin et al., 1999). Another example involves the human telomerase reverse transcriptase (hTERT), which is part of a complex that maintains genome stability by capping chromosome ends. hTERT is released from the nucleoli in response to transformation or DNA damage, and captured by the nucleoli in response to ionizing radiation. Regulation of the subnuclear localization of hTERT by the nucleolus modulates its access to telomeric substrates (Wong et al., 2002). Adenosine Deaminase that Acts on RNA 2 (ADAR2) is an adenosime deaminase that affects the splicing pattern of mRNA.

Figure 7. Nucleolar sequestration as a novel mechanism of post-translational regulation. (A) Capture of a protein by the nucleolar architecture (red) interrupts its functional interaction with substrate molecules (green) and thus modulates the pathway involved. **(B)** List of the main proteins reported to be regulated by nucleolar sequestration.

A



B

Symbol	Full name	Stimulus	Nucleolar response	Reference
VHL	von Hippel Lindau tumour supressor	Extracellular acidosis	Capture	Mekhail et al., 2004
MDM2	Murine double minute 2 homolog	Ribosomal stress DNA damage	Capture	Webert et al., 1999 Lorum et al., 2000 Bernardi et al., 2004
Hsp70	Heat Shock Protein 70 kDa	Heat shock	Capture	Welch and Feramisco, 1984
CDC14	Cell division cycle 14 (C. cerevisiae)	Anaphase	Release	Shou et al., 1999 Visintin et al., 1999
hTERT	Human telomerase reverse transcriptase	Transformation, DNA damage Ionizing radiation	Release Capture	Wong et al., 2002
ADAR2	Adenosine deaminase that acts on RNA 2	Ribosomal stress	Release	Sansam et al., 2003
RelA	p65 subunit of transcription factor NF-kappaB	Aspirin, serum withdrawal, UV-C radiation	Capture	Stark and Dunlop, 2005
Hand1	Heart and neural crest derivatives expressed protein 1	Cell differentiation	Release	Mardindill et al., 2007

Under standard conditions, ADAR2 is localized in the nucleoli and away from the sites of mRNA transcription. In response to selective inhibition of rRNA synthesis, release of ADAR2 from the nucleoli to the nucleoplasm is associated with an increase in mRNA editing (Sansam et al., 2003). c-myc is a nuclear transcription factor and potent oncogene which is regulated by proteosomal degradation. Proteosome inhibition or elevated levels of c-myc result in its accumulation in nucleoli (Arabi et al., 2003). RelA, also known as p65, is a transcription factor thato is part of the NF-κB complex. In response to proapototic signals such as aspirin treatment, serum withdrawal, and UV-C radiation, RelA is sequestered in the nucleoli, which reduces NF-κB driven transcription and activates apoptosis (Stark and Dunlop, 2005). Hand1 (Heart- and neural crest derivatives-expressed protein 1) is a transcription factor involved in placentation and cardiac morphogenesis in the developing embryo. Release of Hand1 from the nucleolus acts a molecular switch that commits proliferating cells to a differentiated giant-cell fate (Martindill et al., 2007). Most importantly, the best-known and characterized example of nucleolar sequestration involve the von Hippel Lindau (VHL) protein in response to anaerobic metabolism, the heat shock protein 70 kDa (hsp70) in response to heat shock, and the murine double minute 2 homolog (MDM2) in response to ribosomal stress.

1.4.3. Nucleolar sequestration of the von Hippel Lindau protein

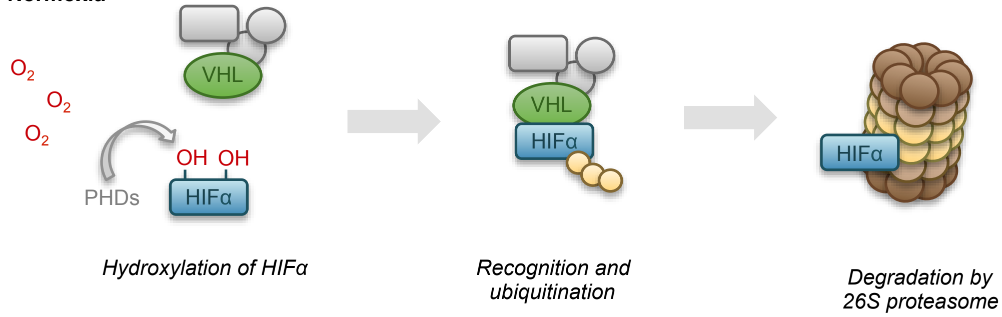
VHL is a tumour suppressor protein that will be used as the main model for nucleolar sequestration in this thesis. Therefore, its clinical relevance and role in regulating the hypoxic response will be briefly discussed.

VHL disease: VHL disease is an inherited, autosomal dominant disorder caused by allelic mutations in the VHL tumour suppressor gene, with an incidence of 1 in 36,000 births. In accordance with the Knudson two-hit hypothesis of tumorigenesis, this loss of heterozygosity (LOH) predisposes affected patients to the development of several tumours. Point mutations in the unaffected allele, referred to as "second hits", result in a complete loss of VHL activity and an oncogenic phenotype. The most frequent tumours observed are retinal and central nervous system (CNS) haemanglioblastomas, pancreatic islet tumours, endolymphatic sac tumours, phaeochromocytomas and renal cell carcinomas (RCC) (Lonser et al., 2003; Maher et al., 1990; Richard et al., 2004), most of which are highly vascularized (Khacho and Lee, 2009). VHL disease accounts for 1/3 of CNS haemanglioblastomas, >50% of retinal angiomas, 50% of isolated familial phaeochromocytomas, 11% of sporadic phaeochromocytomas and 1% of RCCs (Maher et al., 2011). VHL disease is not the only cause of VHL-mediated RCC, as many non-hereditary (sporadic) cancers are linked with VHL mutations. More than 300 such mutations have been reported, in up to 75% of RCCs (Gallou et al., 1999; van Houwelingen et al., 2005). While the majority of these tumours are benign, VHL-associated RCC is malignant and affects 4000 Canadians a year, 1100 of whom will die from it. Worldwide, RCC results in over 100,000 deaths yearly.

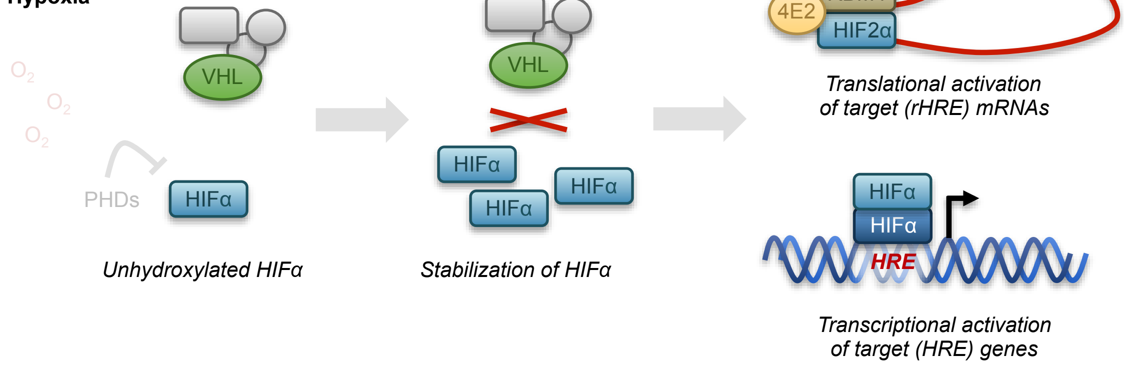
Oxygen-dependent regulation of HIF by VHL: Although of variety of mutations have been reported in VHL (e.g. chromosome 3p deletion, suppressed expression, truncation, loss-of-function base substitution), they all result in a constitutive activation of the hypoxia-inducible pathway (Patel et al., 2006). VHL is the

recognition motif for a Cullin-2-contaning E3 ubiquitin ligase complex involved in the response of the cell to hypoxic stress (Iwai et al., 1999; Kibel et al., 1995; Lisztwan et al., 1999; Lonergan et al., 1998; Pause et al., 1997; Pause et al., 1999). Its primary function is to mediate the oxygen-dependent ubiquitylation and proteasomal degradation of the α-subunit of the hypoxia inducible transcription factors (HIF1α and HIF2α) (Kamura et al., 2000; Maxwell et al., 1999; Ohh et al., 2000; Tanimoto et al., 2000). HIF is a heterodimeric transcription factor involved in the hypoxic response and composed of a highly regulated alpha subunit and constitutively expressed beta subunit. Under normal oxygen tension (normoxia), HIFα is hydroxylated at key proline residues by prolyl hydroxylase (PHD) enzymes, allowing VHL to recognize it and to target it for ubiquitylation and subsequent degradation by the 26S proteasome **(Figure 8A)**. However, under low oxygen tension (hypoxia), the hydroxylation of HIFα is prevented by the absence of molecular oxygen (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). This suppresses the recognition and subsequent degradation of HIFα, allowing it to stabilize and to bind to Hypoxia Response Elements (HRE) within the regulatory regions of target genes **(Figure 8B)**. Binding of HIF promotes the recruitment of transcriptional coactivators and thus induces expression. The number of HIF target genes is evaluated at 1-5% of the human genome (Semenza, 2003) and includes the vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1) and transforming growth factor-α (TGF-α) (Gunaratnam et al., 2003; Harris, 2002; Kaur et al., 2005; Semenza, 2000; Semenza, 2003). This cellular response to hypoxia is essential to normal physiology,

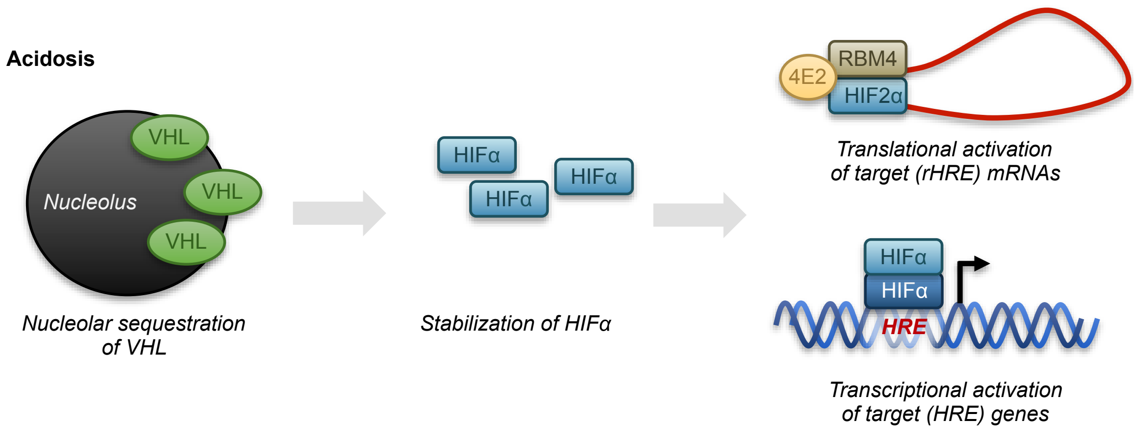
Figure 8. **Oxygen and pH-dependent regulation of HIFα by VHL. (A)** Under normal oxygen tension (normoxia), prolyl hydroxylase (PHD) enzymes hydoxylize HIFα on key proline residues using molecular oxygen. This post-translational modification allows VHL, the recognition motif of a Cul2-containing E3 ubiquitin ligase complex, to recognize HIFα and to target it for degradation by the 26S proteasome. **(B)** Under hypoxia, PHDs are inactivated and unmodified HIFα escapes recognition by VHL. This allows it to stabilize, dimerize with the constitutively expressed β subunit, and activate the transcription of its target HRE genes. HIF2α is also a translation factor that activates the translation of rHRE containing mRNAs. **(C)** In acidosis, VHL is captured by the nucleolus, allowing HIFα to evade recognition and degradation, regardless of its hydroxylation status.



A. Normoxia
O_2
O_2
O_2
VHL
OH OH
PHDs
HIFα
Hydroxylation of HIFα
VHL
HIFα
Recognition and ubiquitination
HIFα
Degradation by 26S proteasome



B. Hypoxia
O_2
O_2
O_2
VHL
PHDs
HIFα
Unhydroxylated HIFα
VHL
HIFα
HIFα
HIFα
Stabilization of HIFα
RBM4
4E2
HIF2α
Translational activation of target (rHRE) mRNAs
HIFα
HIFα
HRE
Transcriptional activation of target (HRE) genes



C. Acidosis
VHL
VHL
VHL
Nucleolus
Nucleolar sequestration of VHL
HIFα
HIFα
HIFα
Stabilization of HIFα
RBM4
4E2
HIF2α
Translational activation of target (rHRE) mRNAs
HIFα
HIFα
HRE
Transcriptional activation of target (HRE) genes

and is central to the development of tumours, muscle stress and ischemic disorders (Khacho and Lee, 2009). In addition to its role in transcription, HIF2α is a translation factor that regulates the activation of a hypoxia-specific translation initiation complex (Franovic et al., 2007; Uniacke et al., 2012). Under normal oxygen tension, translation is initiated by the binding the eukaryotic initiation factor 4E (eIF4E) to the 7-methylguanosine (m^7-GpppG) 5' cap of mRNA (Gebauer and Hentze, 2004). However, under hypoxia, eIF4E is sequestered by eIF4E binding protein (eIF4EBP), thereby inhibiting standard cap-mediated translation initiation (Braunstein et al., 2007; Brugarolas et al., 2004). This absence of eIF4E-mediated translation is compensated by the formational of an alternative complex, composed of HIF2α, the RNA binding protein RBM4 and the eIF4E-homolog eIF4E2. This complex is recruited by a conserved RNA motif, the RNA hypoxia response element (rHRE), that if found on a wide range of mRNAs, including that encoding the epidermal growth factor receptor (EGFR), the insulin-like growth factor 1 (IGF1) and the XIAP-associated factor 1 (XAF1), an inhibitor of apoptosis (Uniacke et al., 2012) **(Figure 8B)**. By repressing HIFs, VHL controls two oncogenic pathways: a transcriptional program that mediates adaptation to hypoxia, as well as a translational program that promotes autonomous growth and apoptosis resistance.

HIF activation by pH-dependent nucleolar sequestration of VHL: Oxygen concentration is not the only physiological variable that regulates the activity of VHL. As hypoxic cells increase the uptake of glucose and promote anaerobic metabolism, lactic acid builds up as the end product of fermentation. To maintain homeostasis in response to this metabolic adjustment, cells respond by pumping out

excess H+ ions, inevitably causing an acidification of the extracellular milieu (acidosis). In 2004, our group reported that extracellular acidosis regulates VHL by triggering its nucleolar relocalization. Nucleolar sequestration of VHL allows HIFα to evade degradation and to activate its target genes, even in the presence of oxygen (Mekhail et al., 2004a) **(Figure 8C)**. These results suggest the evolution of a second, independent mechanism of HIF activation whereby the cell responds to changes in oxygen tension by sensing extracellular pH. However, the existence of this alternative mechanism raises the question of its redundancy; if extracellular acidosis is the result of hypoxia, the HIF pathway should theoretically already be activated, making the status of VHL irrelevant. Several hypotheses have been proposed to justify the existence of an acidosis-specific pathway. One model speculates that pH-dependent nucleolar sequestration of VHL serves as a mechanism of hypoxic cell memory (Mekhail et al., 2004b). Extracellular acidosis persists for an extended period of time, even after a return to normal oxygen levels, allowing HIF and its target genes to remain elevated. Moreover, in scenarios of intermittent ischemia, acidosis would ensure a continuous activation of the HIF pathway. In fact, acidosis has been known to protect cells from several different toxic effects of ischemic injuries, suggesting a significant benefit from acidosis-mediated hypoxic cell memory (Currin et al., 1991; Giffard et al., 1990b; Gladden, 2001; Kaku et al., 1993; Morimoto et al., 1997; Nielsen et al., 2001). Another possibility involves the acidification of the extracellular milieu by hyperactive, normoxic cells. For instance, cancer cells have been shown to undergo a change in metabolism that favours glycolysis and the production of lactic acid. This

phenomenon, known as the Warburg Effect, accounts for the acidity of tumours that have not necessarily reached hypoxia (Warburg, 1956; Warburg et al., 1927). Extracellular acidosis, and therefore HIF activation by nucleolar sequestration of VHL, might help explain the central role played by HIFs in human cancers (Beasley et al., 2002; Franovic et al., 2007; Franovic et al., 2009; Franovic and Lee, 2010; Keith and Simon, 2007; Semenza, 2003; Semenza, 2010; Talks et al., 2000; Zhong et al., 1999).

1.4.4. Nucleolar sequestration of hsp70

Heat shock, also known as hyperthermia, is a stress that is triggered by a temperature increase of a few degrees above the normal physiological settings of an organism. This applies to mammalian cells, yeasts and even thermophilic microorganisms living at extreme temperatures (Brown and Lupas, 1998; D'Amico et al., 2006; Takai et al., 1998). Proteins have evolved to be conformationally flexible at their optimal physiological temperature and are easily destabilized by thermal fluxtuations (Richter et al., 2010). Therefore, it appears that the cell does not respond to temperature per se, but instead to the deleterious effects of heat, which include protein unfolding, entanglement and unspecific aggregation, as well as general damage to its internal organization (Courgeon et al., 1984; Michel and Starka, 1986; Welch and Suhan, 1985; Yura et al., 1984). In fact, the cellular "heat shock response" is deployed in response to a variety of stresses, including oxidation, heavy metals or ethanol (Heikkila et al., 1982; Michel and Starka, 1986; Richter et al., 2010). For that reason, the study of heat shock has provided answers that have

been relevant to a variety of clinical settings. This heat shock response involves primarily the induction of a series of chaperone proteins. Heat shock 70 kDa protein (Hsp70, also known as HspA1) is the main protein in the 70 kDa heat shock protein family (Kampinga et al., 2009). It is composed of three domains: a N-terminal ATPase domain that drives conformational changes in the other two domains, a substrate binding domains with an affinity for neutral and hydrophobic residues, and a C-terminal domain that regulates interactions with substrates. Together with co-chaperones such as Hsp40, Hsp70 guides pathways of protein folding, translocation and degradation (Beckmann et al., 1990; Chiang et al., 1989; Chirico et al., 1988; Saliba et al., 2002). By preventing protein aggregation and stabilizing partially unfolded polypeptides, Hsp70 protects the cell from thermal and oxidative stress (Ben-Zvi and Goloubinoff, 2001; Johnston and Kucey, 1988; Mayer and Bukau, 2005; Mosser et al., 1997; Mosser et al., 2000; Ohtsuka and Hata, 2000; Riabowol et al., 1988; Velazquez and Lindquist, 1984). One interesting aspect of Hsp70 is its accumulation in the nucleoli of heat shock cells. It is the first, and possibly best known example of nucleolar sequestration (Alastalo et al., 2003; Hatayama et al., 1993; Milarski and Morimoto, 1989; Morcillo et al., 1997; Ohtsuka et al., 1986; Welch and Feramisco, 1984; Xu et al., 2003; Xu et al., 1998; Yamane et al., 1995; Zeng et al., 2004). In spite of the interest it has gathered, the mechanism and significance of this nucleolar translocation remains poorly understood. It has been suggested that nucleolar localization of Hsp70 in heat shock serves to accelerate the recovery of nucleolar morphology (Pelham, 1984) or to protect cells from single-strand DNA breaks (Kotoglou et al., 2009). Interestingly, the translocation of Hsp70

to the nucleolus under heat shock is associated with a modulation of nucleolar structure and function (Simard and Bernhard, 1967; Welch and Suhan, 1985). This suggests that heat shock may induce a important nucleolar response, perhaps one that is central to the systemic adaption of cells to thermal stress.

1.4.5. Nucleolar sequestration of the murine double minute 2 protein

The murine double minute 2 homolog (MDM2). MDM2 is a proto-oncogene that physically interacts with, and is a negative regulator of, the tumour suppressor protein p53. In cells that are not subjected to stress, MDM2 functions as an E3 ubiquitin ligase that targets p53 for degradation. However, in response to ribosomal stress or DNA damage, MDM2 relocalizes to the nucleolus, allowing p53 to evade degradation and to activate its target genes (Bernardi et al., 2004; Lohrum et al., 2003; Poyurovsky et al., 2003; Weber et al., 1999). Nicknamed the "guardian of the genome", p53 coordinates a number of DNA repair, cell cycle and apoptosis pathways, making its activation by the nucleolus of central importance from a clinical point of view (Hollstein et al., 1991; Oren, 2003; Soussi et al., 1994; Vazquez et al., 2008; Vousden and Lane, 2007).

1.5. THE NUCLEOLAR DETENTION PATHWAY

1.5.1. The Nucleolar Detention Signal

Subcellular trafficking of proteins is mediated by discrete amino acid sequences that target them to their site of action. Such sequences are responsible for the localization of protein to the nucleus, cytoplasm, Golgi or plasma membrane (Conti

and Izaurralde, 2001; Kutay and Guttinger, 2005; Lee and Helmann, 2006; Shikano et al., 2005). Localization of proteins to subnuclear domains, such as PML bodies, Cajal bodies or nucleoli is particularly interesting in that these compartments are not enclosed by a membrane. Instead, retention of macromolecules within these regions relies on high affinity interactions with scaffolding elements (Chubb and Bickmore, 2003; Isogai and Tjian, 2003; Misteli, 2001; Misteli, 2004; Zimber et al., 2004). Mapping of nucleolus-associated proteins has revealed the existence of several nucleolar-targeting sequences. They are typically qualified as nucleolar localization signals (NoLS) (Catez et al., 2002; Hiscox, 2002; Weber et al., 2000) or nucleolar retention signals (NoRS) (Reed et al., 2006; Tsai and McKay, 2005). These motifs usually range between 6 to 30 amino acids in length and are rich in positively-charged amino acids such as arginine and lysine residues (Emmott and Hiscox, 2009). (R/K)(R/K)X(R/K), for instance, is a common nucleolar targeting sequence (Horke et al., 2004). However, these signals do not pertain to nucleolar sequestration as defined here. For one, they are usually found in proteins that remain functional and dynamic within the nucleolus, unlike sequestered proteins. Secondly, they are not stimulus-responsive, and constitutively localize to the nucleolus. KKLKKRNK for example, which has been identified as the NoLS of MDM2 (Lohrum et al., 2000), localizes to the nucleoli under normal conditions, even though MDM2 has been shown to be both stress-responsive (Bernardi et al., 2004; Weber et al., 1999) and statically detained (Mekhail et al., 2005). These elements suggest the existence a second type localization signal that would be both stimulus-responsive and capable of mediating static detention. Preliminary mapping of VHL revealed a

30-amino acid segment (100-130; TLPPGTGRRIHSYRGHLWLFRDAGTHDGLLV) that is capable of reversibly immobilizing a GFP marker in the nucleolus in response to acidosis (Mekhail et al., 2005). Further mapping established a high-order, position independent and predictive code referred to as nucleolar detention signal regulated by H+ (NoDS-H+) and defined as $[R\text{-}R\text{-}(I/L)\text{-}X_3\text{-}r]_{(n,n>1)}$+ $[L\text{-}(\Phi/N)\text{-}(V/L)]_{(n,n>1)}$. It must be composed of an arginine motif $[R\text{-}R\text{-}(I/L)\text{-}X_3\text{-}r]$ (e.g. RRIHSYR, RRLDIVR) followed by two or more hydrophobic motifs [L-(Φ/N)-(V/L)] where Φ symbolizes any hydrophobic residue (e.g. LWL, LLV, LFV, LQV). Complete loss of nucleolar targeting is obtained when arginines are replaced with lysines, suggesting that arginine function in the NoDS cannot be recapitulated with charged amino acids. This is a significant difference between the NoDS and a typical NoLS where arginines and lysines are usually interchangeable. In order to determine if this signal was a common code found in multiple proteins, a bioinformatic study was performed using the SwissProt database (Bairoch et al., 2004) and the nucleolar sequestration code as defined (Mekhail et al., 2007). A large list of candidate proteins was generated, including RING finger protein ubiquitin ligase/transcription regulator RNF8, inhibitor of apoptosis cIAP2 and DNA polymerase delta catalytic subunit p125/POLD1 **(Figure 9)**. Both GFP-tagged full length proteins and their predicted targeting sequences were statically detained in the nucleoli in response to acidosis **(Figure 9, bottom)**. The identification of a common NoDS indicates that nucleolar sequestration under acidosis is a general process, and not a VHL-specific phenomenon.

Figure 9. Nucleolar Detention Signal regulated by H+ (NoDS-H+). VHL, RNF8, cIAP2 and POLD1, among many others, contain an NoDS-H+. NoDS-H+ is composed of an arginine motif [RR(I/L)X_3r] (red) along with two or more hydrophobic repeats [L(Φ/N)(V/L)]$_{(n,n>1)}$ (blue). NoDS-H+ containing proteins are captured by the nucleolus in response to acidosis. Images: Mekhail *et al.*, 2007.

