

**DNA methylation, cellular stress response and expression
of inner nuclear membrane proteins**

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ABSTRACT

Human diseases such as Hutchinson-Gilford Progeria Syndrome (HGPS) are classified as Laminopathies. Specifically, HGPS is described as a mutation within the lamin A gene giving rise to progerin a truncated protein which accumulates in the nucleus contributing to premature aging. Through a mechanism still poorly understood, this will affect the epigenetic control of the cell. Epigenetic control such as DNA methylation relies on DNA methyltransferase (DNMT) enzymes which usually results in transcriptional silencing of genes. Exposure to environmental stress such as heat shock implies modifications on cell structure and function induced for survival. Human cells display a stress-defense mechanism through the formation of ribonucleoprotein aggregates known as nuclear stress bodies (nSBs). During nSB assembly, heterochromatic silent satellite III sequences become actively transcribed into stable RNAs which associate with RNA-binding proteins including the serine-arginine rich (SR) protein SF2/ASF. The objectives of this study were to determine if cells displaying distinct epigenetic status (i.e.: varying states of DNA methylation) respond differently to environmental stresses. Moreover, to verify if nuclear proteins such as Lamin A and others are affected by similar factors. Five cell lines including four HCT116 clonal lines known to possess varying epigenetic status were examined by targeting SF2/ASF as a marker for nSB formation as well as other nuclear proteins to assess nuclear shape and integrity changes. Prior to heat shock, SF2/ASF is uniformly distributed within the nucleus. Western blot analysis reveals significant differences in the expression of SF2/ASF between cell lines before heat shock. However, following a one hour heat shock at 42°C we detect varying response coefficients (Rc) and numbers of nSBs between clones. In addition, a significant

reduction in SF2/ASF expression is noted. Together, results show that epigenetic modifications do not impede stress response. However, the extent of the response may be affected. In addition, results demonstrate that epigenetic modifications may not impede the functions of most examined nuclear antigens before and after HS. More evidence has come through detailing Lamin A is not the limiting factor in nuclear stiffness. In fact it is most likely the sum of interactions at the interface of the inner nuclear membrane and nuclear Lamina that results in nuclear strength.

RÉSUMÉ

Les maladies humaines telles que Hutchinson-Gilford Progeria Syndrome (HGPS) sont classées comme Laminopathies. Plus précisément, HGPS se décrit comme une mutation dans le gène de la lamine A donnant lieu à la progerin une protéine tronquée qui s'accumule dans le noyau et qui contribue à un vieillissement prématuré. Par l'entremise d'un mécanisme encore mal connu, cela affecte le contrôle épigénétique de la cellule. Le contrôle épigénétique tel que la méthylation de l'ADN est possible grâce aux enzymes ADN méthyltransférases (DNMT). L'activité de ces enzymes résulte habituellement en la répression transcriptionnelle des gènes. L'exposition à des stress environnementaux tel que le choc thermique (HS) implique également des modifications de la structure et des fonctions cellulaires nécessaires pour la survie. Les cellules humaines présentent un mécanisme de défense au stress par la formation d'agrégats ribonucléoprotéiques appelés corps de stress nucléaires (nSBs). Lors de l'assemblage nSBs, les séquences de type satellite III de l'hétérochromatine péricentromérique sont activement transcrites en ARN stable qui s'associe à des protéines liant l'ARN, y compris le facteur de transcription riche en sérine-arginine (SR) SF2/ASF. Les objectifs de cette étude étaient de déterminer si des cellules présentant un statut épigénétique distinct (ex: divers états de méthylation de l'ADN) réagissent différemment au HS. Nous voulions également vérifier si des protéines tel que la lamine A, entre autres, sont affectées. Cinq lignées cellulaires clonales dont quatre de la lignée HCT116 connues pour posséder divers états épigénétiques ont été examinées en ciblant SF2/ASF comme marqueur pour la formation des nSBs ainsi que d'autres protéines nucléaires pour évaluer la forme et un éventuel changement de l'intégrité du noyau. Avant le HS, SF2/ASF est uniformément répartie dans le noyau.

L'analyse par Western blot révèle des différences significatives dans l'expression de SF2/ASF entre les lignées cellulaires avant le HS. Cependant, suite à un choc d'une heure à 42 ° C, nous détectons divers coefficients de réponse (Rc). De plus, une réduction significative de l'expression de SF2/ASF est notée. En somme, les résultats montrent que les modifications épigénétiques ne gênent pas la réponse au stress. Toutefois, l'intensité de la réponse peut être affectée. Aussi, les résultats démontrent que les modifications épigénétiques ne font pas entrave aux fonctions de la plupart des antigènes nucléaires examinés avant et après HS. Nous apportons des preuves supplémentaires comme quoi l'expression réduite de la lamin A n'est pas le seul facteur limitant la rigidité nucléaire. En fait, il est plus probable que la somme des interactions à l'interface de la membrane nucléaire interne et la lamina permettent la rigidité et l'intégrité du noyau.

ABREVIATIONS

α-MEM	Alpha-minimum essential medium
BAF	Barrier to autointegration factor
CY3	Indocarbocyanine
DNA	Desoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate mix
ECL	Enhanced chemiluminescence
EDMD	Emery Dreifuss Muscular Dystrophy
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FCS	Fetal calf serum
FISH	Fluorescence <i>in-situ</i> hybridization
GCL	Germ cell less
HCT116	Human colorectal cancer cell line 116
HEPES	N- [2-Hydroxyethyl] piperazine-N'-[2-ethansulfonic acid]
HGPS	Hutchinson Gilford Progeria Syndrome
hpHS	Hours post heat shock
HS	Heat shock
INM	Inner nuclear membrane
kb	Kilobases
kDa	Kilodaltons
LAP2	Lamina-associated polypeptide 2
LBR	Lamin B receptor
LEM	<u>LAP2</u> , <u>Emerin</u> , <u>MAN 1</u>
M_r	Relative mobility
mRNA	Messenger RNA
NCBI	National center for biotechnology information
NE	Nuclear envelope
NOR	Nucleolar organizer region
NPC	Nuclear pore complex
nSB	Nuclear stress body
ONM	Outer nuclear membrane
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween
RNA	Ribonucleic acid
SatIII	Satellite III
SB	Sample Buffer
SDS	Sodium Dodecyl sulfate
SR	Serine-Arginine
SSC	Sodium chloride
UV	Ultra-violet

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I. INTRODUCTION

1. The nuclear envelope

The nucleus is delimited by the nuclear envelope (NE) comprised of a double lipid envelope which consists of the outer and inner nuclear membranes (ONM and INM) and separates the nucleoplasm from the cytoplasm (Gruenbaum *et al.*, 2000). This double envelope is punctured by ~120 000 kDa proteinic nuclear pore complexes (NPC) which periodically join the inner and outer membranes and are the sites of signal-mediated import and export of substrates through the NE (Grant and Wilson, 1997, King *et al.*, 2006). Moreover, it also comprises a perinuclear space located between the two membranes (Gruenbaum *et al.*, 2000). The outer membrane and the perinuclear space are in continuity with the rough endoplasmic reticulum (ER), which is covered with ribosomes (Gruenbaum *et al.*, 2000). This area, paired with the perinuclear space, is the site of protein translation and modification (Gruenbaum *et al.*, 2000). The INM is viewed as a specialized region of the ER where specific transmembrane proteins link the nuclear lamina which resides inside the nucleus (Georgatos *et al.*, 1994, Grant and Wilson, 1997, Tsuchiya, 2007). This structure provides a highly stable framework for the nuclear envelope and is the site of heterochromatin anchoring which occurs through DNA-binding proteins including Barrier-to-Autointegration-Factor (BAF) and other regulatory proteins (Tsuchiya, 2007; Mansharamani and Wilson, 2005; Delbarre *et al.*, 2006). It is composed of a fibrous meshwork of intermediate filament proteins including lamins A/C, B1 and B2 in mammals (Worman, 1988).

2. Inner nuclear membrane proteins

The number of proteins known to be specifically associated with the INM is rapidly increasing. This is mostly due to the fact that several genetic diseases have been associated with INM protein expression and conformation problems. All INM proteins possess at least one transmembrane domain and interact with nuclear lamins or DNA. In light of these interactions, INM proteins are thought to have an impact on chromatin organization and remodeling.

2.1 LEM bearing proteins

Numerous proteins are integral to the INM and can be targeted to study nuclear organization (Fig 1). The LEM proteins are great candidates for studying the interfaces flanked by the nuclear envelope and the nuclear lumen given their localization and most importantly since they varied widely in form and function and are found within most species from *C. elegans* to humans. LEM proteins compose a family of proteins bearing a 40-50 residue domain termed the LEM domain. This domain is appropriately defined as an acronym utilizing the first three proteins found to contain this domain at their N-terminus; Lamina-associated polypeptide 2 (LAP2) isoforms, emerin, and MAN1. LEM proteins are part of a group of proteins which serve to link the nuclear envelope to the chromatin through protein-protein interactions or protein-DNA interactions (Mansharamani and Wilson 2005; Lin *et al.*, 2000). Other members of the family include otefin (Wagner *et al.*, 2004), LEM-3 (Lee *et al.*, 2000 and Lee and Wilson, 2004), Bocksbeutel α and β isolated from *Drosophila* and others (Wagner *et al.*, 2004).

2.1.1 Lamina-Associated Polypeptides

The LAP2 isoforms (α , β , γ , δ , ϵ and ζ) initially described as thymopoietins, are products of alternative splicing, (Harris *et al.*, 1995; Berger *et al.* 1996). Consequently they have very similar structures belonging to the type II integral proteins of the INM. All isoforms possess one transmembrane domain; a second domain located at the N-terminus, which varies in length and possesses the LEM domain. This second domain serves as the functional area of the protein and is located in the nucleoplasm. Finally, all isoforms possess a short luminal C-terminus domain which does not appear to have any functional properties (Dechat *et al.*, 2000 and Lin *et al.*, 2000) (Fig 1). All LAP2 isoforms bind to chromatin either directly or through an intermediate protein termed Barrier to Auto-integration Factor (BAF) proposing an ability to regulate gene expression. This is achieved through two distinct mechanisms. First, BAF possesses the ability to bind the transcription repressor germ-cell less (GCL) and second, it is able to down regulate effects of the E2F-DP complex (Worman and Courvalin, 2000; Cai *et al.*, 2001 and Nili *et al.*, 2001). Conversely, LAP2 α and ζ lack the transmembrane domain thus are not integrated to the INM but are rather nucleoplasmic isoforms (Lin *et al.*, 2000). Moreover, a LEM-like domain is found between residues 1-88 on LAP2 isoforms allowing similar interactions as with the LEM domain i.e. chromatin and BAF (Lin *et al.*, 2000; Cai *et al.*, 2001). Finally, it is through Lamin binding that LAP2 isoforms distinguish themselves. LAP2 β and γ are capable of binding Lamin B1 linking it to the INM while LAP2 α will bind to Lamins A and C anchoring them to the nucleoplasmic interface of the INM (Foisner and Gerarce, 1993 and Dechat *et al.*, 2000).

2.1.2 Emerin

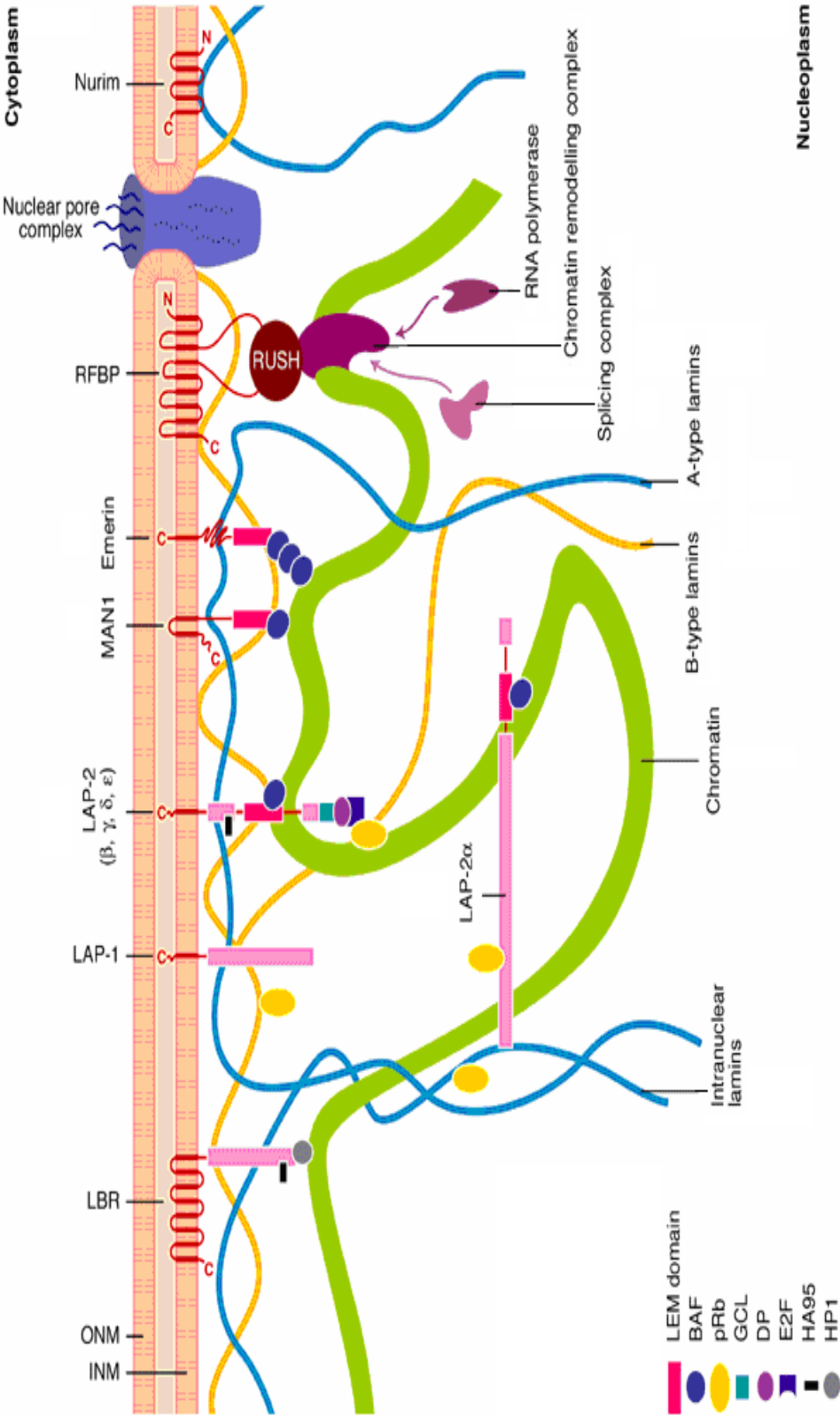
Emerin is an INM protein with a relative mobility (M_r) of 34 kDa comprising a single transmembrane domain (Manilal *et al.*, 1996). Similarly to the LAP2 isoforms, the functional amino-terminal LEM domain is found in the nucleoplasm. Moreover, emerin binds nuclear Lamins A and B through its middle region (Tews, 1999; Lin *et al.*, 2000, Shimi *et al.*, 2003) as well as BAF which once more competes with Germ cell less (GCL) (Holaska *et al.*, 2003). Thus emerin is found forming stable complexes with either Lamin A-BAF or Lamin A-GCL. Additionally, emerin was initially characterized as a missing protein in individuals who are affected by the X-linked form of Emery-Dreifuss muscular dystrophy (EDMD). This alternative form of the muscle weakening disease differs from the autosomal form in several aspects. Classically, the autosomal form of muscular dystrophy, is described as a mutation within the lamin A (LMNA) gene which codes for lamin A and Lamin C thus making it a Laminopathy (Worman, 2005; Mansharamani and Wilson, 2005). However, EDMD patients possess a mutation within the emerin gene that renders it absent from the INM. Moreover, microarray analysis has demonstrated significant up-regulation of 28 genes related to the loss of emerin (Tsukahara, T., 2002; reviewed by Bengtsson, L., 2007). In light of this discovery and of its several gene regulating binding partners, emerin has since been proposed to be an essential protein for normal development.

2.1.3 MAN 1

MAN1, a protein with an approximate M_r of 100 kDa, was found to be a target for an autoantibody isolated from the serum of a patient afflicted with a collagen vascular

disease (Lin, F., *et al.*, 2000; reviewed by Bengtsson, 2007). It possesses two transmembrane segments: an amino and a carboxy terminus, both facing the nucleoplasm (Worman, 2005; Worman, 2006; Lin, F., *et al.* 2000). The LEM domain at the amino-terminus of MAN1 interacts with Lamin A, Lamin B1 and emerin (Worman, 2005). Other interactions of MAN1 resemble those of emerin and include transcription regulators and other DNA-binding proteins such as BAF (Worman, 2005; Mansharamani and Wilson, 2005; Bengtsson, L., 2007). It has been proposed that MAN1 and emerin share certain functions; however it remains unclear whether a redundancy exists (Bengtsson, L., 2007). Recent publications elaborate newfound possible roles for MAN1. There is evidence showing that MAN1 is able to antagonize transforming growth factor β /bone morphogenic protein (TGF β /BMP) known as pleiotropic cytokines which possess several different functions, mainly: the stimulation and/or inhibition of cell proliferation; the synthesis and/or disintegration of extra-cellular matrices and epithelial and/or mesenchymal interactions during embryogenesis as well as during wound healing. (Osada, S., *et al.*, 2003; Raju, G.P., *et al.*, 2003; Hellemans, J., *et al.*, 2004; Lin, F., *et al.* 2005; Pan, D., *et al.*, 2005; Reviewed by Bengtsson, L., 2007). Moreover, MAN1 binds regulatory Smads (R-Smad) including Smad 1-5 and Smad 8 which in response to the binding of TGF β or BMP, will be phosphorylated and bind to Smad 4 a co-Smad acting as transcription factors for other genes (Derynck, R., *et al.*, 1998; Massagué, J., *et al.*, 2005). In short, deregulation of MAN 1 within the INM leads to several developmentally related illnesses, cancers, fibrosis and immune deficient disorders.

Figure 1: Schematization of the NE delimiting the nucleus with a simplified version of the proteinaceous nuclear pore complex (NPC). Major proteins such as BAF, chromosomal factor; E2F, transcription factor; germ-cell less (GCL), transcription factor; heterochromatin protein 1 (HP1), inner nuclear membrane protein; Lamina associated protein 1 and 2 (LAP1,2), emerin and MAN 1 making up the initial LEM domain protein family, Lamin A, Lamin B and retinoblastoma protein pRB, functioning within the INM are represented (from: Maidment and Ellis, 2002).



2.2 The nuclear lamina

There are four nuclear lamins in mammals: lamins A (72 kDa), C (64 kDa), B1 and B2 (both 67 kDa) (Worman *et al.*, 1988). All four lamins form the nuclear Lamina which gives the nucleus its strength and support (Worman *et al.*, 1988). Lamins A and C are expressed in differentiated cells as alternative splicing products from a single gene termed *LMNA*. Lamins B1 and B2 are expressed in all cells from two genes *LMNB1* and *LMNB2* (Worman and Bonne, 2007; Delbarre *et al.*, 2006). Lamins are partly responsible for nuclear dynamics. It was recently discovered that lamins A and C are largely responsible for nuclear stiffness (Lammerding *et al.*, 2006). However, B type lamins are reported to be regulators of nuclear integrity but not of stiffness (Delbarre *et al.*, 2006, Lammerding *et al.* 2006). lamin B has also been shown to form microdomains that have the ability to bind epigenetically marked DNA and physically link it to the nuclear Lamina (Makatsori *et al.*, 2004). Lastly, new evidence has recently been reported that lamins themselves through their interactions with INM and other nuclear proteins or lack thereof, can greatly affect epigenetically controlled pathways within the nucleus (Vlcek and Foisner, 2007).

2.3 Other nuclear proteins

2.3.1 2H12

2H12 was first described as a protein with a M_r of 38-40 kDa human isoelectric variant of B23, a nucleolar protein with cell cycle dependent expression levels (Paulin-Levasseur, M. *et al.*, 1995). Unlike 2H12 which is believed to be expressed at similar levels throughout the cell cycle, B23 is most prominent during interphase and found to have

very strong affinity for the nuclear matrix as well as having shuttling properties between the nucleus and the cytoplasm (Paulin-Levasseur, M. *et al.*, 1995). Conversely, 2H12 was shown through human-mouse heterokaryons to remain tightly bound to nucleoli, the site of ribosomal RNA (rRNA) gene transcription, during interphase but diffused in the cytoplasm at other key points of the cell cycle (Paulin-Levasseur, M. *et al.*, 1995). Further investigation of this finding by Paulin-Levasseur *et al.* (1995) demonstrated a phosphorylation dependent delocalization of the 2H12 antigen from the nucleolar matrix. Functional analysis through a series of one-dimensional and two dimensional immunoblots as well as localization and co-localization of the 2H12 antigen with other known nucleolar components has demonstrated a highly probable role in nucleolar or karyoskeletal architecture maintenance during interphase and retention of key functional molecules within the ribonucleoprotein complexes.

2.3.2 Fibrillarin

An autoimmune serum from a patient with scleroderma revealed in 1985 a novel protein with a M_r of 34 kDa found exclusively in both the dense fibrillar and fibrillar center of the fibrillar region of the nucleolus recognized as the largest transcription factory as well as vital for the synthesis of ribosomal subunits (Ochs, R. L. *et al.*, 1985; Bartova, E. *et al.*, 2010). More specifically, fibrillarin was found to localize on chromosomal nucleolar organizer regions (NORs) during metaphase and anaphase and also reported to be a marker of newly forming nucleoli in telophase (Ochs, R. L. *et al.*, 1985). Moreover, similarly to B23 and the later 2H12 antigen, fibrillarin has been shown through RNase and DNase assays to be a common non-histone protein possessing a structural and/or

functional role within nucleoli (Ochs, R. L. *et al.*, 1985). More recent publications pertaining to the function of this protein relates to the small nuclear RNA components U3, U8 and U13 (Nicol *et al.*, 2000; reviewed by Bartova, E., *et al.*, 2010) which suggests an important implication in the processing of pre rRNA.

3. Epigenetic control through DNA methylation

Epigenetic control is a process that allows cells to undergo different biochemical pathways without altering the genetic code. More specifically, it allows for heritable changes in gene function to occur without changing DNA sequences (Lomberk, 2007). DNA methylation is a basic form of epigenetic control, which relies on the enzyme DNA methyltransferase (DNMT) (Karpf and Matsui, 2005; Rhee *et al.*, 2000). In mammals, DNMT will add a methyl group to carbon 5 of cytosine residues found in CpG islands, transcriptionally silencing regions of the genome (Croas and Bird, 1995; Rhee *et al.*, 2000). The silencing occurs in two different but related events. First, methylation sterically hinders DNA-binding proteins required for transcription; and second, the recruitment of histone deacetylases and chromatin remodeling complexes will result in euchromatin compacting into heterochromatin (Croas and Bird, 1995; Bird and Wolffe, 1999).

