

The splicing inhibitor isoginkgetin leads to defects in
multiple phases of the cell cycle

by

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Abstract

Isoginkgetin is a naturally occurring compound from *Ginkgo biloba* trees that has anti-neoplastic activities related to inhibition of spliceosome assembly. The p53-tumour suppressor plays a prominent role in cell G1 and G2 cycle checkpoints and p53 is strongly activated in response to transcriptional stresses. Therefore, we determined the effect of p53 and isoginkgetin treatment on cell cycle progression. Wildtype p53 expressing cells exhibited a remarkable S phase defect while the p53-null subline accumulated with 4C DNA content. Despite the pronounced difference in these isogenic strains, there was no pronounced p53 transcriptional response as assessed by qRT-PCR and preliminary microarray analysis. Instead, isoginkgetin leads to altered pre-mRNA splicing of S and M phase-specific transcripts. These results suggest that cell cycle alterations are the direct result of abnormal splicing of cell cycle regulatory mRNAs and not p53 dependent checkpoints.

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Statement of Contributions

Several undergraduate students contributed data to this thesis.

Erin Vanzyl assisted with the microarray experiments and their validation by real time RT-PCR. Specifically, Erin collected RNA samples and performed qRT-PCR on these samples.

Victoria Tolls analysed the microarray data as part of her honour's thesis project. A small part of the preliminary data is presented here in support of the predominant findings in the present work (Figures 10-13 and Table 2).

Erin Vanzyl also stained samples for BrdU analysis during the writing the writing of this thesis.

Erin Vanzyl and two first year summer students, Teeghan Rambo and Hadil Sayed, performed Western Blots under my supervision.

Table of Contents

Abstract.....	ii
Acknowledgements.....	iii
Statement of Contributions	iv
List of Tables	vii
List of Illustration	viii
List of Abbreviations	ix
1. Introduction	1
1.1 The Spliceosome	3
1.1.1 Protein diversity.....	3
1.1.2 snRNPs	6
1.1.3 Important sequences on the pre-mRNA.....	7
1.1.4 Splicing occurs co-transcriptionally	9
1.1.5 Spliceosome assembly	9
1.2 Alternative splicing and cancer.....	12
1.3 Splicing Inhibition as a potential cancer therapeutic.....	15
1.3.1. Isoginkgetin.....	18
1.4 Cell Cycle Control	20
1.4.1 Cell Cycle Progression, Overview and Cyclins.....	20
1.4.2 Checkpoints.....	24
1.5 p53 response to transcription stalling and inhibiting spliceosome components.....	27
1.6 Rationale and Hypothesis	28
2. Methods.....	30
2.1 Cell Culture.....	30
2.2 Flow Cytometry.....	30
2.2.1 One Parameter Flow Cytometric Analysis	30
2.2.2 Two Parameter Flow Cytometric Analysis	31
2.3 Immunoblotting	32
2.4 RNA collection and analysis	32
3. Results.....	34
3.1 Isoginkgetin affects cell viability/apoptosis	44
3.2 The effect of isoginkgetin on cell cycle distribution	44

3.3 Preliminary Microarray Analysis	46
3.4 Isoginkgetin does not cause a p53 response	46
4. Discussion.....	48
4.1 Isoginkgetin induces apoptosis	48
4.1.1 The connection between splicing and apoptosis.....	49
4.2 The Effect of isoginkgetin on the Cell cycle	51
4.3 Preliminary Microarray analysis.....	58
4.4 Isoginkgetin had an unexpected p53 response	59
Conclusions	61
References	62

List of Tables

Table 1 Table 1 Partial list of known small molecular pre-mRNA splicing inhibitors	17
Table 2 Changes in splicing index in response to isoginkgetin 30 μ M after 8h	57

List of Illustration

Figure 1 pre-mRNA splicing.....	4
Figure 2 Intron and exon boundaries on the pre-mRNA	8
Figure 3 Site of action of isoginkgetin and structure.....	19
Figure 4 Overview of the cell cycle	21
Figure 5 Sensitivity of colon cancer cells to isoginkgetin	34
Figure 6 Isoginkgetin does not significantly alter cell cycle distribution in HCT 116 cells.....	35
Figure 7 Isoginkgetin led to significant alteration in cell cycle distribution in HCT 116 p53 ^{-/-} cells	36
Figure 8 Isoginkgetin treatment led to decreased BrdU incorporation in HCT 116 cells.....	37
Figure 9 Isoginkgetin treatment cells led to decreased BrdU incorporation in HCT 116 p53 ^{-/-} cells	38
Figure 10 Microarray analysis of IGG - induced changes in gene expression in HCT 116	39
Figure 11 Microarray analysis of IGG - induced changes in gene expression in HCT 116 p53 ^{-/-}	40
Figure 12 Microarray analysis of IGG - induced changes in pre-mRNA splicing in HCT 116 ...	41
Figure 13 Microarray analysis of IGG - induced changes in pre-mRNA splicing in HCT 116 p53 ^{-/-}	42
Figure 14 Isoginkgetin did not lead to a pronounced p53 response	43
Figure 15 Cell Cycle Arrest Due to isoginkgetin Treatment.....	56

List of Abbreviations

AdML	Adenovirus major late (AdML) pre-mRNA
Akt or PKB	Protein kinase B
APC	anaphase promoting complex
ATM	Ataxia telangiectasia mutated
ATR	ATM and rad3-related
ATRIP	ATR interacting protein
B*	B active
BAX	Bcl-2 associated X
BCL2L1	B-cell CLL/Lymphoma 2 like 1
BCL-2	B-cell lymphoma 2
BPS	Branch Point Sequence
BrdU	Bromodeoxyuridine
BubR1	Bub1 Related Kinase
Bud31	Bud site selection homology 31
CAK	Cdk-activating kinases
CDC5L	Cell division cycle 5-like
CDK	Cyclin dependent kinases
CDKN1A	Cyclin dependent kinases inhibitor 1A
CDT1	Chromatin licencing and DNA replication factor 1
C-FLIP	cellular FLICE-like inhibitory protein
CHAC1	Glutathione-specific gamma-glutamylcyclotransferase 1
CHK2	Checkpoint kinase 2
COP9	Constitutive photomorphogenesis 9
COP9S5	COP9 signalosome subunit 5
COX-2	Cyclooxygenase 2
CRNKL1	Crooked Neck Pre-mRNA Splicing Factor 1
CTD	C-terminal Domain
CUL1	Cullin1
DBF4	DumbBell Former 4
DDIT3	DNA damage-inducible transcript 3
DMSO	Dimethyl sulfoxide
E complex	Early complex
E2F	E2 transcription factor
EFTUD2	Elongation factor Tu GTP binding domain containing 2
ESI	Exonic splicing inhibitors
EXE	Exonic splicing enhancers
Fas	First apoptosis signal