NoDS-H+: {[RR(I/L)X_3r]$_{(n,n>1)}$+ [L(Φ/N)(V/L)]$_{(n,n>1)}$}

VHL

MPRRAENWDEAEVGAEEAGVEEYGPEEDGGEESGAEESGPEESGPEELGAEEEMEAGRPRPVLRSVNSREPSQV
IFCNRSPRVV**LPV**WLNFDGEPQPYPTLPPGTG**RRI**HSY**R**GH**LWL**FRDAGTHDG**LLV**NQTE**LFV**PS**LNV**DGQPIF
ANIT**LPV**YTLKERC**LQV**VRSLVKPENY**RRL**DIV**R**SLYEDLEDHPNVQKDLERLTQERIAHQRMGD

RNF8

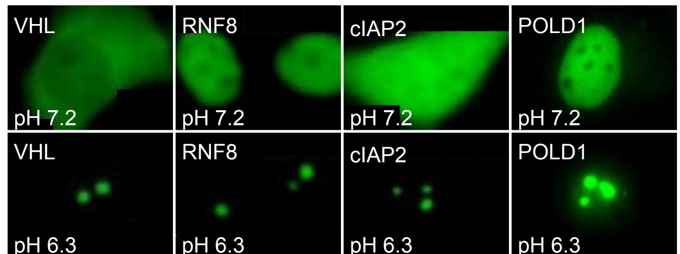
MGEPGFFVTGDRAGGRSWCLRRVGMSAGW**LLL**EDGCEVTVGRGFGVTYQLVSKICPLMISRNHCVLKQNPEGQW
TIMDNKSLNGVWLNRARLEPLRVYSIHQGDYIQ**LGV**PLENKENAEYEYEVTEEDWETIYPCLSPKNDQMIEKNK
ELRTKRKFSLDELAGPGAEGPSNLKSKINKVSCESGQPVKSQGKGEVASTPSDNLDPKLTALEPSKTTGAPIYP
GFPKVTEVHHEQKASNSSASQRSLQMFKVTMSRILRLKIQMQEKHEAVMNVKKQTQKGNSKKVVQMEQELQDLQ
SQLCAEQAQQQARVEQLEKTFQEEEQHLQGLEIAQGEKDLKQQLAQALQEHWALMEELNRSKKDFEAIIQAKNK
ELEQTKEEKEKMQAQKEEVLSHMNDVLENELQCIICSEYFIEAVTLNCAHSFCSYCINEWMKRKIECPICRKDI
KSKTYS**LVL**DNCINKMVNNLSSEVKE**RRI**VLI**R**ERKAKRLF

cIAP2

MNIVENSIFLSNLMKSANTFELKYDLSCELYRMSTYSTFPAGVPVSERSLARAGFYYTGVNDKVKCFCCGLMLD
NWKRGDSPTEKHKKLYPSCRFVQSLNSVNNLEATSQPTFPSSVTNSTHSLLPGTENSGYFRGSYSNSPSNPVNS
RANQDFSALMRSSYHCAMNNENARLLTFQTWPLTFLSPTDLAKAGFYYIGPGDRVACFACGGKLSNWEPKDNAM
SEHLRHFPKCPFIENQLQDTSRYTVSNLSMQTHAARFKTFFNWPSSVLVNPEQLASAGFYYVGNSDDVKCFCCD
GGLRCWESGDDPWVQHAKWFPRCEYLIRIKGQEFIRQVQASYPHLLEQLLSTSDSPGDENAESSIIHFEPGEDH
SEDAIMMNTPVINAAVEMGFSRSLVKQTVQRKILATGENYRLVND**LVL**DLLNAEDEIREEERERATEEKESND**L**
LLIRKNRMALFQHLTCVIPILDSLLTAGIINEQEHDVIKQKTQTSLQARELIDTILVKGNIAATVFRNSLQEAE
AVLYEH**LFV**QQDIKYIPTEDVSD**LPV**EEQL**RRL**QEE**R**TCKVCMDKEVSIVFIPCGH**LVV**CKDCAPSLRKCPICR
STIKGTVRTFLS

POLD1

MDGKRRPGPGPGVPPKRARGGLWDDDDAPRPSQFEED**LAL**MEEMEAEHRLQEQEEEELQSVLEGVADGQVPPSA
IDPRWLRPTPPALDPQTEPLIFQQLEIDHYVGPAQPVPGGPPPSRGSVPVLRAFGVTDEGFSVCCHIHGFAPYF
YTPAPPGFGPEHMGDLQRE**LNL**AISRDSRGGRELTGPAV**LAV**ELCSRESMFGYHGHGPSPFLRITVALPRLVAP
A**RRL**LEQGIRVAGLGTPSFAPYEANVDFEIRFMVDTDIVGCNWLELPAGKYALRLKEKATQCQLEADVLWSDVV
SHPPEGPWQRIAPLRVLSFDIECAGRKGIFPEPERDPVIQICS**LGL**RWGEPEPFLRLALTLRPCAPILGAKVQS
YEKEEDLLQAWSTFIRIMDPDVITGYNIQNFDLPYLISRAQTLKVQTFPFLGRVAGLCSNIRDSSFQSKQTGRR
DTKVVSMVGRVQMDM**LQV**LLREYKLRSYTLNAVSFHFLGEQKEDVQHSIITDLQNGNDQTR**RRL**AVYCLKDAYL
PLRLLERLMVLVNAVEMARVTGVPLSYLLSRGQQVKVVSQLLRQAMHEGLLMPVVKSEGGEDYTGATVIEPLKG
YYDVPIATLDFSSLYPSIMMAHNLCYTTLLRPGTAQK**LGL**TEDQFIRTPTGDEFVKTSVRKGLLPQILENLLSA
RKRAKAELAKETDPLRRQVLDGRQ**LAL**KVSANSVYGFTGAQVGKLPCLEISQSVTGFGRQMIEKTKQLVESKYT
VENGYSTSAKVVYGDTDSVMCRFGVSSVAEAMALGREAADWVSGHFPSPIRLEFEKVYFPYLLISKKRYAGLLF
SSRPDAHDRMDCKGLEAVRRDNCPLVANLVTASL**RRL**LID**R**DPEGAVAHAQDVISDLLCNRIDISQLVITKELT
RAASDYAGKQAHVELAERMRKRDPGSAPSLGDRVPYVIISAAKGVAAYMKSEDP**LFV**LEHSLPIDTQYYLEQQL
AKPLLRIFEPILGEGRAEAVLLRGDHTRCKTVLTGKVGGLLAFAKRRNCCIGCRTVLSHQGAVCEFCQPRESEL
YQKEVSHLNALEERFSRLWTQCQRCQGSLHEDVICTSRDCPIFYMRKKVRKDLEDQEQLLRRFGPPGPEAW



1.5.2. Maintaining energy equilibrium under hypoxia acidosis

Under normal oxygen tension (normoxia), molecular oxygen acts as the final electron acceptor in a long chain of reactions that begins with the glycolytic conversion of glucose to pyruvate and ends with the net production of 36 ATP molecules. This process is referred to as oxidative phosphorylation. Under conditions of low oxygen tension (hypoxia), the cell adapts to a shortage of molecular oxygen by switching to anaerobic respiration, a metabolic process that only produces 2 ATPs per glucose. This sudden drop in ATP supply threatens cell viability by impairing the proper functioning of energy-dependent processes. Such processes include signalling, transcription, translation, trafficking and membrane transport, among many others. In order to maintain homeostasis, the cell must increase energy production and reduce energy consumption as to restore energy equilibrium. ATP supply being limited to glycolysis, glucose update and enzymatic activity are respectively increased. However, this effort is insufficient and energy consumption needs to be decreased. Nuclear sequestration of NoDS-containing proteins appears to offer a rapid, comprehensive and reversible solution to hypoxia-induced energy crises. NoDS containing proteins includes components of the DNA synthesis machinery (POLD1), E3 ubiquitin ligases (VHL, RNF8), chaperones (hsp70, hsc70), cell cycle regulators (Anaphase-promoting complex subunit 2; ANAPC2) and transcription factors (E1A binding protein p300) (Mekhail et al., 2007). Given that sequestered proteins are immobilized and inactivated, this process likely interrupts a broad spectrum of energy-demanding metabolic pathways. In fact, unpublished data from our group demonstrate that acidosis induces an arrest in DNA synthesis,

as measured by BrdU-incoporation, as well as an arrest in cell cycle, through a stabilization of cyclins A and B, and a halt in proliferation. Moreover, whereas hypoxia induces a loss in energy equilibrium and a decrease in cell viability, acidosis restores this balance and allows cells to survive at low oxygen tension (Mekhail et al., 2006). Sequestration under conditions other than acidosis (i.e. heat shock) may induce a separate adaptive program that is tailored to the stress at hand.

1.5.3. Binding to the ribosomal intergenic spacer

NoDS-proteins appear to be retained in the nucleolus through independent mechanisms. Usually, complex networks of protein-protein and protein-nucleic acid interactions mediate nucleolar localization. Many of these interactions are interdependent, and perturbations to the levels of a specific factor can have trickle down effects that disrupt the localization and function of hundreds of proteins (Emmott and Hiscox, 2009). Ultimately, nucleolar accumulation is generally linked to ribosomal biogenesis and regulatory regions of rDNA, pre-rRNA, or their associated factors (Melese and Xue, 1995). In all cases, nucleolar proteins retain a dynamic profile. On the other hand, sequestered proteins are immobilized, which suggests that the molecular interactions at hand are inherently different. Additionally, our group has shown that sequestered VHL interacts with a discrete region of the IGS under acidosis. Though not finely mapped, this interaction appears to table place 28 kb downstream of the rRNA transcription start site, at the heart of the IGS. These results suggest that the IGS, previously assumed to be an inert region of the genome, may hold the key to the mechanism of protein immobilization. How

VHL, and possibly other proteins are targeted to the IGS remains unknown.

SUMMARY AND GENERAL RATIONAL FOR THE PROPOSED STUDY

The nucleolus is primarily known as the site of ribosomal biogenesis, a function that is reflected in its tripartite organization. It is also a plurifunctional organelle that is involved in a range of cellular processes from stress sensing to aging. Of particular interest is the ability of the nucleolus to orchestrate the sequestration of a wide array of proteins in response to specific environmental stimuli, notably extracellular acidosis. This pathway places the nucleolus at the centre of a systemic, post-translational regulatory mechanism that protects the cell under conditions of hypoxia. Despite the physiological relevance of nucleolar sequestration, very little is known about the underlying mechanisms that govern it. Indeed, it seems unexpected that an organelle so specialized and structurally dedicated to ribosomal biogenesis could mediate a response of that scale. Work from our lab suggests that the ribosomal intergenic spacer (IGS) could be at the heart of this pathway, driving the process of nucleolar sequestration from a region of the genome that has historically been assumed to be without function. If this model were correct, it would segregate ribosomal DNA into two functional units: a stretch of DNA focused of ribosome production, and another on protein sequestration. A duality rooted so deeply within the nucleolus would not be without structural and functional consequences for this organelle. I suggest that the ribosomal intergenic spacer regulates protein sequestration and nucleolar function under different physiological conditions.

HYPOTHESIS

The ribosomal intergenic spacer (IGS) regulates protein sequestration and nucleolar function under different physiological conditions.

OBJECTIVES

1. To identify the nucleolar detention signals that regulate the sequestration of proteins under different conditions.

Sequestration under acidosis is mediated by the NoDS-H+ sequence. Using Hsp70 and MDM2 as respective models, I mapped the amino acid sequences that mediate the targeting of proteins to the nucleolus under heat shock and ribosomal stress.

2. To identify the IGS loci involved in protein sequestration

Data from our group suggested that sequestered proteins interact with the IGS. Using chromatin immunoprecipitations, I systematically mapped the IGS in order to identify the exact loci at which proteins are captured under different conditions. I then investigated whether transcription of these loci was involved in this process.

3. To establish the consequences of protein sequestration on the structure and function of the nucleolus.

The nucleolus is a compact organelle that is divided into three distinct compartments involved in ribosomal biogenesis. Using a combination of imaging and biochemistry techniques, I determined the consequences of protein sequestration on the structural organization and functional activity of the nucleolus.

4. To determine whether nucleolar sequestration takes place *in vivo*

Environmental stimuli that trigger protein sequestration, such as extracellular acidosis, are relevant to a variety of physiological settings, including the tumour microenvironment. Using imaging techniques and immunoprecipitation, I investigated whether this process takes place *in vivo* in tumours and differentiated tissues.

IMPORTANCE

Cancer development is a multistep process that involves the acquisition, through genetic mutations, of a series of hallmark traits (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Vogelstein and Kinzler, 2004). These traits can be acquired through multiple genetic alterations, resulting in a complex array of deregulated pathways that is difficult to target therapeutically. Despite this heterogeneity, solid tumours experience a common set of microenvironmental conditions that is different from that of normal tissues. By outgrowing their vasculature, tumours lose normal microcirculation and become characterized by hypoxia and extracellular acidosis (Sutherland, 1988; Vaupel, 1994; Vaupel, 2004; Vaupel and Hockel, 2000; Vaupel et al., 1989). The nucleolar detention pathway thus appears to be at the centre of the cell's adaption to the tumour microenvironment, making it potentially a universal target in the management of cancer. A mechanistic understanding of nucleolar sequestration would be essential to pursue these therapeutic avenues.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

Cell culture, transfection and treatment

MCF-7, U-87 MG, PC-3 and NIH/3T3 cells were grown in monolayer culture in Dulbecco's (modified) Minimum Essential Medium (DMEM, high glucose), supplemented with 5% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. All cultures were maintained at 37 °C and 5% CO_2 in a humidified atmosphere, and passaged every 2-3 days. Cells were plated 24 h before transfection and allowed to grow to 50-60% confluency before transfection with Effectene (Qiagen) as per manufacturer's instructions. Heat Shock was performed by transferring cells to 42 °C for 3 h, unless specified otherwise. Hypoxic-acidosis was obtained by placing cells in acidic (pH 6.0) DMEM at 1% O_2 for 4 h, unless specified otherwise. Stable MCF-7 cells were produce with shRNA targeting the sequences 5'-GGCACTGTATTGCTACTGG (shRNA-22), 5'-TCTAGACAGG CGGGCCTTG (shRNA-28), and 5'-CGTCTTTTTCGAGAGTCTG (shPML)

Plasmids

VHL constructs were amplified from FLAG-VHL-GFP and cloned using ApaI (5') and XhoI (3') into pcDNA3.1 between a N-terminal FLAG and a C-terminal GFP, as previously described (Bonicalzi et al., 2001; Groulx and Lee, 2002). MDM2 constructs were amplified from MDM2-YFP (Galit Lahav, Harvard Medical School, Boston, MA) and cloned using BamHI (5') and NotI (3') into pcDNA3.1 downstream of a N-terminal GFP. Hsp70 and Hsp70ΔRRLRTAC were amplified from MCF-7

genomic DNA and cloned into pcDNA3.1 between a N-terminal FLAG and a C-terminal GFP. shRNA targeted against PML was designed using the following sequence GATCCGCGTCTTTTTCGAGAGTCTGTTCAAGAGACAGACTCTCGAAAAAGACGTTTTTGGAAA. Plasmids expressing VHL-GFP, RNF8-GFP, EGFP-B23 were previously described (Audas et al., 2012a). mCherry-VHL was cloned from plasmid cDNA into an mCherry-c1 empty vector. EGFP-NOM1 (Dr. Kathleen Conklin, University of Minnesota, USA), EGFP-FBL, EGFP-NOPP140, EGFP-PSF, EGFP-RPA40, mCherry-RPA43, EGFP-RPA194, EGFP-NOL1, EYFP-PES1, EYFP-RPL27 and EGFP-RRP1B (Dr. Angus Lamond, University of Dundee, UK) were kindly provided. The following plasmids were obtained from Addgene: EGFP-RPA16 (Tom Misteli (Dundr et al., 2002), Addgene plasmid 17657) and EGFP-SENP3 (Mary Dasso (Yun et al., 2008), Addgene plasmid 34554).

Microscopy and image processing

Figures 10-19: Images were obtained using a Zeiss Axiovert S100 inverted fluorescent microscope (Carl Zeiss Microimaging Inc.) and captured with Northern Eclipse (Empix) software. Figure 20-36: Fluorescence images were collected by confocal microscopy (Zeiss 200 LSM510 META) using a 63x Plan-Apochromat 1.4 NA objective. The system was controlled with Zeiss Zen software. DIC images were collected on a Zeiss AxioObserver D1 microscrope using a 63x Plan-Apochromat 1.4 NA objective. All post-acquisition image processing was performed using Zeiss Zen software. No gamma or other nonlinear adjustments were made to any image.

Fluorescence recovery after photobleaching

Live cells expressing constructs of interest were grown on 35 mm glass-bottom culture dishes and visualized by confocal microscopy (Zeiss 200 LSM510 META) in a 37 °C and 5% CO_2 environmental chamber (Zeiss Incubator XL S1) using a 63x Plan-Apochromat 1.4 NA objective. The system was controlled by Zeiss Zen software for bleaching and image acquisition. Bleached areas (nucleolus) were subjected to 40 iterations at 100% argon laser strength at 488 nm, while imaging used 5% laser strength. Three pre-bleach measurements were taken prior to the photobleaching and 60 to 120 s recovery of the region of interest, as indicated. For nucleolar proteins, a full nucleolus was photobleached. For nucleo-cytoplasmic proteins, a region of the nucleoplasm was bleached instead. Region intensity measurements were performed by Zen software and normalized as previously described (Misteli, 2001). All FRAP experiment data were the average of at least 10 cells.

8-anilino-1-naphthalenessulphonic acid, thioflavin S and congo red imaging

8-anilino-1-naphthalenessulphonic acid (ANS) was purchased from Sigma-Aldrich (A1028) and dissolved in PBS pH 7.4 to a 10 mM stock solution. Culture medium was supplemented with ANS at a concentration of 250 μM for at least 1 h. prior to imaging, as previously described (Hadley et al., 2011). Media were changed prior to imaging. ANS was excited at 405 nm and detected using a band pass filter. Thioflavin S (ThS) was used at a concentration of 1% on fixed (4% formaldehyde) and permeabilized (0.5% Triton X-100, 5 min) cells, as previously described (Hadley et al., 2011). ThS was excited at 488 nm and detected using a long pass filter (505 nm).

Congo red was used at a concentration of 1% on fixed and permeabilized cells, and imaged by brightfield light microscopy.

Immunofluorescence

Cells were seeded onto 20 mm glass coverslips and fixed with CSK (4% formaldehyde) for 15 min. After permeabilization in 0.5% Triton X-100 for 10 min, cells were incubated with primary antibody (1:200), washed 3 times in PBS, and incubated for 1 hour in Alexa Fluor (Invitrogen, CA) secondary antibody (1:400).

Fluorescent In Situ Hybridization

Cells were fixed in methanol for 5 minutes. Samples were blocked in DIG hybridization (Roche) solution and hybridized with 5' and 3' digoxigenin (DIG)-labeled oligonucleotide $IGS_{22}RNA$ anti-sense (5'-DIG-TACTGCATTGTCGCTGAACGTTCTCCCAAAAGGCCAGAAACCCCCTGACTCAGGTCAAGG-DIG), $IGS_{28}RNA$ anti-sense (5'-DIG-CCGGCCTTAACAGTTTATGTTGAAGTCGAGGAGACTTATCGGGGAAATAGGAGAAGTACG-DIG) or IGS sense (5'-DIG-CGTACTTCTCCTATTTCCCCGATAAGTCTCCTCGACTTCAACATAAACTGTTAAGGCCGG-DIG) probes in a 50% formamide/DIG hybridization (Roche) solution at 37°C. Following hybridization, cells were washed in 0.2% SSC and probes were detected with an a-digoxigenin (Roche) antibody.

5-Fluorouridine (5-FU) labeling and detection of nascent rRNA

Live cells were incubated in 1 mM 5-FU (Sigma F5130) for 15 min prior to fixation in CSK (4% formaldehyde). Cells were then permeabilized in 0.5% Triton X-100 and immunostained using an anti-BrdU mouse antibody (Sigma B2531) at 1:500, followed by incubation in Alexa Fluor (Invitrogen, CA) secondary antibody (1:500).

Tissue immunohistochemistry

Mouse tissues were fixed in 3.7% formaldehyde in PBS, pH 7.2, for 24 hours at room temperature, with at least 2 mL solution for every 100 mg of tissue. Samples were then dehydrating in a series of ascending alcohols, cleared in xylene, and embedded in paraffin wax. Bocks were sectioned into 5 μM sections, and sections were placed on glass slide and heated to 65 °C for 20 min, to allow the tissue to bond to the glass. For immunostaining, samples were rehydrated in a series of descending alcohols, and incubated in citrate buffer (10 mM) for 20 minutes at 100 °C. Samples were then blocked in TBS, 10% serum, and immunostained using standard protocol.

Western blotting

Western blot was performed following standard protocol. Briefly, Samples were washed with phosphate buffered saline (PBS) and lysed in 4% sodium dodecyl sulphate (SDS) in PBS and lysates were sheared with a 21-gauge needle. Total protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL.). Equal amounts of protein were resolved by electrophoresis on denaturing polyacrylamide gels containing SDS, and transferred to methanol-

activated polypolyvinylidene difluoride membranes (PVDF; NEN, Boston, MA). Membranes were blocked in 5% (w/v) skimmed dried milk in 0.2% Tween 20-PBS for 1h at room temperature before incubation with the indicated primary antibodies. After washing with 0.2% Tween 20-PBS, membranes were blotted for 1h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and detected by Enhanced Chemiluminescence (Pierce, Rockford, IL). Monoclonal antibodies were used to detect FLAG (Sigma), Hsp70 (Santa Cruz Biotechology) and GADH (GeneTex). Bands were detected by enhanced chemiluminescence (Pierce).

Chromatin immunoprecipirations

Chromatic Immunoprecipitation (ChIP) was performed using the EZ ChIP kit (Upstate Biotechnologies) following the manufacturer's protocol. Briefly, cells were grown to confluence on 15-cm plates and crosslinked with 1% formaldehyde. Lysates were sonicated 120 sec (12x 10 sec) at 8W to obtain ~500 bp DNA fragments. Immunoprecipitation was performed with 1 μg antibody (mouse IgG (Upstate Biotechnologies), M2 (Sigma), or others as mentioned previously). Purified DNA samples were amplified by PCR and compared to 2% input.

Bioinformatic analysis

Search for candidate proteins containing a Nucleolar Detention Signal (NoDS) were performed using ExPASy ScanProsite on the UniProtKB/Swiss-Prot database. Motifs used were R-R-[IL]-X(3)-R for the classical arginine motif, R-R-[IL]-X or R-R-[IL]-

X(0,5)-R for the non-classical arginine motif, as well as L-[AFVQWNPGL]-[LV] for the hydrophobic motifs.

Transmission Electron Microscopy

Cells were treated and fixed with 2% glutaraldehyde in PBS, pH 7.4. Samples were subsequently washed and fixed with 1% osmium tetroxide, before dehydration in a series of ascending alcohols. Specimens were then infiltrated and processed in Spurr's expoxy resin and embedded onto resin-filled BEEM capsule molds. Specimen blocks were ultrathin-sectioned on a Leica EM UC6 ultramicrotome and the resulting sections were stained with uranyl acetate and lead citrate. Samples were imaged with a Jeol 1230 TEM equipped with AMT software.

RNA isolation, reverse transcription PCR (RT-PCR) real time PCR

Total RNA was collected using TriPure reagent (Roche) according to manufacturer's specifications. RT-PCR was performed using the two-step High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) followed by standard PCR conditions. Real Time PCR reactions were performed using iQ SYBR Green SuperMix (BioRad Laboratories) and read with Stratagene MX3005P. Transcripts levels were normalized to β-actin mRNA. Relative fold change in expression is calculated using ΔΔCT method. 45S pre-rRNA and actin mRNA primers were used as previously described (Feng et al., 2010).

Statistical Analysis

P values associated with all comparisons were based on paired two-tailed Student t-tests. Results are mean (n = 3) ± standard error of the mean.

3. Results

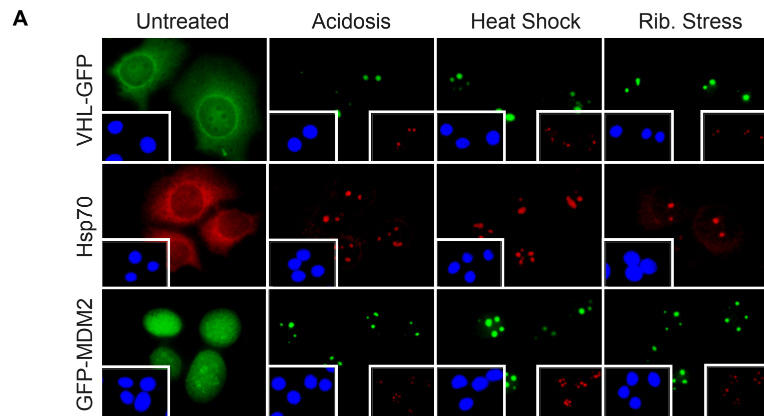
3. RESULTS

3.1. A common NoDS mediates the nucleolar capture of proteins under multiple conditions

3.1.1. VHL, Hsp70 and MDM2 are pluri-responsive proteins

Proteins that are subjected to nucleolar sequestration are usually described as stimulus-specific. VHL, for instance, is specifically sequestered under acidosis (Mekhail et al., 2004a; Mekhail et al., 2005; Mekhail et al., 2004b; Mekhail et al., 2007; Mekhail et al., 2006). In contrast, Hsp70 responds to heat shock (Milarski and Morimoto, 1989; Pelham, 1984) and MDM2 is sequestered under ribosomal stress (Mekhail et al., 2005; Weber et al., 1999). While the literature typically links one protein to one nucleolar stress, the possibility remained that the same protein could be sequestered under more than condition. To test for this possibility, we looked at the subcellular distribution of VHL, Hsp70 and MDM2 under acidosis, heat shock and ribosomal stress. Surprisingly, we found that VHL relocalized to nucleoli under all three stresses **(Figure 10A)**. Hsp70 and MDM2 displayed a similar pattern **(Figure 10A)**. This "pluri-responsiveness" is not universal, as DNA-(5 cytosine)-methyl transferase 1 (DNMT1) and anaphase-promoting complex subunit 2 (APC2) were specific to acidosis and ribosomal stress, respectively **(Figure 10B)**. Other NoDS-proteins also showed some level of specificity **(Table 1)**. These results demonstrate that individual proteins can respond to multiple stresses, and suggest that acidosis, heat shock and ribosomal stress may activate homologous pathways.

Figure 10. VHL, Hsp70 and MDM2 are pluri-responsive proteins. (A) Localization of VHL-GFP, endogenous Hsp70 and GFP-MDM2 in untreated, acidotic, heat shocked or ribosomal stressed MCF-7 cells. **(B)** Localization of EGFP-DNMT1 and APC2-GFP in in untreated, acidotic, heat shocked or ribosomal stressed MCF-7 cells. **(A-B)** DNA was stained with Hoechst 33342 (blue, bottom-left inset), endogenous B23 was immunostained (red, bottom-right inset).


