AN INVESTIGATION INTO THE MOLECULAR AND EPIGENETIC ALTERATIONS ASSOCIATED WITH FUMONISIN B_1-INDUCED TOXICITY IN HUMAN LIVER (HEPG2) CELLS

By

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DECLARATION

I, **Thilona Arumugam**, declare that:

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The research described in this study was carried out in the Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, College of Health Sciences, University of Kwa-Zulu Natal, under the supervision of Professor A.A. Chuturgoon, and Dr T. Ghazi.

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Miss Thilona Arumugam

02/12/2020

Date

DEDICATION

To my parents, **Imantha** and **Erwin Arumugam**, for always believing in me and encouraging me to strive for excellence.

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ABBREVIATIONS

1-deoxySa	1-deoxysphinganine
2-AAF/PH	2-acetylaminofluorene/partial hepatecomy
5-Aza-2-dc	5-Aza-2-deoxycytidine
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5hmU	5-hydroxymethyluracil
5mC	5-methylcytosine
8-OHdG	8-hydroxy-2'-deoxyguanosine
A	Adenosine
AFB_1	Aflatoxin B1
AGO	Argonaute
AID/APOBEC	Activation-induced cytidine deaminase/apolipoprotein B mRNA-editing catalytic polypeptides
AKT	Protein kinase B
Apaf-1	apoptotic protease activating factor-1
ATP	Adenosine triphosphate
CAD	Caspase-activated deoxyribonuclease
CCM	Complete culture media
cDNA	Complementary DNA
ceRNA	Competing endogenous RNA
CERT	Ceramide transport protein
ChIP	Chromatin immunoprecipitation
CpG	Cytosine phosphate guanine
CS	Ceramide synthase
Ct	Comparative threshold cycle
Cul3	Cullin-3 E3-ubiquitin ligase
DDR	DNA damage response
DEHP	di-(2-ethylhexyl) phthalate
DEN	Diethylnitrosamine
DGCR8	DiGerorge Syndrome Critical Region 8
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DON	Deoxynivalenol

DR3	Death receptor 3
EDTA	Ethylenediaminetetraacetic acid
ELEM	Equine leukoencephalomalacia
EMEM	Eagles minimum essentials medium
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ETC	Electron transport chain
EZH2	Enhancer of zeste homolog 2
F6A	N6-formyladenosine
FADD	Fas-associated death domain
FasR	Fas receptor
FB_1	Fumonisin B_1
$GSTP^+$	Glutathione-S-transferase-positive
GGT+	Gamma-glutamyl-transpeptidase-positive
H	Histone
H2DCF-DA	2,7-dichlorodihydrofluorescein-diacetate
H3K4me3	Histone 3 lysine 4 tri-methylation
HAT	Histone acetyl transferase
HepG2	Human liver cell line
HDAC	Histone deacetylase
HFB_1	Hydrolysed FB_1
hm6A	N6-hydroxymethyladenosine
HOTAIR	HOX transcript antisense RNA
HOX11-AS	Homeobox A11 antisense RNA
IARC	International Agency of Research on Cancer
IL	Interleukin
INF-γ	Interferon gamma
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
K	Lysine
KDM	Histone lysine demethylase
Keap1	Kelch-like ECH-associated protein 1
KMT2	Histone lysine methyltransferase
LDH	Lactose dehydrogenase
lncRNA	Long non-coding RNA
LHX8	LIM Homeobox 8
m6A	N-6-methyladenosine

MAPK	Mitogen activated protein kinase
MBD	Methyl-CpG binding domain
MDM2	Murine double minute 2
MeCP2	Methyl CpG binding protein 2
METTL3	Methyltransferase-like-3
METTL14	Methyltransferase-like-14
miRISC	RNA induced silencing complex
miRNA	MicroRNA
MRE	MiRNA Response Element
MRN	MRe11-Rad 50-Nbs1
mRNA	Messenger RNA
ncRNA	Non-coding Ribonucleic acid
NFκB	Nuclear factor kappa B
NIC	Nivalenol
Nrf2	Nuclear factor erythroid 2-related factor 2
nt	Nucleotide
NTD	Neural tube defects
qPCR	Quantitative Polymerase Chain Reaction
PANDA	p21-associated ncRNA DNA damage -activated
PBS	Phosphate buffered saline
PH	Partial hepatectomy
$PHFB_1$	Partially hydrolysed FB¬1
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol-3,4,5-triphosphate
ppm	Part per million
PRC2	Polycomb repressive complex 2
pre-miRNA	Precursor-miRNA
pri-miRNA	Primary-miRNA
PTEN	Phosphatase and tensin homolog
R	Arginine
RBD	Relative band density
RBM	RNA binding protein
RIP	RNA immunoprecipitation
RNA	Ribonucleic acid
RNAP	RNA Polymerase
ROS	Reactive oxygen species

rRNA	ribosomal RNA
RT	Room temperature
Sa	Sphinganine
Sa1p	Sphinganine-1-phosphate
SAM	S-adenosylmethionine
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SET	Su(var)3-9, Enhancer-of-zester and Trithorax
shRNA	Small hairpin RNA
siRNA	Silencing RNA
siR-NC	Negative control siRNA
snRNA	Small nuclear RNAs
snoRNA	Small nucleolar RNAs
So	Sphingosine
So1P	Sphingosine-1-phosphate
SPK1	Sphingosine kinase 1
SPT	Serine palmitoyltransferase
START	Steroidogenic acute regulatory protein-related lipid transfer
TET	Ten-eleven translocation
TDG	Thymine DNA glycolase
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated death domain
TRAILR	TNF-related apoptosis inducing ligand receptor
tRNA	Transfer RNA
TTBS	Tween 20-Tris buffer saline
UHRF	Ubiquitin-like and ring finger domain 1
UPR	Unfolded protein response
UTR	Untranslated region
WHO	World Health Organization
WTAP	Wilm’s tumour 1-associated protein
YTHDC	YT521-B homology domain containing
YTHDF	YT521-B homology domain family

ABSTRACT

The contamination of agricultural commodities with *Fusarium* mycotoxins is a global issue in food safety, with fumonisin B_1 (FB_1) being the most prevalent contaminant. FB_1 is not only phytotoxic, but it induces a wide range of toxic effects in animals and humans and is associated with carcinogenesis in animals and humans. Intense research has uncovered several mechanisms by which FB_1 induces toxicity. Recent evidence suggests that epigenetic mechanisms may also contribute to the toxic effects of FB_1. Epigenetic modifications including DNA methylation, histone methylation, N-6-methyladenosine (m6A) RNA methylation, and non-coding RNAs such as microRNAs (miRNA) and long non-coding RNA (lncRNA) are central mediators of cellular function and cellular stress responses and disruption may be pertinent in FB_1-induced toxicities. This study aimed to determine the epigenetic mechanisms of FB_1-induced hepatotoxicity by specifically investigating changes in DNA methylation, histone 3 lysine 4 trimethylation (H3K4me3), m6A RNA modification, and noncoding RNA in human hepatoma (HepG2) cells. The effect of these FB_1-induced epigenetic modifications on stress responses was further investigated.

FB_1 impairs DNA repair processes via epigenetic mechanism. FB_1 reduced the expression of histone demethylase, KDM5B, which subsequently increased the total H3K4me3 and the enrichment of H3K4me3 at the *PTEN* promoter region; this led to an increase in *PTEN* transcript levels. However, miR-30c inhibited PTEN translation. Thus, PI3K/AKT signaling was activated, inhibiting CHK1 activity via phosphorylation of its serine 280 residue. This hampered the repair of oxidative DNA damage that occurred as a result of FB_1 exposure.

Exposure to FB_1 not only induced oxidative DNA damage but elevated levels of intracellular ROS triggering cell injury. In response to oxidative injury, cells induce Keap1/Nrf2 signaling which is regulated by epigenetic mechanisms. FB_1 elevated global m6A RNA levels which were accompanied by an increase in m6A "writers": *METTL3* and *METTL14*, and "readers": *YTHDF1*, *YTHDF2*, *YTHDF3* and *YTHDC2* and a decrease in m6A "erasers": *ALKBH5* and *FTO*. Hypermethylation occurred at the *Keap1* promoter, resulting in a reduction of *Keap1* transcripts. The hypomethylation of *Nrf2* promoters and decrease in miR-27b expression led to an increase in *Nrf2* mRNA expression. m6A-*Keap1* and m6A-*Nrf2* levels were both elevated; however, protein expression of Keap1 was reduced whereas Nrf2 was increased. Collectively, these epigenetic modifications (promoter methylation, miRNA-27b and m6A RNA) activated antioxidant signaling by reducing Keap1 expression and increasing Nrf2 expression.

If cells are unable to cope with stress, p53-mediated apoptosis is activated. Crosstalk between the lncRNA, HOXA11-AS, miR-124 and DNA methylation can influence p53 expression and apoptosis. FB_1 upregulated HOXA11-AS leading to the subsequent decrease in miR-124 and increase in *SP1* and DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B). This promoted global DNA

methylation and hypermethylation of *p53* promoters, thereby reducing p53 expression and caspase activity. Taken together, the data suggests that FB_1 inhibits p53-dependent apoptosis via HOXA11-AS/miR-124/DNMT axis.

Collectively, this study provides novel insights into additional mechanisms of FB_1-induced toxicities by epigenetically modulating stress response mechanisms.

CHAPTER 1

INTRODUCTION

One of the United Nations sustainable development goals is achieving food safety and security in developing countries. However, almost 30% of global agricultural crops are contaminated by toxic fungal secondary metabolites referred to as mycotoxins (Nesic, Ivanovic et al. 2014, Gbashi, Madala et al. 2018). Annually, over one billion tons of crops are lost due to mycotoxin contamination and it reduces the quality of an already limited food supply (Gbashi, Madala et al. 2018). Contamination frequently occurs in dietary staples that rural and developing communities heavily rely on. These staples include cereal grains such as maize, rice, wheat, oats and sorghum as well as ground nuts, fruit, and their byproducts (Fernández-Cruz, Mansilla et al. 2010, Tolosa, Font et al. 2013, Ferrigo, Raiola et al. 2016, Lee and Ryu 2017). Moreover, the eminent reality of climate change further exasperates the situation as fungal growth and mycotoxin production thrive during weather extremes and plant stress (Magan, Medina et al. 2011). The ingestion of mycotoxin contaminated crops has enormous public health significance because these toxins are usually nephrotoxic, hepatotoxic, immunotoxic, teratogenic and mutagenic (Zain 2011). Over 300 chemically distinct mycotoxins with diverse biological activities have been identified (Nesic, Ivanovic et al. 2014). Among them, fumonisin B_1 (FB_1) is one of the most important in terms of prevalence, contamination levels and toxic effects (Rheeder, Marasas et al. 2002).

FB_1 is a diester that arises from the condensation of two molecules of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethylicosane-3,5,10,14,15-pentol (Alexander, Proctor et al. 2009). *Fusarium verticillioides* and *Fusarium proliferatum* are major FB_1 producers with contamination occurring globally (Rheeder, Marasas et al. 2002). FB_1 is found in abundance in maize, wheat, rice, oats, barley, and millets and has been reported to contaminate numerous food products including vine fruit, asparagus, cornflakes, beers, beef, egg, and milk and canned foods (Gazzotti, Lugoboni et al. 2009, Lee and Ryu 2017, Farhadi, Nowrozi et al. 2019). FB_1 contamination occurs at various points in the food chain including storage and is resistant to many food processing techniques making it difficult to control contamination of foods and feeds as well as human and animal exposure (Kamle, Mahato et al. 2019).

Currently, several countries employ strict regulations to keep levels of FB_1 low in food. Acceptable limits of FB_1 in maize intended for human consumption range from 1 to 2 parts per million (ppm). The Scientific Committee on Food (SCF) and the joint Food and Agriculture Organization (FAO)/ World Health Organisation (WHO) Expert Committee for Food Additives (JECFA) independently established a provisional maximum tolerable daily intake (TDI) of 2 µg/kg body weight/day for FB_1, which was later expanded to include FB_1 alone or in combination with FB_2 and FB_3. This was based on a no-observable-adverse-effects level (NOAEL) in the liver and kidney of rodent models (SCF/EC 2000, FOA/WHO 2002, SCF/EC 2003). Since mycotoxins can be altered by plant defense mechanisms which

often masks their presence during analysis, the European Food Safety Authority (EFSA) established a TDI of 1.0 µg/kg bw per day of FB_1 alone or in combination with FB_2, FB_3, and FB_4 (EFSA 2018). Food that does not reach regulatory limits for human consumption are either used as animal feed or discarded completely. This leads to large annual losses in the agricultural industry (Gbashi, Madala et al. 2018). In many developing countries with a high-cereal consumption, regulation is either lacking or not enforced (Gbashi, Madala et al. 2018). Furthermore, FB_1 contamination and exposure is higher in low income countries, where rural subsistence farming communities are common (Mngqawa, Shephard et al. 2016, Alberts, Rheeder et al. 2019). Young children weaned on maize-based food are also vulnerable to FB_1 exposure that exceed the TDI (Shirima, Kimanya et al. 2013, Chen, Riley et al. 2018).

FB_1 is responsible for several pathological states in humans and animals. It is known to induce leukoencephalomalacia in equine, oedema in porcine and liver and renal toxicities in equine, porcine and rodents (Klarić and Pepeljnjak 2001, Voss, Smith et al. 2007, EFSA 2018). The International Agency for Research on Cancer (IARC) has classified FB_1 as a group 2 carcinogen as it is known to initiate and promote the development of renal, hepatocellular and cholangiocarcinoma in rodents, and is associated with the development of human esophageal (and in one case hepatocellular) carcinomas in regions that have a high maize consumption (Sydenham, Thiel et al. 1990, Dragan, Bidlack et al. 2001, IARC 2002, Sun, Wang et al. 2007, Alizadeh, Roshandel et al. 2012). Due to its structural similarity to sphingoid bases, the primary mechanism by which FB_1 induces its toxicity is through the disruption of sphingolipid metabolism. This inhibitory action interferes with signal transduction, cell cycle regulation and the functioning of lipid containing molecules such as cell membranes (Wang, Norred et al. 1991). FB_1 is known to trigger a host of other toxic responses such as oxidative stress, endoplasmic reticulum (ER) stress, disrupts cell cycle and alterations in immune responses (Chuturgoon, Phulukdaree et al. 2015, Yin, Guo et al. 2016, Arumugam, Pillay et al. 2019, Arumugam, Ghazi et al. 2020, Liu, Zhang et al. 2020). It also disrupts anti-oxidant signaling and cell death mechanisms (Chuturgoon, Phulukdaree et al. 2015, Arumugam, Pillay et al. 2019).

It has become increasingly clear that epigenetic mechanisms may also be exacerbate FB_1 induced toxicities. Epigenetics involves phenotypic variations that are brought about by regulating gene expression rather than altering DNA sequences (Bollati and Baccarelli 2010). Epigenetic modifications are essential for the normal cellular processes and maintenance of gene expression patterns; however, aberrant modifications can affect genome stability or have toxic and carcinogenic effects (Ho, Johnson et al. 2012, Shamsi, Firoz et al. 2017). Epigenetic modifications include changes in DNA methylation, RNA methylation [such as N6-Methyladenosine (m6A)], histone modifications and non-coding RNAs (ncRNA) such as microRNA (miRNA) and long-noncoding RNA (lncRNA) (Bannister and Kouzarides 2011, Moore, Le et al. 2013, Zaccara, Ries et al. 2019, Yang, Liu et al. 2020).

Several studies have investigated the impact of FB_1 on DNA methylation and histone modifications; however, the results are often conflicting. FB_1 induced global hypermethylation of DNA in rat C6

glioma cells and human Caco-2 cells; however, hypomethylation was observed in HepG2 cells and no significant changes occurred in rat liver (Clone 9 cells) and kidney epithelial cells (NRK-52E) (Mobio, Anane et al. 2000, Kouadio, Dano et al. 2007, Chuturgoon, Phulukdaree et al. 2014, Demirel, Alpertunga et al. 2015). Furthermore, FB_1 induced methylation of CpG islands found on the promoter regions of tumor suppressor genes (Demirel, Alpertunga et al. 2015). With regards to histone modification, FB_1 induced H3K9me3 and repressed H4K20me3 (Pellanda, Forges et al. 2012, Sancak and Ozden 2015). FB_1 had little effect on H4K16 and H3K18 acetylation; however, promoted acetylation of H2NK12, H3K9 and H3K23 (Pellanda, Forges et al. 2012, Gardner, Riley et al. 2016). Only one study has evaluated changes in miRNA profiles upon FB_1 exposure (Chuturgoon, Phulukdaree et al. 2014). Thus far, no study has evaluated the impact of FB_1 on m6A modifications and lncRNAs and little is known on the downstream implications of these epigenetic changes. In this study, the impact of FB_1 on DNA methylation, histone methylation (H3K4), m6A RNA methylation, miRNAs and lncRNAs were evaluated. The effect of these changes on response mechanisms to cellular stress were further investigated.

It was previously shown that FB_1 enhanced ROS production, resulting in oxidative stress in HepG2 cells (Arumugam, Pillay et al. 2019). Oxidative stress induced by FB_1 has also been observed in several other *in vivo* and *in vitro* models [extensively reviewed by Arumugam, Ghazi et al. (2020)]. A major consequence of excessive ROS level is oxidative injury to DNA which results in modification to nitrogenous bases and single- and double-stranded DNA breaks. The lesions incurred on DNA are often deleterious or have mutagenic effects (Loft, Høgh Danielsen et al. 2008). Cells are safe guarded by a complex network of DNA damage responses (DDR) with the tumor suppressor, PTEN and checkpoint signaling at the forefront (Dai and Grant 2010). Checkpoint kinase 1 (CHK1), a key transducer in this signaling networking, halts the cell cycle allowing for repair of damaged DNA to occur (Dai and Grant 2010, Patil, Pabla et al. 2013). Loss of the tumor suppressor PTEN generates DNA damage and prevents DNA repair via the inappropriate inactivation of CHK1 (Puc, Keniry et al. 2005, Puc and Parsons 2005). It is possible that PTEN expression is affected by epigenetic changes such as histone modifications and miRNA. Tri-methylation of the fourth lysine residue of histone 3 (H3K4me3) found on the promoter region of PTEN activates its transcription, whereas demethylation has the opposing effect (Shen, Cheng et al. 2018). Furthermore, miRNA, such as microRNA-30c (miR-30c), binds to the 3' untranslated region (3'UTR) of *PTEN* mRNA and inhibits its translation (Hu, Duan et al. 2019). FB_1 in known to affect both miR-30c and H3K4me regulation and may therefore affect DNA damage checkpoint regulation by epigenetically modulating PTEN (Chuturgoon, Phulukdaree et al. 2014, Chuturgoon, Phulukdaree et al. 2014, Sancak and Ozden 2015).

Oxidative stress not only induces oxidative lesions in DNA but it may also induce chemical modifications in RNA (Li, Li et al. 2017, Zhao, Li et al. 2019, Wu, Gan et al. 2020). Over a hundred covalent modifications are known to occur on the various classes of RNA with the most prevalent being

the methylation of the sixth nitrogen of adenosine (m6A) residues found on mRNA and lncRNA (Cantara, Crain et al. 2011, Machnicka, Milanowska et al. 2013, Yue, Liu et al. 2015). m6A marks are installed by "writers" (methyltransferases: METTL3 and METTL14), removed by "erasers" (demethylases: FTO and ALKBH5) and recognized by "readers" [YT521-B homology (YTH) domain family proteins: YTHDF1, YTHDF2, YTHDF3, YTHDC1 and YTHDC2]. M6A "readers" control the fate of m6A modified transcripts by regulating its export, degradation, splicing, and protein translation (Zaccara, Ries et al. 2019). M6A modifications are also influenced by cellular stress and can influence stress responses (Dominissini, Moshitch-Moshkovitz et al. 2012, Engel, Eggert et al. 2018). Global m6A levels are increased in response to oxidative stress; however, m6A modifications to certain transcripts have been shown to influence oxidative stress responses (Li, Li et al. 2017, Zhao, Li et al. 2019, Wu, Gan et al. 2020, Zhao, Wang et al. 2020). For instance, oxidative stress that occurred due to colistin exposure altered m6A levels; however, colistin-induced oxidative stress was diminished by m6A modifications on pri-miR-873. This promoted the generation of mature miR-873-5p and subsequently inhibited Keap1 expression and promoted Nrf2 antioxidant responses (Wang, Ishfaq et al. 2019). It was previously shown that Keap1/Nrf2 signaling is activated in response to FB_1-mediated oxidative stress (Arumugam, Pillay et al. 2019). The activation of Keap1/Nrf2 signaling promotes the transcription of anti-oxidants and other detoxifying enzymes to combat excess ROS (Ray, Huang et al. 2012). It is possible that FB_1-mediated oxidative stress affects global m6A levels and that m6A modifications are a potential factor contributing to Keap1/Nrf2 activation. Furthermore, Keap1 and Nrf2 are also regulated by promoter methylation and microRNA-27b (miR-27b).

When cells are unable to overcome genotoxic and oxidative stress, they initiate p53 mediated apoptosis. While *p53* is considered the most mutated gene in cancer, its expression may also be influenced by epigenetic factors such as lncRNA, miRNA and DNA methylation (Saldaña-Meyer and Recillas-Targa 2011, Chmelarova, Krepinska et al. 2013, Anbarasan and Bourdon 2019). Epigenetic modifications may also work in concert to regulate gene expression. For instance, the lncRNA, homeobox A11 antisense (HOXA11-AS) functions as circulating endogenous RNA (ceRNA) and molecular scaffold to alter DNA methylation patterns (Sun, Nie et al. 2016, Yu, Peng et al. 2017). As a ceRNA, HOXA11-AS binds to miRNAs and inhibits the regulatory interaction between the miRNA and its target mRNA (Khandelwal, Bacolla et al. 2015). By acting as a molecular scaffold, HOXA11-AS modulates the transcription of target genes by recruiting proteins including DNA methyltransferases (DNMTs) to the promoter regions of genes (Wang and Chang 2011). HOXA11-AS sequesters miR-124, which in turn upregulates DNMT3B and SP1, a DNMT1 transcription factor (Chen, Liu et al. 2015). HOXA11-AS may also act as a scaffold for DNMT1 (Sun, Nie et al. 2016). DNMTs are responsible for the methylation of gene promoters and thus inhibition of gene expression (Lyko 2018). It was previously shown that FB_1 impairs the transcription of tumor suppressors via methylation of their promoter regions

(Demirel, Alpertunga et al. 2015). It is possible that p53 expression may be downregulated by methylation of its promoter via the HOXA11-AS/miR-124/DNMT axis.

In this study, the human hepatoma (HepG2) cell line was used to identify epigenetic mechanisms that may contribute to FB_1 induced hepatoxicity. The liver is the initial site for the metabolism and detoxification of food contaminants and is one of the primary organs in which FB_1 accumulates and exerts toxicity (Martinez-Larranaga, Anadon et al. 1999, Kammerer and Küpper 2018). The use of primary hepatocyte cell lines as a toxicity model has many limitations. When primary hepatocytes are cultured they undergo morphological, phenotypic and functional changes in a process known as de-differentiation. Furthermore, liver specific functions such as cytochrome P450 metabolism also declines (Soldatow, Lecluyse et al. 2013). It is for these reasons that the HepG2 cell line was used instead. HepG2 cells have similar physiological functions to primary hepatocytes; however, it retains its functions and morphology in culture. It also displays a metabolic capacity and epigenetic profile similar to intact hepatocytes (Ruoß, Damm et al. 2019). Moreover, no mutations have been found in the *PTEN* or *p53* gene of the HepG2 cell line, making it a reliable model for testing epigenetic changes as a result of FB_1 exposure (Ma, Xu et al. 2005, Lee and Park 2015).

1.1. Aim

The aim of this study was to determine the epigenetic effects of FB_1 and the downstream implications of these epigenetic alterations to stress response pathways in human liver (HepG2) cells.

1.2. Hypothesis

FB_1 modifies the epigenome of HepG2 cells and alters cellular responses to stress which further contributes to its' toxicity.

1.3. Objectives

The objectives of this study were to determine the effects of FB_1 in HepG2 cells by assessing:

- genome integrity, epigenetic regulation of PTEN by miR-30c and H3K4me3 and CHK1.
- ROS levels, global m6A levels and the epigenetic regulation of Keap1/Nrf2 via m6A RNA methylation, miR-27b and promoter methylation.
- epigenetic regulation of p53 via the HOXA11-AS/miR-124/DNMT axis and its effect on apoptosis.

Ethical approval for this study was obtained from the University of Kwazulu-Natal Biomedical Research Ethics Committee (Ethical approval number: BE322/19; Addendum B, Page 209).

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CHAPTER 2

LITERATURE REVIEW

2.1. *Fusarium* Mycotoxins

Among numerous fungal genera, those belonging to *Fusarium* are considered the most significant. *Fusarium* species invade important agricultural crops such as small grain cereals and maize (Escrivá et al., 2015). Under optimal conditions, many of these fungi produce an array of structurally diverse and toxic secondary metabolites. These metabolites are known as mycotoxins and the quantity and type produced is dependent upon factors such as moisture, temperature and insect stress (Nesic et al., 2014, Bakker et al., 2018). Mycotoxins are related to the development of plant diseases resulting in the reduction of global crops by almost 30% (Nesic et al., 2014). Of significant concern is the acute and chronic implications of the consumption of *Fusarium* contaminated commodities on human and animal health (Escrivá et al., 2015). Some *Fusarium* mycotoxins co-contaminate crops and elicit a broad variety of toxic and carcinogenic effects in both humans and animals. Co-exposure to multiple *Fusarium* mycotoxins results in possibly synergistic or additive toxic effects (Grenier and Oswald, 2011). The most relevant *Fusarium* mycotoxins in terms of toxicology and distribution include fumonisins, trichothecenes and zearalenone (Figure 1) (Bakker et al., 2018).

Trichothecenes consist of metabolites containing an epoxide moiety (Figure 2.1A). They are produced by a wide variety of *Fusarium* species, including *F. sporotrichioides, F. poae, F. equiseti,* and *F. acumninatum* (Chain, 2011). More than 150 trichothecenes have been identified and classified into 4 types (A-D) based on substitutions on the core structure of 12,13-epoxytrichothec-9-ene (Escrivá et al., 2015). Toxicologically relevant trichothecenes consist of T-2 toxin, HT-2 toxin, nivalenol (NIV) and deoxynivalenol (DON). Trichothecenes are potent inhibitors of DNA, RNA and protein synthesis and have been associated with damage to the gastrointestinal system, dermatitis, immune suppression and hematologic disorders (Chain, 2011, Nesic et al., 2014).

Zearalenones (Figure 2.1B) are predominantly produced by *F. graminearum*, and *F. cerealis*, in temperate climates with cool temperatures and high humidity (EFSA, 2011). Zearalenones are classified as myco-oestrogens as they bind to cytosolic oestrogen receptors in the uterus, hypothalamus, mammary and pituitary glands resulting in strong hyper-oestrogenic effects (Abbès et al., 2006). Therefore, zearalenones exert their toxicity on the reproductive system by inducing morphological changes to the reproductive tract such as vaginal swelling, testicular atrophy and enlargement of mammary glands; as well as decreased fertility, higher embryo lethal resorptions and precocious puberty (EFSA, 2011, Escrivá et al., 2015). In addition, zearalenone also induces hepatotoxic, immunotoxic, and carcinogenic effects (EFSA, 2011, Escrivá et al., 2015).

Fumonisins are polyketide derived mycotoxins predominantly produced by *F. verticillioides* and *F. proliferatum*. Fumonisins have carcinogenic potential and have been associated with neuro-, hepato-

and renal toxicities (EFSA, 2018). Currently, 28 fumonisins have been identified and categorized into four groups (A, B, C and P). Among these analogues, fumonisin B_1 (FB_1; Figure 2.1C) is regarded as the most relevant due to its wide spread distribution and potent toxicity (Rheeder et al., 2002).

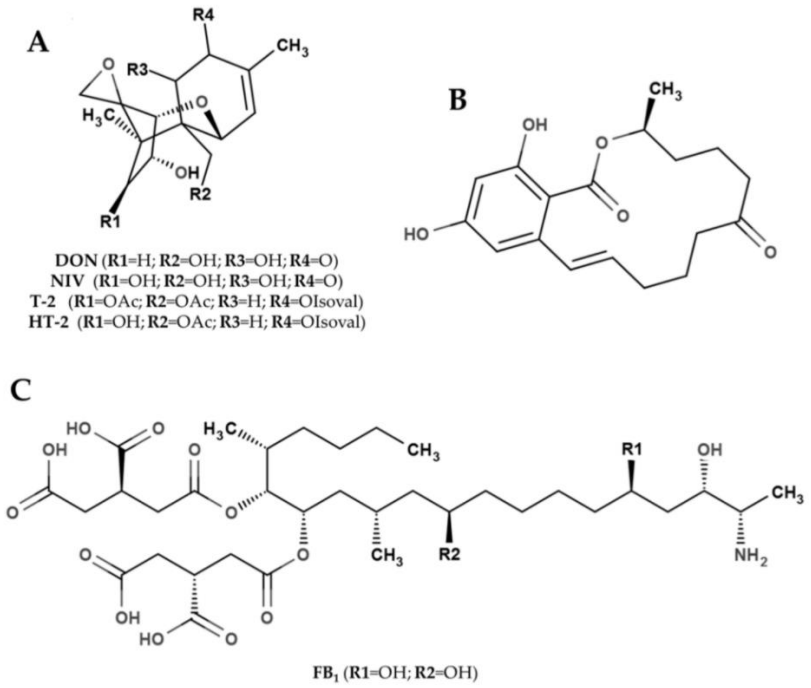

Figure 2.1. Chemical structure of the main *Fusarium* mycotoxins. (A) Trichothecenes; (B) Zearalenone; (C) Fumonisins; OAc = acetyl group; OIsoval = isovalerate group (Ferrigo et al., 2016).

2.1.1. Fumonisin B_1

Approximately 61% of global cereal grains are contaminated with fumonisins (Lee and Ryu, 2017). FB_1 accounts for 70-80% of total fumonisins that naturally infect food and feed samples, making it the most relevant fumonisin analogue (Rheeder et al., 2002). Due to their wide geographical distribution and frequent occurrence on maize, *F. verticillioides* and *F. proliferatum* are considered the most important FB_1 producers (Rheeder et al., 2002). Furthermore, *F. verticillioides* and *F. proliferatum* produce the highest levels of FB_1 reaching levels as high as 17,900 and 31,000 mg/kg of FB_1. 13 additional *Fusariums* have been found to produce FB_1, however, to a much lower extent (7-7,200 mg/kg) (Rheeder et al., 2002).

The production of FB_1 occurs preharvest and during storage and is heavily dependent on agroclimatic conditions. Production is favoured in temperate regions where temperatures are warm and humidity is high. Heat stress, insect damage and drought stress also influence FB_1 production (Ferrigo et al., 2016). It is found in abundance in maize and maize-based products such as corn flakes, flour and oil as well as in small cereal grains such as wheat, rice and oats (Lee and Ryu, 2017). Maize and cereals are dietary staples and developing countries are heavily reliant on them. Moreover, FB_1 production is prominent in rural regions that rely on subsistence farming. Most subsistence farmers do not have the resources to

implement the same agronomic practices seen in commercial settings. Poor agronomic practices exacerbate the incidence of *Fusarium* infection and FB_1 production (Shephard et al., 2019). Due to the high incidence of FB_1 in crops and its resistance to food processing, several countries and organisations have set regulations to limit FB_1 contamination in food and feed. The Joint FAO-WHO Expert Committee (JECFA) have also declared the provisional maximum tolerable intake of FB_1 alone or in combination with FB_2 and FB_3 should be 2 µg/kg bw/day (FOA/WHO, 2002); however, FB_1 intake is exceeded in many developing countries that rely heavily on cereal grains (Sun et al., 2007, Torres et al., 2007).

2.1.1.1. Structure and Biosynthesis

The structure of FB_1 ($C_{34}H_{59}NO_{15}$), consists of linear 20 carbon (C) aminopentol backbone which is substituted with an amine, three hydroxyl, two methyl, and two tricarboxylic acid groups at various positions (Alexander et al., 2009). Genes involved in the biosynthesis of fumonisin have been mapped to one locus in the genome of *F. verticillioides* and *F. proliferatum*. This region is regarded as the FUM cluster and consists of 17 genes (Khaldi and Wolfe, 2011). Genes belonging to the FUM cluster are co-regulated and its expression is influenced by abiotic factors such as water availability and temperature which in turn influence fumonisin production (Medina et al., 2013).

The biosynthesis of FB_1 is initiated by the condensation of nine acetate and two methyl groups to form a linear 18-C long polyketide. This reaction is catalysed by polyketide synthase (FUM 1) (Du et al., 2008, Alexander et al., 2009). Thereafter, the aminotransferase, FUM 8, mediates the condensation of the polyketide to alanine, resulting in a 20-C long backbone with an amine group at C-2, carbonyl group at C-3 and methyl groups at C-12 and C-16 (Du et al., 2008). The resulting polyketide amino acid undergoes hydroxylation at C-14 and C-15 by FUM 6. Thereafter, the carbonyl group is removed at C-3, C-10 is hydroxylated and two tricarboxylic acids are esterified to C-14 and C-15. These three reactions are catalysed by FUM 13, FUM 2 and FUM 10/14, respectively (Alexander et al., 2009). The addition of a hydroxyl group at C-5 by the dioxygenase FUM 3 is responsible for the final step of FB_1 biosynthesis (Figure 2.2) (Ding et al., 2004).

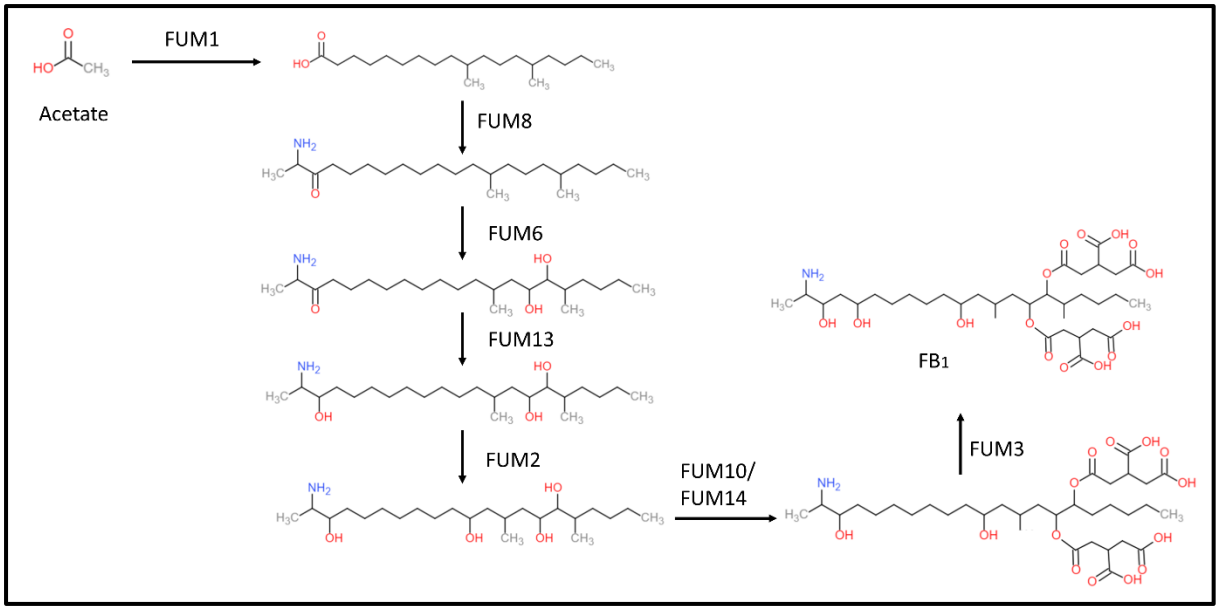

Figure 2.2. FUM mediated biosynthesis of FB_1 (prepared by author).

2.1.1.2. Primary mechanism of toxicity

The primary mechanism by which FB_1 exerts its toxicity is via the disruption of sphingolipid metabolism (Riley and Merrill, 2019). Ceramide synthase (CS) plays a central role in sphingolipid metabolism by catalysing the N-acylation of sphinganine (Sa) during sphingolipid synthesis and the *N*-acylation of sphingosine (So) during sphingolipid turnover (Futerman and Riezman, 2005). The aminopentol backbone of FB_1 bares close structural resemblance to the sphingoid bases: Sa and So, thus, FB_1 competes with sphingoid bases for CS binding. CS recognizes and binds both the amino group and the tricarboxylic acid side chains of FB_1, thereby inhibiting both *de novo* synthesis and degradation pathways of sphingolipid metabolism (Wang et al., 1991). This results in the reduction in the formation of complex sphingolipids such as sphingomyelin and glycosphingolipids. The toxic effects of FB_1 are only partially due to the reduction of complex sphingolipids. The rapid accumulation of sphingoid bases and their phosphorylated counter parts can also trigger cell injury and membrane degradation (Wang et al., 1991, Riley and Merrill, 2019). Reduction of ceramide and the accumulation of phosphosphingolipids disrupt signalling pathways and in turn trigger several toxicologically relevant perturbations such endoplasmic reticulum (ER) stress, accumulation of reactive oxygen species (ROS), altered mitochondrial and immune functioning, and disruption to developmental regulation (Riley and Merrill, 2019). Furthermore, FB_1-induced fluctuations in the levels of sphingoid bases alter rates of cell death and regeneration, which may play a major role in FB_1-mediated tumorigenesis (Wang et al., 1991, Soriano et al., 2005). For a detailed discussion on the impact of FB_1 on disruption of sphingolipid metabolism and the molecular implications, see chapter 3: Molecular and Epigenetic Mechanisms of FB_1 Mediated Toxicity and Carcinogenesis and Detoxification Strategies, pages 74-86.

2.1.1.3. Impact of FB1 on human and animal health

The 1970 field outbreak of equine leukoencephalomalcia (ELEM) in South Africa prompted the discovery and characterization of fumonisins. The disease was associated with the consumption of maize contaminated with *F. verticillioides* (formally *F. moniliforme*); later it was discovered that FB_1 was the main aetiological agent in the outbreak (Marasas, 2001). ELEM affects the central nervous system and is characterized by liquefactive lesions in the subcortical white matter of the cerebrum. Lesions may also develop in the brain stem, spinal cord and cerebellum (Klarić and Pepeljnjak, 2001). This leads to depression, pharyngeal paralysis, lethargy, blind staggering and seizures in affected horses (EFSA, 2018). Death can occur within a week after consuming of contaminated feed and can occur without prior signs (Klarić and Pepeljnjak, 2001). Moreover, hepatic and renal lesions and cardiac defects may develop independently or concurrently with ELEM (Klarić and Pepeljnjak, 2001, EFSA, 2018).

Along with horses, swine are considered the most sensitive domestic animals to FB_1. Swine exposed to FB_1 develop a syndrome termed porcine pulmonary oedema (Haschek et al., 2001). Within 4 to 7 days of exposure, swine present with respiratory distress, cyanosis, hydrothorax and pulmonary oedema. Death occurs rapidly within hours of respiratory distress; however, long term exposure to low doses of FB_1 results in non-lethal oedema (Voss et al., 2007). Pulmonary oedema induced by FB_1 may result from acute left-side heart failure due to changes in So/Sa concentrations which regulate L-type calcium channels. As a result, decreased heart rate, cardiac output and contractility also occur (Haschek et al., 2001). Aside from the pulmonary and cardiac effects, acute liver injury, pancreatic necrosis, formation of oesophageal plaques and depressed immune responses have also been observed (Voss et al., 2007).

The pathological effects of FB_1 have been well established in experimental rodent models. FB_1 predominantly targets the liver and kidney of rat and mouse models however the extent of toxicity is dependent on the species and sex of the animals as well as the dose of FB_1 received (Klarić and Pepeljnjak, 2001). Hepatoxicity is minimal in Sprague Dawley and Fischer 344 rats, whereas the liver is a major target in BD IX rats. However, male rats are more sensitive to the nephrotoxic effects of FB_1 than female rats; while mice are less sensitive to nephrotoxicity than rats (Voss et al., 2007). FB_1-induced hepatotoxicity consisted of necrosis accompanied by changes in the lipid ratios, distortion of liver lobules, and the development of hyperplastic nodules. Nephrotoxicity was characterised by hyperplasia, necrosis of tubules, fatty changes and pyknosis (Klarić and Pepeljnjak, 2001). Impairment of development and congenital malformations in the embryo and foetus are common in dams exposed to FB_1. FB_1 further retards growth and induces developmental abnormalities in these offspring (Lumsangkul et al., 2019). FB_1 has been implicated in the initiation and promotion of carcinogenesis. Cholangiocarcinomas, hepatocellular carcinomas and renal tubular tumours have been observed in male rats; while female mice present with hepatocellular carcinomas and adenomas (Dragan et al., 2001). Tumours tend to be aggressive and often metastasize (Voss et al., 2007). Epigenetic changes in

conjunction with compensatory cell proliferation and apoptosis are the proposed mechanisms by which FB_1 exerts its carcinogenic effects (Dragan et al., 2001, Demirel et al., 2015).

While the carcinogenicity of FB_1 in experimental animals have been well established, evidence of FB_1-carcinogenicity in humans are limited. Therefore, the International Agency for Research on Cancer (IARC) has classified FB_1 as a class 2B carcinogen (IARC, 2002). Epidemiological studies have shown an association between the high incidence of oesophageal cancer and, in one instance, hepatocellular carcinomas in regions with high consumption of FB_1 contaminated maize. Regions of major concern include South Africa, Iran and China (Sydenham et al., 1990, Sun et al., 2007, Alizadeh et al., 2012). Epidemiological studies have also linked the high incidence of neural tube defects along the Mexican-Texan border to the maternal consumption of maize based products contaminated with FB_1 (Missmer et al., 2006). The inhibition of sphingolipid synthesis disturbs cellular membranes and receptors. FB_1 inhibits folate uptake, leading to neural tube defects such as spinal bifida and anencephaly with extremely high exposure leading to foetal death (Marasas et al., 2004). Furthermore, evidence linking fumonisin exposure to the stunting of growth in Sub-Saharan infants and children that consume maize-based weaning foods has been increasing (Shirima et al., 2013, Chen et al., 2018). Finally, only one outbreak of acute mycotoxicosis caused by the consumption of FB_1-contaminated sorghum and corn has been recorded. The outbreak occurred in South India after 2 cyclonic storms which promoted growth of mould. The outbreak affected 27 villages and 1,412 people. Affected individuals reported transient abdominal pain, borborygmus and diarrhoea (Reddy and Raghavender, 2008).

While the disruption of sphingolipid metabolism by FB_1 has been ruled as the primary mechanism for its adverse effects; several emerging evidences suggests that mycotoxins induce epigenetic changes that play a key role in their toxicity. It is plausible to assume that epigenetic changes may also contribute to FB_1-mediated toxicities and pathologies.

2.2. Epigenetics

Although virtually all cells in an organism contain identical DNA sequence, not all cell types share the same phenotype at the same time (Moore et al., 2013). Conard Waddington found that environmental changes during development could induce an alternative phenotype despite their identical sequence. He further observed that these environmentally induced changes could be inherited. He termed this phenomenon as “epigenetics” (Waddington, 1956, Holliday, 2006). Epigenetics encompasses heritable modifications that regulate gene expression and are not associated with changes in DNA sequence (Bollati and Baccarelli, 2010). The complete description of all epigenetic modifications of a cell at any given time is termed the epigenome. Interactions between the epigenome, genome and environment play a critical role in shaping the development and health of an individual (Marczylo et al., 2016).

Several types of epigenetic modifications have been identified. These modifications include: DNA methylation, covalent histone modifications, RNA methylation and non-coding RNAs (ncRNA) (Figure

2.3). DNA methylation and histone modifications influence transcription by altering chromatin structure and accessibility of transcriptional machinery to nucleotide sequences (Bannister and Kouzarides, 2011, Moore et al., 2013). On the other hand, RNA methylation targets posttranscriptional regulation; whereas, ncRNA influence transcriptional and posttranscriptional regulation of genes (Zaccara et al., 2019, Yang et al., 2020).

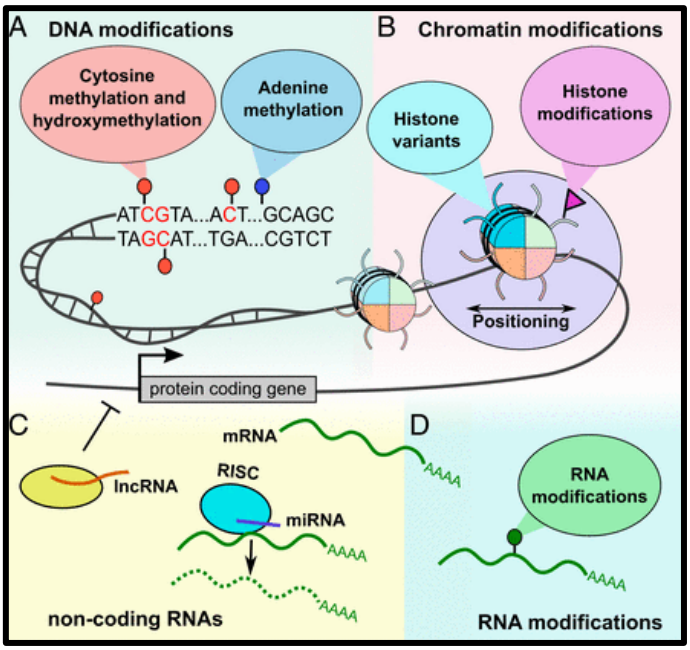

Figure 2.3. The complex epigenetic landscape involves: (A) DNA methylation, (B) histone modifications, (C) ncRNA such as miRNA and lncRNA and (D) RNA modifications such as RNA methylation (Aristizabal et al., 2020)

While the epigenome is stable, it is dynamic and can be influenced by a number of environmental factors (Marczylo et al., 2016). Aberrant changes to the epigenome can induce abnormalities in gene expression and disrupt cellular processes (Kanherkar et al., 2014). Therefore, aberrations in the epigenome have been identified to precede various diseases such as metabolic disorders, autoimmune diseases, neurological disorders and cancers (Shamsi et al., 2017). However, unlike genetic defects, epigenetic deviations are reversible and are thus potential therapeutic targets (Kelly et al., 2010).

2.2.1. DNA Methylation

DNA methylation is the most studied epigenetic mark that involves the covalent transfer of methyl groups from S-adenosylmethionine (SAM) to the fifth carbon in the nitrogenous base of cytosine (5mC) in DNA (Robertson, 2005). It usually occurs on cytosine bases adjacent to guanine bases (CpG site) (Robertson, 2005). Approximately 70% of CpG sites in mammalian DNA are methylated (Cooper and Krawczak, 1989); however, the distribution of CpG sites are not random. Multiple repeats of CpG sites, known as CpG islands, are usually found on gene promoters (Saxonov et al., 2006). CpG islands found on gene promoters are usually unmethylated and are associated with actively transcribing genes (Bird,

1986, Antequera, 2003, Saxonov et al., 2006). In contrast, methylation of promoter associated CpG islands results in the silencing of gene expression (Figure 2.4) (Mohn et al., 2008, Payer and Lee, 2008). Methylation can also occur on intergenic regions, where it prevents the expression of potentially harmful genetic elements (Moore et al., 2013), as well as within the gene body, where a positive correlation with gene expression occurs (Hellman and Chess, 2007, Aran et al., 2011, Jjingo et al., 2012).

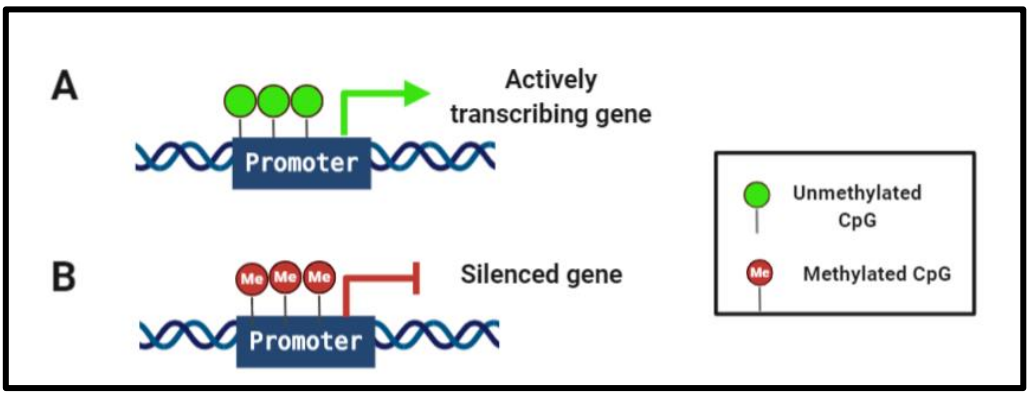

Figure 2.4. Regulation of gene expression via DNA methylation. (A) Genes are actively transcribed when CpG islands are unmethylated; however, (B) methylation of CpG islands on the gene promoter inhibits transcription (prepared by author).

It is clear that DNA methylation is strongly involved in the physiological control of gene expression (Moore et al., 2013). It plays a key role in normal development (Li et al., 1992), compaction of chromatin (Geiman et al., 2004), genomic imprinting (Li et al., 1993), X chromosome inactivation (Csankovszki et al., 2001) and the bulk silencing of viral and transposable elements (Schulz et al., 2006). However, aberrant methylation patterns are associated with a multitude of diseases especially, cancer (Laird and Jaenisch, 1996, Ehrlich, 2002, Robertson, 2005, Jin and Liu, 2018, Kader et al., 2018). For example, CpG sites especially, those found in the promoter region of tumour suppressor genes are hot spots for somatic mutations (Rideout et al., 1990, Greenblatt et al., 1994). DNA methylation can promote increases in mutation rates and forms part of Knudson's two-hit model for tumour formation by causing the heritable silencing of growth regulating genes (Jones, 1996, Moore et al., 2013, Zhou et al., 2020). Furthermore, global hypomethylation accompanied with hypermethylation of tumour suppressor genes are considered a hallmark of cancer and have been observed in several types of cancers (Lin et al., 2001, Yang et al., 2003, Saito et al., 2010, Wu et al., 2010, Hon et al., 2012, Pfeifer, 2018).

2.2.1.1. Regulation of DNA Methylation

DNA methylation is dynamic and involves enzymes that install (methyltransferases), recognize (readers) and remove (demethylases) methyl marks on DNA. DNA methylation is established by the DNA methyltransferase (DNMT) family which includes: DNMT1, DNMT3A, DNMT3B and

DNMT3L (Cheng and Blumenthal, 2008). The DNMT family are structurally similar with large regulatory N-terminal domains and catalytic *C*-terminal domains; however, they vary in functionality (Lyko, 2018).

DNMT1 is known as the maintenance DNMT as it maintains methylation patterns in a cell lineage (Moore et al., 2013). A unique feature of its N-terminal is the replication foci targeting sequence which allows DNMT1 to localize to the replication fork during DNA synthesis (Leonhardt et al., 1992). Here, DNMT1 copies methylation patterns to hemi-methylated daughter strands to precisely mimic the methylation pattern of the parent strand (Hermann et al., 2004). Moreover, DNMT1 accumulates at DNA repair sites and is associated with mismatch repair and DNA damage response machinery (Mortusewicz et al., 2005, Eades et al., 2011, Loughery et al., 2011). Silencing of DNMT1 leads to the significant reduction in DNA methylation, aberrant imprinting and embryonic lethality suggesting that it plays a critical role in dividing cells and cellular differentiation (Li et al., 1992, Li et al., 1993). While DNMT1 maintains methylation patterns, DNMT3A and DNMT3B are responsible for the *de novo* methylation of DNA (Figure 2.5) (Okano et al., 1998). DNMT3A and DNMT3B bare close structural resemblance with the key difference being their expression pattern. DNMT3A is ubiquitously expressed while DNMT3B is poorly expressed in most differentiated tissue (Xie et al., 1999). Furthermore, DNMT3B is essential for early development as knockout of DNMT3B results in embryonic lethality in mice, whereas growth is stunted when DNMT3A is silenced (Okano et al., 1998). The final member of the DNMT3 family, DNMT3L, lacks catalytic activity however it supports *de novo* DNMTs by enhancing their ability to bind to SAM and by stimulating their activity (Kareta et al., 2006). DNMT3L is mainly present during early development where it is required for imprinting, compaction of the X chromosome and methylation of retrotransposons (Bourc'his et al., 2001, Hata et al., 2002, Bourc'his and Bestor, 2004, Zamudio et al., 2011). The exact mechanism by which *de novo* methyltransferases target specific gene sequences is unknown; however, two hypotheses exist. The first suggests that RNA interference directs DNMTs to specific sequences. While this mechanism occurs in plants, the evidence observed in the mammalian genome is insufficient (Morris et al., 2004). The second suggests that transcription factors regulate DNA methylation by either recruiting or blocking DNMTs to specific DNA sequences (Brenner et al., 2005, Straussman et al., 2009). Binding of transcription factors seems to primarily protect CpG islands from methylation and deletion or mutations to transcription factor binding sites results in the *de novo* methylation of CpG islands (Brandeis et al., 1994, Macleod et al., 1994).