FBS	Fetal bovine Serum
FITC	fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-Phosphate dehydrogenase
hBrr2	Human Bad Response to Refrigeration 2
HDAC	histone deacetylase
HIPK2	Homeodomain Interacting Protein Kinase-2
HMEC	human mammary epithelial cells
hnRNP	heterogeneous snRNP
hprp5	human pre-mRNA processing factor 5
IGG	Isoginkgetin
IL-32	Interleukin 32
ISE	Intronic splicing enhancers
ISS	Intronic splicing silencers
L	Long
L-SM	Sm-like
Mad2	Mitotic arrest deficient 2
MCC	Mitotic checkpoint complex
MCM	minichromosome maintenance
MDM2	Mouse double minute 2
MMP-9	matrix metalloproteinase-9
MYC	MYC
MYC-ER	MYC and oestrogen fusion protein (MYC-ER)
NBCS	Newborn Calf Serum
NES	nuclear export signals
NF-KB	Nuclear factor-kappaB
NMD	nonsense mediated decay
NTC	Nineteen Complex
ORC	Origin of Recognition Complex
ORC	Origin of Recognition Complex
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PI3K	phosphatidylinositol 3-kinase
PMAIP1 or NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
Pol	Polymerase
PPT	Polypyrimidine tract
pRB	protein retinoblastoma
Prp	pre-mRNA processing factor

PUMA	p53 upregulated modulator of apoptosis
RBM5	RNA binding motif protein 5
RPA	Replication protein A
R-point	restriction point
S	Short
SAC	Spindle Assembly Checkpoint
SCF	Skp-Cullin-F-box
SEM	Standard error of the mean
SF1/mBBP	Splicing Factor 1/mammalian Branchpoint Binding Protein
Siah-1	Seven in Absentia Homolog 1
Slu7	Step II Splicing Factor 7
SMN	Survival Motor Neuron
sn	small nuclear
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SNRPB	snRNP polypeptide B and B1
SNRPF	small nuclear ribonucleoprotein polypeptide F
Snu114	Small nuclear ribonucleoprotein associated 114
SON	SON DNA binding protein
SR	Serine/Arginine Rich
SR3B1	Splicing Factor 3 Subunit 1
SRPK2	Serine/Arginine rich protein-specific kinase 2
SRSF1	Serine/Arginine –rich splicing factor 1
SS	Splice Sites
TC-NER	transcription coupled nucleotide excision repair
TIMP-1	Tissue inhibitor of metalloproteinases 1
TNRAF2	Tumor necrosis receptor associated factor 2
TUB1	Tubulin 1
U rich	Uridine Rich
U2AF	U2 auxiliary factor
U2AF65	65 kDa subunit of U2AF
UBL5	Ubiquitin-like 5
USP39	Ubiquitin specific peptidase 39
XAB2	XPA binding protein 2
Y	pyrimidines

1. Introduction

Cancer is a group of diseases that are associated with uncontrolled cell proliferation, tissue invasion and often metastasis (Vogelstein & Kinzler, 1993; Vogelstein *et al*, 2013). Two in 5 Canadians will develop cancer in their lifetime (Canadian Cancer Society, 2015) and cancer is the leading cause of death in Canada with 29.9 % of all deaths attributed to these diseases (Canadian Cancer Society, 2015). Cancer develops in a multistep process that is driven by genomic instability (Cahill *et al*, 1999). This instability can occur at the chromosomal level with chromosomal translocations or aneuploidy and gene amplification; and the DNA sequence level with changes in nucleotide sequences (Cahill *et al*, 1999). Sequential acquisition of mutations in genes involved in cell fate, cell survival and genome maintenance pathways are required for oncogenic transformation (Vogelstein *et al*, 2013). This involves inhibition of tumour suppressor genes controlling the cell cycle checkpoints and apoptosis as well as gain of function mutations in oncogenes that control proliferation, invasion and metastasis (Cahill *et al*, 1999; Vogelstein & Kinzler, 1993). The resulting tumour is a heterogeneous population with a drastically different genetic profile than the original genotype of the healthy cell. While there are many differences between each cancer type, they are all thought to share common hallmarks which include: genomic instability and mutation; avoiding growth suppressors; sustaining proliferative signaling; resisting cell death; replication immortality; avoiding immune destruction; tumor-promoting inflammation; angiogenesis; deregulation of cellular energetics; and invasion and

metastasis (Hanahan & Weinberg, 2011). Novel therapeutics aim to target different hallmarks (Hanahan & Weinberg, 2011).

Pre-mRNA splicing is required for the accurate removal of introns to form mature mRNA (Wahl *et al*, 2009). Alternative pre-mRNA splicing plays a prominent role in the regulation of each of the hallmarks of cancer (Oltean & Bates, 2014). Alternative splicing patterns can yield isoforms with different and sometimes antagonistic functions (Biamonti *et al*, 2014). For example, alternative splicing of many apoptotic genes can become pro-apoptotic or anti-apoptotic depending on how the transcript is spliced (Schwerk & Schulze-Osthoff, 2005) and this contributes to cancer cell survival. Aberrant alternative splicing can be the result of mutations at different splice sites, other intronic sequences or other regulatory sequences. In addition, increased expression of splicing regulatory proteins occur across cancer types and are thought to be major contributors to aberrant alternative splicing (Ghigna *et al*, 2008; Silipo *et al*, 2015). Some splicing factors have been identified as proto-oncogenes (Ghigna *et al*, 2008; Silipo *et al*, 2015). Splicing inhibitors are emerging as a potential new cancer therapeutic with many compounds that have been identified (Bonnal *et al*, 2012; Salton & Misteli, 2016), some of which have reported anti-neoplastic properties (Yoon *et al*, 2006). The work presented in the present thesis identifies a novel relationship between inhibition of pre-mRNA splicing and cell cycle progression that is greatly affected by the expression of the p53 tumour suppressor protein.

1.1 The Spliceosome

The spliceosome is a dynamic macromolecular complex responsible for the accurate removal of introns from pre-mRNA to form mature mRNA. This reaction comes through two transesterification reactions at both ends of the introns and the exons are joined together (Figure 1). Although the splicing reaction is simple, the process for this occurs in multiple steps with numerous interactions including: RNA-RNA; RNA-protein; and protein-protein to occur (Wahl *et al*, 2009).