3.1 Mechanism of gene silencing through DNA methylation

The majority of cellular DNA methylation is established and maintained by two subtypes of DNMT enzymes. The establishment of DNA methylation patterns is assured by the *de novo* DNMT3b during embryonic development (Rhee *et al.*, 2000; Rhee *et al.*,

2002). Maintenance DNMT1 preferentially methylates CG sequences that are base-paired to GC sequences which are already methylated (Fig. 2). Other, less common DNMT enzymes such as DNMT3a and 3L which have a minimal role in *de novo* DNA methylation and DNMT2 which methylates RNA sequences are also found within the nucleus (Goll *et al.*, 2006; Jia *et al.*, 2007). Since methylation is restricted to cytosine nucleotides found in CG sequences, which are base-paired to the identical sequence (in the opposite orientation) on the complementary DNA strand, DNA methylation patterns can be inherited by daughter DNA strands during semi-conservative DNA replication (Fig. 2) (Croas and Bird, 1995; Bird and Wolffe, 1999). Moreover, methylation is implicated in embryonic gene regulation. This process dictates which genes are activated during differentiation to generate a committed cell. DNA methylation is therefore essential for normal development and cell function (Bird, 1999; Oligny, 2001; Paulen and Ferguson-Smith, 2001).

Recently, a series of human colorectal cancer cell lines (HCT116) were generated at the John Hopkins School of Medicine (Rhee *et al.* 2000; Rhee *et al.*, 2002) displaying various genotypes pertaining to their ability to methylate DNA. More specifically, a parental or unmodified cell line identified as HCT116#28 was used to generate the other cell lines. The remaining three clones possess two types of deletions or alterations. First HCT116 #38 has a DNMT3b deletion (*DNMT3b*^{-/-}). This type of mutation is known to reduce global methylation by less than 3% (Rhee *et al.*, 2002). HCT116 #30 initially reported to have a DNMT1 knockout only displayed an approximately 20% reduction in total DNA methylation, mainly in pericentromeric regions such as the 9q12 region where satellite III sequences are found (Rhee *et al.*, 2002; Espada *et al.*, 2004; Espada *et al.*,

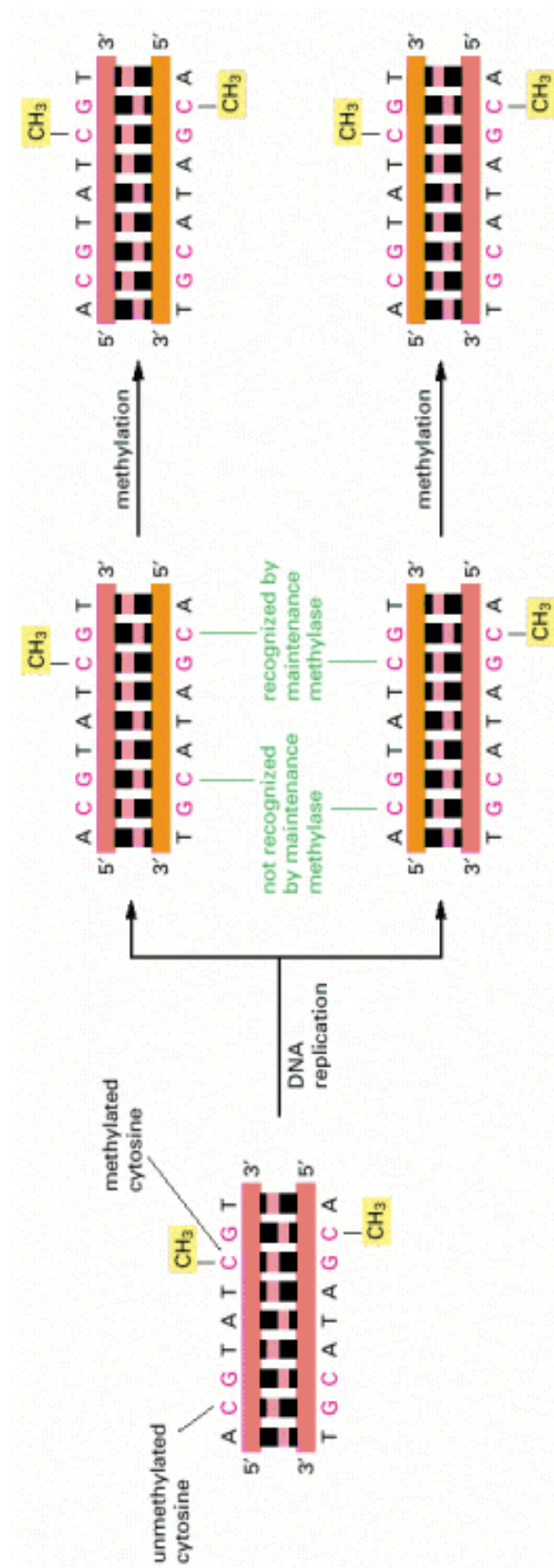
2007). However further studies based on the widely accepted hypothesis stating that DNMT1 is essential for survival, revealed HCT116#30 was not a knockout cell line but rather an alternative form of knockdown known as a hypomorph which expresses a low level of functional truncated DNMT1 (Egger, G., *et al.*, 2006). This mutation was also shown to result in nucleolar architecture disruption (Espada *et al.*, 2004; Espada *et al.*, 2007). Unexpectedly, a double mutation ($DNMT3b^{-/-}$ and $DNMT1^{+/-}$) in HCT116#66 reduced global methylation by ~ 95% (Rhee *et al.*, 2002). This suggests a synergistic effect between the two enzymes in the epigenetic control of genes (Rhee *et al.*, 2002).

4. Nuclear stress response in mammalian cells

4.1 Nuclear stress bodies

Nuclear stress bodies (nSBs) are a category of nuclear structures which appear in the nucleus of any human cell type under stress (Biamonti, 2004). The role of the nSB is still not fully understood. However, we know that these structures arise from the exposure to a variety of physical and chemical stresses (Chiodi *et al.*, 2004). Such conditions will eventually profoundly affect the expression program of genes and the core organization of the cell (Chiodi *et al.*, 2004). In order to resist deleterious effects, cells will activate the expression of heat-shock proteins (HSP), molecular chaperones that will protect against the accumulation of non-native proteins (Chiodi *et al.*, 2004). It is thus a mechanism of defense.

Figure 2: Faithful inheritance mechanism of DNA methylation patterns. In vertebrate DNA, a large fraction of the cytosine nucleotides in the sequence CG are methylated. Because of the existence of a methyl-directed methylating enzyme (the maintenance methyltransferase), once a pattern of DNA methylation is established, each site of methylation is inherited in the progeny DNA as shown. (Reproduced from Alberts, 2004)



4.2 Mechanism of stress body formation

Time-lapse microscopy has shown that short (1h) heat shocks at 42°C, separated by 1 hour recovery periods at 37°C, induce the formation of nSBs recurring in the same nuclear position time and again (Jolly *et al.*, 1997; Jolly *et al.* 1999). In fact, nSBs assemble near nucleoli or the nuclear membrane on chromosomes 9, 12 and 15, for instance where the satellite III locus on the 9q12 region of the genome is found comprising the main site of nSB formation (Denigri *et al.*, 2001; Denigri *et al.*, 2002; Biamonti 2004; Jolly *et al.*, 2002; Metz *et al.*, 2004). The assembly of nSBs has recently been extensively studied in various contexts. Upon administration of a form of stress, Heat Shock Factor 1 (HSF1), a cytoplasmic chaperone protein, trimerizes (Metz *et al.*, 2004). The trimerized complex will then re-localize to the nucleus to bind the promoter region of HSPs leading to their expression (Metz *et al.*, 2004). Interestingly, HSF1 will also bind to the satellite III regions throughout the genome leading to the transcription of stable satellite III RNAs which will remain associated with this region for several hours during recovery (Denigri *et al.*, 2001; Biamonti, 2004; Metz *et al.*, 2004). Non-coding satellite III RNA transcripts are reported to be essential for assembly of nSBs (Biamonti, 2004; Metz *et al.*, 2004). Lastly, it has been shown through immunofluorescence microscopy that the recruitment of the serine/arginine-rich (SR) splicing factor protein SF2/ASF (~33kDa) to the nSBs requires the presence of stress-induced satellite III transcripts (Metz *et al.*, 2004). In fact, targeting of SF2/ASF to the nSB is dependent on its second RNA Recognition Motif (RRM2) (Metz *et al.*, 2004; Chiodi *et al.*, 2004).

Interestingly, individuals who suffer from Hutchinson-Gilford Progeria syndrome (HGPS) have a deleterious mutation in their LMNA gene (Worman, 2005). LA Δ 50 is the resulting protein with a deletion of 50 amino acids (Shumaker *et al.*, 2006). The mutation leads to abnormally shaped nuclei due to the accumulation of LA Δ 50 within the nuclear Lamina, the pathological hallmark of Progeria (Shumaker *et al.*, 2006). As Lamin A is largely responsible for nuclear and cellular mechanics, it is of no surprise to find that a truncated Lamin A leads to a Laminopathy such as Progeria (Lammerding *et al.*, 2006). HGPS is defined as a premature aging disease illustrated by hair loss, growth retardation (average height: 109 cm; average weight: 14.5 kg), lack of subcutaneous fat, aged-looking skin, osteoporosis, arteriosclerosis, and high risk of stroke (Shumaker *et al.*, 2006). Additionally, HPGS cells exhibit a down-regulation of DNMT activity and a subsequent loss of pericentromeric constitutive heterochromatin; as well as histone methyltransferase activity (Paradisi *et al.*, 2005; Shumaker *et al.*, 2006). This is in fact the root cause of misshapen nuclei (Espada *et al.*, 2004). As observed in heat shock response, non heat shocked HPSG cells show an up-regulation of pericentromeric satellite III transcripts observed within nuclear structures due to loss of heterochromatin (Shumaker *et al.*, 2006). These alterations can be detected prior to the changes in nuclear shape (Shumaker *et al.*, 2006). Also, HPGS cells are reported to be very sensitive to heat stress as they take a very long time to recover (Shumaker *et al.*, 2006). Lastly, conditions leading to the formation of nSB have deleterious effects on the cell. Premature aging in HGPS is proposed to be caused by the accumulation of Progerin i.e. LA Δ 50 within the Lamina engaging a relentless “stressed” state of these cells (Shumaker *et al.*, 2006; Russo-Menna, I., and Arancibias, C., 2009).

5. Objectives

The main focus of my research was to study the effects of epigenetic variations on environmental stimuli response in a premature aging disease context such as HGPS. This was achieved taking advantage of four human colorectal tumor cell lines (HCT116) characterized by different states of DNMT activity, thus distinct DNA methylation patterns, mimicking the loss of DNA methylation and nuclear disruption displayed in HGPS cells without the negative effects of a truncated Lamin A protein. The first objective of my study was to determine if cells displaying distinct epigenetic status will respond differently to stress (i.e. heat shock response). This was determined via the induction of cellular stress through a 1 hour heat shock treatment.

The number and localization of nSBs was assessed through immunofluorescence microscopy and compared between HCT116 clones and HeLa control using the SF2/ASF splicing factor as a marker for nSB formation. Since I did not expect to observe nSBs before HS, the distribution of the marker within untreated cells provided a base for comparison. These experiments were accompanied by semi-quantitative Western blotting to detect and compare the expression levels of SF2/ASF between the different clones before and after HS. This goal was achieved by answering the following questions. What is the distribution of the SF2/ASF splicing factor in the different clones before and after HS? How does nSB formation vary between the clones in number, morphology and localization? What is the degree of variation in the expression level of SF2/ASF between clones before and after HS?

As described earlier, the recruitment of satellite III transcripts is a necessary step in the formation of the nSB (Metz *et al.*, 2004). These transcripts remain segregated within nuclear domains for an extended period of time after heat shock. As such, the presence of satellite III transcripts within nuclear aggregates of SF2/ASF served as proof the different clones respond to stress by formation of nSBs. This part of my project was performed using RNA-fluorescent *in-situ* hybridization (RNA-FISH) to localize the transcripts. Data variations between clones and control were analyzed. Here we answered the following question. What is the distribution of satellite III transcripts in the different clones before and after HS?

The second objective of my project is to determine if the composition and organization of INM proteins are affected by the epigenetic status of cells. Moreover, is the expression of INM proteins changed during stress response and are these changes attributable to epigenetic distinctions? As can be expected, modified DNA methylation patterns should result in different gene expression patterns. The INM proteins described in the introduction interact at various levels with the nuclear Lamina and chromatin itself. Moreover, the high response of nucleoli to external stimuli (Bartova, E. *et al.*, 2010) pushed us to also examine internal nucleoli markers such as fibrillarin and 2H12. Modifications in protein composition or organization would certainly imply further consequences on the DNA itself. Therefore, protein expression levels were quantified through Western blots before and after HS. In addition, protein distribution was assessed through immunofluorescence microscopy before and after HS. The data collected was analyzed and compared between clones and control. The specific questions addressed in

this part of our study include. What are the expression levels of the INM proteins before and after HS? How do the levels of INM protein expression vary between clones before and after HS? What are the main differences between the clones in terms of INM protein expression during stress response? What is the distribution of INM proteins before and after HS? How is the distribution of INM proteins affected between clones before and after HS? What are the main differences in INM protein distribution between the clones in terms stress response?

Currently, there has not been any literature published on the effects of epigenetic variations on HS response in HCT116 clones. Moreover, the apparent link existing between epigenetic markers and various illnesses such as Hutchinson Gilford Progeria Syndrome (HGPS) is a great incentive for such a project. Therefore, the initial steps of the study were dedicated to establishing observations pertaining to epigenetic variability and HS response.

II. MATERIALS AND METHODS

1. Materials

General chemical supplies were purchased from BDH (Ville St-Laurent, Québec, Canada), Invitrogen Canada Inc (Burlington, ON, Canada) and Sigma Aldrich (St Louis, MO, USA).

2. Cell culture

HeLa cells and HCT116 clones were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Dr. Bert Vogelstein (Howard Hughes Medical Institute, MD, USA) respectively. HeLa cells were cultured in α -minimum essential medium (α -MEM; Gibco BRL, Burlington, ON, Canada) supplemented with 10% fetal calf serum (FCS; Gibco BRL) and antibiotics (50 U/mL penicillin and 50 μ g/mL streptomycin; Gibco BRL). HCT116 28, 30, 38 and 66 were derived from a human colorectal tumor (Rhee *et al.*, 2000 and Rhee *et al.*, 2002) and cultured in McCoy's 5A medium modified with L-glutamine (Gibco BRL) supplemented with 10% FCS and antibiotics. Clone 30 was selected for resistance to hygromycin by supplementing the medium with 0.1 mg/mL hygromycin B (Invitrogen). All five epithelial monolayer cultures were grown in a humid chamber at 37°C in 5% CO₂ and 95% O₂ atmosphere.

3. Heat Shock

Cell monolayers were incubated for 1 hour at 42°C in a Precision water bath (model 183; Precision Scientific Inc., Chicago, IL USA) with pre-heated complete medium supplemented with 10mM N- [2-hydroxyethyl] piperazine-N'-[2-ethansulfonic acid] (HEPES, BDH) at pH 7.0 and allowed to recover for different times as later detailed within the text in order to evaluate the recovery process on the different clones.

4. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis and immunoblotting

Subsequent to defined treatments, attached cells were washed with tissue culture specific phosphate buffered saline (TC-PBS 1X; 68.44mM NaCl, 1.34mM KCl, 40.4mM Na₂HPO₄, 0.73mM KH₂PO₄) at pH 7.4, trypsinized (0.025% trypsin and 1mM ethylenediamine tetraacetic acid (EDTA) in PBS 1X) for 10 min at 37°C and transferred to centrifuge tubes. Cells were harvested by centrifugation at 1,500g for 5 min. Proteins were then solubilized in sodium dodecyl sulfate (SDS; BIO-RAD Laboratories, Mississauga, ON, Canada) 2X sample buffer (SB; 2% SDS, 10% glycerol, 10mM Tris pH 6.8, 25mM β-mercaptoethanol and 0.005% bromophenol blue) (Laemmli, 1970) in a ratio of 3.0x10⁶ cells/mL. Ten µL were loaded on the gel for analysis by SDS-polyacrylamide gel electrophoresis (PAGE) (5% acrylamide for the stacking gel and 12% acrylamide for the resolving gel) using a BIO-RAD minigel apparatus.

Protein profiles of cells were visualized by Coomassie Blue staining (0.1% Coomassie Blue, 10% acetic acid and 25% methanol) or were electrophoretically transferred to nitrocellulose membranes (Whatman–GE Healthcare, Baie d'Urfé, Québec,

Canada) tested in Ponceau red and processed for immunoblotting as recommended in the Western Blotting Kit (Amersham Canada, Oakville, ON, Canada). Membranes were incubated in 5% milk in PBS-Tween 20 (PBS 1X; 130mM NaCl, 5mM Na₂HPO₄, 1.5mM KH₂PO₄, PBST; 1:200) for 1h followed by: 1) three times 10 min washes in PBST; 2) incubation with primary antibodies in 5% milk/PBST for 1h; 3) three times 10mins washes; and 4) incubation with an appropriate biotinylated secondary antibody in 5% milk/PBST for 1h. Following three times 10min washes with PBST, immunoblots were incubated with streptavidin-conjugated to horseradish peroxidase (1:9 000: Amersham, Canada) for 1h. Blots were developed using the enhanced chemiluminescence (ECL) kit (Amersham Canada) and reactivity was visualized on Hyperfilm ECL (Amersham Canada). Alternatively, blots were processed using the Western-SuperStar™ Immunodetection System (Applied Biosystems, Foster City, CA, USA). Membranes were incubated in Western-SuperStar Blocker for 1h, then with primary antibodies in Western-SuperStar Blocker for 1h, followed by three times 5 min washes in Western-SuperStar Wash Buffer and incubation with the provided appropriate secondary antibody in Western-SuperStar Blocker for 1h. Following two times 5min and two times 2min washes with Western-SuperStar Wash Buffer and Western-SuperStar Assay Buffer respectively, immunoblots were incubated in a solution of 150µL Nitro-Block-II Enhancer and 3mL of Western-SuperStar Substrate for 5min and finally were blotted dry. Reactivity was visualized on Hyperfilm ECL (Amersham).

5. Indirect immunofluorescence staining

HeLa cells and HCT116 clones grown on coverslips were fixed, permeabilized and stained as previously described (Chaly *et al.*, 1984). Fixation was performed with 4% paraformaldehyde (PFA) (MERCK KGaA, Darmstadt, Germany) in PBS 1X for 5 minutes and cells were then washed several times with PBS 1X. Aldehydes were reduced with sodium borohydride (1mg/ml in PBS 1X; BDH) 3 times for 4 minutes each. Cell membrane was permeabilized in 0.2% Triton X-100 (BDH) in PBS 1X for 20 min and cells were washed several times with PBS 1X. Antibody incubations were done at room temperature for 1 hour. DNA staining occurred in bisbenzimidazole 33258 (Hoechst 33258; 1mg/mL; Sigma Aldrich).

6. Fluorescence *in situ* hybridization

Cells grown on coverslips were fixed with 3% (PFA) (MERCK KGaA, Darmstadt, Germany) in PBS 1X for 5 min. Cells were then washed several times with PBS 1X. Aldehydes were reduced with sodium borohydride (1mg/ml in PBS 1X; BDH) 3 times for 4 minutes each. Cell membrane was permeabilized in 0.2% Triton X-100 (BDH) in PBS 1X for 20 minutes and cells were washed several times in PBS 1X and 2X SSC. Coverslips were incubated overnight in hybridization buffer (2X SSC, 5X Denhardt's solution (Sigma-Aldrich), 25% formamide and 1µg/mL yeast tRNA (Sigma-Aldrich)) containing 5ng of the appropriate Dig-labelled probe (Invitrogen). Coverslips were washed twice in 2X SSC and PBS 1X, incubated in a secondary anti-Dig-fluorescein antibody and counterstained to reveal the SF2/ASF protein as described above. Staining of DNA occurred in bisbenzimidazole 33258 (Hoechst 33258; 1mg/mL; Sigma Aldrich). A

total of 3 RNA directed probes (forward, reverse and control) were utilized as described by Rizzi *et al.* (2004).

7. Antibodies

Primary antibodies used in this study are: 1) anti-SF2/ASF (ZYMED, Carlsbad, CA, USA) at a dilution of 1:500 for immunofluorescence and for blotting; 2) anti-actin (Cedarlane[®], Hornby, ON, Canada) at a dilution of 1:500 for blotting; 3) anti-2H12 (Paulin-Levasseur *et al.*, 1995) undiluted for immunofluorescence and for blotting; 4) anti-emerin (Novocastra[™] Leica Microsystems, Wetzlar, Germany) at a dilution of 1:20 for immunofluorescence and 1:500 for blotting; 5) anti-fibrillarin serum (provided by Dr. Ricardo Benavente, University of Würzburg, Germany) at a dilution of 1:20 for immunofluorescence; 6) anti-Lamins A/C (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at a dilution of 1:1000 for immunofluorescence; 7) anti-Lamins A/C (provided by Dr. Georg Krohne, University of Würzburg, Germany) at a dilution of 1:1000 for blotting; 8) anti-Lamin B (Cedarlane[®] Labs Ltd, Burlington, ON, Canada) at a dilution of 1:50 for immunofluorescence and 1:200 for blotting; 9) anti-LAP2 (13D4; provided by Dr. Ricardo Benavente, University of Würzburg, Germany) at a dilution of 1:20 for immunofluorescence and for blotting; 10) fluorescein labeled anti-digoxigenin Fab fragments (Roche Diagnostics GmbH, 68298 Mannheim, Germany) at a dilution of 1:200 for FISH. Secondary antibodies used in this study included: 1) a biotinylated donkey anti-mouse (Amersham, Piscataway, NJ, USA) at a dilution of 1:400 for blotting; 2) a donkey anti-mouse Cy3 conjugate (Jackson ImmunoResearch, West Grove, PA, USA) at

a dilution of 1:400 for immunofluorescence; and 3) a donkey anti-goat Cy3 conjugate (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:400.

III. RESULTS

1. DNA demethylation and the repercussions on the SF2/ASF splicing factor and the assembly of nuclear stress bodies (nSBs).

Several types of environmental stresses result in the formation of nSBs which are thought to be the sites of alternative splicing of a still poorly defined subset of genes in human cells in order to survive and recover (Valgardsdottir *et al.*, 2007). This consideration stems from the recruitment of several proteins such as the SF2/ASF splicing factor within nSBs. Changes in the expression or localization of SF2/ASF before and after HS due to differences in methylation status may offer a glimpse into further understanding of the process and its relevance to cellular recovery after an environmental shock such as HS that is used in this study.