While DNA methylation prevents the binding of transcription factors and thus switched off transcription, DNA methylation “readers” are able to recognize and bind to 5mC bases, further inhibiting transcription factor binding (Moore et al., 2013). Three classes of DNA methylation readers exist: methyl-CpG-binding domain (MBD), ubiquitin-like containing PHD and RING-finger domain (UHRF) and zinc-finger proteins. MBD family consists of Methyl CpG binding protein 2 (MeCP2),

MBD1, MBD2, MBD3, and MBD4. (Fatemi and Wade, 2006). MeCP2, MBD1 and MBD2 contain a transcriptional repression domain that allows them to recruit corepressor complexes such as histone deacetylases to methylated DNA to further silence gene transcription (Nan et al., 1998, Ng et al., 1999, Villa et al., 2006). MeCP2 also plays a role in methylation maintenance by recruiting DNMT1 to hemi-methylated DNA (Kimura and Shiota, 2003). MBD4 has DNA *N*-glycosylase enzymatic activity and is able to recognize and repair guanine : thymine, uracil, or 5-fluorouracil mismatches that occur due to 5mC demethylation processes (Hendrich et al., 1999). Zinc finger proteins (Kaiso, ZBTB4, and ZBTB38) are able to bind to 5mC and act in a similar way to the MBD family by repressing transcription in a DNA methylation-dependent manner (Prokhortchouk et al., 2001, Filion et al., 2006). UHRF promotes DNMT1-targeted methylation of hemi-methylated DNA during DNA synthesis by tethering DNMT1 to chromatin (Bostick et al., 2007).

DNA demethylation is the process of removing methyl marks from 5mC residues in either a passive or active manner. Passive demethylation is the loss of DNA methylation patterns during successive rounds of replication (Kohli and Zhang, 2013). It usually occurs due to loss of DNA methylation maintenance in actively dividing cells (von Meyenn et al., 2016). Active demethylation occurs in both dividing and non-dividing cells and is dependent on three enzyme families (Bhutani et al., 2011, Kohli and Zhang, 2013): (i) ten-eleven translocation (TET) family which can either hydroxylate 5mC to 5-hydroxymethylcytosine (5hmC) or further oxidize it to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Tahiliani et al., 2009, Ito et al., 2011), (ii) Activation-induced cytidine deaminase/apolipoprotein B mRNA-editing catalytic polypeptides (AID/APOBEC) family which is responsible for the deamination of 5mC to thymine or 5hmC to 5-hydroxymethyluracil (5hmU) (Morgan et al., 2004, Guo et al., 2011) and (iii) base excision repair glycosylases such as thymine DNA glycosylase (TDG) which cleaves the products of TET and AID/APOBEC demethylation (5fC, 5caC, thymine, and 5hmU) from the DNA backbone and replaces it with an unmethylated cytosine (Figure 2.5) (Cortellino et al., 2011, He et al., 2011).

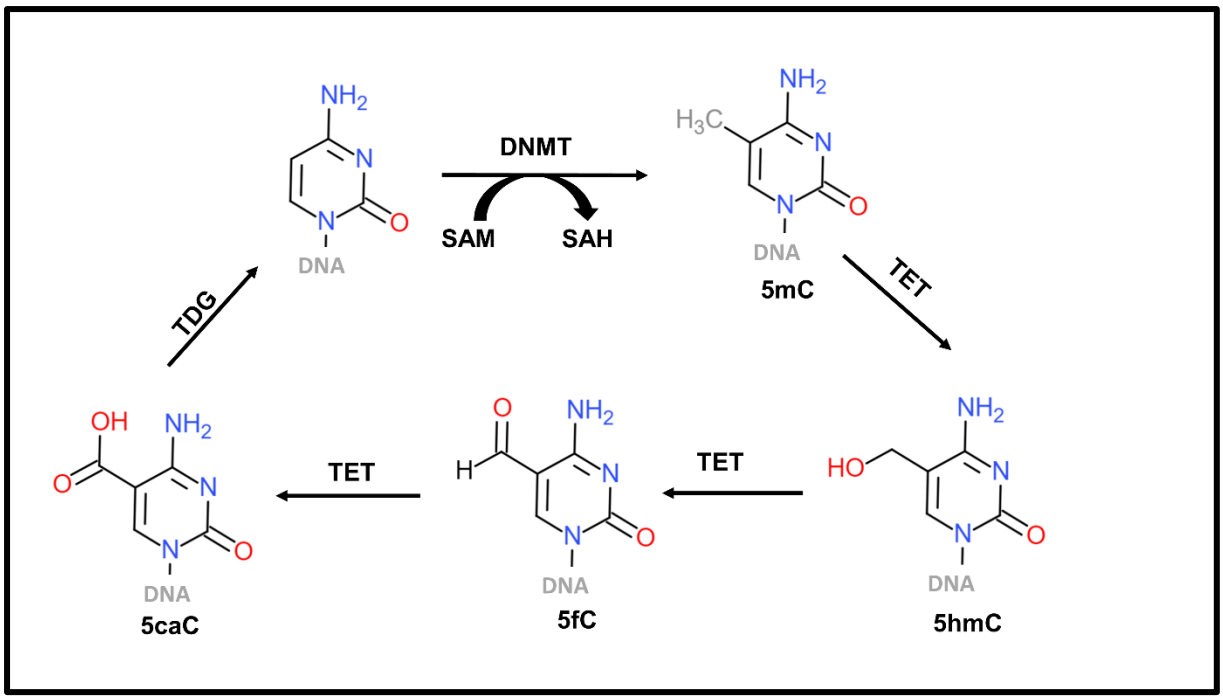

Figure 2.5. Regulation of DNA methylation. DNMT1 maintains DNA methylation patterns via methylation, while DNMT3A and DNMT3B are required for *de novo* methylation by catalysing the transfer of a methyl group from SAM to cytosine forming 5mC. TET plays a central role in DNA demethylation by oxidizing 5mC to hmC and further to 5fC and 5caC. 5caC is excised by TDG and replaced with an unmethylated cytosine (prepared by author).

2.2.2. Histone Modifications

The eukaryotic genome is tightly packaged into chromatin whose functional and structural unit is referred to as the nucleosome. Each nucleosome consists of four core histone proteins (H2A, H2B, H3 and H4) arranged as an octamer around which approximately 200 base pairs of DNA is wrapped (Luger et al., 1997). The tight packaging of DNA by the nucleosome imposes a barrier to protein machinery required for its replication, repair and transcription (Ehrenhofer-Murray, 2004, Eaton et al., 2010, Chambers and Downs, 2012, Voss and Hager, 2014, Li and Zhu, 2015). Like DNA, histones can be modified by the addition or removal of chemical groups to control gene expression; however, histone modifications are not limited to methylation (Bannister and Kouzarides, 2011, Jambhekar et al., 2019). The N-terminal of histone tails can be subjected to several post-translational modifications. Such modifications include the methylation of arginine (R) and lysine (K), phosphorylation of serine and threonine and acetylation, ribosylation, sumoylation or ubiquitination of K (Figure 2.6). These covalent modifications, alter chromatin state, affect nucleosome positioning and influence accessibility to nucleotide base sequences (Bannister and Kouzarides, 2011, Chrun et al., 2017, Jambhekar et al., 2019).

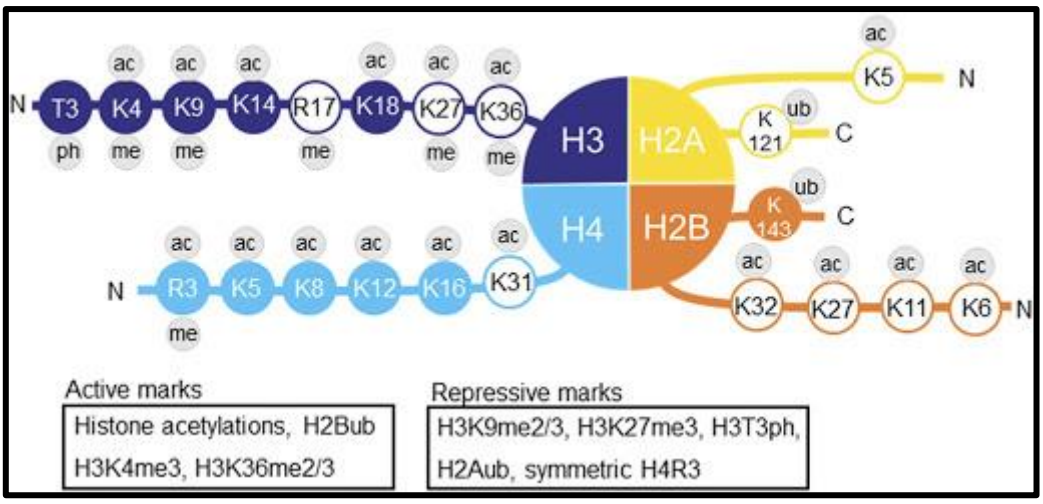

Figure 2.6. Schematic representation of some of the modifications found on histone tails of core histones (H2A, H2B, H3, H4) (Ueda and Seki, 2020).

The most common and well-studied histone marks are acetylation and methylation. Acetylation usually occurs on histone 3 (H3) and histone 4 (H4) and is a dynamic process regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Verdone et al., 2006). The *N*-terminal tail of histones contain highly conserved positively charged K residues that have high affinity to the negatively charged DNA backbone, resulting in a condensed chromatin structure (Müller and Muir, 2015). Acetylation of K residues neutralizes the positive charge; reducing the affinity between histone tail and DNA backbone. This leaves the DNA exposed and more accessible to transcription factors (Müller and Muir, 2015, Zhao and Shilatifard, 2019). Acetylation not only contributes to gene expression by influencing histone-DNA interactions but it is also recognized and bound by bromodomain containing enzymes that can influence transcription and other chromatin-templated processes (Zhao and Shilatifard, 2019).

Histone methylation primarily occurs on K and R residues found on H3 and H4 and is more complex than acetylation (Jambhekar et al., 2019). Methylation does not alter the charge of histone tails, instead histone methylation generates motifs that recruit bromo-, chromo-, and PHD domains of protein containing complexes that regulate gene expression (Strahl and Allis, 2000, Jenuwein and Allis, 2001). The outcome of methylation on gene expression is dependent on the specific residue that is methylated, the degree of methylation and the location of the methylated nucleosome in the genome (Jambhekar et al., 2019). There are three major forms of methylated R: mono-methyl-R, symmetrical di-methyl-R, and asymmetric-di-methyl-R, which are regulated by protein arginine N-methyltransferases (PRMTs) and the demethylase – Jumonji Domain-Containing Protein 6 (JmjD6) (Chang et al., 2007, Guccione and Richard, 2019). Several methylation sites have been identified to alter gene expression. The following R modifications have been associated with active transcription: H4R3me2a, H3R2me2s, H3R17me2a, H3R26me2a; while H3R2me2a, H3R8me2a, H3R8me2s, whereas H4R3me2s marks repression of transcription (Blanc and Richard, 2017). On the other hand, K residues of histones can be

mono-, di- or tri- methylated (Jenuwein and Allis, 2001). Di- or tri- methylation of H3K4 at promoters, H3K36 and K3K79 on gene body is typically associated with active transcription (Bernstein et al., 2002, Bannister et al., 2005, Steger et al., 2008), whereas methylation of H3K9, H3K27, and H4K20 is generally gene repressive (Karachentsev et al., 2005, Brykczynska et al., 2010, Ninova et al., 2019).

In this study, the interest is focussed on histone 3 lysine 4 trimethylation (H3K4me3) due to its distinct presence at transcriptional start sites and promoters of actively transcribing genes as well as its possible susceptibility to alteration by genotoxic agents.

2.2.2.1. H3K4me3

H3K4me3 is a highly conserved histone mark occurring in organisms as simple as protozoan to complex organisms such as humans (Woo and Li, 2012, Song et al., 2017). In mammals, H3K4 methylation is facilitated by histone lysine methyltransferase 2 family (KMT2) which consists of six members. Each member contains a catalytic Su(var)3-9, Enhancer-of-zester and Trithorax (SET) domain that is responsible for the transfer of methyl groups from SAM to the fourth lysine reside of H3 (Collins et al., 2019). Each histone methyltransferase operates within a multiprotein complex that produces distinct enzymatic responses (Hyun et al., 2017). Histone methylation functions by recruiting effector proteins that function in chromatin remodelling and regulate gene expression. Interestingly, some H3K4 effectors reside within the enzymatic writer complexes (Collins et al., 2019). For example, H3K4me3 recruits bromodomain PHD finger transcription factor (BPTF), a subunit of the chromatin remodelling complex – nucleosome remodelling factor (NURF), through its PHD fingers. This promotes the accessibility of transcriptional machinery to the chromatin template (Mizuguchi et al., 1997). On the other hand, demethylation of H3K4 makes the chromatin template inaccessible to transcription factors and inhibits transcription (Hyun et al., 2017). This process is regulated by two families of histone lysine demethylases: KDM1 and KDM5. KDM1 family (KDM1A and KDM1B) removes methyl groups from H3K4me1 and H3K4me2 while the KDM5 family (KDM5A, KDM5B, KDM5C and KDM5D) removes methyl groups from H3K4me1, H3K4me2 and H3K4me3 (Collins et al., 2019).

Aside from its role in transcriptional activation, H3K4me3 has been implicated in other nuclear processes, including pre-mRNA splicing (Davie et al., 2015), meiotic DNA recombination (Borde et al., 2009), and DNA repair (Pena et al., 2008, Faucher and Wellinger, 2010). H3K4me3 is essential for cell cycle regulation, development and differentiation (Cui et al., 2009, Grandy et al., 2016, Zhang et al., 2016, Huang et al., 2019b). Dysregulation of H3K4me3 has been associated with intellectual disabilities and developmental disorders (Singh et al., 2016, Zamurrad et al., 2018, Larizza and Finelli, 2019). Moreover, aberrant H3K4me3 and mutations in H3K4 methyltransferases highly increases an individual's susceptibility to various cancers (Rao and Dou, 2015).

2.2.3. RNA methylation: N6-methyladenosine

Chemical modifications are not limited to histones and DNA as over a hundred structurally distinct chemical modifications are known to occur on the various classes of RNA (Cantara et al., 2011, Machnicka et al., 2013). The most prevalent of these RNA modifications is the methylation of the sixth nitrogen of adenosine (m6A) residues found on mRNA as well as lncRNA (Yue et al., 2015). M6A modifications were first identified in the 1970s by researchers evaluating 5' cap structure of mammalian mRNA (Desrosiers et al., 1974, Perry and Kelley, 1974). However, research in the field subsided shortly after due to the lack of methods for detecting m6A sites in RNA. With the establishment of high throughput sequencing methods, interest in the field has now resurfaced. These mapping approaches has revealed that m6A modifications are dynamic, widespread, conserved and occur primarily in DRACH (where D = A/G/U, R= A/G, H=A/C/U) sequence consensus motifs that are located near stop codons, long exonic regions and 3' untranslated regions (3' UTR) (Dominissini et al., 2012, Meyer et al., 2012, Ke et al., 2015). M6A modifications are conserved amongst eukaryotes (Dominissini et al., 2012) but have also been identified in the mRNA of replicating viruses (Krug et al., 1976), and several classes of RNA in bacteria and archaea (Deng et al., 2015, Couturier and Lindås, 2018). The modification functions by affecting mRNA stability, translation, splicing and nuclear export, miRNA biogenesis and lncRNA metabolism (Wang et al., 2014a, Alarcón et al., 2015, Ma et al., 2019, Zaccara et al., 2019).

The m6A epitranscriptome is shaped by m6A writers, readers and erasers (Figure 2.7) (Zaccara et al., 2019). m6A marks are installed during transcription by a multicomponent methyltransferase complex which selectively methylates RNA substrates exhibiting the DRACH consensus (Bokar et al., 1997, Liu et al., 2014, Ping et al., 2014). The complex consists of methyltransferase like 3 (METTL3) (Bokar et al., 1997), methyltransferase like 14 (METTL14) (Liu et al., 2014) and Wilms' tumor 1-associating protein (WTAP) (Ping et al., 2014). METTL3 serves as the catalytic subunit and facilitates the transfer of methyl groups from SAM to adenosine (A) of RNA (Bokar et al., 1997) while METTL14 acts as a support for METTL3 by recognizing RNA substrates and allowing binding to RNA (Wang et al., 2016). Liu et al. (2014) have demonstrated that METTL14 may have catalytic activity as well. Studies have shown that knockdown of either METTL3 or METTL14 led to a concurrent decrease in m6A levels of polyadenylated RNA (Liu et al., 2014). Surprisingly, the knockdown of METTL14 led to a more pronounced decrease in global m6A transcript levels (Place et al., 2008); however, a combination of both methyltransferases drastically enhances methylation efficiency (Wang et al., 2014b). WTAP is the third crucial component; it does not possess catalytic methyltransferase activity, but coordinates the localization of the METTL3-METTL14 heterodimer into nuclear speckles (Liu et al., 2014, Ping et al., 2014). WTAP may also interact with other components such as RNA binding motif protein 15 (RBM15) and RBM15B which bind to uridine-enriched regions and then recruit WTAP/METTL3 complexes to

methylate nearby DRACH motifs (Patil et al., 2016) and Zinc Finger CCCH-Type Containing 13 (Zc3H13) which also plays a role in the nuclear localization (Wen et al., 2018).

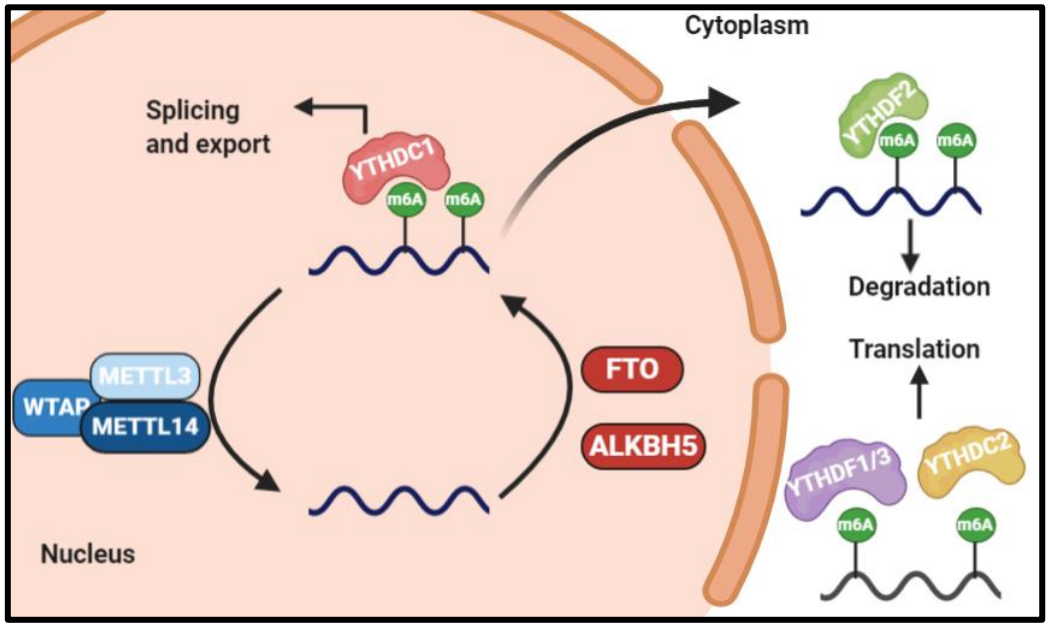

Figure 2.7: m6A modification machinery. The m6A methyltransferase complex (METTL13, METTL14 and WTAP) serves as an m6A "writer", demethylases (e.g., FTO and ALKBH5) serve as m6A "erasers", and a set of m6A "readers" (e.g., YTHDF1/2/3, YTHDC1/2) serve to determine the fate of target m6A-modified mRNA transcripts (prepared by author).

Seeing that m6A modifications are dynamic, demethylation of m6A to adenosine (A) is catalysed by the m6A "erasers" (Jia et al., 2011). Thus far, only two m6A demethylases have been identified, i.e., fat mass and obesity associated protein (FTO) and its homologue ALKBH5 (Jia et al., 2011, Zheng et al., 2013). Both proteins belong to the ALKB subfamily of Fe(II)/α-ketoglutarate-dependent dioxygenases which repair DNA alkylation damage by demethylating DNA and RNA nucleotides that have been alkylated (Fedeles et al., 2015). FTO has been shown to oxidatively demethylate m6A to A in a stepwise manner with N6-hydroxymethyladenosine (hm6A) and N6-formyladenosine (f6A) as intermediates (Fu et al., 2013). In contrast, ALKBH5 directly and oxidatively removes methyl marks with no detected intermediates (Zheng et al., 2013). FTO and ALKBH5 knockout and overexpression have been shown to increase and reduce m6A levels, respectively (Jia et al., 2011, Zheng et al., 2013). Both demethylases are tissue specific and have diverse intracellular localization, thus demethylation in some tissue may be facilitated solely by FTO or ALKBH5 (Zhang et al., 2019).

While writer and eraser proteins are responsible for installing and removing m6A marks, readers control the fate of m6A modified transcripts (Liao et al., 2018). The m6A readers consist of the YT521-B homology (YTH) domain family proteins: YTHDF1, YTHDF2, YTHDF3 and YHT domain containing proteins: YTHDC1 and YTHDC2, which preferentially recognize and bind to m6A sites and confer downstream functions (Liao et al., 2018). Nuclear readers regulate mRNA splicing and other

nuclear processes (Xiao et al., 2016) whereas cytoplasmic readers affect mRNA stability, translation and localization (Zaccara et al., 2019). The localization and function of all known YTH domain containing m6A readers are summarized in Table 2.1.

Table 2.1: The localization and function of YTH domain containing m6A-readers

m6A Reader	Cellular Localization	Effects of Binding to m6A RNA
YTHDC1	Nucleus	Affects splicing and export Preferably binds to ncRNA, may bind to mRNA
YTHDC2	Nucleus and cytoplasm	Implicated in mRNA degradation and initiation of translation
YTHDF1	Cytoplasm	Promotes translation
YTHDF2	Cytoplasm	Promotes degradation
YTHDF3	Cytoplasm	Promotes translation

2.2.4. NcRNA

Although early studies have reported the occurrence of transcription in regions not coding for proteins, it is only recently that researchers have realized that while a vast majority of the genome is transcribed (62.1%); only 2-3% constitute of protein coding genes (Panzeri et al., 2016). Areas of the genome that do not encode for protein, are transcribed to ncRNA. Since ncRNA do not function in protein coding, it was long regarded that ncRNAs were “junk RNAs” or “transcriptional noise”. However, through the development of high-throughput technologies, this idea has been rejected as we now know that ncRNAs play a key role in regulating cellular events and gene expression (Kapranov et al., 2002, Kapranov et al., 2007).

NcRNAs are classified based on their function into housekeeping ncRNAs and regulatory ncRNAs (Wei et al., 2017). Housekeeping ncRNAs include ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). They are usually short (~20-200 nucleotides; nt), constitutively expressed and necessary for the maintenance of normal cellular functions and are involved in protein translation, splice regulation, RNA modifications as well as the transport and insertion of proteins into membranes (Morey and Avner, 2004). On the other hand, regulatory ncRNA consists of both short and long (22 nt to ∼100 kilobases) ncRNAs that are involved in regulating gene expression through various mechanisms (Table 2.2). Transcriptional silencing by ncRNA has been implicated in several diseases including cancer predisposition or status. Among the ever-increasing types of ncRNAs being deciphered, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are the most intensively studied and play a prominent role in epigenetic control (Dai et al., 2019).

Table 2.2: Characteristics and functioning of regulatory ncRNAs.

Type	Symbol	Source	Size (nt)	Function
microRNA	miRNA	pri-miRNA	~22	Gene silencing
Small interfering RNA	siRNA	Long double stranded RNA	19-25	Gene silencing
Piwi interacting RNA	piRNA	Long single chain precursor transcripts	26-31	Transposon silencing and DNA methylation
Long non-coding RNA	lncRNA	Multiple	>200	Transcriptional activation Post-transcriptional regulation X chromosome inactivation Regulation of chromatin remodelling, imprinting, miRNA, methylation and RNA binding proteins

2.2.4.1. MiRNAs

The discovery of miRNAs has revolutionized the field of molecular biology. In 1993, Lee and Whiteman identified the first miRNA, *lin-4* in *Caenorhabditis elegans* (Lee et al., 1993, Wightman et al., 1993). Although miRNAs were identified in the early 1990s, it took almost 10 years until their fundamental roles in gene regulation were recognized (Lagos-Quintana et al., 2001). The field of miRNA research has since grown with over 17,000 miRNAs discovered to date in 142 species (Dwivedi et al., 2019). Today, we know that these small regulatory RNAs, play key roles in developmental and physiological processes in most eukaryotes and are even encoded by some viruses (Pfeffer et al., 2004, Vidigal and Ventura, 2015). However, aberrant expression of miRNAs is associated with many human diseases. Aberrant miRNA profiles have been observed in numerous cancers where they act as either tumour suppressors or oncogenes depending on their mRNA targets (Cui et al., 2019). Therefore, the evaluation of extracellular miRNAs profiles are used as potential biomarkers for a variety of diseases (Paul et al., 2018).

2.2.4.1.1. Biogenesis

The biogenesis of miRNAs begins with its transcription from the host gene (intragenic miRNAs) or independently of the host gene with the use of their own promoter (intergenic miRNAs). miRNAs can be transcribed individually as monocistronic transcripts or as one long transcript (polycistronic transcripts) called clusters which are later processed to individual mature miRNAs. The biogenesis of miRNAs can occur via the canonical or noncanonical pathways (O'Brien et al., 2018).

Processing of miRNAs usually occurs via the canonical biogenesis pathway which involves two ordered endonucleolytic cleavages by RNAse III enzymes (Figure 2.8) (Davis and Hata, 2009). Most miRNAs are transcribed from DNA sequences by RNA polymerase II (RNAP II) as capped and polyadenylated primary miRNAs (pri-miRNA), which undergo processing by the microprocessor complex to form a single hairpin structure termed precursor miRNA (pre-miRNA) (Treiber et al., 2019). The microprocessor complex consists of DiGerorge Syndrome Critical Region 8 (DGCR8), an RNA binding protein that recognizes an N6-methyladenylated GGAC motif within the pri-miRNA and RNase III enzyme Drosha, which cleaves the pri-miRNA duplex. Once pre-miRNA is generated, exportin-5 and Ran-GTP exports it to the cytoplasm where it undergoes cleavage by the RNase III enzyme, Dicer (O'Brien et al., 2018). Processing by Dicer removes the terminal loop giving rise to a double stranded 22 nt product consisting of the mature miRNA guide strand and passenger strand. The double stranded miRNA product is transferred onto RNA binding proteins known as Argonaute (AGO) protein. The passenger strand is usually discarded whereas the guide strand is incorporated into the RNA-induced silencing complex (miRISC) and mediates mRNA degradation or translational inhibition. (Treiber et al., 2019).

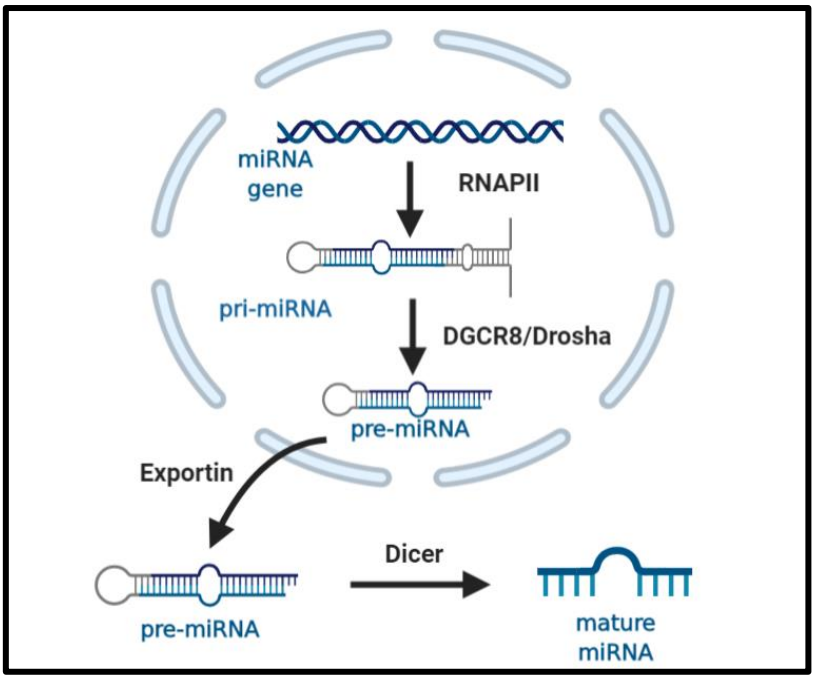

Figure 2.8. The canonical pathway of miRNA biogenesis (prepared by author).

Biogenesis of miRNAs can also occur via several non-canonical pathways. Non-canonical pathways are generally classified into Drosha/DGCR8-independent pathway and Dicer-independent pathways. One class of Drosha/DGCR8-independent miRNAs are known as mitrons which originate from spliced introns and function as pre-miRNAs that do not require cleavage by Drosha/DGCR8 complex. They are immediately exported to the cytoplasm for Dicer processing (Treiber et al., 2019). On the other hand, Dicer-independent miRNAs are relatively rare. They are processed by Drosha from endogenous short hairpin RNA (shRNA) transcripts and are directly recognized by Ago proteins. Therefore, they are produced independently of Dicer (Dai et al., 2019).

2.2.4.1.2. Regulation of gene expression

Generally, miRNAs guide miRISC to recognize a specific complementary seed sequence in the 3'UTR region of the target mRNA and downregulates gene expression by either translational repression or mRNA degradation (Wahid et al., 2010). miRNA binding sites have also been detected in other mRNA regions. miRNA binding to 5' UTR and coding sequences have been reported to have silencing effects whereas binding within promoter regions induces transcription (Place et al., 2008). The mechanism of gene silencing by miRISC depends on the degree of complementarity between the miRNA and a specific sequence on the target mRNA known as the miRNA response element (MRE). A high degree of sequence complementarity enables AGO degradation of target mRNA (Jo et al., 2015). Other mechanisms such as deadenylation, decapping, and exonucleolytic digestion of mRNA are also involved in mRNA degradation (Wahid et al., 2010). However, most miRNA-MRE interactions are not entirely complementary and result in translational repression. The exact mechanism is not well understood but miRNAs are involved in either the inhibition of initiation or elongation stages of translation (Kong et al., 2008).

2.2.4.2. LncRNAs

The first lncRNA, H19, was discovered in the late 1980s during studies investigating genomic imprinting (Jarroux et al., 2017). Since then tens of thousands of lncRNAs have been identified; however, less than 1% of loci identified lncRNA have been experimentally validated (Kopp and Mendell, 2018). lncRNAs share several characteristics with mRNA such as poly-adenylation, 5'-capping and exon-intron splicing. Despite these similarities, lncRNAs tend to have fewer exons and lack open reading frames which prevent its translation (Wang et al., 2017a, DiStefano, 2018). Although lncRNA lack protein coding abilities, they have a broad functional repertoire which include regulation of gene expression, embryonic development, imprinting, chromosomal dynamics, telomere biology, and immune responses (Amaral and Mattick, 2008, Ouyang et al., 2016, Liu et al., 2017, Oliva-Rico and Herrera, 2017). Due to its diverse role in regulating molecular pathways, dysregulation of lncRNA have been implicated in the aetiology of more than 200 diseases including cancer (Bao et al., 2018,

DiStefano, 2018). Therefore, significant research endeavours are being exercised to study the role of lncRNAs in biological processes, and to apply lncRNAs as biomarkers or therapeutic targets.

2.2.4.2.1. Biogenesis

The synthesis of most lncRNA, like mRNA and miRNA, begins with its transcription by RNAP II. They can be transcribed from several different genomic loci and are classified accordingly (Figure 2.9 and Table 2.3) (Khandelwal et al., 2015). Similar to protein coding regions, lncRNA promoters are enriched for active histone modifications (Quinn and Chang, 2016). Many lncRNA transcripts are not end products. To reach their mature forms, they undergo extensive co- and post-transcriptional processing which include 5'capping, 3'-polyadenylation, splicing and RNA editing (Dhanoa et al., 2018). Some lncRNAs undergo alternative processing to distinguish them from other transcripts. For example, back-splicing of linear transcripts produces stable circular RNAs (circRNAs) consisting of non-sequential exon–exon junctions (Lasda and Parker, 2014).

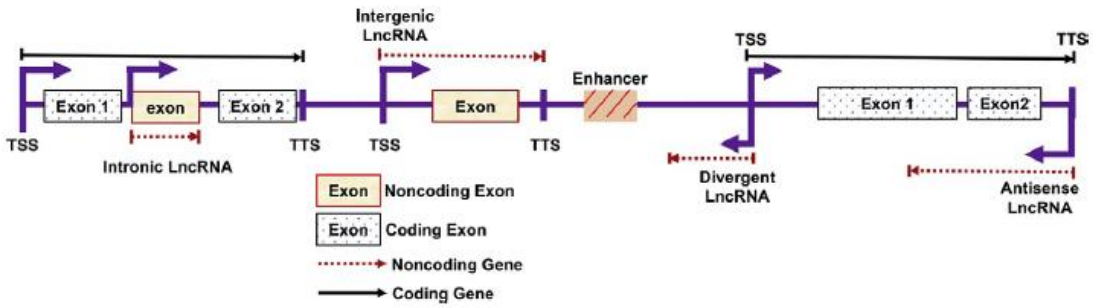

Figure 2.9. Classification of lncRNA based on the location in the genome (Choudhari et al., 2020).

Table 2.3: Classification of lncRNA

Type	Origin	RNA polymerase	Direction of transcription	Additional information
Intergenic lncRNA	Large intervening regions flanked by two protein coding genes	RNAP II or III	Sense or anti-sense	Is 5'-capped and contains 3'-end poly(A) tail. Serves as a precursor to other ncRNAs such as miRNA.
Intronic lncRNA	Intronic regions of protein coding genes	RNAP III or RNAP IV	Sense or anti-sense	Undergoes alternative splicing and contains some exonic sequences and 3'-end poly(A) tail.

Sense lncRNA (exonic/ divergent lncRNA)	Protein coding portions of genes, with exons overlapping those of the companion mRNAs	RNAP II	Sense	Undergoes splicing and lacks open reading frames preventing protein translation
Natural antisense transcripts (NATs)	Antisense strand of proteins coding genes	RNAP III	Antisense	NATs are categorized as cis (occurs on opposite strand of coding gene) or trans (occurs on the opposite strands of the pseudogene)
Bidirectional	(<1 000 bps) to the transcriptional start sites of protein-coding genes	RNAP II	Anti-sense to the protein coding gene	

2.2.4.2.2. Functions

Unlike miRNAs, the functioning of lncRNA cannot be inferred from its sequence or structure. The exact functioning and mechanism of these RNA molecules calls for extensive research; however, we do know that the dynamic functional repertoire of lncRNA includes gene silencing, cell cycle regulation, splicing, chromatin modifications, and differentiation and that lncRNA implement these functions by serving as signalling molecules, molecular decoys, guides or scaffolds (Wang and Chang, 2011, Dhanoa et al., 2018).

The belief that some lncRNA act as signalling molecules stems from the finding that their transcription is tightly controlled and fluctuates in a cell specific manner and is dependent on diverse stimuli and biological events (Figure 2.9A). Signalling lncRNA serve as molecular indicators that reversibly regulate transcriptional and post-transcriptional processes in response to various stimuli (Wang and Chang, 2011). lincRNA-p21 promotes p21 transcription, thereby signalling the repression of p53-dependent genes and the initiation of apoptosis. The main function of a signal lncRNA is to serve as a

molecular signal to regulate transcription in response to various stimuli. Thus, its production and presence can serve as an indicator of transcriptional activity (Huarte et al., 2010).

Recent evidence suggests that like proteins, lncRNA are major players involved in various scaffolding complexes. lncRNAs can also serve as platforms upon which relevant molecular components may be assembled. lncRNA that act as scaffolds are complex and possess different domains that bind to multiple effectors concurrently to regulate gene expression. These effectors can achieve either transcriptional activation or repression in a time and space restricted manner (Figure 2.9B) (Wang and Chang, 2011). For example, the 5'-end of the lncRNA, HOX transcript antisense RNA (HOTAIR) binds to polycomb repressive complex 2 (PRC2) which methylate H3K27 while its 3'-end binds to LSD1 which results in H3K4 demethylation and subsequently gene repression (Tsai et al., 2010).

lncRNA that act as decoys can regulate transcription in a positive and negative manner. Decoy lncRNAs mimic the target binding site of effector molecules on DNA. This prevents the effectors such as transcription factors and chromatin modifiers from gaining access to DNA (Figure 2.9C) (Khandelwal et al., 2015). The lncRNA, p21-associated ncRNA DNA damage activated (PANDA) binds and sequesters the transcription factor NF-YA to limit the expression of pro-apoptotic genes and promote cell survival in response to low levels of DNA damage (Hung et al., 2011).

As molecular guides, lncRNAs bind to proteins and chaperones them to specific targets (Figure 2.9D). This activity can cause changes in gene expression either in cis (on neighbouring genes) or in trans (distantly located genes). For example, the lncRNA, Air recruits the histone methyltransferase, G9a and leads it to their target site where gene silencing is achieved through H3K9 methylation (Nagano et al., 2008).

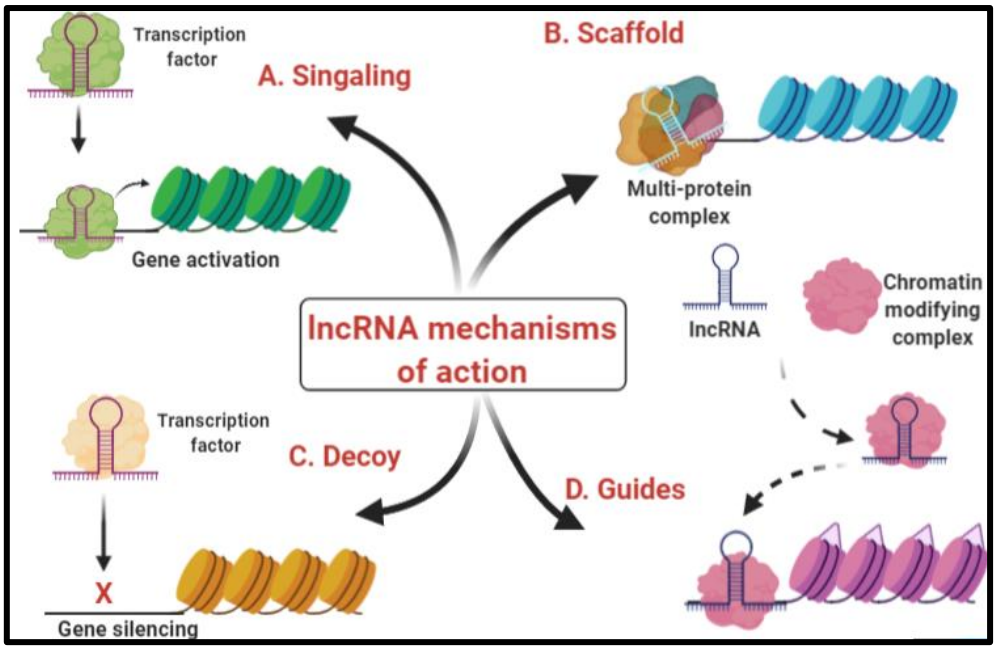

Figure 2.10. General mechanism by which lncRNA function. lncRNAs can act as (A) molecular signals, (B) dynamic scaffolds, (C) decoys and (D) guides (prepared by author).

2.2.4.3. Regulation of miRNA by lncRNAs

As previously discussed, miRNAs sequester their target mRNA through binding of MRE to inhibit translation. lncRNA are able to compete with MRE for miRNA binding. These lncRNA are known as competing endogenous RNAs (ceRNAs) (Wang and Chang, 2011, Tay et al., 2014). They are able to mimic miRNA targets which results in the sequestering of miRNAs at their 3' UTR. This reduces miRNA availability within cells and promotes the translation of their target mRNA (Figure 2.10) (Khandelwal et al., 2015). An example of a lncRNA that functions as a ceRNA is HOXA 11 antisense RNA (HOXA11-AS). HOXA11-AS ceRNA abilities have been observed in various cancers. In non-small-cell lung cancer, HOXA11-AS sequester miR-124 and miR-454, which promotes SP1 and STAT3 expression, respectively (Yu et al., 2017, Zhao et al., 2018). This, in turn, promotes proliferation, invasion and migration of cancer cells. In addition, HOXA11-AS targets miR-125a-5p, miR-130a, miR-140-5p, miR-146-5p, miR-214-3p, miR-215a-5p, miR-241-3p, miR-1297 in various cancers such as hepatocellular, gastric, renal, colorectal cancers and glioma (Wei et al., 2020). Furthermore, HOXA11-AS-miR-124 interactions are involved in fracture healing by inhibiting osteoblast proliferation and enhancing apoptosis (Wang et al., 2017b).

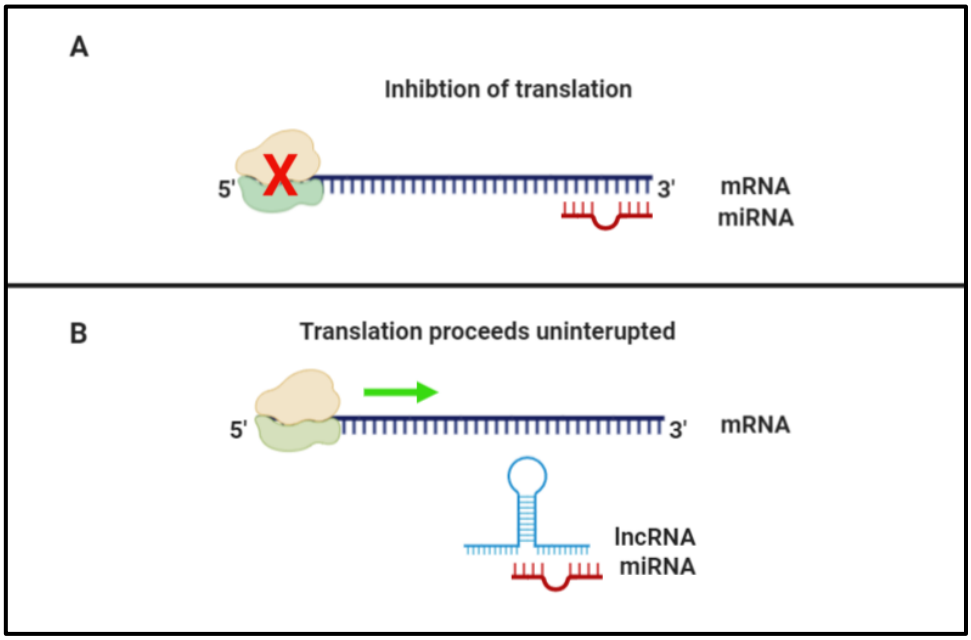

Figure 2.11. Interaction between ncRNA and mRNA. (A) miRNA prevents translation by binding to mRNA. (B) lncRNA sequesters miRNA which allows translation to occur (prepared by author).

2.2.5. The role of epigenetics in Fusarium mycotoxin induced toxicities

The molecular mechanisms by which *Fusarium* mycotoxins induces toxicity have been well documented, however emerging evidence suggests that crosstalk between molecular and epigenetic

modifications play an important role in *Fusarium*-induced toxicities (Huang et al., 2019a, Ghazi et al., 2020a).

It has been suggested that epigenetic modifications may be responsible for Zearalenone's oestrogenic effects. Zearalenone induces the expression of DNMTs and increases global levels of DNA methylation, H3K4me3, H3K9me3 and H3K27me3. These changes have been associated with the disruption of oocyte maturation and early embryonic development associated with zearalenone exposure (Han et al., 2015). Moreover, zearalenone induces CpG methylation of the *LIM Homeobox 8* (*LHX8)* gene, repressing its transcription. LXH8 is the transcription factor responsible for ovarian follicle formation, therefore its downregulation disrupts primordial follicle formation (Zhang et al., 2017). Changes in miRNA profiles have also been attributed to zearalenone's effect on the reproductive system. Zearalenone induces miR-7 expression via protein kinase C and p38. Zearalenone-induced overexpression of miR-7 inhibits follicle stimulating hormone synthesis and secretion (He et al., 2018).

The trichothecenes, T-2 toxin and HT-2 have been shown to induce epigenetic modifications. HT-2 toxin-induced disruption of mouse oocyte maturation via increased global 5mC levels, and decreased H3K9me2 and H3K27me3 levels (Zhu et al., 2016). T-2 toxin induces toxicity through proinflammatory mechanisms. While T-2 toxin increased global DNA methylation, it demethylated the promoters of proinflammatory cytokines which induced cytokine production which in turn induced hepatoxicity (Liu et al., 2019). Furthermore T-2 toxin induces miR-155 expression which disrupts cytokine suppressors (Guo et al., 2020).

Like zearalenone and trichothecenes, the effect of FB_1 on DNA methylation and histone modifications have been thoroughly researched while little research has focused on the role of miRNAs in FB_1-induced toxicity. For instance, several studies have evaluated the effects of FB_1 on DNA methylation. Chuturgoon et al. (2014a) demonstrated that FB_1 induces DNA hypomethylation in HepG2; however, DNA hypermethylation occurred in rat C6 glioma cells and human Caco-2 cells (Mobio et al., 2000, Kouadio et al., 2007). Furthermore, Demirel et al. (2015) found no significant changes in global DNA methylation but hypermethylation occurred at the promoter regions of the tumor suppressors: *c-myc, p15, p16,* and *e-cadherin*. With regards to histone modification, FB_1 induced H3K9me3 and acetylation of H2NK12, H3K9 and H3K23 and repressed H4K20me3 (Pellanda et al., 2012, Sancak and Ozden, 2015, Gardner et al., 2016). The only study to investigate the effect of FB_1 on miRNA found that FB_1 downregulated miR-27b which subsequently increased cytochrome P450 1B1; which may play a role in FB_1-induced hepatic neoplastic transformation (Chuturgoon et al., 2014b). For a detailed discussion on epigenetic mechanisms involved in FB_1 toxicity, see Chapter 3: Molecular and Epigenetic Mechanisms of FB_1 Mediated Toxicity and Carcinogenesis and Detoxification Strategies; pages 86-89.

Little is known on the relationship between *Fusarium* mycotoxins and lncRNA and RNA modifications with the exception of two independent studies that demonstrated that exposure to the mycotoxins fusaric

acid and DON alters the m6A transcriptome (Ghazi et al., 2020b, Zhengchang et al., 2020). While the above-mentioned studies demonstrate that epigenetic modifications play an important role in mycotoxin-induced toxicities, further research should be dedicated to ncRNA and RNA modifications. Moreover, our understanding on the downstream effects of FB_1-induced epigenetic changes is insufficient. Further research should be done to assess the downstream effects of FB_1-induced epigenetic modifications. For instance, epigenetic mechanisms may exacerbate toxicity by dysregulating response mechanism to the stress induced by FB_1. Such stress response mechanisms include DNA damage checkpoint signalling, Keap1/Nrf2 anti-oxidant responses and apoptosis as it well established that FB_1 induces DNA damage and oxidative stress.

2.3. Cellular Response to stress

2.3.1.The DNA damage response

The survival of organisms depends on the preservation of genetic information between cell lineages during replication (Zhou and Elledge, 2000). However, DNA is highly susceptible to damage by endogenous and exogenous agents and mistakes during replication can occur (Chatterjee and Walker, 2017). It is estimated that approximately 10^5 DNA lesions occur in each cell per day. These lesions severely affect important genomic processes such as transcription and replication of damaged DNA result in mutations that induce and propagate carcinogenesis (Giglia-Mari et al., 2011). The timely clearance of genomic injuries is therefore, essential. Cells are equipped with a complex network of DNA damage responses (DDR) which monitor the structure and integrity of the genome, co-ordinate cell cycle arrest and initiate DNA repair (Zhou and Elledge, 2000).

DNA damage checkpoint signalling is a central orchestrator of the DDR network. Checkpoints stall cell division so that effective DNA repair can occur (Dai and Grant, 2010). This signalling network consists of sensors, transducers, mediators and effectors (Figure 2.11) (Zhou and Elledge, 2000). Sensors are multiprotein complexes that detect aberrant DNA structures and initiate the signalling response. The MRe11-Rad 50-Nbs1 (MRN) sensor complex detects double stranded DNA breaks and recruit's ATM to the DNA damage site, while Rad17 and Rad9-Rad1-Hus1/9-1-1 complex generally recognise single strand breaks and localizes ATR to the lesion (Dai and Grant, 2010). ATR and ATM are proximal transducers that have kinase activity. The activation of ATR and ATM phosphorylates and activates mediators (such as 53BP1, MDC1, TopBP1, and claspin etc.) at DNA damage sites which in turn activates the distal transducers: checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2) (Dai and Grant, 2010). Ultimately, ATR transduces signals to CHK1 whereas ATM transduces signals to CHK2. Activated "distal transducers" phosphorylate, degrade or sequester "effectors" Cdc25s (e.g., Cdc25A, B, and C), which in turn inhibit cyclin-dependent kinases (e.g., Cdk1/cdc2 and Cdk2) that are responsible for cell cycle progression (Patil et al., 2013).

This process prevents S-phase entry (G1/S-phase checkpoint), delay S-phase progression (S-phase checkpoint), or halts mitotic entry (G2/M-phase checkpoint) (Dai and Grant, 2010). DNA repair is now able to occur and the type of repair is dependent on the type of DNA damage that occurred (Chatterjee and Walker, 2017). If the damage is irreversible CHK1 and CHK2 trigger p53-dependent or -independent apoptosis (Dai and Grant, 2010).

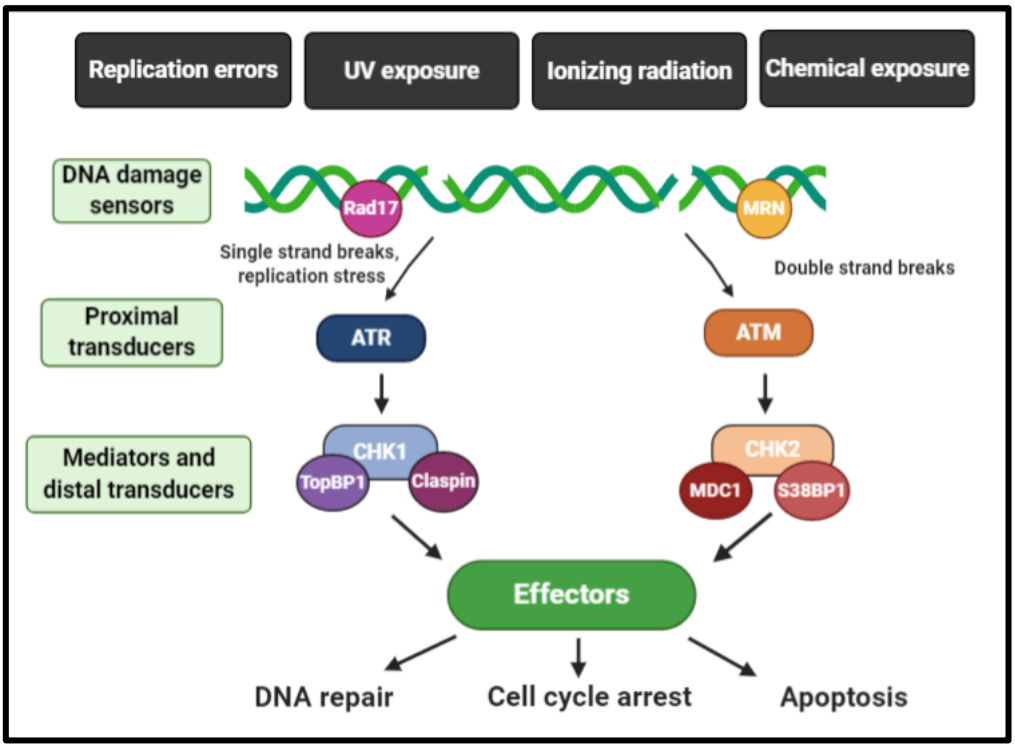

Figure 2.12. DNA damage response network (prepared by author).