A
Untreated
Acidosis
Heat Shock
Rib. Stress
VHL-GFP
Hsp70
GFP-MDM2

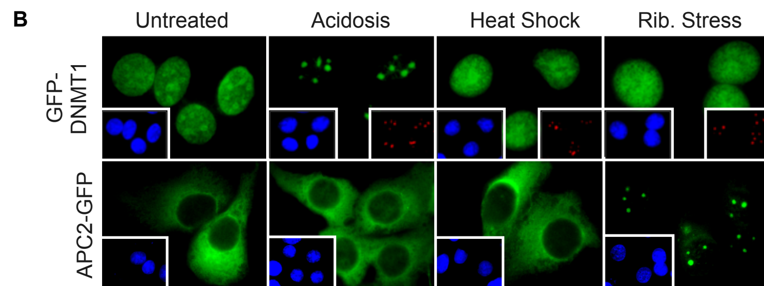
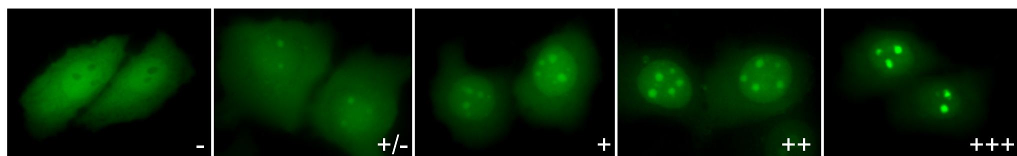

B
Untreated
Acidosis
Heat Shock
Rib. Stress
GFP-
DNMT1
APC2-GFP

Table 1. Stimulus-specificity of NoDS- proteins. The nucleolar relocalization of various NoDS-proteins under acidosis, heat shock and ribosomal stress was scored from – to +++. Representative images are provided below. Note: Data: MJ and TA

Symbol	Full name	Untreated	Nucleolar sequestration Acidosis	Heat Shock	Rib. stress
VHL	von Hippel Lindau protein	-	+++	+++	+++
DNMT1	DNA (cytosine 5)-methyltransferase 1	-	++	-	-
cIAP2	BIRC3 (CIAP2) baculoviral IAP repeat containing 3	-	+/-	+/-	+++
APC2	Anaphase promoting complex subunit 2	-	+/-	+/-	+++
Hsp70	HSPA1A heat shock 70 kDa protein 1A	-	+	+++	+++
RNF8	Ring finger protein 8	-	+++	+++	+++
Tiam1	T-cell lymphoma invasion and metastasis 1	-	+	+/-	+/-
cdh1	Cadherin 1, type 1, E-cadherin	-	+++	+/-	++
SUG1	PSMC5 protease 26S subunit ATPase 5	-	+	+	+
AMPK	PRKAA1 protein kinase, AMP-activated, alpha 1 catalytic subunit	-	++	+	+/-
DAXX	Death domain associated protein	-	++	+	+/-
UVRAG	UV radiation resistance associated	-	+	-	+/-
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	-	-	-	+

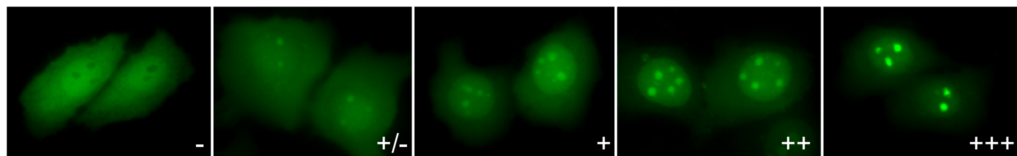


3.1.2. VHL's NoDS mediates its sequestration under multiple conditions

The amino acid sequence responsible for the nucleolar sequestration of VHL under acidosis has been identified as [RR(I/L)X3r](n,n>1)+[L(Φ/N)(V/L)](n,n>1) and is referred to as NoDS-H^+ (Nucleolar Detention Signal regulated by H^+) (Mekhail et al., 2007). How VHL is targeted to the nucleolus under other conditions remains unknown. Different signals could be involved in these processes or, alternatively, the same NoDS-H^+ could be responsible for its sequestration under all conditions. Preliminary mapping of VHL revealed that mutants containing more hydrophobic repeats displayed stronger sequestration, regardless of treatment **(Table 2)**. Moreover, no stimulus-specificity was observed in any mutant, with the exception of VHL 100-130, which showed weak sequestration under acidosis and no sequestration under heat shock or ribosomal stress **(Table 2)**. Based on these observations, we hypothesized that the same NoDS could be responsible for the sequestration of VHL under all conditions. We therefore designed a series of constructs from which we removed either the arginine motif of the NoDS-H^+ (residues 107-120; RRIHSYRGHLWLFR) **(Figure 11)**, or an arginine-rich region found upstream of the NoDS-H^+ (residues 60-87; PVLRSVNSREPSQVIFCNRSPRVV*LPV*) **(Figure 11)**. This region was chosen as a negative control because its sequence was reminiscent of a localization signal. Removal of residues 60-87 had no deleterious effect on the sequestration of VHL 1-170, while removal of residues 107-120, the segment containing the arginine motif of the NoDS, abolished nucleolar relocalization under all conditions **(Figure 11)**. To confirm the pluri-responsiveness of the NoDS-H^+, the same deletions were repeated, this time in a longer construct (1-186) that included the second arginine motif of VHL **(Figure 11)**. In VHL 1-

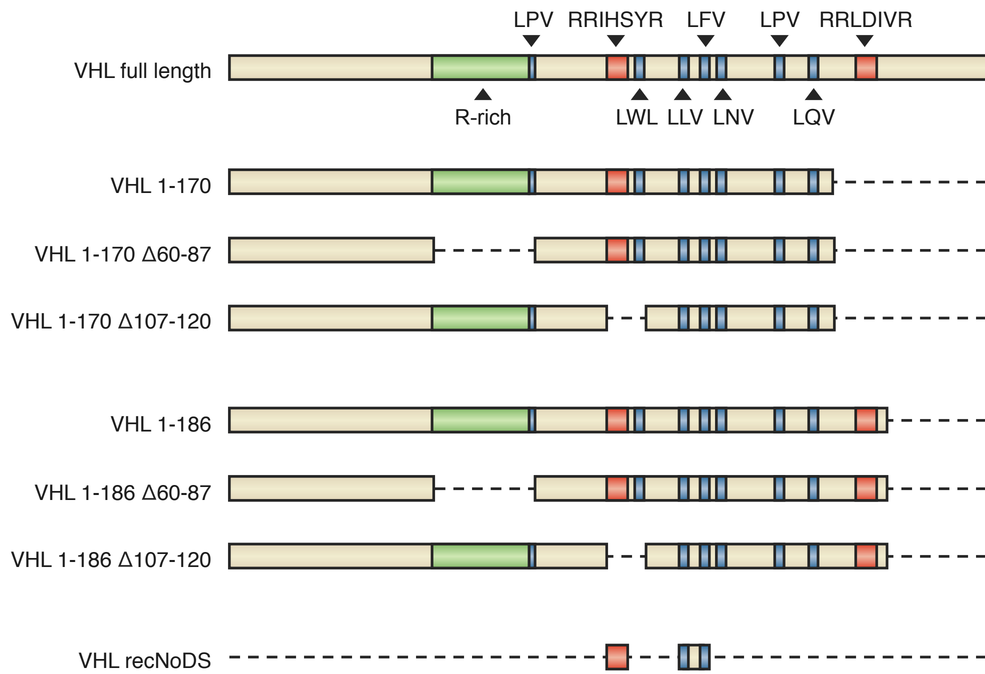
Table 2. Preliminary mapping of VHL. The nucleolar relocalization of various VHL mutants was scored from – to +++. Representative images, as well as the amino acid sequence of VHL, are provided below.

VHL mutant	Nucleolar sequestration			
	Untreated	Acidosis	Heat Shock	Rib. Stress
1-213 (full length)	-	+++	+++	+++
1-109	-	-	-	-
1-133	-	+	+	+
1-156	-	++	++	++
1-170	-	++	++	++
1-186	-	+++	+++	+++
54-213	-	++	++	++
114-213	-	++	+	++
121-199	-	+	+	+
100-130	-	+	-	-
131-150	-	-	-	-
131-170	-	+	+/-	+
152-186	-	+	+	+
135-213	-	+	+/-	+
152-213	-	+	-	+
171-213	-	-	-	-
187-213	-	-	-	-
Δ60-113	-	++	++	++
Δ80-130	-	+	+	++
Δ95-115	-	+	++	++
1-133 Δ59-99	-	+/-	+/-	+/-
1-133 Δ58-77	-	+/-	+/-	+/-
1-133 Δ78-99	-	+/-	+/-	+/-

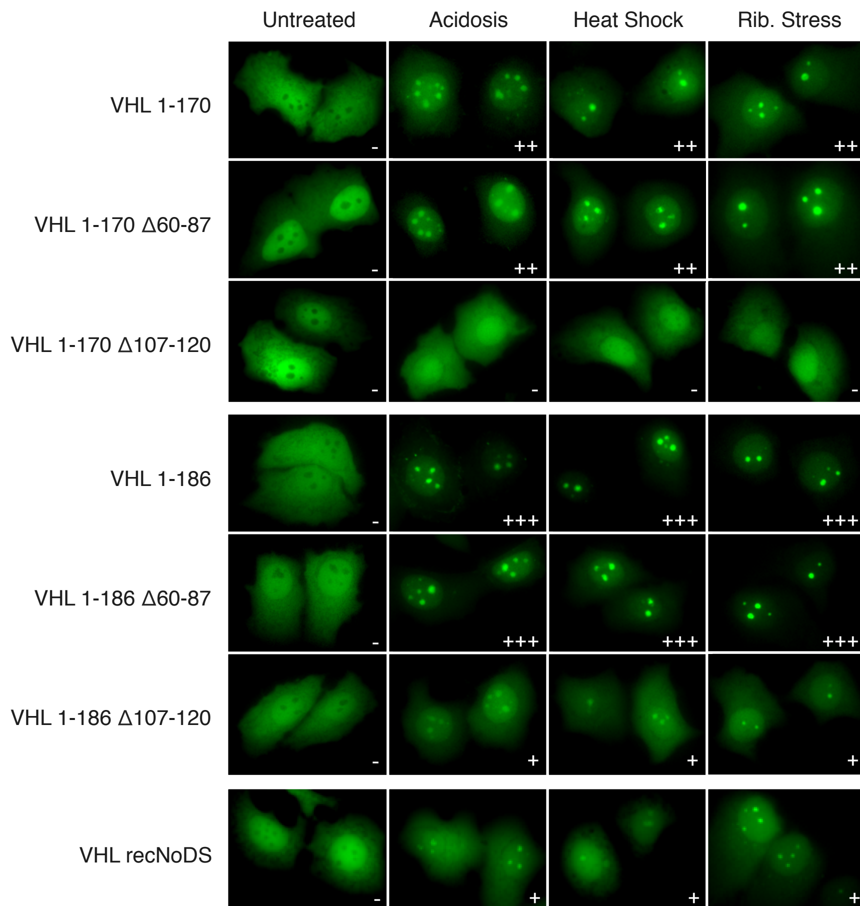


(0) MPRRAENWDE (10) AEVGAEEAGV (20) EEYGPEEDGG (30) EESGAEESGP (40) EESGPEELGA (50)
EEEMEAGRPR (60) PVLRSVNSRE (70) PSQVIFCNRS (80) PRVVLPVWLN (90) FDGEPQPYPT (100)
LPPGTGRRIH (110) SYRGHLWLFR (120) DAGTHDGLLV (130) NQTELFVPSL (140) NVDGQPIFAN (150)
ITLPVYTLKE (160) RCLQVVRSLV (170) KPENYRRLDI (180) VRSLYEDLED (190) HPNVQKDLER (200)
LTQERIAHQR (210) MGD

Figure 11. The NoDS in VHL is required and sufficient to mediate nucleolar sequestration under different conditions. (Top) Schematic representation of the VHL mutants used. Green region correspond to an arginine-rich sequence outside of the NoDS. Red regions correspond to the arginine motifs of the NoDS. Blue regions correspond to the hydrophobic triplets of the NoDS. (Bottom) Lolocalization of GFP-tagged VHL mutants in untreated, acidotic, heat shocked and ribosomal stressed MCF-7 cells. Intensity of nucleolar localization is ranked from – to +++.



LPV
RRIHSYR
LFV
LPV
RRLDIVR
VHL full length
R-rich
LWL
LLV
LNV
LQV
VHL 1-170
VHL 1-170 Δ60-87
VHL 1-170 Δ107-120
VHL 1-186
VHL 1-186 Δ60-87
VHL 1-186 Δ107-120
VHL recNoDS



Untreated
Acidosis
Heat Shock
Rib. Stress
VHL 1-170
VHL 1-170 Δ60-87
VHL 1-170 Δ107-120
VHL 1-186
VHL 1-186 Δ60-87
VHL 1-186 Δ107-120
VHL recNoDS

186, deletion of residues 60-87 had no effect, and removal of residues 107-120 was rescued by the presence of a second, C-terminal arginine motif **(Figure 11)**. Additionally, a reconstituted NoDS-H^+ (recNoDS-H^+) composed of VHL's arginine motif and two hydrophobic repeats (LLV, LNV) relocalized to the nucleoli under all three stresses **(Figure 11)**. These results demonstrate that VHL's NoDS-H^+ is not acidosis-specific, but pluri-responsive. We will hereafter refer to this sequence as simply NoDS.

3.1.3. Hsp70's NoDS mediates its sequestration under multiple conditions

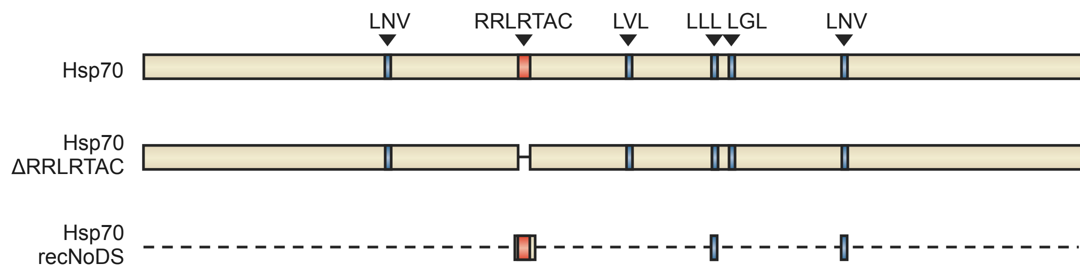
The nucleolar relocalization of Hsp70 under heat shock was first reported in 1984 (Welch and Feramisco, 1984), and has since been one of the most prevalent examples of nucleolar sequestration in the literature. However, the motif responsible for its nucleolar capture remains poorly understood. The domain in question was first described in 1989 as being KRKHKKDISQNKRAVRR (Dang and Lee, 1989), due to its resemblance to the Tat NoLS. Nucleolar relocalization of the KRKHKKRISQNKRAVRR peptide was much weaker than that of the full-length protein, and not triggered by heat shock. The same year, a second group reported that the last 138 amino acids of Hsp70 were required for its nucleolar localization, though they described variable results (Milarski and Morimoto, 1989). In light of these contradictory findings, we analyzed of the sequence of Hsp70 and noticed the presence of an NoDS, composed of an arginine motif (RRLRTAC) and of multiple hydrophobic repeats (LNV, LVL, LLL, LGL, LNV). Knowing that the NoDS of VHL mediates its sequestration under acidosis, heat shock and ribosomal stress, we wanted to know if the same was true of Hsp70. To answer this question, a deletion

mutant was produced in which the arginine motif was removed (Hsp70ΔRRLRTAC). While full-length Hsp70 effectively relocalized to the nucleoli in acidosis, heat shock and ribosomal stress, sequestration of Hsp70ΔRRLRTAC was abolished under all conditions **(Figure 12)**. Additionally, a reconstituted NoDS of Hsp70 was able to relocalized to the nucleoli under all conditions **(Figure 12)**. These results demonstrate that Hsp70 contains an NoDS, which mediates its sequestration under multiple conditions.

3.1.4. Sequestration of MDM2 is mediated by a PML binding site

Contrary to VHL and Hsp70, MDM2 does not contain an NoDS. In fact, different groups have reported that MDM2 is sequestered in the nucleolus by other proteins; namely L11 (Lohrum et al., 2003), Arf in response to Myc activation (Weber et al., 1999), or PML in an Arf-independent fashion in response to DNA-damage (Bernardi et al., 2004). However, there are no explanations as to how these intermediary proteins are recruited to the nucleolus themselves. It has also been suggested that a cryptic nucleolar localization signal (KKLKKRNK) within the C-terminal end of MDM2 is required for its sequestration (Lohrum et al., 2000). The lack of unanimity in the field and the fact that the nucleolar sequestration of MDM2 had never been characterized under acidosis or heat shock encouraged us to perform our own sequential mapping. Different segments of MDM2 were cloned into a GFP-tagged pcDNA vector and transiently transfected in MCF-7 cells treated with ribosomal stress. Results revealed that a region contained within residues 194 to 295 was both sufficient and required for the nucleolar sequestration of MDM2 **(Figure 13A).** All

Figure 12. Hsp70 contains an NoDS that is required and sufficient to mediate nucleolar sequestration under different conditions. (Top) Schematic representation of the Hsp70 mutants used. Red region corresponds to the arginine motif of the NoDS. Blue regions correspond to the hydrophobic triplets of the NoDS. (Bottom) Lolocalization of GFP-tagged Hsp70 mutants in untreated, acidotic, heat shocked and ribosomal stressed MCF-7 cells.


LNV
RRLRTAC
LVL
LLL LGL
LNV
Hsp70
Hsp70
ΔRRLRTAC
Hsp70
recNoDS

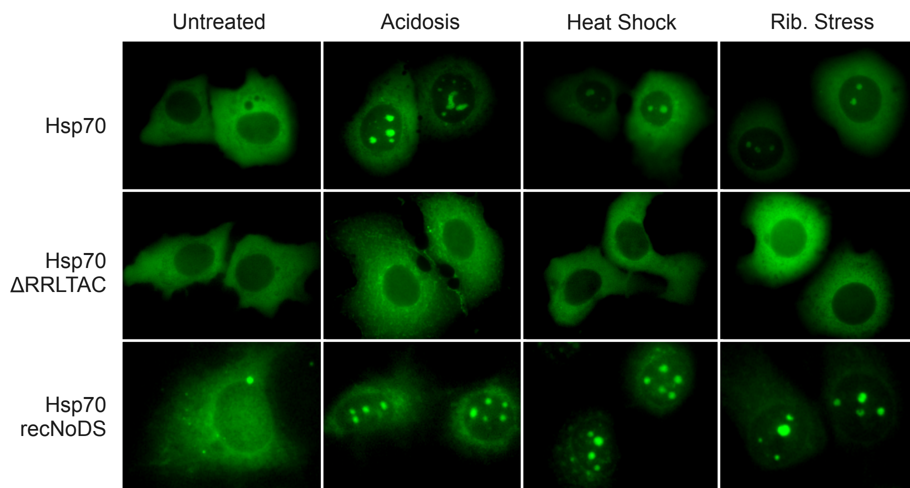
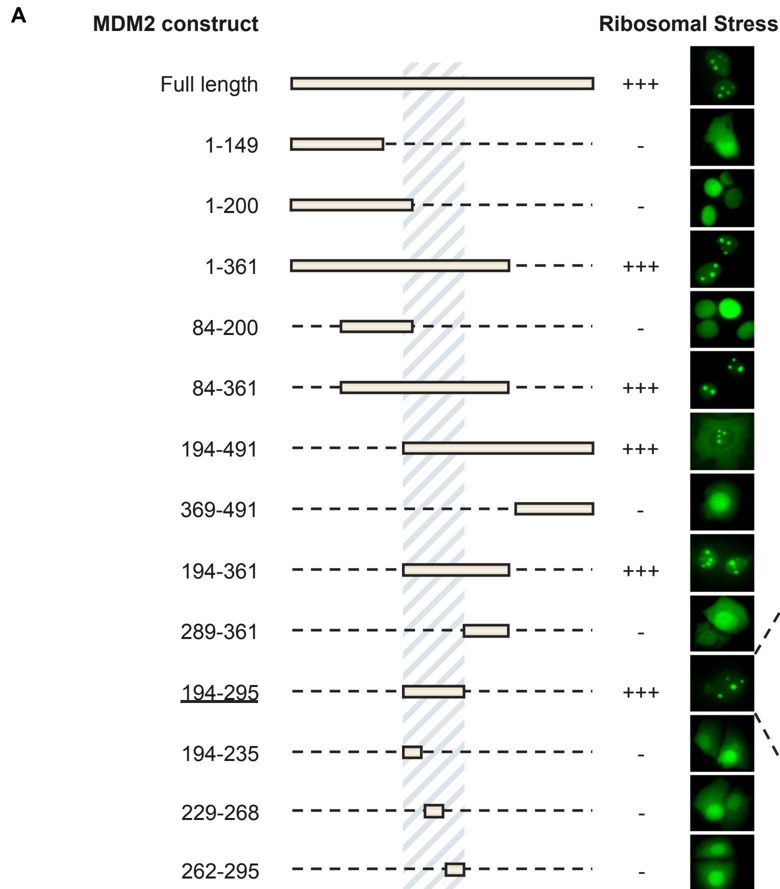
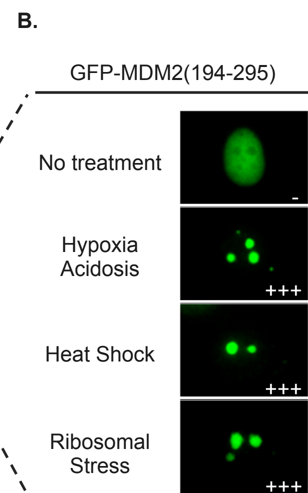
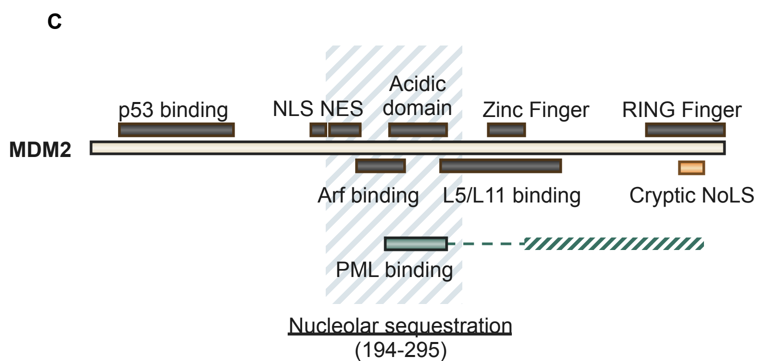
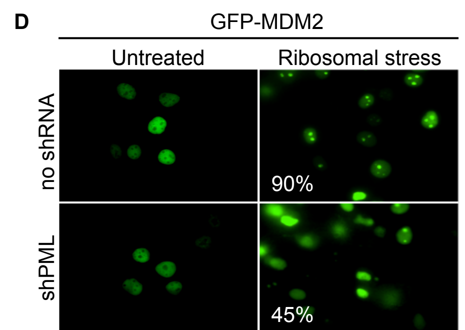

Untreated
Acidosis
Heat Shock
Rib. Stress
Hsp70
Hsp70
ΔRRLTAC
Hsp70
recNoDS

Figure 13. Sequestration of MDM2 under different conditions is mediated by a PML binding site. (A) Schematic representation of the MDM2 mutants used. Amino acid span is indicated on the left. Images of GFP-tagged constructs and intensity of nucleolar relolocalization in ribosomal stressed MCF-7 cells are provided on the right. **(B)** Localization of GFP-MDM2 194-295 (minimal regions required for sequestration) in untreated, acidotic, heat shocked and ribosomal stressed cells. Intensity of nucleolar localization is indicated. **(C)** Schematic of the functional domains of MDM2, including the region spanned by amino acids 194-295. **(D)** Localization of GFP-MDM2 in untreated and ribosomal stressed MCF-7 cells, with and without co-transfection with shPML. Percentage of cells displaying nucleolar sequestration is indicated (bottom right).


A
MDM2 construct
Ribosomal Stress
Full length
+++
1-149
-
1-200
-
1-361
+++
84-200
-
84-361
+++
194-491
+++
369-491
-
194-361
+++
289-361
-
194-295
+++
194-235
-
229-268
-
262-295
-
FDESLALCVIREICCERSSSSESTGTPSNPDLDAGVSEH
SGDWLDQDSVSDQFSVEFEVESLDSEDYSLSEEQELSD
EDDEVYQVTVYQAGESDTDSFEEDP


B.
GFP-MDM2(194-295)
No treatment
-
Hypoxia
Acidosis
+++
Heat Shock
+++
Ribosomal
Stress
+++


C
p53 binding
NLS NES
Acidic
domain
Zinc Finger
RING Finger
MDM2
Arf binding
L5/L11 binding
Cryptic NoLS
PML binding
Nucleolar sequestration
(194-295)


D
GFP-MDM2
Untreated
Ribosomal stress
no shRNA
90%
shPML
45%

mutants that contained this region showed strong relocalization, while those that didn't remained diffused **(Figure 13A).** Furthermore, MDM2 194-295 relocalized to the nucleolus under acidosis and heat shock as well as ribosomal stress, suggesting once again the presence of a single pathway **(Figure 13B)**. Segments within this region (194-235, 229-268, 262-295) did not relocate to the nucleolus, suggesting that 194-295, or at least a substantial portion of it, is the minimal motif **(Figure 14A)**. Surprisingly, our data suggest that the cryptic NoLS previously described (Lohrum et al., 2000) is neither sufficient nor required with our system **(Figure 13 A and C)**. Interestingly, the MDM2 194-295 region does not contain anything resembling a NoDS. Analysis of the functional domains of MDM2 (Wei et al., 2003) revealed that 194-295 is in fact an acidic domain that contains the binding sites to both Arf and PML, though not L11 **(Figure 13C).** PML is particularly relevant in that it contains a NoDS. In fact, of the multiple PML isoforms, only two are capable of localizing to the nucleolus (PML-I and PML-IV). Not surprisingly, these contain the most complete NoDS, with three or more hydrophobic repeats. To confirm the role of PML in the nucleolar sequestration of MDM2 in our system, MCF-7 cells were either transfected with GFP-MDM2 alone, or co-transfected with GFP-MDM2 and shRNA against PML. In cells that expressed shRNA, nucleolar relocalization of GFP-MDM2 under ribosomal stress was reduced from 90% of cells to 45% of cells **(Figure 13D).** These results support Bernardi *et al.*'s claim that PML is required for the nucleolar sequestration of MDM2 (Bernardi et al., 2004). More importantly, they provide additional evidence supporting the universal role of the NoDS.

3.1.5. The NoDS of acidosis-specific protein DNMT1 is pluri-responsive

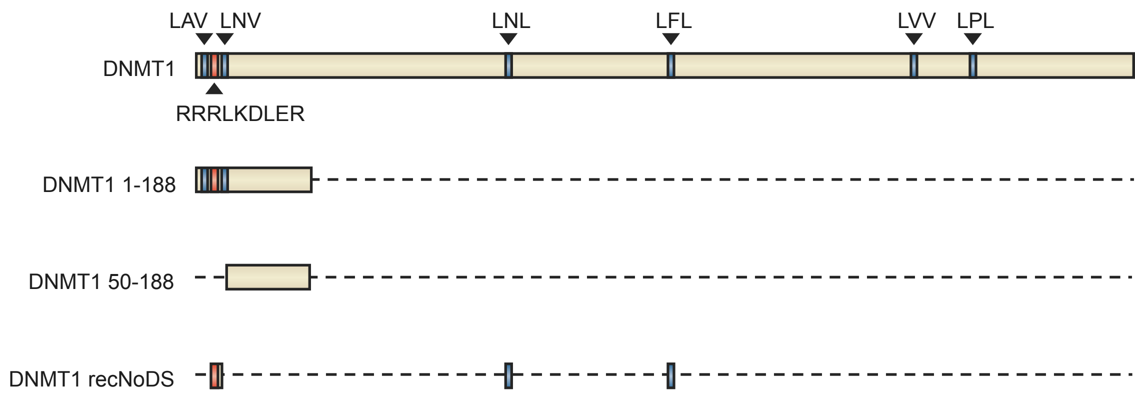
DNMT1 is a NoDS-containing protein that is only sequestered under acidosis **(Figure 10B)**, even endogenously **(Figure 14)**. This observation is unexpected, given the pluri-responsiveness of the NoDS code in other proteins. We proceeded to map DNMT1, and noticed that a truncated mutant that contained the NoDS (1-188) retained acidosis specificity, whereas a reconstituted DNMT1 NoDS displayed pluri-responsiveness **(Figure 14)**. These results suggest that although the NoDS is inherently an pluri-responsive signal, complementary elements within the amino acid sequence of the proteins provide additional levels of regulation. Therefore, the cell is able to fine-tune its response to different stresses around the NoDS.