1.1.1 Protein diversity

The spliceosome is composed of 5 Uridine Rich (U rich) small nuclear ribonucleoprotein (snRNPs) – U1, U2, U4, U5, U6 and are subdivided into the E, A, B, B*(B active) and C complex (Figure 1) (Reviewed by Matera & Wang, 2014; Wahl *et al*, 2009). There have been between 145 (Zhou *et al*, 2002) and 311 (Rappsilber *et al*, 2002) proteins that purify with the human spliceosome and approximately 171 of these are considered to be core spliceosome proteins (Agafonov *et al*, 2011). A study looking at the interactions of 196 splicing proteins using Yeast 2, found that there were 632 different protein-protein interactions identified and 242 of these came from core splicing proteins (Hegele *et al*, 2012). While there are some proteins that are present throughout the splicing process such as U2 associated proteins, there is also a large exchange of proteins that occur between each complex. Some of these proteins are associated directly with the snRNPs and others not associated such as the Nineteen Complex (NTC). Between A and B complex formation, there are approximately 10 proteins that leave and around 65 new proteins added (25 of these from the tri-snRNP).

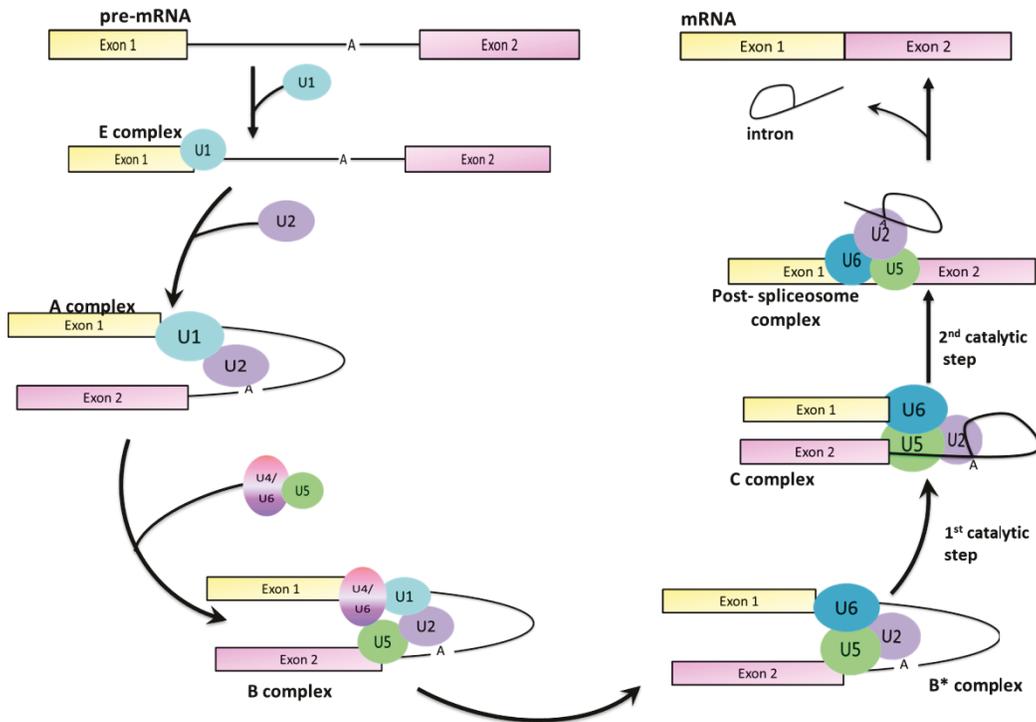


Figure 1 pre-mRNA splicing

The spliceosome assembles in a stepwise manner. The E complex is formed with the binding of U1 snRNP to the 5' Splice Site (SS) of the pre-mRNA. Next, the U2 snRNP binds to the branch point sequence (BPS) and rearrangements bring the 5' SS, 3' SS, and BPS in proximity to each other. The joining of the U4/U6.U5 tri-snRNP forms the B complex, subsequent rearrangements cause the dissociation of U4 and U1 forming the B* (B active) complex. U6 base pairs with the 5' SS and U5 with the 3' exon. The first transesterification reaction (1st catalytic step) occurs between the BP Adenosine and the 5' SS, resulting in a free 5' exon and a lariat intron attached to the second exon (C complex). The second transesterification reaction (2nd catalytic step) occurs between the free exon and the 3' SS, removing the intron and forming a mature mRNA. Figure modified from Wahl et al, 2009.

Between B and C complex formation, there are approximately 35 new proteins and around 35 proteins leave (Wahl *et al*, 2009).

Interestingly, transcripts do not seem to be equally affected by defects in splicing proteins. Mutations in non-essential splicing factors in yeast (which contains fewer introns) resulted in differences in splicing patterns as determined by splicing specific microarrays. A transcript could be affected by the loss of one splicing factor, but would remain unaffected by the loss of another splicing factor. Meanwhile, other transcripts would be differently affected by the loss of each splicing factor (Clark *et al*, 2002; Pleiss *et al*, 2007). This leads to the idea that some splicing proteins are required for the splicing of specific transcripts, and not for others. As well, it might be possible that gene expression regulation could be done through controlling the level of certain spliceosome proteins (Pleiss *et al*, 2007).

The DExD/H-box RNA helicase ATPase family of proteins associate mostly transiently, but are important throughout the spliceosome assembly, catalysis and disassembly process. These proteins are very important in RNA-RNA interaction and RNA-protein rearrangement that occur throughout the splicing process. As described later, human pre-mRNA processing factor 5 (hprp5) helps stabilize the binding of the U2 snRNP to the Branch Point Sequence (BPS). Human Bad Response to Refrigeration 2 (hBrr2 also known as U5-200kDa) is required for the unwinding of the U4/U6 snRNA (Liu & Cheng, 2015). While the exact mechanism is unknown, prp5, prp16 and prp22 have demonstrated proofreading abilities for suboptimal splice sites at the stages in splicing in which they are active. One model suggests that they act as a timer, where there is a

window for the splicing event to occur (for example, binding of U2 to the BPS or splicing of one of the splice sites). If the event takes place, then the ATPase functions as normal and allows the conformational changes needed for the next step of splicing. At suboptimal sites where the event has not occurred, the ATPase becomes antagonistic and leads to a discarding of the substrate. In another model, the DExD/H ATPase senses the suboptimal substrate due to the stability of the spliceosome at the site or due to some sort of regulation of the DExD/H ATPase (Reviewed by Semlow & Staley, 2012).