2.3.1.1. CHK1

As a central regulator in DNA damage checkpoint signalling, the role of CHK1 is not limited to the interphase of the cell cycle. CHK1 enables spindle checkpoint which delays anaphase onset in cells with mitotic spindle defects (Dai and Grant, 2010). CHK1 facilitates DNA damage-induced transcriptional repression via the phosphorylation of threonine residues on histone 3 and loss of histone acetylation (Patil et al., 2013). In addition to its regulation of p53, CHK1 suppresses caspase-3-dependent apoptosis and blocks caspase-2-dependent apoptotic responses. Furthermore, CHK1 mediates DNA repair by targeting repair kinases (e.g., DNA-PK) important for the repair double stranded DNA breaks, homologous repair and Fanconi Anemia(FA)/BRCA-mediated DNA repair pathway (Patil et al., 2013).

Diminished activity or expression of CHK1 abrogates its essential function and therefore it should be tightly regulated. As discussed previously, CHK1 is activated via mediators in response to DNA damage (Dai and Grant, 2010). Activation occurs via the phosphorylation of two conserved sites, serine-317 and serine-345; however, it's not well understood how exactly phosphorylation activates CHK1 (Patil

et al., 2013). One model suggests that the C-terminal domain of CHK1 interacts with its kinase domain to mask the active site, and that the phosphorylation at serine-317 and serine-345 dissociates these two domains leading to CHK1 activation (Chen et al., 2000). Phosphorylation of CHK1 can also have inhibitory effects. Downregulation of the tumour suppressor, phosphatase and tensin homolog (PTEN), inactivates CHK1 activity and promotes the accumulation of DNA damage due to its loss of control over phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signalling. PI3K/AKT signalling induces phosphorylation of the serine-280 residue of CHK1 which subsequently impairs CHK1 activation by DNA damage and promotes genomic instability (King et al., 2004, Puc et al., 2005).

2.3.2. Keap1/Nrf2 anti-oxidant signalling

ROS are produced during normal physiological reactions and are involved in a number of signalling pathways (Finkel, 2011). The rapid accumulation of ROS by dysfunctional endogenous or exogenous sources overwhelms the antioxidant system of cells and oxidative stress ensues (Thannickal and Fanburg, 2000). This results in cellular injury in the form of lipid peroxidation, protein carbonylation and DNA damage and eventually, the development of cancer, neurodegeneration, and diabetes. It is, therefore, necessary that cellular redox signalling is tightly controlled (Thannickal and Fanburg, 2000, Finkel, 2011).

The Kelch-like ECH-associated protein 1 (Keap1)/ Nuclear factor erythroid 2-related factor 2 (Nrf2) signalling pathway is the master regulator of cytoprotective responses to oxidative and electrophilic stress. The key players are the redox sensitive transcription factor, Nrf2 and the cysteine rich repressor protein Keap1 (Kansanen et al., 2013). In a redox balanced environment Keap1 interacts with the cullin-3 E3-ubiquitin ligase (Cul3) which serves as a platform for the ubiquitination and proteasomal degradation of Nrf2 by 26S. (Baird and Yamamoto, 2020). On exposure to oxidative or xenobiotic stress, excess ROS interacts with the redox sensitive cysteine residues on Keap1 resulting in conformational changes to Keap1. The binding affinity between Nrf2 and Keap1 is reduced and the ubiquitination system of Nrf2-Cul3 is disrupted (Kansanen et al., 2013). The stabilized Nrf2 translocates to the nucleus where it dimerizes with small maf proteins and subsequently binds to the anti-oxidant response element (ARE) found on genes involved phase II and III detoxification, cellular regeneration, xenobiotic metabolism, and ROS detoxification (antioxidants) (Figure 2.12) (Ray et al., 2012). On recovery of the redox balance, Nrf2 is dissociated from the ARE sequence. Keap1 enters into the nucleus and escorts Nrf2 to the cytoplasm for degradation (Kansanen et al., 2013).

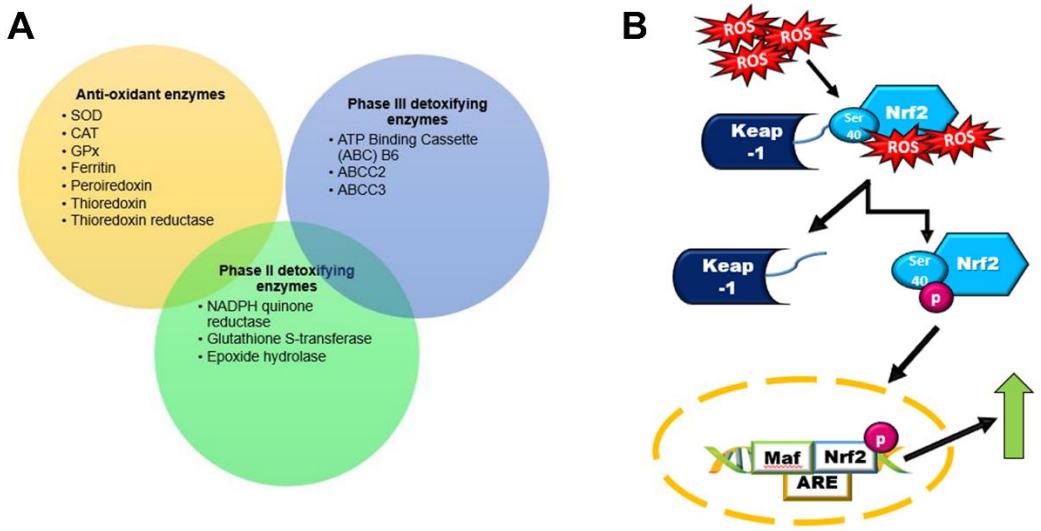

Figure 2.13. (A) Nrf2 promotes the transcription of antioxidants as well as phase II and III detoxifying enzymes. (B) Oxidative stress triggers Nrf2 dissociation from Keap1 and induces transcription of ARE genes (Arumugam et al., 2020).

Although the cytoprotective effects offered by Nrf2 is essential in cancer prevention, the constitutive activation of Nrf2 promotes the development and chemoresistance of various cancers. Nrf2 hyperactivity incites new characteristics to cancer cells such as avoidance of apoptosis, excessive proliferation and chemoresistance. There are several mechanisms by which Nrf2 signalling is activated in cancer cells: i) somatic mutations in *Keap1*, Cul3 or *Nrf2* disrupting Keap1/Nrf2 interactions, (ii) Nrf2 transcription facilitated by the oncogenes Myc, K-Ras, and B-Raf mutation via mitogen-activated protein kinases (MAPKs), (iii) Keap1 competing proteins that disrupt Keap1/Nrf2 interactions and (iv) epigenetic changes that amplify Nrf2 levels and reduce Keap1 (Wu et al., 2019).

2.3.2.1. Epigenetic regulation of Keap1/Nrf2

Research into Epigenetic modifications involved in Keap1/Nrf2 regulation have only recently become wide spread. Interest in the field was initiated by Guo et al. (2012) who observed that hypermethylation of *Keap1* promoters in lung cancer prevented SP1 binding and thus *Keap1* transcription. Since then, several studies have evaluated the effect of DNA methylation, histone modifications, and ncRNA on Keap1 and Nrf2 (Cheng et al., 2016, Bhattacharjee and Dashwood, 2020). Table 2.4 summarizes the effects of these epigenetic mechanisms on Keap1 and Nrf2.

Table 2.4: Epigenetic regulation of Keap-1 and Nrf2

Target	Epigenetic modification	Effect on target	Reference
Nrf2	DNA methylation	Gene silencing	(Khor et al., 2014)
	DNA demethylation	Transcriptional activation	(Kang et al., 2014)
	H3k27me3	Gene silencing	(Li et al., 2014)
	miR-27a, 34, 93 153, 142-5p, 144	Degrade Nrf2 mRNA	(Bhattacharjee and Dashwood, 2020)
	lncRNA: UCA1, MEG3, NRA2	Promotes Nrf2 translation by sponging miRNA that targets Nrf2	(Bhattacharjee and Dashwood, 2020)
Keap1	DNA methylation	Gene silencing	(Guo et al., 2012)
	DNA demethylation	Transcriptional activation	(Palsamy et al., 2012)
	H3K4me3	Transcriptional activation	(Mishra et al., 2014)
	miR-7, 141, 200, 432, 455, 873	Degrades Keap1 mRNA	(Bhattacharjee and Dashwood, 2020)
	lncRNA: MALAT	Downregulates Keap1	(Bhattacharjee et al., 2020)

The role of m6A modifications in Keap1/Nrf2 regulation have also been investigated but not as thoroughly as other epigenetic modifications. One study showed that colistin-induced oxidative stress was attenuated by the accumulation of m6A modifications on pri-miR-873. This promoted the generation of mature miR-873-5p which in turn inhibited Keap1 expression and promoted Nrf2 antioxidant responses (Wang et al., 2019). Oxidative stress was also shown to elevate m6A-*Nrf2* levels in di-(2-ethylhexyl) phthalate (DEHP) exposed rats; however, the authors hypothesized that m6A-*Nrf2* inhibits Nrf2 signalling (Zhao et al., 2020).

2.3.3. Apoptosis

Apoptosis is a form of cell death that involves the controlled dismantling of intracellular components while avoiding inflammation and damage to neighbouring tissue (McIlwain et al., 2013). It is a homeostatic process that secures normal development and aging and controls cell populations by removing surplus, damaged, and cancerous cells (Shen and White, 2001). Apoptosis is also a defence

mechanism that responds to various noxious stimuli and stresses such as DNA damage, cell cycle dysfunctions and oncogene activation (Shen and White, 2001, Elmore, 2007). Considering that apoptosis responds to both physiological and pathophysiological stimuli, aberrant regulation of apoptosis can result in Alzheimer's disease, rheumatoid arthritis, defects in embryonic development and cancer.

Various morphological changes occur during apoptosis (Figure 2.13). The onset apoptosis is characterized by cell shrinkage followed by pyknosis – chromatin condensation and nuclear shrinkage; while the latter stages are typified by membrane blebbing, karyorrhexis (nuclear and DNA fragmentation) and the containment of cell fragments into apoptotic bodies (Saraste and Pulkki, 2000, Elmore, 2007). The apoptotic bodies are tightly packed with intact organelles and nuclear fragments of the apoptotic cells. These bodies are subsequently engulfed by phagocytes such as macrophages and parenchyma (Saraste and Pulkki, 2000). Degradation occurs within phagolysosomes; however, if phagocytosis does not occur cells will undergo degradation which resembles necrosis (cell death via rapid swelling and rupturing of cells) in a process called secondary necrosis (Saraste and Pulkki, 2000) The containment of apoptotic cells in apoptotic bodies and rapid engulfment by phagocytes prevents apoptotic cells from releasing their cellular content into the neighbouring tissue. This prevents the occurrence of inflammation and necrosis to the surrounding tissue (Elmore, 2007).

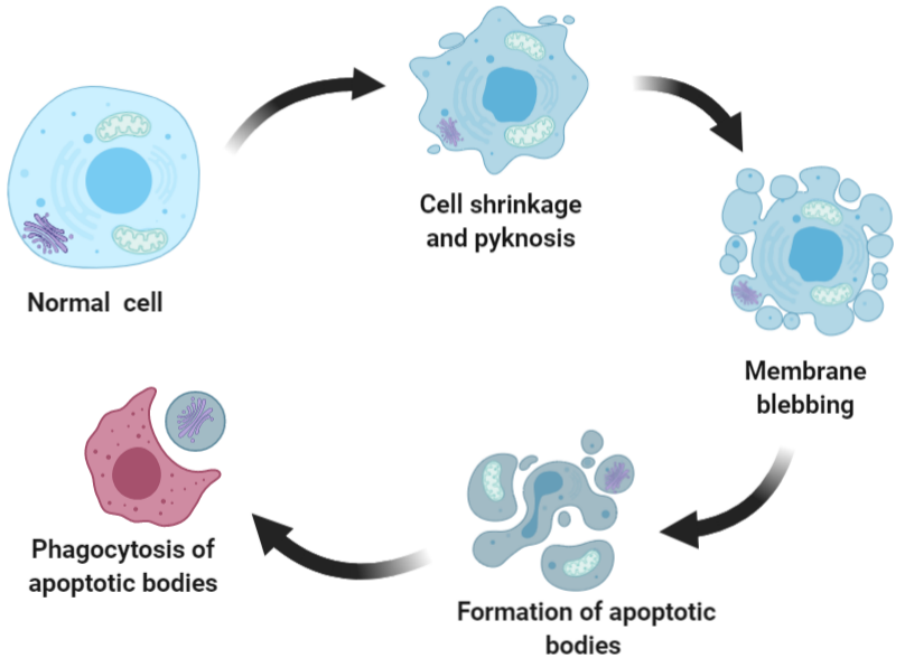

Figure 2.14. Morphological changes that occur during apoptosis (prepared by author).

These morphological hallmarks of apoptosis are dependent on highly complex and sophisticated molecular and biochemical events necessary for the proper execution of apoptosis (Shen and White, 2001). Apoptosis occurs via two main pathways: intrinsic or mitochondrial pathway and extrinsic or

death receptor pathway (Elmore, 2007). Both pathways rely on the activation of a family of endo-proteases known as caspases (McIlwain et al., 2013).

2.3.3.1. Caspase

Caspases are a family of evolutionary conserved cysteinyl aspartate proteinases that are responsible for the morphological changes that occur during apoptosis. Presently, 14 caspases have been identified and have been broadly classified according to their functions in apoptosis and inflammation (McIlwain et al., 2013). All caspases consist of an active site cysteine and can cleave substrates after an aspartic acid residue. Caspases are initially expressed as inert monomeric proenzymes or procaspases (McIlwain et al., 2013). Procaspases consist of an N-terminal prodomain, p10 and p20 domains and activation of caspases can occur via three general mechanisms: induced proximity, formation of a holoenzyme or processing by an upstream caspase (Hengartner, 2000).

Induced proximity involves the aggregation of multiple procaspases resulting in their cross-activation; while activation by holoenzyme is mediated by conformational changes rather than proteolytic cleavage. These two mechanisms are involved in the activation of short domain initiator caspase-8 and caspase-9, respectively (Hengartner, 2000). The activation of procaspase by an upstream caspase is responsible for the activation of most caspases and is the most effective method for executioner caspase (caspase-3, -6, and -7) activation. Initiator caspases cleave executioner procaspases at the aspartate residue between the N-terminal prodomain and p20 and between p20 and p10 domains. These executioner caspases are workhorses of the caspase family. Once activated, an executioner caspase can activate other executioner procaspases (McIlwain et al., 2013). The activation of procaspases by mature caspases is known as the caspase cascade and is an effective method of amplifying apoptotic signalling resulting in rapid cell death (Elmore, 2007).

2.3.3.2.. Pathways of apoptosis

Various pathways exist to execute apoptotic cell death. They are easily distinguished by their adaptors and initiator caspases. However, there are two distinct yet converging pathways that play a key role in the apoptotic program of mammals. These pathways are referred to as the intrinsic and extrinsic pathways and both pathways rely on the activation of the caspase cascade to execute apoptosis.

2.3.3.2.1. Intrinsic apoptotic program

The intrinsic pathway of apoptosis is also known as the mitochondrial pathway as it depends on factors released from the mitochondria. It is activated by an array of cellular stresses such as toxins, free radicals, radiation and viral factors or via developmental signals such as the absence of growth factors or hormones that usually suppress death programs (Elmore, 2007).

These stimuli trigger the activation of the proapoptotic protein, Bim. Bim sequesters the antiapoptotic protein Bcl-2 and promotes the formation of Bak-Bax oligomers within the outer membrane of the

mitochondria (Nakajima and Kuranaga, 2017). This results in the opening of the mitochondrial permeability transition pore and the release of cytochrome c from the mitochondria (Elmore, 2007). The release of cytochrome c into the cytosol promotes the formation of the signalling platform known as the apoptosome (Nakajima and Kuranaga, 2017). The binding of cytochrome c and subsequent binding of deoxyATP to apoptotic protease activating factor-1 (Apaf-1) induces conformational changes that activate Apaf-1 (McIlwain et al., 2013). Seven activated Apaf-1 monomers oligomerize and recruit procaspase-9. This complex is known as the apoptosome and its formation induces conformational changes required for the activation of procaspase-9, which consequently activates executioner caspases, resulting in apoptotic cell death (Figure 2.14) (McIlwain et al., 2013).

2.3.3.2.2. Extrinsic Apoptotic program

The extrinsic pathway or death receptor pathway is triggered by extracellular signals in the form of ligands binding to death receptors. Death receptors involved in apoptosis include tumor necrosis factor (TNF) receptor 1 (TNFR1), TNF-related apoptosis inducing ligand receptor 1 (TRAILR1), TRAILR2, Fas receptor (FasR) and death receptor 3 (DR3) (McIlwain et al., 2013). The binding of ligands to their respective death receptors triggers the multimerization of death receptors and recruitment of adapter proteins [TNFR-associated death domain (TRADD) and Fas-associated death domain (FADD)] via their death domains, forming an intracellular death-inducing signalling complex known as DISC (Li and Yuan, 2008). The N-terminal of procaspase-8 also contains a death domain, thus DISC can recruit procaspase-8 to the complex. An accumulation of procaspase-8 results in its dimerization and activation (McIlwain et al., 2013). Depending on the cell type, caspase-8 can directly cleave and activate executioner caspases (type I cells) or activate intrinsic apoptosis (type II cells). To activate intrinsic apoptosis, caspase-8 cleaves and activates the proapoptotic protein bid to tBid. tBid localizes to the mitochondria to activate downstream intrinsic pathways (Figure 2.14) (Li and Yuan, 2008).

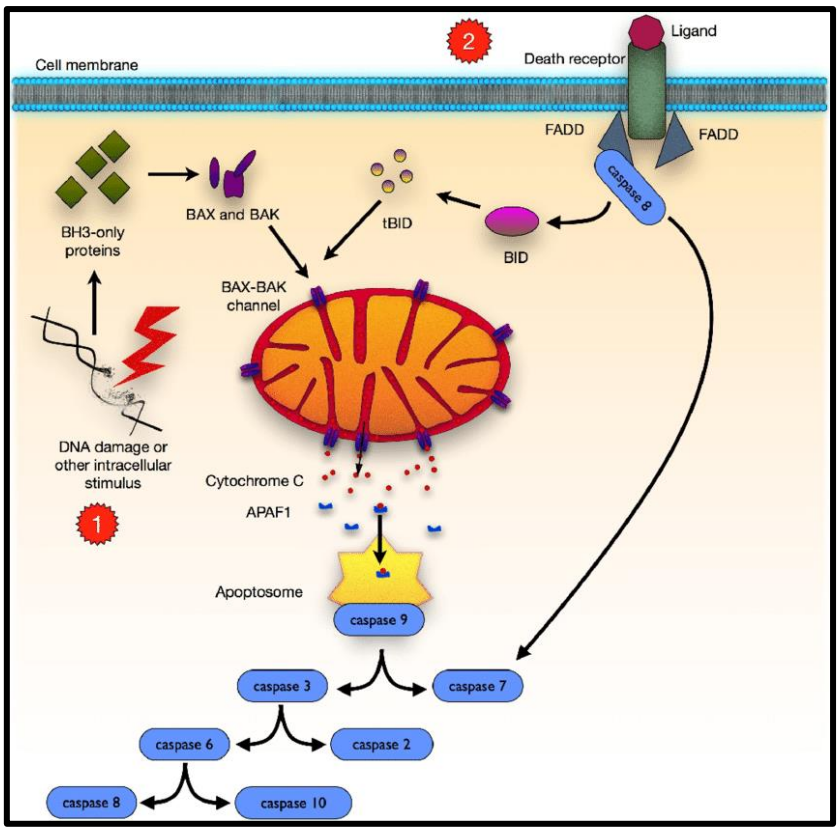

Figure 2.15. Intrinsic and extrinsic signalling of apoptosis (Glowacki et al., 2013).

2.3.3.2.3. Execution of apoptosis

Both intrinsic and extrinsic apoptosis terminate with the activation of executioner caspases (Caspases-3, -6 and -7) (McIlwain et al., 2013). Executioner caspases execute apoptosis via the cleavage and subsequent activation of substrates such as cytoplasmic endonucleases and proteases, which degrade nuclear material and cytoskeletal proteins respectively (Elmore, 2007). The cleavage of various substrates results in the morphological changes that occur in apoptotic cells. For example, caspase-activated deoxyribonuclease (CAD) is responsible for chromatin condensation and degradation of chromosomal DNA during apoptosis. In proliferating cells, CAD is inactivated as it is complexed to the inhibitor, ICAD. Caspase-3 cleaves ICAD thereby activating CAD and chromatin condensation (Enari et al., 1998).

2.3.3.3. p53

The tumour suppressor, p53 is widely regarded as the guardian of the genome and is the master regulator of cellular stress responses (Anbarasan and Bourdon, 2019). p53 is responsible for maintaining tissue homeostasis and responds to a variety of stress signals (such as DNA damage, nutrient deprivation and oncogenic activation) by mediating surveillance of genome integrity, cell cycle checkpoint regulation, DNA repair and apoptosis (Figure 2.15). Loss of p53 expression or function promotes checkpoint defects, genomic instability and the continued proliferation of damaged cells (Fridman and Lowe,

2003). Unfortunately, almost 50% of cancers have been reported to contain a mutated or inactive p53 (Anbarasan and Bourdon, 2019). On the other hand, chronic activation of p53 is associated with degenerative disorders such as arthritis and sclerosis. It is, therefore, imperative that expression and activity of p53 should be tightly regulated (Fierabracci and Pellegrino, 2016).

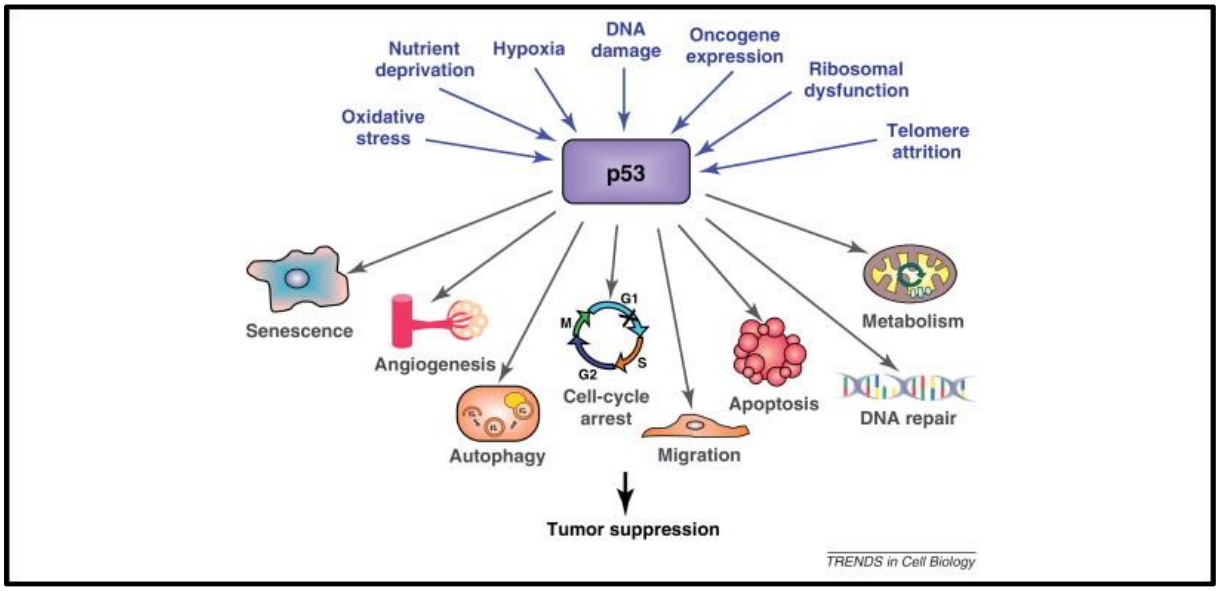

Figure 2.16. p53 responds to a plethora of stress signals and regulates diverse responses (Bieging and Attardi, 2012).

As the central player in stress response, p53 needs to be tightly regulated. During homeostatic conditions, p53 is maintained in an inactive state via proteasomal degradation by Mouse double minute 2 homolog (MDM2). Cellular stress signals inhibit MDM2 degradation of p53 or induce posttranslational modifications (such as acetylation, proliferation) to p53. These changes allow for the accumulation and activation of p53 (Aubrey et al., 2018).

p53 is also regulated via epigenetic mechanisms such as promoter methylation. At the transcriptional level, hypermethylation of the *p53* gene promoter prevents the binding of transcriptional machinery and reduces *p53* transcription. However, hypomethylation of the p53 promoter, enables the binding of transcriptional machinery, and promotes p53 expression (Chmelarova et al., 2013). *In vitro* studies using reporter gene constructs found that DNA methylation reduced *p53* gene expression by 90% in mice and by 85% in rats (Saldaña-Meyer and Recillas-Targa, 2011). In cancer cells tumour suppressor genes are frequently silenced via epigenetic mechanisms Hypermethylation of the *p53* gene promoter and subsequent loss of p53 function was observed in the majority of patients with hepatocellular carcinomas, 51.5% of patients with ovarian cancer, 40% of patients with chronic lymphocytic leukaemia and 30% of patients with acute lymphoblastic leukaemia (Saldaña-Meyer and Recillas-Targa, 2011, Chmelarova et al., 2013). At the post-transcriptional level, p53 is regulated by a variety of miRNAs. miRNAs such as miR-125a, miR-125b, miR-504, miRNA-25 are responsible for the degradation of p53 (Saldaña-Meyer and Recillas-Targa, 2011).

2.3.3.3.1. p53-mediated apoptosis

p53 is a transcription factor that has the ability to transactivate genes involved in promoting apoptosis (Aubrey et al., 2018). The Bcl2 family are important players in regulating apoptosis. The Bcl2 family consists of both pro-apoptotic (Bax, Bak, Bim, Bid, Noxa, Puma and Bcl-x_s) and anti-apoptotic (Bcl-2 and Bcl-x_L) members that interact with one another to control apoptosis especially intrinsic apoptosis (Shen and White, 2001). Such interaction includes the binding and inactivation of anti-apoptotic Bcl2 by proapoptotic Bim (Nakajima and Kuranaga, 2017). Genes encoding for several pro-apoptotic Bcl2 members (Bax, Bid, Puma, and Noxa) harbour consensus p53 response elements which allows for p53 binding. p53 binding to these sequences promotes the transcription of these apoptotic genes (Figure 2.16) (Fridman and Lowe, 2003).

Furthermore, p53 is also involved in the transactivation of apoptotic machinery involved in the extrinsic pathway (DR5, FasR, Fas ligand) and the intrinsic pathway (Apaf-1) and executioner caspase-6. While most studies focus on p53 transactivation function, p53 also suppresses transcription. The inhibitor of apoptosis, survivin is one of the targets of p53-transrepression (Fridman and Lowe, 2003).

p53 can drive the expression of several other genes to inhibit survival pathways. For example, the PI3K/AKT pathway is involved in the phosphorylation and subsequent activation of proteins that promote survival. p53 induces PTEN expression which in turn negatively regulates PI3K/AKT and survival signals (Fridman and Lowe, 2003). p53 induces miR-34 expression, which in turn represses proapoptotic Bcl2 translation (Aubrey et al., 2018). p53 may also regulate apoptosis via transcription-independent mechanisms, however it is not as established as transcriptional-dependent mechanisms. p53 accumulates in the mitochondria in response to DNA damage and this redistribution may play a role in cytochrome c release and caspase activation (Fridman and Lowe, 2003). p53 plays an essential; role in co-ordinating apoptosis. While it may not induce apoptosis directly; it sensitizes cells so that apoptosis can be triggered more easily in response to stimuli that activates cell death (Aubrey et al., 2018).

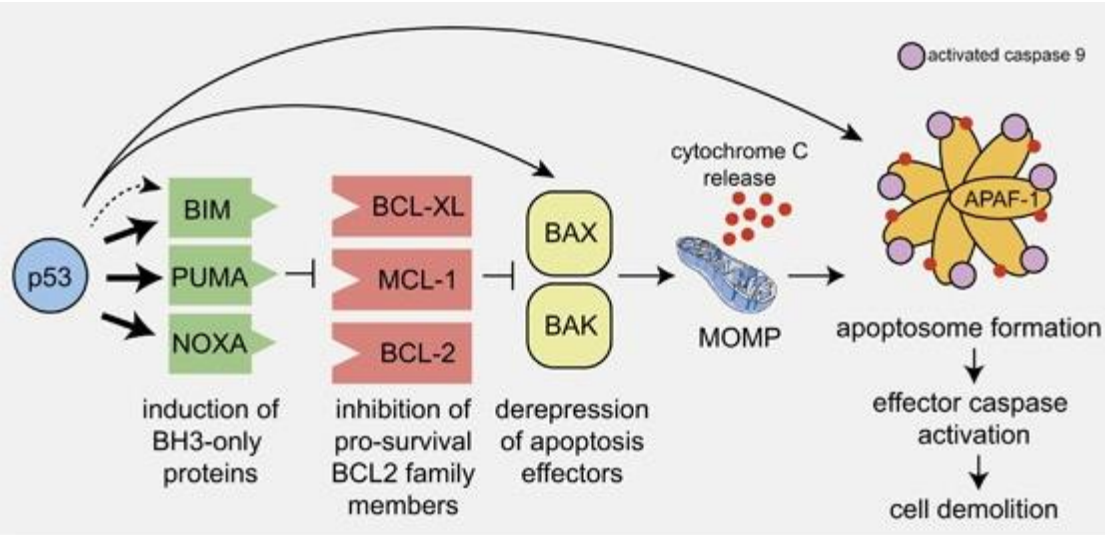

Figure 2.17. Mechanisms of p53-mediated apoptosis via Bcl2-regulated pathway (Aubrey et al., 2018).

Recently, changes to the epigenome have been associated with exposure to FB_1. However current research on the association between FB_1 and epigenetic modifications are often conflicting. Furthermore, the downstream effects of these FB_1-induced epigenetic changes have not been adequately assessed. It is well established that FB_1 induces oxidative stress and DNA damage. FB_1-induced epigenetic changes may dysregulate responses (checkpoint signalling, Keap1/Nrf2 and apoptosis) to oxidative stress and/or DNA damage. This may further exacerbate toxicity induced by FB_1. Therefore, this study aimed to determine the epigenetic effects of FB_1 and the downstream implications of these epigenetic alterations to stress response in human liver (HepG2) cells.

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CHAPTER 3

Molecular and Epigenetic Modes of FB_1 Mediated Toxicity and Carcinogenesis and Detoxification Strategies

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Abstract

Fumonisin B_1 (FB_1) is a natural contaminant of agricultural commodities that has displayed a myriad of toxicities in animals and humans. Moreover, it is known to be a hepatorenal carcinogen in rodents and may be associated with oesophageal and hepatocellular carcinomas in humans. The most well elucidated mode of FB_1-mediated toxicity is its disruption of sphingolipid metabolism; however, enhanced oxidative stress, endoplasmic reticulum stress, autophagy and alterations in immune response may also play a role in its toxicity and carcinogenicity. Alterations to the host epigenome may impact on the toxic and carcinogenic response to FB_1. Seeing that the contamination of FB_1 in food poses a considerable risk to human and animal health, a great deal of research has focused on new methods to prevent and attenuate FB_1-induced toxic consequences. The focus of the present review is on the molecular and epigenetic interactions of FB_1 as well as recent research involving FB_1 detoxification.

Key Words

Fusarium, Mycotoxins, Fumonisin B_1, Toxicity, Oxidative Stress, ER Stress, Immunotoxicity, Epigenetics, Mycotoxin Detoxification

Introduction

Fumonisins are a ubiquitous group of secondary fungal metabolites (mycotoxins) which are produced by the *Fusarium* genus, particularly *F. verticillioides* and *F. Proliferatum* (Rheeder et al., 2002). The discovery of fumonisins were prompted by a field outbreak of equine leukoencephalomalacia (ELEM) in 1970, South Africa. After extensive research it was concluded that the causative agent of this neurotic disease was associated with mouldy maize that was predominately contaminated with *F. verticillioides* (formally, *F. moniliforme*) (Kellerman et al., 1972). Almost a decade later, *F. verticillioides* contaminated maize was found to be linked with the high incidence of oesophageal cancer in South Africa's former Transkei region, where maize is a dietary staple (Marasas et al., 1981). Experimental studies also showed that *F. verticillioides* induced ELEM in horses as well as pulmonary oedema in swine (Kriek et al., 1981a). In rats, the fungi were found to be hepatotoxic, cardiotoxic and induced primary hepatocellular carcinomas and cholangiocarcinoma (Kriek et al., 1981b, Marasas, 2001). Several mycotoxins were identified to be metabolites of *F. verticillioides,* but the causative agent of these incidents remained elusive until fumonisins were finally isolated and characterized in 1988 (Gelderblom et al., 1988b).

Since then, at least 28 fumonisins have been identified and grouped into one of four classes (A, B, C and P) of which fumonisin B_1 (FB_1) is regarded as the most abundant and toxicologically relevant homologue (Rheeder et al., 2002). FB_1 persistently contaminates the food supply of both animals and humans across the world. Maize and maize-based products are one of the most common foods infected by FB_1 (Lee and Ryu, 2017). It is also found in abundance in other cereals such as wheat, rice, oats, barley, and millet (Lee and Ryu, 2017), and has been reported to contaminate numerous food products

including vine fruit (Varga et al., 2010), asparagus (Waskiewicz et al., 2010), beers (Piacentini et al., 2017), and milk (Gazzotti et al., 2009). FB_1 contamination of crops can occur pre- and/or post-harvest, making it difficult to control contamination. Factors favouring *Fusarium* growth and FB_1 production include heat stress, insect damage, and high humidity (Ferrigo et al., 2016). Furthermore, improper storage conditions that are not moisture and temperature-controlled account for a large amount of FB_1 contamination (Phokane et al., 2019). Due to regional climatic variations, the Americas have the highest incidence of FB_1 contamination (96%), followed by Africa and Asia (62%) (Lee and Ryu, 2017). Moreover, the rise in average temperatures and humidity due to climate change may potentially give rise to increased levels of FB_1 in agricultural products. It is expected that additional regions may begin to experience issues with FB_1 contamination while countries with existing FB_1 contamination may expect higher levels in their crops (Magan et al., 2011).

Developed countries have set federal regulations to limit FB_1 contamination of foods and feeds. For example, the United States Food and Drug Administration set the maximum tolerable limit for FB_1 in maize products at 2 ppm while the European Union regulation of FB_1 levels in maize is 1 ppm (Wild and Gong, 2010). In 2000, the Scientific Committee on Food established a maximum daily intake of 0.2 mg/kg body weight (bw) based on no observed adverse effects in the liver and kidneys of rodents. Later, the limit was expanded to include FB_2 and FB_3. The Joint FAO-WHO Expert Committee (JECFA) has also declared that the provisional maximum tolerable intake of FB_1 alone or in combination with FB_2 and FB_3 should be 2 µg/kg bw/day (FOA/WHO, 2002), however, in developing countries where maize is a dietary staple, intake far exceeds the recommended maximum daily limits. FB_1 intake can range from 2.87–8.14 µg/kg bw/day in Eastern Cape, South Africa (van der Westhuizen et al., 2011); 3.5-15.6 µg/kg bw/day in Guatemala (Torres et al., 2007); 0.1-26 µg/kg bw/day in Tanzanian children (Kimanya et al., 2009); and can reach as high as 10,541.6 µg/kg bw/day in Fusui, China (Sun et al., 2011).

FB_1 contamination is especially prominent in rural areas where subsistence farming is common (Shephard et al., 2019). Most subsistence farmers do not have the resources to implement the same agronomic practices seen in commercial settings. Lack of pest control and crop rotation, use of untreated seeds from previous seasons, maize monoculture, poor sorting and inadequate storage conditions, and general lack of mycotoxin awareness can exacerbate the incidence of fungal infection and FB_1 production in crops (Mboya and Kolanisi, 2014, Alberts et al., 2019, Phokane et al., 2019). FB_1-related adverse health conditions are especially common in rural areas that depend on “homegrown” crops. Areas along the Mexican-American borders have reported that maternal consumption of maize and maize products contaminated with FB_1 during gestation was related to an increased risk of their offspring developing neural tube defects (NTD) such as spinal bifida and anencephaly with extremely high exposure leading to foetal death (Hendricks, 1999, Missmer et al., 2006). In rural Tanzania, infantile exposure to FB_1 contributes to the high growth impairment and developmental issues (Shirima

et al., 2015, Chen et al., 2018). Outbreaks of acute toxicosis presenting with transient abdominal pain, borborygmus, and diarrhoea were reported in South India after the consumption of bread made from FB_1-contaminated sorghum and corn (Reddy and Raghavender, 2008). In addition to the 1981 cohort, several other epidemiological studies have demonstrated a close link between the high incidence of oesophageal carcinomas and FB_1 (Sydenham et al., 1990, Yoshizawa et al., 1994, Wang et al., 2000, Qiu et al., 2001, Sun et al., 2007, Alizadeh et al., 2012). A Chinese cohort also found that FB_1 may be linked with a high incidence of hepatocellular carcinomas (Sun et al., 2007). While FB_1 exposure is a suspected contributing factor for carcinogenesis in humans; FB_1 has both cancer initiating and promoting effects in animal models (Table 3.1). The type of tumour present in these models are both sex and species dependent. After evaluating published epidemiological studies and experimental models that demonstrated a link between FB_1 consumption and cancer occurrence, the International Agency for Research on Cancer (IARC) concluded that there was enough evidence to classify FB_1 as a class 2B carcinogen (IARC, 2002).

The carcinogenic character of fumonisins is not fully understood; however, it has been hypothesized that tumour development could be a result of FB_1 mimicking genotoxic carcinogens by inducing toxicity resulting in compensatory proliferation and survival (Ramljak et al., 2000). The primary mode in which FB_1 induces toxicity is through the disruption of sphingolipid metabolism which can trigger or potentiate a host of toxic responses such as oxidative stress, endoplasmic reticulum (ER) stress, autophagy, and alterations in immune responses. Furthermore, FB_1 can mediate changes in the epigenome, altering the expression of cancer-related genes (Chuturgoon et al., 2014b, Demirel et al., 2015). Therefore, this review focuses on the molecular and epigenetic modes of action involved in FB_1 toxicity and carcinogenicity with emphasis on recent findings. Furthermore, we discuss new strategies related to the detoxification of this harmful mycotoxin.

Table 3.1: Studies evaluating the development of neoplastic lesions in *in vivo* models exposed to *F. verticillioides* and/or FB_1

Model	Target organ	Summary and Findings	Reference
Male BDIX Rats	Liver	In a life-long feeding experiment, BDIX rats were fed diets containing 4% culture of *F. moniliforme*. 80% of rats fed diets containing culture material developed hepatocellular carcinomas; while 63% developed ductular carcinomas. The incidence of both carcinomas increased with increased exposure time and the two distinctive tumours often developed concurrently in the same liver.	(Marasas et al., 1984)

Male F344 Rats	Liver	F344 rats were fed maize naturally contaminated with *F. moniliforme* (MRC 826) for 123 to 176 days. Three distinct lesions: neoplastic nodules, adenofibrosis and cholangiocarcinomas were observed in the liver of all rats in the treatment group.	(Wilson et al., 1985)
Male BD IX Rats	Liver	The cancer-promoting activity of FB_1 isolated from *F. moniliforme* (MRC 826) was evaluated. FB_1 (0.1%) was incorporated into the diet of male rats where cancer was initiated with DEN or not for 4-weeks. There was a marked increase in the formation of GGT^+ foci in both DEN-initiated and non-initiated groups. After 33 days, proliferation and fibrosis of bile ducts were also observed.	(Gelderblom et al., 1988a)
Male BD IX Rats	Liver	Progression of lesions were assessed at 6, 12 and 24 months in male BD IX rats fed a corn-based diet containing 50 mg/kg of purified FB_1 isolated from *F. moniliforme* (MRC 826). All FB_1-fed rats developed regenerative nodules which manifested characteristics of preneoplastic nodules with 93.3% developing cholangiofibrosis. All rats that survived to the terminal end of the study developed cirrhosis and hepatocellular carcinomas. Neoplasms metastasized in the heart and lung in 2 of the rats and in the kidney for one of them.	(Gelderblom et al., 1991)
Male Fischer Rats	Liver	Varying concentrations of FB_1-containing diets (0-500 mg FB_1/kg) were fed to DEN-initiated rats for 21 days. The number of GGT^+ foci were increased in livers of rats fed 100 mg/kg FB_1 and greater. Marked increases in number and size of $GSTP^+$ foci were present in livers fed 50 mg/kg and higher. The cancer-promoting activity of FB_1 was associated with an inhibitory effect on PH-induced regenerative hepatocyte proliferation.	(Gelderblom et al., 1996)
$B6C3F_1$ Mice and F344 Rats	Liver and Kidney	Doses of FB_1 were administered to male (0-150 mg/kg diet) and female (0-80 mg/kg diet) mice as well as male (0-150 mg/kg diet) and female (0-100 mg/kg diet) rats for 104 weeks.	(Howard et al., 1999)

		Mice: Significant tumour incidence was only detected in female mice. At 50 ppm FB_1, 40.4% of the mice had either adenomas or carcinomas, while at 80 ppm FB_1, 86.7% of the mice had either adenomas or carcinomas. Rats: There was no significant FB_1 tumour development in female F344 rats. 4.2% and 14.6% of male F344 fed 50 ppm and 150 ppm developed renal tubule adenomas while 10.4% and 20.8% fed 50 ppm and 150 ppm developed renal tubule carcinomas. Increased renal tubule apoptosis and hyperplasia occurred in livers with lesions.	
$B6C3F_1$ Mice and F344 Rats	Liver and Kidney	Doses of FB_1 were administered to male (0-150 ppm) and female (0-80 ppm) mice and male (0-150 ppm) and female (0-100 ppm) rats for 104 weeks. Mice: Hepatocellular adenomas were present in 36.3% (50 ppm FB_1) and 73.7% (80 ppm FB_1) of female B6C3F1 mice. Hepatocellular carcinomas were also present in 22.5% (50 ppm FB_1) and 23% (80 ppm FB_1) of female mice. Adenomas and carcinomas were also evident in the lower concentration but were not statistically significant. FB_1 did not affect the incidence of neoplasia in male mice. Rats: There was no significant FB_1 tumour development in female F344 rats, while their male counterparts groups dosed with higher concentrations of FB_1 developed renal tubule carcinomas, with 38.1% of rats dosed with 150 ppm developing either adenomas or carcinomas.	(Howard et al., 2001)
$B6C3F_1$ Mice and F344 Rats	Liver and Kidneys	Male and female F344 rats and $B6C3F_1$ mice were fed diets containing 0–150 ppm FB_1 for 104 weeks. Mice: FB_1 increased the incidence of hepatocellular adenomas and carcinomas with 88% of female mice fed 80 ppm FB_1 developing either lesion. Carcinomas were locally invasive and metastatic. Rats: Tumour incidence in female rats was unaffected by FB_1; however, there was a dose-dependent rise in the incidence of renal tumours in males. Renal tubule adenomas	(Voss et al., 2002)

		or carcinomas were present in 26% and 38% of male rats fed 50 ppm and 150 ppm, respectively.	
F344 Rats	Kidney	A 2-year carcinogenicity bioassay was conducted on male and female F344 rats fed 0-150 ppm and 0-100 ppm FB_1, respectively. Nephrotoxicity manifested in a dose-dependent manner. FB_1 induced proximal tubule loss and sustained regeneration which is a risk factor for tumour development. In males, renal tubule tumours were observed at 100 (21%) and 150 (33%) ppm. Atypical tubule hyperplasia, a preneoplastic lesion were found in 8% and 19% of these 2 groups. Tumour development in female rats was statistically insignificant. Furthermore, there was a correlation between proliferative lesions and nephrotoxicity.	(Hard et al., 2001)
Male F344 Rats	Liver	The separate and combined effects of FB_1 and AFB_1 on the cancer initiation and promotion in hepatocarcinogenesis were evaluated in rats. There was a significant increase in the number of large $GSTP^+$ lesions in AFB_1 and FB_1-treated rats subjected to PH promoting treatment. The induction of $GSTP^+$ lesions was also significantly enhanced in rats treated either with AFB_1 or FB_1 without the 2-AAF/PH promoting stimuli. The underlying mechanism that resulted in the significant increase in the size of $GSTP^+$ foci and nodules during the successive AFB_1/FB_1 treatment regimen could be ascribed to the potent cancer promoting potential of FB_1.	(Gelderblom et al., 2002)
F344 Rats	Liver	Male F344 mice were fed diets of AFB_1 (150 µg/kg), FB_1 (250 mg/kg) or AFB_1 and FB_1 sequentially. $GSTP^+$ preneoplastic hepatic foci were evaluated after 8 weeks. The number and mean size of $GSTP^+$ foci were higher in the AFB_1-only group than that of the FB_1-only treated group. Sequential treatment markedly and significantly increased the number and size of $GSTP^+$ foci by approximately 7-fold and 12-fold as compared to the AFB_1 or FB_1 only treatment groups, respectively. This indicates that there is a synergistic	(Qian et al., 2016)

		effect by sequential treatment on preneoplastic foci induction.	

DEN: Diethylnitrosamine; GGT$^+$: gamma-glutamyl-transpeptidase-positive; GSTP$^+$: glutathione-S-transferase-positive; PH: partial hepatectomy; AFB$_1$: aflatoxin B$_1$; 2-AAF/PH: 2-acetylaminofluorene/partial hepatecomy

Overview of literature search

We performed a systematic search of published research studies pertaining to the molecular and epigenetic modes of FB$_1$-induced toxicity and carcinogenicity. We further identified recent studies that assessed strategies to reduce and detoxify FB$_1$ contaminated foods and feeds. To identify eligible studies for this review, the following academic databases and search engines were used: Pubmed, Google scholar and Europe PMC. Keywords searched included a combination of fumonisin b$_1$, toxicity, cancer, sphingolipid metabolism, oxidative stress, endoplasmic reticulum stress, autophagy, immunotoxicity, epigenetics, DNA methylation, histone modifications, microRNA, and detoxification. Moreover, we used the bibliography of papers obtained using the above-mentioned database to identify additional studies. Articles eligible for inclusion in this review included original research studies and review papers that reported an association between FB$_1$ exposure and negative health outcomes in animal models as well as negative effects on cultures cells of human and animal origin. Additionally, we included papers that assessed, developed or improved on methods (physical, chemical or biological) that may possibly reduce FB$_1$ contamination of food and feeds or attenuate the effects of FB$_1$ exposure. Only full text articles published in English in scientific journals with high peer-reviewing standards were included. Following sourcing suitable literature, we assessed the quality of research based on the scientific approach. This included assessing details of methods, validity and reliability of results and accuracy of statistical analysis. The results from the relevant studies are included under the appropriate sections which summarizes and analyses findings on the molecular and epigenetic aspects of FB$_1$ toxicity and as well as recent methods used to detoxify FB$_1$ contaminated foods and feed.

Disruption of Sphingolipid Metabolism

The disruption of sphingolipid metabolism has been identified as a key molecular mode of FB$_1$ toxicity. Sphingolipids are abundant in all eukaryotic cells as they form major components of membranes, lipoproteins, and other lipid-rich structures. They are critical in maintaining the fluidity and structure of membranes and modulating the activity of receptors (Merrill, Schmelz et al. 1997). Bio-active sphingolipids [ceramide, sphinganine (Sa), sphingosine (So) and their phosphorylated counterparts] also mediate vital signalling pathways such as differentiation, cell cycle progression, proliferation, and apoptosis (Merrill, Sullards et al. 2001). Thus, disruptions in sphingolipid metabolism can trigger a chain of events leading to FB$_1$-altered cell growth, differentiation, and cell injury.

The initiation of *de novo* sphingolipid synthesis occurs in the ER where, serine palmitoyltransferase (SPT) catalyses the condensation of serine and palmitoyl Coenzyme A (palmitoyl CoA) to form 3-

ketosphinganine; which is subsequently reduced to Sa (Futerman and Riezman, 2005). Sa is either phosphorylated by sphingosine kinase to form sphinganine-1-phosphate (Sa1p) or acylated to form dihydroceramide by ceramide synthase (CS). Dihydroceramide is desaturated to ceramide, which can then be converted to complex sphingolipids such as glycosphingolipids and sphingomyelin (Futerman and Riezman, 2005). CS is also responsible for reacylation of So to ceramide via the sphingolipid salvage pathway (Kitatani et al., 2008).

FB_1 and its hydrolysed form (HFB_1) bare close structural resemblance to the aminopentol backbone of sphingoid bases (Figure 3.1). Due to this similarity, CS recognizes the amino group of FB_1 and HFB_1 as a substrate and allows it to compete with sphingoid bases for the same binding site (Wang et al., 1991). CS is also able to recognize the tricarboxylic acid side chain of FB_1 as an analogue of fatty acyl CoA and can thus obstruct the fatty acyl-CoA binding site of CS (Wang et al., 1991). *In vitro* assessment showed that FB_1 blocks the incorporation of serine into the So backbone, completely inhibits the formation of sphingolipids and depletes the total mass of cellular sphingolipids (Wang et al., 1991, Yoo et al., 1992, Merrill et al., 1993). Accumulation of free sphingoid bases and their phosphorylated counterparts are evident in affected tissues, serum, and urine of animals exposed to contaminated feed [summarised by Riley et al. (2001)].

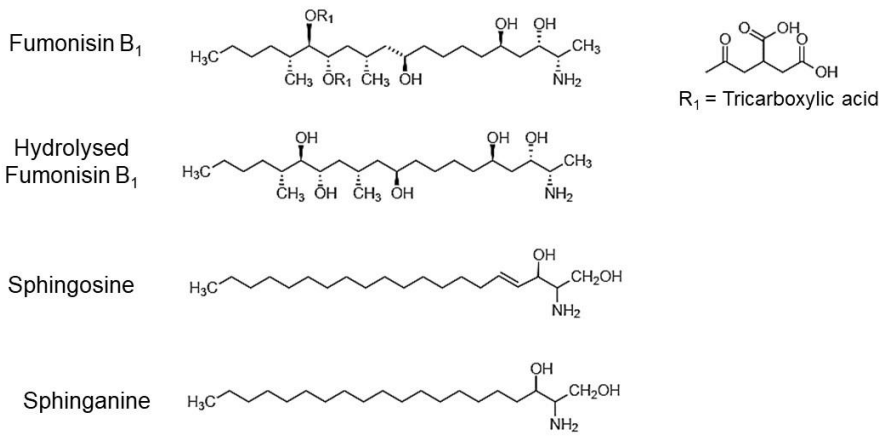

Figure 3.1. The molecular structure of FB_1, HFB_1 and the sphingoid bases, Sa and So. The aminopentol backbone of FB_1 and HFB_1 bare close structural resemblance to that of Sa and So.

HFB_1 is considered a weak disruptor of sphingolipid metabolism and is not as toxic in comparison to FB_1. The lack of tricarboxylic acid side chains reduces the potency of HFB_1 as a ceramide synthase inhibitor by almost 10-fold (Howard et al., 2002, Collins et al., 2006, Hahn et al., 2015, Harrer et al., 2015). Rats fed hydrolysed *Fusarium* culture material containing hydrolysed fumonisins but not FB_1 presented with liver and kidney lesions and demonstrated hepatic tumour promoting activity (Hendrich

et al., 1993, Voss et al., 1996). In contrast, studies on pregnant rats have found no evidence of tissue lesions or changes in sphingoid bases (Collins et al., 2006); while studies in female mice fed HFB_1 found no signs of hepatic lesions but altered sphingolipid metabolism was observed (Howard et al., 2002). A recent study found that exposure to HFB_1 or partially hydrolysed FB_1 ($PHFB_1$) did not affect Sa/So ratios and slightly increased the number of lesions observed in the kidney of exposed rats; while a significant increase in Sa/So ratios and number of lesions were observed in FB_1 exposed rats (Hahn et al., 2015). Regardless, HFB_1 can undergo acylation by CS to form cytotoxic *N*-acylated HFB_1 metabolites (C_n-HFB_1). The type of metabolite produced is dependent on the isoform of CS and the acyl CoA chain used (Seiferlein et al., 2007). Humpf et al. (1998) found that the *N*-acyl derivative, *N*-palmitoyl-HFB_1 (C16-HFB_1) was only half as effective as FB_1 in inhibiting CS but caused significantly greater accumulation of Sa and toxicity in human colonic (HT-29) cells. Seiferlein et al. (2007) also investigated the impact *N*-acyl-HFB_1 derivatives; incubation of rat liver microsomes with HFB_1 and either nervonoyl-CoA or palmitoyl-CoA resulted in the formation of *N*-nervonoyl-HFB_1 (C24:1-HFB_1) and *N*-palmitoyl-HFB_1 (C16-HFB_1), respectively. *In vivo* assessment of these derivatives in HT-29 cells were undertaken to determine toxicity and its ability to inhibit CS. There was a 50% reduction in cell viability after a 24-hour treatment with 25 µM of C24:1-HFB_1 and C16-HFB_1. These results suggest that the *N*-acylated metabolites are more potent than FB_1 and HFB_1 in HT-29 cells (Schmelz et al., 1998, Seiferlein et al., 2007). Furthermore, just 1 µM of C24:1-HFB_1 and C16-HFB_1 inhibited CS activity by 30%, while up to 80% inhibition was observed at 10 µM (Seiferlein et al., 2007). An *in vitro* assessment showed that the most prevalent metabolites were the HFB_1-acyl compounds containing long-chain fatty acids (C24, C24:1, C22 and C20) in rats dosed with HFB_1 (52, 115 and 230 µg/day for 5 days); however, gross and microscopic examinations of the liver and kidneys of these animals found no treatment-related alterations (Seiferlein et al., 2007). It has long been regarded that FB_1 is unable to undergo *N*-acylation due to its tricarboxylic acid side chains; however, *N*-acyl-FB_1 metabolites were recently discovered. Human fibroblasts, hepatoma (Hep3B), and embryonic kidney (HEK293) cells were treated with 20 µM of either FB_1 or HFB_1 for 24 hours; subsequently FB_1 metabolites were then quantified by HPLC-ESI-MS/MS. Similar to HFB_1, the *N*-acylation of FB_1 corresponded to the acyl chain specificity of each of the CS isoforms and the *N*-acyl-FB_1 metabolites were significantly more cytotoxic than FB_1 in cell culture (Harrer et al., 2013). The *in vivo* formation of *N*-acyl-FB_1 were tissue specific and depended on the dominant CS isoform. C_{16} derivatives were dominant in the kidney and C_{24} derivatives were more prevalent in the liver (Harrer et al., 2015). However, further investigation on *N*-acyl-FB_1 toxicity *in vivo* should be undertaken.