3.2. Proteins are sequestered at stimulus-specific loci on the ribosomal intergenic spacer

3.2.1. The IGS contains multiple protein binding sites

Knowing that the Intergenic Spacer (IGS) is involved in protein sequestration (Mekhail et al., 2006), we wanted to finely map its 30 kb in order to identify the exact locus at which proteins interact. To do so, chromatin immunoprecipitations (ChIP) were performed on cells exposed to the three different conditions previously described: acidosis, heat shock and ribosomal stress. The protein models used were VHL, Hsp70 and MDM2. Twelve pairs of primers were designed at 2kb intervals along the IGS, between 16kb and 40kb from the rDNA transcription start site **(Figure 15)**. As expected, ChIP analysis revealed that none of these proteins

Figure 14. The NoDS of acidosis-specific protein DNMT1 is inherently pluri-responsive. (Top) Schematic representation of the DNMT1 mutants used. (Bottom) Localization of endogenous DNMT1, as well as GFP-tagged mutants in untreated, acidotic, heat shocked and ribosomal stressed MCF-7 cells.


LAV
LNV
LNL
LFL
LVV
LPL
DNMT1
RRRLKDLER
DNMT1 1-188
DNMT1 50-188
DNMT1 recNoDS

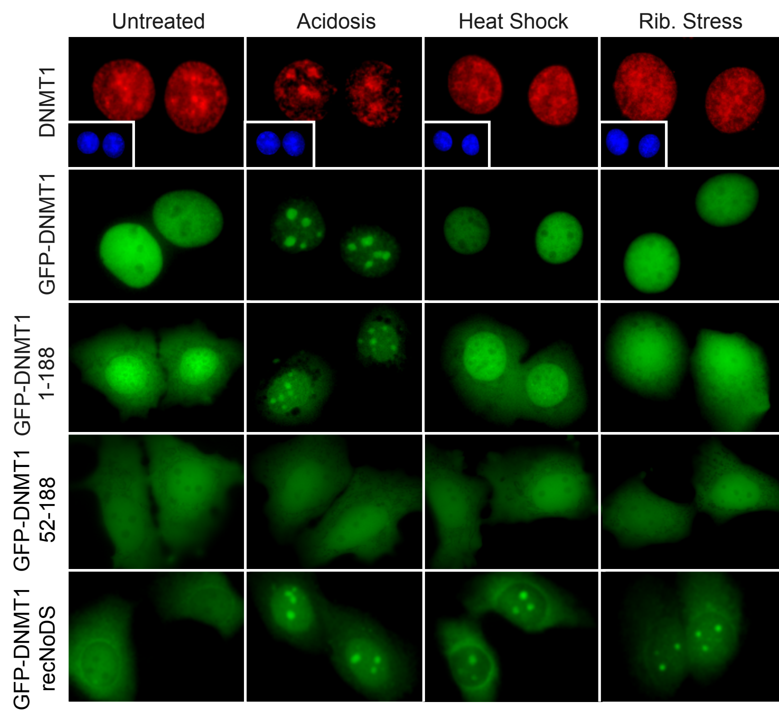
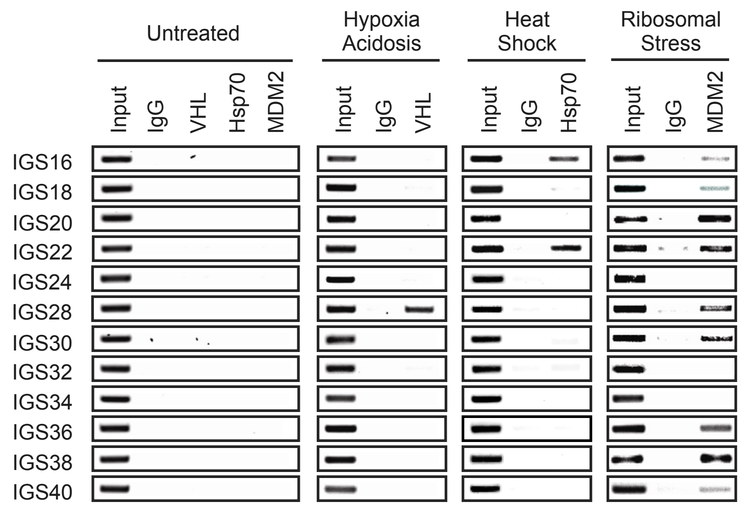
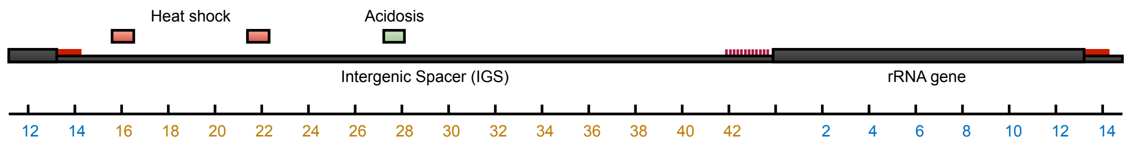

Untreated
Acidosis
Heat Shock
Rib. Stress
DNMT1
GFP-DNMT1
GFP-DNMT1 1-188
GFP-DNMT1 52-188
GFP-DNMT1 recNoDS

Figure 15. Proteins are sequestered at stimulus-specific loci on the ribosomal intergenic spacer. (Top) Chromatin immunoprecipitation of VHL-GFP, endogenous Hsp70 and GFP-MDM2 in untreated, acidotic, heat shocked and ribosomal stressed MCF-7 cells. (Bottom) Diagram of the ribosomal cassette, including the intergenic spacer. Figure adapted from Audas TE, Jacob MD, Lee S. 2012a. Note: Acidosis: TA; Heat shock: MJ.



Untreated
Hypoxia Acidosis
Heat Shock
Ribosomal Stress
Input
IgG
VHL
Hsp70
MDM2
IGS16
IGS18
IGS20
IGS22
IGS24
IGS28
IGS30
IGS32
IGS34
IGS36
IGS38
IGS40



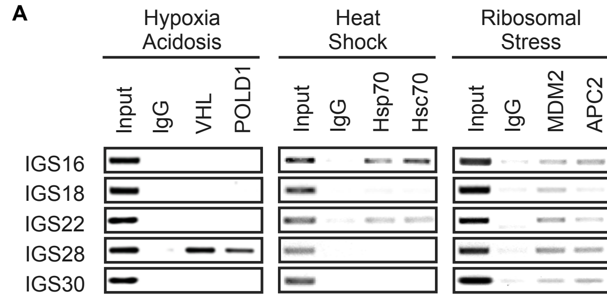
Heat shock
Acidosis
Intergenic Spacer (IGS)
rRNA gene
12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 2 4 6 8 10 12 14

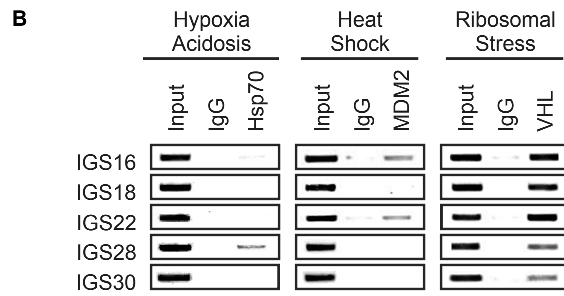
interacted with the IGS in untreated cells **(Figure 15)**. However, under acidosis, VHL was found to interact with a discrete locus located 28 kb downstream from the transcription start site, which we thus refer to as IGS_{28}. This observation is consistent with previously data (Mekhail et al., 2006) and offers a finer mapping of the IGS binding site. Surprisingly, under heat shock, Hsp70 was shown to bind to two distinct regions of the IGS; IGS_{16} and IGS_{22} **(Figure 15)**. MDM2, on the other, hand showed binding throughout the IGS, most notably at IGS_{20}, IGS_{22}, IGS_{28}, IGS_{30}, IGS_{36} and IGS_{38} **(Figure 15)**. These results demonstrate that sequestered protein interact with different loci within the IGS.

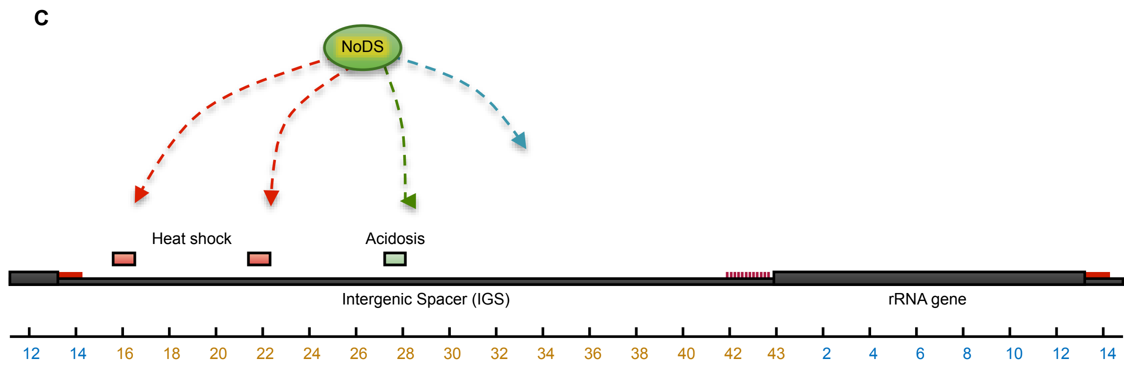
3.2.2. Proteins can be targeted to different IGS loci depending on the stimulus

The IGS loci described above are not protein-specific. Double-ChIPs, in which two proteins are immunoprecipitated simultaneously, revealed that both VHL and POLD1 were captured at the IGS_{28} locus in acidosis **(Figure 16A)**. Likewise, both Hsp70 and Hsc70 were captured at IGS_{16} and IGS_{22} in heat shock **(Figure 16A)**. Finally, both MDM2 and APC2 were captured at multiple loci along the IGS (IGS_{16}, IGS_{18}, IGS_{22}, IGS_{28}, IGS_{30}) in ribosomal stress **(Figure 16A)**. These data suggest that IGS loci are stimulus-specific hubs of sequestration. In fact, Hsp70, which is normally sequestered at IGS_{16} and IGS_{22} in heat shock, interacted with IGS_{28} in acidosis **(Figure 16B)**. MDM2 interacted with IGS_{16} and IGS_{22} in heat shock, whereas VHL bound to IGS_{16}, IGS_{18}, IGS_{22}, IGS_{28} and IGS_{30} in ribosomal stress **(Figure 16B)**. Together, these results demonstrate that IGS loci are stimulus-

Figure 16. Individual proteins can be targeted to different loci on the IGS depending on the stimulus. (A) Double chromatin immunoprecipiration (ChIP) of VHL-GFP/POLD1(endo), Hsp70(endo)/Hsc70(endo) and GFP-MDM2/APC2-GFP in acidotic, heat shocked and ribosomal stressed MCF-7 cells, respectively. **(B)** ChIP of Hsp70, GFP-MDM2 and VHL-GFP in acidosis, heat shock and ribosomal stress, respectively. **(C)** Diagram of the ribosomal cassette, including the intergenic spacer. **(A-C)** Adapted from Audas TE, Jacob MD, Lee S. 2012a. Note: (A) Acidosis: TA, Heat shock: MJ, Ribosomal stress: MJ. (B) Acidosis: MJ, Heat shock: MJ, Ribosomal stress: MJ.


A
Hypoxia
Acidosis
Heat
Shock
Ribosomal
Stress
Input
IgG
VHL
POLD1
Input
IgG
Hsp70
Hsc70
Input
IgG
MDM2
APC2
IGS16
IGS18
IGS22
IGS28
IGS30


B
Hypoxia
Acidosis
Heat
Shock
Ribosomal
Stress
Input
IgG
Hsp70
Input
IgG
MDM2
Input
IgG
VHL
IGS16
IGS18
IGS22
IGS28
IGS30


C
NoDS
Heat shock
Acidosis
Intergenic Spacer (IGS)
rRNA gene
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
43
2
4
6
8
10
12
14

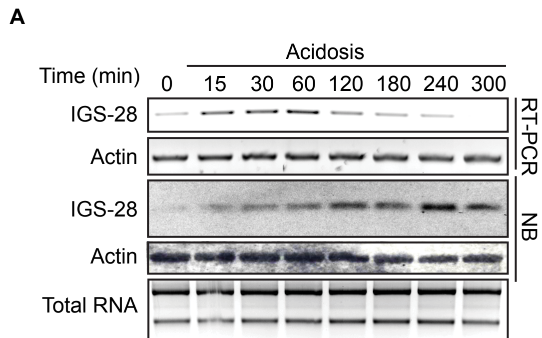
specific hubs of protein sequestration, and that individual proteins can be targeted to different loci depending on the stimulus.

3.2.3. IGS loci are transcriptionally induced in response to their associated stimuli

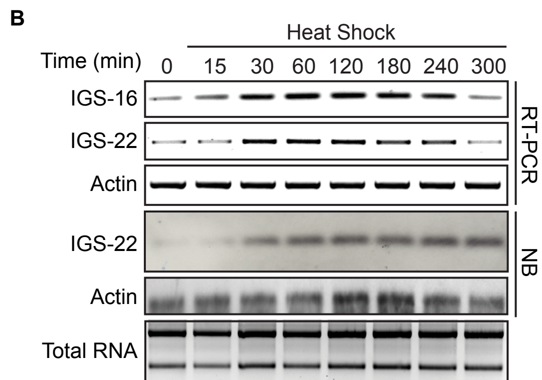
The ability of the IGS to rapidly respond to environmental cues led us to hypothesize that individual loci could be transcriptionally induced. To test for this possibility, we looked for the presence of RNA by RT-PCT and northern blot, using oligonucleotides complementary to our IGS loci of interest. Although IGS_{28} appeared transcriptionally silent under neutral conditions, time-course analysis revealed the presence of a transcript, the levels of which were progressively and temporarily elevated in response to extracellular acidosis **(Figure 17A)** (Audas et al., 2012a). Similarly, we found that IGS_{16} and IGS_{22} were transcriptionally induced in response to heat shock **(Figure 17B)** (Audas et al., 2012a). This unexpected ability of the IGS to produce stimulus-specific RNAs raised the possibility that these transcripts could be required for protein sequestration. We thus proceeded to test the effect of IGS RNA depletion on nucleolar sequestration by establishing several shRNA cell lines that efficiently reduced IGS_{28}RNA and IGS_{22}RNA levels. Whereas IGS RNA knockdown had no effect under neutral conditions, shIGS_{28} cells were incapable of sequestering VHL under acidosis **(Figure 17C)** (Audas et al., 2012a). Similarly, binding of Hsp70 at the IGS22 locus was impaired in shIGS_{22} cells **(Figure 17D)**, considerably delaying nucleolar sequestration under heat shock **(Figure 17E)** (Audas et al., 2012a). These results demonstrate that IGS loci are transcriptionally induced in

Figure 17. IGS loci are transcriptionally induced in response to their associated stimuli. (A) Analysis of RNA levels in acidosis over time over time at the IGS_{28} locus by RT-PCR and northern blot (NB). **(B)** Analysis of RNA levels in heat shock over time at the IGS_{16} and IGS_{22} loci by RT-PCR and NB. **(C)** Localization of VHL-GFP in untreated and acidotic parental, shControl and shIGS-28 MCF-7 cells. **(D)** Chromatin immunoprecipitation of endogenous Hsp70 in heat shocked shIGS22 cells. **(E)** Localization of endogenous Hsp70 in untreated and heat shocked (60, 180 min) shControl and shIGS-22 cells. **(A-E)** Adapted from Audas TE, Jacob MD, Lee S. 2012a. Note: (A, B, C, E): TA. (D): MJ

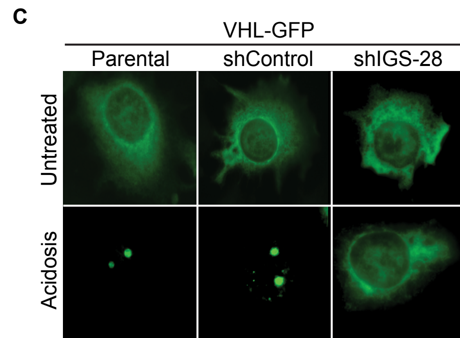
A



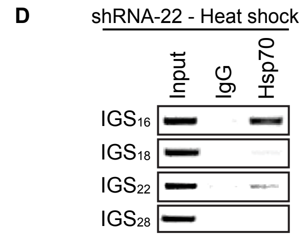
B



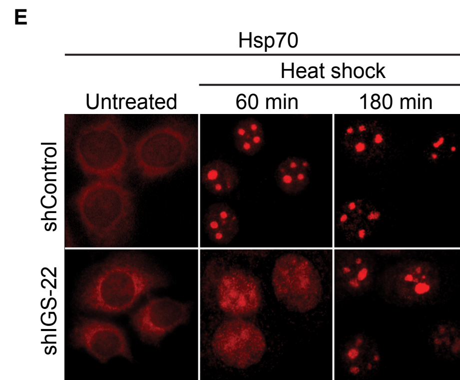
C



D



E



response specific environmental stimuli, and that the resulting transcripts are indispensable for the nucleolar sequestration of proteins.

3.2.4. IGS loci function independently

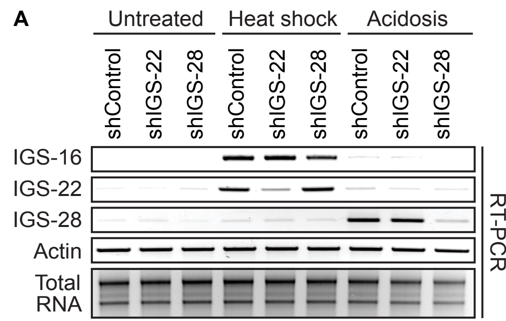
An notable aspect of IGS RNA expression is specificity. IGS_{16}RNA and IGS_{22}RNA are induced in response to heat shock but remain at basal levels under acidosis **(Figure 18A)** (Audas et al., 2012a). Similarly, IGS_{28}RNA accumulates in response to acidosis but not heat shock **(Figure 18A)** (Audas et al., 2012a). These results suggest that IGS loci function independently and that responses within the IGS are highly localized. In fact, we have found that although silencing IGS_{28}RNA inhibits the sequestration of VHL under acidosis, it does not prevent the sequestration of Hsp70 in heat shock or that of MDM2 under ribosomal stress **(Figure 18, B-D)**. Similarly, an IGS_{22}RNA knockdown only affects sequestration under heat shock, leaving the nucleolus competent at other loci (VHL in acidosis, MDM2 in ribosomal stress) **(Figure 18, B-D)**. Together, these findings demonstrate that the IGS is a complex transcriptional unit, composed of different loci that operate independently to capture proteins as a function of environmental stimuli.

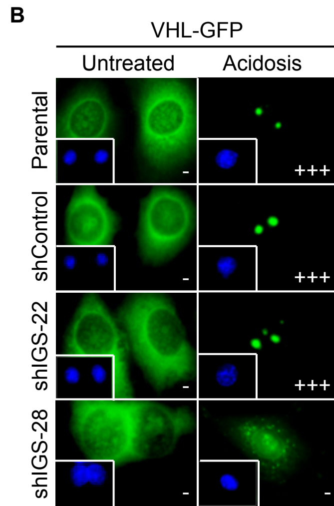
3.3. IGS RNAs induce a structural and functional remodelling of the nucleolus

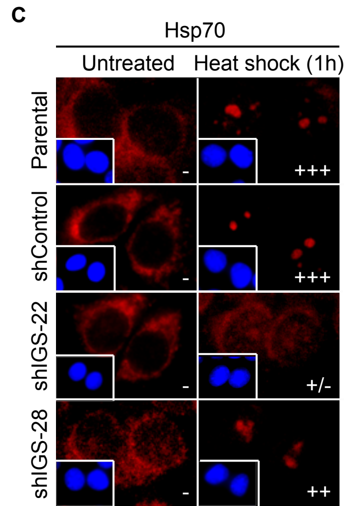
3.3.1. The nucleolus responds structurally to environmental stimuli

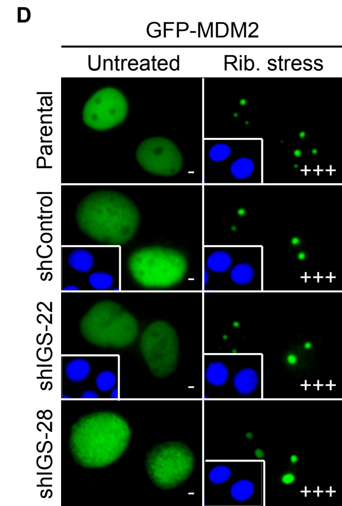
Environmental stimuli, such as heat shock and acidosis, induce the immobilization of a large number of NoDS-containing proteins in the nucleolus. To investigate how the nucleolar architecture adapts to accommodate this influx of proteins, we looked

Figure 18. IGS loci function independently. (A) Levels of IGS_{16}RNA, IGS_{22}RNA and IGS_{28}RNA in untreated, heat shocked and acidotic cells, in parental, $shIGS_{22}$ and $shIGS_{28}$ cells. **(B-D)** Locolization of VHL-GFP (acidosis), Hsp70 (heat shock) and GFP-MDM2 (ribosomal stress) in parental, shControl, shIGS-22 and shIGS-28 cells. DNA was stained with Hoechst 33342 (blue, bottom left). Intensity of nucleolar relocalization is indicated (bottom left). Adapted from Audas TE, Jacob MD, Lee S. 2012a. Note: (A): TA. (B-D): MJ and TA.


A
Untreated
Heat shock
Acidosis
shControl
shIGS-22
shIGS-28
shControl
shIGS-22
shIGS-28
shControl
shIGS-22
shIGS-28
IGS-16
IGS-22
IGS-28
Actin
Total RNA
RT-PCR

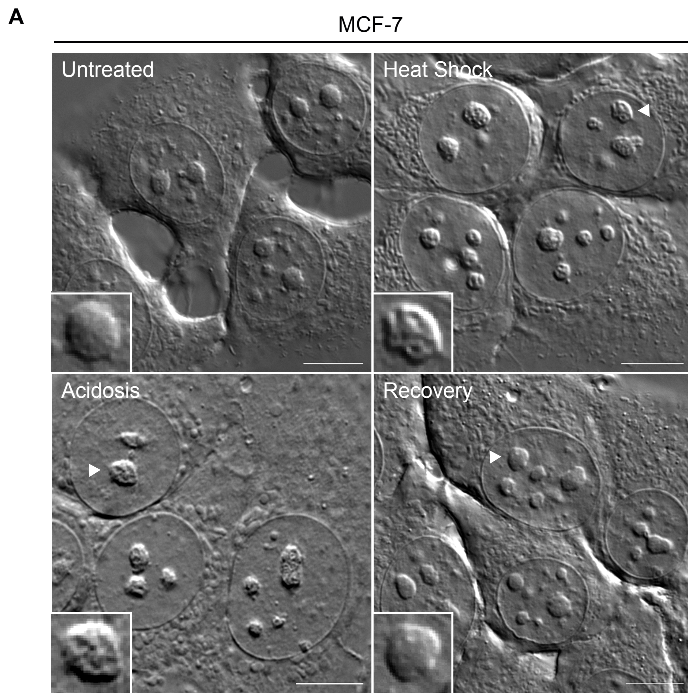

B
VHL-GFP
Untreated
Acidosis
Parental
shControl
shIGS-22
shIGS-28
-
+++
-
+++
-
+++
-
-

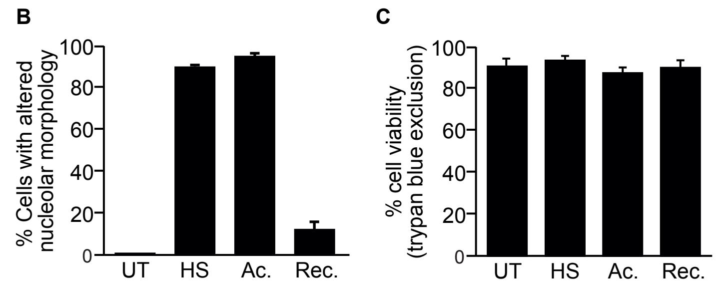

C
Hsp70
Untreated
Heat shock (1h)
Parental
shControl
shIGS-22
shIGS-28
-
+++
-
+++
-
+/-
-
++

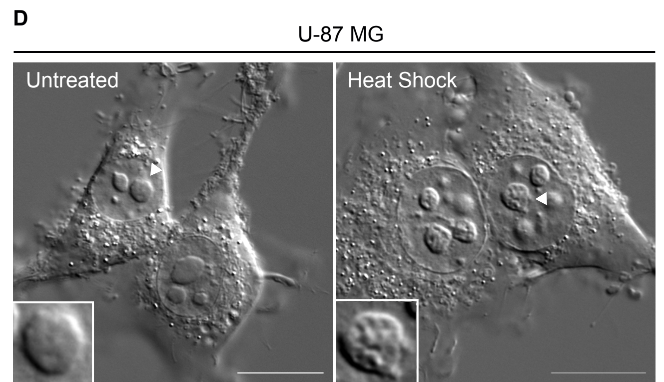

D
GFP-MDM2
Untreated
Rib. stress
Parental
shControl
shIGS-22
shIGS-28
-
+++
-
+++
-
+++
-
+++

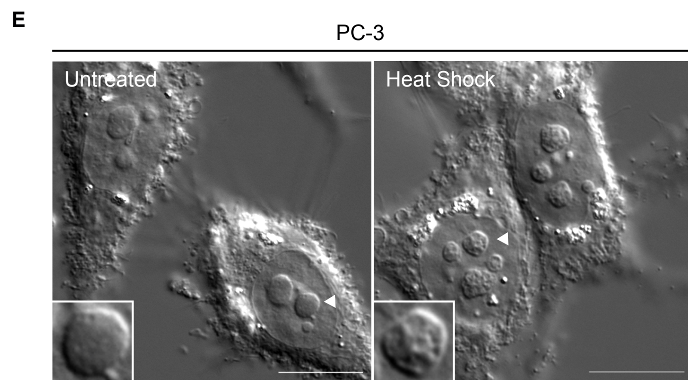
for gross morphological changes by differential interference contrast (DIC) microscopy. Whereas the nucleoli of untreated MCF-7 cells appeared relatively round and smooth, ~90% of cell treated with heat shock or acidosis displayed an altered nucleolar morphology, more irregular in appearance, and perforated with voids **(Figure 19, A and B)**. Upon return to normal condition, nucleoli regained their original form **(Figure 19, A and B).** Cell viability was not affected by these treatments **(Figure 19C)**, and similar structural responses were observed in other mammalian cell lines, including PC-3 **(Figure 19D)**, U-87 MG **(Figure 19E)** and NIH/3T3 **(Figure 19F)**. To gain more insights into this process, we used transmission electron microscopy (TEM) to monitor changes at the ultrastructural level. As expected, we found that the nucleoli of untreated cells displayed their typical tripartite organization, with visible FCs and DFCs distributed throughout the GC **(Figure 20).** Interestingly, these distinctive features were lost in response to heat shock and acidosis. Cells exposed to these treatments possessed a noticeably transformed nucleolar morphology, composed primarily of electron-dense anastomosed sheets in a reticular structure **(Figure 20)**. Again, we found that the nucleolus reverted to its original conformation upon signal termination **(Figure 20).** These results suggest that the nucleolus undergoes a substantial, yet reversible, remodelling in response to changing environmental conditions. (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

Figure 19. The nucleolus responds structurally to environmental stimuli. (A) DIC images of untreated, heat shocked, acidotic and recovered (heat shock followed by 6 h recovery) MCF-7 cells. **(B)** Quantification of untreated, heat shocked, acidotic and recovered MCF-7 cells showing altered nucleolar morphology. **(C)** Quantification of viable untreated, heat shocked, acidotic and recovered MCF-7 cells, assessed by trypan blue exclusion. **(D-F)** DIC images of live untreated and heat shocked U-87 MG, PC-3 and NIH/3T3 cells. **(A, D-F)** Indicated nucleoli (arrow) is enlarged in inset. Scale bar, 10 μm. **(B, C)** Columns, mean (n = 3); error bars; s.e.m.
(A-F) Adapted from Jacob et *al.*, 2013


A
MCF-7
Untreated
Heat Shock
Acidosis
Recovery


B
% Cells with altered nucleolar morphology
100
80
60
40
20
0
UT
HS
Ac.
Rec.
C
% cell viability (trypan blue exclusion)
100
80
60
40
20
0
UT
HS
Ac.
Rec.