1.1 Induction of nSBs.

Multiple sets of experiments have been performed here. Controls to my experiments include a human cervical cancer cell line HeLa as well as the parental HCT116 #28 cell line which expresses normal DNMT activity and was used to generate the different mutant clones (Rhee *et al.*, 2002). Initially, HeLa, HCT116 # 28, HCT116 # 30, HCT116 # 38 and HCT116 # 66 were subjected to a comparative analysis by

immunofluorescence microscopy utilizing α -tubulin as in order to confirm the methodology (data not shown). Subsequently, the SF2/ASF splicing factor served as a marker, and its distribution between the different clones was compared (Fig. 3). Since the SF2/ASF splicing factor was found to be homogeneously distributed within the nucleus and excluded from nucleoli in all clones, as initially expected, HS experiments were performed to examine the formation of nSBs.

Figure 4 depicts different aggregations of the SF2/ASF splicing factor showing what is thought to be classic nSB formation in all cell lines independently of the epigenetic status. However, variations in the number of nSBs per cell as well as in the number of cells that responded to the treatment were noticeable and are detailed in Tables 1 and 2. The number of nSBs found within a cell is directly related to its ploidy (Jolly *et al.*, 1997; Jolly *et al.*, 1999). As such we expected that our different clones would display no more than 1-6 nSBs (Valgardsdottir *et al.*, 2007). As reported by the mode in Table 1, most cell lines assembled between 2 and 6 nSBs, corroborating earlier observations on nSB formation. However, the variation range in numbers of nSBs did not correlate with our expectations. In fact we observed up to 15 nSBs in some cells. Moreover, we observed micronuclei in aging cells (data not shown) suggesting that HCT116 clones do not have a stable genome in culture as it was previously believed. Perhaps this observation is in part due to lack of methylation. Thus we report a higher number of nSBs, which varies not only between clones but also within populations of a single cell line.

Cell Line	Mean \pm S.E.M	Mode	Maximum	Minimum
HeLa	6.15 \rightarrow 6 \pm 2	6	12	2
HCT116 #28	3.60 \rightarrow 4 \pm 2	2	9	1
HCT116 #30	5.22 \rightarrow 5 \pm 3	4	11	2
HCT116 #38	5.81 \rightarrow 6 \pm 4	6	15	1
HCT116 #66	5.59 \rightarrow 6 \pm 4	2	15	1

Table 1. Table showing the differences between the clones pertaining to the formation of nSBs after 1 hour induction of physiological range HS plus 1 hour recovery period. Three blinded independent counts resulting from separate HS treatments were obtained and the averages as well as the mode (i.e. the most recurrent number) of nSBs were assessed.

Cell Line	Cells counted	Positive Response	Negative Response	Response coefficient (Rc)
HeLa	410	231	179	0.56
HCT116 #28	415	191	224	0.46
HCT116 #30	398	281	117	0.71
HCT116 #38	446	232	214	0.52
HCT116 #66	410	137	273	0.33

Table 2. Table showing the differences between the clones pertaining to their response after one hour induction under physiological range HS plus one hour recovery period. Three blinded independent counts resulting from separate HS treatments were obtained and the number of cells which responded to the treatment was assessed.

The response coefficient (Rc) quantifying the degree of positive response to HS (Table 2) varies between clones and seems to be low. However, there seems to be a correlation with SF2/ASF expression and DNA methylation after HS. As a baseline, it seems that HS is only effective on 56% of HeLa cells. Moreover, the innate response of HCT116 cells as outlined by HCT116 #28 seems to be limited to 46% of the population. Peculiarly, 20% and 3% reductions in overall DNA methylation in HCT116 #30 and #38 will respectively induce different Rcs. In general, a higher sensitivity to HS is demonstrated by a higher Rc. However, HCT116 #66, the DNMT1 hypomorph which also displays a mutation in the DNMT3b enzyme and is characterized by a 95% reduction in global methylation seems to demonstrate a resistance to the HS treatment, with a reduced Rc of 33%.

Figure 3: Distribution of the SF2/ASF splicing factor before HS. HeLa and HCT116 clones marked with a mouse anti-SF2/ASF and counterstained with an anti-mouse Cy3 antibody display a homogenous distribution of SF2/ASF within the nucleus. Three independent assays were done from which 100 cells per assay were observed. Note the exclusion of the splicing factor from the nucleoli, which are visible in the SF2/ASF Cy3, the DNA DAPI stain as well as in the phase contrast (PC) pictures. Cells are shown at 1000x magnification and scale bar is 5 μ m

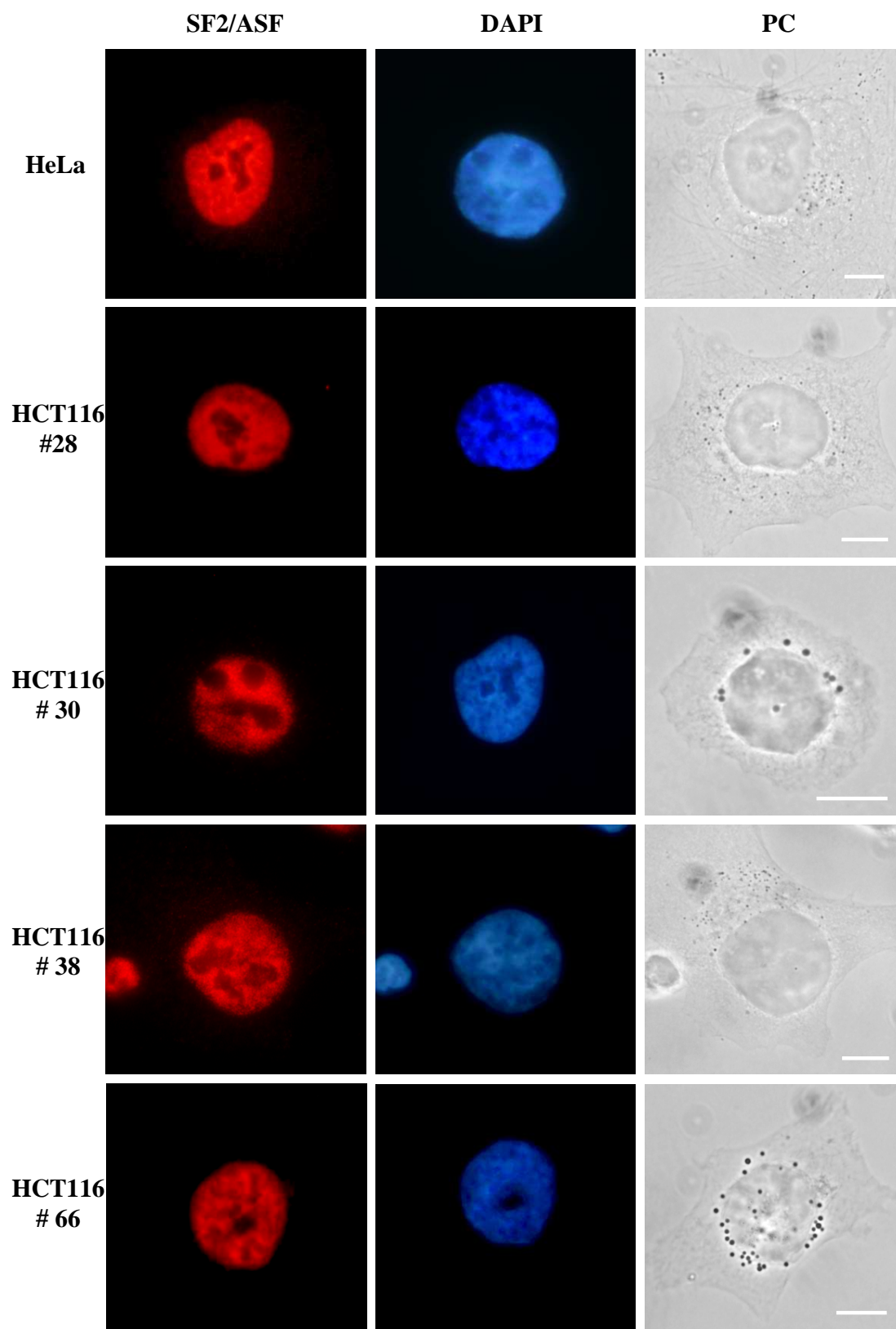
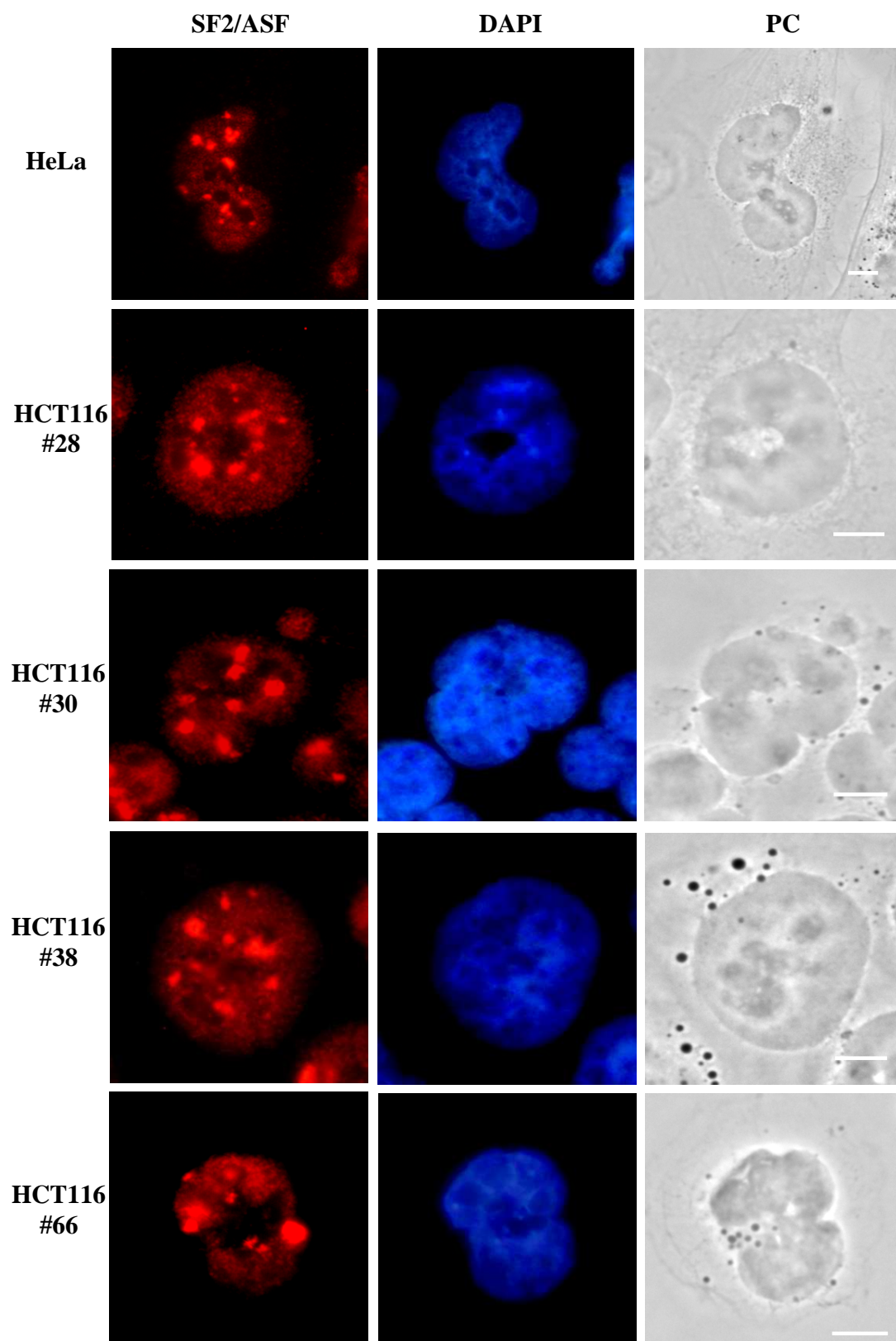


Figure 4: Distribution of the SF2/ASF splicing factor after HS. HeLa and HCT116 clones marked with a mouse anti-SF2/ASF and counterstained with an anti-mouse Cy3 antibody display an alternate distribution pattern of SF2/ASF within nSBs (note variations in numbers, sizes and shapes of nSBs between clones) after a one hour HS treatment and a one hour recovery period. Three independent assays were done from which 100 cells per assay were observed. Once again, the splicing factor is excluded from nucleoli. Cells are shown at 1000x magnification and scale bar is 5 μ m



1.2 Protein levels of SF2/ASF: quantifying a splicing factor.

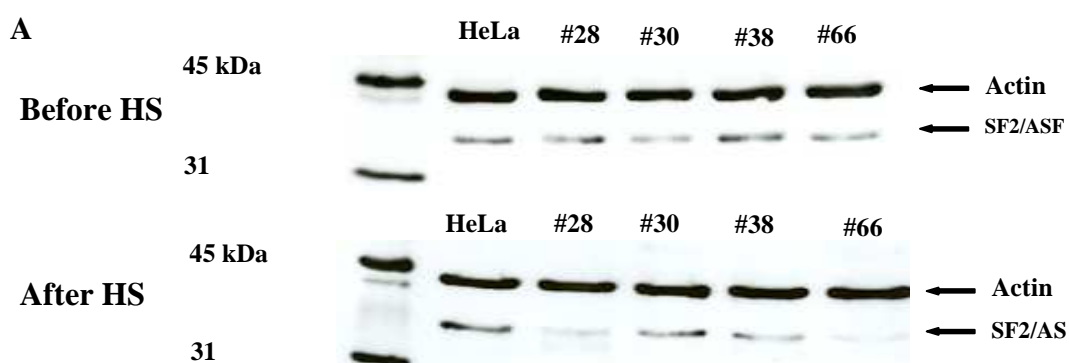
Given that SF2/ASF follows published data pertaining to its localization before and after HS, we proceeded to analyze its levels of expression in the same conditions in order to determine if variations in methylation will affect its expression. Pre-HS and post-HS cellular homogenates of the cell lines were prepared and analyzed by SDS-PAGE gels and Western blotting techniques. The immunoblots targeted the SF2/ASF splicing factor and included an actin loading control. The goal here was to detect any variations in the expression of the splicing factor between clones and between the control cell line, outlined as a possibility in the relative intensity assay (data not shown). This might permit to correlate them to the previously detailed Rc results. Figure 5 illustrates the expression levels of SF2/ASF before and after HS as well as detailing a semi-quantitative measurement on SF2/ASF expression. Results show that the baseline expression of SF2/ASF before HS in the parental cell line does not vary from the external control set at 1. This allows for an easier interpretation of further results since any variation might be directly linked to the DNA methylation states.

The expression of SF2/ASF is variable depending on which DNMT enzyme is deleted before and after HS. Repeated measures ANOVAs have revealed that before HS, HCT116 #38 shows no significant variation compared to the parental cell line while HCT116 #30 and HCT116 #66 demonstrate a significant decrease in expression. After HS, clones #30, #38 and #66 display no significant variations from the parental cell line. One way ANOVAs demonstrate that the innate response to HS is a significant reduction in SF2/ASF expression as observed with the parental cell line HCT116 #28. HCT116 #38

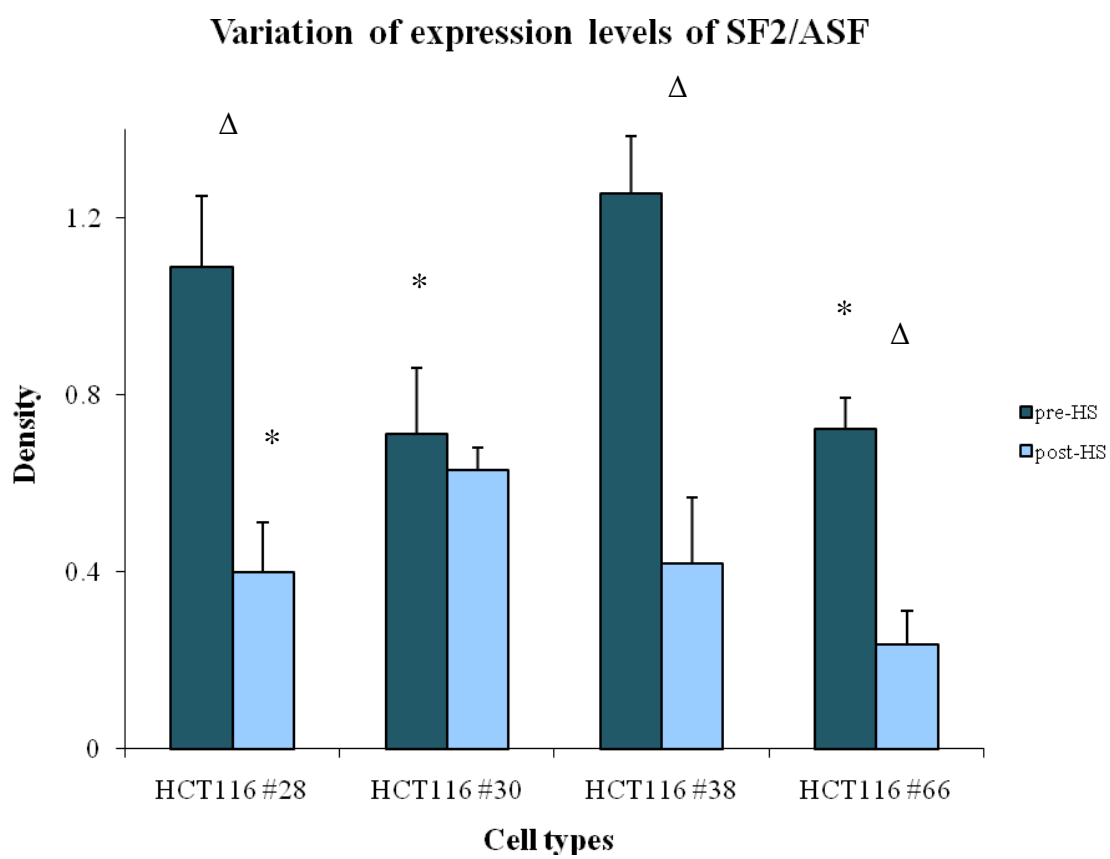
demonstrated an innate response, with a down regulation resembling that of the parental cell line HCT116 #28. The double mutated cell line showed an even stronger decline in expression. Interestingly, HCT116 #30 does not show any significant changes in the expression of the splicing factor.

When compared to Rc results detailed in Table 2, it seems upon further examination that the level of expression of the SF2/ASF splicing factor follows a trend with very similar values of expression versus Rc values. Taken as a whole, these results suggest that the partial loss of function in the DNMT1 enzyme leading to a 20% reduction of overall DNA methylation correlates with a non-native molecular response to HS, i.e different from the parental cell line, while the deletion of DNMT3b seemingly has no effect. In other words, the higher yield of cells displaying nSBs combined with the higher than normal expression of the SF2/ASF splicing factor might indicate a deregulation in the programmed ability to recover from stress.

Figure 5: Expression profile of the SF2/ASF splicing factor before and after heat shock. **A)** Three independent cell homogenates of 3 million cells/mL were subjected to an SDS-PAGE gel analysis then transferred to a nitrocellulose membrane and exposed to a primary mouse anti-actin and anti-SF2/ASF, then a secondary biotinylated anti-mouse and streptavidin-HRP. **B)** Quantification of SF2/ASF where the star represents significant differences from the parental cell line and triangles significant differences for before and after HS. An actin loading control was established for semi-quantification. A repeated measures ANOVA ($\alpha=0.05$) before HS, reveals that there is a significant reduction of expression in clone #30 (p -value=0.001) compared to #28 and clone #66 (p -value= 0.001) compared to clone #28. Clone #38 displays a non-significant increase (p -value=0.372) compared to the parental cell line. After HS, clone #30 shows a non significant increase (p -value=0.173) from the parental cell line. Clone #38 does not display significantly different levels of SF2/ASF from the parental cell line (p -value=1.000). Lastly, clone #66 depicts non significant reduction from the parental cell line (p -value=0.713). One way ANOVAs comparing pre and post HS data for each cell line reveal that there are significant expression reductions in clones #28 (p -value=0.001), #38(p -value=0.000) and #66 (p -value=0.000) but not in #30 (p -value=0.500).



B



1.3 Localization of Sat III sequences the initial step to nSB formation.

1.3.1 Cellular localization of SatIII sequences

In light of the results that were obtained, with the knowledge that nSB formation is always initiated by the expression of a collection of pericentromeric satellite DNA sequences termed SatIII, we proceeded to track the localization of SatIII RNAs with respect to the HS response. Thus we ensued to perform RNA fluorescence *in situ* hybridization (RNA FISH) using probes known to co-localize with the transcripts under several types of environmental stresses (Valgardsdottir *et al.*, 2007). Additionally, we opted to include a co-localization step identifying SF2/ASF in the same cells in order to certify that we were indeed observing nSBs and that any signal generated by the RNA probes was localized within nSBs. As previously described by (Valgardsdottir *et al.* 2007), SatIII sequences are rich in G and C nucleotides. As such, one strand is said to be C rich while the other is by default G rich. Previous studies led by Valgardsdottir *et al.* in 2007, demonstrated that the expression of SatIII sequences is biased and thus more G rich RNAs are produced. This led to the conclusion that these RNA strands lead to nSB formation more so when HS is involved as a form of stress. As such, we opted to examine nSB formation with probes against G rich SatIII RNAs.

Before HS, there was no clear signal of the forward probe in any of the five cell lines indicating that SatIII DNA is generally not expressed or only transcribed in negligible amounts since it is for the most part methylated (Fig. 6). Moreover, no signal was detected with the reverse and control probes (data not shown). However, HCT116 #30, #38 and #66 encompass demethylation in regions that include the SatIII sequences. This had initially led to the belief that SatIII expression might be observed and could

even lead to nSB-like formations within the nucleus before HS. Ultimately this last hypothesis was rejected since there is no co-localization of SatIII-SF2/ASF complexes. Nevertheless, a molecular analysis using RT-PCR might reveal an up-regulation in SatIII expression before HS since these regions are said to be demethylated.

After HS, minuscule speckles illustrating aggregation of SatIII RNAs are clearly noted in all cell lines independently of their total methylation state when hybridized with the forward probe (Fig. 7) while no signal was detected for the reverse and control probes (data not shown). Moreover, when overlapped, the images of SatIII and SF2/ASF show clear co-localization of both targets. This thus confirms that what has been observed so far are nSBs but more importantly that these nSBs assemble in a classical way as initially described, independently of which DNMT enzyme has been deleted if any.