Computational modelling has revealed that FB_1 disruption of sphingolipids goes beyond inhibition of CS. While ceramide synthesis occurs in the ER, the formation of the complex sphingolipid - sphingomyelin occurs in the Golgi apparatus (Futerman and Riezman, 2005). Ceramide transport protein (CERT) mediates the non-vesicular transport of ceramide from the ER to the Golgi via the

steroidogenic acute regulatory protein-related lipid transfer (START) domain (Hanada et al., 2003). Through docking simulations, Dellafiora et al. (2018) demonstrated that *N*-acyl derivatives of HFB_1 might fit the START binding site depending on the fatty acid chain length. *N*-capryl- and *N*-palmitoyl-HFB_1 might compete with ceramides for CERT-dependent ER-to-Golgi transport, although polar/hydrophobic mismatch may limit binding into the START pocket. Nevertheless, disruptions to CERT mediated ceramide transport may be a contributing factor in reduced sphingomyelin synthesis that is observed post FB_1 exposure (He et al., 2006). Dellafiora et al. (2018) also demonstrated that HFB_1 was able to fit the enzyme pocket of sphingosine kinase 1 (SPK1), the enzyme responsible for the conversion of So to sphingosine-1-phosphate (So1P) (Maceyka et al., 2002). The calculated fit of HFB_1 was similar to that calculated for known SPK1 inhibitors. This stimulation contradicted work done by He et al. (2006) and collaborators who observed an increase in SPK1 activity, and several other studies have demonstrated the accumulation of So1p and Sa1p during FB_1 exposure (Gelineau-van Waes et al., 2012, Riley et al., 2015a, Riley et al., 2015b, Gardner et al., 2016).

FB_1 not only induces the accumulation of Sa, So and its phosphorylated counterparts, but also results in the accumulation of 1-deoxysphinganine (1-deoxySa). 1-deoxySa is formed when SPT utilizes alanine instead of serine in the initial steps of sphingolipid synthesis. *In vivo* and *in vitro* exposure to FB_1 results in the accumulation of this atypical sphingoid base. *In vitro* experimentation also revealed that the cytotoxicity of 1-deoxySa was greater than or equal to Sa (Zitomer et al., 2009). 1-DeoxySa can also undergo acylation by CS; however, these acylated derivates are unable to produce complex sphingolipids and function as membrane disruptors (Jiménez-Rojo et al., 2014). In summary, the inhibition of CS by FB_1 and HFB_1 results in: 1. reduced levels of dihydroceramide, ceramide, and complex sphingolipids; 2. accumulation of sphingoid bases and phosphorylated sphingoid bases; 3. elevation in 1-deoxySa bases; and 4. the accumulation of cytotoxic *N*-acylated HFB_1/FB_1 metabolites (Figure 3.2). These changes result in several toxicologically relevant perturbations such as ER stress, accumulation of ROS, altered mitochondrial and immune functioning, and disruption to developmental regulation (Riley and Merrill, 2019). Furthermore, FB_1-induced alterations in sphingolipid signalling pathways will lead to altered rates of cell death and regeneration, which may play a major role in FB_1-mediated tumorigenesis via continuous compensatory regeneration of cells as a response to the apoptosis induced by FB_1 (Riley et al., 2001, Soriano et al., 2005).

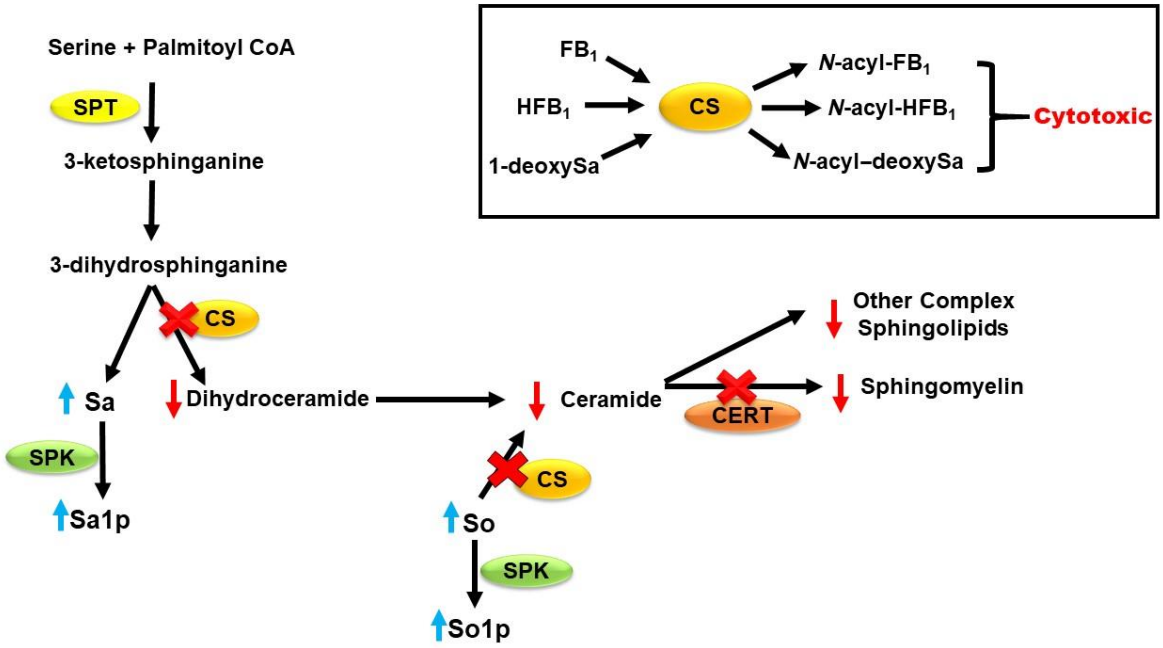

Figure 3.2. An overview of the effect of FB_1 and its metabolites on sphingolipid metabolism. A) Sphingolipid biosynthesis begins in the ER, where serine and palmitoyl-CoA are incorporated into 3-ketosphinganine before sphinganine (Sa), followed by acylation to dihydroceramides by ceramide synthase (CS). Likewise, 1-deoxysphinganine (1-deoxy-Sa) is made from alanine (not shown). Dihydroceramide is desaturated to ceramide and subsequently incorporated into complex sphingolipids. The formation of some complex sphingolipids such as sphingomyelin occurs in the golgi apparatus and requires ceramide transport protein (CERT) mediated trafficking of ceramide. Sphingolipid degradation occurs to release Sphingosine (So) and is recycled via CS phosphorylated by sphingosine kinase (SPK1) to sphingosine-1-phosphate (So1p). SPK1 can also phosphorylate Sa to sphinganine-1-phosphate (Sa1p). FB_1 and/or its metabolites disrupts sphingolipid metabolism by inhibiting CS and CERT, altering levels of sphingolipid metabolites. The metabolites with the blue arrow are generally elevated when CS is inhibited by FB_1 while the metabolites with the red arrow are reduced. B) FB_1, HFB_1 and deoxy-1Sa act as substrates for CS, releasing cytotoxic N-acylated metabolites.

Oxidative Stress

Reactive oxygen species (ROS) are radical and nonradical derivatives of oxygen; formed predominantly during normal aerobic respiration (Andreyev et al., 2005). Low basal levels of ROS mediate several biological processes such as cell proliferation, apoptosis, cell cycle, phosphorylation of proteins, activation of transcription factors and immune regulation (Pizzino et al., 2017). Contrarily, excessive ROS levels and a diminished capacity of cells to detoxify excess ROS results in oxidative stress (Phaniendra et al., 2015). This disturbance in redox homeostasis inflicts damage to macromolecules and can trigger the onset or progression of diseases such as cancer, diabetes, metabolic disorders, atherosclerosis, and cardiovascular diseases (Phaniendra et al., 2015, Pizzino et al., 2017).

Mitochondria metabolize carbohydrates and fatty acids via the electron transport chain (ETC) to produce ATP. During this process, unpaired electrons leak into the mitochondrial matrix, where it reduces oxygen to form ROS (Ma, 2013). Unwarranted production of ROS from the ETC can be stimulated by several factors, such as the inhibition of complexes of the ETC (Bratic and Larsson, 2013). Domijan and Abramov (2011) have reported that FB_1 inhibits complex I of the ETC. FB_1 inhibited state 4 respiration in the presence of substrates for complex I. This resulted in the enhanced generation of mitochondrial ROS and subsequent mitochondrial depolarization. The activation of cytochrome P450 (CYP450) enzymes by FB_1 may also be a driving force in ROS production as seen in spleen mononuclear cells of Wistar rats and colonic tissue of ICR mice (Mary et al., 2012, Kim et al., 2018). Several other studies reported elevated levels of ROS after FB_1 exposure in rodent GT1-7 hypothalamic cells, C6 glioblastoma, and spleen mononuclear cells as well as in human fibroblast, U-118MG glioblastoma, and HepG2 hepatocellular carcinoma cells (Galvano et al., 2002, Stockmann-Juvala et al., 2004a, Mary et al., 2012, Arumugam et al., 2019); with only one study showing that low doses of FB_1 had the opposite effect on ROS levels in human oesophageal carcinoma cells (SNO) (Khan et al., 2018).

A major consequence of ROS overproduction is oxidative injury to macromolecules and organelles (Phaniendra et al., 2015). Besides disrupting sphingolipid metabolism, FB_1 can indirectly disrupt lipid homeostasis through the oxidative degradation of lipids. Lipid peroxidation results in the formation of lipid peroxyl radicals that can accelerate the peroxidation of other unsaturated fatty acid moieties, disrupt membrane receptor signalling as well as membrane permeability (Ayala et al., 2014). Malondialdehyde (MDA), is a cytotoxic and tumorigenic by-product of lipid peroxidation that is often used as a biomarker in determining oxidative stress (Ayala et al., 2014). Varying concentrations and treatment periods showed that FB_1 is a potent inducer of lipid peroxidation and elevates MDA levels (Abado-Becognee et al., 1998, Mobio et al., 2003, Stockmann-Juvala et al., 2004b, Stockmann-Juvala et al., 2004a, Kouadio et al., 2005, Domijan et al., 2007a, Domijan et al., 2008, Theumer et al., 2010, Domijan and Abramov, 2011, Mary et al., 2012, Minervini et al., 2014, Hassan et al., 2015, Arumugam et al., 2019). Interestingly, SNO cells were more resistant to lipid peroxidation when exposed to low doses of FB_1 (Khan et al., 2018).

A strong correlation between elevated ROS levels and structural damage to proteins in the form of protein carbonyls have also been made in the presence of FB_1 (Domijan et al., 2007a, Domijan et al., 2007b, Mary et al., 2012, Arumugam et al., 2019). HepG2 cells were extremely sensitive to FB_1 as indicated by the 11.9-fold increase in protein carbonyls (Arumugam et al., 2019). The carbonylation of proteins alters polypeptide confirmation which can impair protein functioning. This may have various downstream consequences such as disrupting signalling pathways, modifying enzyme activity, and impairing other protein functions including binding of transcription factors to DNA (Gonos et al., 2018). Moreover, protein carbonyls can inhibit proteasomal activity which is necessary for the degradation of

carbonylated proteins. Thus, protein carbonylation can result in cellular dysfunction and eventually contribute to the aetiology and progression of disease states (Dalle-Donne et al., 2006).

The threat of oxidative damage is particularly significant to nucleic acids. Elevated levels of ROS can induce strand breaks, protein-DNA crosslinking and has mutagenic potential (Loft et al., 2008). Several studies have demonstrated the genotoxic potential of FB_1 in humans and animals. With the use of the micronuclei test, Ehrlich et al. (2002), Theumer et al. (2010) and Karuna and Rao (2013) assessed genotoxic potential of FB_1. Micronuclei are formed when there are breakages in chromosomes or when spindle assembly is disturbed. A dose-dependent formation of micronuclei occurred in FB_1-exposed HepG2 cells (Ehrlich et al., 2002) and Wistar rats (Theumer et al., 2010). Conversely, FB_1 failed to induce micronuclei in BALB/C mice (Karuna and Rao, 2013). DNA strand breaks and fragmentation were studied *in vivo* and *in vitro*. These studies found that DNA fragmentation and strand breaks occurred as a consequence of FB_1-induced oxidative stress (Atroshi et al., 1999, Mobio et al., 2003, Stockmann-Juvala et al., 2004b, Theumer et al., 2010, Hassan et al., 2015). 8-hydroxy-deoxyguanosine (8-OHdG) is a predominant oxidative DNA lesion, and thus widely used as a critical biomarker for oxidative stress and carcinogenesis (Valavanidis et al., 2009). FB_1-mediated the oxidation of guanine in both *in vivo* and *in vitro* studies (Mobio et al., 2003, Mary et al., 2012, Arumugam et al., 2020). Only one study found that DNA damage occurred independent of ROS levels (Galvano et al., 2002)

The detoxification capacity of cells is also affected by FB_1-induced ROS. Kelch-like ECH-associated protein 1 (Keap1)/Nuclear factor erythroid 2-related factor 2 (Nrf2) signalling pathway is activated in response to excess ROS production. Antioxidant defence depends on the disassociation of the antioxidant transcription factor, Nrf2, from Keap1 degradation. Surplus ROS alters Keap1 conformation and activates phosphorylation pathways which in turn phosphorylate Nrf2. These changes trigger the dissociation of Nrf2 from Keap1 degradation and promotes anti-oxidant transcription (Huang et al., 2002, Nguyen et al., 2009). In response to FB_1-induced ROS, HepG2 cells significantly upregulate phosphorylation of Nrf2 leading to the transcription of major antioxidants: superoxide dismutase 2 (SOD2), catalase (CAT), and glutathione peroxidase (GPx) (Arumugam et al., 2019). Nrf2 was also activated in SNO cells but antioxidant expression did not correspond (Khan et al., 2018). Furthermore, FB_1 reduced antioxidant status in BALB/C mice, Wistar rats and bovine peripheral blood mononuclear cells (PBMC) exposed to FB_1 (Domijan et al., 2007a, Bernabucci et al., 2011, Abbès et al., 2016). However, subchronic exposure of Wistar rats with FB_1 boosted SOD2 and CAT activity (Theumer et al., 2010). The use of antioxidants is being investigated as a method to reduce FB_1 toxicity. Antioxidants such as *N*-acetylcysteine, coenzyme Q10, L-carnitine, vitamin E (α-tocopherol) and selenium were shown to attenuate FB_1-mediated oxidative stress and toxicity (Abel and Gelderblom, 1998, Atroshi et al., 1999, Zhang et al., 2018). In summary, FB_1 promotes ROS generation and alters antioxidant status, which results in oxidative injury to cells (Figure 3.3). The use of antioxidants may be a promising approach to minimize the effects of FB_1 on cellular redox status and subsequently cytotoxicity.

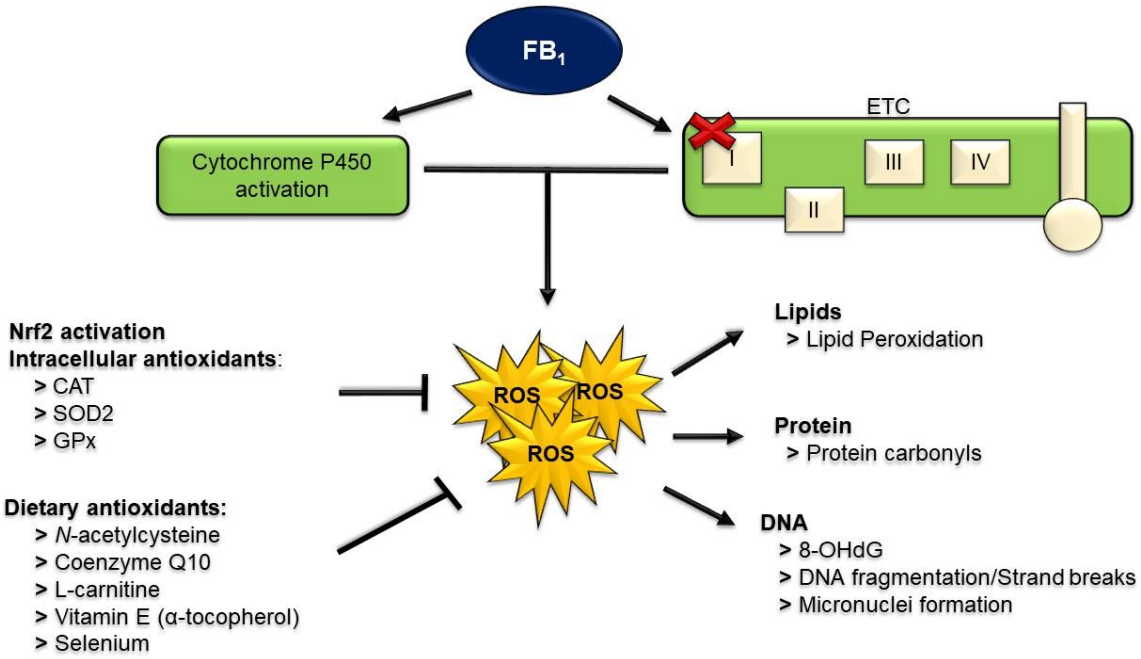

Figure 3.3. FB_1 disrupts redox homeostasis. High levels of ROS are generated through the activation of cytochrome P450 enzymes and inhibition of the electron transport chain (ETC) by FB_1. Reduced capacity of intracellular antioxidants to detoxify ROS leads to oxidative injury to lipids, protein and DNA. The use of dietary antioxidants may normalize ROS levels.

Endoplasmic Reticulum Stress and Autophagy

The role of the ER is not exclusive to sphingolipid synthesis. It is a highly dynamic organelle responsible for protein folding, free calcium storage, carbohydrate metabolism, synthesis of other lipids and assembly of lipid bilayers (Koch, 1990, Stevens and Argon, 1999, Hebert and Molinari, 2007, Bravo et al., 2013, Schwarz and Blower, 2016, Jacquemyn et al., 2017). The ER also has tissue-specific functioning; liver ER contain cytochrome P450 enzymes that can metabolize and detoxify hydrophobic drugs and carcinogens (Kwon et al., 2020); whereas in the muscle, specialized ER (sarcoplasmic reticulum) regulate calcium flux to execute muscle contraction and relaxation (Guerrero-Hernandez et al., 2010). Despite its dynamic role, the ER is sensitive to a multitude of intracellular and microenvironmental changes. Cellular stressors such as imbalances in redox and calcium homeostasis or defects in lipid metabolism or protein folding can cause unfolded or misfolded proteins to accumulate in the ER. This phenomenon is known as ER stress (Senft and Ronai, 2015). The accumulation of damaged proteins in the ER can lead to irreversible damage to cellular functioning and pose a threat to cell survival. Fortunately, eukaryotes have developed several signalling mechanisms to sense and ameliorate the effects of ER stress and restore ER homeostasis and functioning (Bravo et al., 2013). Principal pathways involved in this response include the unfolded protein response (UPR), ER-associated degradation (ERAD), autophagy, hypoxia signalling and mitochondrial biogenesis. These

pathways work in concert to determine whether cells re-establish ER homeostasis or activate cell death mechanisms (Senft and Ronai, 2015).

In unstressed conditions, the master regulator – binding immunoglobin protein (GRP78) sequesters and maintains UPR sensors in an inactive state. During UPR, the ER lumen binds to GRP78, releasing UPR sensors. Together, these sensors [protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring protein 1 (IRE1α)] and their respective transducers [activating transcription factor 4 (ATF4), cleaved ATF6, and X-Box Binding Protein 1 (XBP1)] suppress protein translation and folding, facilitate ERAD to degrade misfolded proteins and mediate cell death and survival (Chakrabarti et al., 2011, Senft and Ronai, 2015). ER stress is also a potent trigger for autophagy, a self-degradative process that has both pro-survival and pro-apoptotic functioning (Yorimitsu et al., 2006, Glick et al., 2010). Both UPR signalling and autophagy are interconnected with the 3 canonical arms of UPR regulating autophagy during ER stress (Kouroku et al., 2007, Margariti et al., 2013, Li et al., 2014, Kabir et al., 2018).

Several *in vivo* and *in vitro* investigations have revealed that FB_1 induces ER stress through the disruption of sphingolipid metabolism and subsequent accumulation of sphingoid bases and intracellular ROS (Yin et al., 2016, Singh and Chul, 2017, Kim et al., 2018, Liu et al., 2020, Yu et al., 2020). Autophagy was also observed in these studies; however, the activation and role of autophagy differed. FB_1-induced autophagy was first observed in MARC145 green monkey kidney cells. Yin et al. (2016) showed a dose-dependent increase in the phosphorylation and activation of ER stress markers [IRE1α, eIF2AK2 and eIF2S1] after exposure to FB_1 for 48 hours. IRE1α mediated mitogen-activated protein kinase 8/9/10 (MAPK8/9/10) autophagy in response to ER stress as numerous autophagic vacuoles and increased LC3 I/LC3 II conversion was observed. Inhibition of IRE1α via RNA interference or chemical inhibition attenuated MAPK activity, LC3 conversion as well as autophagy confirming the role of IRE1α/MAPK8/9/10 in FB_1-mediated autophagy (Yin et al., 2016).

In colon tissue of male mice, both IRE1α and PERK levels were upregulated after exposure to 2.5 mg/kg bw FB_1 for 24 to 96 hours. Rather than MAPK8//9/10 activation, IRE1α activated JNK, which led to the subsequent elevation in autophagy markers (beclin, ATG5, ATG7) and LC3 I conversion in all FB_1 treated mice (Kim et al., 2018). Most recently, Yu et al. (2020) found that human gastro-intestinal epithelial (GES-1) cells were also sensitive to FB_1-mediated ER stress autophagy via the PERK/CHOP pathway. All 3 of these studies reported that pro-death mediated autophagy and apoptosis occurred in response to FB_1 (Yin et al., 2016, Kim et al., 2018, Yu et al., 2020). With the use of the SPT inhibitor, myriocin in the presence of FB_1, levels of free sphingoid bases diminished which in turn reduced ER stress biomarkers and abolished FB_1-mediated autophagy apoptosis (Yin et al., 2016, Yu et al., 2020). This data strongly suggests that disruptions in sphingolipid metabolism is an essential event for FB_1 to trigger autophagic cell death.

However, *in vitro* and *in vivo* assessment by Singh and Chul (2017) and Liu et al. (2020) proved that FB_1 mediated autophagy is a pro-survival mechanism in the liver. ER stress activated PKC, PERK and JNK, which lead to the activation of autophagy related gene 5 (ATG5), ATG7, and LC3 conversion. Mammalian target of rapamycin (mTOR) signalling was suppressed resulting in the dissociation of pro-autophagic Beclin from B-cell lymphoma 2 (Bcl2). Both research groups found that FB_1 mediated ER stress activated PERK and IRE1α but concluded the main mechanism of autophagy was facilitated via the IRE1α/JNK pathway. The pre-treatment of HepG2 cells with the autophagy inhibitor 3-methyladenine (3-MA) followed by FB_1 significantly reduced cell viability with respect to the control and individual treatment with 3-MA and FB_1. Only 20% loss in viability was observed after 24 hours, with proliferation occurring after 12 hours. The inhibition of autophagy using RNA interference led to increased cell death in mouse liver cells, while the autophagy inducer rapamycin protected the liver cells from FB_1-induced cell death. (Liu et al., 2020) Taken together, these results suggest that FB_1-mediated autophagy protects cells from hepatic injury (Singh and Chul, 2017, Liu et al., 2020).

The difference in the role of autophagy can be explained by the type of cell (cancerous versus non-cancerous), duration of FB_1 exposure (acute versus prolonged), and the duration and extent of autophagy (Sun et al., 2013, Linder and Kögel, 2019). The extent and duration of autophagy may have been greater in kidney, gastric, and colon tissue. This could be due to the prolonged exposure to FB_1 in these cells in comparison to the acute exposure received by the liver. Furthermore, the HepG2 cell line is cancerous and FB_1 is known to induce cancer in hepatic tissues in mice. Autophagy is used as a pro-survival mechanism in the latter stages of tumorigenesis to cope with metabolic stress, hypoxia, nutrient deprivation, and ER stress (Sun et al., 2013, Linder and Kögel, 2019). As previously mentioned, FB_1 has been shown to upregulate sphingosine kinase activity. Recently, sphingosine kinases were shown to play a role in ER stress mediated through the inhibition of mTOR signalling via Sa1P. This is a pro-survival phenomenon used by cancer cells and should be further investigated in relation to FB_1 (Lépine et al., 2011). But what we do know is that FB_1-induced ER stress mediates autophagy through either the IRE1α/MAPK8/9/10, IRE1α/JNK or PERK/CHOP pathway (Figure 3.4). The outcome of autophagy depends on several factors.

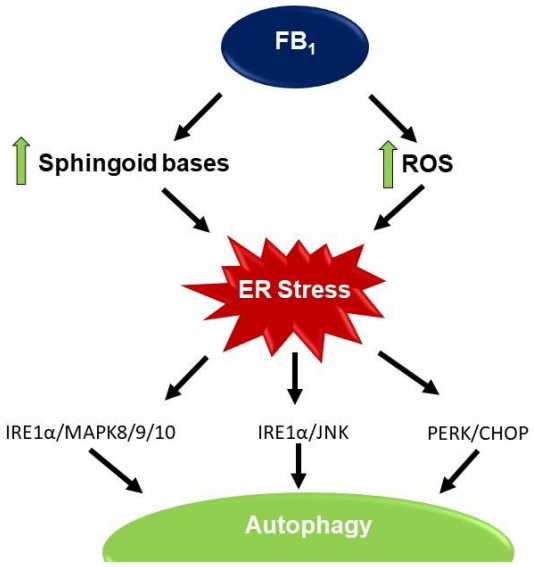

Figure 3.4. FB1-induced ER stress mediates autophagy. The accumulation of sphingolipids and ROS in the ER after exposure to FB1 triggers ER stress. Cells cope with stress by activating UPR signalling and autophagy via IRE1α/MAPK8/9/10, IRE1α/JNK, or PERK/CHOP pathways. FB1-induced autophagy can be either pro-death or pro-survival depending on several factors.

Immunotoxicity

The immune system is a major defence mechanism in animals and humans, protecting them from invading micro-organisms and foreign chemicals and its' effectiveness is an important determinant of animal and human health (Surai and Mezes, 2005). Mycotoxins are major immuno-suppressive agents and FB_1-induced immunotoxicity is an area of active research (Surai and Mezes, 2005). Current studies have observed diverse immunomodulatory effects of FB_1, which include altered inflammatory, cellular, and humoral responses (Oswald et al., 2005).

Inflammation is a non-specific response that acts by removing harmful stimuli and initiating repair through the activation of phagocytes. The activated phagocytes secrete cytokines that act as chemical messengers between other immune cells (Oswald et al., 2005). They stimulate or inhibit the growth and activity of various immune cells, which mediate and regulate immunity and inflammation. Proinflammatory cytokines mediate inflammation via receptor activation, which can trigger intracellular signalling pathways such as MAPK, nuclear factor kappa B (NFκB), and Janus kinase/Signal transducer and activator of transcription (JAK/STAT). While inflammation plays an important role in immune response, excessive production of inflammatory cytokines can lead to cytotoxicity and tissue damage (Chen et al., 2017).

Alterations in proinflammatory cytokine profiles have been shown to be one of the factors that influence toxicity. Localized network of key proinflammatory cytokines: tumour necrosis factor alpha (TNF-α),

interferon gamma (INF-γ) and interleukin-12 (IL-12) are involved in FB_1-induced hepatotoxicity in mice (Bhandari et al., 2002). Knockout of TNFα and IFN-γ or their receptors greatly reduced toxicity in the liver of mice (Sharma et al., 2000, Sharma et al., 2001, Sharma et al., 2003). The differential hepatotoxic response to FB_1 in male and female mice can also be attributed to the difference in proinflammatory cytokine profiles (Bhandari et al., 2001). Several studies have investigated the immunomodulatory effect of FB_1 in porcine intestinal systems as the intestine is the first physical barrier to protect against ingested FB_1. Furthermore, the results obtained from these studies may be valid for humans due to the similarities between the porcine and human intestinal system. In porcine intestinal epithelial (IPEC-J2) cells, both non-cytotoxic (20 µM) and cytotoxic (40 µM) concentrations of FB_1 significantly increased the expression of inflammatory cytokines [monocyte chemoattractant protein (MCP-1), TNF-α, IL-1β, IL-6, and IL-8]; however, 40 µM had no significant effect on IL-1α (Wan et al., 2013). Gu et al. (2019) investigated the effects of FB_1 and HFB_1 in a co-culture of IPEC-J2 and porcine PBMCs that had been stimulated with lipopolysaccharide (LPS) and Deoxynivalenol (DON). FB_1 significantly increased intestinal permeability and reduced barrier integrity. This may be due to disruptions in sphingolipid metabolism and depletion of glycosphingolipids which act as a structural component of tight junctions. FB_1 exacerbated proinflammatory responses through the upregulation of IL-8, MCP-1 and C-C Motif Chemokine Ligand 20 (CCL20) in the presence of LPS/DON compared to only LPS/DON treatments. The use of HFB_1 leads to decreased cytokine expression; however, the effect of HFB_1 on IPEC-J2 cell viability and barrier integrity was comparable to that of FB_1. Thus, FB_1 degradation could be an effective strategy to reduce intestinal inflammation. Moreover, FB_1 but not HFB_1 provoked PBMC cell death in the presence of LPS/DON. In another study, FB_1 reduced IL-2 expression and inhibited porcine PBMC proliferation via blockage of G0/G1 transition of $CD2^+$, $CD4^+$, $CD8^+$ and immunoglobulin$^+$ (Ig^+) lymphocyte subsets (Marin et al., 2007).

In humans, cytokine profiles were investigated in lymphocytes, gastric adenocarcinoma (AGS) and colon cancer (SW742) cells. FB_1 stimulated the synthesis of TNF-α, IL-1β, inhibited IL-8 expression, and reduced cell viability in a dose-dependent manner in all 3 cell lines. The changes in cytokine profiles were more evident in SW742 cells than AGS cells; this higher sensitivity of colon cells might be due to FB_1 having a greater inhibitory effect on CS in the colon compared to the stomach (Mahmoodi et al., 2012). FB_1 was found to be immunosuppressive in human cancer patients. Lymphocytes and neutrophils, harvested from the circulation of healthy subjects and patients with breast or oesophageal cancer, were dosed with 20 µg/ml to 100 µg/ml for 0 to 24 hours. Ultrastructure visualization of exposed lymphocytes and neutrophils showed cell membrane disruption, damage to cytoplasmic organelles and loss of nuclear integrity. The extensive cellular damage observed in all 3 populations correlated with enhanced apoptosis in exposed cells (Odhav and Bhoola, 2008). In some cancer therapies, cytokines are used to activate the immune system of cancer patients (Conlon et al., 2019). FB_1 downregulated TNF-α and GCSF receptors on lymphocytes and neutrophils, inhibiting cytokine signalling.

Furthermore, FB_1 increased expression of IL-1 and decreased IL-10 in lymphocytes of breast cancer patients and decreased IL-6 in oesophageal cancer patients (Odhav and Bhoola, 2008). Taken together, this data suggests FB_1 suppresses immune functioning in a population that is already immunocompromised. Not only does FB_1 diminish immune response to cancer but also raises susceptibility to infectious diseases. Pig weanlings were given 0.5 mg/kg bw FB_1 for 6 weeks before being orally inoculated with a septicaemic *Escherichia coli* (*E. coli*) strain. FB_1 facilitated intestinal colonization of septicaemic *E. coli* and its translocation. Bacterial translocation was prominent in mesenteric lymph nodes and lungs and to a lesser extent in the liver and spleen (Oswald et al., 2003). FB_1 also prolonged intestinal infection of enterotoxigenic *E. coli* in pigs. This was achieved through the impairment of antigen-presenting cells maturation by downregulating IL-12p40 and major histocompatibility complex class II molecules (MHC-II) (Devriendt et al., 2009). Antigen processing and presentation was also affected in human gastric epithelium (GES-1) cells. FB_1 reduced expression of antigen processing complexes: transporter associated with antigen processing 1 (TAP1) and low molecular weight peptide (LMP2), which contributed to reduced expression of human leukocyte antigen (HLA)-class I expression. This may also lead to $CD8^+$ T cells resistance (Yao et al., 2010).

Finally, FB_1 can affect humoral immune response by diminishing the specific antibody response built during vaccination. IL-4 plays a key role in the development of the humoral immune response and antibody production (Yang et al., 2017). Prolonged exposure (8 mg FB_1/kg; 28 days) to FB_1 significantly decreased the expression of IL-4 in porcine lymphocytes, which in turn diminished antibody response after vaccination against *Mycoplasma agalactiae (Taranu et al., 2005).* A decrease in the specific antibody production was also observed in rodents immunized with sheep red blood cells (Martinova and Merrill, 1995, Tryphonas et al., 1997). However, exposure of piglets for up to 4 months to FB_1-contaminated feed had no significant effect on antibody production against Aujeszky's disease (Tornyos et al., 2003). In summary, exposure to FB_1 activates proinflammatory networks, impairs maturation of antigen-presenting cells and affects immune cell viability and responses. These immunosuppressive effects increase susceptibility to infectious diseases, affects the treatment of diseases such as cancer, and diminishes vaccine efficacy.

FB1-mediated changes to the epigenome

Exogenous stimuli such as mycotoxins are prominent disrupters to the epigenome (Huang et al., 2019). They can induce phenotypic changes by differentially regulating gene expression rather than altering DNA sequences. Epigenetic modifications are essential for the normal cellular processes and maintenance of gene expression patterns. In contrast, aberrant alterations to the epigenome can affect genome stability and may activate transcription of various genes, such as oncogenes, or silence the expression of tumour suppressor genes (Sharma et al., 2010, Ho et al., 2012, Peschansky and Wahlestedt, 2014). Epigenetic mechanisms include DNA methylation, histone modifications and the production of non-coding RNA transcripts such as microRNA (miRNA) and long non-coding (lncRNA)

(Lennartsson and Ekwall, 2009, Smith and Meissner, 2013, Peschansky and Wahlestedt, 2014). These modifications play an important role in the toxicity and sometimes carcinogenicity of mycotoxins. Epigenetic alterations in response to FB_1 have been investigated *in vivo* and *in vitro* (Mobio et al., 2000, Kouadio et al., 2007, Pellanda et al., 2012, Chuturgoon et al., 2014a, Chuturgoon et al., 2014b, Demirel et al., 2015, Sancak and Ozden, 2015, Arumugam et al., 2020).

DNA methylation

DNA methylation is the most widely studied epigenetic modification. It is facilitated by DNA methyltransferases (DNMTs), which catalyses the transfer of methyl groups to selective cytosine and to a lesser extent adenine of mammalian DNA (Lyko, 2018). DNA methylation usually occurs in CpG islands of gene promoters although non-CpG methylation can also occur. Hypermethylation of CpG islands in gene promoter regions inhibit the binding of transcription factors and suppress gene transcription (Moore et al., 2013). FB_1 (9 and 18 µM) induced significant DNA hypermethylation in rat C6 glioma cells after 24 hours; however, failed to induce hypermethylation at higher concentrations (27 and 54 µM). It was suggested that the lack of DNA methylation in higher concentrations could be due to higher toxicity and DNA damage inflicted by FB_1 (Mobio et al., 2000). Hypermethylation is known to play a role in the regulation of DNA replication and gene expression in cell division and differentiation processes (Moore et al., 2013). Hypermethylation observed at 9-18 µM may have resulted in the hypermethylation of gene promoters involved in protein synthesis, DNA synthesis and cell cycle regulation which may explain the impairment of G0/G1 transition, DNA and protein synthesis and the low percentage of cells observed in the S phase of the cell cycle (Mobio et al., 2000). In human intestinal Caco-2 cells, FB_1 (10, 20, 40 µM for 24 hours) was also shown to significantly increase DNA methylation from 4.5% in control cells to 9%, 9.5% and 8% at concentrations of 10, 20 and 40 µM of FB_1, respectively (Kouadio et al., 2007). Moreover, Demirel et al. (2015) evaluated the effect of FB_1 on both global DNA methylation and candidate gene methylation. While no significant changes to global DNA methylation occurred in rat liver (Clone 9 cells) and kidney epithelial cells (NRK-52E); CpG promoter methylation occurred in selective tumour suppressor genes. CpG islands of VHL and e-cadherin promoters were methylated in both cell lines. In addition, c-Myc was found methylated exclusively in Clone 9 cells and methylation of p16 gene occurred in NRK-52E cells (Demirel et al., 2015). Hypermethylation of tumour suppressor genes inhibits the transcription of these genes aiding carcinogenesis (Sharma et al., 2010). Global DNA hypomethylation is also characteristic of cancer cells and is found in early carcinogenesis and during tumour progression (Sheaffer et al., 2016). In HepG2 cells, FB_1 (200 µM; 24 hours) induced significant global DNA hypomethylation which was accompanied by decreased expression of DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) and increased expression of DNA demethylase, MBD2 (Chuturgoon et al., 2014a). A major consequence of global DNA hypomethylation is the lack of sufficient ability to maintain genomic stability and activate appropriate DNA damage responses (Sheaffer et al., 2016). Global

hypomethylation by FB_1 leads to the loss of genomic integrity which was observed by the increased comet tail lengths induced by FB_1 (Chuturgoon et al., 2014a). The inconsistencies in global methylation across these 4 studies maybe due to a number of factors: i) heterogeneity of the cells used – DNA methylation regulates gene expression in a cell and tissue specific manner; ii) doses of FB_1 used – low doses seemed to favour hypermethylation; whereas high dose favoured hypomethylation. Hypermethylation of tumour suppressor genes observed by Demirel et al. (2015) in combination with a gross loss of global DNA methylation witnessed by Chuturgoon et al. (2014a) may be one of the mechanisms responsible for FB_1-related carcinogenesis.

Histone Modifications

Modifications to histones are another means in which FB_1 can affect chromatin architecture and gene expression. Histone modifications are covalent post-translational modifications that can influence chromatin structure and subsequently the transcriptional status of genes. Histone modifications include the methylation, acetylation, phosphorylation, sumoylation and ubiquitination of specific amino acid residues (Cosgrove et al., 2004). In FB_1-treated NRK-52E cells (25, 50 and 100 µM), a global increase in di- and tri- methylation of lysine 9 on histone 3 (H3K9me2/3) was accompanied by an increase in H3K9 histone methyltransferase (HMT). However, high doses (50 and 100 µM, 24 hours) and prolonged exposure (25 µM, 27 and 96 hours) to FB_1 significantly reduced methylation of lysine 20 of histone 4 (H4K20) (Sancak and Ozden, 2015). Similar results in H3K9me3 and H4K20me3 were observed in the foetus of methyl deficient dams exposed to FB_1 (Pellanda et al., 2012). Both H3K9me3 and H4K20me3 establishes a condensed and transcriptionally inert chromatin conformation that contributes to the maintenance of genome stability (Saksouk et al., 2015). Loss of H4K20me3 provokes genome instability and is considered a hallmark of cancer (Van Den Broeck et al., 2008); The rise in H3K9me3 might be the defence mechanism promoting the cell to resist heterochromatin disorganization by FB_1 (Pellanda et al., 2012). These changes in H3K9 methylation are associated with closed chromatin and inhibition of transcription, further pointing to the probability that FB_1 silences genes especially, tumour suppresser genes (Sharma et al., 2010). However, the study by Chuturgoon et al. (2014a) indicated that FB_1 significantly increased the expression of two histone demethylase genes *KDM5B* and *KDM5C*, which may promote H3K4me3/me2 demethylation. But this was not the case in NRK-52E cells and in a recent study which used HepG2 cells (Sancak and Ozden, 2015, Arumugam et al., 2020). Regarding histone acetylation, FB_1 had little effect on H4K16 and H3K18 acetylation (Pellanda et al., 2012, Gardner et al., 2016). A dose and time-dependent decrease was observed in the H3K9ac levels in response to FB_1, while histone acetyl transferase activity was only inhibited as a consequence of prolonged exposure (96 hours) (Sancak and Ozden, 2015). In LM/Bc embryonic fibroblasts, the elevation in Sa1P after FB_1-mediated inhibition of CS, inhibited histone deacetylase activity, promoting histone acetylation of H2NK12, H3K9 and H3K23 (Gardner et al., 2016) The results of this study along with Pellanda et al. (2012), provides a potential mechanism for the failure of neural tube closure

observed in mice and humans following FB_1 exposure. However, further *in vitro* studies should be undertaken to confirm this hypothesis. Histone phosphorylation also contributes to the toxicity of FB_1. Downregulation in the phosphorylation of γ-H2AX was observed upon FB_1 (200 µM, 24 hours) exposure in HepG2 cells (Chuturgoon et al., 2015). Poor phosphorylation of γ-H2AX provokes genome instability and prevents appropriate responses to DNA damage leading to gene mutations and tumorigenesis (Podhorecka et al., 2010).

MicroRNA profiles

Only two studies has investigated the effect of FB_1 on miRNA profiles (Chuturgoon et al., 2014b, Arumugam et al., 2020). MiRNAs are a class on small non-coding RNAs that target mRNAs to induce mRNA degradation and translational repression (O'Brien et al., 2018). Quantitative polymerase chain reaction array-based profiling of miRNA and hierarchical cluster analysis by Chuturgoon et al. (2014b) found that miR-135b, miR-181d, miR-27a, miR-27b, and miR-30c were significantly downregulated. They further investigated miR-27b and found a 10-fold decrease that correlated with increased expression of cytochrome 1B1, which mediates the bioactivation of procarcinogens (Chuturgoon et al., 2014b). A recent study found that FB_1 induced miR-30c expression which altered H3K4me as well as inhibited the translation of the tumour suppressor, phosphatase and tensin homolog (PTEN) leading to diminished response and repair of FB_1-induced oxidative DNA lesions (Arumugam et al., 2020). By evaluating all the previous data, it is evident that epigenetic modifications are involved in FB_1 toxicity and possibly the aetiology of diseases such as neural tube defects and cancer. Nevertheless, further research should be undertaken to fully explore the effect of FB_1 on the epigenome as a whole and to elucidate the impact of gene-specific epigenetic modifications in relation to a particular toxicological phenotype.

Current strategies in minimizing FB1 toxicity

Considering that FB_1 contamination of agricultural staples is unavoidable and the negative impact it has on human health, a great deal of research has focused on strategies to mitigate FB_1 contamination and toxicity. The implementation of good agricultural, storage and processing practices can reduce FB_1 contamination and subsequent exposure to humans and animals (Okabe et al., 2015). Several new approaches are being investigated to detoxify FB_1 contaminated foods and feeds. These strategies include the use of physical, chemical or biological means to remove FB_1 or attenuate its effects. Below we review some recent research investigating FB_1 detoxification.

Physical methods

Although FB_1 is relatively heat stable, the use of extrusion cooking (high temperature/high pressure) has been shown to be an effective method of reducing FB_1 levels in maize [reviewed by Jackson et al. (2012)]. At the right temperature and pressure, extrusion cooking can reduce FB_1 concentration by 64% in grits; however, cooking grits with the addition of glucose along with extrusion can eliminate 99% of

FB_1 from this maize-based porridge. Furthermore, this cooking technique prevented the disruption of sphingolipid metabolism and development of kidney lesions in male rats fed diets consisting of FB_1 contaminated grits that have undergone extrusion and glucose supplementation (Voss et al., 2011). Nixtamalization is an alternative cooking method of corn and other grains. This ancient cooking process involves cooking and steeping grains in an alkaline solution (calcium hydroxide) to improve nutritional value and possibly reduce toxin contamination (Voss et al., 2017). However, the fate of FB_1 during nixtamalization is not fully understood and potentially toxic reaction products, including matrix-associated "masked" FB_1 might remain in nixtamalized corn (Voss et al., 2013). Nixtamalization involves the removal of one or both tricarboxylic acid groups from FB_1 yielding $pHFB_1$ or HFB_1, respectively (Voss et al., 2017). De Girolamo et al. (2016) found that while cooking without an alkaline solution did reduce the levels of FB_1 and $pHFB_1$; HFB_1 levels remained the same. However, the use of an alkaline solution reduced FB_1 and $pHFB_1$ by converting it to HFB_1. This confirms the role of alkaline in releasing matrix associated FB_1. No evidence of "masked" FB_1 was found in another study that investigated the role of nixtamalization on FB_1 detoxification. Moreover, nixtamalization not only reduced FB_1 levels in the feed of Sprague Dawley rats but also lowered Sa and So levels and reduced the number of renal lesions in comparison to rats fed uncooked corn (Voss et al., 2013).

Chemical methods

Organic and inorganic compounds can be used to bind or adsorb mycotoxins from the gastrointestinal tract preventing their entry into circulation. Calcium montmorillonite (NovaSil), a dioctahedral smectite clay, is an affective aflatoxin binder and is considered safe in humans. Robinson et al. (2012) evaluated the effectiveness of NovaSil with regards to FB_1 in male F344 rats and humans. NovaSil reduced rat urinary FB_1 levels by 20% in the first 24 hours and 50% after 48 hours. In a clinical trial, 3 g/day NovaSil eliminated 90% of FB_1. The protonation of the amino group of FB_1 in acidic conditions like that of the stomach allows for its binding to the negatively charged surfaces of the clay. Nanosilicate platelets exfoliated from montmorillonite have a large surface area and high density which may allow for effective FB_1 binding. Nanosilicate platelets lowered FB_1 levels in circulation, reversed sphingolipid perturbations and prevented abnormalities in mice dams fed FB_1 contaminated diets. It also lowered the incidence of neural tube defects in their offspring (Liao et al., 2014). Two studies independently evaluated the effects of novel nanocellulose compounds on FB_1. Jebali et al. (2015) modified nanocellulose with polylysine (NMPL); which has a high affinity to the carboxyl groups of FB_1; while Zadeh and Shahdadi (2015) coated nanocellulose with free fatty acids which bind to the hydrophobic tail of FB_1. Both studies found that the nanocellulose compounds effectively adsorbed FB_1 and reduced toxicity in mouse liver cells (Jebali et al., 2015, Zadeh and Shahdadi, 2015). However, NMPL is sensitive to changes in pH (Jebali et al., 2015), and both compounds should be tested *in vivo*. Lastly, 2-5 g/kg of relatively new mycotoxin inactivator, Adidetox™ moderately reduced FB_1 toxicity in Sprauge

Dawley rats; however, it did not fully avoid a significant accumulation of sphingolipids (Denli et al., 2015).

Biological Methods

Certain micro-organism form part of normal gut flora and its consumption is associated with a range of health benefits, including improved immune function, antioxidant capacity and prevention of cancer (Hullar et al., 2014). Consequently, the role of these micro-organisms as mycotoxin detoxification agents are being investigated and inclusion of such microbes in the diet may decrease availability and absorption of FB_1 in the gastrointestinal tract. 12 *Lactobacillus* bacterial strains and 6 *Saccharomyces cerevisiae* yeast strains significantly reduced FB_1 levels by 62-77% and 67-74%, respectively (Chlebicz and Śliżewska, 2020). FB_1 binds to the micro-organism's cell wall through weak noncovalent interactions. The interactions need as little time as a minute, suggesting that neither FB_1 cell entry nor metabolism may occur. Further, they can absorb FB_1 and aflatoxin simultaneously without changes in their efficiency (Pizzolitto et al., 2012). The use of *Lactobacillus delbrueckii* and *Pediococcus acidilactici* as probiotics ameliorated FB_1-induced hepatorenal toxicity and genotoxicity in rats by normalizing kidney function, restoring redox homeostasis and reducing DNA fragmentation (Khalil et al., 2015, Abdellatef and Khalil, 2016). Antioxidant capabilities of probiotics against FB_1 were also demonstrated by *Lactobacillus paracaeseu* which upregulated antioxidant capacity, inhibited lipid peroxidation, increased free radical scavenging and reduced DNA fragmentations. It also had protective effects against immunotoxicity induced by FB_1 (Abbès et al., 2016). The use of recombinant carboxylesterase, *FUMD,* from *yeast (Pichia pastoris)* has been shown to degrade FB_1 in the gastrointestinal tract of pigs. *FUMD* is responsible for the removal of the tricarboxylic acid side chains of FB_1, forming HFB_1 (Masching et al., 2016). As shown previously, HFB_1 can undergo *N*-acylation forming toxic derivatives, thus deamination is necessary for effective detoxification. *FUMD* along with *FUM1* were shown to be the genes responsible for the degradation of FB_1 by the bacterium *Sphingopyxis* sp. MTA144. *FUMD*, was responsible for the desertification; while *FUM1*, an aminotransferase, deaminated FB_1 and HFB_1. HFB_1 only has 1 amino group therefore, the product of these reactions can no longer inhibit CS activity. The authors believe the product to be 2-keto-HFB_1; however, the products need to undergo characterization (Heinl et al., 2010).

Conclusion

Fumonisin contamination of global agricultural produce is unavoidable and unpredictable. This poses a unique challenge to food quality and safety. The most potent and abundant class of fumonisins is FB_1, which is the cause of several species-specific toxicities and is involved in carcinogenesis. Therefore, it is necessary to investigate the mode of action of FB_1 as well as interventions that aide in detoxification. As discussed above, the main mode of FB_1 toxicity is via the disruption of sphingolipid metabolism. This results in the accumulation of sphingoid bases in the ER, which disrupts signalling pathways and results in ER stress and autophagy. FB_1 also enhances ROS production leading to oxidative damage to

cells and alters immune responses. Furthermore, FB_1 induces epigenetic changes that affect cell cycle regulation, DNA and protein synthesis as well as promotes cancer via the inhibition of tumour suppressor genes and activation of procarcinogens. Considering only a handful of studies have investigated the impact of FB_1 on the epigenome, it is necessary that more accurate epigenetic mechanisms of FB_1-induced toxicity are explored. Through proper crop management and storage, FB_1 levels in crops can be minimized. Dietary interventions that eliminate or detoxify FB_1 in the gut, such as the use of chemical adsorbents or probiotics may also be crucial in mitigating the unpleasant consequences of FB_1 (Figure 3.5).