1.1.2 snRNPs

Each snRNP in the spliceosome contains a specific small nuclear RNA (snRNA) from 160-1175 nucleotides long and a core of 7 Sm or 7 Sm-like (L-Sm) as well as specific proteins for that unit. These snRNPs are processed and matured between the nucleus and cytoplasm, with the exception of U6 (Matera & Wang, 2014).

snRNAs with the exception of the one from U6 are transcribed by Polymerase (Pol) II in the nucleus, processed, capped and exported to the Survival Motor Neuron (SMN) complex. Here, 7 Sm proteins (B/B', D3, G, E, F, D1, and D2) assemble around a conserved site on the snRNA. It is then imported back into the nucleus where the final maturation and addition of specific small nuclear (sn) proteins is thought to occur in the Cajal bodies, substructures in the nucleus (Matera & Wang, 2014).

U6 snRNPs are transcribed and assembled differently from other snRNPs. The U6 snRNP is transcribed by RNA Pol III and the rest is assembled and matured between the nucleolus and Cajal bodies. Instead of Sm proteins it has 7 L-Sm proteins (L-Sm2-8) (Kiss,

2004). Mature snRNP as well as splicing specific proteins are found in splicing speckles in the interchromatin region (Matera & Wang, 2014).

1.1.3 Important sequences on the pre-mRNA

There are some sequences found along the exon/intron border as well as along the intron that are loosely conserved and required for the spliceosome assembly (Figure 2). The Splice Sites (SS) are found at the 5' and 3' end of the intron, the BPS usually contains 21-34 nucleotides (Gao *et al*, 2008) upstream of the 3'SS and is followed by a polypyrimidine tract (PPT). The metazoan 5'SS sequence is GURAGU (Wahl *et al*, 2009), 3'SS is YAG (Wahl *et al*, 2009) and the human BPS is YUNAY (Gao *et al*, 2008), with the adenosine being conserved and important in the first splicing reaction. Y represents pyrimidines, R purines and N is any nucleotide. These sequences are degenerated in metazoans (Wahl *et al*, 2009) so additional factors are required for intron and splice site selection.

There are a variety of *cis* and *trans* acting elements that are also important in splice site selection. Selection alone is not determined by the snRNPs. *Cis* acting elements are sequences found on the pre-mRNA that act to enhance or repress splicing. These sequences include exonic splicing enhancers or inhibitors (ESE or ESS) and intronic splicing enhancers or silencers (ISE or ISS) (Matera & Wang, 2014; Wahl *et al*, 2009).

Trans acting elements are proteins that bind to *cis* acting sequences.

Serine/Arginine (SR) proteins contain one or two RNA binding domains and a serine-arginine rich domain that interacts with proteins. This family of proteins bind to ESE and

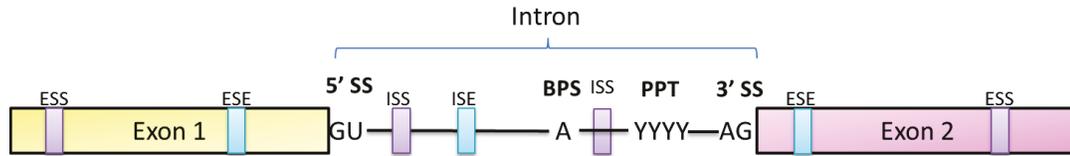


Figure 2 Intron and exon boundaries on the pre-mRNA

Specific Sequences on the pre-mRNA are important for the binding of spliceosome components and spliceosome associated proteins. Exonic Splicing Enhancers (ESE) and Exonic Splicing Silencers (ESS) located on the exons, allow proteins to bind that will allow them to either promote splice site (SS) selection, or prevent the SS selection. On the intron, there is the 5' SS and 3' SS at the boundaries, as well as the branch point sequence (BPS) which contains a conserved adenosine (A) followed shortly by the polypyrimidine tract (PPT) (pyrimidines represented as Y). There are also Intronic Splicing Enhancers (ISE) and Intronic Splicing Silences (ISS) which further promote or repress SS selection respectively. Figure modified from (Matera & Wang, 2014).

promote spliceosome formation. Less commonly, they have a repressive role if they bind to an intronic sequence (Matera & Wang, 2014). Heterogeneous RNPs (hnRNP)s typically bind to ESS to suppress splicing (Matera & Wang, 2014). It is usually the accumulation of different enhancers and suppressors that determine if splicing will occur.

1.1.4 Splicing occurs co-transcriptionally

Splicing occurs co-transcriptionally through an interaction with the C-terminal Domain (CTD) of RNA Pol II and some splicing components and splicing factors. Splicing efficiency and splice site selection are impacted by RNA Pol. Splicing occurs less efficiently with RNA Pol II lacking a CTD (McCracken *et al*, 1997). A decrease in the elongation rate can result in the selection of a weak 3'SS over a stronger 3'SS located downstream of it (Merkhofer *et al*, 2014). There are several sites that can be phosphorylated in the CTD, including Ser5 and Ser2. Ser5 tends to be phosphorylated at the promoter and in early transcription while Ser2 is phosphorylated in transcription elongation (Reviewed by McKay, 2014; Merkhofer *et al*, 2014). RNA Pol II also pauses at the 3'SS on an intron and is hyperphosphorylated on Ser5 and phosphorylated on Ser2. The pausing appears to be due more to the splicing event than the actual RNA sequence (Alexander *et al*, 2010). Therefore, the CTD of RNA pol II is another important factor in spliceosomal SS selection and efficiency.

1.1.5 Spliceosome assembly

The spliceosome assembles along the intron in a stepwise manor ensuring proper site recognition and pairing (Figure 1). The Early complex (E complex) is the first

commitment to splicing. During this step, the 5'SS, BPS, PPT, and 3'SS are recognized (Michaud & Reed, 1993) and components bind independently of ATP (Michaud & Reed, 1991). U1C subunit of U1 snRNP binds to the 5'SS (Du & Rosbash, 2002) followed by the U1 snRNA base pairing with this sequence (Seraphin *et al*, 1988). Splicing Factor 1/mammalian Branchpoint Binding Protein (SF1/mBBP) binds to the BPS (Berglund *et al*, 1997) and U2 auxiliary factor (U2AF) bind to the PPT (Wahl *et al*, 2009). The 65 kDa subunit of U2AF (U2AF65) binds to SF1/BBP and the PPT (Wahl *et al*, 2009) and U2AF35 binds to the 3'SS (Wu *et al*, 1999). In addition there is evidence that SMN complex proteins are present in the E complex (Makarov *et al*, 2012). As well lower amounts of U2 snRNP are present in the E complex but not bound in the intron like it will in the A complex (pre-spliceosome) but needs to be loosely associated (Das *et al*, 2000).