Figure 6: Fluorescence *in situ* hybridization (FISH) of pericentromeric Satellite III sequences before HS. Three independent samples of HeLa and HCT116 clones harvested before HS were initially exposed to a forward digoxigenin labelled Sat III probe. Secondly, fluorescein labeled anti-digoxigenin Fab fragments were utilized to allow visualization of Sat III transcripts. This initial preparation was then subjected to an immunolocalization assay with SF2/ASF and Cy3 labelled secondary antibodies. As previously 100 per sample were observed and no nSBs were observed before HS. Moreover, no aggregates of Sat III are observed. Magification is 1000x and scale bar is 5 μ m.

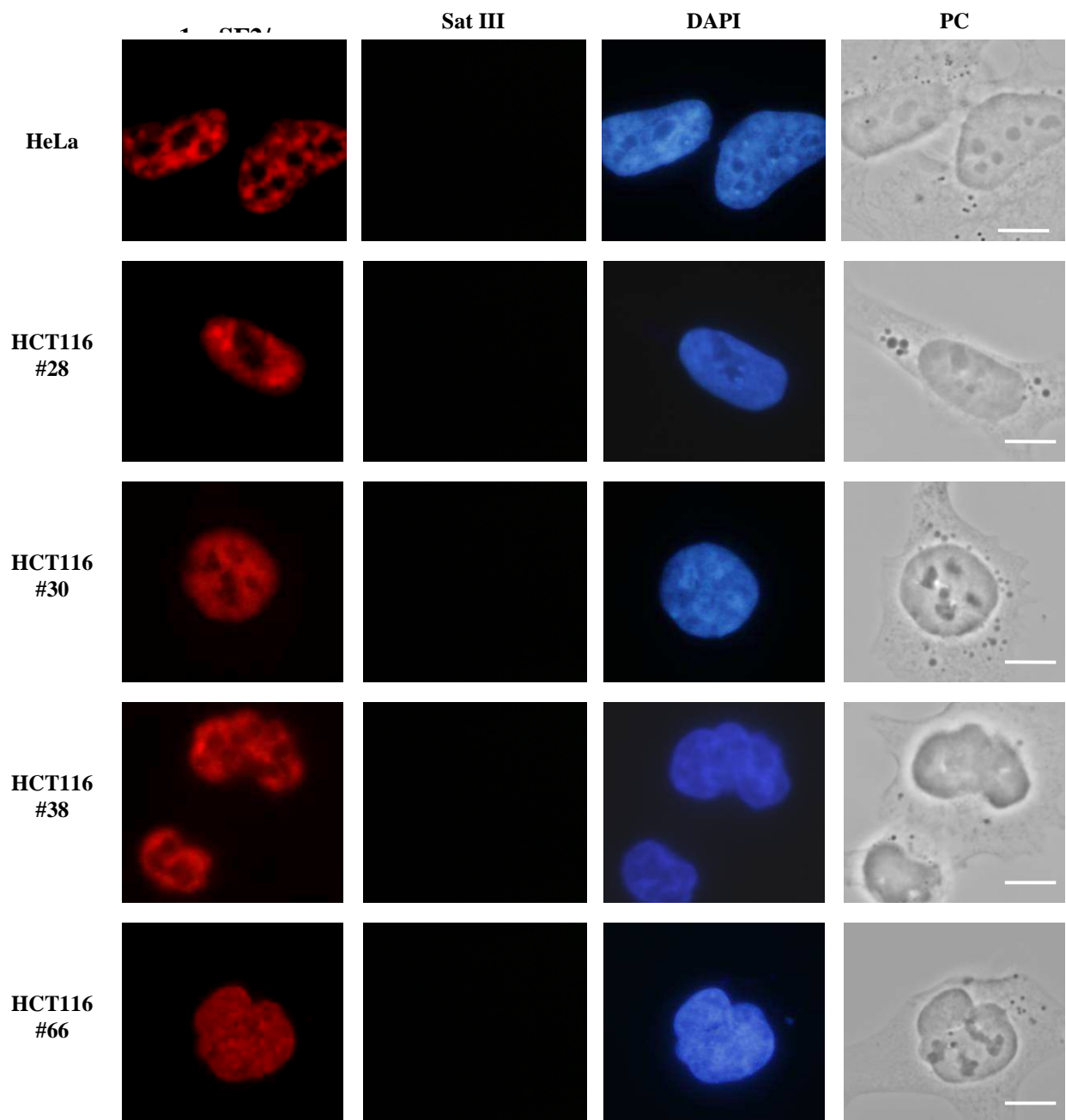
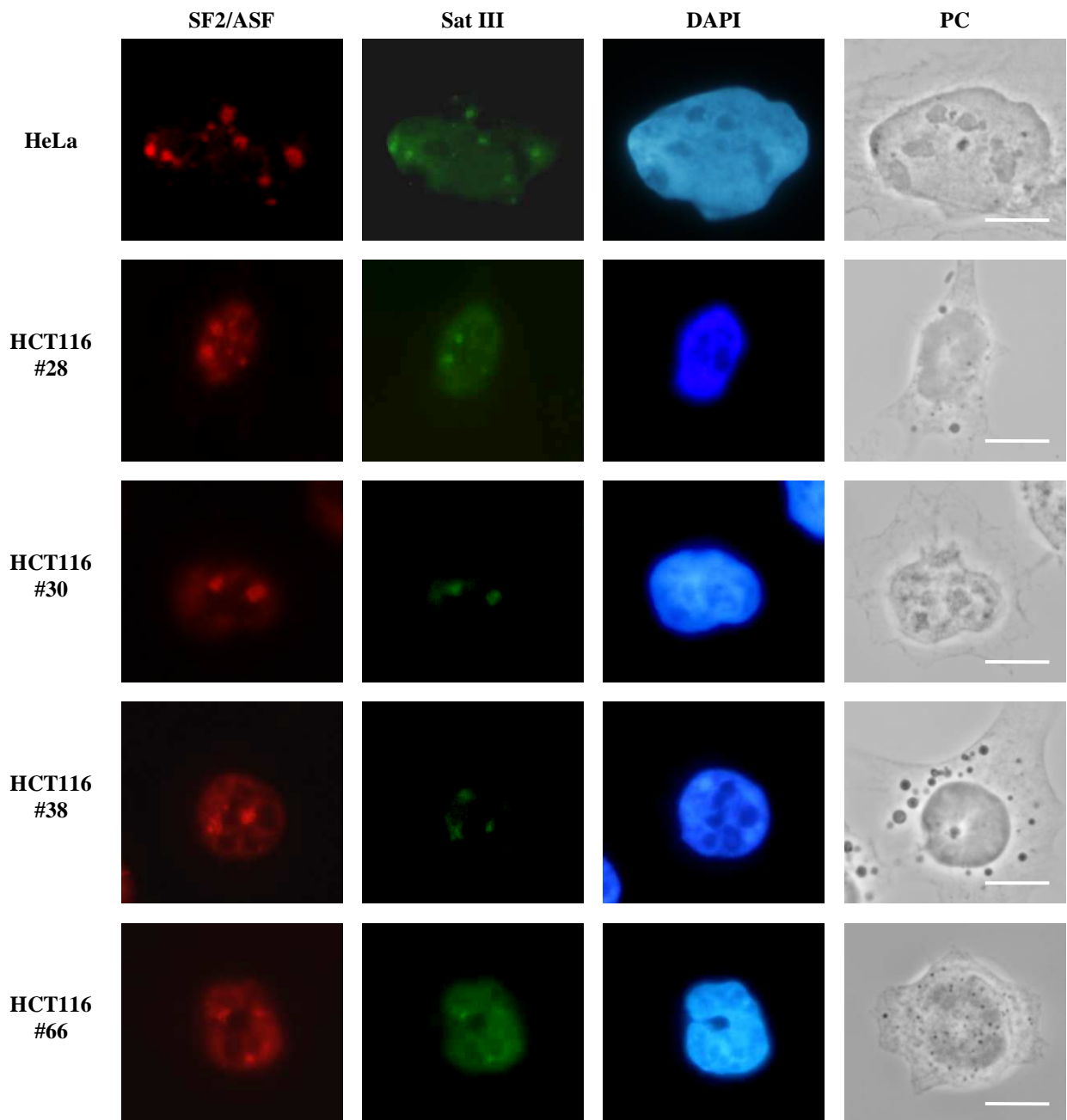


Figure 7: Fluorescence *in situ* hybridization (FISH) of pericentromeric Sat III sequences after HS. HeLa and HCT116 clones were harvested and subjected to a HS treatment. Three independent preparations were exposed as above to a forward Sat III probe and fluorescein labeled anti-digoxigenin Fab fragments which allowed visualization of Sat III transcripts and 100 cells per sample were observed. As previously, an immunolocalization assay of SF2/ASF counterstained with Cy3 labelled mouse anti-SF2/ASF was performed. Here, confirmation of nSB formation is obtained through co-localization of Sat III sequences and SF2/ASF in ribonucleoprotein aggregates. Cells are shown at 1000x magnification and scale bar is 5 μ m.



2 Expression of nuclear antigens and their localization in relation to DNA demethylation

Subsequently, we proceeded to analyze the effects of DNA methylation on the expression of nucleolar and INM proteins. It is common knowledge that in mammals, most genes rely on a methylation/demethylation process in order to be available or unavailable for expression. As described earlier, deregulation in the expression of these proteins has been shown in many cases to compromise the integrity as well as the strength of the entire nucleus and can be the root cause for several developmentally related diseases. Moreover, HS will induce a generalized demethylation process, which could further disturb normal protein metabolism. The methodology adopted for this part of the study is straightforward but has proven in the past to be very effective at the cellular and/or tissue level. First, SDS-PAGE paired with semi-quantitative immunoblotting analyses of the nuclear antigens of interest were completed and followed by indirect immunofluorescence staining and microscopy to locate the antigens and examine nuclear shape. Primarily, we observed the effect of demethylation on nucleolar antigens such as 2H12 and fibrillarin followed by two subclasses of INM proteins; the LEM proteins emerin and LAP2 and nuclear Lamins A and B.

In the past, similar studies had been performed using HeLa cells. As such we proceeded to compare the parental cell line with HeLa, which is known to express normal protein levels, to determine if any significant changes are seen. Subsequently, we proceeded to compare the modified clones to the parental cell line in order to determine if epigenetic modifications have an impact on the expression of detailed antigens.

2.1 Nucleolar antigens

Nucleolar proteins such as 2H12 and fibrillarin possess mainly structural roles pertaining to nucleoli but can also in the case of fibrillarin possess RNA processing capabilities by association with small nuclear RNA components (Bartova *et al.*, 2010). In this part of the study we tried to determine if modifications within the normal methylation metabolism of a cell would alter the expression of such nucleolar proteins and thus possibly further modify the cellular dynamics due to inconsistent nucleolar strength, conformation or function. Moreover, we proceeded to analyze if further demethylation due to HS create, worsen or has no effect on these pre-existing conditions.

Figures 8 – 12 illustrate the expression and localization of the 2h12 and fibrillarin antigens before and after heat shock. Observations of interphase cells demonstrate that the 2H12 antigen depicted in figures 8 and 9 is found as expected within nucleoli. Moreover, a subtle signal is depicted around nucleoli. Results are similar before and after HS in all cell lines. However, the expression levels of the 2H12 antigen detailed in figure 10 is variable between cell lines. When comparing the parental cell line to HeLa before HS, the expression of 2H12 is up-regulated by a factor of approximately 1.2 though this difference is not significant. A decrease in total DNA methylation however small, as marked by clones #30, #38, translates into an increase in total 2H12 expression when compared to the parental cell line. However, this increase is not significant. Conversely, clone #66 does not display any apparent changes in expression when compared parental cell line.

After HS, no significant variations between cell lines are notable. However, when observing the response to HS, differences do occur. The natural expression of 2H12 after

HS as observed with the parental cell line is a significant reduction to a level approximately 0.7 times that of HeLa cells. This result is duplicated in clone #38 but not in clones #30 and 66. Finally, it seems no permanent damage is caused by HS given protein levels seem to have reverted to pre HS levels in a 24 hour post HS analysis (data not shown).

Figures 11 and 12 depict the localization of the fibrillarin antigen before and after HS. Similarly to 2H12, the localization of fibrillarin does not vary much within interphase cells before and after HS. In fact this antigen is found within and loosely around nucleoli in both situations. However, results pertaining to the expression levels of this antigen are not available due to the lack of an antibody that performs properly in quantitative analyses. As such the protein levels of fibrillarin were not assessed during this study. Nonetheless, our results show that, both nucleolar antigens, regardless of their expression levels, are found within the expected region. As such we propose they are most certainly capable of performing their normal functions pertaining to the maintenance of nucleolar shape and structure and, in the case of fibrillarin, RNA processing.

FIGURE 8: Distribution of the 2H12 nucleolar antigen before HS. Three independent samples of HeLa and HCT116 clones harvested before HS were labelled with a mouse anti-2H12 and counterstained with a Cy3 labelled secondary antibody. Observations done on 100 cells per sample showed all cell lines displaying a specific distribution of 2H12 within the nucleolus. Note the exclusion of 2H12 from other areas of the nucleus and its faint distribution around the nucleolus. Cells are shown at 1000x magnification and scale bar is 5 μ m.

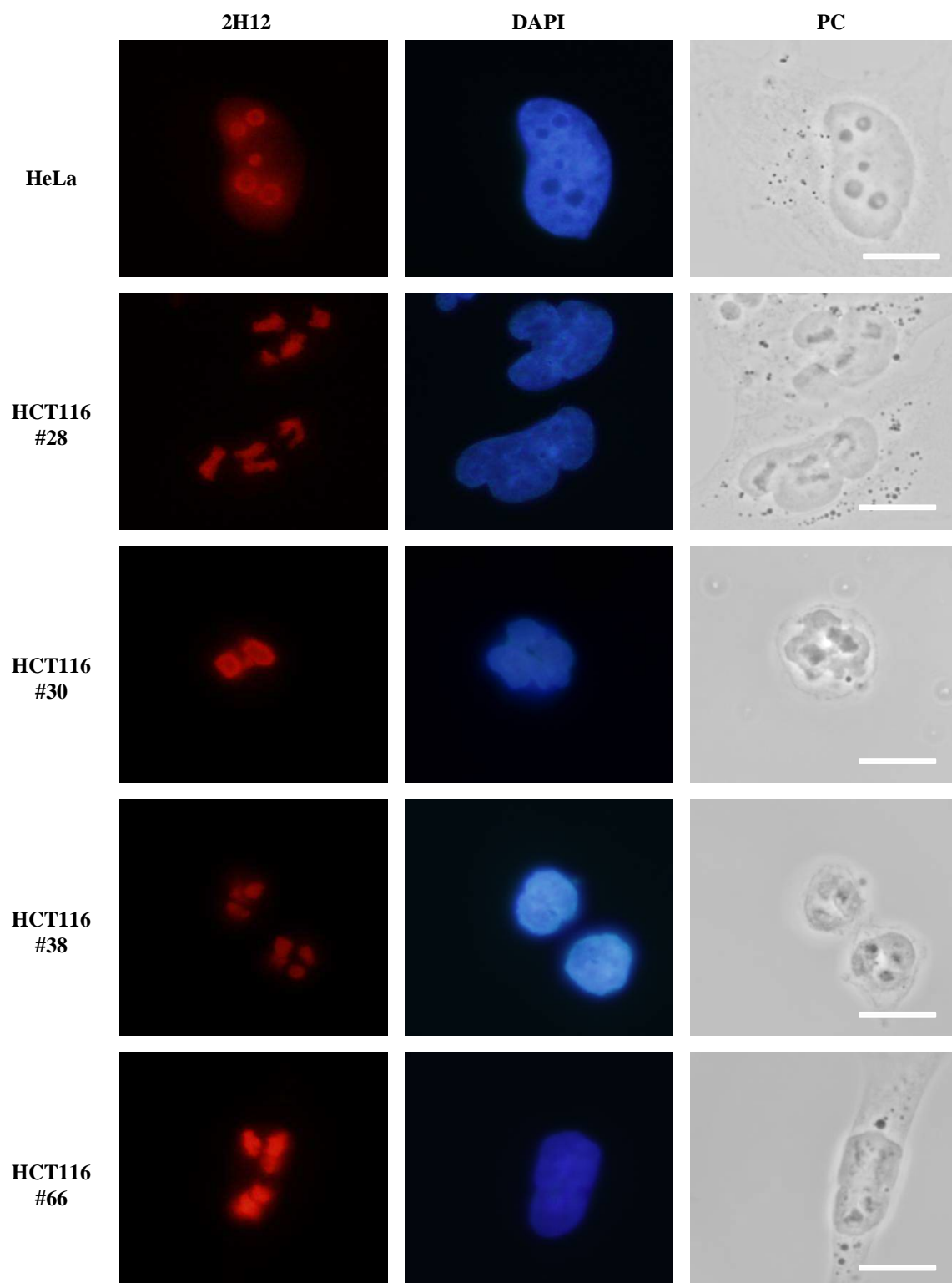


FIGURE 9: Distribution of the 2H12 nucleolar antigen after HS. HeLa and HCT116 clones harvested after a HS treatment were labelled as previously described. Similarly to pre HS results, three independent samples of 100 cells per cell line display a specific distribution of 2H12 within and faintly around the nucleolus. Cells are shown at 1000x magnification and scale bar is 5 μ m.

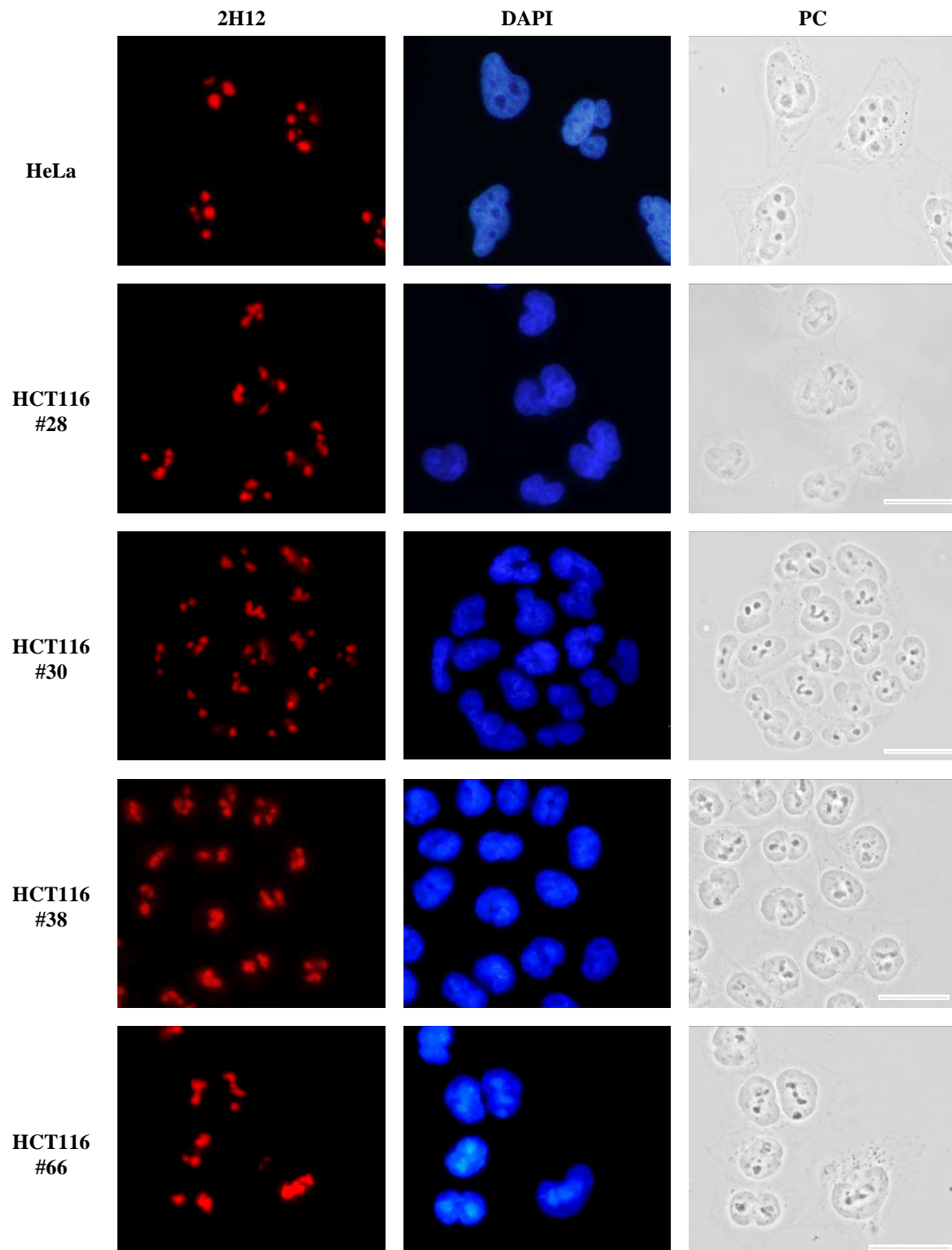
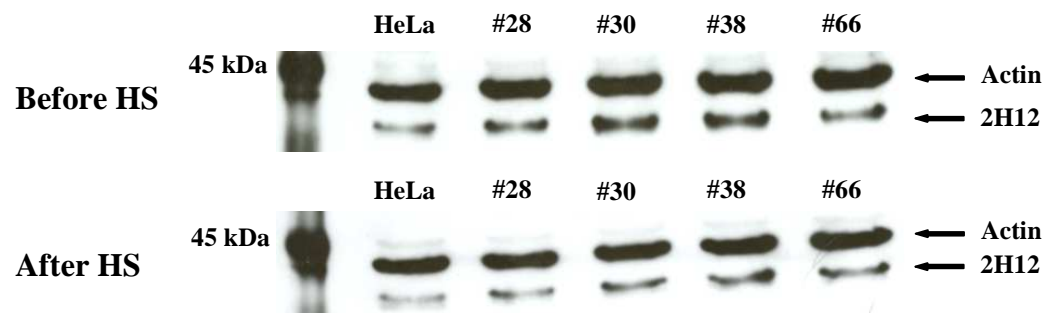


FIGURE 10: Immunoblotting analysis and expression levels of 2H12 antigen before and after HS. **A)** Cell homogenates (3 million cells/mL) were subjected to an SDS-PAGE gel analysis, transferred to a nitrocellulose membrane and exposed to a primary mouse anti-actin and anti-2H12. **B)** Quantification of 2H12 where the star represents significant differences from the parental cell line and triangles significant differences for before and after HS. A repeated measures ANOVA ($\alpha=0.05$) before HS, reveals an up-regulation by a factor of 1.2 times in the parental cell line when compared to HeLa. However, this is not significant (p -value=1.000). Clone #30 displays a non significant increase compared to the parental cell line (p -value=0.255). Clone #38 also depicts a non significant up-regulation when compared to the parental cell line (p -value=0.096). Clone #66 displays no significant up-regulations (p -value=1.000). After HS, Clone#30 displays no significant changes from clone #28 (p -value=1.000). The same is observed for clone #38 (p -value=1.000) and clone #66 (p -value=1.000). However, when observing the response to HS, a one way ANOVA shows differences do occur. The parental cell line depicts a significant reduction to a level approximately 0.7 times that of HeLa cells (p -value=0.019). This result is duplicated in clone #38 (p -value=0.004) but not clones #30 (p -value=0.077) and 66 (p -value=0.100).