D
U-87 MG
Untreated
Heat Shock


E
PC-3
Untreated
Heat Shock

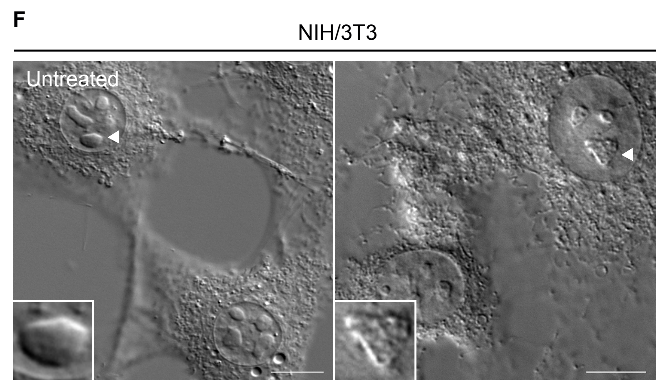
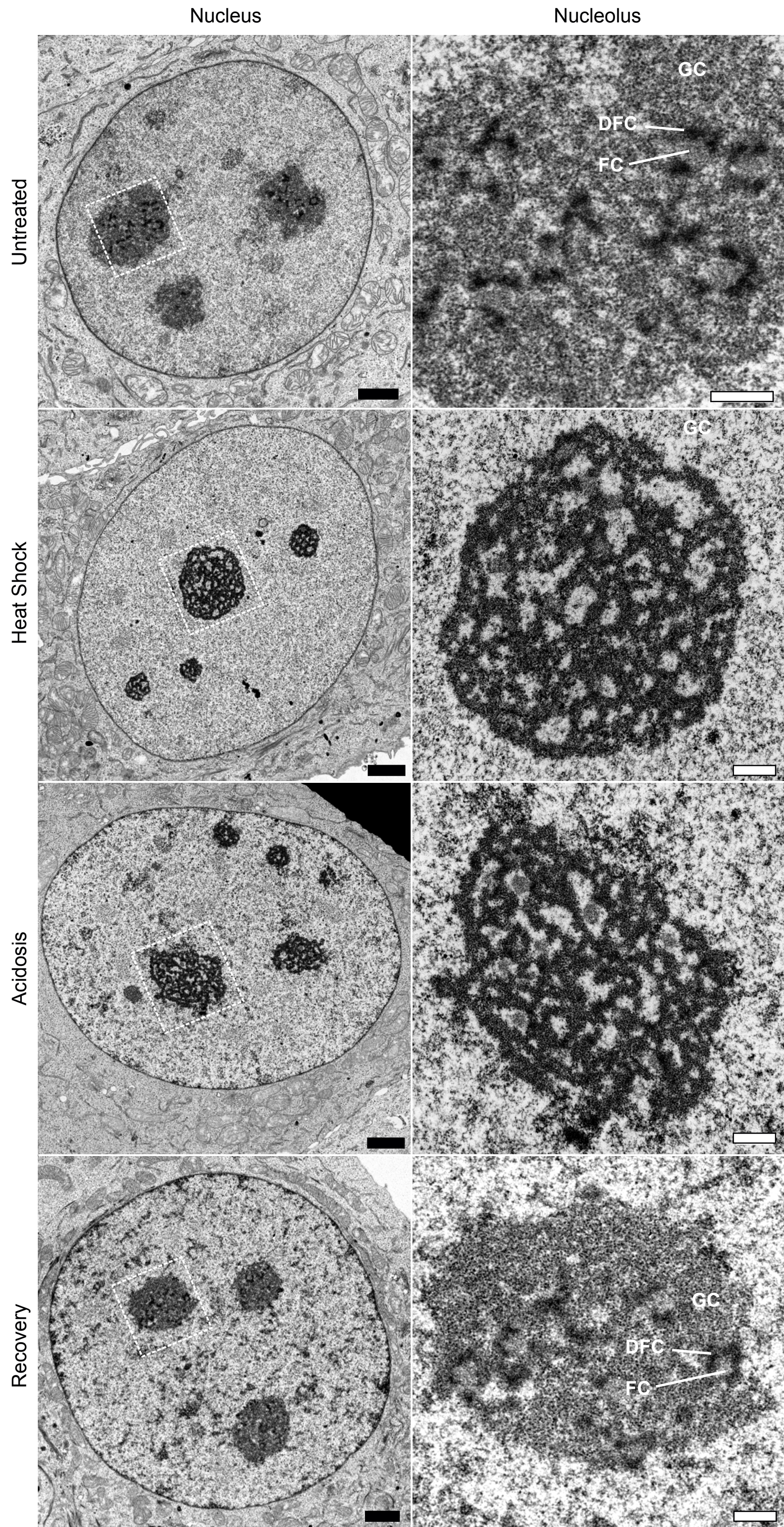

F
NIH/3T3
Untreated

Figure 20. The nucleolus responds ultra-structurally to environmental stimuli. Transmission electron microscopy images of untreated, heat shocked, acidotic and recovered (heat shocked followed by 6 h recovery). Indicated nucleoli (dotted square) is enlarged on right panel. Black scale bars, 2 μm; white scale bars, 0.5 μm. Subnucleolar compartments (GC, DFC, FC) are indicated. Adapted from Jacob et *al.*, 2013.

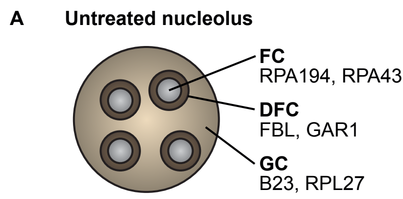


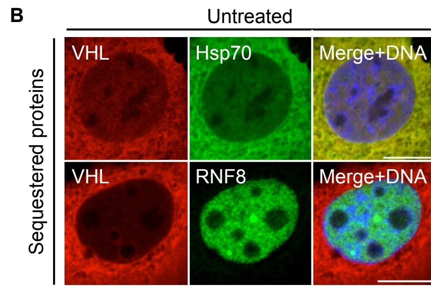
Nucleus
Nucleolus
Untreated
Heat Shock
Acidosis
Recovery
GC
DFC
FC
GC
GC
DFC
FC

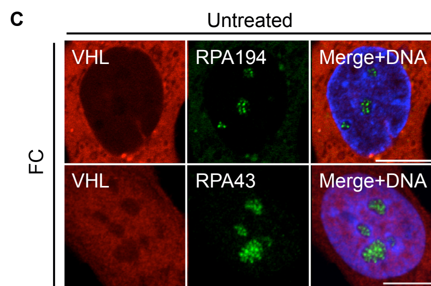
3.3.2. The nucleolus is reorganized around the detention center

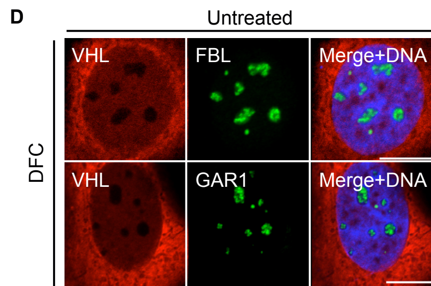
The structural adaptation described above correlates with an influx of NoDS-containing proteins. To understand the consequences of this process on the functional organization of the nucleolus, we compared the localization of VHL, a well-characterized marker of sequestration, with that of several resident nucleolar proteins. RNA polymerase I subunits 43 and 194 were used as markers of the FC, fibrillarin (FBL) and GAR1 as markers of the DFC, and B23 and 60S ribosomal protein L27 (RPL27) as markers of the GC **(Figure 21A)**. Under standard growth conditions, VHL displayed its normal nucleo-cytoplasmic distribution, whereas RPA43, RPA194, FBL, GAR1, B23 and RPL27 localized predominantly to their respective nucleolar compartments **(Figure 21, B-E)**. However, in response to heat shock, VHL accumulated at the core of the nucleolus where it formed a large and irregular structure that colocalized with several other sequestered proteins including Hsp70 and RNF8 **(Figure 21B)**. Surprisingly, this region did not colocalize with any of the known nucleolar compartments, as markers of the FC, DFC and GC appeared to have migrated to the nucleolar periphery **(Figure 21, C-E)**. Upon return to normal conditions, VHL was released from the nucleolus and FBL regained its punctuated localization **(Figure 21F)**. This redistribution was also observed in three dimensions **(Figure 21G)**, as well as with endogenous Hsp70 and FBL in both MCF-7 and U-87 MG cells **(Figure 21, H-J)**. Next, we looked at the dynamic profile of the different compartments, as most nuclear and nucleolar proteins are known to be highly mobile (Borer et al., 1989; Lander et al., 2001; Misteli, 2001; Misteli, 2004; Phair and Misteli, 2001). Using fluorescence recovery after photobleaching (FRAP),

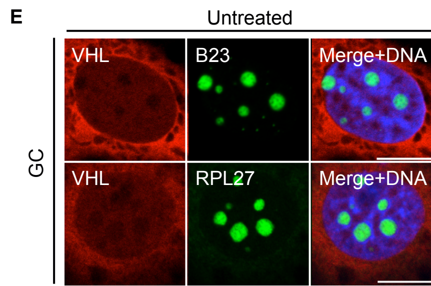
Figure 21. The nucleolus is reorganized around the detention center. (A) Schematic representation of the nucleolus, indicating markers of the three subnucleolar compartments. **(B-E)** Localization of mCherry-VHL in untreated and heat shocked MCF-7 cells, relative to other sequestered proteins (B; Hsp70, RNF8) and as well as markers of the GC (C; B23, RPL27), DFC (D; FBL, GAR1) and FC (E; RPA194, RPA43). **(F)** Localization of mCherry-VHL relative to EGFP-FBL in recovered cells (heat shock followed by 6 h recovery). **(G)** 3D reconstruction of the localization of VHL, B23, GAR1 and FBL in untreated and heat shocked MCF-7 cells. **(H-I)** Localization of endogenous Hsp70 relative to endogenous FBL in untreated and heat shocked MCF-7 **(H)** and U87MG **(I)** cells. **(J)** Diagram of the remodelled nucleolus, including the detention center. **(B-I)** Scale bar, 10 μm. **(H, I)** Indicated nucleolus (dotted square) is enlarged in inset. **(A-J)** Adapted from Jacob et *al.*, 2013.

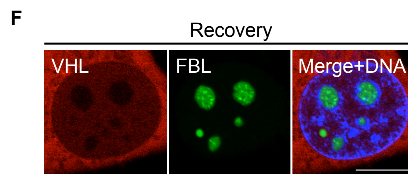

A
Untreated nucleolus
FC
RPA194, RPA43
DFC
FBL, GAR1
GC
B23, RPL27

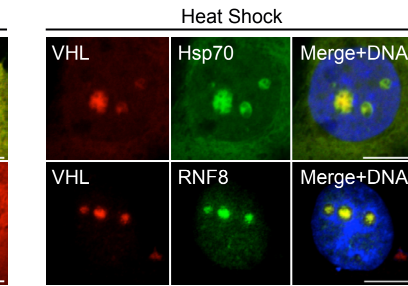

B
Untreated
Sequestered proteins
VHL
Hsp70
Merge+DNA
VHL
RNF8
Merge+DNA

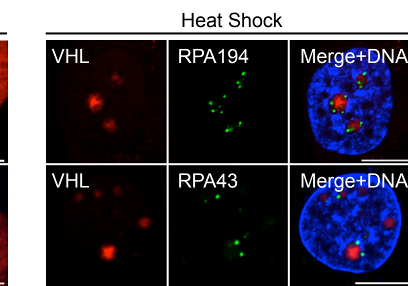

C
Untreated
FC
VHL
RPA194
Merge+DNA
VHL
RPA43
Merge+DNA

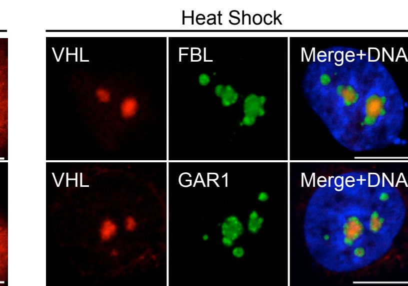

D
Untreated
DFC
VHL
FBL
Merge+DNA
VHL
GAR1
Merge+DNA

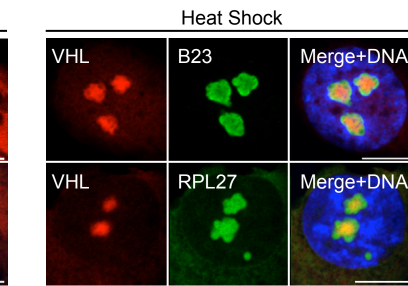

E
Untreated
GC
VHL
B23
Merge+DNA
VHL
RPL27
Merge+DNA

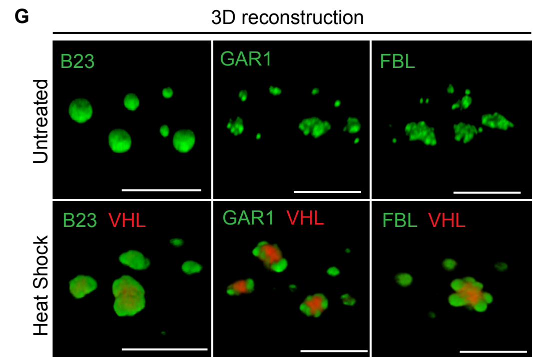

F
Recovery
VHL
FBL
Merge+DNA

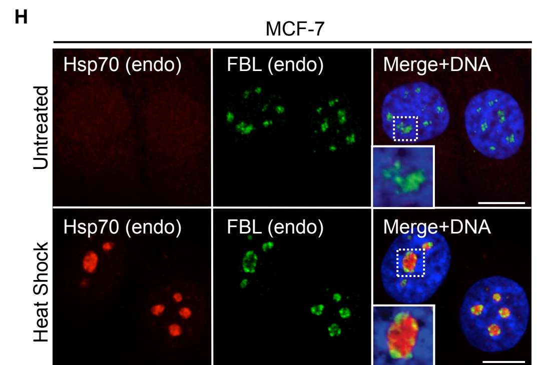

Heat Shock
VHL
Hsp70
Merge+DNA
VHL
RNF8
Merge+DNA

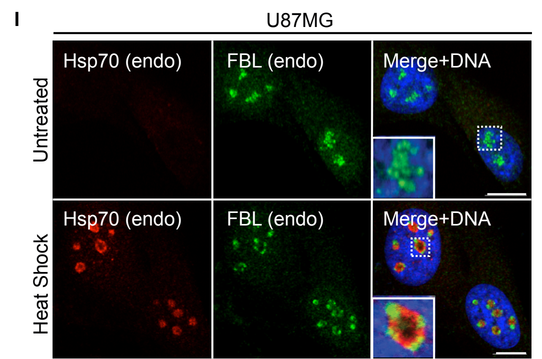

Heat Shock
VHL
RPA194
Merge+DNA
VHL
RPA43
Merge+DNA

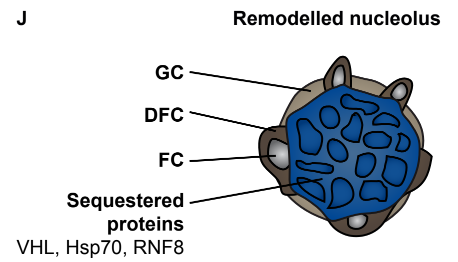

Heat Shock
VHL
FBL
Merge+DNA
VHL
GAR1
Merge+DNA


Heat Shock
VHL
B23
Merge+DNA
VHL
RPL27
Merge+DNA


G
3D reconstruction
Untreated
B23
GAR1
FBL
Heat Shock
B23 VHL
GAR1 VHL
FBL VHL


H
MCF-7
Untreated
Hsp70 (endo)
FBL (endo)
Merge+DNA
Heat Shock
Hsp70 (endo)
FBL (endo)
Merge+DNA


I
U87MG
Untreated
Hsp70 (endo)
FBL (endo)
Merge+DNA
Heat Shock
Hsp70 (endo)
FBL (endo)
Merge+DNA

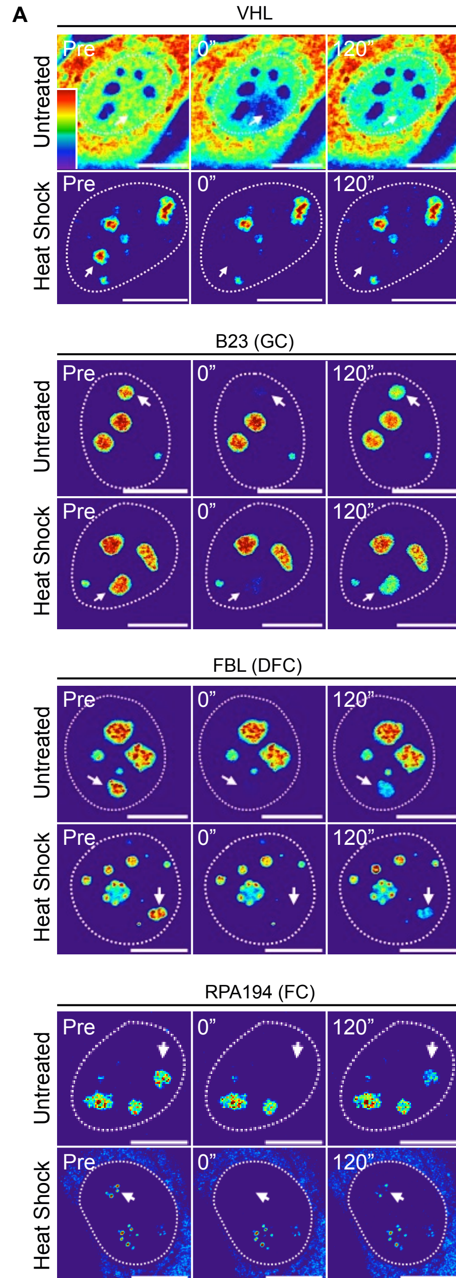

J
Remodelled nucleolus
GC
DFC
FC
Sequestered proteins
VHL, Hsp70, RNF8

we observed that VHL transition from highly dynamic in the nucleus under normal conditions to immobilized in the nucleolus under heat hock and acidosis **(Figure 22, A and B)**. In stark contrast, resident nucleolar proteins remained mobile under all conditions, in spite of their spatial redistribution **(Figure 22, A and B)**. Together, these results demonstrate that protein sequestration is associated with a reorganization of the nucleolus, and that the region in which protein are immobilized is spatially and dynamically distinct. We will hereafter refer to this region as the detention center (DC). (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

3.3.3. The detention center is characterized by a hydrophobic signature

The immobility of protein in the DC suggests that the organization of this compartment relies on a distinctive set of biochemical properties. VHL and Hsp70 extracted from heat shocked and acidotic cells migrated as monomers by SDS-PAGE, even in the absence of reducing agents (2-mercaptoethanol, DTT) **(Figure 23A)**, suggesting that covalent bonds were not involved in this structure. Therefore, we hypothesized that the DC could rely on hydrophobic interactions, a possibility that would be consistent with the requirement for hydrophobic triplets in the NoDS. Using 8-anilinonaphlene-1-sulphonate (ANS), a fluorescent and membrane permeable dye that highlights misfolded and hydrophobic protein deposits (Hadley et al., 2011), we stained untreated and treated cells. Untreated cells incubate with ANS displayed elevated levels of fluorescence in the cytoplasm and an absence of signal in the nucleus **(Figure 23B)**. This pattern is consistent with previous reports,

Figure 22. The detention center is dynamically distinct from the rest of the nucleolus. (A) LUT images representing the fluorescence recovery after photobleaching (FRAP) profile of the DC (VHL-GFP), GC (EGFP-B23), DFC (EGFP-FBL) and FC (EGFP-RPA194). Scale bar, 10 μm. **(B)** Quantification of the FRAP profile of VHL-GFP, EGFP-B23, EGFP-RPL27, EGFP-FBL, EGFP-RPA194 and mCherry-RPA43 in untreated, heat shocked and acidotic cells. n = 10. **(A-B)** Adapted from Jacob et *al.*, 2013.


A
VHL
Untreated
Heat Shock
Pre
0”
120”
B23 (GC)
FBL (DFC)
RPA194 (FC)

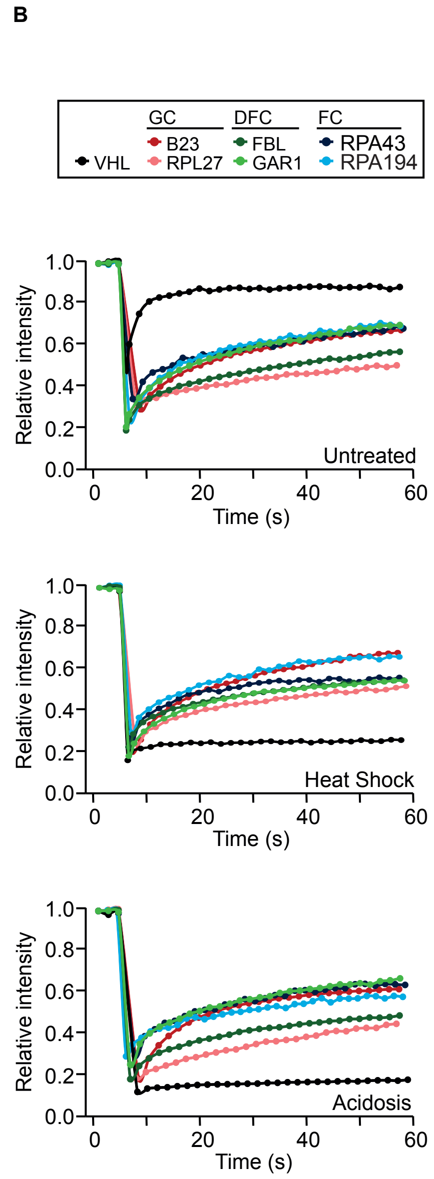
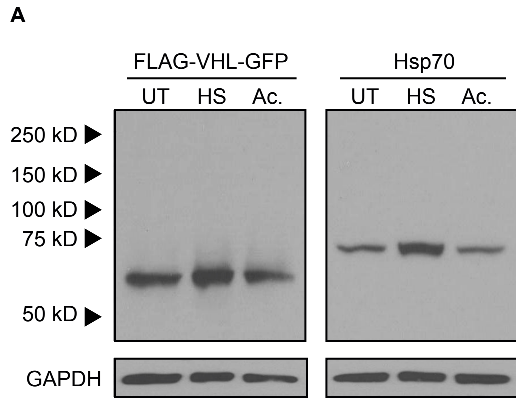
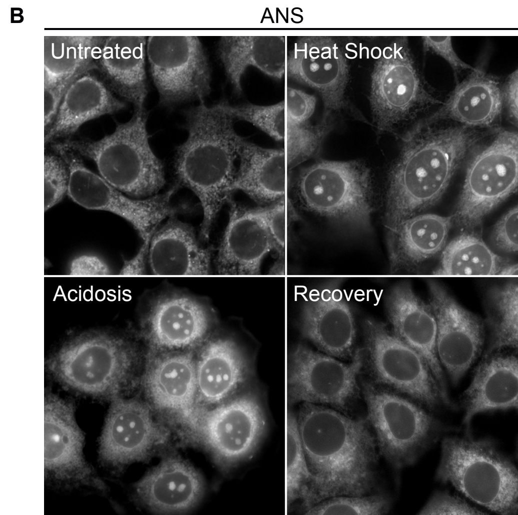
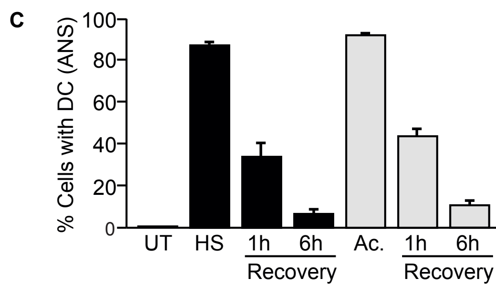
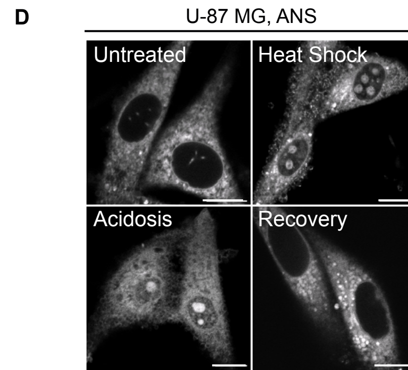

B
GC
DFC
FC
VHL
B23
RPL27
FBL
GAR1
RPA43
RPA194
Relative intensity
Time (s)
Untreated
Heat Shock
Acidosis

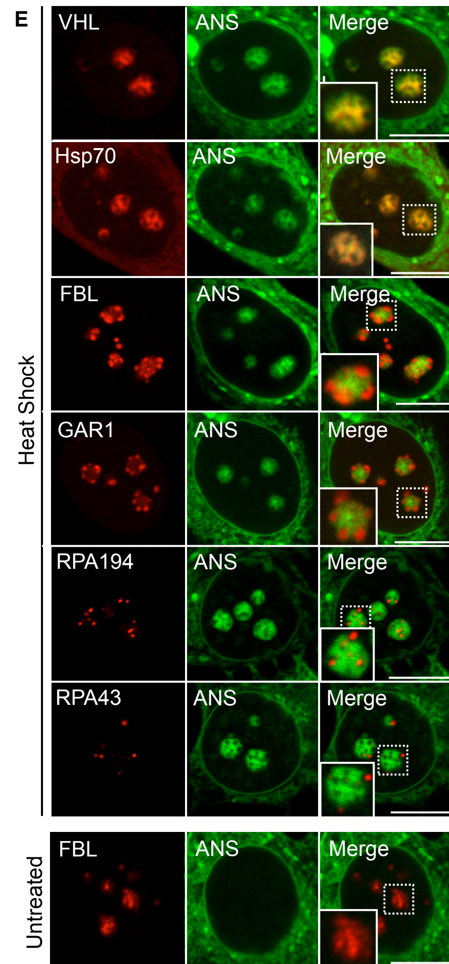
Figure 23. The detention center is characterized by a hydrophobic signature. **(A)** Western blot of VHL-GFP and endogenous Hsp70 in untreated, heat shocked and acidotic MCF-7 cells. GAPDH was used as a loading control. No reducing agents (DTT, 2-mercaptoethanol) were used. **(B)** Live untreated, heat shocked, acidotic and recovered (heat shock followed by 6 h recovery) MCF-7 cells stained with 8-anilino-1-naphthalenesulfonic acid (ANS). **(C)** Quantification of untreated, heat shocked, acidotic and recovered (1 h, 6 h) cells showing ANS-positive nucleoli. Columns, mean ($n = 3$); error bars, s.e.m. **(D)** Live untreated, heat shocked, acidotic and recovered U87MG cells stained with ANS. **(E)** Localization of VHL-GFP, Hsp70-GFP, EGFP-FBL, EGFP-GAR1, EGFP-RPA194 and mCherry-RPA43 relative to ANS-positive region in heat shocked cells. Localization of EGFP-FBL relative to ANS in untreated cells. **(D-E)** Scale bar, 10 μm. **(A-E)** Adapted from Jacob et *al.*, 2013.