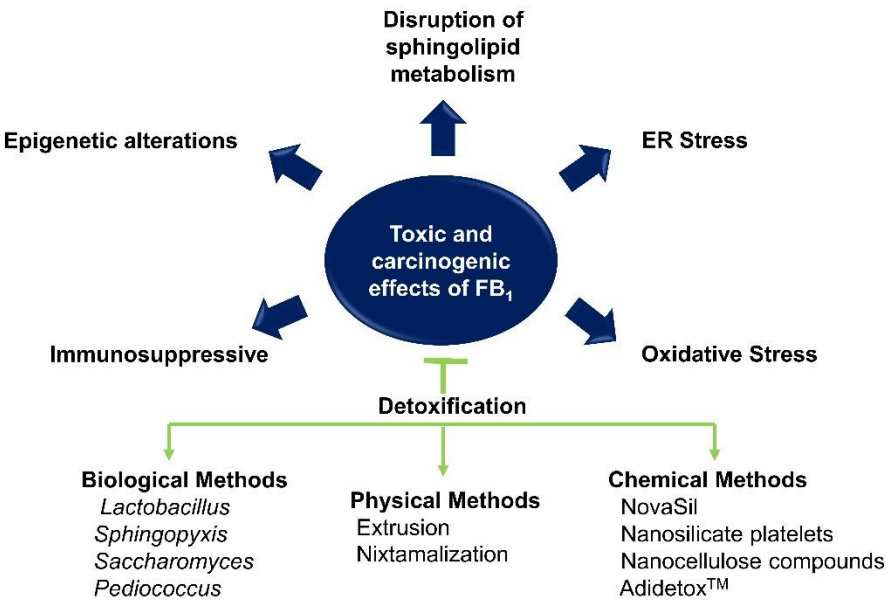

Figure 3.5. An overview of the toxic and carcinogenic modes of action by FB1 as well as strategies involved in its detoxification

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CHAPTER 4

Fumonisin B_1 Epigenetically Regulates PTEN Expression and Modulates DNA Damage Checkpoint Regulation in HepG2 Liver Cells

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Abstract

Fumonisin B_1 (FB_1), a *Fusarium*-produced mycotoxin, is found in various foods and feeds. It is a well-known liver carcinogen in experimental animals; however, its role in genotoxicity is controversial. The current study investigated FB_1-triggered changes in the epigenetic regulation of PTEN and determined its effect on DNA damage checkpoint regulation in human liver hepatoma G2 (HepG2) cells. Following treatment with FB_1 ($IC_{50:}$ 200 µM; 24 h), the expression of miR-30c, KDM5B, PTEN, H3K4me3, PI3K, AKT, p-ser473-AKT, CHK1, and p-ser280-CHK1 was measured using qPCR and/or Western blot. H3K4me3 enrichment at the PTEN promoter region was assayed via a ChIP assay and DNA damage was determined using an ELISA. FB_1 induced oxidative DNA damage. Total KDM5B expression was reduced, which subsequently increased the total H3K4me3 and the enrichment of H3K4me3 at PTEN promoters. Increased H3K4me3 induced an increase in PTEN transcript levels. However, miR-30c inhibited PTEN translation. Thus, PI3K/AKT signaling was activated, inhibiting CHK1 activity via phosphorylation of its serine 280 residue preventing the repair of damaged DNA. In conclusion, FB_1 epigenetically modulates the PTEN/PI3K/AKT signaling cascade, preventing DNA damage checkpoint regulation, and induces significant DNA damage.

Keywords: Fumonisin B_1; DNA damage; epigenetics; PTEN; H3K4me3; Checkpoint Kinase 1

Key Contributions

Fumonisin B_1 (FB_1) induces oxidative damage to DNA and alters the epigenetic status of cells. This study confirms the genotoxic potential of FB_1 and provides novel insight into the impairment of DNA damage responses by FB_1 via the epigenetic downregulation of PTEN; which in turns inhibits DNA damage checkpoint regulation via the PI3K/AKT/CHK1 axis. The diminished repair of FB_1-induced oxidative DNA lesions may contribute to the cytotoxic effects of FB_1.

Introduction

Fumonisins are major food-borne mycotoxins produced by fungi belonging to the *Fusarium* genus [1,2]. Presently, 28 fumonisin homologues have been characterized into the following groups: fumonisins A, B, C, and P [2]. Over 70% of fumonisins produced are fumonisin B_1 (FB_1), making it the most prevalent and toxicologically relevant homologue [3]. FB_1 contamination is common in maize and cereal-related products in several countries throughout the world, with concentrations reaching as high as 30,000 µg/kg [4]. Poor food processing, handling, and storage conditions aide FB_1 contamination, thereby increasing the risk of exposure for both animals and humans [5]. The effect of FB_1 in animals is sex-dependent and has species-specific toxicity, with the liver, kidney, and nervous system being the most common targets [6–11]. The International Agency for Research on Cancer (IARC) has classified FB_1 as a class 2B carcinogen [12]. Studies on rodents have demonstrated that FB_1 can initiate and promote cancer [1,13], while the consumption of FB_1-contaminated commodities has been associated with increased incidence of hepatocellular and/or esophageal carcinomas [14,15]. Earlier studies have

dismissed FB_1 as a mutagen and reported that FB_1 is a weak genotoxin [16] or that it showed no signs of genotoxicity [17,18]. Irrespective of these earlier studies, numerous studies have since observed that a consequence of FB_1 exposure is extensive DNA damage through strand breaks, micronuclei induction, and fragmentation [19–21].

Cells are equipped with a complex network of DNA damage responses (DDRs) that coordinate DNA repair and consequently cell fate [22]. The tumor suppressor phosphatase and tensin homolog (PTEN) controls multiple cellular processes including growth and differentiation by opposing the phosphoinositide 3-kinases (PI3K)/protein kinase B (AKT) signaling cascade [23,24]. Emerging evidence has demonstrated the unique role PTEN plays in maintaining genomic stability and DNA repair [25,26]. PTEN responds to DNA damage by inhibiting the PI3K/AKT cascade and preventing the inhibitory phosphorylation of checkpoint kinase 1 (CHK1). This activates checkpoint regulation and induces cell cycle arrest, which allows for the repair of DNA [27,28]. Underlining the important role of PTEN, poor expression of PTEN is a common risk factor in the occurrence of liver pathologies [29,30]. Studies have elucidated that poor expression of PTEN may be due to epigenetic alterations [31]. Small non-coding RNAs, known as microRNAs (miRNA), such as miR-19a and miR-21, reduce PTEN gene expression by binding to the 3′ untranslated region (3′UTR) of *PTEN* mRNA and inhibits its translation [32,33], while the trimethylation of lysine 4 residues of histone 3 (H3K4me3) on the promoter region of *PTEN* is associated with active transcription [34].

While the role of PTEN in cellular functioning has been well established, further research should be undertaken to determine the epigenetic mechanisms in which PTEN is regulated. Moreover, the epigenetic effects of FB_1 in humans have only recently begun to be uncovered and no study to date has determined the effects FB_1 has on PTEN [21,35]. Previously, Chuturgoon et al. [35] conducted miRNA profile arrays in human hepatoma G2 (HepG2) cells following FB_1 exposure and found miR-30c to be one of the major miRNAs affected. Through computational prediction analysis, we found a possible link between miR30c, PTEN, and the histone lysine demethylase 5B (KDM5B). KDM5B catalyzes the removal of methyl groups from histone 3 lysine 4 (H3K4) [36]. H3K4me3 is predominantly found at transcriptional start sites, where it promotes gene transcription [37]. Therefore, we proposed that together miR-30c and KDM5B mediate the epigenetic regulation of PTEN. The current study determined the consequences of FB_1 exposure on DNA damage and DNA damage checkpoint regulation via the PTEN/PI3K/AKT network. Further, we determined FB_1 epigenetic regulation of PTEN via miR-30c and H3K4me3 in human liver (HepG2) cells.

Method and Materials

Materials

FB_1 *(Fusarium moniliforme*, 62580) was purchased from Cayman Chemicals (Michigan, MI, USA). The HepG2 cell line (HB-8065) was procured from the American Type Culture Collection (ATCC).

Cell culture consumables were purchased from Whitehead Scientific (Johannesburg, South Africa). Western blot reagents were obtained from Bio-Rad (California, CA, USA). All other reagents were purchased from Merck (Massachusetts, MA, USA), unless otherwise stated.

Cell Culture and Treatments

HepG2 cells (passage 3; 1.5×10^6) were cultured in complete culture media [CCM: Eagle's Minimum Essentials Medium (EMEM) supplemented with 10% fetal calf serum, 1% penicillin–streptomycin–fungizone, and 1% L-glutamine] at 37°C in a 5% CO_2 humidified incubator until 80% confluent. Thereafter, cells were treated with varying concentrations of FB_1 (5, 100, and 200 µM) for 24 h. These FB_1 concentrations were obtained from the crystal violet assay (Supplementary Figure S4.1) and represented 90%, 70%, and 50% cell viabilities, respectively. An untreated control was prepared along with the FB_1 treatments. Data obtained using 200 µM FB_1 (IC_{50}) are shown in the main text. The results for all assays conducted using 5 and 100 µM FB_1 are available in the Supplementary material (Supplementary Figure S4.2–S4.7). Results were verified by performing two independent experiments in triplicate.

DNA Damage

DNA was isolated using the FlexiGene DNA isolation kit (Qiagen, Hilden, Germany, 512608). Extracted DNA was used to determine 8-OHdG levels using the DNA damage ELISA kit (Enzo Life Sciences, New York, USA, ADI-EKS-350), as per the manufacturer's instructions.

RNA Isolation and Quantitative Polymerase Chain Reaction (qPCR)

RNA was isolated according to the method described by Ghazi et al. (2019) [38]. For miRNA expression, cDNA was synthesized using the miScript II RT Kit (Qiagen, Hilden, Germany, 218161), as per the manufacturer's instructions. The expression of miR-30c was analyzed using the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany, 218073) and the miR-30c primer assay (Qiagen, Hilden, Germany, MS00009366), as per the manufacturer's instructions. Samples were amplified using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the following cycling conditions: initial denaturation (95 °C, 15 min), followed by 40 cycles of denaturation (94°C, 15 sec), annealing (55°C, 30 sec), and extension (70°C, 30 sec).

For mRNA expression, cDNA was prepared using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, Waltham, MA, USA, K1652), as per the manufacturer's instructions. The expression of *KDM5B*, *PTEN*, *AKT*, and *CHK1* was determined using the Powerup SYBR Green Master Mix (Thermo-Fisher Scientific, Waltham, MA, USA, A25742), as per the manufacturer's instructions. Samples were amplified using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the following cycling conditions: initial denaturation (95°C, 8

min), followed by 40 cycles of denaturation (95°C, 15 sec), annealing (Temperatures: Table 4.1, 15 sec), and extension (72°C, 30 sec).

Table 4.1. The annealing temperatures (°C) and primer sequences for the genes of interest.

Gene	Annealing Temperature (°C)	Primer	Sequence
KDM5B	55	Sense	5′-CGA CAA AGC CAA GAG TCT CC-3′
		Anti-sense	5′-CTG CCG TAG CAA GGC TATTC-3
PTEN	56.6	Sense	5′-TTT GAA GAC CAT AAC CCA CCA C-3′
		Anti-sense	5′-ATT ACA CCA GTT CGT CCC TTT C-3′
AKT1	55	Sense	5′-GCC TGG GTC AAA GAA GTC AA-3′
		Anti-sense	5′-CAT CCC TCC AAG CTA TCG TC-3′
CHK1	59.1	Sense	5′-CCA GAT GCT CAG AGA TTC TTC CA-3′
		Anti-sense	5′-TGT TCAACA AAC GCT CAC GAT TA-3′
GAPDH	Same as gene of interest	Sense	5′-TCCACCACCCTGTTGCTGTA-3′
		Anti-sense	5′-ACCACAGTCCATGCCATCAC-3′

Relative gene expression was determined using the method described by Livak and Schmittgen [39]. $2^{-\Delta\Delta Ct}$ represents the fold change relative to the untreated control. miRNA and mRNA of interest were normalized against the house-keeping genes, *RNU6* (Qiagen, Hilden, Germany, Ms000033740) and *GAPDH*, respectively.

Chromatin Immunoprecipitation Assay

H3K4me3 at the *PTEN* promoter region was determined using the chromatin immunoprecipitation (ChIP) assay. Histones were crosslinked to DNA by incubating (37°C, 10 min) the cells in 37% formaldehyde. Cells were washed in cold 0.1 M PBS (containing protease inhibitors), mechanically lysed and centrifuged (2000 rpm, 4°C, 4 min). The DNA pellet was re-suspended in sodium dodecyl

sulphate (SDS)–lysis buffer (200 µl; 1% SDS, 10 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM Tris; pH 8.1) and sheared by homogenization. Samples were centrifuged (13,000 rpm, 4°C, 10 min) and supernatants were diluted with ChIP dilution buffer [0.01% SDS, 1.1% Tritonx-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), and 167 mM NaCl]. The diluted supernatants were split into equal fractions. Anti-H3K4me3 (Abcam, Cambridge, UK, ab12209) was added to one fraction, while no antibody was added to its counterpart. Both fractions were incubated overnight at 4°C. A 50% slurry of Protein A agarose and salmon sperm DNA (Merck, Kenilworth, NJ, USA, 16-157) was added to all samples and incubated (4°C, 1 h) with gentle rotation. Thereafter, samples were centrifuged (1000 rpm, 4 °C, 1 min), and pellets were washed once with the following buffers: low salt immune complex wash buffer (0.1% SDS, 1% Tritonx-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 150 Mm NaCl), high salt immune complex wash buffer [0.1% SDS, 1% Tritonx-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl], Lithium chloride immune complex wash buffer (0.25 M LiCl, 1% IGEPAL, 1% deoxycholic acid, 1 mM EDTA, and 10 mM Tris; pH 8.1), and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). DNA was eluted using elution buffer (1% SDS, 0.1 M $NaCHO_3$) for 15 min (gentle rotation, RT). Samples were centrifuged (1000 rpm, 4°C, 1 min) and elution was repeated on the protein A agarose/ssDNA pellet. Eluates were combined and incubated in 5 M NaCl (65°C, 4 h) to reverse crosslinks. DNA was further purified using a DNA Clean & Concentrator-5 kit, as per the manufacturer's instructions (Zymo research, Irvine, CA, USA, D4003).

H3K4me3 immunoprecipitated chromatin was used in a RT-qPCR reaction (as previously described) to determine H3K4me3 at the *PTEN* promoter (Sense: 5′- CGC CCA GCT CCT TTT CCC-3′; Anti-sense: 5′- CTG CCG CCG ATT CTT AC-3′). The fold enrichment method was used to normalize data obtained from the ChIP-qPCR.

Protein Isolation and Western Blotting

Protein was isolated using Cytobuster reagent (Merck, Kenilworth, NJ, USA, 71009-3) supplemented with protease and phosphatase inhibitors (Roche, Basel, Switzerland, 05892791001 and 04906837001, respectively). Cells were mechanically lysed, and centrifuged (13,000 rpm, 4°C, 10 min). Supernatants were used to quantify protein concentration via the bicinchoninic acid assay (BCA). Proteins were standardized to 1 mg/mL. The expression of KDM5B (Abcam, Cambridge, UK, ab19884), H3K4me3 (Abcam, Cambridge, UK, ab12209), PTEN (Cell Signalling Technologies, Danvers, MA, USA, 9552S), p-ser473-AKT (Cell Signaling Technologies, Danvers, MA, USA, 9271S), AKT (Cell Signaling Technologies, Danvers, MA, USA 9272S), PI3K (Cell Signaling Technologies, Danvers, MA, USA, 4249S), p-ser280-CHK1 (Cell Signaling Technologies, Danvers, MA, USA, 23475), and CHK1 (Cell Signaling Technologies, Danvers, MA, USA, 2360S) were determined using Western blotting as previously described [43]. The Image Lab Software version 5.0 (Bio-Rad, Hercules, CA, USA) was used to measure band densities of expressed proteins. Protein expression is represented as

relative band density and calculated by normalizing the protein of interest against the housekeeping protein, β-actin.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego CA, USA). The unpaired *t* test was used for all assays. One-way ANOVA with Dunnet's post-test was used to evaluate the significant effect of FB_1 in all Supplementary Figures. All results are presented as the mean ± standard deviation, unless otherwise stated. A value of $p < 0.05$ was considered to be statistically significant.

Results

FB_1 Induces DNA Damage in HepG2 Cells

FB_1 negatively impacts redox homeostasis, which results in oxidative damage to cellular structures. We assessed FB_1-mediated DNA damage by evaluating levels of the oxidative DNA damage biomarker—8-hydroxy-2'-deoxyguanosine (8-OHdG). FB_1 significantly increased the level of 8-OHdG (2.68-fold) compared with the control ($p = 0.0061$; Control: 1.04 ± 0.0641 vs. FB_1: 2.68 ± 0534; Figure 4.1.).

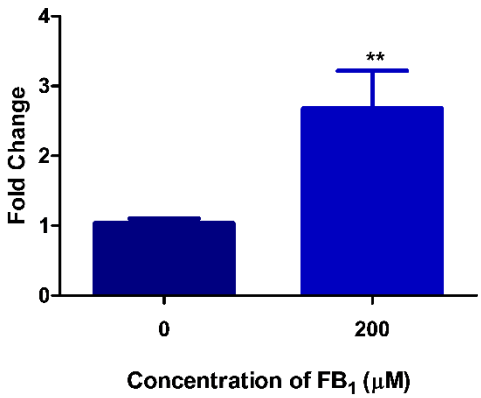

Figure 4.1. Fumonisin B_1 (FB_1) significantly increased the oxidative DNA damage biomarker, 8-OHdG, in human hepatoma G2 (HepG2) cells (** $p < 0.01$).

FB_1 Increases miR-30c Expression in HepG2 Cells

Since PTEN initiates DNA damage responses and miR-30c has been shown to disrupt DNA damage responses, we investigated the epigenetic regulation of PTEN [26,40]. miR-30c is involved in regulating cell cycle transition, proliferation, and lipid metabolism. FB_1 (IC_{50}; 200 μM) significantly upregulated miR-30c by 1.47-fold ($p = 0.0023$; Control: 1.04 ± 0.0642 vs. FB_1 1.47 ± 0.149; Figure 4.2a).

Target Scan version 7.2 (http://www.targetscan.org/vert_72/) was used to identify putative mRNA targets of miR-30c. miR-30c has complimentary base pairs with *PTEN* (at positions 3957–3963, 5018–5029, and 5880–5886 in the 3′UTR) and *KDM5B* (at positions 432–438 in the 3′UTR) (Figure 4.2b)

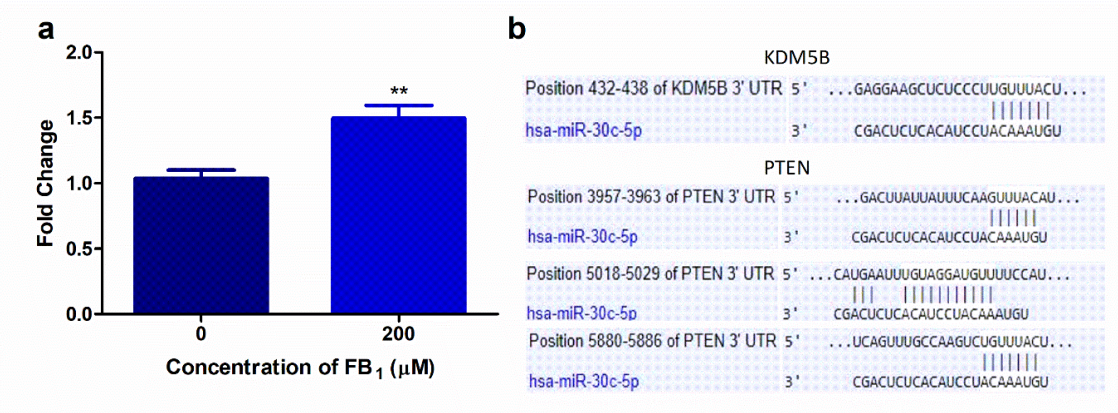

Figure 4.2. The effect of FB_1 on miR-30c levels in HepG2 cells and potential miR-30c targets. (**a**) FB_1 significantly elevated miR-30c expression (** $p \leq 0.01$). (**b**) Target Scan analysis of miR-30c with the 3′ untranslated region (3′UTR) of *KDM5B* and *PTEN*.

FB_1 Induces H3K4me3 by Downregulating KDM5B in HepG2 Cells

Since FB_1 altered the expression of miR-30c (which has a complimentary sequence to KDM5B 3' UTR), we evaluated the gene and protein expression of KDM5B. FB_1 decreased *KDM5B* transcript levels by 9.86-fold ($p < 0.0001$; Control: 1.04 ± 0.0642 vs. FB_1: 9.86 ± 1.15; Figure 4.3a). KDM5B protein expression (Figure 4.3b) was reduced slightly ($p = 0.2966$) by FB_1 (1.47 ± 0.117 RBD) in comparison with the control (1.70 ± 0.142 RBD).

KDM5B is a negative regulator of H3K4me3; hence, we determined the effect of FB_1 on H3K4me3. FB_1 (3.00 ± 0.0589 RBD) induced a considerable increase ($p < 0.0001$) in total H3K4me3 compared with the control (0.585 ± 0.00423 RBD; Figure 4.3c).

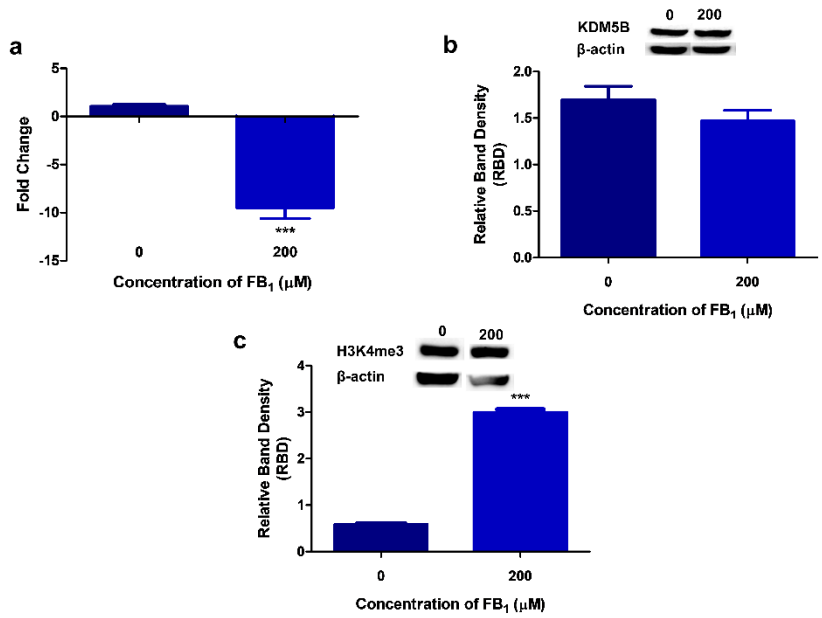

Figure 4.3. The effect of FB_1 on KDM5B and H3K4me3 levels in HepG2 cells. FB_1 reduced both the transcript (**a**; *** $p \leq 0.0001$) and protein (**b**; $p > 0.05$) expression of KDM5B. This may have led to the subsequent increase in total H3K4me3 (**c**; *** $p \leq 0.0001$).

FB_1 Alters PTEN Expression in HepG2 Cells

PTEN expression may be influenced by KDM5B and miR-30c. In addition to the total H3K4me3 levels, FB_1 also induced a significant 2.5-fold upregulation of H3K4me3 at *PTEN* promoter regions (p = 0.0052; Control: 1.04 ± 0.0641 vs. FB_1: 2.15 ± 0.273; Figure 4.4a).

H3K4me3 at promoter regions is associated with active transcription. The FB_1-induced increase in H3K4me3 corresponded with active transcription of the *PTEN* gene with a 1.46-fold increase (p = 0.0039; Control: 1.04 ± 0.0641 vs. FB_1: 1,46 ± 0,0354; Figure 4.4b). However, PTEN protein expression was significantly downregulated (p = 0.0001) by FB_1 (1.67 ± 0,0110 RBD) compared with the control (2,31 ± 0,0749 RBD; Figure 4.4c).

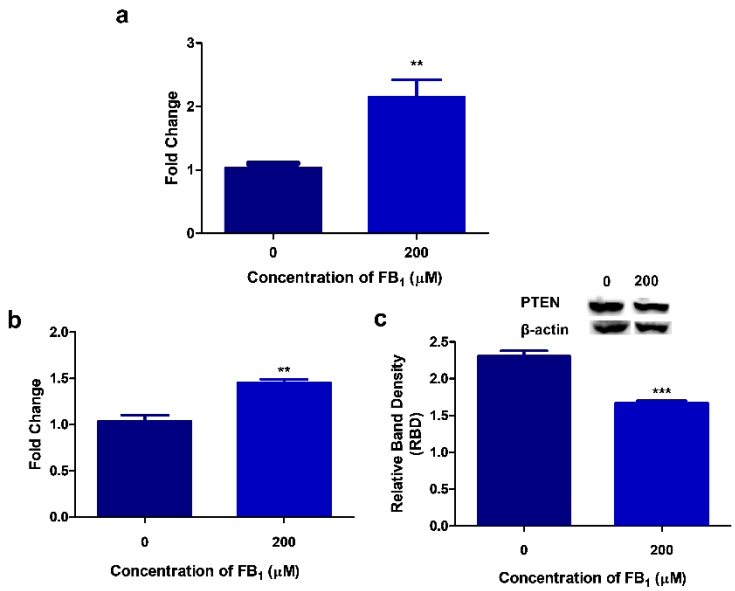

Figure 4.4. FB_1-induced KDM5B and miR-30c modulates PTEN expression. PTEN expression is influenced by both KDM5B and miR-30c. FB_1 increased H3K4me3 at *PTEN* promoter regions (**a**; ** $p < 0.01$), which resulted in significantly higher levels of PTEN transcripts (**b**; ** $p < 0.01$). However, miR-30c negatively influenced PTEN translation/protein expression (**c**; *** $p < 0.0001$).

FB_1 Affects PI3K/AKT Signaling in HepG2 Cells

Numerous biological processes are regulated by the PTEN/PI3K/AKT signaling network. PI3K protein expression ($p = 0.0014$; Figure 4.5) was 2.44-fold greater in FB_1-exposed cells (1.08 ± 0.126 RBD) compared with the control (0.443 ± 0.0600 RBD).

Total AKT protein expression was slightly increased ($p = 0.4200$; Figure 4.5) by FB_1 (Control 1.61 ± 0.0148 RBD vs. FB_1 1.82 ± 0.396 RBD). AKT is activated by the phosphorylation of serine 473 within the carboxy terminus. FB_1 significantly increased the phosphorylation of AKT ($p = 0.001$, 0.973 ± 0.0350 RBD; Figure 4.5) compared with the control (0.604 ± 0.0661 RBD).

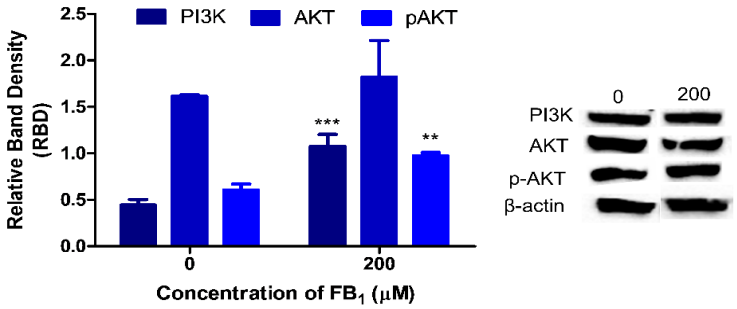

Figure 4.5. The effect of FB_1 on the PI3K/AKT signaling cascade. The protein expression of PI3K, AKT, and pAKT in HepG2 cells was evaluated using western blotting. FB_1 increased PI3K (*** $p < 0.0001$), AKT ($p > 0.05$), and p-ser473-AKT (** $p < 0.01$) protein expression. PI3K and AKT expression was normalized against β-actin, and p-ser473-AKT was normalized against AKT.

FB_1 Modulates CHK1 Expression and Activity in HepG2 Cells

CHK1 is critical in coordinating DDR and cell cycle checkpoints. FB_1 elevated *CHK1* transcript levels by 1.79-fold ($p = 0.0209$; Figure 4.6a). Western blotting revealed an increase in total CHK1 protein expression ($p = 0.0008$; Control 0.540 ± 0.105 RBD vs. FB_1 1.18 ± 0.0614 RBD; Figure 4.6b). Active PI3K/AKT signaling phosphorylates serine 280 of CHK1 and inactivates it. FB_1 significantly elevated ($p = 0.0314$; 1.54 ± 0.179 RBD) p-ser280-CHK1 expression in comparison with the control (1.09 ± 0.162 RBD; Figure 4.6c). This suggests that FB_1 inactivates CHK1 via the PI3K/AKT signaling pathway.

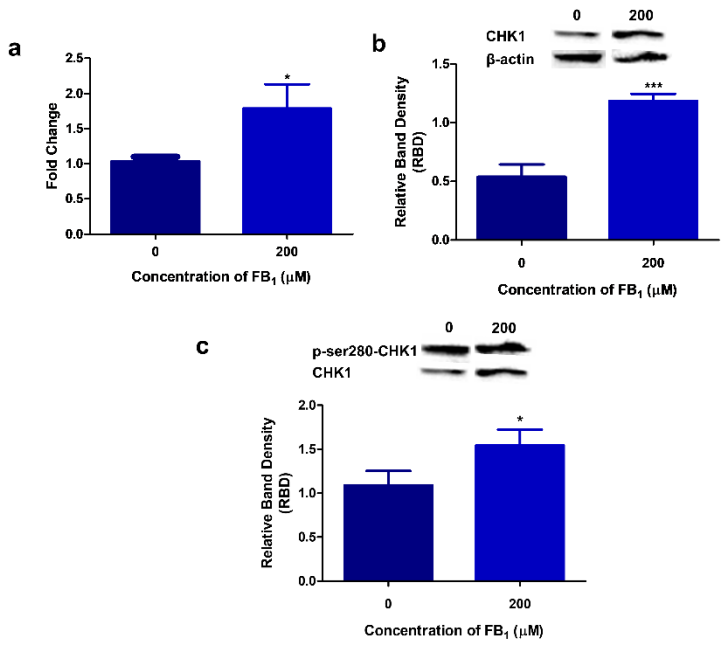

Figure 4.6. The effect of FB_1 on CHK1 expression. FB_1 significantly increased *CHK1* transcript levels (**a**; $*p < 0.05$), CHK1 protein expression (**b**; *** $p < 0.0001$), and p-ser280-CHK1 (**c**; * $p < 0.05$). CHK1 expression was normalized against β-actin and p-ser280-CHK1 was normalized against CHK1.

Discussion

Considering that FB_1 contamination of agricultural products is common throughout the world, it is necessary to evaluate the health hazards FB_1 poses to humans and animals. Several studies have attributed oxidative stress as one of the mechanisms in which FB_1 exerts its toxicity [41–45]. Excessive production of reactive oxygen species (ROS) results in oxidative damage to cells and macromolecules including DNA [44]. While some studies have disputed the genotoxic potential of FB_1 [17,18], others have reported chromosomal aberrations and oxidative DNA damage triggered by FB_1 exposure [16,41,47,48]. Apart from inducing DNA damage, FB_1 may disrupt DDR network and repair processes. One potential mechanism could be through the PTEN/PI3K/AKT/CHK1 axis.

To better understand the genotoxic potential of FB_1, we set out to determine if FB_1 induces DNA damage and if it alters DNA damage checkpoint regulation via the PTEN/PI3K/AKT/CHK1 network. Seeing that poor PTEN expression is common in toxicity, we further determined the effects of FB_1 on the epigenetic regulation of PTEN via miR-30c and H3K4me3 in human hepatoma G2 (HepG2) cells. The liver is one of the primary organs in which FB_1 is thought to accumulate, and is usually the initial site for the metabolism and detoxification of food and food contaminants [49,50]. Due to the limitations of primary hepatocytes such as poor availability, short life span, inter-donor variability, loss of hepatic function, and early phenotypic changes, we opted to use the HepG2 cell line for this study [51,52]. The DNA of HepG2 cells is less sensitive to damage caused by xenobiotics than intact hepatocytes [53,54].

Moreover, no mutations have been found in the PTEN gene of the HepG2 cell line, making it an apt model for testing genotoxicity and epigenetic changes that may occur as a result of FB_1 exposure [55]. The effect of FB_1 on HepG2 cell viability was conducted using a crystal violet assay in accordance with Feoktistova et al. [56] (Supplementary Figure S4.1). FB_1 reduced HepG2 cell viability in a dose-dependent manner (5, 50, 100, 200 µM). For subsequent assays, HepG2 cells were exposed to 5, 100, and 200 µM FB_1 as they represented 90%, 70%, and 50% cell viabilities, respectively. Results obtained for 5 and 100 µM can be found in the supplementary materials (Supplementary Figures S4.2–S4.7).

We evaluated the genotoxic potential of FB_1 by determining if FB_1 inflicted damage on DNA. Previously, we showed that at 200 µM FB_1 enhanced ROS production, resulting in oxidative stress [45]. Thus, in the present study we measured 8-OHdG levels as a marker of oxidative DNA damage. The low redox potential of guanine makes it the most vulnerable base and its product (8-OHdG) the best characterized oxidative lesion [57]. We found a significant 2.63-fold increase in 8-OHdG levels in the DNA of FB_1-exposed cells (Figure 4.1). The incorporation of 8-OHdG into DNA can generate double strand breaks, making this a harmful lesion [58]. Several other *in vivo* and *in vitro* studies observed DNA fragmentation as a consequence of FB_1 exposure, proving that FB_1 is genotoxic [19–21,42].

While the impact FB_1 has on DNA damage has been thoroughly researched, little is known on the impact it may have on DNA damage responses. Hence, we investigated the effect of FB_1 on the PTEN/PI3K/AKT/CHK1 axis and further determined if FB_1 effects the epigenetic regulation of PTEN. Currently, only a few studies have demonstrated the effects of FB_1 on epigenetic modifications in humans. Previously, Chuturgoon et al. (2014) screened for alterations in the miRNA expression profile of HepG2 cells exposed to 200 µM FB_1. miR-30c was one of the miRNAs shown to be dysregulated [35]. MiR-30c is an important regulator of hepatic liver metabolism, apoptosis, cell cycle transition, proliferation, and differentiation [59–61]. We found that the expression of miR-30c was significantly increased after exposure to 200 µM FB_1 (Figure 4.2a). Using an online computational prediction algorithm (TargetScan version 7.2), miR-30c was found to possibly target PTEN and KDM5B (Figure 4.2b). miRNAs silence their mRNA targets through mRNA cleavage or translational repression [62–64]. FB_1 reduced KDM5B transcript and protein levels in HepG2 cells (Figure 4.3a, b). While FB_1 reduced KDM5B mRNA levels by 9.86-fold, only a slight decrease in protein expression was observed. A previous study did find a minor increase in *KDM5B* transcript levels at 200 µM FB_1; however, these results were not statistically significant [35]. Further studies using miR-30c inhibitors and mimics need to be conducted to validate miR-30c regulation of KDM5B expression.

FB_1 can also induce epigenetic changes through the post-translational modifications of histones, but no study to date has investigated these changes in humans [65–67]. Here, we identified changes to H3K4 methylation. Although there was a slight decrease in KDM5B, we found a significant increase in global H3K4me3 (Figure 4.3c). H3K4me3 is predominantly found at transcriptional start sites, where it regulates the binding of transcription factors and activates gene transcription [68,69]. Thus, we

determined H3K4me3 levels at the *PTEN* promoter region using the ChIP assay; FB_1 significantly increased H3K4me3 at the *PTEN* promoter region (Figure 4.4a). These results correspond to the substantial elevation in *PTEN* transcript levels; however, the protein expression of PTEN was decreased (Figure 4.4b, c). PTEN may be post-transcriptionally regulated by miR-30c, as the decrease in PTEN protein expression corresponded to the increased miR-30c levels. Hence, miR-30c may act as a possible inhibitor of PTEN translation.

PTEN functions in regulating several cellular processes by antagonizing the PI3K/AKT signaling cascade [70]. Emerging evidence has revealed that PTEN is central in maintaining the DNA integrity by regulating DDR pathways via its interaction with CHK1 [27,28]. Additionally, PTEN regulates the activity of CHK1 via the PI3K/AKT axis [71–74]. Briefly, PTEN dephosphorylates the primary product of PI3K, phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 activates AKT via its phosphorylation at serine residue 473 [71]. Downregulation of PTEN permitted PI3K/AKT signaling to proceed undisturbed as PI3K and p-ser473-AKT expression was upregulated (Figure 4.5). FB_1 inhibits ceramide formation and promotes the formation of spingoid bases [75]. This may explain the activation of AKT by FB_1, as ceramide inhibits PI3K and promotes the dephosphorylation of AKT on serine 473 [76,77]. Furthermore, sphingosine-1-phosphate activates PI3K/AKT signaling by binding to G_I-coupled receptors [78].

AKT, in its activated form, inhibits CHK1 functioning by phosphorylating serine 280 of CHK1 [71,73,74]. Activated PI3K/AKT signaling impaired CHK1 function via increased p-ser-280-CHK1 after FB_1 exposure (Figure 4.6). During DDR, CHK1 arrests cells at the G1/S, S, and G2/M phases by phosphorylating the cdc25 family of phosphatases [79,80]. This allows for DNA repair to occur prior to determining cell fate. Although we did not analyze changes in cell cycle, previous studies have shown that FB_1 disrupts G1/S blockade; however, increased G2/M arrest was observed [81–83]. Nonetheless, the inhibitory phosphorylation of CHK1 coincided with DNA damage after FB_1 exposure in HepG2 cells, as cell cycle checkpoints were disrupted, inhibiting repair.

In addition to 200 µM FB_1, the effects of 5 and 100 µM FB_1 were investigated (Supplementary Figures S4.2–S4.7). While cells exposed to 5 and 200 µM FB_1 responded in a similar manner, the effect at 200 µM FB_1 was exacerbated. Additionally, we observed that 100 µM FB_1 generally had the opposite effect on 8-OHdG levels, H3K4 trimethylation on the PTEN promoter, and the expression of miR-30c, KDM5B, PTEN, PI3K, p-ser423-AKT, CHK1, and p-ser-280-CHK1 in HepG2 cells in comparison with the 5 and 200 µM FB_1. As with many toxins, this suggests that FB_1 is associated with a biphasic dose response [84].

Conclusions

This study further confirms the genotoxic potential of FB_1, and that the inhibition of DNA damage checkpoint regulation may allow cells to evade DNA repair. FB_1 epigenetically downregulates the

expression of PTEN via miR-30c. The downregulation of PTEN inhibits DNA damage checkpoint regulation via the PI3K/AKT signaling network, preventing the repair of oxidative DNA lesions induced by FB_1 (Figure 4.7). Needless to say, further investigation should be conducted using miRNA inhibitors and mimics, and on whether the outcome of FB_1-induced DNA damage and impaired DNA damage checkpoint regulation contributes to its cytotoxicity or carcinogenicity.

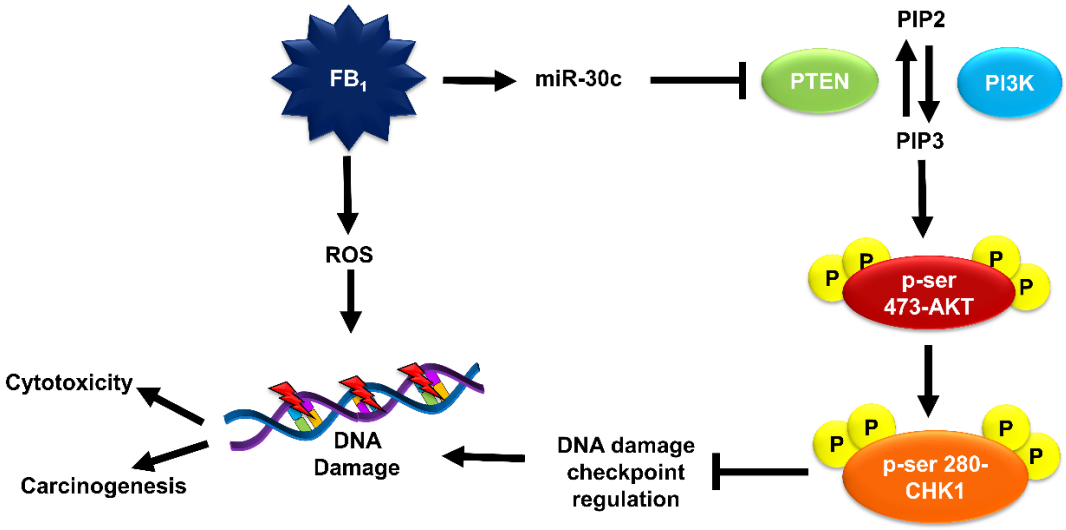

Figure 4.7. FB_1 induces oxidative DNA damage. It further impairs DNA damage checkpoint regulation pathways via the PTEN/PI3K/AKT/CHK1 axis by epigenetically regulating PTEN. FB_1 upregulates miR-30c, which inhibits PTEN translation, allowing for the phosphorylation of PIP2 to PIP3 by PI3K. This triggers the phosphorylation of AKT and subsequent phosphorylation of ser-280-CHK1, inhibiting CHK1 activity. Inhibition of CHK1 inhibits DNA damage checkpoint regulation. The resulted DNA damage may either contribute to FB_1-mediated cytotoxicity or carcinogenicity.

Ethics Approval: Approval was received from the University of Kwa-Zulu Natal's Biomedical Research Ethics Committee. Ethics number: BE322/19.

Author Contributions: T.A., T.G., and A.C. conceptualized and designed the study. T.A. conducted all laboratory experiments, analyzed the data, and wrote the manuscript. T.G. and A.C. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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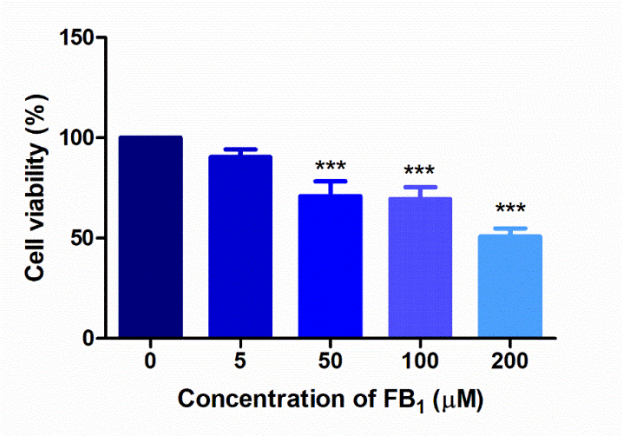
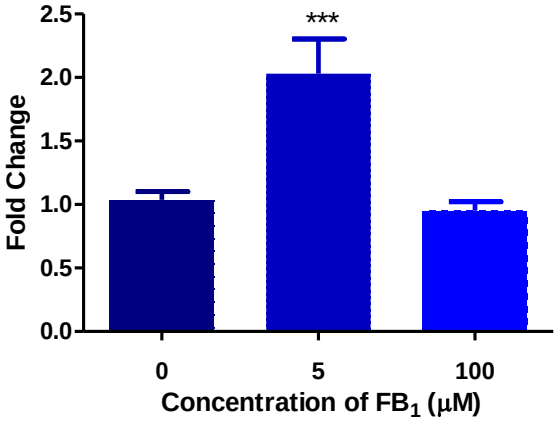
Conflicts of Interest: The authors declare no conflicts of interest.

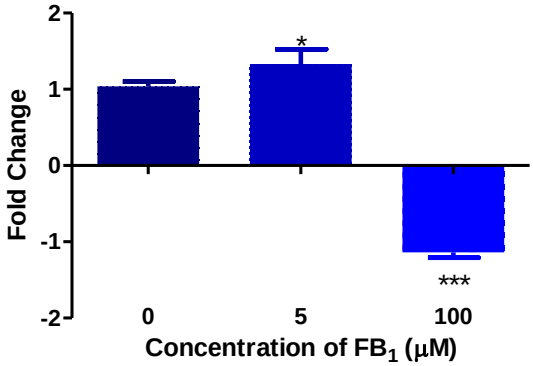
Supplementary Information



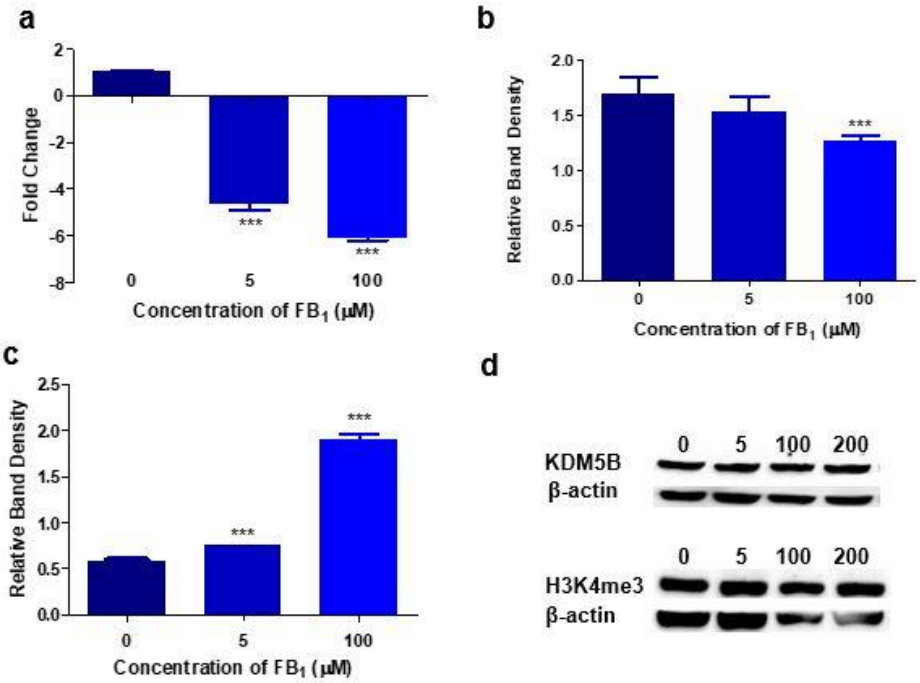
Supplementary Figure S4.1. The cytotoxic effects of FB_1 on HepG2 cells. HepG2 cells were treated with 0, 5, 50, 100 and 200 µM FB_1 for 24h. Cell viability was determined using the crystal violet assay and expressed as a percentage of the untreated control. Control viability was taken as 100%. FB_1 significantly altered the cell viability of HepG2 cells. Data is represented as mean percentage cell viability ± SD (n=3) (*** $p \leq 0.001$; one-way ANOVA with the Dunnet: compare all columns to control post-test).



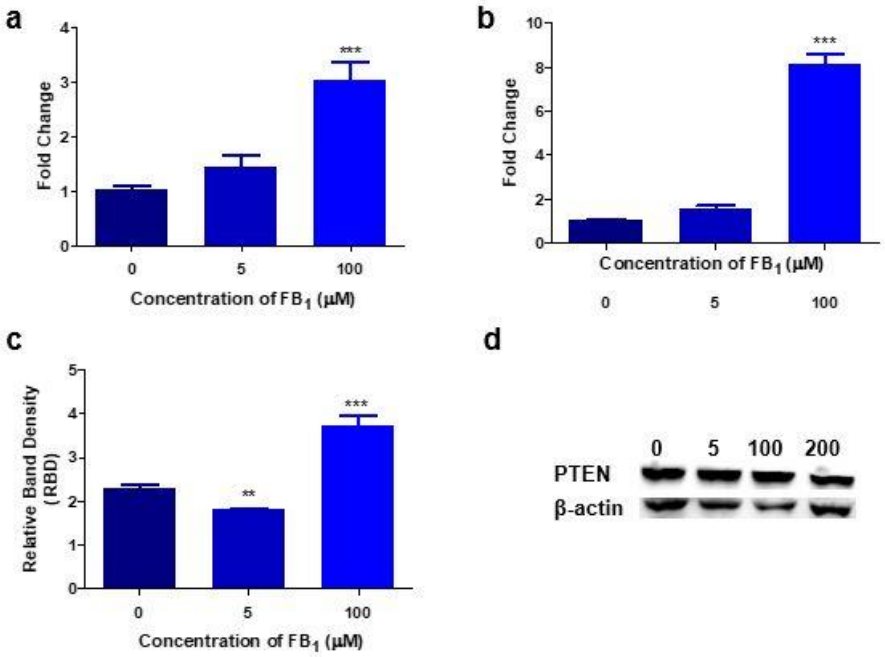
Supplementary Figure 4.2. FB_1 induced 8-OHdG levels in HepG2 cells. 8-OHdG levels were measured as a marker of oxidative DNA damage. FB_1 significantly altered 8-OHdG levels in HepG2 cells (***p = 0.0007). Data is represented as mean fold change ± SD (n=3) (*** $p \leq 0.001$; one-way ANOVA with the Dunnet: compare all columns to control post-test).



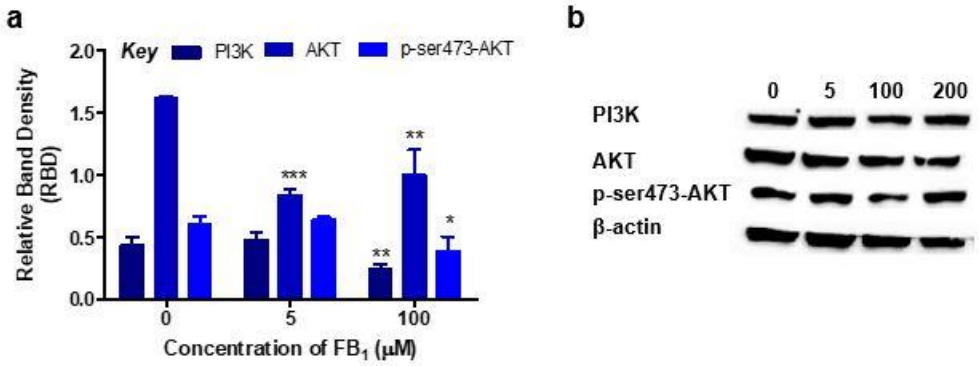
Supplementary Figure S4.3: FB_1 altered miR-30c expression in HepG2 cells. qPCR analysis of miR-30c showed that FB_1 significantly altered miR-30c expression (***$p < 0.0001$). Results are represented as mean fold-change ± SD (n=3) (*$p < 0.05$, ***$p < 0.0001$; one-way ANOVA with the Dunnet: compare all columns to control post-test).



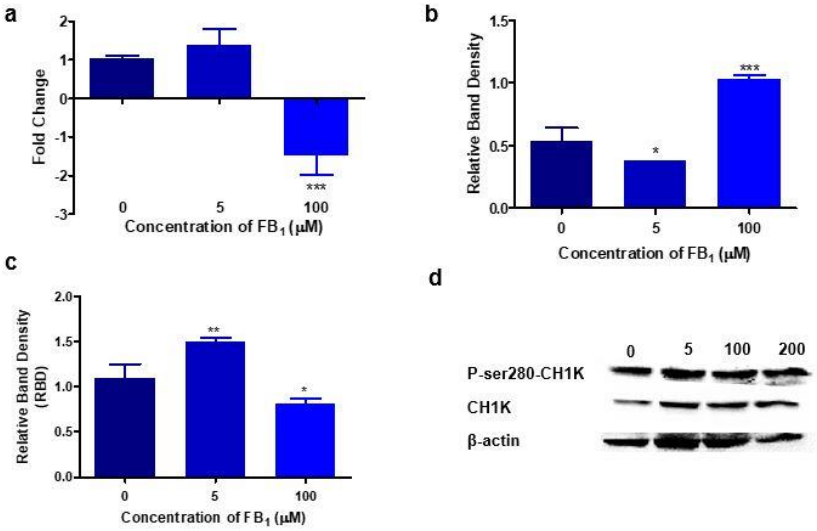
Supplementary Figure S4.4: The effect of FB_1 on KDM5B and H3K4me3 expression in HepG2 cells. FB_1 reduced both the transcript (**a**; ***$p < 0.0001$) and protein (**b**; *p= 0.0106) expression of KDM5B. There was a dose-dependent increase in total H3K4me3 (**c**; *** $p < 0.0001$). Western blot images of KDM5B and H3K4me3 (**d**). KDM5B and H3K4me3 expression was normalized against β-actin. Results are represented as mean fold-change ± SD (n=3) for gene expression and mean relative band density ± SD (n=3) for protein expression (***$p < 0.0001$; one-way ANOVA with the Dunnet: compare all columns to control post-test).



Supplementary Figure 4.5: FB_1 induced KDM5B and miR-30c modulates PTEN expression. PTEN expression is under the influence of both KDM5B and miR-30c. **(a)** Low levels of KDM5B allowed for the increased H3K4me3 at *PTEN* promoter regions (*** $p < 0.0001$). **(b)** This resulted in significantly higher levels of *PTEN* transcripts (*** $p < 0.0001$). **(c)** However, miR-30c inhibited PTEN translation/protein expression at 5 μM FB_1 but increased PTEN translation at 100 μM FB_1 (*** $p < 0.0001$). **(d)** Western blot images of PTEN. PTEN expression was normalized against β-actin. Results are represented as mean fold-change ± SD (n=3) for gene expression and mean relative band density ± SD (n=3) for protein expression (*$p < 0.05$, ***$p < 0.0001$; one-way ANOVA with the Dunnet: compare all columns to control post-test).