The E complex (Figure 1) is necessary for the formation of the A complex. During the transition from E to A complex, any excess U1 on alternative 5'SS are removed (Hodson *et al*, 2012). The formation of the A complex is ATP dependent and requires the DExD/H helicase proteins Prp5 (Liu & Cheng, 2015; Wahl *et al*, 2009) and UAP65 (Fleckner *et al*, 1997; Liu & Cheng, 2015) for conformational changes. U2AF65 recruits UAP65 which also helps with U2-BPS interaction (Fleckner *et al*, 1997). U2 binds to the BPS, the process is stabilized by Prp5, (Xu *et al*, 2004) and U2 snRNA base pairs with this sequence (Wu & Manley, 1989). This causes a bulging of a conserved adenosine (Query *et al*, 1994). The 2'OH of this adenosine will act as a nucleophile in the first splicing reaction. p14 replaces SF1/BBP and Splicing Factor 3 Subunit 1 (SF3B1) binds with

U2AF65 (Wahl *et al*, 2009). Prp5 interacts with U1 and U2 and causes the two snRNPs to interact, (Xu *et al*, 2004) bringing the 5'SS, BPS and 3'SS in close proximity to each other.

For introns longer than 200 nt, it is thought that an exon-defining complex occurs first before an intron defining complex (equivalent to the A complex) is formed. The exon defined complex occurs with U1 binding on the 5'SS downstream of the exon and U2 and U2AF binding to the BPS and polypyrimidine tract. The mechanisms for the switch from exon to intron defined complex is not known (Wahl *et al*, 2009).

Following the creation of the A complex, the pre-assembled tri U4/U6. U5 snRNP are recruited and form the spliceosomal B complex (Figure 1). In humans, phosphorylation of Prp28 by Serine/Arginine rich protein-specific kinase 2 (SRPK2) is required in order for incorporation of the tri-snRNP (Mathew *et al*, 2008). The U4 and U6 snRNA are wound together and at this stage, and the spliceosome is still inactive. Another series of conformational changes requiring hBrr2, Small nuclear ribonucleoprotein associated 114 (Snu114) and Prp2 result in the B active (B*) complex (Wahl *et al*, 2009). U1 is displaced from the 5'SS by hPrp28 (Staley & Guthrie, 1999) U5-200kDa (also called hBrr2) is responsible for U4/U6 snRNA unwinding (Laggerbauer *et al*, 1998) exposing the active site on U6 snRNA which binds to the 5'SS after U1 displacement (Sawa & Shimura, 1992). The U4 snRNA is also released from the spliceosome. The 3' end of U6 snRNA and 5'end of the U2 snRNA base pair with each other (Madhani & Guthrie, 1992). U5 snRNA contain sequences complimentary to those in the 5' and 3' exons (Newman & Norman, 1992), however in B*, U5 is only base paired with the 5'exon and not with the 3' exon (Newman & Norman, 1992) .

The prp19/cell division cycle 5-like (cdc5L) complex or NTC is important during the catalytic stages of the spliceosome. It binds during the B complex (Makarova *et al*, 2004) and helps stabilize the spliceosome upon the disassociation of the U1 and U4 snRNPs. It assists U6 and U5 in stably binding to the spliceosome as well as the 5' exon (Chan *et al*, 2003). In addition, it helps destabilize the L-Sm proteins from U6 snRNA (Chan *et al*, 2003). The complex becomes stably associated with the core complex during the B to C complex transition (Makarov *et al*, 2002).

The B* complex carries out the first transesterification reaction (1st catalytic step, Figure 1). The 2'OH of the branch point adenosine acts as a nucleophile and attacks the phosphodiester bond the 5'SS resulting in a free 5' exon and a lariat intron (5'SS to the adenosine) attached to the 3'SS (Wahl *et al*, 2009). Next, further conformational changes requiring Prp8, Prp16, Step II Splicing Factor 7 (Slu7) occur to form the C complex (Wahl *et al*, 2009). U5 snRNA binds to the 3' exon while still bound to the 5' exon, helping to align the two exons (Newman & Norman, 1992). The second splicing reaction occurs with the 5' exon 3'OH targeting the phosphodiester bond of the 3'exon and the two exons are joined together. The lariat intron is released as are the remaining spliceosome components which are recycled for further splicing (Wahl *et al*, 2009). Both splicing reactions have been shown to be reversible (Tseng & Cheng, 2008).

1.2 Alternative splicing and cancer

Transcripts can be spliced in a variety of ways resulting in different isoforms from a single gene. Approximately 95% of human genes containing multiple exons can be alternatively spliced (Pan *et al*, 2008). Different types of alternative splicing include:

exon skipping, mutually exclusive exons, and alternative 5'SS, alternative 3'SS and intron retention. The resulting transcript can result in: different protein isoforms that can have different or antagonistic functions; changes to the transcript reading frame; and transcripts that undergo nonsense mediated decay (NMD). Alternative splicing varies between tissues, occurs during development and cell differentiation but is commonly dysregulated in cancer and other diseases (Cartegni *et al*, 2002).

Alternative splicing is common across all cancers, but the genes affected and the type of AS event differs between cancer types. Transcriptome wide analysis across 27 different cancers and 37 corresponding healthy tissue types showed over 15,000 cancer specific splice variants (He *et al*, 2009). It also showed that the number of cancer specific splice variant per gene was smaller than that of the normal tissue specific variants (He *et al*, 2009). The cancer specific variants most commonly used alternative 3' or 5' SS and lower levels of alternative intron retention or alternative exons. There is variation between the cancer types, and there are examples of all alternative splice types being used in cancer. Alternative splicing in cancer can allow for beneficial isoforms that promote cancer progression, resistance to apoptosis, growth, angiogenesis, and others. For example, BCL-X, encoded by BCL2L1 gene (B-cell CLL/Lymphoma 2 like 1) has alternative 5'SS to form two isoforms; the pro-apoptotic short BCL-X_s or a longer anti-apoptotic BCL-X_L. (A review by Oltean & Bates, 2014 highlight the effects of alternative splicing on cellular processes considered to be the hallmarks of cancer).