A
FLAG-VHL-GFP
Hsp70
UT HS Ac.
UT HS Ac.
250 kD
150 kD
100 kD
75 kD
50 kD
GAPDH


B
ANS
Untreated
Heat Shock
Acidosis
Recovery

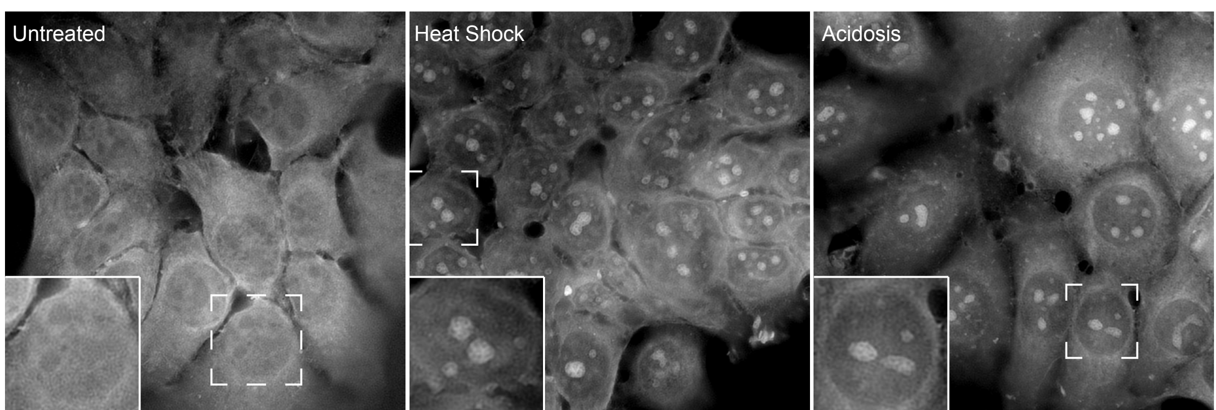

C
% Cells with DC (ANS)
0
20
40
60
80
100
UT
HS
1h
6h
Recovery
Ac.
1h
6h
Recovery

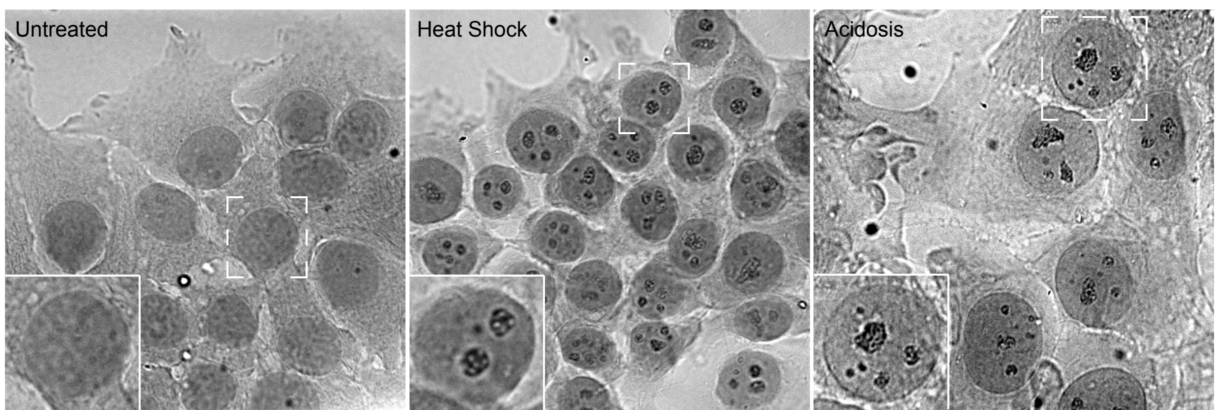

D
U-87 MG, ANS
Untreated
Heat Shock
Acidosis
Recovery


E
Heat Shock
VHL
ANS
Merge
Hsp70
FBL
GAR1
RPA194
RPA43
Untreated

as the Golgi, endoplasmic reticulum and lysosomes are rich is misfolded proteins and ANS-binding sites (Hadley et al., 2011). In contrast, ~90% of MCF-7 cells treated with heat shock and acidosis displayed strong ANS-positive nucleoli **(Figure 23, B and C)**, a pattern that was also present in U-87 MG cells **(Figure 23 D)**. Upon signal termination, nucleoli lost their ANS-positive signature **(Figure 23, B-D)**. Given the correlation between DC formation and ANS signal, we compared the localization of ANS with that of different nucleolar proteins. We found that immobilized VHL and Hsp70 colocalized with ANS, whereas markers of the FC, DFC and GC were excluded from the ANS-positive region **(Figure 23E)**. These data indicate that the DC is characterized by a hydrophobic signature, further highlighting its unique biochemical profile. This signature allows ANS to be used a marker of protein sequestration and nucleolar remodelling. Interestingly, ANS is also known to recognize beta-amyloid deposits (Hadley et al., 2011), suggesting that the DC could indeed be composed of such structures. To further test this possibility, we stained cells with two standard markers of amyloid plaques: thioflavin S (ThS) and congo red. Untreated cells displayed minimal staining, whereas heat shocked and acidotic cells contained highly visible nucleoli, both with ThS and congo red **(Figure 24)**. Furthermore, the structures highlighted possessed a circumvallated appearance that was characteristic of the DC. These results suggest that the DC could be a regulated, intracellular beta-amyloid structure. (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

Figure 24. The detention center is highlighted by several markers of amyloid deposits. Untreated, heat shocked and acidotic MCF-7 cells stained with thioflavin S (top) and congo red (bottom). Indicated cells (square) are enlarged (inset).

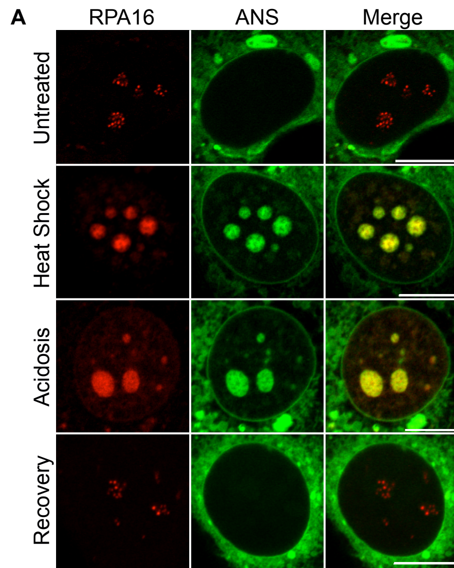

Thioflavin S
Untreated
Heat Shock
Acidosis

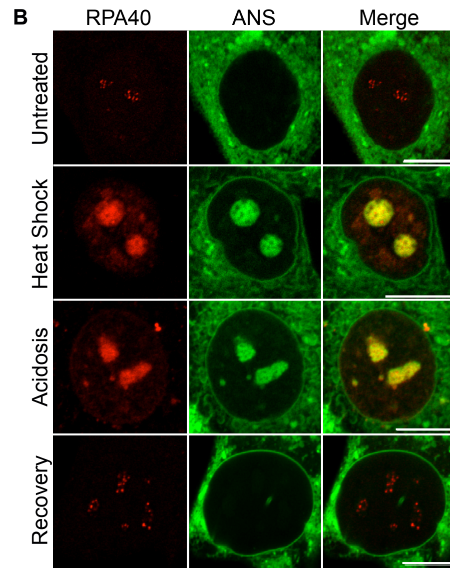

Congo red
Untreated
Heat Shock
Acidosis

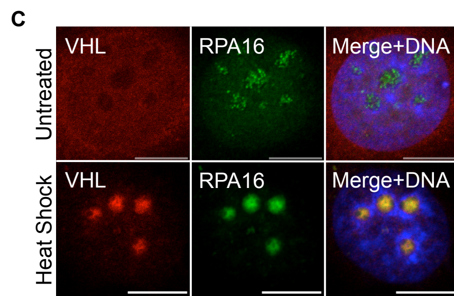
3.3.4. Components of the ribosomal biogenesis machinery relocalize in the DC

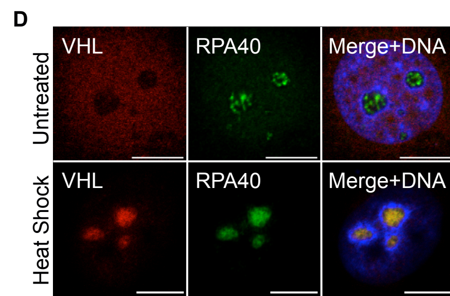
The DC is composed of a variety of immobilized factors, including E3-ubiquitin ligases, chaperones, cell cycles regulators and DNA replication enzymes (Audas et al., 2012b). Surprisingly, we found that several components of the ribosomal biogenesis machinery were also targets of this pathway. RPA16 and RPA40, two essential subunits of RNA pol I and III complexes, relocalized from the FCs to the DC in response to heat shock and acidosis, as indicated by their colocalization with ANS and VHL **(Figure 25, A-D)**. Upon return to standard conditions, these proteins were released from the DC and regained their punctuated FC localization **(Figure 25, A and B)**. As expected, RPA16 and RPA40 reversibly lost their mobility in the DC **(Figure 25, E-H)**. In addition to these subunits of RNA pol I, several GC were also found to be immobilized in the DC in response to heat shock and acidosis, including NOP2 nucleolar protein homolog (NOL1), nucleolar protein with MIG4Gmain 1 (NOM1), ribosomal RNA processing protein 1 homolog A (NOP52), ribosomal RNA processing protein 1 homolog B (RRP1B), pescadillo (PES1), SUMO1/sentrin/SMT3 specific peptidase 3 (SENP3) **(Figure 26, A-C and E-G; Table 3)**. The ability of certain nucleolar proteins, such as FBL, B23 and NOPP140 to retain mobility and evade capture by the DC further highlights the specificity of this intranucleolar reorganization process **(Figure 26, D and H)**. (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

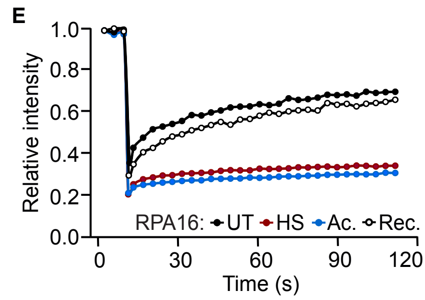
Figure 25. Subunits of the RNA polymerase I complex relocalize from the FC to the detention center. (A-D) Localization of EGFP-RPA16 and EGFP-RPA40 in untreated, heat shocked, acidotic and recovered (heat shock followed by 6 h recovery) MCF-7 cells, relative to 8-anilino-1-naphthalenesulfonic acid (ANS) **(A, B)** and mCherry-VHL **(C, D)**. **(E-H)** Nucleolar FRAP analysis of the dynamic profile of EGFP-RPA16 and EGFP-RPA40 in untreated, heat shocked, acidotic and recovered cells. **(A-D, F-H)** Scale bars, 10 μm. **(A-H)** Adapted from Jacob et *al.*, 2013.

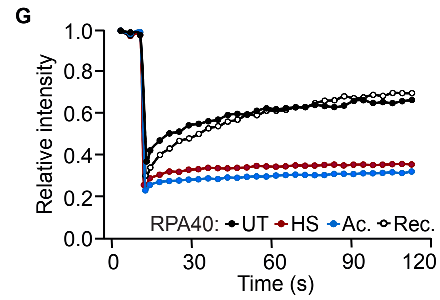

A
RPA16
ANS
Merge
Untreated
Heat Shock
Acidosis
Recovery

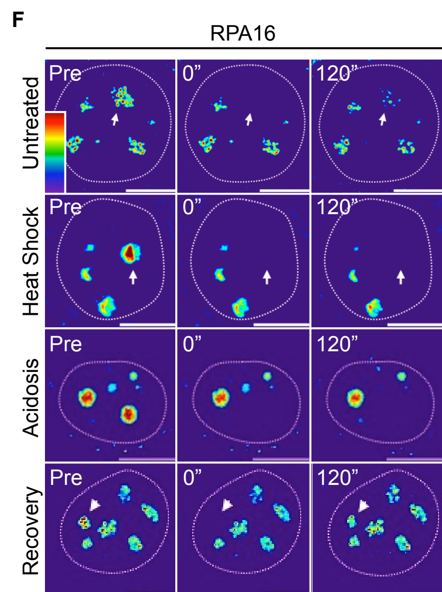

B
RPA40
ANS
Merge
Untreated
Heat Shock
Acidosis
Recovery


C
VHL
RPA16
Merge+DNA
Untreated
Heat Shock


D
VHL
RPA40
Merge+DNA
Untreated
Heat Shock


E
Relative intensity
RPA16: UT HS Ac. Rec.
Time (s)


G
Relative intensity
RPA40: UT HS Ac. Rec.
Time (s)


F
RPA16
Pre
0”
120”
Untreated
Heat Shock
Acidosis
Recovery

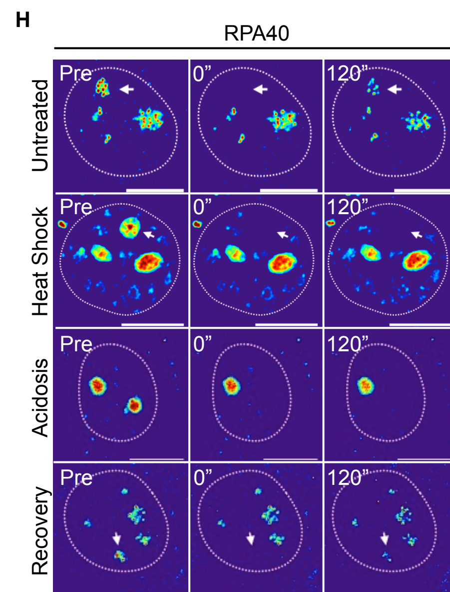
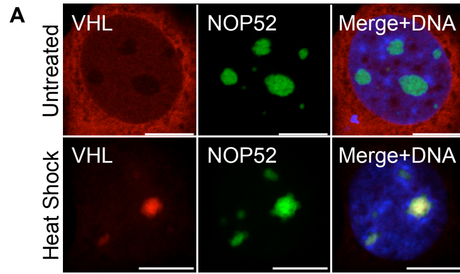
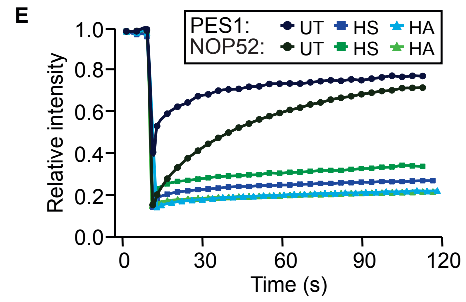
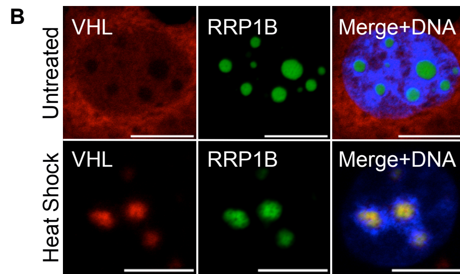
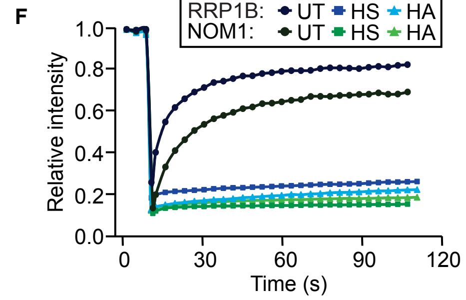

H
RPA40
Pre
0”
120”
Untreated
Heat Shock
Acidosis
Recovery

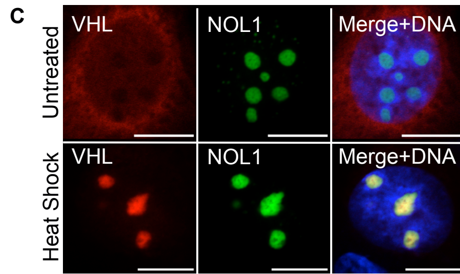
Figure 26. Components of the ribosomal biogenesis machinery relocalize from the GC to the detention center. (A-D) Localization of EGFP-NOP52 **(A)**, EGFP-RRP1B **(B)**, EGFP-NOL1 **(C)** and EGFP-NOPP140 **(D)** relative to mCherry-VHL in untreated and heat shocked MCF-7 cells. DNA was stained with Hoechst 33342. Scale bars, 10 um. **(E-F)** Nucleolar FRAP analysis of the dynamic profile of EYFP-PES1, EGFP-NOP52, EGFP-RRP1B, EGFP-NOM1, EGFP-NOL1, EGFP-SENP3 and EGFP-NOPP140 in untreated, heat shocked and acidotic cells. **(A-F)** Adapted from Jacob *et al,* 2013.

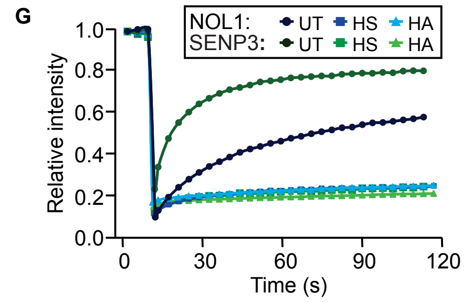

A
Untreated
Heat Shock
VHL
NOP52
Merge+DNA

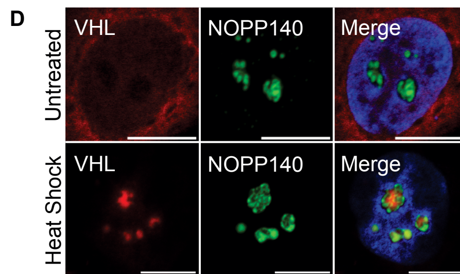

E
PES1: UT HS HA
NOP52: UT HS HA
Relative intensity
Time (s)


B
Untreated
Heat Shock
VHL
RRP1B
Merge+DNA


F
RRP1B: UT HS HA
NOM1: UT HS HA
Relative intensity
Time (s)


C
Untreated
Heat Shock
VHL
NOL1
Merge+DNA


G
NOL1: UT HS HA
SENP3: UT HS HA
Relative intensity
Time (s)


D
Untreated
Heat Shock
VHL
NOPP140
Merge

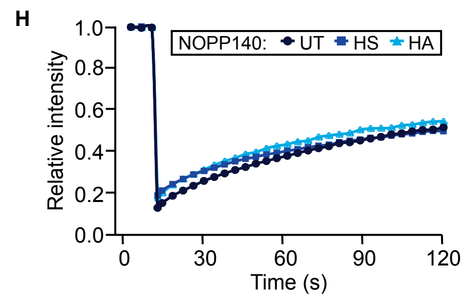

H
NOPP140: UT HS HA
Relative intensity
Time (s)

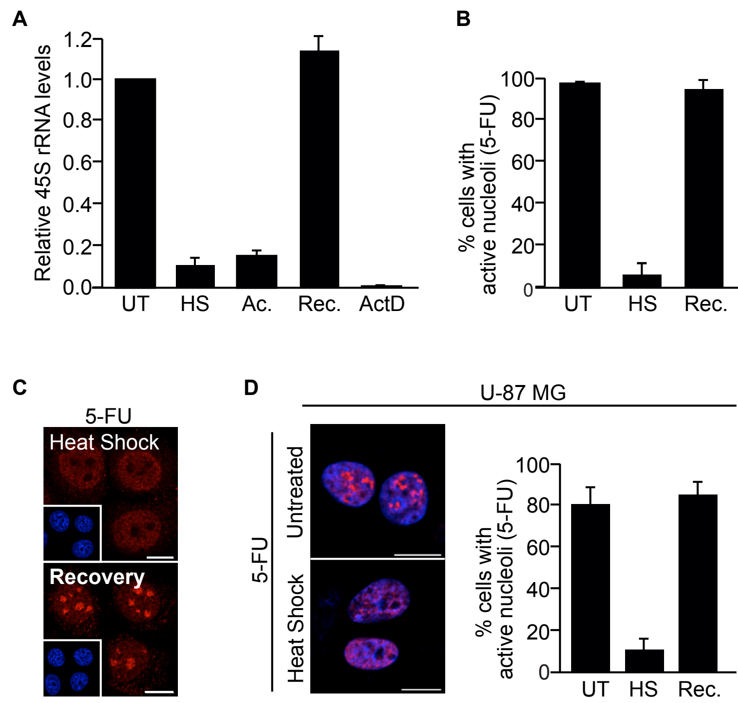
Table 3. Spatio-dynamic profile of nucleolar proteins in untreated, heat shocked and acidotic cells. Localization was assessed by confocal imaging, mobility was measured by FRAP. The data presented here was consolidated from figures 21, 25 and 26.

Symbol	Full name	Intranucleolar localization		
		Untreated	Heat Shock	Acidosis
RPA194	Polymerase (RNA) I polypeptide A, 194 kDa	FC (mobile)	FC (mobile)	FC (mobile)
RPA40	Polymerase (RNA) I polypeptide C, 40 kDa	FC (mobile)	DC (immobile)	DC (immobile)
RPA16	Polymerase (RNA) I polypeptide D, 16 kDa	FC (mobile)	DC (immobile)	DC (immobile)
RPA43	Polymerase (RNA) I polypeptide subunit 43 kDa	FC (mobile)	FC (mobile)	FC (mobile)
FBL	Fibrillarin	DFC (mobile)	DFC (mobile)	DFC (mobile)
GAR1	GAR1 ribonucleoprotein	DFC (mobile)	DFC (mobile)	DFC (mobile)
NOPP140	Nucleolar and coiled body phorosphoprotein 1	DFC (mobile)	DFC (mobile)	DFC (mobile)
B23	Nucleophosmin B23	GC (mobile)	GC (mobile)	GC (mobile)
NOL1	NOP2 nucleolar protein homolog	GC (mobile)	DC (immobile)	DC (immobile)
NOM1	Nucleolar protein with MIF4G domain 1	GC (mobile)	DC (immobile)	DC (immobile)
NOP52	Ribosomal RNA processing protein 1 homolog A	GC (mobile)	DC (immobile)	DC (immobile)
PES1	Pescadillo	GC (mobile)	DC (immobile)	DC (immobile)
RPL27	60S ribosomal protein L27	GC (mobile)	GC (mobile)	GC (mobile)
RRP1B	Ribosomal RNA processing protein 1 homolog B	GC (mobile)	DC (immobile)	DC (immobile)
SENP3	SUMO1/sentrin/SMT3 specific peptidase 3	GC (mobile)	DC (immobile)	DC (immobile)

3.3.5. The remodelled nucleolus is transcriptionally inactive

Ribosomal biogenesis relies on a complex and compartmentalized assortment of dynamic factors in which structure and function are intimately linked (Dundr et al., 2002; Hernandez-Verdun, 2006). Given the drastic remodelling of the nucleolar architecture and the immobilization of several key nucleolar proteins, we asked whether rRNA synthesis could be sustained under conditions of nucleolar sequestration. We used real-time reverse transcription PCR (qPCR) analysis to measure the levels of 45S pre-rRNA transcripts in untreated and treated cells. Results revealed an ~85% decrease in both heat shocked and acidotic cells, relative to untreated cells **(Figure 27A)**. 45S pre-rRNA levels returned to their original values upon extracellular signal termination **(Figure 27A)**. In addition to qPCR, we labelled de novo RNA transcription sites with 5-fluorouridine (5-FU). We observed an absence of nucleolar incorporation in ~95% of heat shocked cells **(Figure 27, B and C)**, with a return to normal values following signal **termination (Figure 27, B and C)**. Similar results were observed in U-87 MG cells **(Figure 27D)**. 5-FU incorporation is impractical under acidotic conditions, due to the pH of the media. These results suggest that remodelling of the nucleolus is associated with a severe, yet reversible, inhibition of ribosomal biogenesis. (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

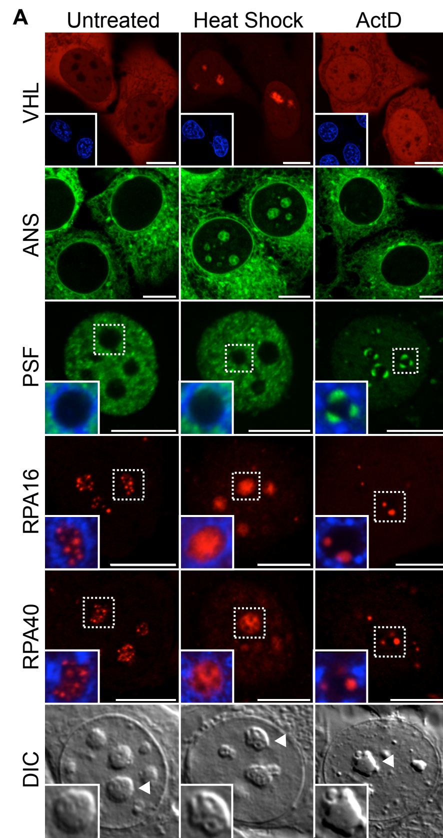
Figure 27. The remodelled nucleolus is transcriptionally inactive. (A) Relative levels of 45S pre-rRNA in untreated, heat shocked, acidotic and recovered (heat shock followed by 6 h recovery) MCF-7 cells, measured by real time quantitative PCR. Actinomycin D (ActD, 2 h, 50 nM) was used to selectively inhibit RNA polymerase I-dependent transcription. **(B)** Quantification of 5-fluorouridine (5-FU) incorporation in the nucleoli of untreated, heat shocked and recovered MCF-7 cells. **(C)** Localization of 5-FU incorporation in heat shocked and recovered MCF-7 cells. DNA was stained with Hoechst 33342 (inset). **(D)** Localization (left) and quantification (right) of 5-FU incorporation in untreated, heat shocked and recovered U87MG cells. **(A, B, D)** Column, mean (n = 3); error bars, s.e.m. (**C, D)** Scale bar, 10 µm. **(A-D)** Adapted from Jacob et *al.*, 2013.