A



B

Variation of expression levels of 2H12

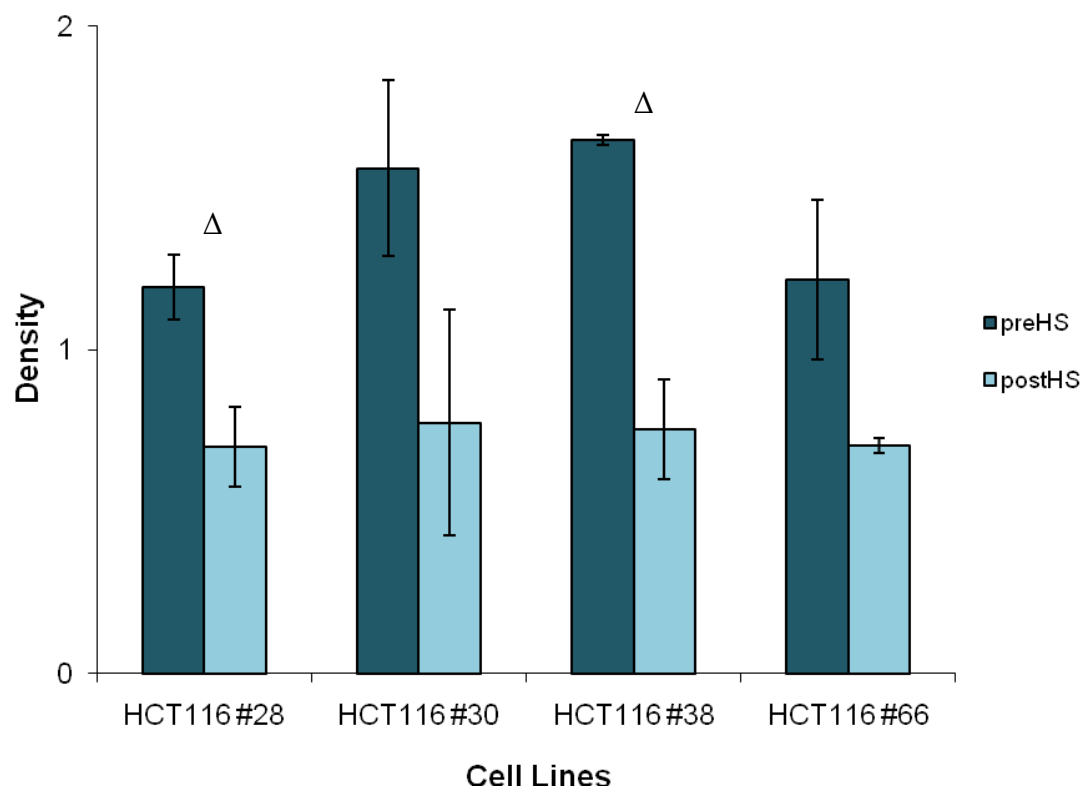


FIGURE 11: Distribution of the fibrillarin nucleolar antigen before HS. HeLa and HCT116 clones harvested in three independent samples per cell line before HS were labelled with anti-fibrillarin and Cy 3 labelled specific primary and secondary antibodies respectively. Comparably to the previous 2H12 assay, 100 cells per sample depict all cell lines display a specific distribution of fibrillarin within the nucleolus excluded from other areas of the nucleus. Again, faint distribution around the nucleolus is visible. Cells are shown at 1000x magnification and scale bar is 5 μ m.

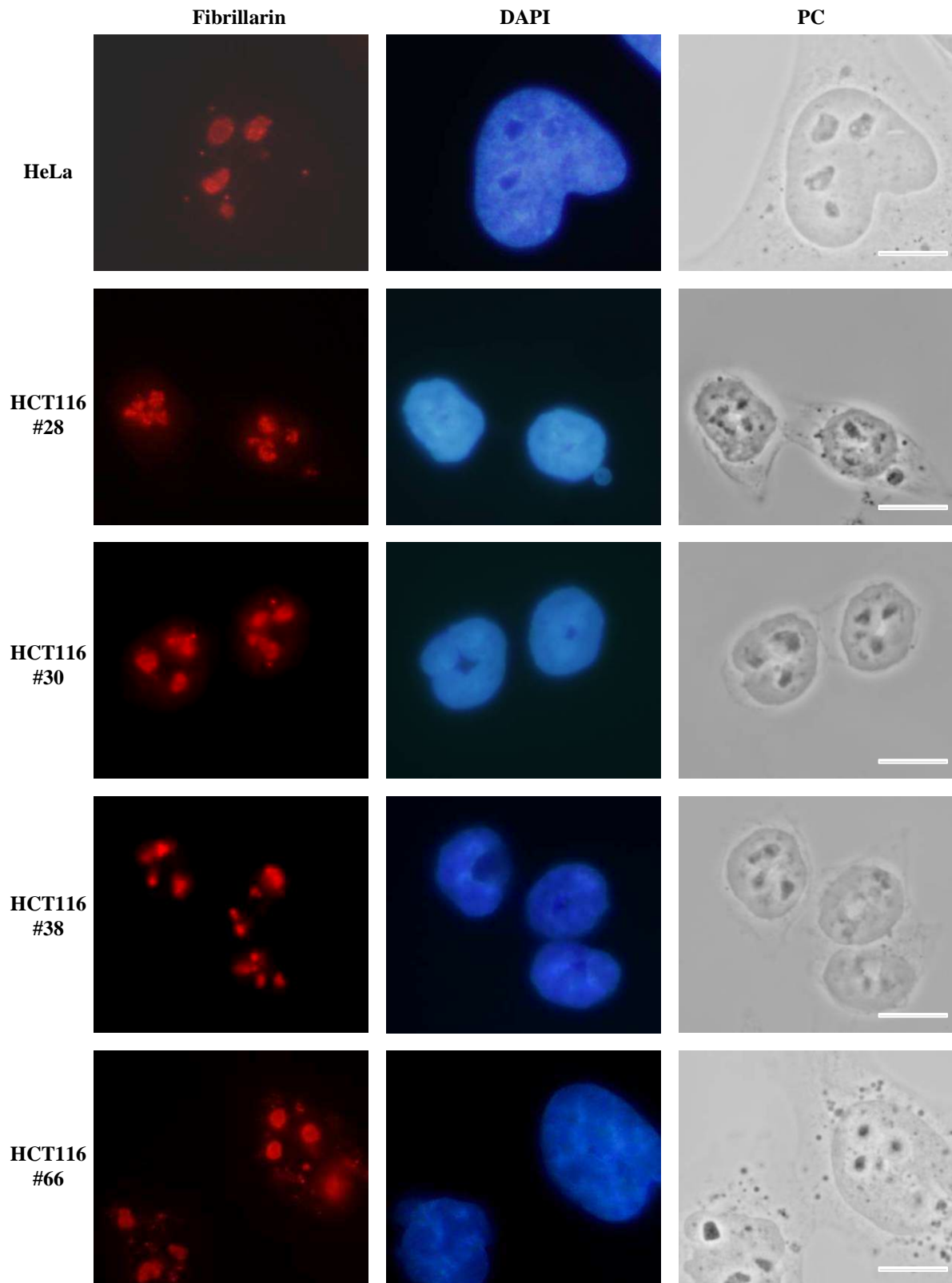
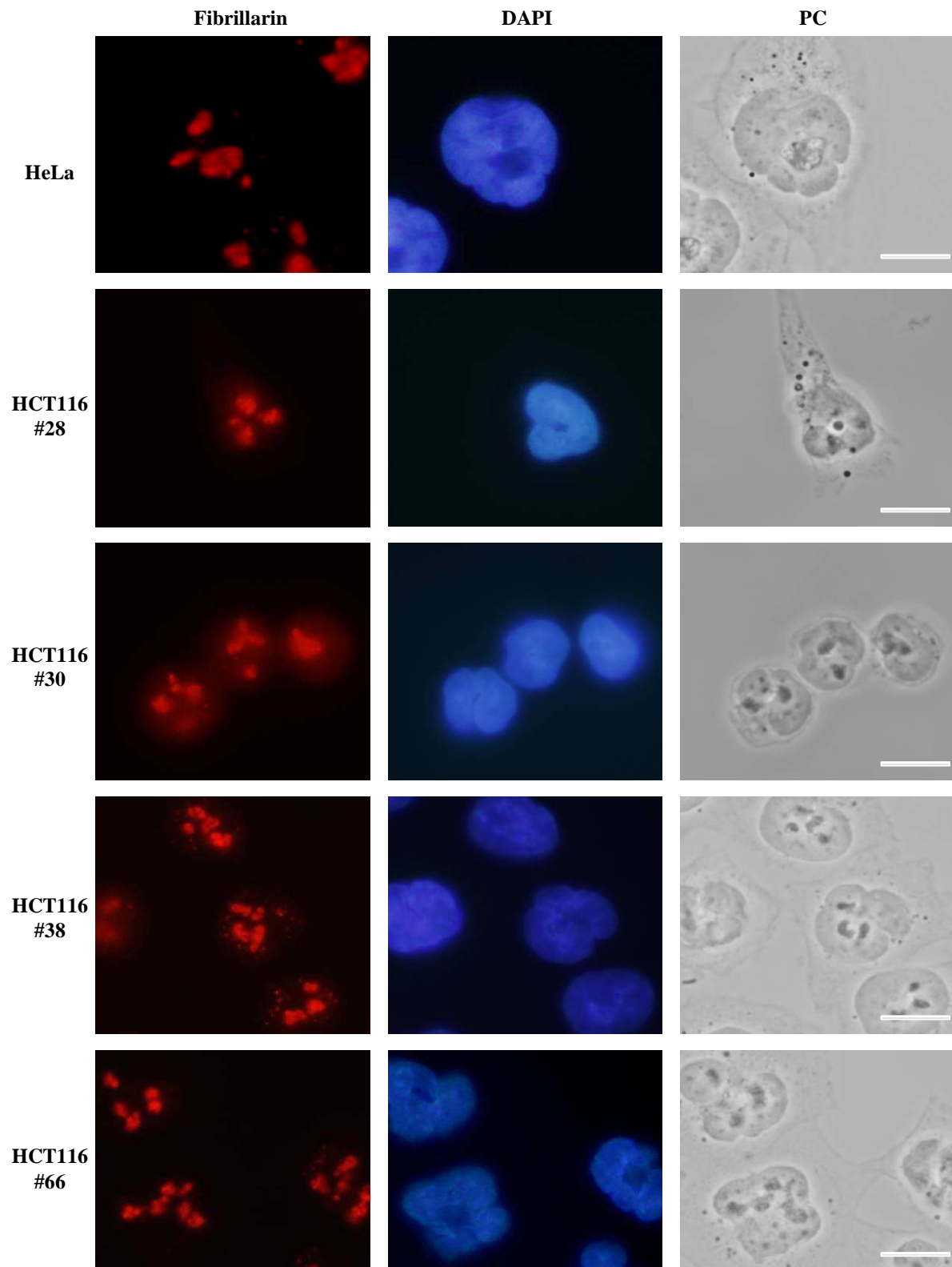


FIGURE 12: Distribution of the fibrillarin nucleolar antigen after HS. Three different samples from all five cell lines harvested after a HS treatment were labelled as above. Results from 100 cells per cell line show no differences are notable and are similar to what is described in terms of nucleolar localization and exclusion of other nuclear regions. Cells are shown at 1000x magnification and scale bar is 5 μ m.



2.2 Inner nuclear membrane antigens

Following the study of nucleolar components and the effects of HS on these proteins, we moved on to assess the possibility that a deregulation in the normal methylation patterns and methyltransferase enzymes might induce discrepancies within the normal metabolism i.e. expression and localization of INM proteins as well as nuclear shape and deduced integrity. As detailed earlier, this was done for two sub-classes of proteins; LEM proteins as well as Lamins A and B; following the same methodology as with the nucleolar proteins.

2.2.1 LEM proteins

LEM proteins are, as detailed earlier, part of the basic components that lead to the correct development and function of cells, more specifically, nuclear dynamics. Their mutual interactions, as well as interactions with DNA and other proteins render LEM proteins relevant in the study of the effects of DNA methylation in HS and HS recovery. This part of the study focuses on the effect of HS on the expression of LAP2 and emerin antigens. Figures 13 to 18 illustrate and quantify the distribution and expression levels respectively of these two antigens before and after HS. Moreover, this part of the study encompasses the first glimpse into the effects of DNA demethylation on nuclear shape, a clue as to the overall health of the nucleus. HGPS cells, as for many other disease-derived cell lines, demonstrate general demethylation and misshapen nuclei. However, in the case of HGPS cells it is not known whether demethylation or accumulation of progerin, truncated Lamin A, is the cause. Thus, we opted to include such observations in our analysis.

Figures 13 and 14 depict the localization of emerin before and after HS. In both instances, the protein is clearly localized within the constraints of the nuclear membrane as expected. However, the shape of the nucleus varies between the in-group and out-group control cell lines and the different HCT116 clones. In fact, the nucleus of clones #30, #38 and #66 are not spherical as expected but rather are irregular in shape and size and seem lobulated similarly to a nucleus about to bleb. The irregular shape is at times such that the task of obtaining a clear image of the nucleus was very difficult. This is the case mainly in clones #30 and #66 which both have in common a truncated version of DNMT1 and 20% and 95% reductions in methylation, respectively.

The expression levels and immunoblotting analysis of emerin before as well as after HS are found in figure 15. Before HS, the parental cell line expresses emerin to a similar level as HeLa cells, which leads to the idea that HCT116 cells express emerin at normal levels in a stable environment. When examining the expression in the modified clones, it seems a 20% loss of methylation due to a truncated maintenance DNMT enzyme (DNMT1) leads to a non-significant increase in the expression of emerin in clone #30. Similarly, a 3% loss of methylation due to the lack of the developmentally vital DNMT3b enzyme in clone #38 leads to a non-significant increase in expression. Interestingly, a synergy seems to arise with clone #66 where the increase in emerin expression is significant from the parental cell line. After HS, the parental cell line expresses emerin to a level significantly lower than HeLa cells. Moreover, modified clones express emerin at levels comparable to the parental cell line.

FIGURE 13: Distribution of the emerin INM antigen before HS. Three independent samples of all five cell lines were treated as described and labelled with anti-emerin. 100 cells per sample were observed. Note the localization of the protein exclusively at the periphery of the nucleus and excluded from within. Also note the nuclear shape and size, which vary between cell lines. Cells are shown at 1000x magnification and scale bar is 5 μ m.

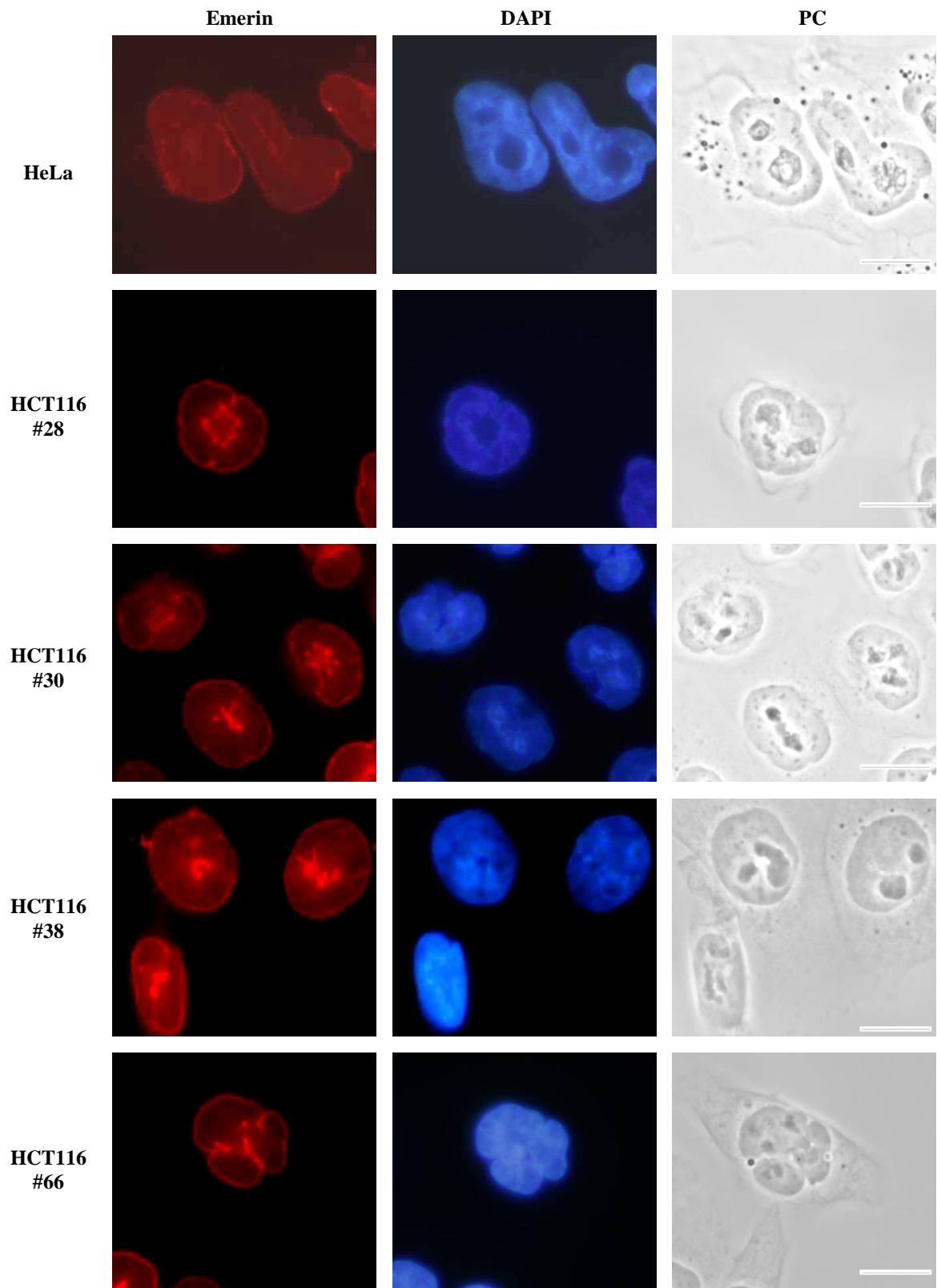


FIGURE 14: Distribution of the emerin INM antigen after HS. Cell lines were harvested in three independent samples after HS and labelled with anti-emerin. Observations made on 100 cells per cell line show as above the localization of the protein is limited to the nuclear periphery which in turn is variable in shape between cell lines. Cells are shown at 1000x magnification and scale bar is 5 μ m.

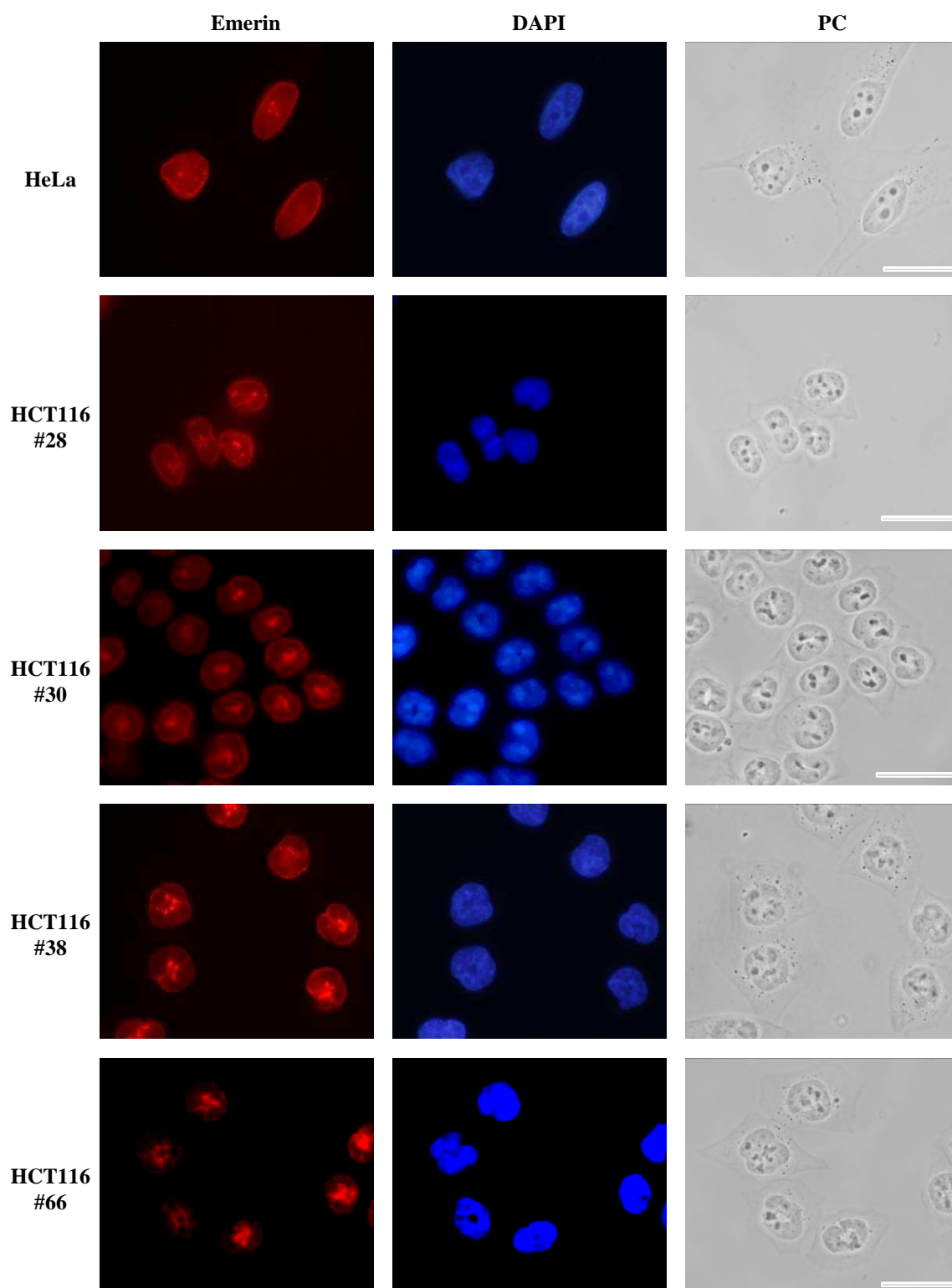
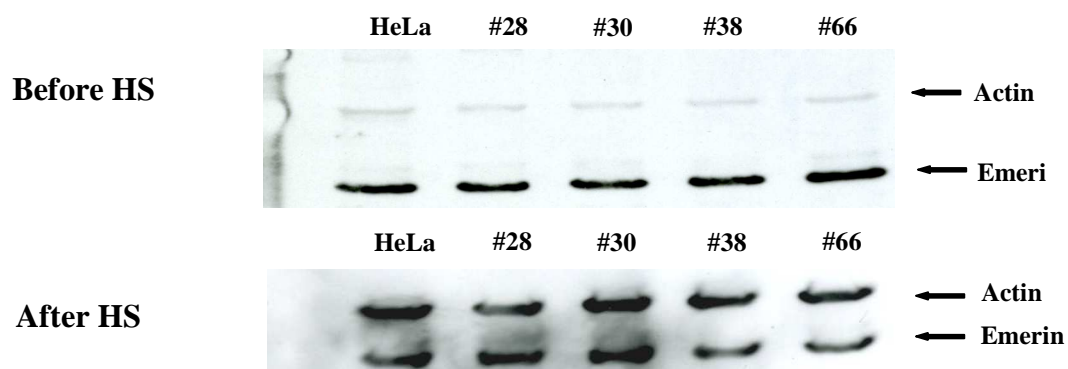