As mentioned, *cis* acting elements and *trans* acting proteins are involved in the determination of splice site selection. Adding to the complexity is that there are other

regulatory proteins that can affect the splicing of an intron even after the splice site selection. A specific example is the putative tumour suppressor RNA binding motif protein 5 (RBM5), which is downregulated in many cancers. In an RNAi experiment it was determined that RBM5 is involved in the alternative splicing of first apoptosis signal (Fas) cell surface death receptor exon 6 (Bonnal *et al*, 2008). When absent, it resulted in the retention of exon 6 which is a membrane bound pro-apoptotic form, and when present, exon 6 was skipped resulting in a soluble anti-apoptotic protein. RBM5 interacts with U2AF65 in the early spliceosome and is believed to either prevent the transition from cross exon to cross intron definition at this exon or interacts with an SR protein to promote exon skipping. It also alternatively splices cellular FLICE-like inhibitory protein (c-FLIP) to c-FLIP short(S) an inhibitor of FAS when RBM5 is present and decreases the expression of c-FLIP long (L), an activator of the FAS pathway (Bonnal *et al*, 2008). RBM5 is loosely associated with the spliceosome so it may have some a few other targets. It is likely that other proteins loosely associated with the spliceosome (Agafonov *et al*, 2011) are required for the splicing or alternative splicing of specific pre-mRNA (Wahl *et al*, 2009).

Sequence mutations can lead to alternative SS selection, exon skipping or intron retention. Single point mutations in the 5'GT or 3'AG alone often will result in exon skipping or less commonly a cryptic splice site will be used instead. A cryptic splice site is a sequence that is almost identical to a 5' or 3' SS but is not used as a SS under normal circumstances (Cartegni *et al*, 2002; Krawczak *et al*, 1992). Mutations alone, cannot account for all the alternative splicing occurring in cancer as more studies are finding

that these splicing events are occurring without mutations in the gene. Dysregulation of splicing factors has been observed in many different tumour types (Reviewed by Grosso *et al*, 2008). Many factors affected are members of the SR and hnRNP protein families and more appear to be upregulated than downregulated. The splicing effect due to dysregulation of these factors is not known for all. Serine/Arginine –rich splicing factor 1 (SRSF1) is a proto-oncogene when upregulated has been demonstrated to affect alternative splicing in genes to promote tumorigenesis (Grosso *et al*, 2008; Karni *et al*, 2007).

1.3 Splicing Inhibition as a potential cancer therapeutic

The spliceosome is a potential novel cancer therapeutic. The isoform switches that occur in some cancers that drive tumour survival and progression make splicing a potential target to prevent these alternative splicing events (Salton & Misteli, 2016).

MYC is an oncogene transcription factor that activates genes involved in growth and differentiation. A synthetic lethal screen was performed using shRNA to identify genes that were sensitive to MYC hyperactivity. This was done in human mammary epithelial cells (HMEC) with an inducible MYC and oestrogen fusion protein (MYC-ER). Bud site selection homology 31 (Bud31), a component of the spliceosome was identified as a target, was synthetic lethal within the MYC-induced cells, but did not affect viability in non-induced HMEC (Hsu *et al*, 2015). Further analysis into the properties of BUD31 revealed that it was a core component of the spliceosome across the complexes. It interacted with 79 of the 134 core spliceosome components (Hsu *et al*, 2015). The increase in pre-mRNA in MYC hyperactivated cells may increase pressure on the

spliceosome. When the spliceosome is inhibited, there is an increase in intron retention and a decrease in mature mRNA. Some of the genes affected in this study include those involved in DNA replication and repair, mitotic spindle, unfolded protein response and RNA splicing (Hsu *et al*, 2015). An increase in apoptosis was also observed in MYC hyperactivated HMEC with partial depletion of other spliceosome components including SF3B1, U2AF1, Elongation factor Tu GTP binding domain containing 2 (EFTUD2) and small nuclear ribonucleoprotein polypeptide F (SNRPF) (Hsu *et al*, 2015).

Similarly, SD6, a small molecular inhibitor of SF3B1, inhibited tumor growth, viability and induced apoptosis. Overexpression of MYC is common in triple negative breast cancers (ER-, HER2-, and PR-) that are also metastatic. Triple negative breast cancer line MDA-MP-231-LM2 and SUM159 treated with SD6 or inhibiting BUD31 affected cell viability. In xenograph mice, SD6 or BUD31 KD resulted in reduced metastasis (Hsu *et al*, 2015). In a screen of 72 breast cancer cell lines looking for cell viability, MYC dependent cancers were more sensitive with shRNA affecting core spliceosome components (Hsu *et al*, 2015).

Small molecular inhibitors of splicing are clinically a simpler method of delivery over RNAi or oligonucleotides. There are many compounds that have been identified through screens that are pre-mRNA splicing inhibitors and have anti-cancer properties. Many current compounds target SF3B1 which is a subunit of the U2 snRNP (Reviewed by Bonnal *et al*, 2012; Salton & Misteli, 2016). (See table 1 for a partial list of known compounds).

Compound Name	Target and Effects
FR901464 Spliceostatin A	Binds and inhibits SF3B1, has inhibited growth in xenograph mouse models
Pladienolides GEX1	Inhibits SF3B1, inhibits growth of tumours
Isoginkgetin	Inhibits A to B complex formation through unknown target, other known anti-cancer properties include inhibition of MMP-9 preventing tumour invasion
Meamycin B Sudemycin E7107	SFB1 inhibitors , Spliceostatin A analogs, inhibition of tumour growth in xenograph mouse models SF3B1 inhibitor and platiénolide derivative, currently in Phase I clinical trials
Madrain	Inhibits the first stage of splicing, causes cell cycle arrest in HEK293 and HeLa cell culture
Clotrimazole Flunarizine Chlorhexidine	Splicing modulators but do not appear to be general splicing inhibitors, each drug appears to be targeting specific genes. Chlorhexidine inhibits CDC2-like kinase preventing phosphorylation of SR protein splicing factors
1,4 Heterocyclin 4-Naphthoquinone	Inhibits splicing at second step preventing the release of the intron lariat
Tetrocarcin A Indole Naphthazarin	Inhibits the first step of splicing Inhibits the second step of splicing
Amilioride	Inhibits the splicing of certain genes, including some involved in cancer such as BCL-X

Table 1 Partial list of known small molecular pre-mRNA splicing inhibitors Table modified from Salton & Misteli, 2016