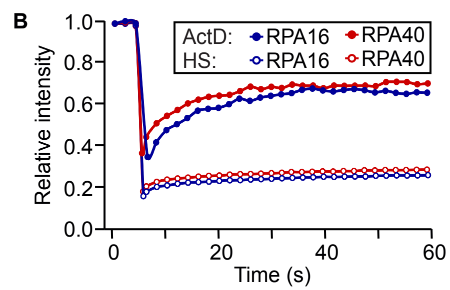

A
Relative 45S rRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
UT
HS
Ac.
Rec.
ActD
B
% cells with
active nucleoli (5-FU)
100
80
60
40
20
0
UT
HS
Rec.
C
5-FU
Heat Shock
Recovery
D
U-87 MG
5-FU
Untreated
Heat Shock
% cells with
active nucleoli (5-FU)
100
80
60
40
20
0
UT
HS
Rec.

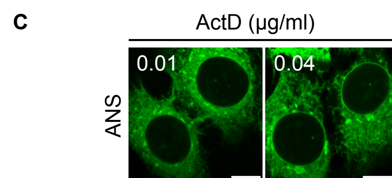
3.3.6. Environment-induced remodelling of the nucleolus is not a by-product of transcriptional inhibition

Transcriptional inhibition has been shown to induce several alterations to the nucleolar architecture, including the formation of nucleolar caps (Reynolds et al., 1964; Shav-Tal et al., 2005) and the flux of a large number of different factors (Andersen et al., 2005). Given the correlation between structure and transcriptional activity under heat shock and acidosis, we wanted to know if transcriptional inhibition was sufficient to induce some of the traits described thus far. We found that ActD treatment (5 μg/mL) failed to induce DC formation, as evidenced by the lack ANS-positive nucleoli and the diffuse nucleocytoplasmic distribution of VHL **(Figure 28A)**. Furthermore, using polypyrimidine tract-binding protein-associated-splicing factor (PSF), a common marker of nucleolar caps, we observed that only ActD-mediated transcriptional arrest triggered the formation of these cap structures **(Figure 28A)**. This suggests that this form of reorganization is distinct from that observed in response to environmental stresses, such as heat shock. Additionally, while RPA16 and RPA40 were immobilized in the DC under heat shock conditions **(Figure 28A)**, both proteins migrated to the nucleolar periphery and retained their mobility during transcriptional arrest **(Figure 28, A and B).** Lower levels of ActD that specifically inhibit rRNA synthesis (0.04 μg/mL) also failed to induce DC formation **(Figure 28C)**. These results demonstrate that the remodelling observed in response to environmental cues, such as heat shock, are not simply a by-product of transcriptional inhibition, as ActD appears to induce a different reorganizational program. Our data further suggest that the impairment in rRNA synthesis **(Figure**

Figure 28. The remodelled nucleolus is not a by-product of transcriptional inhibition. (A) Localization of mCherry-VHL, ANS signal, EGFP-PSF, EGFP-RPA16 and EGFP-RPA194 in live untreated, heat shocked, and ActD-treated (5 ug/mL) MCF-7 cells. VHL, PSF, RPA16 and RPA194: cells were stained with Hoechst 33342 to visualize DNA (blue). Bottom: DIC images of untreated, heat shocked and Actinomycin D (ActD, 5 ug/mL)-treated cells. **(B)** Nucleolar FRAP analysis of the dynamic profile of RPA16 and RPA40 in ActD-treated cells. **(C)** ANS staining in cells treated with low (RNA pol I-specific) levels of ActD (0.01 and 0.04 ug/mL). **(A, C)** Scale bar, 10 µm. Adapted from Jacob et *al.*, 2013.


A
Untreated
Heat Shock
ActD
VHL
ANS
PSF
RPA16
RPA40
DIC


B
ActD:
HS:
RPA16
RPA40
RPA16
RPA40
Relative intensity
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
Time (s)


C
ActD (μg/ml)
0.01
0.04
ANS

27, A-D) is likely a consequence, not a cause, of the nucleolar detention pathway. (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

3.3.7. Formation of the detention center correlates with the induction of IGS lncRNA

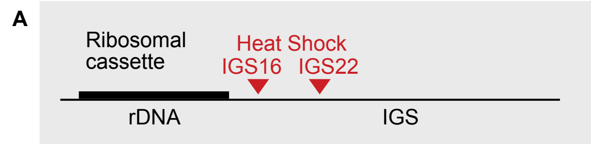
Sequestered proteins are immobilized on the ribosomal DNA cassette by several stimuli-specific IGS RNAs, including IGS_{16}RNA and IGS_{22}RNA during heat shock (Audas et al., 2012a) **(Figure 29A)**. These transcripts rapidly accumulate in response to environmental cues (Audas et al., 2012a) **(Figure 29B)** and return to basal levels upon signal termination **(Figure 29C)**. Induction of IGS lncRNA readily correlates with the remodelling of the nucleolus, the rapid sequestration of endogenous Hsp70, the formation of ANS-positive DCs and the interruption of nucleolar transcription **(Figure 29D)**. Upon signal termination, IGS lncRNA expression returns to basal levels **(Figure 29D)** and the nucleoli regained their original morphology, as indicated by Hsp70 release, loss in nuclear ANS signal and resumption in nucleolar 5-FU incorporation **(Figure 29D)**. (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

3.3.8. IGS lncRNAs are localized in the detention center

Given the temporal correlation between IGS lncRNA induction and DC formation, we asked whether these transcripts were localized within the DC itself. Using RNA fluorescence *in situ* hybridization (FISH) and probes specific to IGS_{22}RNA, we detected the presence of IGS_{22}RNA in the nucleoli of heat shocked cells **(Figure**

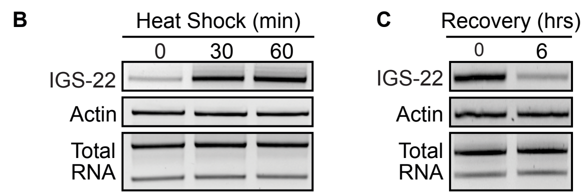
Figure 29. Formation of the Detention Center correlates with the induction of IGS lncRNA. (A) Schematic diagram of the ribosomal DNA cassette with the sites of protein immobilization during heat shock treatment (IGS_{16}, IGS_{22}). **(B)** Semi-quantitative RT-PCR analysis of the IGS_{22}RNA levels in MCF-7 cells heat shock treated for the indicated times. **(C)** Semi-quantitative RT-PCR analysis of the IGS_{22}RNA levels in heat shocked and recovered (6 h) cells. **(D)** DIC images of live untreated, heat shocked (30 min, 60 min) and recovered (6 h) cells. Localization of endogenous Hsp70, ANS signal and 5-FU incorporation in untreated, heat shocked (30 min, 60 min) and recovered (6 h) cells. Scale bar, 10 µm. Adapted from Jacob et *al.*, 2013.

A

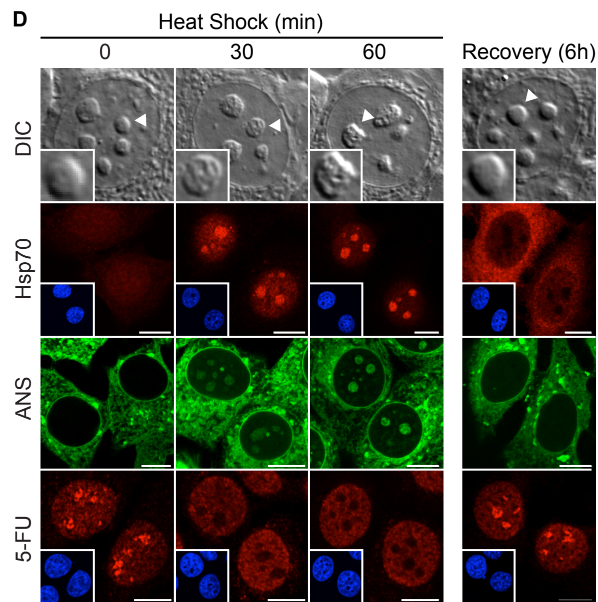


B

C



D



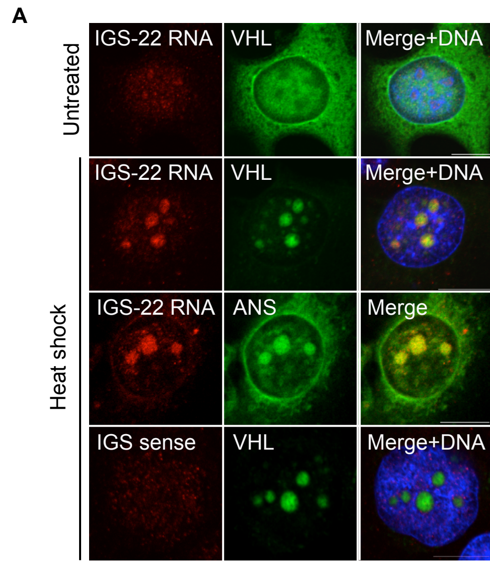
30A). $IGS_{22}RNA$ localized in the detention center, as evidence by colocalization with VHL and ANS **(Figure 30A)**. Untreated cells possessed basal levels of nucleolar $IGS_{22}RNA$, an observation that is consistent with RT-PCR data **(Figure 29B)**. As expected, IGS sense probes failed to produce a signal **(Figure 30A)**. Acidosis yielded similar results, with a nucleolar accumulation of $IGS_{28}RNA$ **(Figure 30B)**. As $IGS_{22}RNA$, $IGS_{28}RNA$ localized with VHL **(Figure 30B)**. Together these results demonstrate that the DC correlates spatially and temporally with the presence of IGS lncRNA. (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

3.3.9. IGS lncRNA is required to remodel the nucleolus

Given the presence of IGS lncRNA in the DC, we asked whether these transcripts were required to mediate the nucleolar reorganization process described here. Despite the intricacy of the IGS as a transcriptional unit, we found that cells stably expressing shRNA targeting $IGS_{22}RNA$ **(Figure 31A)** effectively delayed the sequestration of proteins during heat shock treatment (Audas et al., 2012a), with a near complete inhibition at the 30 minute time point. Stable knockdown of $IGS_{22}RNA$ did not affect the proper localization of the nucleolar factors; B23, FBL, RPA16 and RPA194 **(Figure 31B)**, the ultrastructure of the nucleolus (Jacob 2013), or the expression level of rRNA under standard conditions **(Figure 31C)** but impaired the sequestration of Hsp70 during heat shock treatment (Audas et al., 2012a) **(Figure 31D)**. We asked whether formation of the DC would also be compromised by inhibiting $IGS_{22}RNA$ expression. $ShIGS_{22}$ cells failed to produce any of the previously characterized hallmarks of DC formation. During heat shock

Figure 30. IGS lncRNA localizes within the detention center. (A) Fluorescent *in situ* hybridization (FISH) of IGS-22 RNA in untreated and heat shocked MCF-7 cells, relative to VHL-GFP and ANS. IGS sense probes were used a negative control **(B)** FISH of IGS-28 RNA in untreated and acidotic cells, relative to VHL-GFP. IGS sense probes were used a negative control. **(A-B)** DNA was stained with Hoechst 33352 (blue). Scale bars, 10 μm. Adapted from Jacob et *al.*, 2013.

A



B

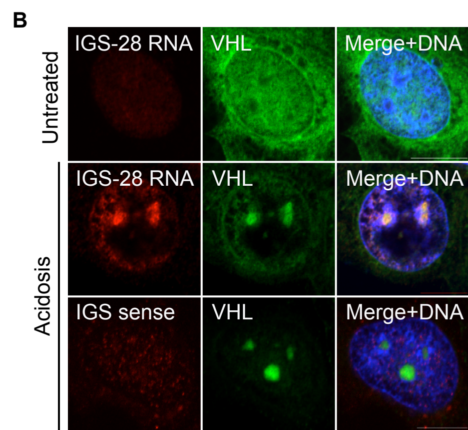
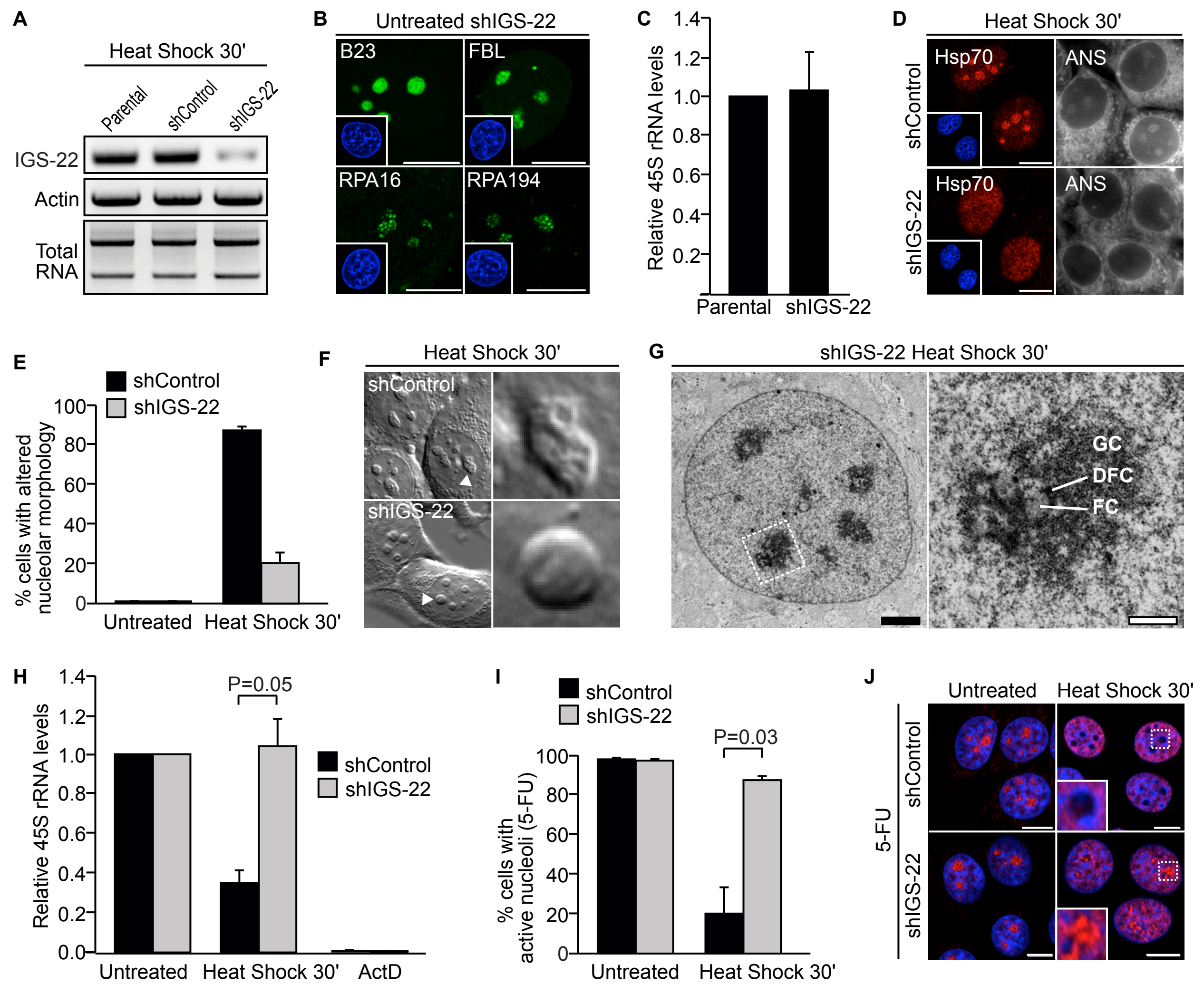


Figure 31. IGS lncRNA is required to remodel the nucleolus. (A) Parental and MCF-7 cells expressing shRNA targeted to a scrambled sequence (shControl) or IGS_{22}RNA (shIGS-22) were exposed to heat shock to induce transcription of the IGS_{22} locus prior to semi-quantitative RT-PCR. **(B)** Localization of EGFP-B23, EGFP-FBL, EGFP-RPA16 and EGFP-RPA194 in live untreated shIGS-22 cells. **(C)** Relative levels of 45S pre-rRNA in untreated parental and shIGS-22 cells measured by real-time reverse transcription PCR. **(D)** shControl and shIGS-22 cells were heat shocked for 30 min and either immunostained for Hsp70 or stained with ANS (live). **(E)** Quantification of untreated and heat shocked (30 min) shControl and shIGS-22 cells showing altered nucleolar morphology by DIC. **(F)** DIC images of live heat shocked (30 min) shControl and shIGS-22 cells. Indicated nucleoli are enlarged on right panel. **(G)** Transmission electron micrograph of an $shIGS_{22}$ cell after a 30 min heat shock treatment. Subnucleolar compartments (GC, DFC, FC) are indicated. Black scale bars, 2 μm; white scale bars, 0.5 μm. **(H)** Relative levels of 45S pre-rRNA in untreated and heat shocked (30 min) shControl and shIGS-22 cells measured by real-time reverse-transcription PCR. Actinomycin D (2 h, 50 nM) was used as a control for inhibition of RNA polymerase I-dependent transcription. **(I)** Quantification of untreated and heat shocked (30 min) shControl and shIGS-22 cells showing nucleolar incorporation of 5-FU. **(J)** *De novo* rRNA synthesis visualized by incorporation of 5-FU in untreated and heat shocked (30 min) shControl and shIGS-22 cells. **(B, D, J)** Cells were stained with Hoechst 33342 to visualize DNA (blue). Scale bar, 10 μm. **(C, E, H, I)** Columns, mean (n = 3); error bars, s.e.m. **(A-J)** Adapted from Jacob et *al.*, 2013.



A
Heat Shock 30'
Parental
shControl
shIGS-22
IGS-22
Actin
Total RNA
B
Untreated shIGS-22
B23
FBL
RPA16
RPA194
C
Relative 45S rRNA levels
Parental
shIGS-22
D
Heat Shock 30'
shControl
shIGS-22
Hsp70
ANS
E
shControl
shIGS-22
% cells with altered nucleolar morphology
Untreated
Heat Shock 30'
F
Heat Shock 30'
shControl
shIGS-22
G
shIGS-22 Heat Shock 30'
GC
DFC
FC
H
P=0.05
Relative 45S rRNA levels
shControl
shIGS-22
Untreated
Heat Shock 30'
ActD
I
shControl
shIGS-22
P=0.03
% cells with active nucleoli (5-FU)
Untreated
Heat Shock 30'
J
Untreated
Heat Shock 30'
5-FU
shControl
shIGS-22

treatment these cells did not acquire ANS-positive sub-nuclear structures **(Figure 31D)** and most of the nucleoli retained their original appearance by DIC imaging **(Figure 31, E and F).** These observations were supported by transmission electron micrographs that show that the majority of $shIGS_{22}$ cells retained their native nucleolar morphology, with clearly visible FCs, DFCs and GC **(Figure 31G)**. Finally, we tested whether an absence of reorganization would allow the nucleolus to retain its transcriptional activity during extracellular stimulation. Using qPCR analysis of the 45S pre-rRNA transcript, we found a 65% decrease in the levels of pre-rRNA in control cells within 30 min of heat shock treatment, but no changes in $shIGS_{22}$ cells **(Figure 31H)**. Consistently, 5-FU incorporation revealed that $shIGS_{22}$ cells were unable to fully repress nucleolar transcription during heat shock treatment, as 88% of shIGS22-expressing cells retained nucleolar incorporation upon treatment compared to 20% of the control cells **(Figure 31, I and J).** These data demonstrate the requirement for IGS RNA in both the structural and functional remodelling of the nucleolus. (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

3.3.10. Transiently expressed IGS lncRNAs induce the formation of ANS-positive structures

A notable property of IGS RNAs is their ability to immobilize proteins outside of the nucleolar environment when exogenously expressed (Audas et al., 2012a). We thus wanted to know if these molecules were sufficient to promote the formation of DC-like, ANS-positive structures in unstressed cells. IGS RNA transcripts expressed from a pcDNA vector partially localize outside of the nucleus and are therefore secluded

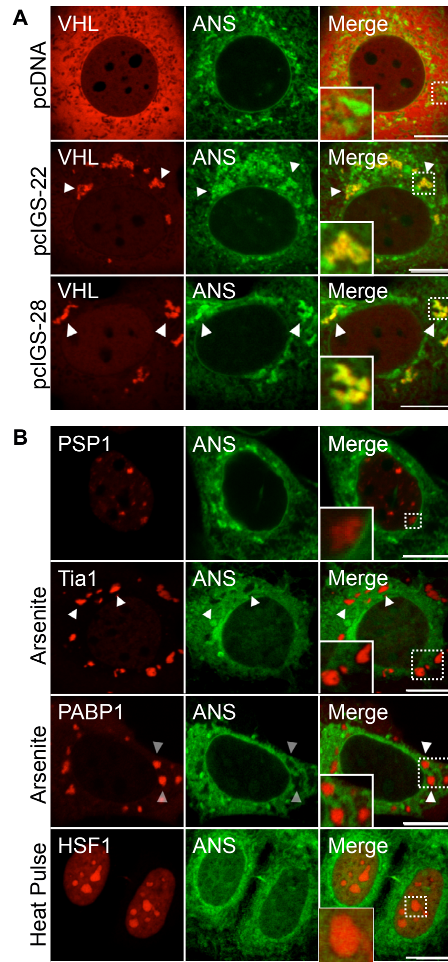
from the nucleolus. Using VHL as a marker, we observed that transiently expressed IGS_{22}RNA and IGS_{28}RNA were sufficient to induce the formation of large ANS-positive structures in the cytoplasm **(Figure 32A)**. These structures were distinct from other RNA-containing complexes. For instance, NEAT1 lncRNA-containing paraspeckles, indicated by the paraspeckles protein 1 (PSP1), were ANS-negative **(Figure 32B)**. Similarly, mRNA-containing stress granules, indicated by the polyadenylate-binding protein 1 (PABP1), Tia1 and heat shock factor 1 (HSF1) also excluded ANS. **(Figure 32B)**. These results highlight the architectural capabilities of IGS RNAs. Though these transcripts can operate autonomously, under physiological conditions their activity is contained within the nucleolus.

3.4. The nucleolar detention pathway is activated in tumours and tissues

3.4.1. POLD1 interacts with the IGS in mouse xenografts in an IGS lncRNA-dependent manner

Tumours regularly outgrow their vasculature and are often characterized by a hypoxic and acidotic core (Hockel and Vaupel, 2001; Jordan et al., 2007). To survive in this hostile environment, cancer cells must adapt by adjusting their metabolism. Given our findings on acidosis and sequestration, we asked whether nucleolar sequestration could be triggered by the tumour microenvironment. We performed xenograft studies by injecting shControl and $shIGS_{28}$ cells in the flank of nude mice and allowing for the formation of tumours. $shIGS_{28}$ cells formed tumours 5-fold larger than that of shControl cells, suggesting that an absence of acidosis-responsive IGS lncRNA promotes proliferation. To ensure that this dramatic effect was due to

Figure 32. Transient expression of IGS lncRNA induces the formation of ANS-positive structures. (A) Localization of VHL-GFP relative to ANS in cells expressing an empty pcDNA vector, pcIGS-22 or pcIGS28. **(B)** Localization of PSP1, Tia1 (arsenite treated), PABP1 (arsenite treated) and HSF1 (10 min heat pulse) relative to ANS. **(A, B)** Regions of interest (arrows, dotted squares) are enlarged (inset). Scale bar, 10 μm.



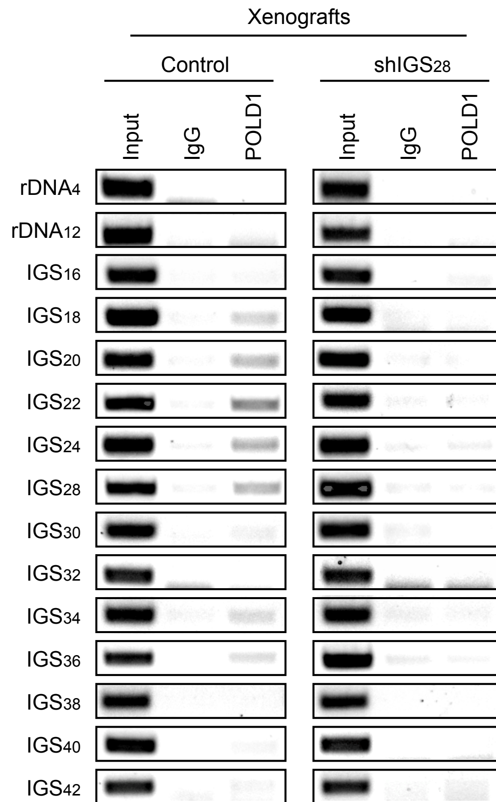
A
VHL
ANS
Merge
pcDNA
pcIGS-22
pcIGS-28
B
PSP1
Tia1
PABP1
HSF1
Arsenite
Heat Pulse

an absence of sequestration, we proceeded to perform ChIP on shControl and $shIGS_{28}$ xenografts. Results revealed that endogenous POLD1 interacted with the IGS of shControl cells, at the IGS_{18} to IGS_{28} loci **(Figure 33)**. In contrast, POLD1 did not interact with the IGS of $shIGS_{28}$ xenografts **(Figure 33)**. These results suggest that the tumour microenvironment induces the capture of NoDS-containing proteins on the IGS, in an IGS lncRNA-dependent fashion.

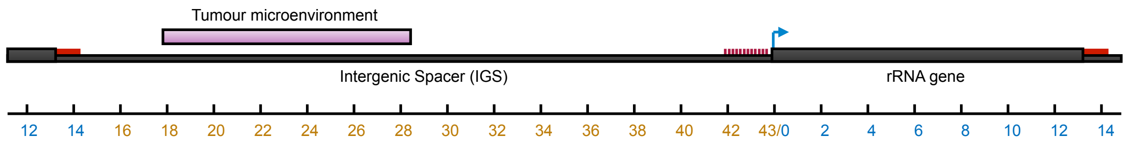
3.4.2. Select mouse tissues display ANS-positive nucleoli

Nucleolar sequestration can be induced in response to a wide range of stimuli, and is not limited to heat shock and acidosis. In fact, it is possible that differentiated and metabolically dormant tissues could exploit this pathway. To obtain insights as to the frequency of nucleolar sequestration in normal tissues, we sectioned a variety of mouse tissues and stained them with ANS. We found that some tissues, such as the lungs, displayed a staining pattern similar to untreated cultured cells: strong cytoplasmic signal and an absence of nuclear signal **(Figure 34)**. Other tissues, such as the spleen or the brain, were more similar to heat shocked and acidotic tissues in that they displayed strong nucleolar ANS in the majority cells **(Figure 34)**. These results suggest that select mouse tissues could employ nucleolar sequestration as a means to regulate their metabolism. Further experiments may validate these observations.

Figure 33. POLD1 interacts with the ribosomal intergenic spacer in an RNA-dependent manner in xenografts. Chromatin immunoprecipitation of POLD1 and a shControl (left) and an shIGS-28 (right) xenograft. A diagram of the ribosomal cassette, including the intergenic spacer, is provided (bottom).