Supplementary Figure 4.6: The effect of FB_1 on the PI3K/AKT signalling cascade. (a) Western blotting was used to determine the effect of FB_1 on the PTEN/PI3K/AKT signalling network. FB_1 significantly altered PI3K (*** $p < 0.0001$), AKT (*** $p = 0.0004$) and p-ser473-AKT (*$p < 0.0174$) protein expression. **(b)** Western blot images of PI3K, AKT and pAKT. p-ser473-AKT expression was normalized against AKT and PI3K and AKT expression was normalized against β-actin. Data is represented as mean RBD ± SD (n=3), (* $p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$; one-way ANOVA with the Dunnet: compare all columns to control post-test).



Supplementary Figure 4.7: The influence of FB_1 on CHK1 expression in HepG2 cells. FB_1 significantly altered *CHK1* gene expression (**a**; ***$p = 0.0001$), CHK1 protein expression (**b**; *** $p < 0.0001$) and p-ser280-CHK1 (**c**; ***$p = 0.0006$). **(d)** Western blot images of CHK1 and p-ser280-CHK1. CHK1 expression was normalized against β-actin and p-ser280-CHK1 was normalized against CHK1. Gene expression is represented as fold changes ± SD relative to the control and protein expression is represented as mean RBD ± SD (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$; one-way ANOVA with the Dunnet: compare all columns to control post-test).

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CHAPTER 5

Fumonisin B_1 Alters Global m6A RNA Methylation and Epigenetically Regulates Keap1/Nrf2 Signaling in Human Hepatoma (HepG2) Cells

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Abstract

Fumonisin B_1 (FB_1) is a common contaminant of cereal grains that affects human and animal health. It has become increasingly evident that epigenetic changes are implicated in FB_1 toxicity. N6-methyladenosine (m6A) is the most abundant post-transcriptional RNA modification that is influenced by fluctuations in redox status. Since oxidative stress is a characteristic of FB_1 exposure, we determined if there is cross talk between oxidative stress and m6A in FB_1 exposed HepG2 cells. Briefly, HepG2 cells were treated with FB_1 (0, 5, 50, 100, 200 µM; 24h) and ROS, LDH and m6A levels were quantified. qPCR was used to determine expression of m6A modulators, *Nrf2*, *Keap1* and miR-27b while western blotting was used to quantify Keap1 and Nrf2 protein expression. Methylation status of *Keap1* and *Nrf2* promoters was assessed and RNA immunoprecipitation quantified m6a-*Keap1* and m6A-*Nrf2* levels. FB_1 induced an accumulation of intracellular ROS ($p \leq 0.001$) and LDH leakage ($p \leq 0.001$). Elevated m6A levels ($p \leq 0.05$) were accompanied by an increase in m6A "writers" [METLL3 ($p \leq 0.01$) and METLL14 ($p \leq 0.01$)], and "readers" [YTHDF1 ($p \leq 0.01$), YTHDF2 ($p \leq 0.01$), YTHDF3 ($p \leq 0.001$) and YTHDC2 ($p \leq 0.01$)] and a decrease in m6A "erasers" [ALKBH5 ($p \leq 0.001$) and FTO ($p \leq 0.001$)]. Hypermethylation and hypomethylation occurred at *Keap1* ($p \leq 0.001$) and *Nrf2* ($p \leq 0.001$) promoters, respectively. MiR-27b was reduced ($p \leq 0.001$); however, m6A-*Keap1* ($p \leq 0.05$) and m6A-*Nrf2* ($p \leq 0.01$) levels were upregulated. This resulted in the ultimate decrease in Keap1 ($p \leq 0.001$) and increase in Nrf2 ($p \leq 0.001$) expression. Our findings reveal that m6A RNA methylation can be modified by exposure to FB_1, and a cross talk between m6A and redox regulators does occur.

Keywords

Fumonisin B_1, epigenetics, m6A RNA Methylation, Oxidative Stress, Keap1, Nrf2

Introduction

As one of the most toxic mycotoxins produced by the *Fusarium* fungal species, fumonisin B_1 (FB_1, $C_{34}H_{59}NO_{15}$) is a highly problematic agricultural contaminant in developing countries (Idahor, 2010, Kamle et al., 2019). Not only does it affect food quality in regions that have already inadequate food supplies but it also impinges on human and animal health. FB_1 has been conjectured to be a major factor is hepato-, nephro- and neuro-toxicity (Domijan, 2012, Müller et al., 2012, Singh and Kang, 2017, Szabó et al., 2018). It has been implicated in carcinogenesis of the liver and kidney in animals and may play a role in esophageal carcinogenesis in humans (Gelderblom et al., 2001, Alizadeh et al., 2012, Müller et al., 2012). While it is universally acknowledged that inhibition of sphingolipid metabolism is the major mechanism of FB_1 toxicity (Riley and Merrill, 2019), mounting evidence suggests that changes to the epigenetic landscape may also be critically involved in its toxicity. Although changes in DNA methylation, microRNA (miRNA) profiles and histone modifications have already been linked to FB_1-induced toxicity (Mobio et al., 2000, Kouadio et al., 2007, Chuturgoon et al., 2014a, Chuturgoon

et al., 2014b, Demirel et al., 2015, Arumugam et al., 2020); the link between RNA methylation and FB_1-induced hepatotoxicity remains uncharted territory.

RNA methylation accounts for over 60% of all RNA modifications and has been identified on all four ribonucleic acid bases (Cantara et al., 2010, Roundtree et al., 2017). However, methylation to the sixth nitrogen of adenosine, known as N6-methyladenosine (m6A), is the most prevalent modification that occurs on mammalian messenger RNA (mRNA) and long non-coding RNA (lncRNA) (Desrosiers et al., 1974, Pan, 2013). It functions in various biological processes by controlling the fate of m6A modified-RNA through splicing, export, translation, and degradation (Zaccara et al., 2019). Transcriptome-wide analysis revealed that m6A sites are preferentially distributed within long exons, in 3' untranslated regions (3'UTR) and adjacent to stop codons of mRNA and non-coding RNAs in various eukaryotes and some nuclear replicating viruses (Dominissini et al., 2012, Meyer et al., 2012, Yue et al., 2015, Kennedy et al., 2016).

M6A "writers", "erasers" and "readers" are responsible for this dynamic and reversable modification (Zaccara et al., 2019). M6A sites are methylated by "writers" [which include methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14) and Wilm's tumour 1-associated protein (WTAP)] (Schwartz et al., 2014, Wang et al., 2016) whereas "erasers" [such as ALKB homolog 5 (ALKBH5) and fat mass and obesity-associated protein (FTO)] are responsible for its demethylation (Jia et al., 2011, Zheng et al., 2013). Furthermore, m6A-modified transcripts are specifically recognized by "readers" namely, the YT521-B homology domain containing proteins 1 and 2 (YTHDC1 and YTHDC2) and the YT521-B homology domain family proteins 1, 2, and 3 (YTHDF1, YTHDF2, and YTHDF3) which bind to m6A within the consensus DRACH (where D = A/G/U, R= A/G, H=A/C/U) sequence to regulate the expression and function of specific mRNAs and proteins (Dominissini et al., 2012, Zaccara et al., 2019).

Aberrant m6A patterns contribute to defective physiological processes, unusual immune responses, abnormal metabolism, neurodegeneration and have been implicated in hepatic diseases, rheumatoid arthritis, osteoporosis, type 2 diabetes mellitus, obesity, neurodegenerative complications, infectious diseases and various cancers (Shen et al., 2015, Lan et al., 2019, Xu et al., 2019, Han et al., 2020, Paramasivam et al., 2020). Of particular interest, studies have suggested that oxidative stress may be prevalent in altering m6A methylation levels and that m6A modifications may in turn affect oxidative stress through changes in the expression of redox regulating mRNA (Li et al., 2017, Zhao et al., 2019, Wu et al., 2020, Zhao et al., 2020a).

We previously found that FB_1 enhanced ROS production which led to liver cell injury. We further observed activation of Kelch-like ECH associated protein 1 (Keap1)/ nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) antioxidant signalling to counter the oxidative effects of FB_1 (Arumugam et al., 2019). Under physiological conditions, Keap1 maintains Nrf2 in an inhibitory state through

ubiquitination, tagging it for proteasomal degradation. Changes in redox status triggers Nrf2 release allowing it to translocate to the nucleus where it promotes the transcription of anti-oxidants and other detoxifying enzymes (Kobayashi et al., 2006). However, whether FB_1-mediated oxidative stress affects m6A levels and if m6A modifications are a potential factor contributing to FB_1-mediated oxidative stress is unknown. Thus, the aim of this study was to investigate the effects of FB_1 on m6A RNA methylation and its crosstalk with oxidative stress responses in human hepatoma (HepG2) cells. We further examined FB_1-mediated alterations in the epigenetic regulation of Keap1/Nrf2 expression by evaluating changes in promoter methylation, m6A-*Nrf2*, m6A-*Keap1* and miRNA levels.

Method and Materials

Materials

The HepG2 cell line (HB-8065) was obtained from the American Type Culture Collection (ATCC) and cell culture consumables were purchased from Whitehead Scientific (Johannesburg, South Africa). MiR-27b-3p mimic (MSY0000419), miR-27b-3p inhibitor (MIN0000419), and attractene transfection reagent (301005) were purchased from Qiagen (Hilden, Germany). Western blot reagents were purchased from Bio-Rad (Hercules, CA, USA) while primary antibodies: anti-Nrf2 (#12721S), anti-Keap1 (#8047S); horse-radish peroxidase (HRP)-conjugated secondary antibody: goat anti-rabbit (#7074S) were obtained from Cell Signalling Technologies (Danvers, MA, USA) and β-actin was obtained from Sigma-Aldrich (A3854; St. Louis, MO, USA). All other reagents were purchased from Merck (Boston, MA, USA), unless otherwise stated.

Cell Culture

HepG2 cells (1.5 X 10^6, passage 3) were seeded in 25 cm^3 polystyrene tissue culture flasks containing Eagle's Minimum Essentials Medium (EMEM) supplemented with 10% heat-inactivated foetal calf serum, 1% penicillin-streptomycin-fungizone, and 1% L-glutamine and maintained in a 5% carbon dioxide (CO_2) atmosphere at 37°C. At 80% confluency, cells were exposed to various concentrations of FB_1 (5, 50, 100 and 200 µM) for 24 hours (h) (Arumugam et al., 2020). An untreated control (containing supplemented EMEM) was prepared along with FB_1 treatments. All experiments were repeated in two independent experiments and triplicate for reproducibility of results.

Detection of Intracellular Reactive Oxygen Species

Intracellular ROS was quantified using the 2,7-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) assay, as previously described (Arumugam et al., 2019).

Measurement of Lactic Acid Dehydrogenase Leakage

Membrane damage to HepG2 cells were assessed through the measurement of lactic acid dehydrogenase (LDH) leakage. Medium collected from control and FB_1 treated cells were centrifuged (400xg, 24°C, 10 min) and dispensed (100 µl/well) in triplicate into a 96-well microtiter plate. An equal

volume of LDH reagent (11644793001, Sigma Aldrich, St. Louis, MO, USA) was added to each well. The plate was incubated for 30 min at room temperature (RT) in the dark. Absorbance was read with a spectrophotometer (Bio-Tek µQuant, Winooski, VT, USA) at 500 nM. Results are represented as relative fold change.

Transfection of HepG2 cells with MiR-27b Mimic and MiR-27b Inhibitor

MiR-27b is an oxidative stress responsive miRNA that targets Nrf2. To assess the effects of miR-27b on Nrf2 mRNA and protein expression, cells were transfected with the mimic (Syn-hsa-miR-27b, MYS0000419, Qiagen, Hilden, Germany) and inhibitor (Anti-hsa-miR-27b-3p, MIN0000419, Qiagen, Hilden, Germany) to miR-27b. HepG2 cells were seeded in 25 cm^3 polystyrene tissue culture flasks until 80% confluent. Lyophilised miRNA mimic and inhibitor (5 nmol) was reconstituted to 20 µM in nuclease-free water. For the transfection, miR-27b mimic or inhibitor (15 µl) was added to EMEM (72 µl) and attractene (3 µl) in microcentrifuge tubes. Samples were then incubated for 15 min at RT to allow complex formation. Cells were rinsed with PBS and supplemented EMEM (2,940 µl) was added to the flasks. The transfection complex was dispensed in a drop-wise fashion into the appropriate flask with gentle swirling to ensure uniform distribution. All treatments were then incubated for 24 h (37°C, 5% CO_2) and utilised for RNA and protein isolation.

RNA Isolation

RNA extraction from HepG2 cells was carried out using Qiazol reagent (79306, Qiagen, Hilden, Germany). Once treatments were removed, HepG2 cells were rinsed thrice with PBS (0.1M) and incubated with Qiazol and 0.1M PBS for 5 min. Cells were lysed with the cell scraper, and lysates were incubated (-80°C, overnight). Thereafter, chloroform (100 µl) was dispensed into thawed samples and centrifuged (12,000xg, 4°C, 15 min). Supernatants were transferred to sterile microcentrifuge tubes and incubated with 500 µl isopropanol (-80°C, overnight). Subsequently, samples were centrifuged (12,000xg, 4°C, 20 min), supernatants were discarded and residual salts from the RNA-containing pellets were removed with 75% ice-cold ethanol and thereafter centrifuged (7,400xg, 4°C, 15 min). RNA pellets were air-dried (30 min, RT) and resuspended in nuclease-free water (10 µl). RNA concentration and purity were assessed using the Nanodrop2000 spectrophotometer (Thermo Scientific, Waltham, USA). RNA with a 260:280 absorbance ratio between 1.8 and 2 was used for subsequent assays and concentration was adjusted accordingly.

Quantification of Global m6A RNA Methylation

Global m6A RNA methylation was determined using the m6A RNA methylation quantification kit (ab185912, Abcam, Cambridge, UK). Briefly, total RNA, together with m6A standards (0 – 0.1 ng/µl) were bound to strip wells using a high-affinity RNA binding solution. Thereafter, m6A levels were detected using an m6A capture and detection antibody. The detected signal was enhanced, and the absorbance was measured at 450 nM using a spectrophotometer (Bio-Tek µQuant, Winooski, VT,

USA). The mean absorbance of the standards was used to construct a standard curve from which the percentage m6A in each sample was determined. Results are presented as relative fold change.

Quantitative Polymerase Chain Reaction

qPCR was used to compare the changes in the expression of *METLL3, METLL14, FTO, WTAP, YTHDF1, YTHDF2, YTHDF3, YTHDC2, Nrf2, Keap1* and miR-27b. For mRNA expression, cDNA was prepared from RNA (1000 ng/µl) using the Maxima H Minus First Strand cDNA Synthesis Kit according to manufacturers' protocol. qPCR was performed using the PowerUp™ SYBR™ Green Master Mix (A25742, Thermo-Fisher Scientific, Waltham, MA, USA) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the following cycling conditions: initial denaturation (95°C, 8 min), followed by 40 cycles of denaturation (95°C, 15 s), annealing (Supplementary Table S5.1, 40 s), and extension (72°C, 30 s). Primer sequences and annealing temperatures are listed in Supplementary Table S5.1.

For miRNA expression, cDNA synthesis was performed with 1000 ng/µl RNA, using the miScript II RT Kit (218161, Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-qPCR was performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the miScript SYBR Green PCR Kit (218073, Qiagen, Hilden, Germany) and miR-27b miScript primer assay (MS00009247, Qiagen, Hilden, Germany) according to the manufacturer's protocol with the following cycling conditions: initial denaturation (95°C, 15 min), followed by 40 cycles of denaturation (94°C, 15 s), annealing (55°C, 30 s), and extension (70°C, 30 s).

GAPDH and *RNU6* were used as endogenous controls for mRNA and miRNA expression, respectively and relative expression was calculated using the comparative threshold cycle ($2^{\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001).

RNA Immunoprecipitation

Quantification of m6A-*Nrf2* and m6A-*Keap1* levels were determined using RNA immunoprecipitation. Briefly, RNA (1000 ng/µl) were incubated with m6A-primary antibody (1:100; ab208577, Abcam, Cambridge, UK) overnight at 4°C. Thereafter, the RNA-antibody complex was precipitated using protein A beads [20 µl 50% bead slurry (Cell Signalling Technology, #9863), 4°C, 3 h]. Samples were centrifuged (2,500xg, 4°C, 60s), washed twice in RNA immunoprecipitation buffer (150 mM KCl, 25 mM Tris-Cl (pH 7.4), 5mM EDTA, 0.5mM DTT, 0.5% IGEPAL, 100 U/ml SUPERase IN RNase Inhibitor (Thermo-Fisher Scientific, AM2694), protease and phosphatase inhibitors (A32961, Thermo-Fisher Scientific)], washed once in nuclease free water and resuspended in nuclease free water (10 µl). Immunoprecipitated RNA was standardised to 200 ng/µl, and reverse transcribed into cDNA as described above. The expression of m6A-*Nrf2* and m6A-*Keap1* was then determined using qPCR as mentioned above. Primer sequences and annealing temperatures are listed in Supplementary Table S5.1.

DNA Isolation and Promoter Methylation Analysis

Genomic DNA was isolated from HepG2 cells as previously described (Ghazi et al., 2020b). Isolated DNA was standardized to 4 ng/µl and used to determine methylation status at *Nrf2* and *Keap1* promoter regions. This was done using the OneStep qMethyl Kit (5310, Zymo Research, 5310) as per manufacturer's instructions. Primer sequences and annealing temperatures are listed in Supplementary Table 1. Cycling conditions were as follows: digestion by methyl sensitive restriction enzymes (37°C, 2 h), initial denaturation (95°C, 10 min), followed by 45 cycles of denaturation (95°C, 30s), annealing (Supplementary Table S5.1, 60s), extension (72°C, 60s), final extension (72°C, 60s), and a hold at 4°C. Results are represented as a fold-change relative to the control.

Protein Isolation and Western Blotting

The western blotting technique was used to determine protein expression of Nrf2 and Keap1. Protein was isolated and quantified as previously described (Arumugam et al., 2019). The standardized protein extracts (1 mg/ml) were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes which were then blocked in 5% non-fat dry milk (1 h) before incubation with the primary antibodies, anti-Nrf2 (1:1000; #12721S, Cell Signalling Technologies, Danvers, MA, USA) and anti-Keap1 (1:1000; #8047S, Cell Signalling Technologies, Danvers, MA, USA) overnight at 4°C. Membranes were washed thrice in Tween 20-Tris buffer saline (TTBS: 150 mmol/l NaCl, 3 mmol/l KCl, 25 mmol/l Tris, 0.05% Tween 20, dH2O, pH 7.5) and thereafter incubated with horse-radish peroxidase-conjugated goat anti-rabbit (1:5000; #7074S, Cell Signalling Technologies, Danvers, MA, USA) secondary antibody for 2 hours. Thereafter, membranes were washed thrice with TTBS and protein expression was visualised using the Clarity Western ECL Substrate Kit (1705060, Bio-Rad, Hercules, CA, USA) with the Chemidoc gel documentation system (Bio-Rad, Hercules, CA, USA). β-actin served as a housekeeping control and protein expression was determined using the Image Lab Software version 5.0 (Bio-Rad, Hercules, CA, USA) which measured band densities of expressed proteins. Protein expression is represented as relative band density and calculated by normalising the protein of interest against β-actin.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as the mean ± standard deviation and analysis of variance (ANOVA) with Dunnet's post-test was used to determine the statistical differences among the groups. A p value of less than 0.05 was considered statistically significant.

Results

FB_1 Enhanced ROS Production and Cell Membrane Damage

The effect of FB_1 on ROS generation was evaluated using the H_2DCF assay. FB_1 altered the redox status of HepG2 cells by inducing a significant dose-dependent increase in ROS levels (p= 0.0005; Figure. 5.1a). Excessive production of ROS leads to cellular injury and hepatoxicity. Upon damage to cellular membranes, cells release the enzyme LDH. As depicted in Figure 5.1b, exposure to FB_1 for 24 h promoted LDH leakage in a significant dose-dependent manner ($p < 0.0001$) indicating severe cell damage occurred.

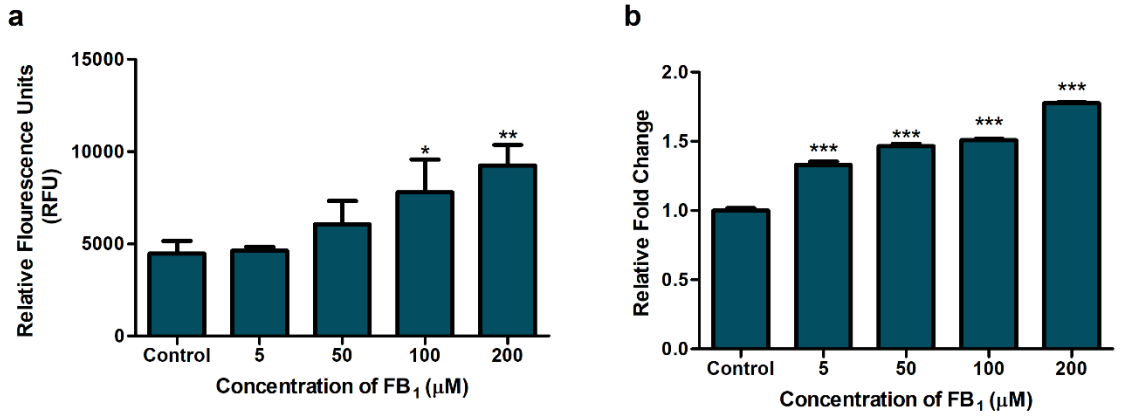

Figure 5.1. FB_1-induced hepatotoxicity in HepG2 cells. HepG2 cells were cultured with varying concentrations of FB_1 for 24 h. Intracellular ROS generation was examined by an oxidation sensitive fluorescent probe and ROS generation was significantly accelerated upon FB_1 exposure (a; *** $p \leq 0.001$). LDH leakage was used as an indicator of hepatic injury and was found to be significantly increased at all FB_1 concentrations tested (b; *** $p \leq 0.001$).

FB_1 Altered Global M6A Levels and Expression of M6A Regulatory Elements

To determine whether FB_1-prompted oxidative stress has the potential to induce aberrant m6A modifications, levels of total m6A-modified RNA in FB_1-treated HepG2 cells were detected. In Figure 5.2a, the m6A levels of the FB_1-treated groups increased, but only cells treated with 200 µM showed significant changes in m6A compared to the control (p= 0,0132).

M6A modifications are regulated by methyltransferases and demethylases; therefore, we set out to determine if changes in the expression of m6A-modifing enzymes were responsible for the changes in m6A levels observed in FB_1-exposed cells. There was a significant concentration-dependant increase in the mRNA levels of m6A methyltransferase (Figure 5.2b) *METTL3* (p = 0,0017), while METTL14 was reduced at 5 µM FB_1 and upregulated at the higher (50-200 µM) concentration of FB_1 tested (p = 0,0043). Conversely, a significant dose-dependent decrease in the m6A demethylases (Figure 5.2c), *FTO* ($p < 0.0001$) and *ALKBH5* ($p < 0.0001$) were observed in the presence of all FB_1 treatments.

Specific m6A readers recognize m6A-modified RNA and regulate gene expression through various mechanisms. Thus, we determined if FB_1 had any effects on the expression of the m6A readers (Figure 5.2d); and found that FB_1 significantly increased the expression of *YTHDF1* (p = 0,0038), *YTHDF3* (p = 0,0005) and *YTHDC2* (p = 0,0064) in HepG2 cells in comparison to the untreated cells. *YTHDF2* expression was reduced at 5 µM FB_1 and elevated at the higher (50-200 µM) concentration of FB_1 tested (p = 0,0021).

Taken together, the data suggests that FB_1-induced oxidative stress increased m6A methylation, possibly, through mediating dysregulation of m6A regulatory genes.

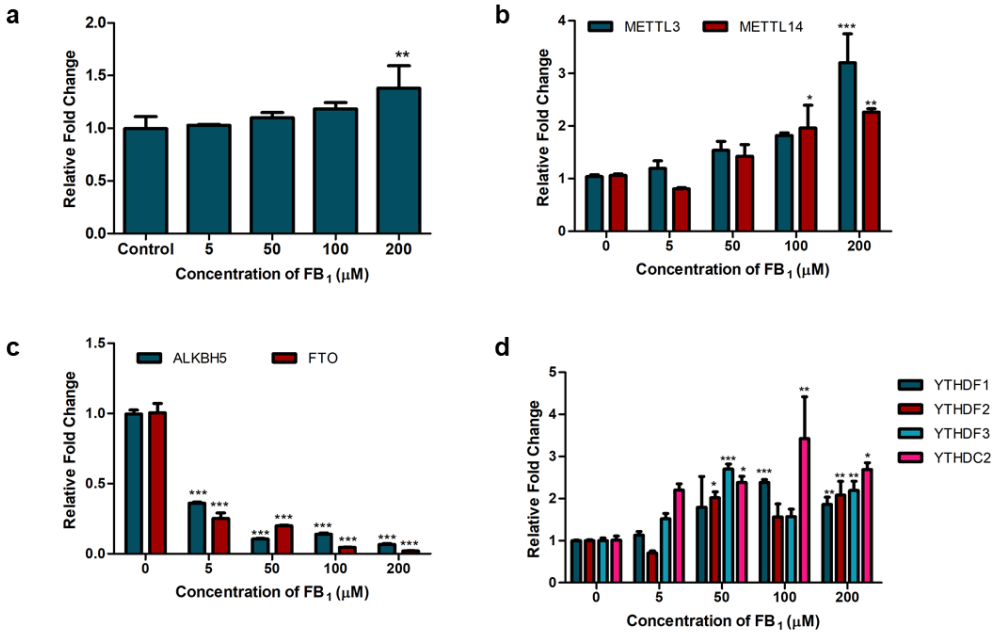

Figure 5.2. Aberrant m6A modifications induced by FB_1 in HepG2 cells. FB_1 increased global m6A RNA modifications (a; *p ≤ 0.05) and induced changes in m6A writers [b: *METLL3* (** p ≤ 0.01) and *METLL14* (** p ≤ 0.01)], erasers [c: *ALKBH5* (*** p ≤ 0.001) and *FTO* (*** p ≤ 0.001)] and readers [d: *YTHDF1* (** p ≤ 0.01), *YTHDF2* (** p ≤ 0.01), *YTHDF3* (*** p ≤ 0.001) and *YTHDC2* (** p ≤ 0.01)].

FB_1 Epigenetically Regulates Keap1 Expression

In response to xenobiotic stress, cells activate the Keap1/Nrf2 pathway. Inactivation of Keap1 is required for Nrf2-mediated activation of the antioxidant response to oxidative stress (Kobayashi et al., 2006). Furthermore, it was recently observed that m6A modifications may also regulate Keap1/Nrf2 expression (Wang et al., 2019, Zhao et al., 2020a). Thus, we evaluated the epigenetic regulation of Keap1 through both post-transcriptional (RNA methylation) and transcriptional (DNA methylation) mechanisms.

FB_1 has previously been shown to induce changes in the methylation status of promoter regions in genes (Demirel et al., 2015). We observed significant dose-dependent hypermethylation of CpG islands at the *Keap1* ($p < 0.0001$; Figure 5.3a), this led to a corresponding significant decrease in *Keap1* mRNA expression ($p< 0.0001$; Figure 5.3b).

Since FB_1 altered global m6A RNA levels, we employed the m6A site predictor SRAMP to identify m6A sites on *Keap1* mRNA (Zhou et al., 2016). The results showed 29 possible m6A sites including 7 possible m6A sites with high confidence and 1 with very high confidence. Figure 5.3c represents the m6A consensus sequence motif of *Keap1* (AGACU or GGACU) depicted as sequence logo obtained by the WebLogo 3 server (weblogo.threeplusone.com/create.cgi). The height of each stack indicates the degree of conservation (bits). The height of the letters represents the relative frequency of the base.

Changes in m6A-*Keap1* levels were then evaluated via RNA immunoprecipitation. In Figure 5.3d, exposure to varying concentrations of FB_1 lead to a significant dose-dependent increase in m6A-*Keap1* levels (p= 0,0125). Furthermore, we assessed changes in Keap1 protein expression and found it to be dose-dependently reduced by FB_1 ($p < 0.0001$; Figure 5.3e).

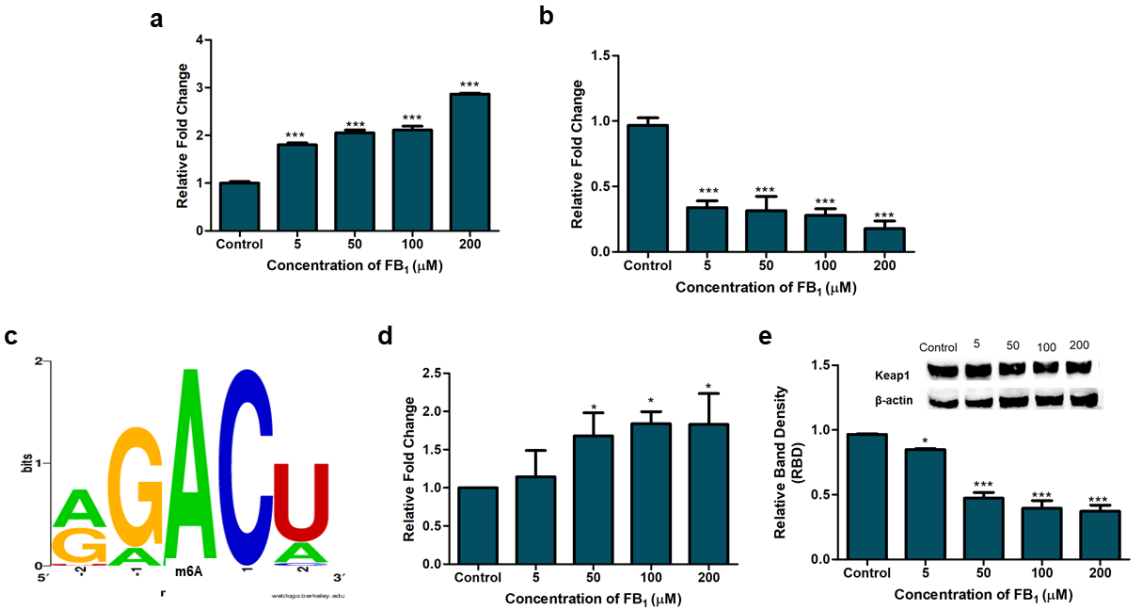

Figure 5.3. The epigenetic effects of FB_1 on Keap1 expression in HepG2 cells**.** FB_1 induced hypermethylation at *Keap1* promoters (a; *** $p \leq 0.001$) resulting in reduced *Keap1* gene expression (b; *** $p \leq 0.001$). A consensus sequence for possible m6A modifications on *Keap1* transcripts was constructed (c). RNA immunoprecipitation with m6A antibodies revealed that FB_1 upregulated m6A-*Keap1* (d; * $p \leq 0.05$) while western blotting found downregulation in Keap1 protein expression (e; *** $p \leq 0.001$).

FB_1 Promoted Nrf2 Expression Through Epigenetic Modifications

DNA methylation, miR-27b and m6A-modifications are just a few of the epigenetic factors that play a role in Nrf2 regulation, thus it was evaluated accordingly (Kang et al., 2014, Xu et al., 2017, Zhao et al., 2020a).

First, methylation status of *Nrf2* promoters was evaluated in control and FB_1 treated HepG2 cells. FB_1 induced a significant dose-dependent hypomethylation of *Nrf2* promoters (Figure 5.4a; $p < 0.0001$). Next, posttranscriptional regulation of Nrf2 was determined. MiR-27b was previously shown to directly target Nrf2 (Xu et al., 2017). This was further confirmed using the bioinformatics prediction algorithm software, TargetScan (version 7.1), where miR-27b was found to have complementary base pairs with *Nrf2* at positions 62-68 in humans (Agarwal et al., 2015). Thus, miR-27b expression was determined in FB_1 treated HepG2 cells using qPCR. The HepG2 cells were also treated with a miR-27b mimic and inhibitor which acted as a positive and negative control, respectively. Here, miR-27b levels were diminished at all concentrations of FB_1 tested (Fig. 4b; $p < 0.0001$). The expression of miR-27b in HepG2 cells treated with the mimic and inhibitor were increased and decreased, respectively (Figure 5.4b; $p < 0.0001$).

Nrf2 gene expression was also determined by qPCR. FB_1 increased *Nrf2* expression in HepG2 cells. Treatment of HepG2 cells with the miR-27b mimic and inhibitor resulted in a decrease and increase, respectively in Nrf2 levels (Figure 5.4c).

SRAMP was also used to predict m6A sites on *Nrf2* transcripts. A total of 54 m6A sites were predicated with 15 high confidence and 2 very high confidence sites. Figure 5.4d represents the consensus motif of m6A modification on *Nrf2* which is GGACU. We further tested m6A-*Nrf2* levels and found that like Keap1, FB_1 significantly upregulated m6A-*Nrf2* levels ($p = 0{,}0018$; Fig. 4e). Moreover, western blotting analysis revealed that FB_1 significantly increased Nrf2 protein expression in a dose-dependent manner ($p < 0.0001$; Figure 5.4f). Treatment of HepG2 cells with the miR-27b mimic and inhibitor resulted in a decrease and increase, respectively in Nrf2 protein levels (Figure 5.4f); further validating that Nrf2 is a target of miR-27b.

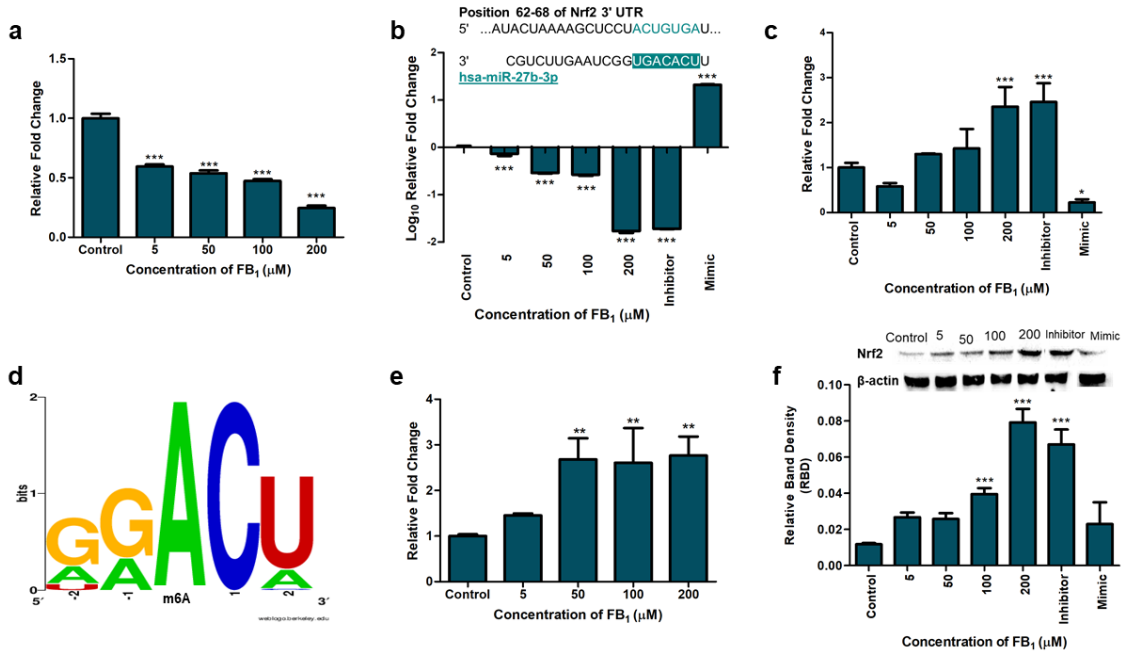

Figure 5.4. FB_1 epigenetically regulates Nrf2 expression in HepG2 cells. FB_1 induced hypomethylation at *Nrf2* promoter regions (a; *** $p \leq 0.001$) and reduced miR-27b (b; *** $p \leq 0.001$) expression; which led to the subsequent increase in *Nrf2* mRNA levels (c; *** $p \leq 0.001$). A consensus sequence for possible m6A modifications on *Nrf2* transcripts was constructed (d). m6A-*Nrf2* (e; $p \leq 0.01$) and Nrf2 protein expression (f; *** $p \leq 0.001$) were significantly increased.

Discussion

FB_1 is a well-known hepatotoxin and hepatocarcinogen (Gelderblom et al., 2001, Singh and Kang, 2017). It induces its toxicity via the disruption of sphingolipid metabolism, resulting in oxidative stress, endoplasmic reticulum stress and autophagy (Liu et al., 2019). However, epigenetic changes also play a critical role in its toxicity and carcinogenicity. For instance, miR-27b is an important regulator of cholesterol and lipid metabolism, and prevents the bioactivation of procarcinogens via the suppression of cytochrome 1b1 (Tsuchiya et al., 2006, Vickers et al., 2013). However, the downregulation of miR-27b by FB_1 and concurrent increase in cytochrome 1b1 facilitates neoplastic transformation observed in FB_1 exposed liver cells (Chuturgoon et al., 2014b). Furthermore, FB_1 specifically methylates CpG islands found on the promoters of tumour suppressor genes and induces global hypomethylation which are both common hallmarks of cancer (Chuturgoon et al., 2014a, Demirel et al., 2015). More recently, FB_1 prompted changes in miRNA-30c and histone methylation which led to the loss the tumour suppressor, phosphatase and tensin homolog (PTEN) and diminished response and repair of oxidative DNA lesions (Arumugam et al., 2020). While alterations in DNA methylation, histone modifications and miRNA profiles have been shown to play a part in FB_1-mediated hepatopathologies, little has been uncovered about the potential role of RNA methylation in these pathologies.

With more than 100 identified RNA modifications, m6A remains the most prevalent epitranscriptomic marker (Cantara et al., 2010). Changes in redox homeostasis have been shown to affect m6A levels and m6A modifications in turn may affect oxidative stress through regulating redox-associated genes (Li et al., 2017, Zhao et al., 2019, Wu et al., 2020, Zhao et al., 2020a). Therefore, in this study, we explored the effects of m6A modifications to further analyse the mechanisms by which FB_1 induces its toxicity. We evaluated changes in ROS, global m6A RNA levels and expression of m6A regulatory genes in HepG2 cells exposed to varying concentrations of FB_1 for 24 h. We further examined the epigenetic regulation of Keap1/Nrf2 signalling by assessing changes in promoter methylation, m6A-*Nrf2*, m6A-*Keap1* and miR-27b levels.

In order to characterize oxidative stress induced by FB_1, intracellular ROS production was quantified using the fluorometric H_2DCF assay. As presented in Figure 5.1a, exposure to FB_1 for 24 h enhanced intracellular ROS levels in a dose-dependent manner. Excessive levels of ROS inflict cellular injury. We previously showed that FB_1 (200µM, 24h) accelerated the production of ROS inducing severe damage to lipids and proteins, contributing to its toxicity in HepG2 cells (Arumugam et al., 2019). Here, we found that FB_1-induced ROS inflicted severe cellular damage as LDH leakage was significantly increased at all FB_1 concentrations tested (Figure 5.1b). Taken together these results confirm that FB_1 induces hepatotoxicity through an accumulation of intracellular ROS.

Environmental stimuli including heat shock and ultra-violet radiation have been shown to alter m6A patterns in HepG2 cells (Dominissini et al., 2012). To determine whether FB_1 may have an impact on m6A patterns, we first determined whether FB_1 altered global m6A levels. Analysis of total RNA revealed that m6A levels were elevated in a dose-dependent manner by FB_1; however, they were only significantly elevated at the highest concentration of FB_1 tested (200 µM; Figure 5.2a). Previous reports have indicated that other *Fusarium* toxins that naturally co-occur with FB_1 can alter m6A methylation patterns. Deoxynivalenol (DON) differentially regulated genes related to the tumour necrosis factor alpha inflammatory pathway through aberrant m6A patterns (Zhengchang et al., 2020), while fusaric acid reduced p53 expression through the reduction of m6A-*p53* levels (Ghazi et al., 2020a). Of particular interest, Wu et al. (2020) demonstrated that ROS-mediated increases in m6A RNA methylation may be a potential mechanism of aflatoxin B_1-induced hepatotoxicity. Although the observed trends were different in these studies, the results suggest that m6A modifications are involved in the toxic effects of these mycotoxins. In addition, m6A modifications promote hepatic growth and aberrant m6A RNA levels in liver have been associated with liver pathologies such as hepatocellular carcinogenesis, viral hepatitis and non-alcoholic fatty liver disease. Therefore, we speculate that increases in m6A modification may be related to the toxic nature of FB_1 in the liver.

Ideally, increased expression of m6A methyltransferases and reduced expression of m6A demethylases should result in the elevated m6A levels that were observed. Thus, we determined if FB_1 altered the expression of m6A regulatory genes. M6A marks are installed by the methyltransferase complex

consisting of the catalytic unit METLL3 and structural components METTL14 and WTAP. FB_1 dose-dependently increased the expression of *METTL3* and *METTL14* (Figure 5.2b); however, like global m6A levels, results were only significant at the higher FB_1 concentrations tested. M6A marks are removed by the demethylases: FTO and ALKBH5. Exposure to FB_1 resulted in the drastic decrease in m6A-demethylases at all concentrations tested (Figure 5.2c). The extremely low levels of FTO may also contribute to the toxic nature of FB_1 as *FTO* knock down was shown to contribute to chromosomal instability and cell cycle arrest (Huang et al., 2019). The results suggest that together m6A writers and erasers are involved in regulating global m6A levels; however, METLL3 may play a more prominent role as its expression pattern closely matched total m6A levels induced by FB_1.The expression of m6A readers were also determined as they recognize and govern the fate of m6A modified transcripts. For instance, YTHDF1, YTHDF3 and YTHDC2 promote the translation of m6A marked transcripts; while YTHDF2 accelerates the degradation of m6A-modified transcripts. FB_1 increased the mRNA levels of m6A "readers" in HepG2 cells; however, 200 µM FB_1 was the only concentration to significantly increase the expression of all m6A "readers" (Figure 5.2d). The differential expression in m6A regulating enzymes may also contribute to abnormal lipid metabolism and immune profiles in the liver (Zhao et al., 2020b).

FB_1-induced increases in m6A levels may lead to the altered expression of important genes involved in its toxicity. Since FB_1 triggered abnormal ROS production, we decided to focus on Keap1 and Nrf2 as the Keap1/Nrf2 signaling plays a critical role in responding to xenobiotic and electrophilic stress. Not only did we set out to determine changes in m6A-*Keap1* and m6A-*Nrf2* but we also evaluated other epigenetic changes that might affect their expression.

The most extensively studied epigenetic modification to eukaryotic genomes is DNA methylation which occurs primarily at CpG sites. Methylation of CpG islands found in gene promoters prevents the binding of transcription factors, silencing transcription. As seen in Figure 5.3a, FB_1 induced significant hypermethylation at the *Keap1* promoter, inhibiting *Keap1* transcription (Figure 5.3b). Before assessing m6a-*Keap1* levels, a sequence based m6A site predictor (SRAMP) was used to define potential m6A sites on *Keap1* mRNA (Zhou et al., 2016). 29 possible m6A sites were predicted on *Keap1* transcripts including 7 possible m6A sites with high confidence and 1 with very high confidence. Furthermore, the consensus motifs (GGACU and AGACU) matched DRACH motif (Figure 5.3c). The results suggest that m6A-modified *Keap1* maybe be involved in its translation. Using RNA immunoprecipitation and western blotting, we determined changes in m6A-*Keap1* and Keap1 protein expression, respectively. Although m6A modified *Keap1* levels were increased (Figure 5.3d); there was a severe loss in Keap1 protein expression (Figure 5.3e). The m6A-reader YTHDF2 may be responsible for this. The aromatic cage of YTHDF2 specifically targets m6A modified RNA to cytoplasmic decay sites and accelerates the degradation of marked transcripts (Wang et al., 2014). The high levels of *YTHDF2* observed post FB_1 treatments may be involved in *Keap1* degradation. Furthermore, colistin-induced oxidative stress

was attenuated by the overexpression of METTL3 and diminished Keap1 levels. METLL3, enhanced m6A modifications on pri-miR-873, promoted the generation of mature miR-873-5p which in turn inhibited Keap1 expression (Wang et al., 2019). Cells may be responding to FB_1-mediated oxidative stress in a similar manner, however this needs to be further investigated.

Not only is Nrf2 expression regulated by DNA and RNA methylation but also by miRNA-27b (Kang et al., 2014, Xu et al., 2017). As mentioned earlier, FB_1 downregulated miR-27b expression (Chuturgoon et al., 2014b). It was previously shown that miR-27b regulates Nrf2 expression (Xu et al., 2017) and this was further confirmed using TargetScan version 7.1 (Agarwal et al., 2015). Hypomethylation of *Nrf2* promoters (Figure 5.4a), coupled with the gross loss of miR-27b (Figure 5.4b) resulted in elevated *Nrf2* mRNA expression (Figure 5.4c). Moreover, 54 possible m6A sites with 15 high confidence and 2 very high confidence sites were predicted using SRAMP. The consensus motif (GGACU) also matched the DRACH motif (Figure 5.4d). FB_1 significantly increased m6A-*Nrf2* levels (Figure 5.4e) and Nrf2 protein expression (Figure 5.4f). It is possible that the increase in YTHDF1, YTHDF3 and/or YTHDC2 may be responsible for elevated Nrf2 protein expression as these readers promote the translation of targeted transcripts (Wang et al., 2015). Oxidative stress was also shown to elevate m6A-*Nrf2* levels in di-(2-ethylhexyl) phthalate (DEHP) exposed rats, however, the fate of m6A-tagged *Nrf2* transcripts were not further investigated. The authors speculated that Nrf2 protein expression would be decreased, however, the opposite could be true; like our study YTHDC2 was also elevated after DEHP exposure.

To our knowledge, this is the first study to identify that FB_1 alters global and transcript-specific m6A methylation levels. While several studies have noted the accumulation of ROS enhances m6A RNA levels, we cannot say for certain that the observed changes were due to FB_1 effect on ROS generation (Li et al., 2017, Zhao et al., 2019, Wu et al., 2020, Zhao et al., 2020a). The use of positive and negative controls such as hydrogen peroxide and an antioxidant such as *N*-acetylcysteine would have given a more definitive answer. However, it is evident that FB_1 does significantly alter the expression of m6A modulator genes especially m6A demethylases. It would be interesting to further explore if the differential expression of these m6A regulating genes may play a role in FB_1 toxicity aside from m6A regulation as these genes have been shown to regulate metabolism and immune profiles in the liver (Xu et al., 2019). Further, FB_1 epigenetically regulates Keap1 and Nrf2 expression, through changes in promoter methylation, RNA methylation and miR-27b levels. The downregulation of Keap1 and upregulation of Nrf2 by FB_1 suggests that antioxidant signalling pathways have been activated. An increase in Nrf2 regulated anti-oxidants were previously observed in response to FB_1-induced oxidative stress (Arumugam et al., 2019). However, the activation of Nrf2 antioxidant signalling may not be sufficient to counter the accumulation of ROS induced by FB_1 as severe cellular injury occurred. Furthermore, prolonged activation of Nrf2 signalling supports a cancerous phenotype through ROS detoxification and tumorigenesis (Wu et al., 2019). Epigenetic changes such hypermethylation at *Keap1*

promoters, hypomethylation at *Nrf2* promoters and altered miRNA profiles have shown to be involved in deregulation of Keap1/Nrf2 in various cancers (Eades et al., 2011, Barbano et al., 2013, Kang et al., 2014, Fabrizio et al., 2018). We can only speculate that this may be a possible mechanism by which FB_1 promotes hepatocarcinogenesis. However, further studies should be conducted to test this hypothesis. The use of longer exposure times and comparing differences in the epigenetic profiles linked to Keap1/Nrf2 dysregulation in normal and cancerous cells may be key.

Conclusion

The results of this study revealed that FB_1 induces hepatotoxicity as observed by ROS accumulation and loss of cell membrane integrity. Global m6A levels were increased in response to changes in the expression of m6A-modulating genes (Figure 5.5a). Further, we observed hypermethylation of *Keap1* promoters, hypomethylation of *Nrf2* promoters, reduction in miR-27b and increase in m6A-*Keap1* and m6A-*Nrf2*, which ultimately led to the activation of Keap1/Nrf2 signalling (Figure 5.5b-c). This study provides new evidence that m6A modifications may play a pivotal role in FB_1-induced oxidative stress and hepatocarcinogenesis.

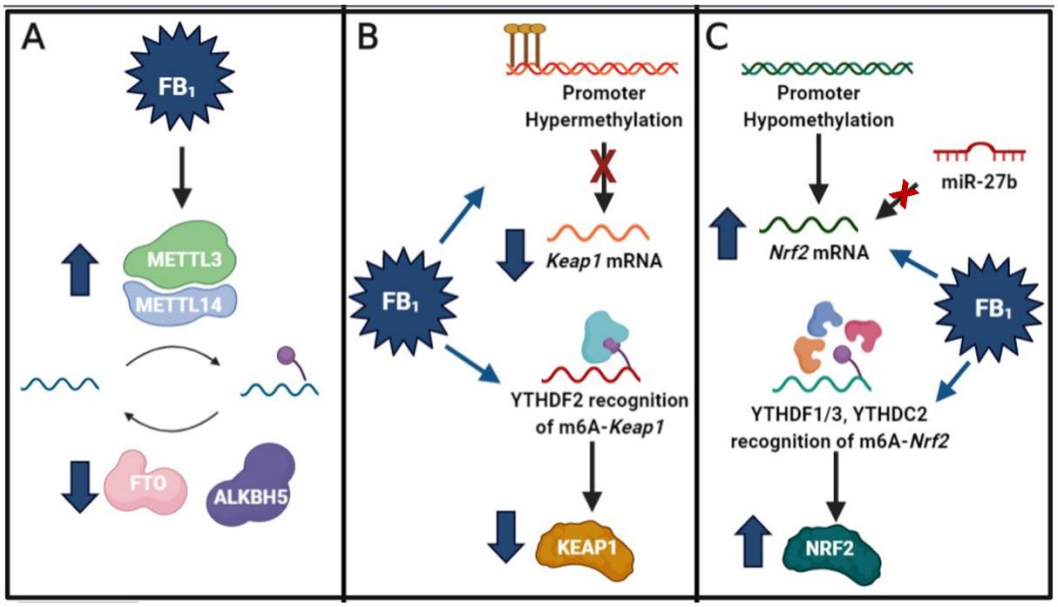

Figure 5.5. FB_1 alters global m6A RNA methylation and epigenetically regulates Keap1-Nrf2 signaling. (a) FB_1 induced changes to global m6A RNA methylation by mediating changes in m6A "writers" (*METLL3* and *METLL14*) and m6A demethylases (*FTO* and *ALKBH5*). (b) FB_1 epigenetically downregulates Keap1 through hypomethylation of *Keap1* gene promoters and degradation of m6A-*Keap1* transcripts via *YTHDF2*. (c) Nrf2 is epigenetically upregulated by FB_1 via hypomethylation of *Nrf2* promoters, reduced miR-27b and increased recognition of m6A-*Nrf2* transcripts by *YTHDF1*, *YTHDF2* and *YTHDC2*.

Declarations

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Conflicts of interest

The authors declare that they have no conflicts of interest

Ethics approval

Ethic was received from the University of Kwa-Zulu Natal's Biomedical Research Ethics Committee. Ethics number: BE322/19.

Author Contributions

TA, TG, and AC conceptualised and designed the study. TA conducted all laboratory experiments, analysed the data and wrote the manuscript. TG and AC revised the manuscript. All authors have read the manuscript prior to submission.

Supplementary Information

Supplementary Table S5.1: Primer sequences and annealing temperatures used in qPCRs

Gene	Sense Primer 5'→ 3'	Anti-sense Primer 5' → 3'	Annealing Temperature (°C)
qPCR			
METTL3	TTGTCTCCAACCTTCCGTAGT	CCAGATCAGAGAGGTGGTGTAG	56
METTL14	GAACACAGAGCTTAAATCCCCA	TGTCAGCTAAACCTACATCCCTG	56
FTO	GCTGCTTATTTCGGGACCTG	AGCCTGGATTACCAATGAGGA	56
ALKBH5	ATCCTCAGGAAGACAAGATTAG	TTCTCTTCCTTGTCCATCTC	60
YTHDF1	ATACCTCACCACCTACGGACA	GTGCTGATAGATGTTGTTCCCC	56
YTHDF2	CCTTAGGTGGAGCCATGATTG	TCTGTGCTACCCAACTTCAGT	56
YTHDF3	TCAGAGTAACAGCTATCCACCA	GGTTGTCAGATATGGCATAGGCT	56
YTHDC2	CAAAACATGCTGTTAGGAGCCT	CCACTTGTCTTGCTCATTTCCC	60
Keap1	CTGGAGGATCATACCAAGCAGG	GGATACCCTCAATGGACACCAC	57
Nrf2	TCAGCGACGGAAAGAGTATGA	CCACTGGTTTCTGACTGGATGT	58

GAPDH	TCCACCACCCTGTTGCTGTA	ACCACAGTCCATGCCATCAC	Same as gene of interest
Promoter Methylation			
Keap1	TTAGTTATTTAG-GAGGTTGT	AACCCCCCTTCTCACTA	54
Nrf2	TGAGATATTTTGCACATCCGATA	ACTCTCAGGGTTCCTTTACACG	54
RNA Immunoprecipitation			
Keap1	CTGGAGGATCATACCAAGCAGG	GGATACCCTCAATGGACACCAC	57
Nrf2	TCAGCGACGGAAAGAGTATGA	ACCACAGTCCATGCCATCAC	58

	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 62-68 of NFE2L2 3' UTR	5' ...AUACUAAAAGCUCCUACUGUGAU...
	|||||||
hsa-miR-27b-3p	3' CGUCUUGAAUCGGUGACACUU

Supplementary Figure S5.1. TargetScan analyses of miR-27b to the 3' UTR of *NFE2L2 (Nrf2)* in humans. MiR-27b has complementary base pairs with the 3' UTR of *Nrf2* at positions 62-68 in humans.