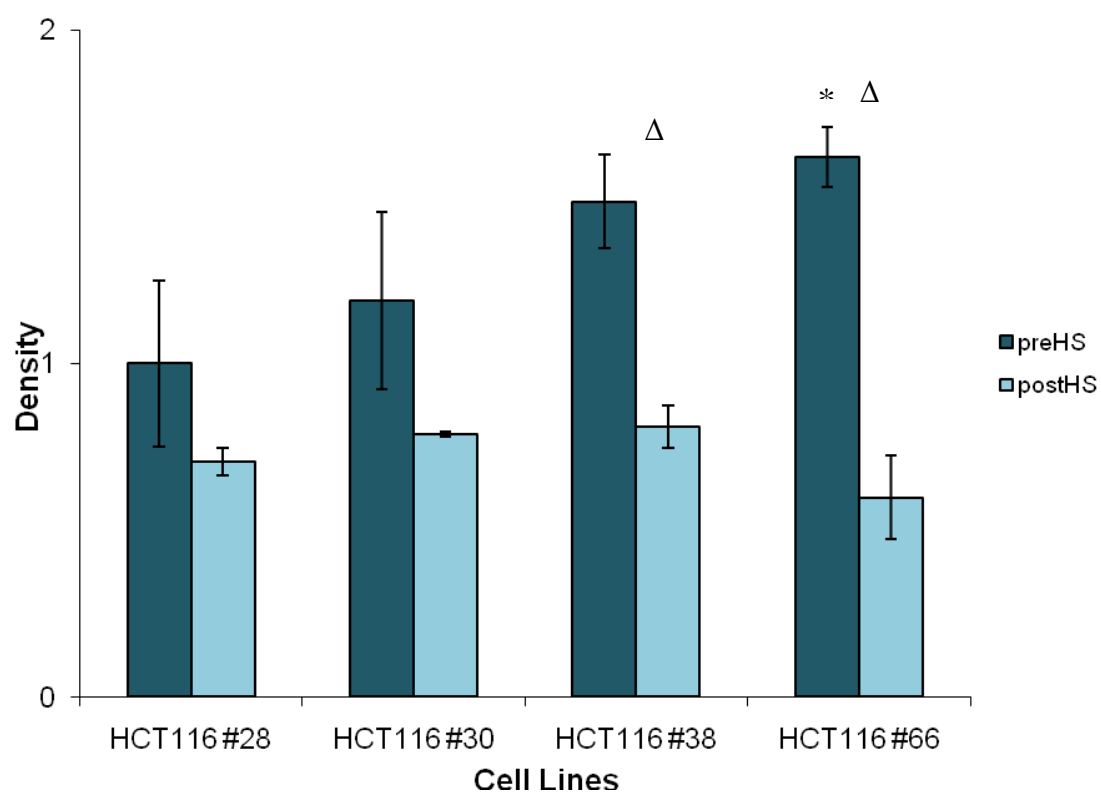
FIGURE 15: Immunoblotting analysis and expression levels of emerin before and after HS. **A)** Cell homogenates were subjected to an SDS-PAGE gel analysis then transferred to a nitrocellulose membrane and exposed to a primary anti-actin and anti-emerin. **B)** Quantification of emerin where the star represents significant differences from the parental cell line and triangles significant differences for before and after HS. A repeated measures ANOVA ($\alpha=0.05$) before HS, reveals there is no significant difference in expression in the parental cell line (p -value=1.000) compared to HeLa. Clone #30 displays no significant change in expression compared the parental cell line (p -value=1.000). Similarly, clone #38 shows a no significant differences from its parental cell line (p -value=0.062). However, clone #66 displays significant up-regulation when compared to the parental cell line (p -value=0.008) respectively. After HS, the parental cell line displays a significant reduction when compared to HeLa (p -value=0.001). Clones #30, #38 and #66 display similar results when compared to the parental cell line (p -value=1.000), (p -value=0.753) and (p -value=0.763) respectively. When observing the response to HS, a one way ANOVA shows the parental cell line and clone #30 show no significant reductions (p -value=0.242) and (p -value=0.167) respectively. However, a significant reduction in expression is noted in clones #38 (p -value=0.025) and clone #66 (p -value=0.002) respectively.

A



B

Variation of expression levels of emerin



After analyzing the expression and localization of emerin, we moved on to perform the same experiments to analyze LAP2. LAP2 is found in several isoforms of varying lengths. As mentioned before, these isoforms are all found within the INM except for LAP2 α that lacks the trans-membrane domain and is thus found bound within the nuclear Lamina at the interface of the INM and the nuclear lumen. For the purpose of simplifying the methodology and for reasons of time constraints, we localized the LAP2 α , β and γ isoforms with an isoform non-specific antibody but quantified expression of only the LAP2 γ with an isoform specific antibody. Figures 16 and 17 depict the localization of the aforementioned LAP2 isoforms. There is no method for discriminating between the INM and the nuclear Lamina. As such, we assume before and after HS, LAP2 isoforms are observed within known constraint proper to each isoform's biochemical properties. This assumption is justified since the signal is clean and undiffused to other areas of the nucleus. Although these results were expected, it should be noted that variations in the localization of LAP2 or of other nuclear proteins would potentially have serious consequences for the cell.

We then proceeded to analyze the expression levels of LAP2 γ before and after HS as shown in Figure 18. Interestingly, unlike other nuclear antigens analyzed in this study, the expression of LAP2 remains relatively stable between cell lines. In fact, the parental cell line shows a significantly increased expression of LAP2 compared to HeLa cells, before HS. This increase is also seen in all derivatives of this parental cell line. Moreover, clone #30 depicts a significant increase in expression when compared to the parental cell line. After HS, the parental cell line and clone #30 show significant reductions in expression. Clones #38 and #66 also show a decrease in expression

FIGURE 16: Distribution of LAP2 INM antigens before HS. Three independent samples of all cell lines harvested before HS were labelled with anti-LAP2. Results from 100 cells per sample display similar results to emerin; LAP2 is exclusively limited to the periphery of the nucleus and is excluded from within. Once more nuclear shape variations are notable. Cells are shown at 1000x magnification and scale bar is 5 μ m.

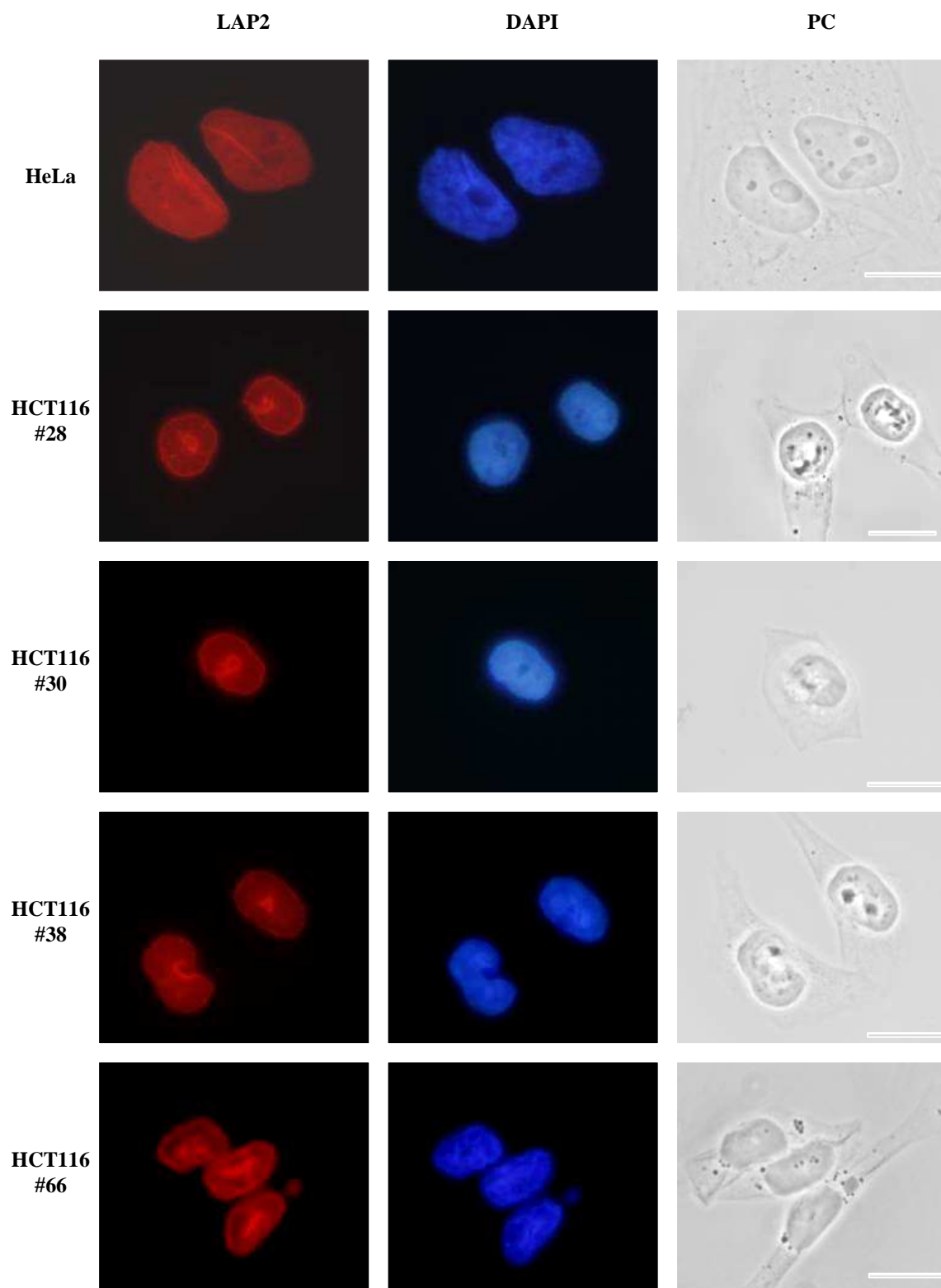


FIGURE 17: Distribution of LAP2 INM antigens after HS. Independent samples of HeLa and HCT116 clones harvested after HS were labelled with anti-LAP2. Results of 100 cells per sample show similar nuclear shape variations between cell lines as well as localization of the antigen as before HS. Cells are shown at 1000x magnification and scale bar is 5 μ m.

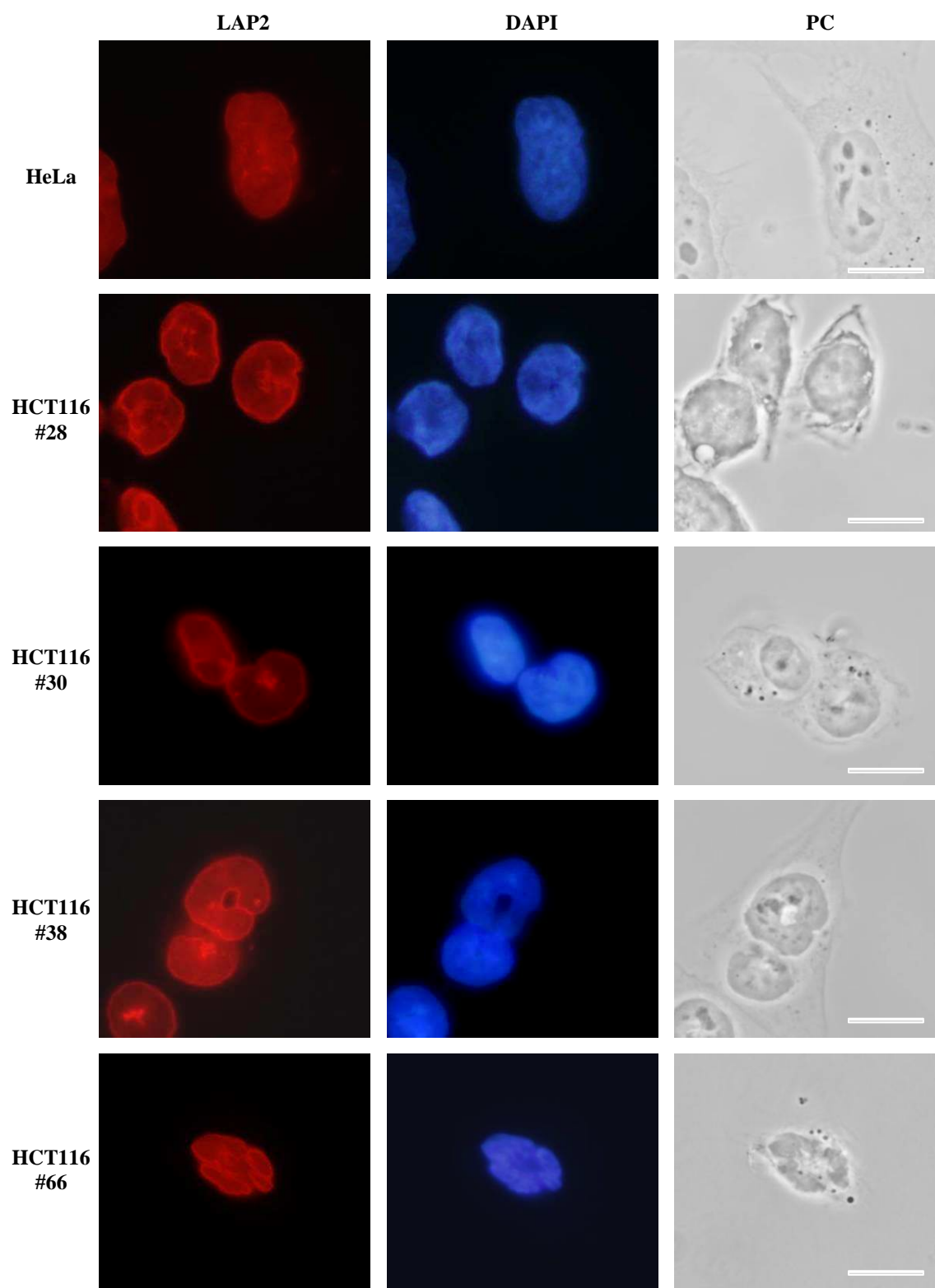
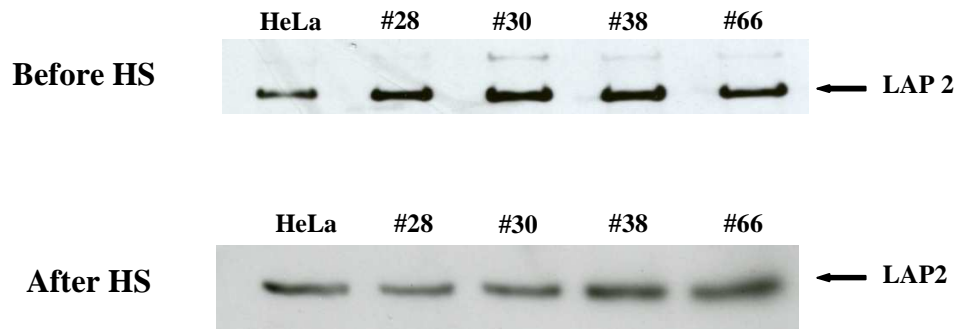


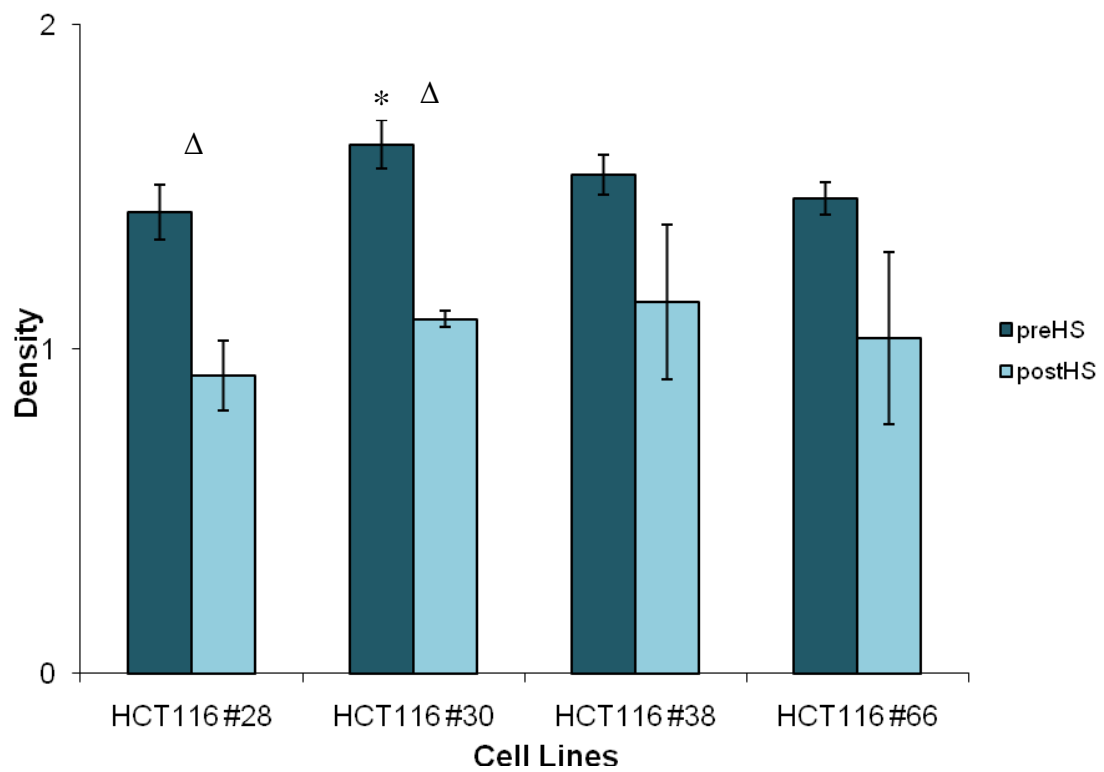
FIGURE 18: Immunoblotting analysis and expression levels of LAP2 before and after HS. **A)** Cell homogenates were subjected to an SDS-PAGE gel analysis, transferred to a nitrocellulose membrane and exposed to primary anti-actin and anti-LAP2 antibodies. **B)** Quantification of LAP2 γ where the star represents significant differences from the parental cell line and triangles significant differences following HS. A repeated measures ANOVA ($\alpha=0.05$) in absence of HS, reveals significant up-regulations in the expression on LAP2 γ in the parental cell line (p -value=0.000) when compared to HeLa. When compared to the parental cell line, only clone # 30 is significantly up-regulated (p -value=0.019) while clone #38 and #66 are not (p -value=0.362) and (p -value=1.000) respectively. After HS, the parental cell line displays no significant changes from HeLa (p -value=1.000). When compared to the parental cell line, clones #30, #38 and #66 do not display significant changes (p -value=1.000) in all cases. When observing the response to HS, a one way ANOVA shows the parental cell line and clone #30 show significant reductions in expression of LAP2 (p -value=0.035) and (p -value=0.011) respectively. However, no significant reduction in expression is noted in clones #38 (p -value=0.152) and clone #66 (p -value=0.149) respectively.

A



B

Variation of expression levels of LAP 2



compared to the parental cell line. However these decreases did not reach statistical significance.

2.2.2 Nuclear Lamins

In this final section of our study, in light of the results we have obtained pertaining to nuclear shapes and sizes, we proceeded to evaluate the integrity of nuclei of our epigenetically modified cell lines. As detailed earlier, nuclear shape is a strong indicator of general cellular health. Nuclei of healthy cells are generally smooth, circular or semi circular structures. However, in many cases, the cell line derivatives used in this study had smaller, misshapen nuclei accompanied by derived nuclear structures. Together these observations suggest the integrity of the nucleus has been affected or at the very least is not stable. Here, we describe the localization and the expression levels of Lamins A and B, which are responsible for nuclear strength and integrity.

Figures 19 through to 22 illustrate the localization of Lamins A and B before and after HS. Although not integrated directly into the INM, Lamins A and B represent a major part of the nuclear Lamina which is situated inside the nucleus directly associated to the INM and other proteins, as described earlier. Both before and after HS, images show similar localization of the Lamin A and Lamin B antigens. This observation is important for two reasons. First, it suggests the modified clones express essential nuclear Lamina proteins and second that these are well localized within their expected constraints. However, our goal here was more geared to observe nuclear shape which is an indicator of nuclear and cellular health. HeLa nuclei are smooth and circular, thus

healthy cells. Although smaller, nuclei from the parental cell line are similar in morphology to HeLa nuclei. Nonetheless, the shape and texture of nuclei from a vast majority of the epigenetically modified clones are notably different. Moreover, many of these cells also demonstrated a type of nuclear blebbing (data not shown), which can suggest two things. First, the genome of these cells may not be very stable. As cells age in culture, the nucleus will bleb to form derived structures. Second, the levels at which Lamins A and B are expressed may not be sufficient to support a strong nucleus and as such it will collapse and form alternative structures.

In order to further elucidate this situation, we proceeded to study the levels at which Lamins A and B are expressed before and after HS as well as 24hrs post HS (data not shown) as depicted in figures 23 and 24. Before HS, the expression of Lamin A in the parental cell line is not significantly changed from HeLa cells. In clones #30 and #38, a significant reduction in the expression of Lamin A is observed compared the parental cell line. Conversely, clone #66 expresses Lamin A at levels comparable to the parental cell line. After HS, a significant reduction in the expression of Lamin A in the parental cell line is observed. Clone #30 depicts a significant reduction when compared to the parental cell line. However, clone #38 displays a non-significant increase in expression. Conversely, clone #66 displays a significant increase in the expression of Lamin A compared to the parental cell line. However the expression is comparable to levels before HS in this clone. The expression of Lamin A in both of these clones does not seem to be affected by HS to the same level as with other proteins or in other clones.