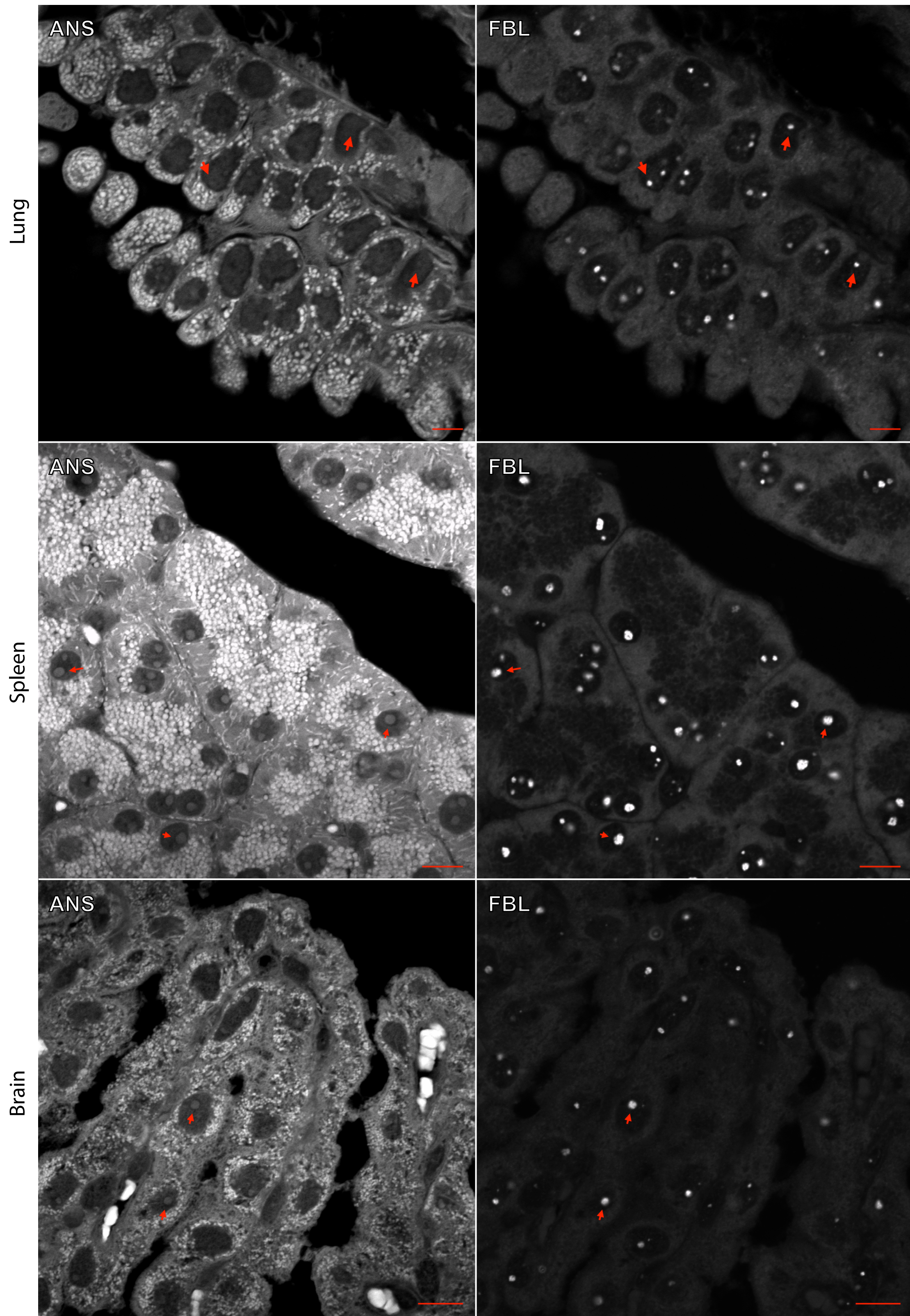


Xenografts
Control
shIGS28
Input
IgG
POLD1
Input
IgG
POLD1
rDNA4
rDNA12
IGS16
IGS18
IGS20
IGS22
IGS24
IGS28
IGS30
IGS32
IGS34
IGS36
IGS38
IGS40
IGS42



Tumour microenvironment
Intergenic Spacer (IGS)
rRNA gene
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
43/0
2
4
6
8
10
12
14

Figure 34. Select mouse tissues display ANS-positive nucleoli. Lung, spleen and brain mouse tissues stained with ANS (left) and immunostained against FBL (right). Scale bar, 10 μm. Nucleoli of interest are indicated with red arrow.


ANS
FBL
Lung
Spleen
Brain

4. Discussion

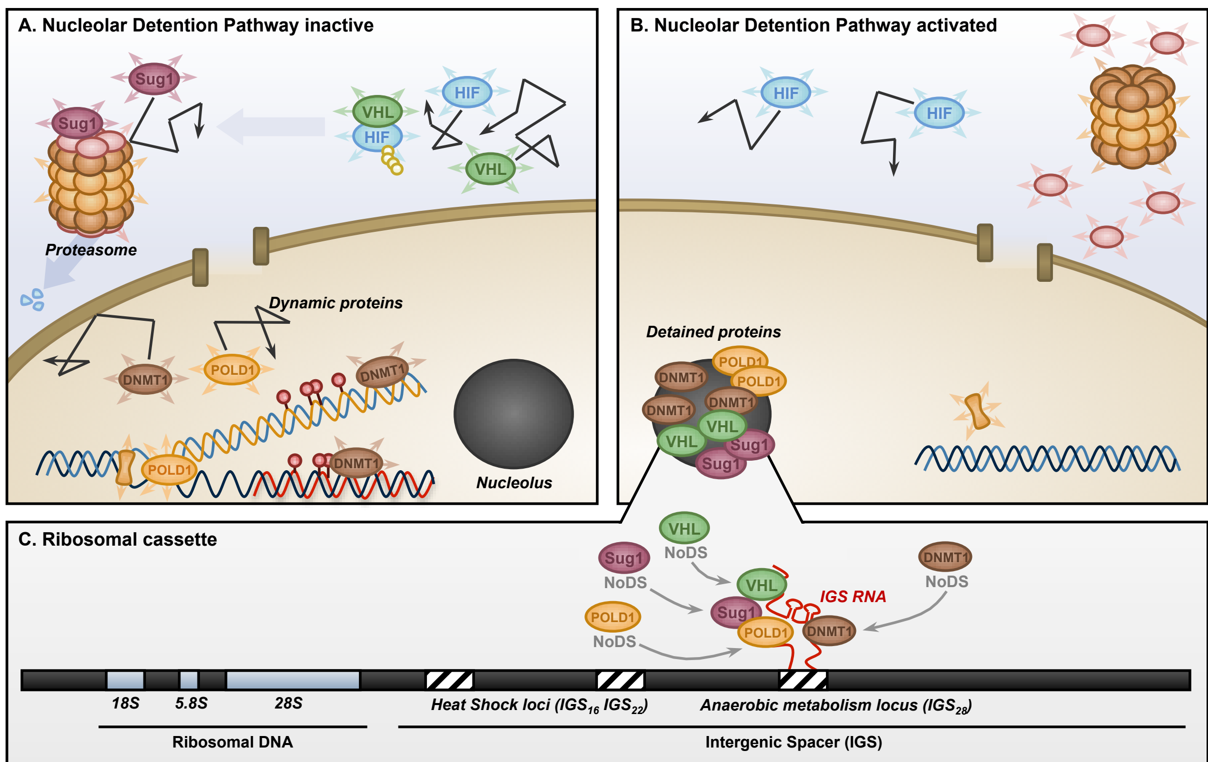
4. DISCUSSION

4.1. Summary of findings

In this thesis, we characterize a mechanism of post-translational gene regulation that is relevant to multiple physiological and stress conditions. We show that the intergenic spacer (IGS) of the ribosomal cassette is a complex transcriptional unit that contains multiple stimulus-specific sites of protein sequestration. We have shown that IGS_{28} is an acidosis-specific locus, whereas IGS_{16} and IGS_{22} are specific to heat shock. These loci are transcriptionally induced in response to their associated stimuli, and the resulting lncRNAs are responsible for the process of protein immobilization. Targeted proteins are characterized by the presence of a universal nucleolar detention signal (NoDS). This code is either contained within the targeted protein itself, or can be found in one of its binding partners **(Figure 35)**. The process of NoDS-protein sequestration on the IGS is not without consequences for the nucleolus. We show that the nucleolus readily alternates between two distinct and environment-dependent morphologies: a tripartite, transcriptionally active conformation under normal conditions, and a remodelled, transcriptionally inert conformation. The latter is characterized by the presence of the Detention Center, a region composed of immobilized proteins that is distinct from the other nucleolar compartments (FC, DFC and GC). These two architectures differ functionally as well as structurally. The former provides the cell with the output of rRNA required to sustain protein synthesis under growth conditions. The latter operates as a molecular prison, detaining cellular proteins away from their associated pathways.

Figure 35. Model: Regulation of protein mobility by IGS lncRNA. (A) Under normal conditions, NoDS-proteins are highly dynamic and maintain networks of functional interactions. Examples include Sug1 in the proteasome pathway, VHL in the degradation of HIF, POLD1 in DNA synthesis and DNMT1 in DNA methylation. **(B)** In responses to specific environmental stimuli (e.g. acidosis, heat shock), NoDS-proteins are captured and immobilized by the nucleolus. This loss of mobility prevents their interactions with downstream effectors and induces a collapse of the associated pathways. **(C)** Capture of NoDS-proteins by the nucleolus is mediated by stimulus-specific loci on the ribosomal intergenic spacer that induce the expression of lncRNA in response to their associated cues.

Adapted from Audas TE, Jacob MD, Lee S. 2012b.



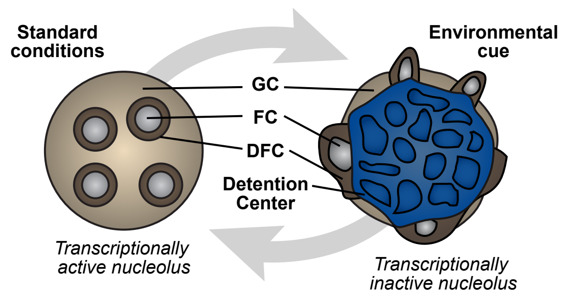
A. Nucleolar Detention Pathway inactive
Sug1
Sug1
VHL
HIF
HIF
VHL
Proteasome
Dynamic proteins
DNMT1
POLD1
DNMT1
POLD1
DNMT1
Nucleolus
B. Nucleolar Detention Pathway activated
HIF
HIF
Detained proteins
DNMT1
POLD1
POLD1
DNMT1
DNMT1
VHL
VHL
Sug1
Sug1
C. Ribosomal cassette
VHL
NoDS
Sug1
NoDS
DNMT1
NoDS
VHL
IGS RNA
Sug1
POLD1
NoDS
POLD1
DNMT1
18S
5.8S
28S
Heat Shock loci (IGS_{16} IGS_{22})
Anaerobic metabolism locus (IGS_{28})
Ribosomal DNA
Intergenic Spacer (IGS)

This transition is regulated by the stimulus-induced IGS lncRNAs, which operate as a macromolecular switch **(Figure 36)**. Through the inactivation of molecular networks and the interruption of rRNA synthesis, the remodelled nucleolus likely contributes to cell viability under conditions of stress (Audas et al., 2012b; Boulon et al., 2010; Mayer and Grummt, 2005; Olson, 2004). Together, our results provide a physiological rationale for the inherent plasticity of the nucleolar architecture.

4.2. A link between heat shock and acidosis

One of the first findings presented in this thesis is the unexpected parallel between heat shock in acidosis, as both stimuli induce the capture of proteins by IGS lncRNAs. Although some NoDS-proteins evade sequestration in heat shock (e.g. DNMT1), the majority of targets appear to be pluri-responsive, suggesting that the nucleolus inactivates the same pathways in response to both stresses. This is perhaps not surprising given that both hyperthermia and hypoxia lead to a drop in ATP levels (Lambowitz et al., 1983; Patriarca and Maresca, 1990) as well as to the activation of the unfolded protein response (Benjamin et al., 1990; Feldman et al., 2005; Ferriero et al., 1990; Giffard et al., 2004). In fact, much like hypoxia-acidosis, heat shock triggers an arrest in cell cycle progression and an interruption of growth and proliferation (Lindquist, 1980; Yost and Lindquist, 1986; Zeuthen, 1971). In light of these findings, it is tempting to speculate that nucleolar sequestration serves as a general response to physiological stress; one that allows the cell to rapidly depress its metabolism, interrupt energetically demanding processes and suspend cell cycle progression. This initial response would then be followed by a more

Figure 36. Model: Functional remodelling of the nucleolus. The nucleolus readily alternates between two distinct and environment-specific morphologies: a tripartite, transcriptionally active conformation and a remodelled, transcriptionally inert architecture. This transition is regulated by IGS lncRNAs. Adapted from Jacob *et al*. 2013.


Standard conditions
Environmental cue
GC
FC
DFC
Detention Center
Transcriptionally active nucleolus
Transcriptionally inactive nucleolus

tailored adaptation that would involve the expression or activation of specialized stress factors (Morimoto, 1993; Morimoto, 1998; Morimoto et al., 1992; Pirkkala et al., 2001; Trinklein et al., 2004).

4.3. The NoDS as a universal signal of protein sequestration

First identified by our group in 2007, the NoDS sequence was initially described as acidosis-specific (Mekhail et al., 2007). However, a number of additional proteins known to localize in the nucleolus under a variety of stresses also contain an NoDS. These include PML (Bernardi et al., 2004), hTERT (Wong et al., 2002), Plk-5 (Andrysik et al., 2010), Hsp70 (Welch and Feramisco, 1984), and TIP5 (Mayer et al., 2006), among others. The pluripartite nature of the NoDS makes its identification through mapping challenging, which is likely why it has not been reported for these proteins; although some groups have narrowed their search to a motif that contains it (Banski et al., 2010). Similarly, the two PML isoforms that are capable of localizing to the nucleolus are the ones that contain the full NoDS sequence (Condemine et al., 2007). This generality of the NoDS is supported by the results presented in this thesis. We have found that heat shock and ribosomal stress, two of the most commonly studied nucleolar stresses, rely on the NoDS sequence for the sequestration of proteins. Furthermore, most NoDS-containing proteins, such as VHL and Hsp70, respond to all stimuli equally. These findings, along with the fact that over 2000 protein-coding genes contain an NoDS, suggest that the nucleolar detention pathway is of central importance to the cell. The observation that several NoDS-proteins are stimulus-specific suggests that the cell is capable of tailoring its

response to stress. How some proteins, like VHL, are targeted to the nucleolus under multiple conditions whereas others, like DNMT1, respond exclusively to acidosis is unknown. Given the high variability that exists across the different NoDS sequences, it seems plausible that a "code within the code" could mediate specificity. In fact, unpublished data from our group demonstrate the arginine residues of VHL are methylated under standard conditions. This observation is particularly relevant in that arginine methylation is known to regulate subcellular localization through affinity to RNA (Bedford and Richard, 2005; Gary and Clarke, 1998; Liu and Dreyfuss, 1995; Smith et al., 2004). Future research may establish arginine methylation as the fine-tuner of the nucleolar detention pathway, and perhaps even identify the amino acid sequences that recruit the right methylases under the right conditions.

4.4. A functional role for intergenic DNA

98% of the human genome does not code for proteins, and has thus been historically referred to as "noncoding" or "junk" DNA. In fact, the function of these genomic regions is mostly unknown and represents one of the most fundamental mysteries in cellular biology. Recent collaborative efforts, grouped under the ENCODE (ENCyclopedia Of DNA Elements) project consortium, have proposed functions for that up to 80% of the noncoding genome (de Souza, 2012; Pennisi, 2012). Such functions include the recruitment of transcription factors and enhancers, the modulation of chromatin remodelling, the epigenetic regulation of RNA processing, the three dimensional organization of the genome, and of course the expression of

noncoding RNAs (Arvey et al., 2012; Djebali et al., 2012; Gerstein et al., 2012; Neph et al., 2012; Sanyal et al., 2012; Thurman et al., 2012). The results presented in this thesis contribute an importance piece to this puzzle. The IGS is the noncoding spacer region of the nucleolus, and as such has been assumed to be functionally inert. We show that the IGS is in fact a complex transcriptional unit that is capable of inducing the expression of several lncRNAs in response to specific environmental triggers. Not only are transcripts produced from the IGS, they perform an essential role by mediating the immobilization of over 2000 potential NoDS-proteins. Unpublished data from our group demonstrates that silencing IGS lncRNA expression in mouse xenografts prevents cell dormancy in the hypoxic tumor microenvironment and leads to much larger tumors (Audas *et al*. Manuscript in preparation). This remarkable phenotype illustrates the biological relevance of the IGS as a post-translational regulatory hub, and provides an unexpected function for intergenic DNA.

4.5. A family of lncRNAs

The IGS transcripts presented here differ from all other lncRNAs, and thus represent a distinct class of functional transcripts. Although other RNAs, such as NEAT1 and satellite III, are capable of targeting proteins to distinct subnuclear foci (Bond and Fox, 2009; Clemson et al., 2009; Jolly et al., 2004; Mao et al., 2011; Metz et al., 2004; Sasaki et al., 2009; Sunwoo et al., 2009), IGS lncRNAs are unique in their ability to immobilize proteins. Indeed, protein components of paraspeckles and other RNA-containing bodies are known to remain highly mobile (Bond and Fox, 2009; Fox et

al., 2002; Fox and Lamond, 2010). Furthermore, whereas other nucleating transcripts recruit primarily a select number of RNA transcription and processing factors, IGS lncRNAs target a much broader range of unrelated proteins, including VHL, POLD1, DNMT1, Hsp70, HSC70, RNF8, APC2 and AMPK (Audas et al., 2012a; Audas et al., 2012b). The exact mechanism by which IGS transcripts mediate the immobilization of these proteins is unclear, although it is known that they interact directly with NoDS-protein and induce a conformational change (Audas et al., 2012a). Another interesting feature of IGS RNAs is their processing. Both $IGS_{28}RNA$ and $IGS_{22}RNA$ are initially synthesized by RNA pol I as longer transcripts, which are then trimmed at both termini for form shorter, ~300 nt RNAs. $IGS_{22}RNA$ even undergoes an internal processing event that results in the excision of a long "intronic" region. The unprocessed variant of $IGS_{22}RNA$ is not functional, whereas the processed variant is (Audas et al., 2012a; Jacob et al., 2012). This observation suggests that a processing pathway is utilized in the nucleolus. This is unexpected given that the spliceosome is not operation in this region of the nucleus (Rino and Carmo-Fonseca, 2009; Will and Luhrmann, 2011). Therefore, it is likely that an alternate machinery is utilized, perhaps a variation from the rRNA processing pathway. Future research may provide valuable answers. The most remarkable property of IGS lncRNAs may be their architectural capabilities. The localized recruitment of proteins at IGS loci has tremendous structural and functional consequences for the nucleolus (Jacob et al., 2013). As discussed previously, not only do IGS lncRNAs mediate the *de novo* formation of a large cellular structure, they

induce a profound remodelling of the nucleolar architecture. Therefore, IGS lncRNAs are emerging as central players in the functional organization of the nucleus.

4.6. Additional levels of regulation

The ability of ectopically expressed IGS lncRNA to immobilize proteins in the cytoplasm of unstressed cells suggests that, at least in some settings, the simple presence of the transcript can be sufficient to mediate the formation of DC-like structures (Audas et al., 2012a). However, IGS transcripts are also present at basal levels in the nucleoli of untreated cells, yet they do not capture proteins and do not interfere with the normal organization of the nucleolus (Audas et al., 2012a; Jacob et al., 2013). This observation raises the possibility that additional levels of regulation may control the interaction between IGS lncRNA and NoDS-proteins. In fact, as mentioned above, arginine methylation is though to one of these mechanisms, as it could regulate the affinity of NoDS-proteins to the nucleolus. In addition to this post-translational pathway, the processing of IGS lncRNAs is also known to be of central importance. Since unprocessed $IGS_{22}RNA$ fails to immobilize proteins (Audas et al., 2012a), it appears that the post-transcriptional processing of this transcript serves as a method of activation. This would explain why RNA polymerase I can synthesize IGS lncRNA without being immediately captured. It is also possible that chaperon complexes prevent the aggregation of NoDS-proteins in non-permissive conditions. In fact, ectopically-induced VHL aggregates are immobile in the cytoplasm but mobile in the nucleus, suggesting that mechanisms may exist in the nucleus to repress the process of protein immobilization. These same mechanisms could be

inactivated in response to heat shock and acidosis, only to be re-induced upon signal termination. Finally, the questions of the detection of environmental cues and of the signaling cascades that synchronize the process of protein immobilization remain to be addressed.

4.7. The Detention Center as a cellular compartment

The cell is divided into organelles and dedicated subunits that assume specific functions. Mitochondria, for instance, are often described as "cellular power plants", for that they generate usable energy in the form of ATP. Ribosomes are likened to "factories" that produce the different proteins needed to sustain cellular biochemistry. The Golgi is considered a logistic hub that modifies, sorts and packages macromolecules for cell secretion. In the same way, the Detention Center is a "prison for proteins". It is a region of the cell that temporarily and reversibly holds proteins captive under periods of cellular stress. It is characterized by a distinct spatial localization, dynamic profile, protein composition, hydrophobic signature and cellular function. Although the DC is very obvious under the right circumstances (by DIC, TEM and immunofluorescence), it is an evasive compartment that is absent under normal culture conditions. This may be the reason why it has gone unnoticed, despite its functional significance. The ability of the DC to form rapidly, and in response to a transcriptional event, is one of its most defining properties. The induction of IGS lncRNA is the molecular switch that activates the assembly of the DC and the reorganization of the nucleolus that accompanies it.

4.8. The amyloid properties of the Detention Center

Amyloids are defined as insoluble, fibrous aggregates of misfolded proteins. They typically form in the extracellular space, when otherwise soluble proteins accumulate into insoluble structures, leading to cell death and tissue degeneration. In total, 18 different proteins have been linked to amyloid-associated pathologies, including PrP (prion protein) in spongiform encephalopathy and Aβ in Alzheimer's disease. All amyloid deposits share a common set of properties that are used for their clinical diagnosis: they are protease-resistant, and stain positive with planar aromatic dyes such as Thioflavin and Congo Red (Kelly, 1996; Kelly, 1998; Ramirez-Alvarado et al., 2000; Serpell et al., 1997). Surprisingly, DCs share some of these properties, since they stain positive with both Thioflavin S and Congo red. The nature of the interaction between NoDS-protein in the DC is unknown, notably the presence or absence of cross-beta sheet quaternary structures; though it is known that NoDS-proteins undergo a conformational change when sequestered (Audas et al., 2012a). Additionally, the requirement for hydrophobic motifs in the NoDS code suggests that hydrophobic associations could be at play. In fact, it is possible that immobilized NoDS-proteins could convert soluble NoDS-proteins to their insoluble conformation, in a prion-to-scrapie fashion. Such a model would provide an explanation the rapidity of the sequestration process, and would be consistent with reports that structured RNAs stimulate the prion-to-scrapie conversion process (Adler et al., 2003; Deleault et al., 2003). Ultimately, obtaining *in vitro* NoDS-protein complexes would be the method of choice to fully understand their structural properties of the DC, by crystallography and/or electron microscopy. Demonstrating

that DCs are homologous to amyloid deposits would be highly relevant medically, since we know that the cell has evolved mechanisms to rapidly disassemble them. Once identified and characterized, the molecular pathways involved in DC disassembly could be harnessed to treat pathological amyloid deposits.

4.9. A mechanism of ribosomal biogenesis regulation

The relocalization of several components of the ribosomal biogenesis machinery into the DC, including key subunits of the RNA polymerase I complex, represents a unexpected mechanism of regulation. As discussed previously, rRNA synthesis is generally thought to be controlled through transcription factors and chromatin remodelling complexes (Leary and Huang, 2001; Lempiainen and Shore, 2009; Santoro and Grummt, 2001). Even pRNA, a noncoding transcript originating from the promoter region of rDNA, functions by recruiting chromatin remodelling factors (Mayer et al., 2008; Mayer et al., 2006). Here, show that selected proteins can relocalize from one nucleolar compartment to another, and lose mobility in the process. In fact, at the molecular level, factors that interact with rDNA, such as RPA16 and RPA40, migrate from one region of the ribosomal cassette (rDNA) to another (IGS_{16}, IGS_{22} or IGS_{28}). In essence, the profound reorganization of the nucleolus described here could be the most dramatic method employed by the cell to inactivate, or reactivate, the production of ribosomal subunits.

4.10. Clinical relevance of nucleolar sequestration

Ongoing ribosomal biogenesis is essential to sustain the growth and proliferation of tumours (Lempiainen and Shore, 2009). In fact, rRNA synthesis is often elevated in cancer, and processes that promote this up-regulation have been described as oncogenic, whereas those that repress it are considered tumour suppressing (Arabi et al., 2005; Budde and Grummt, 1999; Dai and Lu, 2008; Grandori et al., 2005; Lempiainen and Shore, 2009; Montanaro et al., 2008; Montanaro et al., 2012; Sugimoto et al., 2003; van Riggelen et al., 2010; White, 2005). For this reason, enlarged nucleolar morphology is indicative of malignancy (Derenzini et al., 2000; Derenzini et al., 1998; Maggi and Weber, 2005), and the RNA pol I machinery is emerging as a therapeutic target for the treatment of cancer (Drygin et al., 2010). Interestingly, the nucleolar stresses described in this study are relevant to a variety of pathological conditions, including cancer. Extracellular acidosis, the outcome of anaerobic metabolism, is encountered both in the core of tumours (Engin et al., 1995; Tannock and Rotin, 1989), during development and in ischemic tissues, where it has been shown to have protective effects (Currin et al., 1991; Giffard et al., 1990a). Similarly, hyperthermia has been reported in some metabolically hyperactive tumours (Jayasundar and Singh, 2002) and, perhaps not coincidently, is also correlated with neuroprotection of ischemic brain tissues (Laptook et al., 1994; Thoresen et al., 1995). In light of these reports, it is tempting to speculate that the tumour microenvironment may inhibit cellular proliferation by inducing the nucleolar detention pathway. In fact, this model is supported by some of our most recent results. First, we have shown that NoDS-proteins (POLD1) interact with the

IGS in an IGS lncRNA-dependent manner within mouse xenografts. Furthermore, unpublished results from our group demonstrate that silencing IGS_{28}RNA inhibits cellular dormancy, and promotes the formation of tumours multiple fold larger (Audas *et al*. Manuscript in preparation). Therefore, it appears that IGS lncRNA play a tumour suppressing role within the tumour microenvironment. (Paragraph adapted from first author publication; Jacob *et al.*, 2013)

4.11. Concluding remarks

In conclusion, the data presented here demonstrate the existence of a family of lncRNAs that regulate the structure and function of the nucleolus. These transcripts function by immobilizing a variety of proteins within a compartment of the nucleolus, the detention center. Preliminary data from our lab suggest that this process is relevant to a variety of physiological processes, notably the regulation of proliferation within hypoxic tumours. Future research may link IGS lncRNA-dependent remodelling of the nucleolus to various physiological or pathological conditions.

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APPENDIX

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B. Publications

Environmental Cues Induce a Long Noncoding RNA-dependent Remodelling of the Nucleolus.
Jacob MD, Audas TE, Uniacke J, Trinkle-Mulcahy L, Lee S.
Mol Biol Cell. 2013. 24(18):2943-53

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Mol Cell. 2012. 45(2):147-57.