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CHAPTER 6

Fumonisin B_1 inhibits p53-dependent apoptosis via HOXA11-AS/miR-124/DNMT axis in human hepatoma (HepG2) cells

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Abstract

FB_1 is a hazardous mycotoxin that induces toxic and carcinogenic effects in humans and animals. FB_1 induces changes to the epigenome which may provide insight into its toxic and carcinogenic nature. The lncRNA, HOXA11-AS influences the epigenome by modulating DNA methylation functioning as a competing endogenous RNA (ceRNA) or molecular scaffold. However, the role of HOXA11-AS in FB_1-toxicity is unknown. Therefore, we investigated the effect of FB_1 on p53-dependent apoptosis via the HOXA11-AS/miR-124/DNMT axis. HepG2 cells were treated with various concentrations of FB_1 (0, 5, 50, 100 and 200 µM; 24 h). qPCR and/or western blotting was used to determine expression of HOXA11-AS, miR-124, SP1, DNMT1, DNMT3A, DNMT3B and p53. Global DNA methylation and p53 promoter methylation was assessed, whilst luminometry was used to measure caspase activity. FB_1 upregulated HOXA11-AS ($p \leq 0.05$) leading to the subsequent decrease in miR-124 ($p \leq 0.01$) and increase in SP1 ($p \leq 0.001$), DNMT1 ($p \leq 0.001$), DNMT3A ($p \leq 0.001$) and DNMT3B ($p \leq 0.001$). This promoted global DNA methylation ($p \leq 0.05$) and hypermethylation of p53 promoters ($p \leq 0.001$) thereby reducing p53 expression ($p \leq 0.001$) and caspase activity ($p \leq 0.001$). Taken together the data suggests that FB_1 inhibits p53-dependent apoptosis via HOXA11-AS/miR-124/DNMT axis in HepG2 cells.

Keywords

Fumonisin B_1, Epigenetics, HOXA11-AS, miR-124, DNA Methylation, p53.

Introduction

Our life long development is not only dictated by our genetic code but also a dynamic network regulating DNA methylation, covalent histone modifications, RNA modifications and non-coding RNA (Kanherkar et al., 2014). This network is known as the epigenome. Together these modifications regulate gene expression and bring about phenotypic variations without altering the genetic code (Marczylo et al., 2016). However, changes to the epigenome brought about by environmental factors such as mycotoxins can lead to adverse health outcomes (Marczylo et al., 2016, Huang et al., 2019). Mycotoxins are toxic secondary metabolites produced by various fungi (Bennett, 1987). They chronically contaminate agricultural foods that are intended for human and animal consumption and elicit a wide variety of detrimental effects (Bennett and Klich, 2003, Eskola et al., 2020). The mechanisms by which mycotoxins induce their toxicity vary; however, over the past decade epigenetic changes have been implicated in various mycotoxin-related diseases and toxicities in humans and animals (Huang et al., 2019). The most toxicologically relevant mycotoxins include aflatoxins, ochratoxins, trichothecenes and fumonisins (Fung and Clark, 2004). Fumonisins are naturally produced by *Fusarium verticillioides* and *Fusarium proliferatum* (Ross et al., 1990, Ross et al., 1992). Due to poor agricultural practices and storage conditions, fumonisins mainly contaminate cereals and cereal-based-products thereby, posing a serious threat to human and animal health (Mashinini and Dutton, 2006, Stępień et al., 2011, Ferrigo et al., 2016, Alberts et al., 2019, Phokane et al., 2019). Among the

28 identified fumonisin analogues, fumonisin B_1 (FB_1) is regarded as the most relevant due to its potent toxicity and widespread distribution (Rheeder et al., 2002).

Epigenetic changes have been linked to FB_1 toxicity. For instance, FB_1-induced changes in miRNA profiles and covalent histone modifications have been linked to genetic instability, and may be potential mechanisms for FB_1-related carcinogenesis and neural tube defects (Chuturgoon et al., 2014b, Sancak and Ozden, 2015, Gardner et al., 2016, Arumugam et al., 2020). Moreover, the effects of FB_1 on global DNA methylation have been thoroughly investigated by several research groups (Mobio et al., 2000, Kouadio et al., 2007, Chuturgoon et al., 2014a), with Demirel et al. (2015) demonstrating that FB_1 may exert its carcinogenic effects by modulating the promoter methylation of specific tumour suppressor genes. While the effects of FB_1 on DNA methylation, histones modification and miRNA have been explored, no study has evaluated the impact FB_1 may have on long non-coding RNAs (lncRNAs). LncRNAs were long considered irrelevant and thought of as merely "transcriptional noise" (Kung et al., 2013). However, with recent advances in sensitive, high-throughput genomic technologies and next-generation sequencing, their true potential is finally being recognized (Atkinson et al., 2012, Zhu et al., 2016). LncRNAs influence chromatin structure and gene expression thereby, regulating several biological processes such as apoptosis, proliferation differentiation and cell cycle regulation (Hu et al., 2011, Han and Chang, 2015, Nötzold et al., 2017, Yang et al., 2018c, Li et al., 2019); thus, dysregulation of lncRNAs have been associated with several pathological states such as, neurodegenerative disorders, chronic liver diseases, renal failure and numerous cancers (Prensner and Chinnaiyan, 2011, Sun et al., 2018a, Tang et al., 2019, Kim et al., 2020).

One such lncRNA is homeobox A11 antisense (HOXA11-AS), a highly conserved lncRNA located in the HOXA gene cluster on chromosome 7p15 (Wei et al., 2020). By acting as a circulating endogenous RNA (ceRNA) and molecular scaffold, HOXA11-AS contributes to the ever-changing epigenome (Wei et al., 2020). As a ceRNA, HOXA11-AS sequesters miRNA with complementary binding sites such as miR-148, miR-200 and miR-124 and blocks the regulatory interaction between the miRNA and its target mRNA (Chen et al., 2017, Bai et al., 2019). By acting as a molecular scaffold, HOXA11-AS modulates the transcription of target genes by recruiting proteins including DNA methyltransferases (DNMTs) and transcription factors to the promoter regions of genes (Sun et al., 2016). Furthermore, Yu et al. (2017b) demonstrated that HOXA11-AS "sponging" of miR-124 upregulates SP1, a DNMT1 transcription factor (Kishikawa et al., 2002). MiR-124 is also responsible for DNMT3B regulation (Chen et al., 2015). Thus, it is possible that HOXA11-AS may play a role in FB_1-mediated changes in both global and gene-specific methylation by regulating DNMT expression. Using bioinformatic prediction analysis and laboratory-based methods, this study evaluated the potential role of HOXA11-AS in FB_1 toxicity and DNA methylation. We assessed the relationship between HOXA11-AS and miR-124 and how it may impact DNMT expression, global DNA methylation and promoter methylation via DNMT regulation. We looked specifically at *p53* promoter methylation as it is a multifaceted tumour

suppressor and transcription factor that plays a pivotal role in facilitating stress responses (Shieh et al., 1999, Yin et al., 1999, Vousden and Prives, 2009). Such stresses include oxidative stress, DNA damage and cell cycle abnormalities (Shieh et al., 1999, Yin et al., 1999). We recently found that FB_1 induced oxidative DNA damage and inhibited DNA damage checkpoint regulation (Arumugam et al., 2020). It is possible that p53 may play a role in responding to FB_1-mediated stress. Therefore, the aim of this study was to determine the effects of FB_1 on HOXA11-AS and the downstream effects it may have on global and *p53* promoter methylation via HOXA11-AS/miR-124/DNMT axis.

Method and Materials

Materials

FB_1 (*Fusarium moniliforme*) was purchased from Cayman Chemicals (62580, Ann Arbor, MI, USA). Silencing RNA (siRNA) against HOXA11-AS (SI03654588), siRNA negative control (0001027281), miR-124 mimic (MSY0004591), miR-124 inhibitor (MIN0004591), and attractene transfection reagent (301005) were purchased from Qiagen (Hilden, Germany). The DNA methylation inhibitor, 5-Aza-2-deoxycytidine (5-Aza-2-dc; A3653) was purchased from Sigma-Aldrich (A3854, St. Louis, MO, USA) and the human heptoma (HepG2) cell line (HB-8065) was procured from the American Type Culture Collection (ATCC). Cell culture consumables were obtained from Whitehead Scientific (Johannesburg, South Africa). Western blot reagents were purchased from Bio-Rad (Hercules, CA, USA) while primary and secondary antibodies were obtained from Cell Signalling Technologies (Danvers, MA, USA) and β-actin was obtained from Sigma Aldrich (A3854, St. Louis, MO, USA). A detailed list of the antibodies used in this study is included in Supplementary Table S6.1. All other reagents were purchased from Merck (Boston, MA, USA), unless otherwise stated.

Cell culture

HepG2 cells were grown in complete culture medium [CCM: Eagle's Minimum Essentials Medium (EMEM) supplemented with 10% foetal calf serum, 1% penicillin-streptomycin fungizone, and 1% L-glutamine] under the following conditions: pH 7.4, 37°C, 5% CO_2 and 95% relative humidity. For experiments, cells (1.5×10^6, passage 3) were seeded in 25 cm^3 sterile tissue culture flasks. When 80% confluency was achieved, cells were treated with a range of FB_1 concentrations (5, 50, 100 and 200 µM) (Arumugam et al., 2020). 5-Aza-2-dc, an inhibitor of DNA methylation, was used as a negative control. To induce DNA hypomethylation, cells were exposed to 10 µM of 5-Aza-2-dc (Ahn et al., 2013). An untreated control containing CCM only was also prepared. All treatments occurred for 24 hours (h) and experiments were repeated two independent times and in triplicate for reproducibility of results.

Transfection with siRNA and miRNA mimic and inhibitors

To assess the effect of HOXA11-AS on miR-124 levels and DNMT1 scaffolding, HepG2 cells were transfected with the siRNA-against HOXA11-AS (siR-HOXA11-AS) and a negative control siRNA (siR-NC). HepG2 cells also underwent transfection with miR-124 mimic and miR-124 inhibitor in an effort to assess the effects of miR-124 on DNMT3B and SP1 expression.

HepG2 cells were grown as described above to 80% confluency in 25 cm^3 cell culture flasks. The lyophilized siRNAs (20 nmol) and miR-124 mimic and inhibitor (1 nmol) were reconstituted in nuclease-free water to a concentration of 20 µM. The transfection complex consisting of siRNA or miRNA mimic or inhibitor (15 µl), CCM (72 µl) and attractene (3 µl) was prepared and incubated (15 min, RT). Thereafter, cells were washed with PBS and EMEM (2,910 µl) was added to yield a final concentration of 100 nM of siRNAs, mimic and inhibitor. The transfection complex was added in a dropwise manner with gentle swirling to allow even distribution. The cells were then incubated (37°C, 5% CO_2, 24 h).

RNA isolation

Total RNA was isolated from control and treated HepG2 cells. Cells were washed with 0.1M PBS and incubated (5 min, RT) with Qiazol reagent (79306, Qiagen, Hilden, Germany) and 0.1M PBS (1:1) before being mechanically lysed. Cell lysates were stored at -80°C overnight. Chloroform (100 ul) was added to the thawed lysates to promote phase separation, and samples were centrifuged (12,000xg, 4°C, 15 min). RNA in the aqueous phase was precipitated overnight (-80°C) using isopropanol (500 µl). Once thawed, samples were centrifuged (12,000xg, 4°C, 20 min). The RNA-containing pellets were washed with 75% ice-cold ethanol and centrifuged (7,400xg, 4°C, 15 min). RNA pellets were air dried (30 min, RT) and resuspended in nuclease-free water (10 µl). Extracted RNA was quantified using the Nanodrop2000 spectrophotometer (Thermo Scientific, Waltham, USA) and RNA purity was assessed using the A260/A280 absorbance ratio. RNA was standardized to 1000 ng/µl in nucleus free water unless otherwise stated.

Quantification of HOXA11-AS levels

HOXA11-AS expression was determined via real time quantitative polymerase chain reaction (RT-qPCR). cDNA was prepared from standardized RNA using the RT^2 First Strand Kit (330404, Qiagen, Hilden, Germany). Residual genomic DNA was removed from standardized RNA using the Genomic DNA elimination mix for 5 min at 42°C prior to cDNA synthesis using the reverse transcriptase mix. Thermocycler conditions for cDNA synthesis were as follows: 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and a final hold at 4°C. Thereafter, cDNA underwent preamplification using the RT^2 PreAMP cDNA Synthesis Kit (330451, Qiagen, Hilden, Germany) and RT^2 lncRNA PreAMP Primer Mix (330741, Qiagen, Hilden, Germany) as per manufacturer's protocols. The expression of HOXA11-AS was determined using the RT^2 SYBR Green qPCR Master Mix (330503, Qiagen, Hilden, Germany) and RT^2 lncRNA qPCR Assay for Human HOXA11-AS (LPH14348A, Qiagen, Hilden, Germany). GAPDH

(LPH31725A-200, Qiagen, Hilden, Germany) was used as housekeeping control and run simultaneously with HOXA11-AS. Relative changes in gene expression was determined using the comparative threshold cycle (Ct) method as described by Livak and Schmittgen (2001).

Quantification of miR-124 expression

miR-124 expression was determined using RT-qPCR. The miScript II RT Kit (218161, Qiagen, Hilden, Germany) was used to reverse transcribe standardized RNA to cDNA. miR-124 expression was determined using the miScript SYBR Green PCR Kit (218073, Qiagen, Hilden, Germany) and Hs_miR-124*_1 10X miScript Primer Assay (MS00008547, Qiagen, Hilden, Germany), as per manufacturer's instructions. Human RNU6 (Qiagen, MS000033740, Qiagen, Hilden, Germany) was used as the housekeeping gene to normalize miRNA expression. Amplification was conducted using the CFX96 Real Time PCR System (Bio-Rad, Hercules, CA, USA) and analysed using the Bio-Rad CFX Manager Software version 3.1. Relative changes in gene expression was determined using the method described by Livak and Schmittgen (2001).

Quantification of mRNA levels

cDNA was synthesized using standardized RNA and the Maxima H Minus First Strand cDNA Synthesis Kit (K1652, Thermo-Fisher Scientific, Waltham, MA, USA) as per manufacturer's instructions. The gene expression of *SP1*, *DNMT1*, *DNMT3A*, *DNMT3B* and *p53* was assessed using the PowerUp SYBR Green Master Mix (A25742, Thermo-Fisher Scientific, Waltham, MA, USA) and the CFX96 Real Time PCR System (Bio-Rad, Hercules, CA, USA) with the following cycling conditions: initial denaturation (95°C, 8 min), followed by 40 cycles of denaturation (95°C, 15 s), annealing (Supplementary Table S6.2, 40 s), and extension (72°C, 30 s). Primer sequences and annealing temperatures are listed in Supplementary Table S6.2. The housekeeping control, GAPDH was run alongside the target and mRNA expression was normalized against *GAPDH*. Relative changes in gene expression was determined using the Ct method as described by Livak and Schmittgen (2001).

RNA immunoprecipitation

RNA immunoprecipitation was performed to assess HOXA11-AS binding to DNMT1. DNMT1 antibody (1:100; 5032S, Cell Signalling Technologies, Danvers, MA, USA) was incubated with standardized RNA (1000 ng/µl), overnight at 4°C. Protein A beads [20 µl, 50% bead slurry (#9863, Cell Signalling Technology), 4°C, 3 h] were used to precipitate the RNA-DNMT1 complex. Samples were centrifuged (2,500xg, 4°C, 60s) and washed twice in RNA immunoprecipitation buffer [150 mM KCl, 25 mM Tris-Cl (pH 7.4), 5mM EDTA, 0.5mM DTT, 0.5% IGEPAL, 100 U/ml SUPERase IN RNase Inhibitor (AM2694, Thermo-Fisher Scientific), protease and phosphatase inhibitor (A32961, Thermo-Fisher Scientific)]. Samples were washed once in nuclease free water and resuspended in nuclease free water (10 µl). Immunoprecipitated RNA was standardised to 200 ng/µl, and reverse transcribed into cDNA as described above. The expression of DNMT1-HOXA11-AS was then determined using qPCR

as mentioned above. Primer sequences and annealing temperatures are listed in Supplementary Table S6.2.

DNA isolation

Genomic DNA was isolated from HepG2 cells and used to assess global DNA methylation levels and methylation status of p53 promoter region. Once treatments were removed, cells were washed thrice with 0.1M PBS and incubated (RT, 15 min) in cell lysis buffer [0.5 M EDTA (pH 8.0), 1 M Tris-Cl (pH 7.6), and 0.1% SDS] before being mechanically lysed. Potassium acetate (5 M potassium acetate and glacial acetic acid) was added to samples which were then invert mixed (8 min). Samples were centrifuged (13,000 x g, 5 min, 24°C) and isopropanol was added to the aqueous phase to precipitate DNA. Sample were then invert mixed (6 min) before being centrifuged (13,000 x g, 5 min, 24°C). DNA-containing pellets were washed with 100% cold ethanol to remove residual salts. Samples were centrifuged (13,000 x g, 5 min, 24°C), ethanol removed and pellets were left to air dry for 30 min. Once dried, pellets were resuspended in TE buffer [10 mM EDTA (pH 8.0) and 100 mM Tris-Cl (pH 7.4)] and heated (65°C, 15 min). DNA concentration was quantified using the Nanodrop2000 spectrophotometer and adjusted as required.

Quantification of global DNA methylation

Isolated DNA was standardized to 100 ng/µl and used to quantify global DNA methylation levels through the Colorimetric Methylated DNA quantification Kit (ab117128, Abcam, Cambridge, UK) as per manufacturers' protocol.

p53 promoter methylation

Isolated genomic DNA was standardized to 4 ng/µl and used in the OneStep qMethyl Kit (5310, Zymo Research, Irvine, CA, USA) to asses promoter methylation of *p53*. Primer sequences used were as follows; p53 promoter sense: 5'- GTGGATATTACGGAAAGT-3' and p53 promoter anti-sense: 5'-AAAATATCCCCGAAACC-3'. Cycling conditions were as follows: digestion by methyl sensitive restriction enzymes (37°C, 2 h), initial denaturation (95°C, 10 min), followed by 45 cycles of denaturation (95°C, 30s), annealing (54°C, 60s), extension (72°C, 60s), final extension (72°C, 60s), and a hold at 4°C. Results are represented as a fold-change relative to the control.

Protein expression

HepG2 cells were lysed with Cytobuster reagent (71009-3, Merck, Kenilworth, NJ, USA) which contained protease and phosphatase inhibitors (A32961, Thermo-Fisher Scientific). The protein concentration was measured using the bicinchoninic acid assay (Walker, 1994) and standardized to 1 mg/ml. The protein expression of DNMT1, DNMT3A, DNMT3B, and p53 were determined using western blotting as previously described (Arumugam et al., 2019). Protein expression is represented as relative band density (RBD) and calculated by normalizing the protein of interest against the

housekeeping protein, β-actin. A list of antibodies and dilutions used can be found in supplementary table S6.1.

Caspase activity

The activity of caspases -3/7, -6, -8, and -9 were assessed using the Caspase-Glo luminometry assays (G8090, G0970, G8200, and G8210, Promega, Madison, WI, United States). Control and treated cells (20,000 cells/well) were dispensed into an opaque 96-well microtiter plate in triplicate and incubated with the respective Caspase-Glo reagent (20 µl) in the dark for 30 min at RT. Luminescence was quantified using the Modulus microplate luminometer (Turner Biosystems) and the results were expressed as relative light units (RLU).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Prism Software Inc.). Data was analysed using the one-way Analysis of Variance (ANOVA) with Dunnet's post-test. The results were represented as the mean ± standard deviation (SD) and a p value of less than 0.05 was considered statistically significant.

Results

FB_1-induced HOXA11-AS sponges miR-124, regulating SP1 and DNMT expression

The RT^2 lncRNA PCR Array Human IncFinder (LAHS-001Z, Qiagen, Hilden, Germany) was used to identify differentially expressed lncRNA in HepG2 cells exposed to 200 µM FB_1 [IC_{50} (Arumugam et al., 2020)]. HOXA11-AS was identified as one of the most upregulated lncRNA (Supplementary Figure S6.1). The expression of HOXA11-AS was then validated in HepG2 cells using a range of FB_1 concentrations (5, 50, 100 and 200 µM) and the downstream effects of HOXA11-AS was determined.

HOXA11-AS expression was increased in response to increasing concentrations of FB_1 ($p \leq 0.001$; Figure 6.1a). To validate the relationship between FB_1 and HOXA11-AS, HepG2 cells were transfected with siRNA against HOXA11-AS. Cells were transfected with silencing RNA against HOXA11-AS which acted as a negative control and used to gain insight into potential downstream effects of HOXA11-AS. Cells were also transfected with siR-NC to test the efficiency of transfection. HOXA11-AS expression was effectively knocked down in cells treated with siR-HOXA11-AS ($p \leq 0.05$; Figure 6.1a); however, expression for the siR-NC treated cells was similar to the control, suggesting that transfection was successful (Figure 6.1a).

It was previously shown that HOXA11-AS acts as a ceRNA for miR-124 (Lu et al., 2017). This was confirmed using online bioinformatics prediction algorithm software, starBase v2.0 (Li et al., 2014). MiR-124 was reduced at all FB_1 concentrations, but significantly reduced at 100 µM and 200 µM FB_1 (Figure 6.1b; $p \leq 0.01$). Furthermore, we found miR-124 to be significantly upregulated in cells treated with siR-HOXA11-AS confirming the relationship between these 2 RNA species ($p \leq 0.001$; Figure

6.1b). In addition to siRNA, HepG2 cells were transfected with a mimic and inhibitor against miR-124 which acted as a positive and negative control, respectively. The expression of miR-124 in HepG2 cells treated with the mimic and inhibitor were increased and decreased, respectively (Figure 6.1b; $p \leq 0.001$).

TargetScan (version 7.1), an online bioinformatics prediction software that predicts miRNA-mRNA interactions was used to determine possible targets of miR-124 (Agarwal et al., 2015). MiR-124 was shown to potentially regulate DNA methylation as it was found to have complementary base pairs with the DNA methyltransferase, *DNMT3B* at positions 1363-1369 and the DNMT1 transcription factor, *SP1* at positions 524-530, 4149-4155 and 4520-4526 (Figure 6.1c).

Due to the decreased expression in miR-124 observed by FB_1, we then evaluated the expression of SP1 and DNMT3B. FB_1 significantly increased gene expression of *SP1* (Figure 6.1d; $p \leq 0.001$) and *DNMT3B* (Figure 6.1d; $p \leq 0.001$). DNMT3B protein expression (Figure 6.1e; $p \leq 0.001$) was increased in response to 50-200 µM FB_1; yet, it was reduced at 5 µM FB_1. *SP1* gene and DNMT3B gene and protein expression was also significantly increased in cells treated with miR-124 inhibitor and significantly reduced in miR-124 mimic and siR-HOXA11-AS treated cells; confirming the relationship between HOXA11-AS and miR-124 with SP1 and DNMT1.

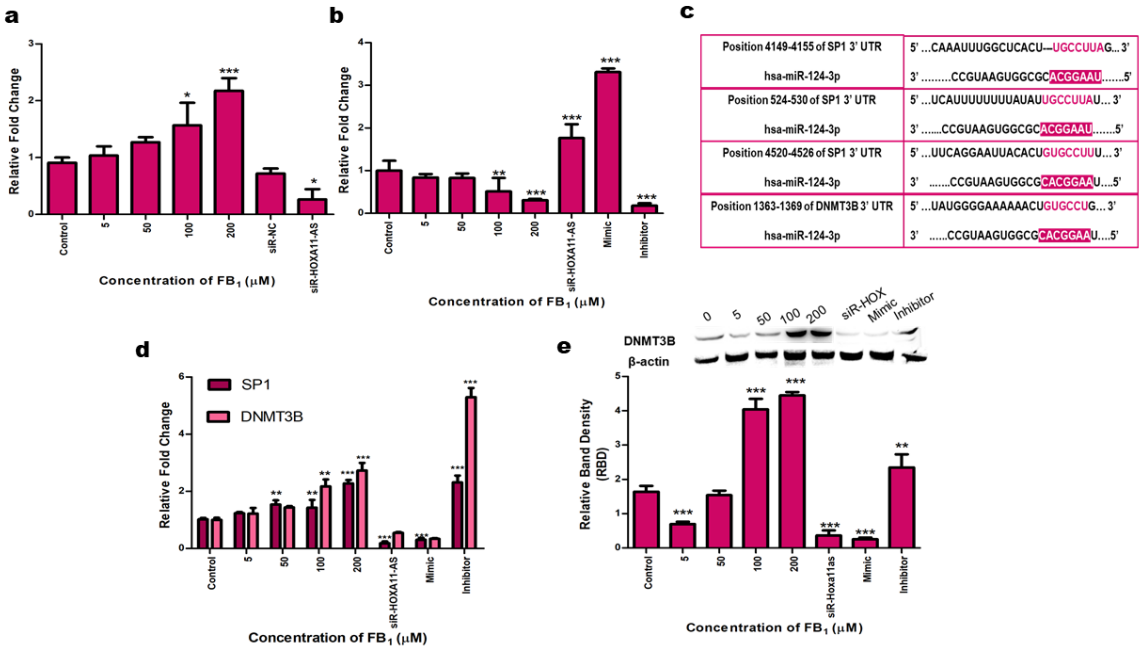

Figure 6.1. FB_1 upregulated HOXA11-AS levels (a; ***$p \leq 0.001$) which negatively regulated miR-124 (b; *** $p \leq 0.001$). Bioinformatic prediction revealed that the 3' UTR of *SP1* and *DNMT3B* contains binding sites for miR-124 (c). *SP1* gene (d; ***$p \leq 0.001$) and *DNMT3B* gene (d; ***$p \leq 0.001$) and protein (f; ***$p \leq 0.001$) expression was altered by FB_1 treatment.

FB_1 elevates DNMT1 expression and promotes HOXA11-AS-DNMT1 binding

SP1 activates the transcription of DNMT1 (Kishikawa et al., 2002), thus, DNMT1 expression was assessed. qPCR and western blotting analysis revealed a significant increase in DNMT1 mRNA (Figure 6.2a; $p \leq 0.001$) and protein expression (Figure 6.2b; ≤ 0.001), respectively.

In addition to its function as a ceRNA, HOXA11-AS acts as a scaffold for DNMT1 by recruiting it to gene promoters (Sun et al., 2016), therefore, HOXA11-AS-DNMT1 binding was evaluated by performing RNA immunoprecipitation. There was a slight reduction in the interaction at 5 µM FB_1, however, increased at the all other concentrations tested (Figure 6.2c; $p \leq 0.001$). HOXA11-AS-DNMT1 interactions were significantly reduced in siR-HOXA11AS treated cells ($p \leq 0.01$; Figure 6.2c).

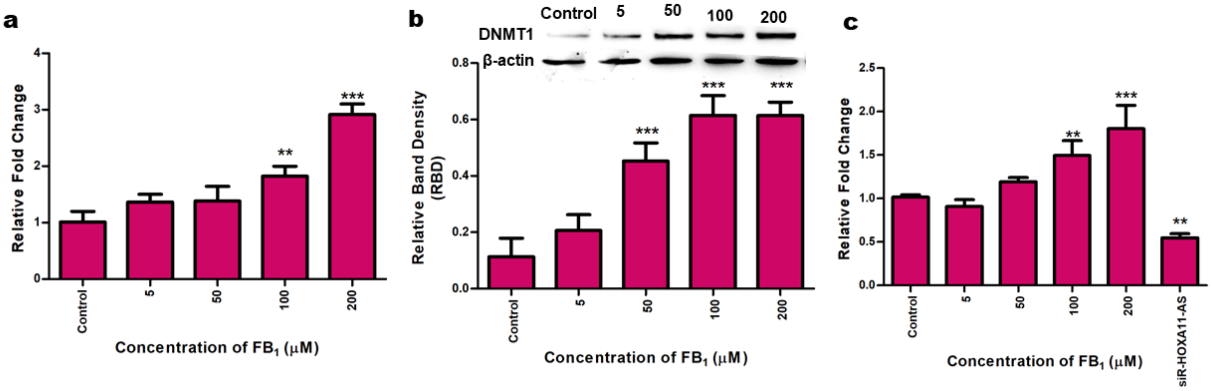

Figure 6.2. FB_1 significantly upregulated *DNMT1* mRNA (a; ***$p \leq 0.001$) and DNMT1 protein (b; ***$p \leq 0.001$) expression and altered HOXA11-AS-DNMT1 binding (c; ***$p \leq 0.001$) in HepG2 cells.

FB1 altered DNMT3A expression and global DNA methylation status of HepG2 cells

Although DNMT3A is not regulated by HOXA-11AS or miR-124, it does play an important role in DNA methylation., FB_1 significantly increased *DNMT3A* gene (Figure 6.3a, $p \leq 0.001$) and protein (Figure 6.3b; $p \leq 0.001$) expression at all concentrations investigated.

Since FB_1 differentially regulated DNMT expression, we next determined whether FB_1 affected global DNA methylation levels (Figure 6.3c). Along with FB_1 treatments, cells were treated with 5-Aza-2-dc, a known DNA methylation inhibitor. Naturally, 5-Aza-2-dc treatment significantly reduced total methylation levels in HepG2 cells ($p \leq 0.05$; Figure 6.3c). In contrast, FB_1 increased total methylation of DNA; however, these results were not significant at any of the tested concentrations ($p > 0.05$; Figure 6.3c).

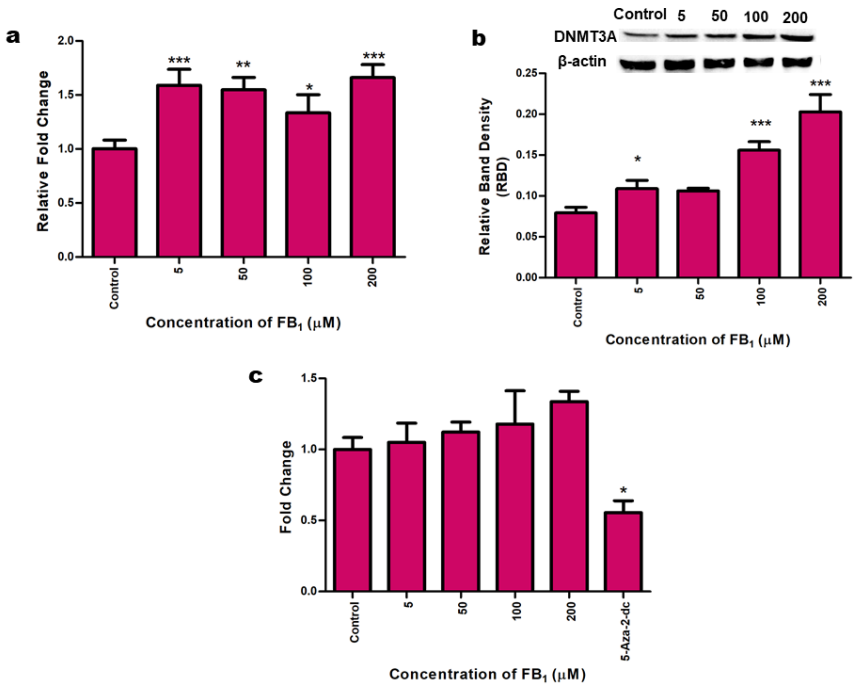

Figure 6.3. qPCR and western blot quantification revealed that *DNMT3A* gene (a; ***$p \leq 0.001$) and DNMT3A protein (b; *** $p \leq 0.001$) expression was significantly elevated in FB_1-exposed HepG2 cells. Methylation of cytosine in the DNA of HepG2 cells were also increased following FB_1 treatment (c; * $p \leq 0.05$).

FB_1 reduced p53 expression via hypermethylation of gene promoter

In addition to global methylation levels, we evaluated gene-specific methylation in HepG2 cells. Methylation of specific CpG islands on gene promoters silences their transcription. We assessed promoter methylation of the tumour suppressor, p53. p53 promoters of HepG2 cells were significantly hypermethylated in response to FB_1 (Figure 6.4a; $p \leq 0.001$). This led to significant decreases in *p53* gene (Figure 6.4b; $p \leq 0.001$) and p53 protein (Figure 6.4c; $p \leq 0.001$) expression.

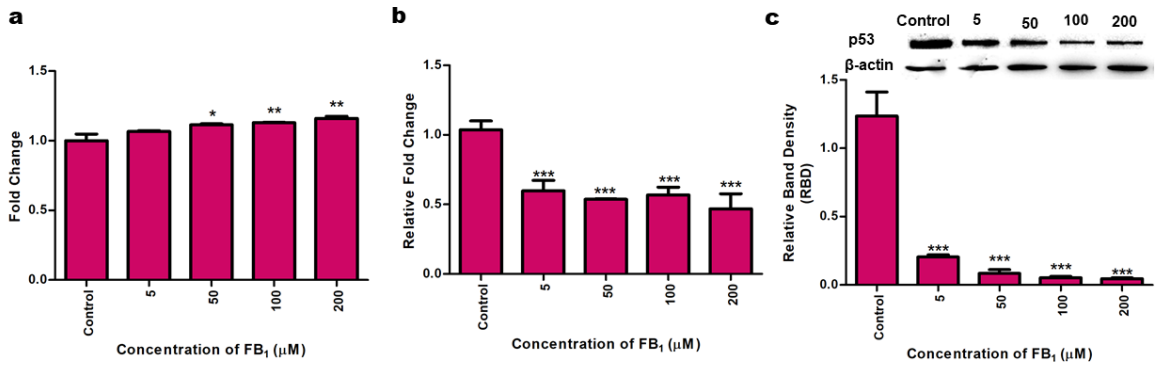

Figure 6.4. Increasing doses of FB_1 led to increasing hypermethylation at *p53* promoter regions (a; *** $p \leq 0.001$) in HepG2 cells. This led to a significant dose-dependent decrease in *p53* gene (b; *** $p \leq 0.001$) and p53 protein (c; *** $p \leq 0.001$) expression.

FB_1 inhibits caspase dependent apoptosis

The p53 tumour suppressor protein plays a major role in apoptosis. One of the mechanisms by which it does this is through caspase activation (Schuler et al., 2000). There was a significant dose-dependent decline in the activity of caspases -3/7 (Figure 6.5a; $p \leq 0.001$), -6 (Figure 6.5b; $p \leq 0.001$), -8 (Figure 6.5c; $p \leq 0.001$) and -9 (Figure 6.5d; $p \leq 0.001$) in the presence of FB_1.

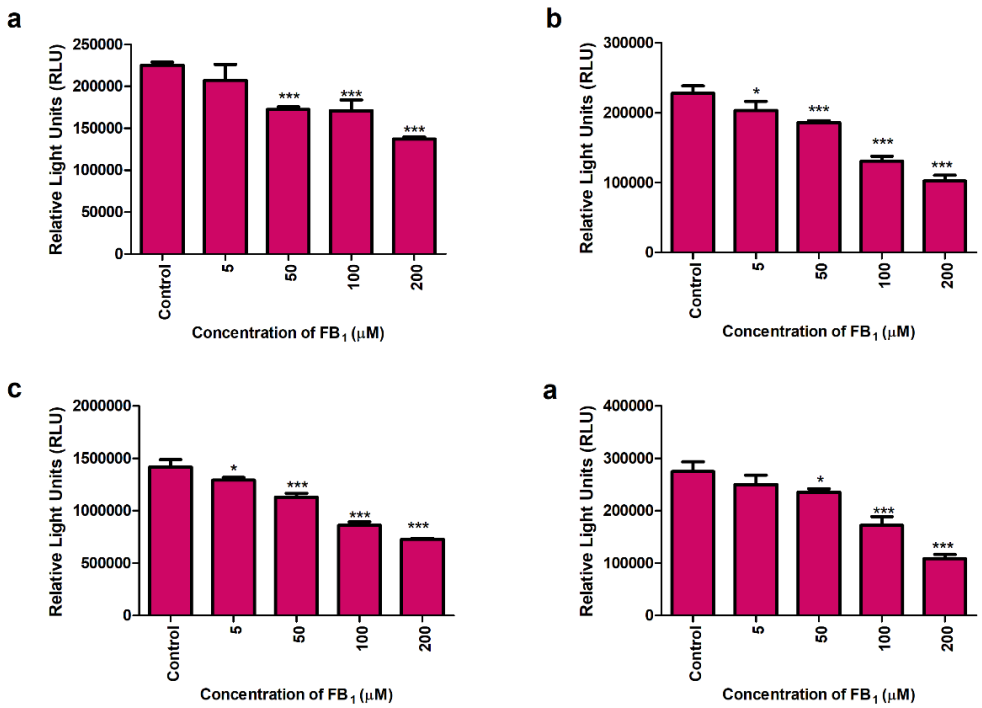

Figure 6.5. The activity of caspases -3/7 (a; *** $p \leq 0.001$), -6 (b; *** $p \leq 0.001$), -8 (c; *** $p \leq 0.001$) and -9 (d; *** $p \leq 0.001$) were decreased in FB_1 treated HepG2 cells.

Discussion

The epigenetic landscape is critical in modulating functional pathways such as apoptosis, proliferation and differentiation; however, it is continuously changing in response to external stimuli such as mycotoxin insult (Marczylo et al., 2016, Huang et al., 2019). FB_1 is regarded as one of the most important mycotoxins as it abundantly contaminates agricultural staples and adversely affects human and animal health (Idahor, 2010, Kamle et al., 2019). FB_1 impacts the epigenetic landscape of humans and animals which may play a role in its toxicity (Mobio et al., 2000, Chuturgoon et al., 2014a, Chuturgoon et al., 2014b, Demirel et al., 2015, Sancak and Ozden, 2015, Gardner et al., 2016, Arumugam et al., 2020). One mechanism studied is the alteration of DNA methylation patterns which contributes to genomic instability as well as effects the expression of genes regulating protein and DNA synthesis, cell cycle, proliferation and apoptosis (Mobio et al., 2000, Kouadio et al., 2007, Chuturgoon et al., 2014a, Demirel et al., 2015). Thus, in this study we evaluated the anti-apoptotic effects of FB_1 by assessing the epigenetic regulation of p53 via the HOXA11-AS/miR-124/DNMT axis.

Once considered irrelevant, lncRNAs are gaining increasing advertence due to our new understanding of the functional role they play (Kung et al., 2013). Although over 146,000 lncRNAs have been documented to date; most have only been predicted and studied via computational analysis (Volders et al., 2015). To determine if FB_1 affected lncRNA profiles of HepG2 cells, we used a lncRNA array and evaluated changes in the expression of 84 lncRNA using an untreated control and IC_{50} [200 µM $FB_{1;}$ (Arumugam et al., 2020)]. We found that FB_1 significantly dysregulated the lncRNA profiles of HepG2 cells and HOXA11-AS was amongst the most upregulated lncRNA (Supplementary Figure S6.1). Thus, we validated HOXA11-AS expression and further investigated its downstream effects following FB_1 exposure.

HOXA11-AS has mainly oncogenic functions, influencing the proliferation, invasion and migration of various cancers such as hepatocellular carcinomas, oesophageal cancer, renal cancer and melanomas (Lu et al., 2017, Yu et al., 2017a, Sun et al., 2018b, Yang et al., 2018a, Liu et al., 2019, Zhang et al., 2019). Conversely, it has tumour suppressor capabilities in epithelial ovarian cancer (Richards et al., 2015). In addition to its carcinogenic effects, HOXA11-AS influences gene expression by modulating epigenetic modifications by functioning as a ceRNA and molecular scaffold (Sun et al., 2016, Wang et al., 2017).

As a ceRNA or RNA “sponge”, HOXA11-AS is able to bind to certain miRNAs blocking the interaction between the miRNA and its target mRNA. This reduces the negative regulatory impact that miRNAs have on their target mRNA. For instance, Lu et al. (2017) demonstrated that HOXA11-AS positively regulated enhancer of zeste homolog 2 (EZH2) expression by sequestering miR-124 and preventing miRNA-124 degradation of EZH2 mRNA. We confirmed the relationship between HOXA11-AS and miR-124 in the liver by using an online bioinformatics prediction algorithm, starBase v2.0

(Supplementary Figure S6.2) (Li et al., 2014). We further validated the ceRNA capabilities of HOXA11-AS by determining the expression of HOXA11-AS and miR-124 in HepG2 cells treated with various concentrations of FB_1 (0, 5, 50, 100 and 200 µM). HOXA11-AS was significantly upregulated in the presence of FB_1 which resulted in the concurrent decrease in miRNA-124 levels (Figure 6.1a, b). This relationship was confirmed using relevant controls as miR-124 expression was significantly elevated in cells where HOXA11-AS was knocked down.

To explore the downstream targets of miR-124, the online bioinformatics tool Targetscan (version 7.2) was employed (Agarwal et al., 2015). We found that miR-124 may influence DNA methylation as we uncovered complementary binding sites between miR-124 and the 3'UTR of *SP1* at positions 524-530, 4149-4155 and 4520-4526 and the 3'UTR of *DNMT3B* at positions 1363-1369 (Figure 6.1c).

DNMT3B directly regulates DNA methylation (Okano et al., 1998, Hervouet et al., 2018); while SP1 indirectly influences DNA methylation as it binds to the cis-element of *DNMT1* gene promoter, activating its transcription (Kishikawa et al., 2002). FB_1-induced HOXA11-AS prevented the degradation of miR-124 targets as DNMT3B gene and protein expression (Figure 6.1d, e) as well as *SP1* gene expression (Figure 6.1d) were significantly upregulated. The use of appropriate controls confirmed this relationship as miR-124 knockdown resulted in a significant increase of its targets; while the use of miR-124 mimic and siR-HOXA11-AS independently downregulated DNMT3B and SP1 expression. Furthermore, several other studies confirmed that HOXA11-AS sequesters miR-124 with one study revealing that HOXA11-AS positively regulates SP1 by sponging miRNA-124 (Cui et al., 2017, Xu et al., 2017, Yu et al., 2017b, Yang et al., 2018b, Jin et al., 2019, Zhang et al., 2019). Since FB_1 altered the expression of *SP1*, we determined if DNMT1 expression was also altered. In agreement with the upregulation of *SP1*, DNMT1 expression was also elevated both at the gene and protein levels (Figure 6.2a, b). Apart from its ceRNA capability, HOXA11-AS can also serve as a molecular scaffold that recruits chromatin modifying proteins such as EZH2, LSD1 and DNMT1 to the promoter region of genes thus modulating their transcription (Wei et al., 2020). For example, HOXA11-AS interacts with DNMT1 and EZH2, recruiting these proteins to the promoter regions of miR-200b and mediating methylation silencing of miR-200b in non-small cell lung cancer cells (Chen et al., 2017). Using RNA immunoprecipitation, we determined if FB_1 influences HOXA11-AS-DNMT1 binding and found that HOXA11-AS-DNMT1 interactions were significantly higher in the presence of FB_1 and reduced with HOXA11-AS knockdown (Figure 6.2c). However, this interaction should be further investigated at specific gene promoters.

Four members make up the DNMT family with DNMT1, -3A and -3B having catalytic capabilities (Hervouet et al., 2018). Seeing as FB_1 altered the expression of DNMT1 and DNMT3B, we determined if FB_1 affects DNMT3A and found the mRNA and protein expression to be significantly upregulated (Figure 6.3a, b). DNMTs are responsible for the transferring methyl groups from S-adenosyl-methionine (SAM) to the 5-position of cytosine residues in DNA (Hervouet et al., 2018). We found that

the increase in DNMT expression corresponded with an increase in total DNA methylation levels (Figure 6.3c); however, our results were not significant and opposed the results of another study which investigated in the effects of 200 µM FB_1 in HepG2 cells (Chuturgoon et al., 2014a). Chuturgoon et al. (2014a) found that FB_1 reduced the expression of DNMTs which resulted in global hypomethylation, however similar to our study, DNA hypermethylation occurred in human intestinal Caco-2 cells and rat C6 glioma cells after 24 hours (Mobio et al., 2000, Kouadio et al., 2007).

While several studies have investigated the effects of FB_1 on global DNA methylation only one other study has looked at its effects on gene-specific methylation (Demirel et al., 2015). Demirel et al. (2015) assessed CpG promoter methylation of tumour suppressor genes in rat liver (clone 9) cells and kidney epithelial (NRK-52E) cells. CpG islands of *VHL* and *e-cadherin* promoters were methylated in both cell lines; while, the *c-Myc* promoter was methylated exclusively in Clone 9 cells and methylation of the *p16* gene occurred in NRK-52E cells. Thus, we investigated the role of DNA methylation on the tumour suppressor p53.

p53 responds to cellular stress such as DNA damage, oxidative stress and cell cycle abnormalities by inducing cell cycle arrest, apoptosis or autophagy (Shieh et al., 1999, Yin et al., 1999, Vousden and Prives, 2009). FB_1 is a known inducer of oxidative stress, DNA damage and altered cell cycle checkpoint regulation (Mobio et al., 2000, Galvano et al., 2002, Stockmann-Juvala et al., 2004, Domijan et al., 2007, Kim et al., 2018, Arumugam et al., 2019, Arumugam et al., 2020). Furthermore, HOXA11-AS is known to repress p53 expression; however, the mechanism is unknown (Connell et al., 2009). Therefore, we evaluated CpG island methylation at *p53* promoters and subsequently p53 expression. We found that the promoter region of *p53* was significantly hypermethylated (Figure 6.4a), leading to the subsequent decrease in p53 transcription and translation (Fig. 4b, c). It is possible that p53 activity may be also be disrupted by FB_1. p53 is activated via a host of post-translational modifications, including phosphorylation (Sakaguchi et al., 1998). FB_1 was shown to inhibit checkpoint kinase 1 (Arumugam et al., 2020), a kinase responsible for p53 phosphorylation and activation (Ou et al., 2005).

One mechanism by which p53 initiates apoptosis is through the activation of the caspase cascade. p53 activates initiator caspases (caspase- 8 and -9) and subsequently downstream effector caspases (caspase-3/7) (Ding et al., 1998, Schuler et al., 2000). p53 is also responsible for the transactivation of effector caspase-6 (Ehrnhoefer et al., 2014). We found a significant decrease in the activity of all 4 of the above-mentioned caspases (Figure 6.5). Taken together, our results suggest that FB_1 inhibits p53-dependent cell death. FB_1 was also found to inhibit apoptosis in HepG2 cells through the upregulation of anti-apoptotic Birc-8/ILP-2 and decrease in apoptotic Smac/DIABLO (Chuturgoon et al., 2015). Together the inhibition of p53 and activation of Birc-8 prevents caspase-dependent apoptosis in the presence of FB_1. However, several other studies have observed stress-induced apoptosis in response to FB_1 exposure (Tolleson et al., 1996, Tolleson et al., 1999, Seefelder et al., 2003). The difference could be due to the models used to examine toxicity. The HepG2 cell line is a cancerous model and thus prefers prosurvival

mechanisms. It is possible that inhibition of p53-dependent apoptosis via the HOXA11-AS/miR-124/DNMT axis may be responsible for promoting FB_1-induced carcinogenesis. However, further studies using cancerous and primary liver cell lines should be conducted to test this hypothesis. Nevertheless, this study provides novel insight into the relationship between HOXA11-As, DNA methylation and p53 expression, which was previously unknown and adds to our understanding on the impact of FB_1 on the human epigenome.

Conclusion

This study revealed that FB_1 upregulated the lncRNA, HOXA11-AS, which in turn sequesters and inhibits miR-124, leading to an increase in SP1, DNMT1, DNMT3A and DNMT3B expression. The increase in DNMTs not only elevated global methylation of FB_1 exposed HepG2 cells but also hypermethylation of *p53* promoters. This led to a decrease in p53 expression and ultimately diminished caspase activity. Therefore, FB_1 inhibits p53-dependent cell death via the HOXA11-AS/miR-124/DNMT axis (Figure 6.6).

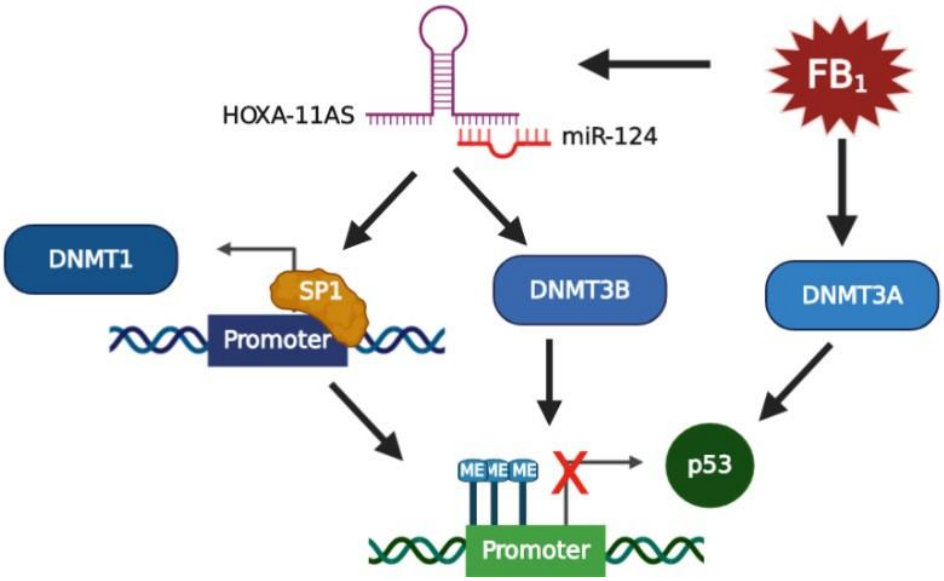

Figure 6.6. FB_1 inhibits p53 via HOXA11-AS/miR-124/DNMT axis. FB_1 enhances HOXA11-AS levels. HOXA11-AS inhibits miR-124, thus preventing the interaction between miR-124 and its target mRNAs (SP1 and DNMT3B). The resulting upregulation of SP1 promotes DNMT1 expression. Moreover, FB_1 enhances DNMT3A levels. The increase in DNMT expression facilitates promoter hypermethylation of p53, reducing p53 transcription and expression.

Declarations

Funding

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethics approval

EthicS was received from the University of Kwa-Zulu Natal's Biomedical Research Ethics Committee. Ethics number: BE322/19.

Availability of data and material

All datasets generated in this study are available from the corresponding author on reasonable request.

Author Contributions

TA, TG, and AC conceptualised and designed the study. TA conducted all laboratory experiments, analysed the data and wrote the manuscript. TG and AC revised the manuscript. All authors have read the manuscript prior to submission.