FIGURE 19: Distribution of the Lamin A nuclear antigen before HS. HeLa and HCT116 clones were harvested into three independent samples before HS and labelled with anti-Lamin A. As previously with emerin and LAP2, the localization of the protein is exclusive to the periphery of the nucleus as noted through the observation of 100 cells per sample. However here, Lamin A is depicted loosely around the nuclear membrane, nonetheless excluded from within. Variations in nuclear shape can also be seen here. Cells are shown at 1000x magnification and scale bar is 5 μ m

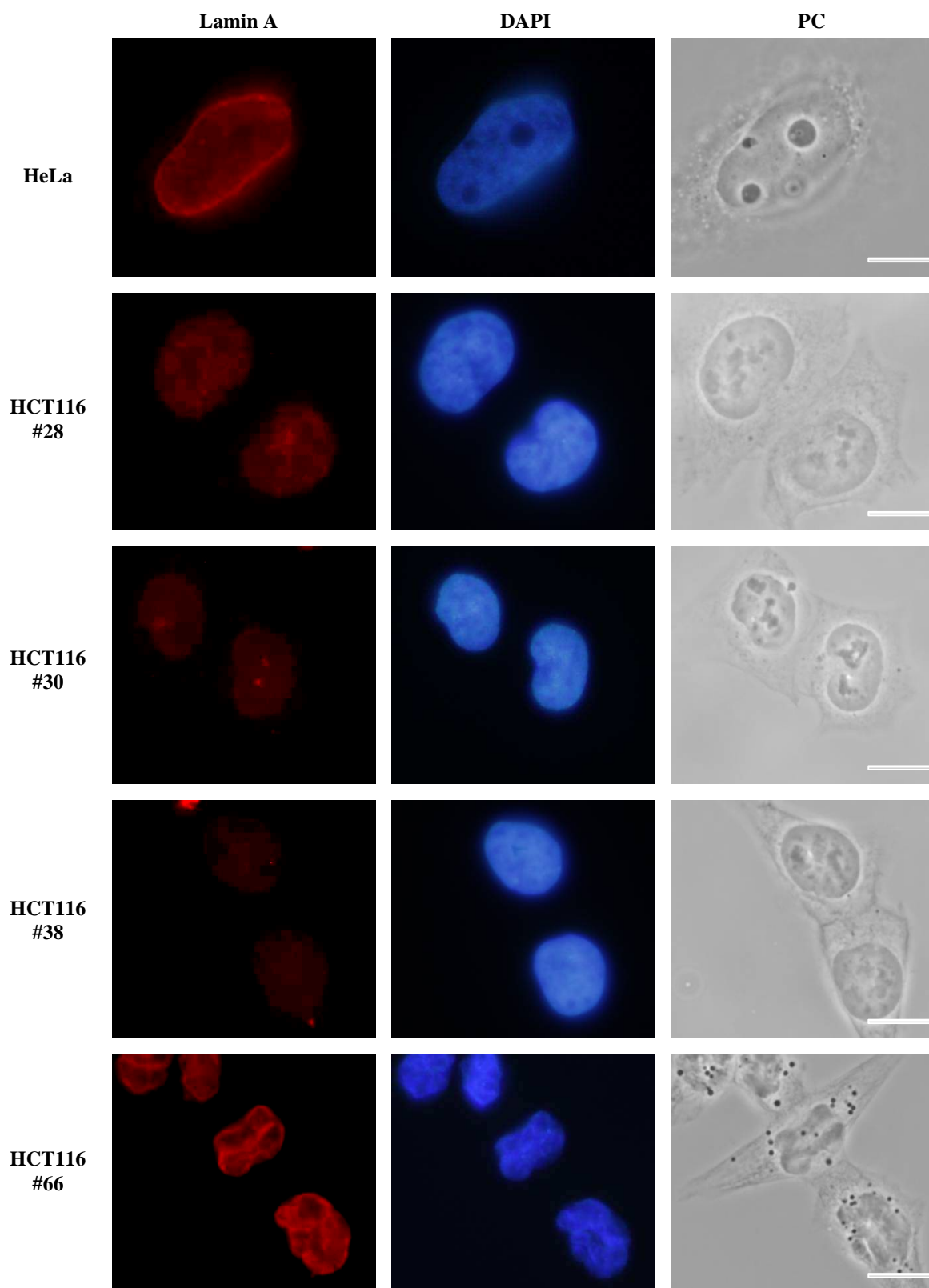


FIGURE 20: Distribution of the Lamin A nuclear antigen after HS. HeLa and HCT116 clones were harvested after HS and treated as above. After HS results are similar in terms of nuclear shape and protein localization. Cells are shown at 1000x magnification and scale bar is 5 μ m.

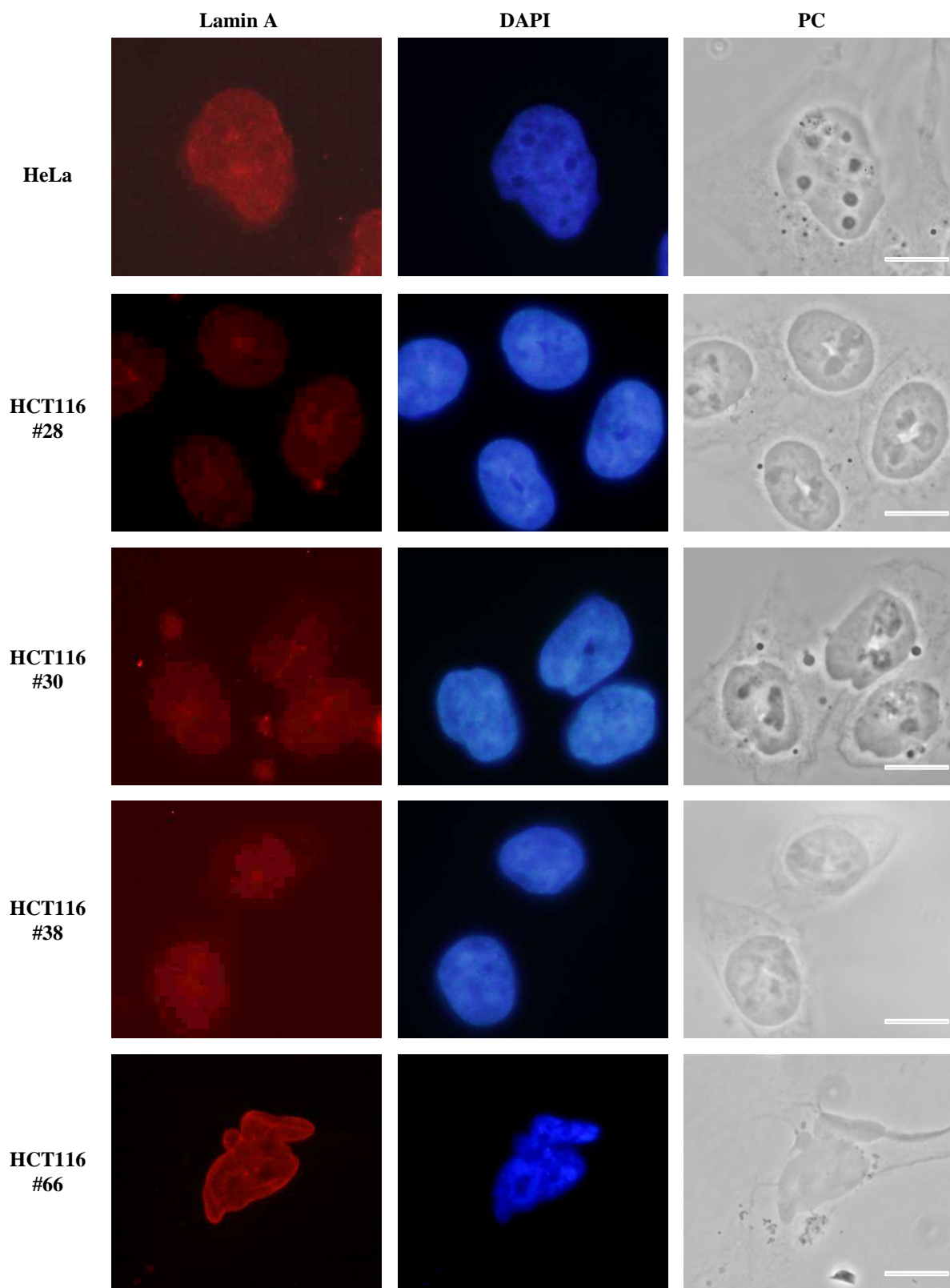


FIGURE 21: Distribution of the Lamin B nuclear antigen before HS. HeLa and HCT116 clones harvested before HS labelled with anti-Lamin B. 100 cells from 3 independent samples per cell line show that similarly to Lamin A, Lamin B is depicted at the nuclear membrane excluded from within. Unlike Lamin A, the localization is clean at the nuclear periphery. Once more, variations in nuclear shapes are visible. Cells are shown at 1000x magnification and scale bar is 5 μ m

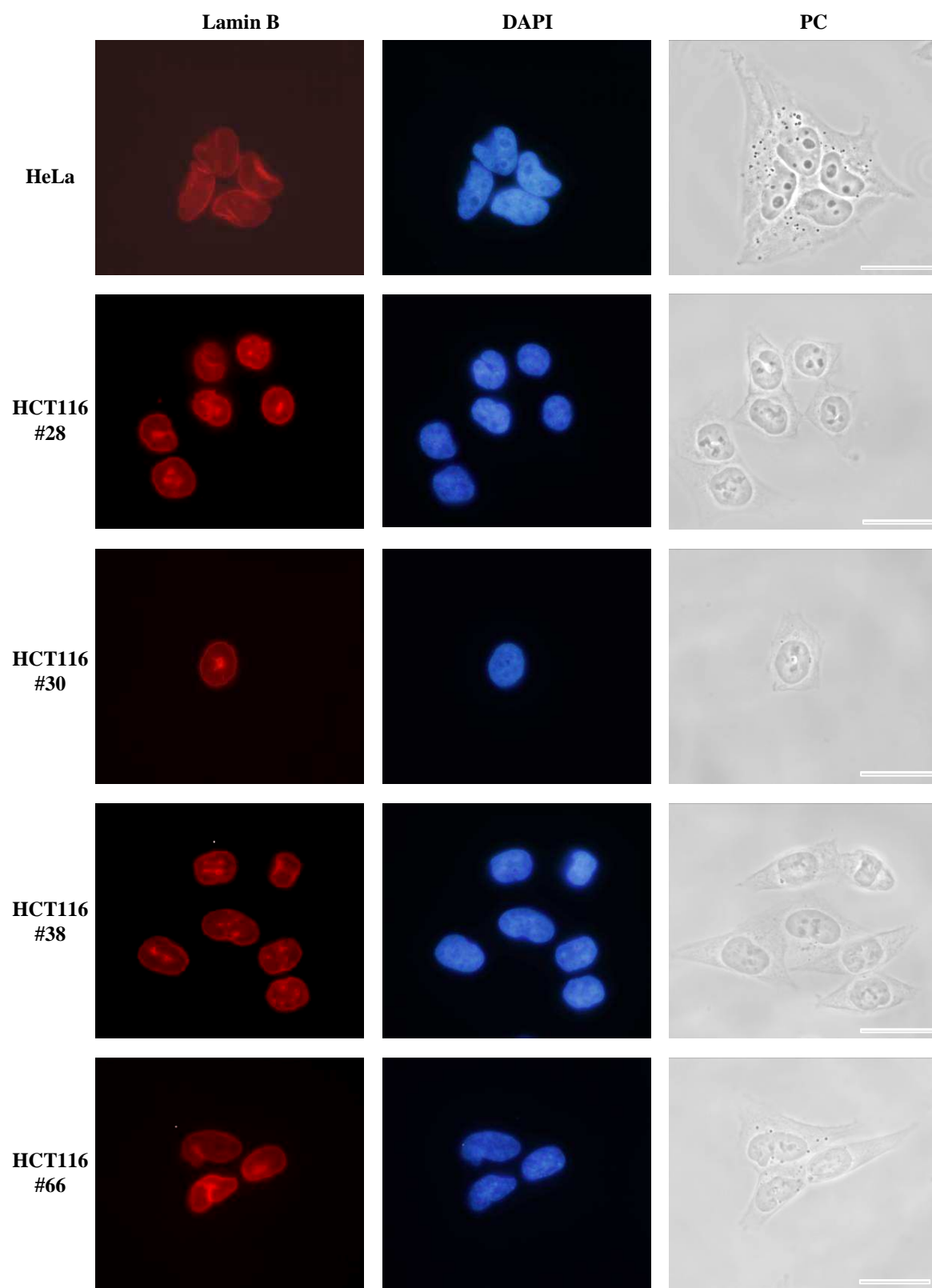


FIGURE 22: Distribution of the Lamin B nuclear antigen after HS. Described cell lines harvested after HS were labelled with anti-Lamin B. Results from 100 cells per sample from 3 independent samples for each cell line after HS are comparable to what is observed and described previously with Lamin B before HS. Cells are shown at 1000x magnification and scale bar is 5 μ m.

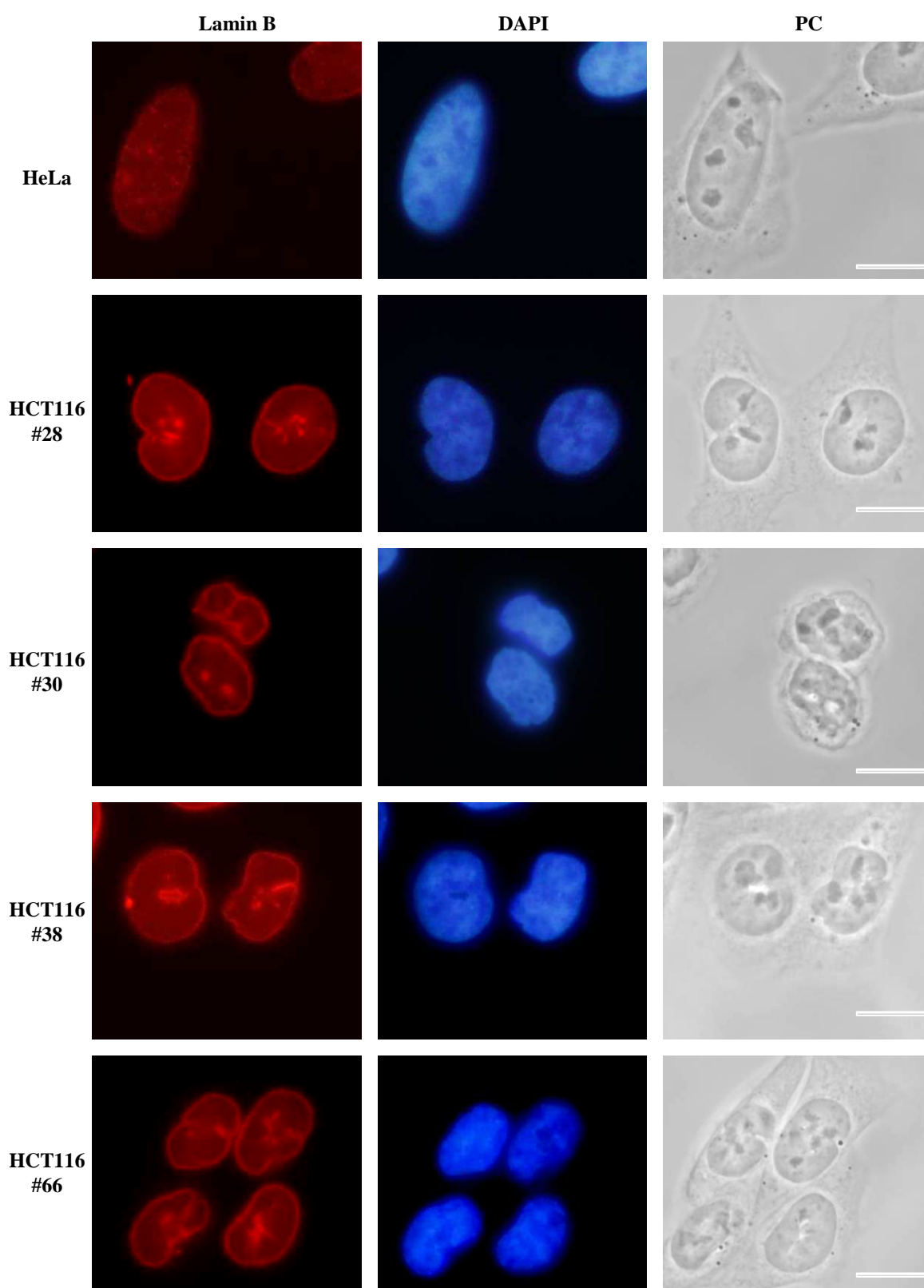
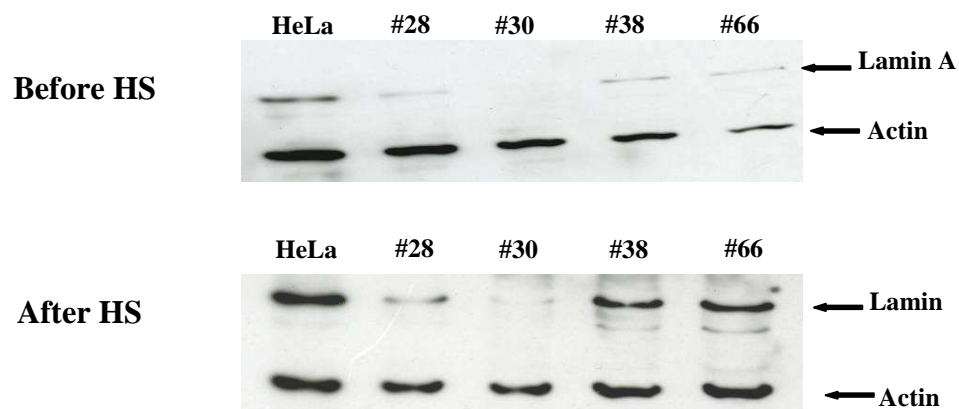


FIGURE 25: Immunoblotting analysis and expression levels of Lamin A before and after HS. **A)** Cell homogenates were subjected to an SDS-PAGE gel analysis then transferred to a nitrocellulose membrane and exposed to anti-actin and anti-Lamin A. **B)** Quantification of Lamin A where the star represents significant differences from the parental cell line and triangles significant differences for before and after HS. A repeated measures ANOVA ($\alpha=0.05$) before HS, reveals no significant difference of expression in the parental cell line (p -value=1.000) compared to HeLa. Clone #30 displays significant down regulation compared to parental cell line (p -value=0.005). Clone #38 also depicts a significant down regulation when compared the parental cell line (p -value=0.008). Clone #66 displays a non-significant up-regulations when compared the parental cell line (p -value=1.000). After HS, the parental cell line displays a significant reduction when compared to HeLa (p -value=0.002). Clone#30 displays a non-significant down regulation from clone #28 (p -value=1.000). Clone #38 displays no significant changes when compared to the parental cell line (p -value=0.211). Clone #66 depicts a significant up-regulation when compared to the parental cell line (p -value=0.006). When observing the response to HS, a one way ANOVA shows clone #28 depicts a significant reduction in expression (p -value=0.004) while clones #30, #38 and # 66 do not (p -value=0.057) (p -value=0.326) (p -value=0.317) respectively.

A



B

Variation of expression levels of Lamin A

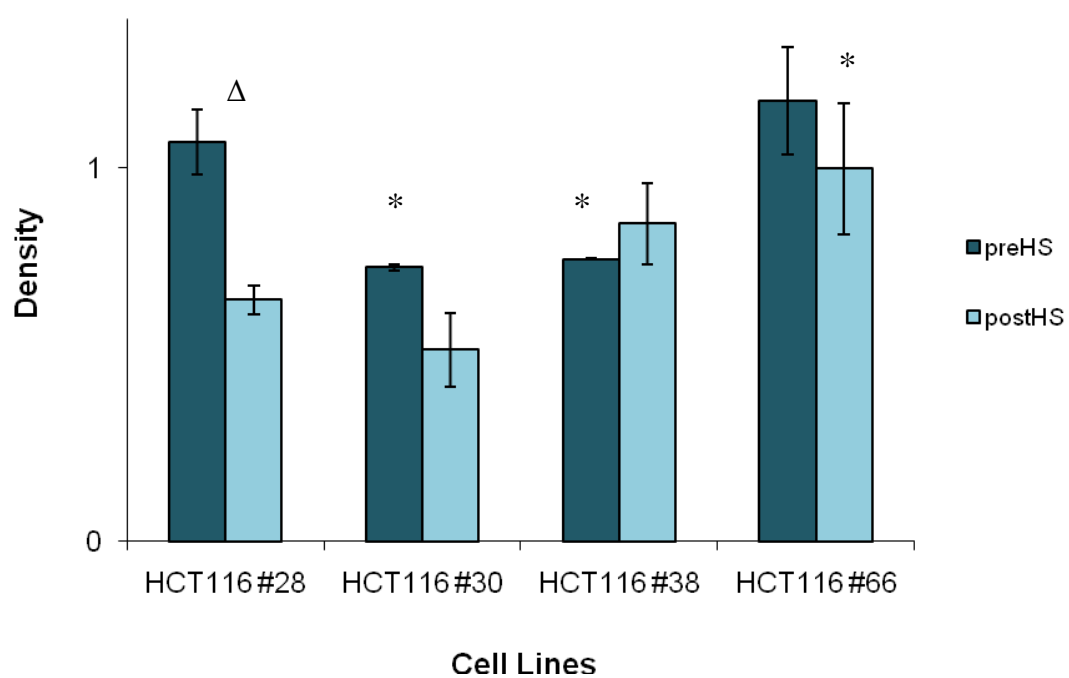
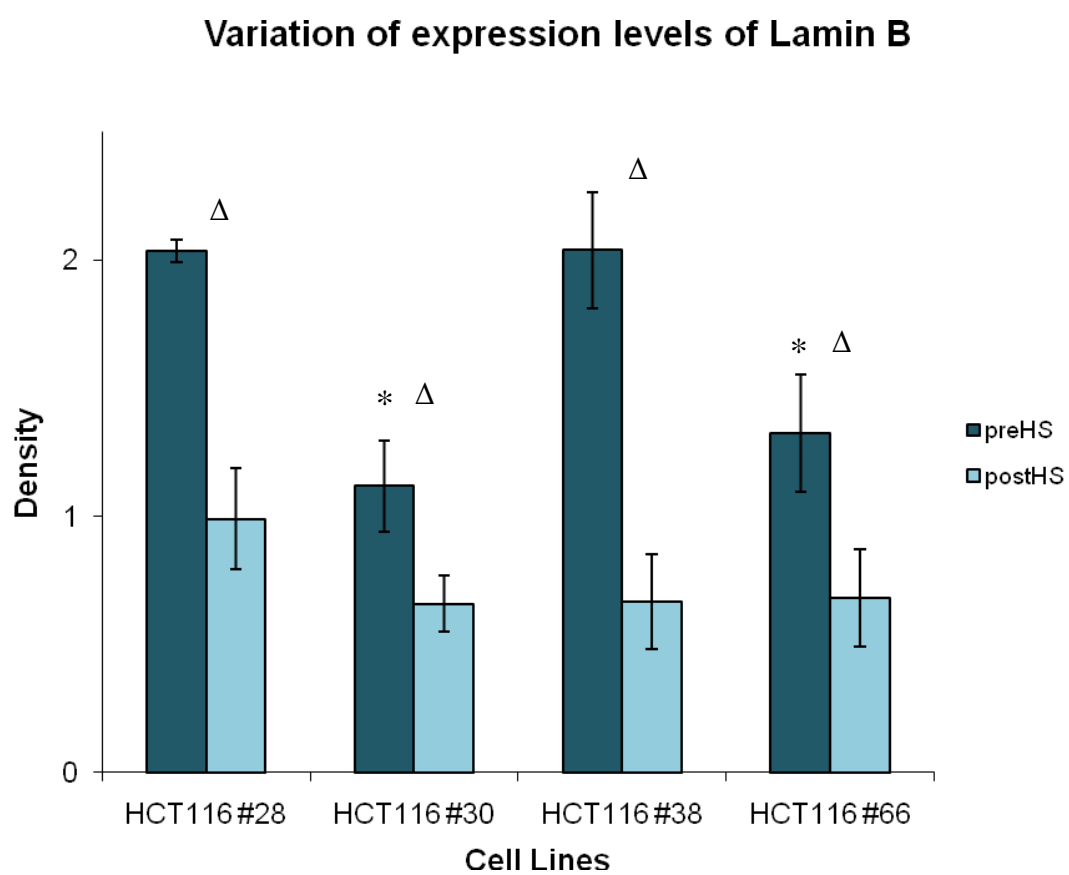


FIGURE 24: Immunoblotting analysis and expression levels of Lamin B before and after HS. **A)** Cell homogenates were subjected to an SDS-PAGE gel analysis then transferred to a nitrocellulose membrane and exposed to anti-actin and anti-Lamin B. **B)** Quantification of Lamin B where the star represents significant differences from the parental cell line and triangles significant differences for before and after HS. A repeated measures ANOVA ($\alpha=0.05$) before HS, reveals a significant difference of expression in the parental cell line (p -value=0.000) compared to HeLa. Clone #30 displays a significant down-regulation from the parental cell line (p -value=0.000). Clone #38 depicts a no significant changes from the parental cell line (p -value=1.000). Clone #66 displays a significant reduction when compared to the parental cell line (p -value=0.001). After HS, the parental cell line displays results similar to HeLa (p -value=1.000). Clone#30 displays non significant down regulation from clone #28 (p -value=0.149). Clone #38 displays no significant changes when compared to the parental cell line (p -value=0.169). Clone #66 depicts no significant change from the parental cell line (p -value=0.213). When observing the response to HS, a one way ANOVA shows clone #28, #30, #28 and #66 depict a significant reduction in expression (p -value=0.001), (p -value=0.020), (p -value=0.001) and (p -value=0.020) respectively.