1.3.1. Isoginkgetin

Isoginkgetin (IGG) is a naturally occurring biflavonoid found in *Ginkgo biloba* leaves and other plants. It is a pre-mRNA splicing inhibitor and has anti-cancer and anti-inflammation properties (Kwak *et al*, 2002; O'Brien *et al*, 2008; Yoon *et al*, 2006; Zhou *et al*, 2011). The splicing inhibiting properties of IGG were first identified using a Luciferase splicing reporter in HEK293 cells (O'Brien *et al*, 2008). Follow up experiments on a few other genes with semiquantitative RT-PCR regulated in the accumulation of pre-mRNA with increased dose (0-100 μ M) and accumulation of pre-mRNA was detectable within 3 hours (O'Brien *et al*, 2008). *In vitro* in HeLa nuclear extract, it was determined that the IC₅₀ is 30 μ M and full splicing inhibition at 50 μ M (O'Brien *et al*, 2008). The HeLa nuclear extract was treated with 70 μ M IGG to determine where splicing inhibition was occurring using an Adenovirus major late (AdML) pre-mRNA. There was an accumulation of pre-mRNA with complex A and little to none in complex B or C, indicating that IGG prevents the transition from the A to B splicing complex where the U4/U5/U6 tri-snRNP would normally be recruited (O'Brien *et al*, 2008) (Figure 3). The exact mechanism and target of inhibition is unknown. Treatment also resulted in growth arrest but did not affect cell viability. The effect of IGG is reversible; upon removal of the drug, splicing and growth resumed (O'Brien *et al*, 2008).

IGG also has other anti-cancer properties. It inhibits expression of matrix metalloproteinase-9 (MMP-9) which is involved in tumor invasion and metastasis by degrading type IV collagen of the basement membrane (Yoon *et al*, 2006). It is thought to regulate this through the phosphatidylinositol 3-kinase (PI3K)/ Protein kinase B (PKB

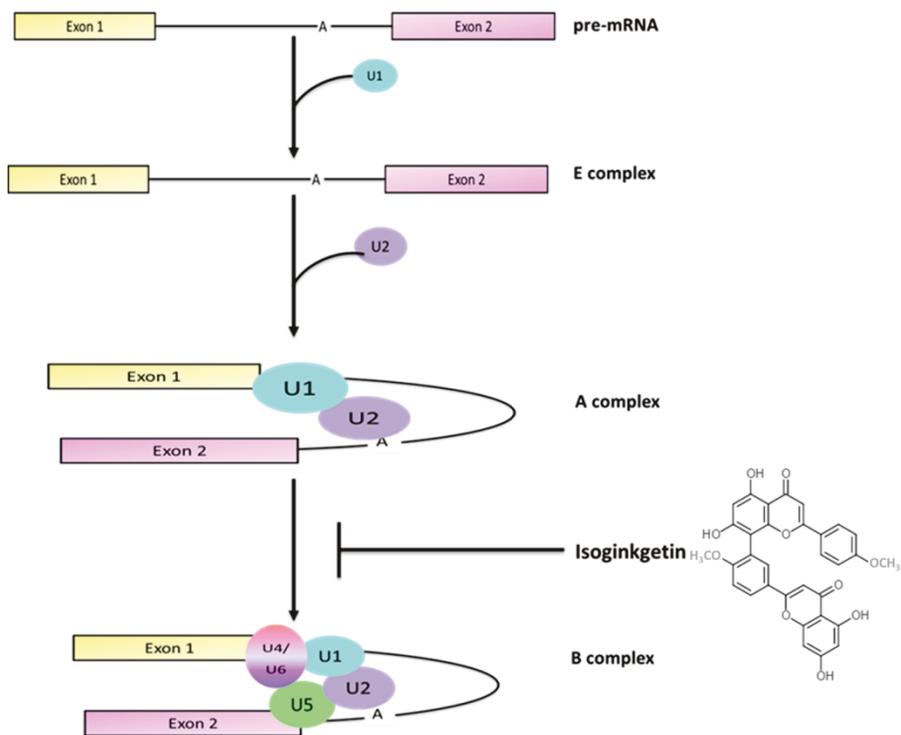


Figure 3 Site of action of isoginkgetin
Isoginkgetin blocks the transition from the A to B complex.

or Akt)/Nuclear factor-kappaB (NF-KB) pathway. IGG treatment resulted in decreased Akt activity and phosphorylation. Tissue inhibitor of metalloproteinases 1 (TIMP-1) expression increased and NF-KB activity decreased (Yoon *et al*, 2006). In a mixture with ginkgetin, it inhibited prostaglandin E2 production partially through downregulation of Cyclooxygenase 2 (COX-2) (Kwak *et al*, 2002). Other studies have also shown that IGG inhibits NF-KB activity (Zhou *et al*, 2011). It is not known if these effects are related to its splicing inhibition function. Although inhibition of splicing leads to alternative expression of cell cycle mRNAs (Hsu *et al*, 2015), the effect of IGG on cell cycle distribution has never been studied.

1.4 Cell Cycle Control

1.4.1 Cell Cycle Progression, Overview and Cyclins

The cell cycle is tightly regulated to prevent premature division or prevent cell with damaged DNA from replicating. The cell cycle is divided between interphase and mitosis. The cell spends most of its time in interphase which can be subdivided into 3 stages, G₁, S and G₂ (Figure 4). The cells divide in mitosis, first with nuclear division and then separate in cytokinesis into two daughter cells. The coordination of cell cycle is carried out by cyclins and cyclin dependent kinases (CDK) through the phosphorylation of specific proteins in the cell cycle. Cyclin levels rise and fall at specific times during the cell cycle. CDKs levels are not affected but lack a substrate to be active (Obaya & Sedivy, 2002). The activity of CDKs is dependent on a cyclin to bind as well as phosphorylation by Cdk-activating kinases (CAK) to activate. Cell division cycle 25 (CDC25) A, B and C