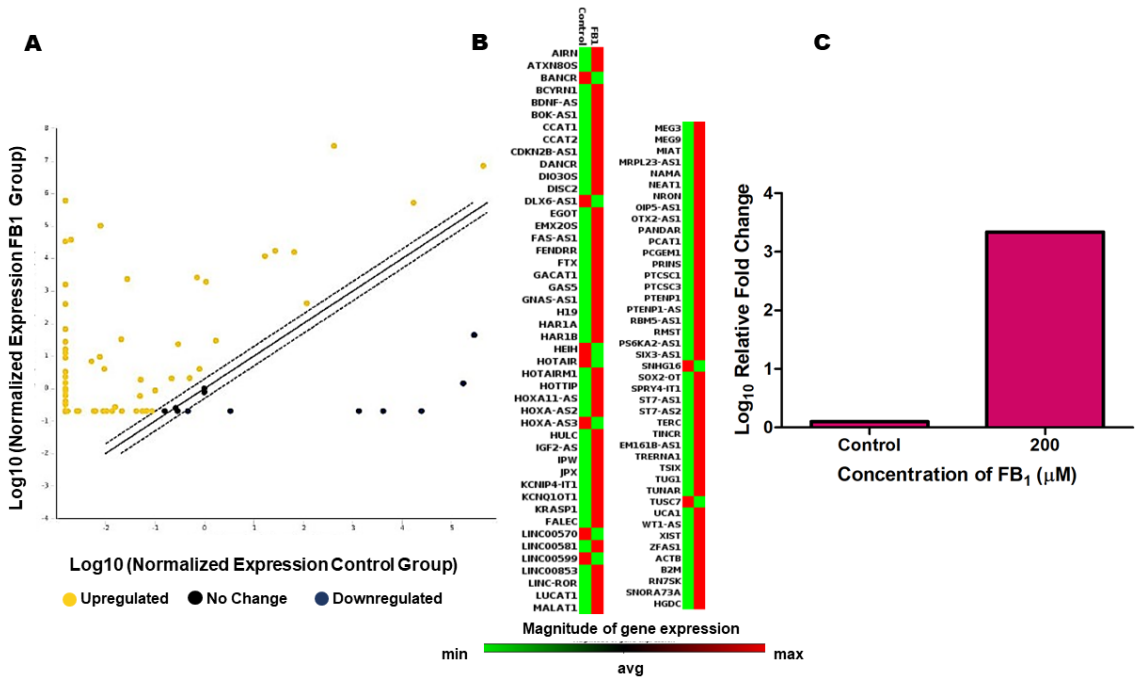
Supplementary Information

Supplementary Table S6.1: Antibodies with dilutions used for western blotting

Antibody	**Dilution**	**Catalogue number (Cell Signaling Technologies)**
Primary Antibodies		
Rabbit-Anti-DNMT1	1:250	5032S
Rabbit-Anti-DNMT3A	1:250	3598S
Rabbit-Anti-DNMT3B	1:250	57868S
Mouse-Anti-p53	1:500	2524S
Secondary Antibodies		
Goat-Anti- Rabbit	1:5000	#7074S
Goat-Anti-Mouse	1:5000	#7076P2

Supplementary Table S6.2: Primer sequences and annealing temperatures used in qPCRs

Gene	Sense Primer 5'→ 3'	Anti-sense Primer 5' → 3'	Annealing Temperature (°C)
qPCR			
DNMT1	ACCGCTTCTACTTCCTCGAGGCCTA	GTTGCAGTCCTCTGTGAACACTGTGG	60
DNMT3A	GGGGACGTCCGCAGCGTCACAC	CAGGGTTGGACTCGAGAAATCGC	58
DNMT3B	CCTGCTGAATTACTCACGCCCC	GTCTGTGTAGTGCACAGGAAAGCC	58
SP1	CTTGGTATCATCACAAGCCAGTT	TCCCTGATGATCCACTGGTAGTA	56
p53	ACTTGTCGCTCTTGAAGCTAC	GATGCGGAGAATCTTTGGAACA	58
GAPDH	TCCACCACCCTGTTGCTGTA	ACCACAGTCCATGCCATCAC	Same as gene of interest
Promoter Methylation			
p53	GTGGATATTACGGAAAGT	AAAATATCCCCGAAACC	54
RNA Immunoprecipitation			
HOXA11-AS	GAGTTTGAAGCCGTGGATGT	AGATGAGGGGAGAGGTGGAT	56



Supplementary Figure S6.1. FB_1 alters lncRNA profiles in HepG2 cells. **A:** Scatter plot showing normalized expression of lncRNA in FB_1 treated HepG2 cells. Yellow dots represent upregulated lncRNA, blue dots represent downregulated lncRNA and black dots represent unchanged lncRNA in FB_1 treated cells compared to the control. **B:** Heatmap showing expression profile of all lncRNA assessed using the array. **C:** Normalized expression of HOXA11-AS using the lncRNA array.

miRNA	GeneID	GeneName	GeneType	TargetSite	Alignment	Class	AgoExpNum	CleaveExpN
hsa-miR-124-3p	ENSG00000240990	HOXA11-AS	antisense	chr7:27225522-27225542[+]	Target: 5' ccgaagCGCUUUAGUGCCUUc 3' |:|: : ||||||| miRNA : 3' ccguaaGUGGCG-CACGGAAu 5'	7mer-m8	1	0

Supplementary Figure S6.2. starBase v2.0 analyses of HOXA11-AS interaction humans.

	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 524-530 of SP1 3' UTR hsa-miR-124-3p.1	5' ...UCAUUUUUUUUAUAUUGCCUUAU... |||||| 3' CCGUAAGUGGCGCACGGAAU
Position 4520-4526 of SP1 3' UTR hsa-miR-124-3p.1	5' ...UUCAGGAAUUACACUGUGCCUUU... ||||||| 3' CCGUAAGUGGCGCACGGAAU
Position 4149-4155 of SP1 3' UTR hsa-miR-124-3p.1	5' ...CAAAUUUGGCUCACU---UGCCUUAG... |||| |||||| 3' CCGUAAGUGGCGCACGGAAU

	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 1363-1369 of DNMT3B 3' UTR hsa-miR-124-3p.1	5' ...UAUGGGGAAAAAACUGUGCCUUG... ||||||| 3' CCGUAAGUGGCGCACGGAAU

Supplementary Figure S6.3. TargetScan analyses of miR-124 to the 3' UTR of *SP1 and DNMT3B* in humans. MiR-124 has complementary base pairs with the 3' UTR of *SP1* at positions 524-530, 4149-4155 and 4520-4526 and *DNMT3B* at positions 1363-1369 in humans.

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CHAPTER 7

CONCLUSION

7.1. General conclusions

Epigenetic modifications are necessary for normal development and health; however, environmental factors such as mycotoxin exposure disrupts the epigenome of cells often leading to toxicity (Huang et al., 2019). Many studies have focused on the health implications of FB_1 as well as molecular mechanisms involved in its toxicity (Wang et al., 1991, Yin et al., 2016, Kouzi et al., 2018, Arumugam et al., 2019, Arumugam et al., 2020, Liu et al., 2020). Furthermore, some studies have evaluated epigenetic changes that occur due to FB_1 exposure but these studies mainly focused on DNA methylation and histone modifications and most failed to assess the downstream implications of these epigenetic changes (Mobio et al., 2000, Kouadio et al., 2007, Pellanda et al., 2012, Chuturgoon et al., 2014a, Chuturgoon et al., 2014b, Demirel et al., 2015, Sancak and Ozden, 2015, Gardner et al., 2016).

This study, for the first time, demonstrates that FB_1 not only alters the epigenetic landscape in HepG2 cells; but that these epigenetic modifications affect cellular responses to FB_1 mediated stress. Furthermore, it is the first study to evaluate the effect of FB_1 on the m6A epitranscriptome and lncRNAs.

FB_1 induced oxidative damage to DNA of HepG2 cells. PTEN is vital in maintaining genomic stability and DNA repair, while its inactivation or downregulation promotes DNA instability and damage (Ming and He, 2012, Bassi et al., 2013). Downregulation of PTEN activates PI3K/AKT signaling which inhibits CHK1 activity and DNA damage checkpoint signaling (Puc et al., 2005, Puc and Parsons, 2005). Therefore, epigenetic modifications that affect PTEN expression were evaluated in the presence of FB_1. FB_1 reduced the expression of histone demethylase, KDM5B which in turn resulted in the significant increase in global H3K4me3. H3K4me3 was also elevated at the promoter region of *PTEN*, where it activated *PTEN* transcription. While there was a significant increase in *PTEN* mRNA levels, FB_1 reduced the protein expression of PTEN. PTEN is post-transcriptionally regulated by miR-30c (Hu et al., 2019). FB_1 upregulated miR-30c, which inhibited the translation of *PTEN*, resulting in reduced PTEN protein expression. PTEN is a negative regulator of PI3K/AKT signaling (Cantley and Neel, 1999). The downregulation of PTEN permitted PI3K/AKT signaling to proceed undisturbed, resulting in the inhibitory phosphorylation of serine-280-CHK1. Inhibition of CHK1 prevents DNA repair and promotes genomic instability. This may contribute to the toxicity and carcinogenicity of FB_1.

Alterations to the m6A epitranscriptome have been linked to the toxic effects of some *Fusarium* mycotoxins (Ghazi et al., 2020, Zhengchang et al., 2020). Furthermore, m6A is influenced by cellular stresses such as oxidative stress and may in turn regulate responses to oxidative stress (Zhao et al., 2020). Intracellular ROS and global m6A levels were both elevated in HepG2 cells exposed to FB_1. Furthermore, FB_1 upregulated m6A methyltransferases (*METTL3* and *METTL14*) and downregulated m6A demethylases (*FTO* and *ALKBH5*); contributing to the elevation of global m6A levels observed.

FB_1-induced increases in m6A levels may lead to the altered expression of important genes involved in its toxicity. Considering that there was an accumulation of intracellular ROS, the effect of m6A on Keap1/Nrf2 signaling was determined. Additional epigenetic changes to Keap1/Nrf2 were also evaluated. FB_1 induced hypermethylation of the *Keap*1 promoter region, which inhibited *Keap*1 transcription; 29 possible m6A sites with the consensus motifs: GGACU and AGACU, were predicted on *Keap1* transcripts. M6A-*Keap*1 levels were upregulated; however, Keap1 protein expression was reduced. FB_1 increased the m6A reader *YTHDF2*, which may be responsible for inhibiting Keap1 translation. Hypomethylation of Nrf2 promoters together with decreased miR-27b upregulated *Nrf2* mRNA levels in HepG2 cells exposed to FB_1; 54 possible m6A sites with the consensus motif GAACU were predicated on *Nrf2* transcripts. FB_1 elevated m6A-*Nrf2* and Nrf2 protein expression. The increase in m6A readers *YTHDF1*, *YTHDF3* and *YTHDC2* may be responsible for promoting Nrf2 translation. The downregulation of Keap1 and upregulation of Nrf2 activates antioxidant responses, which was previously observed (Arumugam et al., 2019). However, severe cellular injury occurred in cells exposed to FB_1, suggesting that the activation of Nrf2 antioxidant signaling may not be sufficient to counter the accumulation of ROS. Furthermore, prolonged activation of Nrf2 by epigenetic changes may support the cancerous phenotype observed in some models exposed to FB_1.

The tumor suppressor, p53 is activated by cellular stress such as genotoxic and oxidative stress. When activated, p53 regulates several stress responses such as cell cycle arrest, DNA repair and apoptosis (Fridman and Lowe, 2003). However, p53 inactivation by epigenetic modifications inhibits its response to stress and promotes carcinogenesis (Saldaña-Meyer and Recillas-Targa, 2011, Chmelarova et al., 2013). FB_1 elevated the expression of the lncRNA, HOXA11-AS. HOXA11-AS sequestered miR-124, inhibiting its regulation of DNMT3B and SP1. Therefore, FB_1 upregulated the expression of DNMT3B and the DNMT1 transcription factor, SP1 as well as the expression of DNMT1 and DNMT3A. The increase in DNMT expression facilitated global DNA hypermethylation and *p53* promoter hypermethylation. This led to the decrease in both p53 gene and protein expression. p53 is known to activate caspase-dependent apoptosis during cellular stress. The decrease in p53 inhibited caspase-mediated apoptosis as observed by the decrease in the activity of initiator caspases-8 and -9 as well as executioner caspases-3/7 and -6. It is possible that the inhibition of p53-dependent apoptosis via the HOXA11-AS/miR-124/DNMT axis may be responsible for promoting FB_1-induced carcinogenesis.

Taken together, this study suggests that FB_1 induces hepatoxicity in the form of DNA damage and oxidative stress. FB_1 also alters the epigenome of liver cells by affecting DNA methylation, m6A RNA methylation, H3K4me3, miRNA (miR-30c, miR-27b and miR-124) and lncRNA (HOXA11-AS). These epigenetic changes in turn disrupt the DNA damage and anti-oxidant response mechanisms further exacerbating FB_1-induced hepatotoxicity. Furthermore, the epigenetic downregulation of the tumor suppressor proteins PTEN and p53, together with inhibition of DNA repair, activation of Nrf2

and dysregulation of apoptosis, provides a potential mode of action by which FB_1 may induce or promote hepatocellular carcinomas.

7.2. Limitations, shortcomings and recommendations

This study provides novel mechanisms for FB_1-induced hepatotoxicity at the epigenetic level using an *in vitro* model that was acutely exposed (24 hours) to FB_1. However, the following limitations and shortcomings were found:

- *In vitro* models usually consist of a single cell type (in this study, HepG2 cells) grown in monolayer and are therefore not exact dissociated replicates of their *in vivo* counterparts. This limits our interpretations of epigenetic patterns and interactions between the various cell types found in a multicellular organism. The use of *in vivo* models may express different patterns of epigenetic changes with different outcomes on stress response signaling that may be more accurate than the use of an *in vitro* model.
- Maize is considered a staple in many developing countries and thus may be consumed on a daily basis. Humans and animals that are heavily reliant on maize are recurrently exposed to FB_1. The use of an acute model such as the one used in this study (24 hours) may not provide realistic epigenetic patterns. Additionally, while HepG2 cells were exposed to a range of FB_1 concentrations (0-200 µM) which included an IC_{50}, it may not provide realistic results that are pertinent to humans.
- While we can conclude that hepatoxicity induced by FB_1 may be a result of its epigenetic properties, we cannot say with confidence that epigenetic mechanisms identified in this study also contribute to the carcinogenic nature of FB_1. This is because a cancerous cell line was used in this study. The use of a primary cell line along with a cancerous cell line should be used to evaluate whether these FB_1-induced epigenetic alterations to stress responses contributes to its carcinogenicity.

Taking the limitations of this study into consideration, chronic exposure (greater than 24 h) to FB_1 or the use of an *in vivo* model may exhibit different patterns of epigenetic changes with different outcomes on stress response signaling. The outcomes observed may provide more realistic results than the ones found in the current study. Furthermore, the concentration of FB_1 used in experiments should be calculated based on the average daily intake of FB_1 and not a range based on the IC_{50}. Hence this study provides insight for future epigenetic studies using longer exposure times to FB_1, more accurate concentrations or *in vivo* models.

7.3. Final remarks

Collectively, this study suggests that FB_1 possesses epigenetic properties which dysregulate cellular responses to FB_1-induced stress, further exacerbating its toxicity and possibly carcinogenicity.

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ADDENDUM A

The following study titled, **"Fumonisin B_1-induced oxidative stress triggers Nrf2-mediated antioxidant response in human hepatocellular carcinoma (HepG2) cells"** set the foundation for this study.

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ORIGINAL ARTICLE

Fumonisin B_1-induced oxidative stress triggers Nrf2-mediated antioxidant response in human hepatocellular carcinoma (HepG2) cells

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Abstract

Fumonisin B_1 (FB_1), a causative agent for animal-related mycotoxicoses, has been implicated in human and animal cancer. FB_1 induces oxidative stress but the related survival responses are not well established. Central to this response is the transcription factor, nuclear factor erythroid 2 p45–related factor 2 (Nrf2). The effects of FB_1 on Nrf2-related survival responses in human hepatoma (HepG2) cells were investigated. HepG2 cells were treated with 200 μmol/l FB_1 (IC_{50}–24 h). Cellular redox status was assessed via the quantification of intracellular reactive oxygen species (ROS), lipid peroxidation, protein oxidation and the antioxidant glutathione (GSH). The protein expression of oxidative stress and mitochondrial stress response proteins [Nrf2, phosphorylated-Nrf2 (pNrf2), superoxide dismutase 2 (SOD2), catalase (CAT), sirtuin 3 (Sirt 3) and Lon-protease 1 (Lon-P1)] were quantified by western blotting, while gene expression levels of *SOD2*, *CAT* and *GPx* were assessed using quantitative polymerase chain reaction (qPCR). Lastly, the fluorometric, JC-1 assay was used to determine mitochondrial polarisation. FB_1 significantly increased ROS ($p \leq 0.001$), and induced lipid peroxidation ($p < 0.05$) and protein carbonylation ($p \leq 0.001$), which corresponded with the increase in GSH levels ($p < 0.05$). A significant increase in pNrf2, SOD2, *SOD2*, CAT ($p < 0.05$), *CAT* ($p \leq 0.01$) and *GPx* ($p \leq 0.001$) expression was observed; however, total Nrf2 ($p > 0.05$) was reduced. There was also a minor reduction in the mitochondrial membrane potential of HepG2 cells ($p < 0.05$); however, the expression of Sirt 3 and Lon-P1 ($p \leq 0.001$) were upregulated. Exposure to FB_1 induced oxidative stress in HepG2 cells and initiated Nrf2-regulated transcription of antioxidants.

Keywords Fumonisin B_1 · Oxidative stress · Reactive oxygen species · Antioxidants · Nuclear factor erythroid 2–related factor 2

Introduction

Maize forms a vital part of the African staple diet due to its high yields, adaptability to different climates, versatile uses and storage capabilities. However, it is commonly contaminated by fungi, which produce toxic secondary metabolites known as mycotoxins (Fandohan et al. 2003). *Fusarium verticillioides* and *F. proliferatum* are amongst the most common maize–associated fungi and the most abundant producers of the fumonisin family of mycotoxins. Fumonisin B_1 (FB_1) is the most frequent and toxic of the 28 fumonisin analogues that have been identified (Rheeder et al. 2002).

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FB_1 exerts toxicity by disrupting the de novo biosynthesis of sphingolipids and altering plasma membrane composition, signal transduction and cell cycle regulation. As a structural analogue of sphingoid bases, FB_1 can competitively inhibit ceramide synthase, the enzyme responsible for the acetylation of sphingoid bases. This inhibitory action leads to the accumulation of sphinganine and sphingosine to cytotoxic levels (Riley et al. 2001).

The liver and kidney are major targets of FB_1 toxicity in almost all animal species tested (Riley and Voss 2006). Additional species specific effects such as equine leukoencephalomalacia, porcine pulmonary oedema and the development of carcinomas in rodents have also been reported (Marin et al. 2013; Ross et al. 1990). Furthermore, epidemiological studies in humans have shown a correlation between high consumption of maize and incidence of oesophageal and hepatocellular carcinomas (Rheeder 1992; Shephard et al. 2007; Sun et al. 2007). Exposure to FB_1 is also associated with the high prevalence of neural tube defects and the induction of oxidative stress leading to DNA, lipid and protein damage (Marasas et al. 2004; Mary et al. 2012; Stockmann-Juvala and Savolainen 2008).

Oxidative stress occurs when the balance between reactive oxygen species (ROS) and antioxidants shifts towards ROS. The electron transport chain (ETC), found within the mitochondria, leaks unpaired electrons into the mitochondrial matrix during respiration (Turrens 2003). These electrons react with molecular oxygen to form ROS (Lenaz and Genova 2010; Sena and Chandel 2012). FB_1 disrupts mitochondrial respiration by inhibiting complex I of the ETC, elevating ROS generation (Domijan and Abramov 2011). Previous studies have identified oxidative stress as a consequence of FB_1 exposure. However, the role of the ensuing antioxidant response in the context of FB_1 toxicology has not been well established (Khan et al. 2018; Mary et al. 2012; Wang et al. 2016).

The first line of defence against oxidative stress in cells is the induction of antioxidants, which scavenge ROS and dampen oxidative damage to macromolecules (Birben et al. 2012). Nuclear factor erythroid 2 p45–related factor 2 (Nrf2) is a transcription factor that activates the antioxidant response element (ARE), a regulatory element found in the promoters of several cytoprotective and antioxidant genes including glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) (Nguyen et al. 2009). Under basal physiological conditions, Nrf2 is sequestered in the cytoplasm and undergoes constant degradation through Kelch-like ECH-associated protein 1 (Keap-1) ubiquitination. The Nrf2-ECH homologue h2 (Neh2) domain of Nrf2 contains several lysine residues that are targets for Keap-1 ubiquitination via cullin 3 (CUL3) ubiquitin ligase (Rojo de la Vega et al. 2016). This prevents Nrf2 from translocating to the nucleus and activating antioxidant transcription. When cells experience oxidative stress, the Keap1-Nrf2 stress response pathway is activated. Elevated levels of ROS oxidise specific cysteine residues in Keap-1, weakening its ability as a ligase adapter. This leads to the dissociation of Keap-1 from the Neh2 domain, allowing the accumulation of Nrf2 in the cytosol. Nrf2 is thus free to enter the nucleus, where it dimerises with small Maf proteins and binds to the ARE; promoting the transcription of antioxidant genes (Fig. 1) (Bellezza et al. 2018; Buendia et al. 2016; Furukawa and Xiong 2005; Itoh et al. 1997; Itoh et al. 1999; Kensler et al. 2007; Ma 2013; Nguyen et al. 2003; Ray et al. 2012; Valko et al. 2007).

Considering that FB_1 inhibits the ETC, which may enhance the production of ROS within the mitochondria; survival responses related to the mitochondria may be noteworthy (Domijan and Abramov 2011; Stockmann-Juvala and Savolainen 2008). Sirtuin 3 (Sirt 3) and the mitochondrial Lon-protease 1 (Lon-P1) help maintain homeostasis within the mitochondria during oxidative stress (Bause and Haigis 2013; Pinti et al. 2015). Lon-P1 dampens the effects of oxidative stress by degrading oxidised proteins, while Sirt 3 deacetylates antioxidant proteins such as SOD2 and CAT, increasing their capacity to detoxify ROS (Ngo et al. 2013; Weir et al. 2013).

Although a number of studies have investigated, the effect of FB_1 on ROS production and oxidative damage, the Nrf2-antioxidant response has not been thoroughly investigated. This study focussed on the effect of FB_1 on Nrf2-related survival responses in human hepatoma (HepG2) cells.

Materials and methods

Materials

FB_1, isolated from *Fusarium verticillioides*, was obtained from Sigma-Aldrich (St Louis, MO, USA). The HepG2 cell line was acquired from Highveld Biologicals (Johannesburg, South Africa). Cell culture reagents and supplements were purchased from Lonza Bio-Whittaker (Basel, Switzerland). Western blot reagents were procured from Bio-Rad (Hercules, CA, USA) and anti-bodies were purchased from Abcam (Cambridge, UK), Sigma-Aldrich (St Louis, MO, USA), Cell Signalling Technologies (Danvers, MA, USA) and Santa Cruz (Dallas, TX, USA). All other reagents were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

Cell culture

HepG2 cells were cultured in monolayer (10^6 cells per 25 cm^3 culture flask) with complete culture media [CCM: Eagle's Essential Minimal Media (EMEM) supplemented

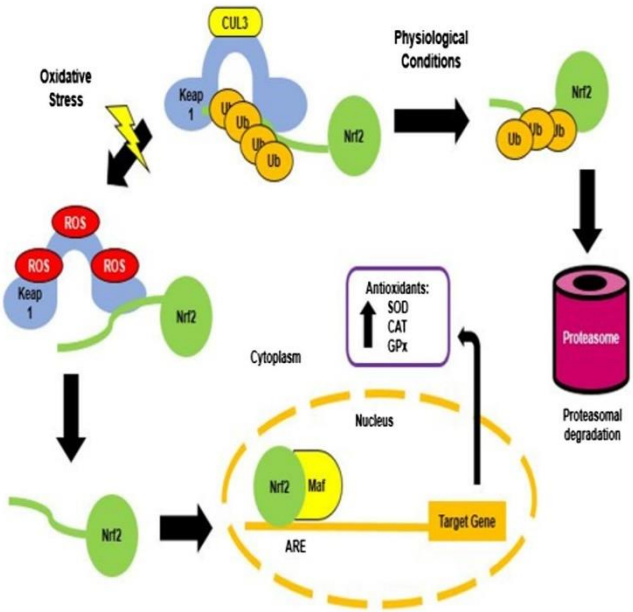

Fig. 1 The Keap-1-Nrf2-mediated antioxidant response. Under physiological conditions, Nrf2 is ubiquitinated (Ub) by Keap-1-CUL3 system and degraded within the proteasome. Exposure to high levels of ROS disrupts Keap-1-CUL3 ubiquitination of Nrf2. This triggers the release and subsequent translocation of Nrf2 to the nucleus, where it dimerises with Maf and promotes the transcription of antioxidants such as SOD, CAT and GPx

with 10% foetal calf serum, 1% penstrepfungizone and 1% L-glutamine] at 37 °C in a humidified incubator. Cells were allowed to reach 80% confluence in 25 cm^3 flasks before treatment with an IC_{50} of 200 μmol/l FB_1 in CCM for 24 h (Chuturgoon et al. 2015). An untreated control, containing only CCM, was also prepared.

Reactive oxygen species analyses

Intracellular ROS was quantified using the fluorometric 2′,7′-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) assay. Control and treated cells (50,000 cells per treatment) were incubated in 500 μl of 5 μmol/l H_2DCF-DA stain (30 min, 37 °C). The stain was removed via centrifugation (400×*g*, 10 min, 24 °C) and cells were washed twice with 0.1 mol/l phosphate buffer saline (PBS). Cells were resuspended in 400 μl of 0.1 mol/l PBS and seeded in triplicate (100 μl/well) in a 96-well opaque microtiter plate. A blank consisting of only 0.1 mol/l PBS was plated in triplicate as well. Fluorescence was measured with Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA) using a blue filter with an excitation wavelength (λex) of 503 nm and emission wavelength (λem) of 529 nm. The fluorescence of each sample was calculated by subtracting the average fluorescence of the blank from the fluorescence of each sample.

Lipid peroxidation assessment

The thiobarbituric acid reactive substances (TBARS) assay measured lipid peroxidation by-products—malondialdehyde (MDA) and other TBARS as a measure of oxidative damage to lipids. TBARS assay was conducted as per the method described by Sheik Abdul et al. (2016). Absorbance of the samples was read using a spectrophotometer, λ = 532/600 nm. The TBARS content was expressed in terms of MDA-TBA adduct.

Protein isolation

Protein was isolated using 200 μl of cell lysis buffer (50 mmol/l HEPES, 1% Triton ×100, 10% glycerol, 50 mmol/l NaCl) for the protein carbonyl assay and 200 μl of Cytobuster™ (Novagen, USA) supplemented with protease and phosphatase inhibitors (Roche, 05892791001 and 04906837001, respectively) for western blotting.

Cells were incubated in the respective lysis solutions on ice for 10 min, then mechanically lysed and decanted into microcentrifuge tubes. The cell lysate was centrifuged (13,000×*g*, 10 min, 4 °C) to obtain crude protein; which was quantified using the bicinchoninic acid (BCA) assay. Bovine serum albumin standards (0–1 mg/ml) were prepared and 25 μl of the standards and samples (triplicate) were dispensed into a 96-well microtiter plate. BCA working solution (196 μl BCA 4 μl

$CuSO_4$ per well) was dispensed into each well, followed by a 30 min incubation at 37 °C. The optical density of the samples was measured at 562 nm using a spectrophotometer (Bio-Tek μQuant, Winooski, VT, USA). The mean absorbance values of the standards were used to construct a standard curve, which determined the protein concentration of the samples. Quantified proteins were standardised to 1 mg/ml.

Protein carbonyl analysis

Protein oxidation was measured via the quantification of intracellular protein carbonyl groups. Standardised protein was incubated at room temperature (RT) for 1 h with 2,4-dinitrophenylhydrazine (DNPH) (800 μL). A blank, which consisted of standardised protein from control cells and 2.5 mol/l HCl (800 μl) was also prepared. Proteins were precipitated with 20% Trichloroacetic acid (1 ml), vortexed and centrifuged (2000×*g*, 10 min, 24 °C). The pellet was washed twice with 1 ml ethanol-ethyl acetate (1:1) and dissolved in 6 mol/l guanidine-HCl (1 ml). Samples were incubated (10 min, 37 °C) before any insoluble material was removed with centrifugation (2000×*g*, 10 min, 24 °C). The supernatant was collected and dispensed in triplicate in 96-well plate (100 μl/well). Absorbance was measured at $\lambda = 370$ nm with a spectrophotometer. The corrected absorbance was calculated by subtracting the mean absorbance of the blank from the absorbance of samples. The concentration of protein carbonyls was obtained by dividing the corrected absorbance by the absorption co-efficient of DNP (22,000 l mol^{-1} cm^{-1}). Results were expressed in nanomoles per milligram.

Protein expression

The protein expressions of pNrf2, Nrf2, SOD2, CAT, Sirt 3 and Lon-P1 were determined by western blotting. Standardised protein samples were boiled in Laemmeli buffer [dH_2O, 0.5 mol/l Tris-HCl (pH 6.8), glycerol, 10% sodium dodecyl sulphide (SDS), b-mercaptoethanol, 1% bromophenol blue] for 5 min. Proteins (25 μl) were separated by electrophoresis on SDS-polyacrylamide electrophoresis gels (4% stacking gel; 10% resolving gel) and electro-transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in Tween 20-Tris buffer saline (TTBS 150 mmol/l NaCl, 3 mmol/l KCl, 25 mmol/l Tris, 0.05% Tween 20, dH_2O, pH 7.5) for 1 h, and incubated with primary antibody [pNrf2 (ab76026); Nrf2 (ab31163); SOD2 (HPA001814); CAT (C0979), Sirt3 (C73E3), Lon-P1 (HPA002034)] in 5% BSA in TTBS (1:1000 dilution) overnight at 4 °C. Following overnight incubation, membranes were equilibrated to RT and washed with TTBS (5 times, 10 min). Membranes were subsequently probed with horseradish peroxidase-conjugated secondary antibody [Rabbit (sc-2004); Mouse (sc-2005)] in 5% BSA in TTBS (1:10,000) for 1 h at RT. Thereafter, membranes were washed with TTBS (5 times, 10 min) and immunoreactivity was detected (Clarity Western ECL Substrate) with the Bio-Rad Chemidoc gel documentation system. After detection, membranes were quenched with 5% H_2O_2 for 30 min, incubated in blocking solution (5% BSA for 1 h at RT), rinsed thrice in TTBS, and probed with HRP-conjugated anti-β-actin (housekeeping protein). Protein expression was analysed by the Image Lab Software version 5.0 (Bio-Rad) and the results were expressed as relative band density (RBD). The expression of proteins of interest was normalised against β-Actin.

Glutathione analysis

The GSH status of HepG2 cells was measured using the GSH-Glo™ Glutathione assay. Cells were dispensed in an opaque microtiter plate (50 μl of 20,000 cells/well in 0.1 mol/l PBS) in triplicate. GSH standards (0–50 μmol/l) were prepared from a 5 mmol/l stock of GSH using 0.1 mol/l PBS and dispensed in triplicate. GSH-Glo reaction solution (50 μl) was added to each well and the plate was left in the dark (RT, 30 min). After the 30-min incubation, luciferin detection reagent (100 μl) was dispensed into each well and the plate was incubated (RT, 15 min). The luminescence emitted by the cells was measured by a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA). The GSH standards were used to prepare a standard curve, which was used to facilitate conversion of luminescence (RLU) to GSH concentration (μmol/l).

RNA analysis

Total RNA was isolated according to the method described by Chuturgoon et al. (2014). Isolated RNA was quantified (Nanodrop 2000, ThermoScientific, Waltham, USA) and standardised to 1000 ng/μl. cDNA was synthesised from standardised RNA using the iScript cDNA synthesis kit (Bio-Rad). Thermocycler conditions for cDNA synthesis were 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min and a final hold at 4 °C (Nagiah et al. 2015).

Gene expression was analysed using the SsoAdvanced™ Universal SYBR® Green Supermix kit (Bio-Rad). The mRNA expressions of *CAT*, *SOD2* and *GPx* were investigated using specific forward and reverse primers (Table 1). Reaction volumes which consisted of the following were prepared: SYBR green (5 μl), forward primer (1 μl), reverse primer (1 μl), nuclease free water (2 μl) and cDNA template (1 μl). All reactions were carried out in triplicate.

The samples were amplified using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The initial denaturation occurred at 95 °C (4 min). Thereafter, 37 cycles of denaturation (15 s, 95 °C), annealing (40 s; temperatures—

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Table 1 The annealing temperatures and primer sequences for the genes of interest

Gene	Annealing temperature	Primer	Sequence
CAT	58 °C	Forward	5′-TAAGACTGACCAGGGCATC-3′
		Reverse	5′-CAACCTTGGTGAGATCGAA-3′
GPx	58 °C	Forward	5′-GACTACACCCAGATGAACGAGC-3′
		Reverse	5′-CCCACCAGGAACTTCTCAAAG-3′
SOD	57 °C	Forward	5′-GAGATGTTACACGCCCAGAT AGC-3′
		Reverse	5-AATCCCCAGCAGTGGAATAAGG-3′
GAPDH		Forward	5′-TCCACCACCCTGTTGCTGTA-3′
		Reverse	5′-ACCACAGTCCATGCCATCAC-3′

Table 1) and extension (30 s, 72 °C) occurred. The method described by Livak and Schmittgen (2001) was employed to determine the changes in relative mRNA expression, where $2^{-\Delta\Delta Ct}$ represents the fold change relative to the untreated control. The expression of the gene of interest was normalised against the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was amplified simultaneously under the same conditions.

Mitochondrial membrane potential

The mitochondrial membrane potential (Δψm) was measured by the JC-1 stain (Zheng et al. 2013). Control and treated cells (50,000 cells per treatment) were incubated in 200 µl of 5 µg/ml JC-1 stain (BD Biosciences, San Jose, NJ, USA) (20 min, 37 °C). The stain was removed via centrifugation (400×*g*, 10 min, 24 °C) and the cells were washed twice with JC-1 staining buffer. Cells were re-suspended in 400 µl of JC-1 staining buffer and seeded in an opaque 96-well plate in triplicate (100 µl/well). A blank, which consisted of only JC-1 staining buffer, was plated in triplicate as well (100 µl/well). Fluorescence was quantified on a Modulus™ microplate reader (Turner Biosystems, Sunnyvale, CA). JC-1 monomers were measured with a blue filter (λex = 488 nm, λem = 529 nm) and JC-1 aggregates were measured with a green filter (λex = 524 nm, λem = 594 nm). The Δψm of the HepG2 cells was expressed as the fluorescence intensity ratio of JC-1 aggregates and JC-1 monomers (Zheng et al. 2013).

Statistical analysis

GraphPad Prism version 5.0 (GraphPad Software Inc., California) was used to perform all statistical analyses. The unpaired *t* test was used for all assays. All results were represented as the mean ± standard deviation unless otherwise stated. A value of $p < 0.05$ was considered statistically significant.

Results

Assessment of oxidative stress

Oxidative stress parameters were quantified in HepG2 cells post-FB_1 exposure. The H_2DCF-DA assay revealed a highly significant ($p = 0.0002$) 3.34-fold increase (Fig. 2a) in intracellular ROS generated by FB_1 exposure (116,000 ± 9020 RFU) compared to control cells (34,700 ± 5740 RFU). As shown in Fig. 2b, the concentration of MDA-TBA adducts were significantly higher ($p = 0.0205$) in FB_1 exposed cells (FB_1 0.186 ± 0.007 µmol/l) compared to the control (0.152 ± 0.014 µmol/l). FB_1 also induced protein oxidation (Fig. 2c),

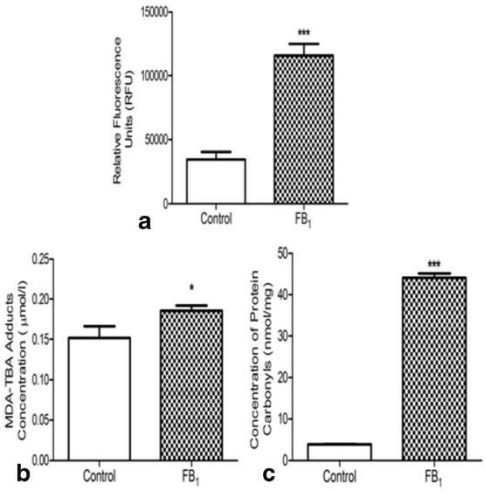

Fig. 2 Effects of FB_1 on cellular oxidation. **a** Intracellular ROS levels represented as relative light units (RLU) produced after H_2DCF-DA staining in control and FB_1-treated HepG2 cells. **b** Concentration (µmol/l) of MDA-TBA adducts. **c** Concentration (ng/mol) of proteins carbonyls formed after 24 h-exposure to FB_1, where a single asterisk represents significance $p < 0.05$ and a triple asterisk represents significance $p < 0.001$

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as evidenced by a significant ($p < 0.0001$) 11.3-fold elevation in the formation of protein carbonyls in FB_1 (44.1 ± 1.10 nmol/mg) exposed cells in relation to control cells (3.89 ± 0.120 nmol/mg). The observed increase in intracellular ROS and corresponding increase in lipid peroxidation and protein carbonylation indicated that oxidative stress was induced in HepG2 cells following FB_1 exposure.

The antioxidant response

Antioxidant regulation

Elevated ROS generated by FB_1 altered the antioxidant status in HepG2 cells. The transcription factor Nrf2 is the master regulator of endogenous antioxidants (Vomund et al. 2017). Western blot analysis revealed that the expression of total Nrf2 (Fig. 3a) was slightly reduced ($p = 0.111$) after a 24-h exposure to FB_1 (0.246 ± 0.037 RBD) when compared to control cells (0.371 ± 0.100 RBD). High concentrations of ROS normally activate phosphorylation pathways which in turn results in phosphorylation and nuclear translocation of Nrf2 (Bo et al. 2015). A significant 1.9-fold increase in the expression of active pNrf2 was observed in cells treated with FB_1 ($p = 0.0311$; control 7.24 ± 0.857 RBD vs FB_1 13.7 ± 3.33 RBD—Fig. 3b).

Superoxide detoxification

The transcription of the mitochondrial detoxification enzyme, SOD2, is regulated by Nrf2 (Bo et al. 2015). SOD2 expression was significantly elevated at both mRNA ($p = 0.0172$; control $1.00 \pm 6.08 \times 10^{-6}$ fold vs FB_1 1.76 ± 0.335 fold—Fig. 4a) and protein levels ($p = 0.004$; control 0.924 ± 0.083 RBD vs FB_1 4.48 ± 0.848 RBD—Fig. 4b).

Detoxification of peroxides

Hydrogen peroxide is detoxified by CAT and GPx (Murphy 2009; Turrens 2003). CAT mRNA levels (Fig. 5a) were significantly ($p = 0.009$) upregulated 1.5-fold in FB_1 treatments.

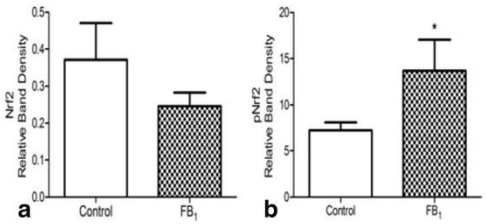

Fig. 3 Effect of FB_1 on Nrf2 and pNrf2. **a** Protein expression of total Nrf2. **b** Protein expression of pNrf2, where a single asterisk represents significance $p < 0.05$

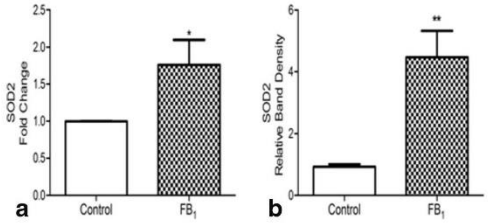

Fig. 4 Levels of SOD2 in HepG2 cells exposed to FB_1. **a** mRNA levels. **b** Protein expression, where a single asterisk represents significance $p < 0.05$ and a double asterisk represents significance $p < 0.01$

This was further confirmed by an increase in *CAT* protein expression after FB_1 exposure ($p = 0.073$; control 0.272 ± 0.092 RBD vs FB_1 0.492 ± 0.128 RBD—Fig. 5b).

The qPCR results for *GPx* (Fig. 5c) showed a highly significant ($p = 0.0001$) 1.9-fold upregulation in FB_1-exposed cells. The concentration of GSH ($p = 0.012$; Fig. 5d), a cofactor for GPx, was 2.27-fold greater in FB_1-treated cells (17.5 ± 3.75 μM) in relation to control cells (7.71 ± 1.14 μM).

Mitochondrial stress responses

Mitochondrial health and function can be determined by measuring the mitochondrial membrane potential (Δmψ) (Sakamuru et al. 2016). The JC-1 assay was used to determine Δmψ and found that it was slightly reduced in FB_1-treated

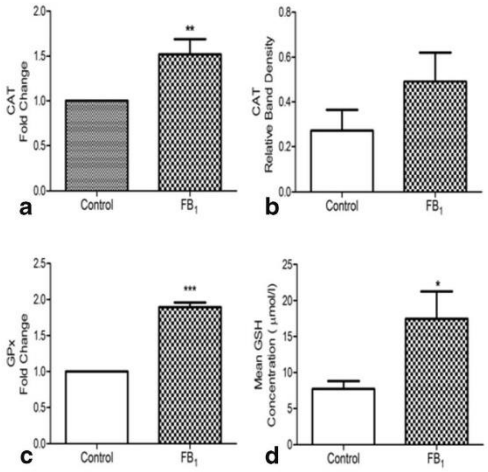

Fig. 5 Effects of FB_1 on the expression of antioxidants involved hydrogen peroxide detoxification. **a** CAT mRNA expression. **b** CAT protein expression. **c** GPx mRNA expression. **d** GSH concentration post-FB_1 exposure, where a single asterisk represents significance $p < 0.05$, a double asterisk represents significance $p < 0.01$ and a triple asterisk represents significance $p < 0.001$

cells ($p = 0.205$; control 0.044 ± 0.01 JC-1 fluorescence ratio vs FB_1 0.027 ± 0.009 JC-1 fluorescence ratio—Fig. 6a).

Mitochondrial stress response proteins, Sirt 3 and Lon-P1, were highly expressed during oxidative and mitochondrial stress. Western blot analysis of Sirt 3 (Fig. 6b) revealed a significant ($p = 0.0003$) 2.03-fold increase in FB_1-treated cells (7.63 ± 0.003 RBD) relative to the control (3.76 ± 0.577 RBD). The protein expression of the protease, Lon-P1 (Fig. 6c), was significantly increased 1.72-fold in cells exposed to FB_1 ($p = 0.0004$; control 0.189 ± 0.012 RBD vs FB_1 0.324 ± 0.017 RBD).

Discussion

The mycotoxin, FB_1, is a world-wide contaminant of maize and maize-based products (Marasas 2001; Shephard et al. 1996). It is nephrotoxic, cytotoxic and hepatotoxic to both animals and humans (Ross et al. 1990). Although FB_1 is poorly absorbed in humans, a major portion of absorbed FB_1 is distributed to the liver (Voss et al. 2002). The liver is the oxidative hub for many metabolic and detoxification reactions. Hepatocytes have a high density of mitochondria, increasing the risk of oxidative insult (Johannsen and Ravussin 2009).

The primary function of the mitochondria is to generate ATP via the ETC (Brand et al. 2013). Normal mitochondrial metabolism contributes to the generation of ROS, by leaking unpaired electrons into the mitochondrial matrix (Turrens 2003). Unpaired electrons react with oxygen to form superoxide; which is converted to hydrogen peroxide (Apel and Hirt 2004). Unwarranted production of ROS from the ETC can be stimulated by a number of factors, including the inhibition of complex I of the ETC (Lenaz and Genova 2010; Sena and Chandel 2012). Domijan and Abramov (2011) have reported that FB_1 inhibits complex I of the ETC, resulting in the enhanced generation of ROS and mitochondrial depolarisation. Hence, complex I inhibition may explain the observed mitochondrial depolarisation and increased levels of intracellular ROS (Fig. 2a) following FB_1 exposure in HepG2 cells. Previous studies confirm that FB_1 triggered the generation of intracellular ROS in mouse GT1-7 hypothalamic cells, rat C6 glioblastoma cells, human U-118MG glioblastoma cells and human SH-SY5Y neuroblastoma cells (Domijan and Abramov 2011; Stockmann-Juvala et al. 2004a; Stockmann-Juvala et al. 2004b).

One consequence of uncontrolled production of ROS is the peroxidation of lipids, which yield by-products such as MDA (Ayala et al. 2014). FB_1 significantly increased extracellular MDA-TBA adducts in HepG2 cells as evidenced by the TBARS assay (Fig. 2b). This is supported by findings in a number of different studies involving human cell lines and animal in vivo and in vitro models (Bernabucci et al. 2011; Kouadio et al. 2005; Wang et al. 2016).

Additional downstream repercussions of elevated ROS include nucleic acid and protein oxidation. FB_1 has been implicated in both these outcomes as evidenced in a study by Mary et al. (2012), where a significant increase in the formation of protein carbonyls and mis-incorporation of 8-oxoG in the DNA of rat spleen mononuclear cells was observed after a 48-h incubation with FB_1. A 24-h exposure to FB_1 in this study also resulted in significant increase in protein oxidation in form of protein carbonyls in HepG2 cells (Fig. 2c). This finding confirms protein oxidation is a biochemical hallmark of FB_1 exposure despite a significantly shorter exposure period.

The antioxidant defence system is responsible for detoxifying and neutralising the effects of excess intracellular ROS (Birben et al. 2012). Redox homeostasis relies on the disassociation of the antioxidant transcription factor, Nrf2, from Keap-1 (Bo et al. 2015). The cysteine residues of Keap-1 are targets for ROS (Schieber and Chandel 2014). Oxidative modification of these cysteine residues results in structural modifications to Keap-1, weakening its activity as a ligase adaptor (Sporn and Liby 2012). This leads to the dissociation of Keap-1 from the Neh domain, allowing the accumulation of Nrf2 in the cytosol. Modifications to Nrf2 such phosphorylation of the serine 40 residue also induce the dissociation of Nrf2 from Keap-1 (Nguyen et al. 2009). This study found that the expression of total Nrf2 was slightly reduced post-FB_1 exposure though the expression of Nrf2 with a phosphorylated serine 40 residue was significantly elevated (Fig. 3). Most transcription

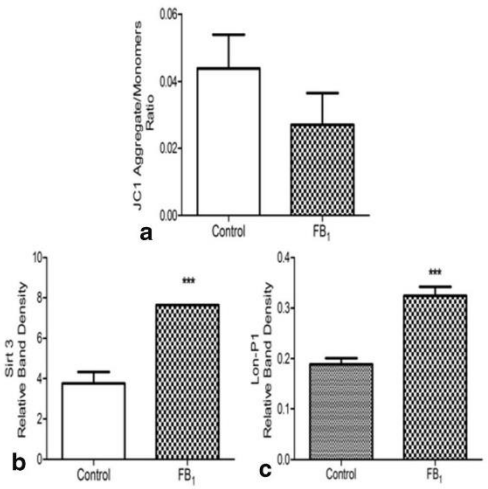

Fig. 6 Mitochondrial response to FB_1. **a** $\Delta m\psi$ represented as a ratio of JC-1 aggregates and JC-1 monomers. **b** Protein expression of Sirt 3. **c** Protein expression of Lon-P1, where a triple asterisk represents significance $p < 0.001$

factors—including Nrf2—are regulated by phosphorylation (Huang et al. 2002; Whitmarsh and Davis 2000). Excess ROS activates phosphorylation pathways such as mitogen-activated protein kinase (MAPK) and protein kinase c (PKC), which in turn participate in the phosphorylation and activation of the Nrf2-ARE (Bo et al. 2015). Phosphorylation of Nrf2 triggers its disassociation from Keap-1 ubiquitination, allowing translocation to the nucleus and subsequent transcription of antioxidant genes (Huang et al. 2002). Studies have shown that FB_1 activated both the MAPK and PKC pathways which may contribute further to Nrf2 phosphorylation (Pinelli et al. 1999; Yeung et al. 1996).

Nrf2 promotes the transcription of major antioxidants such as SOD2, CAT and GPx (Bo et al. 2015; Ma 2013). These antioxidant enzymes are the first line of defence against ROS (Wang et al. 2016). Surplus superoxide radicals, produced by dysregulated ETC, are detoxified to hydrogen peroxide by SOD2. Hydrogen peroxide is further detoxified by CAT and GPx to water and oxygen (Weir et al. 2013). The expression of SOD2 (Fig. 4), CAT and GPx (Fig. 5a–c) was all upregulated in HepG2 cells after exposure to FB_1. The expression of these antioxidants, however, were reduced in Balb/c mice and peripheral blood mononuclear cells (PBMC) exposed to FB_1 (Abbes et al. 2016; Bernabucci et al. 2011). Most cells in vivo are exposed to low oxygen concentrations; however, HepG2 cells were grown under 95% oxygen and 5% carbon dioxide. Therefore, more oxygen may have been available to react with electrons leaked from the mitochondria in HepG2 cells, which may have resulted in a higher production of ROS and more rigorous antioxidant response (Halliwell 2003).

An alternative non-enzymatic mechanism for hydrogen peroxide detoxification was also investigated. Glutathione is a major intracellular antioxidant in hepatocytes that protects against oxidative damage and is involved in detoxification of xenobiotics (Chen et al. 2013). This tripeptide is often referred to as the body's master antioxidant. It can be present in its reduced state—GSH or oxidised state (GSSG) (Filomeni et al. 2002). GSH directly quenches hydroxyl radicals and other oxygen-centred free radicals. It also acts a cofactor for the enzymatic antioxidant, GPx, in the detoxification of peroxides (Birben et al. 2012; Lushchak 2012).

After a 24-h incubation with FB_1, the concentration of GSH was significantly elevated in HepG2 cells (Fig. 5d). This is in agreement with results obtained by Domijan and Abramov (2011), who showed a significant increase in the concentration of GSH in SH-SY5Y cells after a 24-h incubation with FB_1. Long-term exposure to FB_1, however, lowered GSH concentration (Stockmann-Juvala et al. 2004a). Elevation of GSH could be a result of increased NADPH availability, a cofactor of GSH and component of GSH synthesis. Inhibition of sphingolipid synthesis by FB_1 distorts the structure of membrane receptors such as the folate receptor (Stevens and Tang 1997). Inhibition of folate uptake promotes the conversion of homocysteine to cysteine, a key amino acid required for the synthesis of GSH (Lu 2009; Stevens and Tang 1997).

As discussed previously, ROS produced by ETC resulted in the depolarisation of the mitochondria, which may have led to mitochondrial dysfunction. After observing a mild reduction in $\Delta\psi m$ (Fig. 6a), mitochondrial stress responses to FB_1 was assessed.

Proteins within the mitochondrial matrix are at great risk to oxidative insult. The clearance of oxidised proteins within the mitochondria is essential as oxidised proteins form aggregates and crosslinks, resulting in mitochondrial toxicity (Ngo et al. 2013). Lon-P1 is responsible for degrading oxidised proteins such as protein carbonyls within the mitochondrial matrix (Gibellini et al. 2014). Several reports have indicated that Lon-P1 expression and activity increased in the presence of high levels of carbonylated proteins (Pinti et al. 2015). The 11.37-fold increase in protein carbonyls by FB_1 may have induced the upregulation in Lon-P1 expression in HepG2 cells (Fig. 6c) (Ngo et al. 2013).

Lon-P 1 is post-transcriptionally activated by the mitochondrial deactylase enzyme, Sirt 3 (Bota and Davies 2016). Sirt 3 expression may have been upregulated to counteract the highly oxidative environment induced by FB_1 (Fig. 6b). Sirt 3 does not have any direct antioxidant capabilities but is able to upregulate the mitochondrial antioxidant capacity via two methods. The first method involves activating the mitochondrial antioxidant, SOD2, via deacetylation. The second method involves the enhancing isocitrate dehydrogenase 2 (IDH2) activity through Sirt3-mediated deacetylation. The activity of IDH2 produces increased levels of NADPH, which facilitates regeneration of GSH from GSSG. Together, increased SOD2 and IDH activity increases the detoxification capacity of the mitochondria (Bause and Haigis 2013).

This study found that exposure to FB_1 induced oxidative stress, as noted by the increase in intracellular ROS and the corresponding increase in oxidative stress bio-markers (MDA and protein carbonyls). Cells responded to the highly oxidative environment created by FB_1, via the upregulation of the antioxidant transcription factor Nrf2 and its associated antioxidants—SOD2, CAT and GPx. Oxidative stress responses in the mitochondria (Sirt 3 and Lon-P1) were also upregulated in response to the elevated ROS induced by FB_1. Although there was an increase in the expression of all antioxidants and stress response proteins investigated, this may not be reflective of enzyme activity of these antioxidants. Further investigation into the enzymatic activities of these antioxidants should be carried to have a better understanding of the overall antioxidant capacity during exposure to FB_1.

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Compliance with ethical standards

Conflict of interest None.

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ADDENDUM B

Ethical Approval Letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

29 May 2019

Ms T Arumugan (213531562)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
cyborglona@gmail.com

Dear Ms Arumugan

Protocol: An investigation into the epigenetic and subsequent biochemical effects of Fumonisin B1in human Liver (HepG2), oesophageal (SNO) and kidney (HEK293) cells.
Degree: PhD **BREC Ref No: BE322/19**

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received 05 April 2019.

Please ensure that site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from **29 May 2019.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **noted** by a full Committee at its next meeting taking place on **11 June 2019.**

Yours sincerely

[redacted]

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc: Postgrad Admin: dudhrajhp@ukzn.ac.za Supervisor: CHUTUR@stu.ukzn.ac.za Nagiah.savania@gmail.com

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