A



B



Before HS the expression levels of Lamin B are variable. In fact before HS the expression in the parental cell line is 2x more elevated than in HeLa cells. Clone #30 expresses Lamin B to a similar level as HeLa cells meaning a significant reduction in expression from the parental cell line. Clone #38 displays similar levels as the parental cell line. Lastly, clone # 66 expresses Lamin B to levels similar to those of clone #30 thus significantly less than the parental cell line. After HS, the expression profile is much less variable. In fact, all cell lines display a significant decrease in the expression of Lamin B. In the parental cell line, the expression drops to a level comparable to HeLa cells. In all other clones, the expression is lower but not significantly different from the parental cell line.

As expected by the images obtained through immunofluorescence microscopy the expression of Lamins A and B seems to be variable between cell lines, which could mean the integrity, and strength of the nucleus is affected. In order to determine if that is the case, a mechanical analysis of the elasticity of the nuclear membrane was performed. This was done in collaboration with the laboratory of Dr. Andrew Pelling, Department of Physics at the University of Ottawa. Appendix 1 depicts the stiffness of the nucleus in kilo Pascals (kPa). Stiffness is a degree of elasticity. By definition, a rigid surface is able to withstand more pressure and thus is more elastic. The graph in Appendix 1 seemingly places HeLa cells and HCT116 cells in two different categories in terms of nuclear membrane rigidity, the latter being less elastic i.e. able to withstand lesser amounts of pressure. That being said, clone #30 and #38 are more rigid than the parental cell line and clone #66. Although small, the differences in nuclear stiffness were highly significant between clones. As detailed in this same appendix, clones #30, #38 and #66

are significantly different from the parental cell line (p -value=0.000), (p -value=0.000), (p -value=0.000) respectively.

There is no clear correlation between stiffness measurements and levels of Lamin expression. Being a descriptive analysis, causality is hard to determine. However, assuming causality, the observations between the expression of these proteins and nuclear stiffness would have been more similar than what is depicted. Nevertheless, immunofluorescence microscopy of HCT116 clones has revealed changes in nuclear sizes, shapes and in some cases numbers. These data lead us to believe that although the adequate expression levels of Lamins A and B are essential for nuclear integrity, perhaps another factor such as nuclear Lamina assembly also plays an important role.

IV. DISCUSSION

In this study, the main goals were to determine if epigenetic modifications would alter the ability of a cell to initiate and maintain a HS response through the formation of nSBs. In addition, we set out to evaluate if changes in methylation levels would have effects on the nucleus. More specifically, measurements of nucleoli and INM proteins were obtained before and after HS in modified HCT116 clones and compared to a non-modified HCT116 cell line. The parental cell line was then compared to the well characterized HeLa cell line in order to identify natural variations between both cell lines. Results lead us to conclude that the epigenetic modifications comprised within HCT116 clones #30 #38 and #66 do not hinder the initiation of a HS response. However, the

extent of the response may be affected as demonstrated by Rc and nSB amounts. Moreover, epigenetic modifications seem to have an impact on nuclear shape. Finally, results also showed epigenetic modifications may have a mild effect on the expression of INM and nuclear Lamin proteins.

1. nSB assembly and expression of splicing factors

In this part of the study, the objective was to determine if epigenetic status will have an impact on the ability of a cell to induce and maintain a HS response. This was also the main focus of the project. In order to assess this, we observed the expression levels and the localization of SF2/ASF as well as localization of SatIII sequences before and after HS.

1.1. SF2/ASF

The localization of SF2/ASF followed expected patterns. It was diffuse in the nucleus before HS and aggregated within granules speculated to be nSBs after HS. As reported earlier, on average, the parental cell line and clones #30 #38 and #66 presented 4, 5, 6 and 6 nSBs respectively. However, most cells displayed 2, 4, 6 and 2 nSBs respectively. Throughout the cell lines, none showed less than one nSB nor more than 15 nSBs. The expression of SF2/ASF before HS was variable. In fact, the parental cell line expresses the splicing factor at a level that is similar to HeLa cells. Furthermore, clone #38 expressed SF2/ASF at a level comparable to the parental cell line. This is not surprising since clone #38 possess a mere 3% reduction in total methylation thus resembles the parental cell line the most. However, clones #30 and #66 depict a

significant reduction in SF2/ASF expression when compared to the parental cell line. It seems partial loss of function of DNMT1 and/or a 20% loss of total methylation translates into a down-regulation of the splicing factor regardless of DNMT3b. After HS, the parental cell line showed a significant reduction in expression compared to HeLa and itself before HS. Clone #38 presents similar expression levels when compared to the parental cell line while clone #66 depicts a significant reduction. However clone #30 shows only relative response to HS expressing SF2/ASF at a level significantly higher than the parental cell line while showing very little down-regulation compared to results before HS. Given the apparent sensitivity of this cell line illustrated by the lower expression level before HS as well as in clone #66, this was not expected. Nevertheless, there seems to exist a correlation between the Rc and the expression of the splicing factor after HS. The higher the Rc the higher are the expression levels. This is an interesting fact because it may mean that HS response is in part dependant on the availability of essential proteins and not on general cellular health or epigenetic status. As such we propose that the more the protein is expressed the more available it is to partake in the HS response and the stronger this response will be. Further inquiries should perform similar experiments with other known nSB constituents such as the HSF 1 transcription factor, and the SRp30 and 9G8 splicing factors among others in order to determine if this correlation is also observed (Biamonti and Vourc'h, 2010). When compared to nSB amounts, there does not seem to exist such a correlation. In light of these results, we conclude that epigenetic modifications do not impede on the ability of a cell to generate stress granules as seen with SF2/ASF aggregates, but the extent at which cells respond through modification in the numbers of Rc and nSB may depend on methylation states.

1.2. SatIII

Initial steps in nSB formation involve DNA rearrangements and demethylation in areas known to contain SatIII DNA. Shortly thereafter, there is transcription of an array of SatIII sequences of variable lengthHS. Sat III sequences have been proposed to be the committing step to nSB formation as a recruiter of secondary constituents such as SF2/ASF which possess RRM and are able to recruit other proteins to the area. The end result is an aggregate of RNA and proteins now termed as nSBs. In this part of the study, the goal was to confirm that what had been observed thus far were in fact nSBs. As such, the localization of SatIII sequences was determined through colocalization studies of SF2/ASF and SatIII. Experiments with forward, reverse and control probes were performed (data not shown for the reverse and control probes). Results show that before HS no SatIII signal is detected in FISH assays. Moreover, as before the SF2/ASF was diffused within the nucleus excluded from nucleoli. However, after HS, punctual aggregates of SatIII are visible. Moreover, the aggregates are perfectly aligned with SF2/ASF aggregates in all cell lines. As such we conclude that what had been observed in the previous part of the study were in fact nSBs.

2. Nuclear antigens in relation to HS

2.1. Nucleolar antigens

Nucleoli are essential for cell survival. In this part of our study we proceeded to analyze the effect epigenetic modifications might have on these regions through the analysis of 2H12 and fibrillarin. These are known to be essential for the structure and integrity of this specialized nuclear region. Specifically, the 2H12 protein is thought to

be essential in nucleolar structure maintenance and retention of key molecules in the ribonucleoprotein complex (Paulin-Levasseur, M. *et al.*, 1995). Fibrillarin is known to be associated with nucleoli, the largest transcription factories of ribosomal subunits, and localized on NORs during metaphase and anaphase and also reported to be a marker of newly forming nucleoli in telophase (OcHS, R. L. *et al.*, 1985; Bartova, E. *et al.*, 2010).

My results had shown very similar localization of 2H12 as well as fibrillarin before and after HS in all clones for both antigens. Before HS, the expression of 2H12 indicates HCT116 #30, #38 and #66 displayed similar levels when compared to the parental cell line which in turn expressed 2H12 at a level similar to HeLa. With respect to the fact that 2H12 studies have been conducted in HeLa cells, it is thought that this cell line possesses a “normal” or well characterized level of 2H12 as such we propose all cell lines express healthy levels of 2H12 (Paulin-Levasseur *et al.*, 1995). After HS, only the parental cell line and clone #38 has a significant reduction in 2H12 expression while clone #30 and #66 show a reduction in expression that is not significantly different from the parental cell line. As such while the significance of results varies between clones after HS, all express similar levels of 2H12 when compared to the parental cell line. These observations lead us to conclude that loss of DNMT enzymes does not affect the integrity of the nucleoli. Moreover, fibrillarin was observed within its normal constraints. This led us to believe epigenetic modifications will not hinder its function. Unfortunately, the unavailability of an antibody against fibrillarin that performs adequately in immunoblotting analyses precluded the assessment of measuring the expression levels.

2.2. LEM proteins

As detailed earlier, emerin is an essential protein for development. In fact its loss is associated with a form of muscular dystrophy. Moreover, it is able to bond with Lamins A and B making emerin a key player in the integrity of the nuclear structure; BAF and GCL thus DNA and MAN1 through BAF (Tews, 1999; Lin *et al.*, 2000, Holaska *et al.* 2003; Shimi *et al.*, 2003). In all cell lines, before and after HS, emerin was observed at the nuclear membrane. Before HS, the expression of emerin in the parental cell line is comparable to HeLa. Moreover clone #30 expresses similar levels of emerin as the parental cell line. Clones #38 and #66 express higher levels when compared the parental cell line. This was significant in the case of #66. In itself we do not view higher levels of emerin to be a negative thing. In fact past studies have only demonstrated a negative impact in cells with no emerin expression i.e. developmental disorders. Conversely, after HS, all three modified clones expressed comparable levels of emerin which resemble that of the parental cell line. Furthermore, the parental cell line expressed emerin to a level significantly lower than HeLa. Lastly, clones #38 and #66 show a significant drop in expression compared to results prior to HS. Although the loss DNMT enzymes seem to have an impact on the expression of emerin before HS, this is not the case after HS. As such we conclude that the expression of emerin post HS in the modified clones is not affected by epigenetic modifications since the parental cell line naturally expresses lower levels of emerin. Nevertheless, in light of the fact that loss or reduction in emerin expression leads to the up regulation of 28 other genes (Tsukahara, T., 2002), the observations made in this section are quite important. It is not known if the above 28 genes or a subset of these are expressed during the establishment of a HS

response. This could be the object of a future study. However, significant reductions in emerin will surely translate in reduced interactions with its binding partners which in turn will induce significant changes in protein-protein and protein-DNA interactions at the nuclear periphery.

LAP2 isoforms are known to interact with several other proteins located at or within the nuclear Lamina. In this part of the study we specifically focused on LAP2 γ which interacts with Lamin B1, BAF as well as DNA. As such, this isoform plays a key role in the attachment of DNA at the nuclear periphery as well as a site of anchor for the nuclear Lamina. Changes in the availability of this protein would have effects on DNA anchoring and anchoring of the nuclear Lamina to the nuclear periphery. Before HS, the parental cell line and all other clones display increases in LAP2 γ expression independently of which DNMT enzyme is available or partly functional. Moreover, clone #30 displays LAP2 γ levels significantly higher than the parental cell line. Given what has been observed in immunofluorescence assays, it is safe to say that DNA methylation does not affect the availability, the location or the function of LAP2 γ . After, HS levels of LAP2 γ are reduced and are similar in all clones to the parental cell line. Moreover, there is a significant loss of expression in the parental cell line and clone #30 while non-significant in clones #38 and #66 when compared to levels before HS. As such, we suggest a drop in LAP2 γ could be an innate response to HS. However, none of the examined cell lines display expression levels that are different from HeLa. As such we conclude that HS seemingly has no effect on the function of LAP2 γ , thus it maintains its role at the interface of the INM and nuclear lumen.

2.3. Nuclear Lamins A and B

The nuclear Lamina is an intricate meshwork of proteins mainly composed of A type and B type Lamins; Lamins A, C and B1, B2 (Worman, 1988 and Worman and Bonne, 2007). The particular functions of Lamins A and B are similar in many aspects but are also very different in others. Lamin A, as detailed earlier, is reported to be primordial in nuclear stiffness (Lammerding *et al.*, 2006). Lamin B, however, is responsible for nuclear integrity and is able to form microdomains with epigenetically marked DNA to anchor it to the Lamina (Makatsori *et al.*, 2004; Delbarre *et al.*, 2006; Lammerding *et al.*, 2006). Most importantly, through their interactions with other INM proteins nuclear Lamins greatly affect epigenetically controlled pathways (Vlcek and Foisner, 2007). It is then not surprising that any modifications in localization or expression of these proteins could potentially spell serious effects on the nucleus itself and general cellular health through environmental stability and changes.

Immunofluorescence assays have demonstrated that before and after HS there is no significant change in the localization of Lamin A or Lamin B. In terms of expression however, some changes are noted. Before HS, the parental cell line expresses Lamin A to a level very similar to HeLa. However, clones # 30 and #38 which have reduced DNMT1 function and loss of DNMT 3b, respectively, show significant reductions in Lamin A expression. Moreover, this reduction is also significantly different from the parental cell line. Reduced Lamin expression can potentially result into a softer nucleus, as the indirect consequence of demethylation. Nonetheless, clone #66, the double mutant expresses Lamin A to a level higher than the parental cell line resembling more the normal levels seen in HeLa cells. This is unexpected and seems to go against the two

preceding observations. As detailed in earlier sections, modified clones possess decreases in overall DNA methylation. It should be noted that only methylation levels were assessed in these clones. No previous studies have examined exactly where methylation is retained. As such, we propose that the location at which DNA methylation is retained may have an impact on the expression of proteins, namely Lamin A.

Nuclear stiffness assays performed before HS reveal the parental cell line and clone #66 possess a nucleus that is softer than clones #30 and #38 which possess a nucleus with similar stiffness. Moreover, the nucleus of the parental cell line is softer than that of HeLa cells. Immediately it is evident that the patterns at which Lamin A is expressed follow the same trend as nuclear stiffness when comparing clones. However, the relationship is inverted. In fact, when comparing the graph for Lamin A expression to the graph for nuclear stiffness, more Lamin A in a clone seems to translate into a softer nucleus. This was not expected since it is in contradiction with known functions for Lamin A. As mentioned, the parental cell line presents a nucleus that is softer than HeLa cells. However, before HS, the parental cell lines express Lamin A to a similar level than HeLa cells. This might offer a clue into why we obtained such contradicting results. The fact that a softer nucleus paired with normal Lamin A expression is observed suggests Lamin A alone is not responsible for nuclear stiffness. As such, we propose more Lamin A does not necessarily translate into a stiffer nucleus. The opposite is also true; Lamins A expressed in lesser amounts does not necessarily translate into a softer nucleus. In sum, these observations lead us to conclude that changes in methylation due to partial or total loss of DNMT1 and DNMT3b respectively translates into modified expression of Lamin A which in turn is not solely related to changes in nuclear stiffness

before HS. This is not very surprising since lamin A possesses many different interaction partners that contribute to a stiff nucleus.

After HS, the expression of lamin A is significantly reduced in the parental cell line, which is expected. The same result is seen with clone #30. However, there is no significant change in the expression of lamin A in clones #38 and #66 contrarily to what was expected. For many other proteins, reductions in expression after HS are proposed to be due to lack of production and destruction of aging proteins. In the present case, lack of reduction post HS is not as easy to explain. Proteins age and are usually replaced accordingly. As such we propose that the loss of DNMT3b does not impede the maintenance of lamin A production after HS as observed with the to lack of down-regulation. How this occurs is not yet understood, however may be subject to further inquiries.

Before HS, the parental cell line expresses lamin B at a significant up-regulation of 2 fold compared to HeLa cells. Moreover, clone #38 expresses lamin B to a similar level as the parental cell line, while clones #30 and #66 display levels similar to HeLa significantly lower than the parental cell. As such we propose that partial loss of DNMT 1 function as seen in clones #30 and #66, leads to these changes in expression. However, the integrity of the nucleus does not seem to be jeopardized since none of the cell lines express levels of lamin B that are lower than HeLa known to have a nucleus with good integrity. After HS, all clones express significantly reduced levels of lamin B comparable to the parental cell line. Moreover, the parental cell line expresses lamin B to a level similar to HeLa cells. Interestingly, all clones express levels that are similar,

independently of pre-HS levels and of epigenetic status. As such we propose that partial loss of DNMT1 function and total loss of DNMT3b, which imply altered/impaired epigenetic modifications, do not alter the integrity of the nucleus after HS.

In terms of their interactions in epigenetically-controlled pathways, Lamins A and B are proposed to be affected at different levels. Evidently if a protein is expressed to lower levels, it is not available to perform the regulatory steps it is usually involved in. For example clones #30 and #38 express lower levels of Lamin A. However, in these two cell lines, Lamin B is expressed at normal or above normal levels. Although nuclear shape is affected in all epigenetically modified clones, they survive well in culture. As such we propose that effects imposed on the expression of Lamins A and B due to epigenetic modifications may be dampened by mutual complementation. As such, regulatory functions of both proteins may the most part be maintained.

General cellular metabolism results in the degradation of aging proteins. When ideal living conditions are met, these proteins are replaced by newer ones on a regular basis. When environmental stress is encountered such as HS, protein metabolism is altered. During that time several proteins i.e. RNAs are thought to be heavily subjected to alternative splicing (SF2/ASF) (Biamonti and Vourc'h, 2010). This is thought to impede on the production translation of proteins that are not essential for the recovery process i.e nSB formation. As such, many proteins usually found at high levels during interphase become much less available as degraded proteins are not replaced. Our observations show most proteins examined in this study demonstrate at least a modest if not significant drop in expression after HS except in one instance where Lamin A is

expressed at similar levels before and after HS due to loss of DNMT3b function. As such we propose these proteins are not vital to the recovery process.

V. CONCLUSIONS

In conclusion, I have demonstrated throughout this study that exposure to stressful environmental conditions such as HS induces a cascade of events independently of epigenetic modifications. More specifically, DNA demethylation is ensued leading to the expression of SatIII sequences and a re-localization of the SF2/ASF splicing factor within nSBs. I have also offered more evidence showing that HS response is in fact a series of non-apoptotic mechanisms which are not dependent on the general methylation state of DNA. Instead, response to HS involves coping and recovery processes that occur through rearrangements of several cellular components. The reduced expression of several proteins is proposed to be the consequence of a general cellular metabolic response. This is contrary to earlier thoughts which stipulate that programmed cell death or apoptosis is the only mechanism by which a cell can cope with non ideal conditions. Furthermore, the functions of the nuclear antigens examined in this study, for the most part, may not be affected by epigenetic modifications or HS. In addition, more evidence has come through suggesting Lamin A expression is not a limiting factor for nuclear stiffness. This is not surprising given the amount of proteins within the INM and the nuclear Lamina with similar interactions. Further studies particularly focusing on the transcription of RNAs leading to the expression of the proteins assessed in this study would provide even stronger arguments toward my findings. Taken as a whole, the results of this study shed

more light on diseases such as HGPS and others which involve general demethylation of DNA and are hypersensitive to environmental stress such as HS (Shumaker *et al.*, 2006). Although epigenetic modifications do not affect the ability to respond to HS, data has demonstrated that the extent of this response, through Rc and nSB amounts, may be affected. Recovery experiments would further elucidate this matter.

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APPENDIX 1 - Mechanically guided analysis of nuclear membrane elasticity;

nuclear strength assessment. In collaboration with Dr. Andrew Pelling, Department of Physics, University of Ottawa, a mechanically driven analysis of nuclear stiffness was conducted where the star represents significant difference from the parental cell line and the triangle from HeLa. HeLa and HCT116 cells were harvested before HS. A total of 25 randomly selected living cells per cell line were measured. Statistical analysis was performed and returned to us by Dr. Pelling's research team.