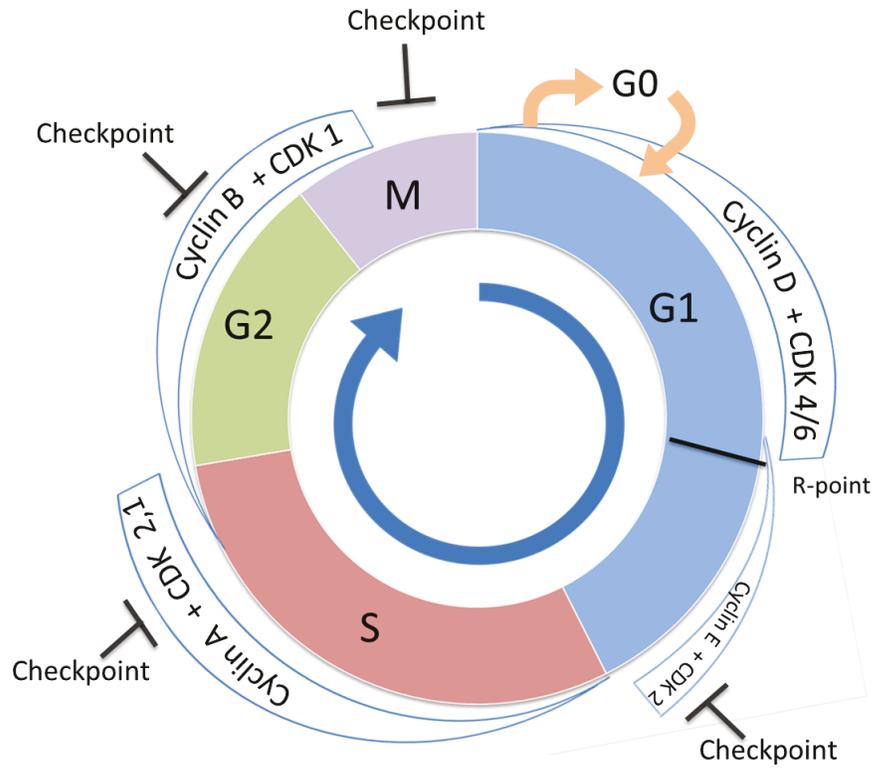


Figure 4 Overview of the cell cycle
 Overview of the cell cycle including changes in Cyclin and Cyclin Dependent Kinase (CDK) levels as well as checkpoints.

activates CDKs by dephosphorylating an inhibitory phosphate (Obaya & Sedivy, 2002).

There are two E3 ubiquitin ligase complexes that target cyclins and some other cell cycle targets. The first is the Skp-Cullin-F-box (SCF) complex and is active from late G_1 to early G_2 (Obaya & Sedivy, 2002). The second is the anaphase promoting complex (APC) active from late G_2 to early G_1 (Peters, 2002; Vodermaier, 2004).

In early G_1 , the cells have the option to become senescent (enter G_0 and not replicate) or continue and divide. Mitogenic signals help initiate the process of cell division. From early to mid G_1 cyclin D increases and binds to CDK 4 and 6 leading to increased activity of both cyclin-CDK complexes. All G_1 cyclin-CDK complexes progressively phosphorylate protein retinoblastoma (pRB). The restriction point (R-point) occurs mid to late in G_1 (Figure 4) and is point at which the cell becomes committed to the cell cycle and no longer responds to mitogenic signals (Obaya & Sedivy, 2002). pRb along with histone deacetylase (HDAC) are bound to the transcription factor E2 transcription factor (E2F) repressing E2F dependent gene expression. CyclinD-CDK4/6 phosphorylation of pRB disrupts HDAC allowing E2F to transcribe Cyclin E (Woo & Poon, 2003). When phosphorylated, pRB can no longer bind to E2F allowing it to be transcriptionally active and target genes involved in S phase. Following the R-point, levels of cyclin D plummet and levels of cyclin E and CDK2 increase at the end of G_1 (Obaya & Sedivy, 2002).

DNA is replicated during the S phase. At the end of G_1 , components of the pre-replication complex are phosphorylated. The Origin of Recognition Complex (ORC) binds to the initiation sequence along with CDC6 and Chromatin licencing and DNA replication

factor 1 (CDT1) and 6 minichromosome maintenance (MCM) family proteins are recruited. CDC7-Dumbbell Former 4 (DBF4) and cyclinE-CDK2 phosphorylate targets allowing for CDC45 which is essential for replication to bind and allows for the recruitment of DNA polymerase (Woo & Poon, 2003). At the end of G₁ the levels of cyclin E increases but subsequently decreases in S phase (Woo & Poon, 2003). The levels of cyclin A rise in S phase. Cyclin A-CDK2 is the main cyclin in early S phase and is responsible for phosphorylating targets involved in DNA replication. It phosphorylates Replication Protein A (RPA) proteins on the single stranded DNA as well as several DNA polymerases involved in elongation and is associated with the replication fork. Cyclin A is also responsible for ensuring that replication only occurs once per cell cycle. One of its targets is CDC6, when it is phosphorylated leaves the nucleus preventing endoreduplication (Bunz *et al*, 1998).

During G₂ the cell will continue to grow and prepare for mitosis. CDK1 replaces CDK2 and associates with Cyclin A in late S. Towards the end of G₂, Cyclin B levels rise and bring the cell into mitosis (Obaya & Sedivy, 2002).

Mitosis can be subdivided into several phases and is followed by cytokinesis. In prophase, chromosomes condense, spindle fibers form and the nuclear envelope disappears. Cyclin A is degraded by the APC after the nuclear envelope is gone. At prometaphase, the spindle fibers attach to kinetochores located at the centromeres. The chromatid align at the metaphase plate during metaphase, the spindle fibers attach the kinetochores to the poles. Cyclin B is degraded between metaphase and anaphase (Obaya & Sedivy, 2002). In anaphase the sister chromatids separate and move to

opposite poles. APC ubiquitinates securin, targeting it for degradation. Separase is derepressed which cleaves the cohesion complex keeping the sister chromatids together (Peters, 2002). In telophase, two new nuclear envelopes reform followed by cell division.

Tight control of the cell cycle is important as errors can lead to genomic instability that can lead to cancer. The cell has many checkpoints in place to prevent DNA damage or unreplicated DNA from propagating to daughter cells (Kastan & Bartek, 2004).

1.4.2 Checkpoints

Checkpoints exist at each stage of the cell cycle and several can be activated in response to DNA damage and stalled replication (Kastan & Bartek, 2004). There are two main protein kinases involved in initiating the signal transduction for activating the checkpoint pathway. Ataxia telangiectasia mutated (ATM) is activated in response to double stranded breaks and ATM and rad3-related (ATR) for the stalled DNA replication and single strand DNA (Kastan & Bartek, 2004). Sensor proteins complexes assemble at the site of DNA damage to activate ATM and ATR. Inactive ATM forms a homodimer and disassociates when active. It appears that can be activated either at the site of DNA damage or indirectly in response to the changes in chromatin structure (Kastan & Bartek, 2004). ATR forms a heterodimer with ATR interacting protein (ATRIP) and is activated at the damage site in order to be activated (Flynn & Zou, 2011). These kinases phosphorylate other targets involved in halting the cell cycle. ATM and ATR

phosphorylate checkpoint kinase 2 (CHK2) and CHK1 respectively and all four can phosphorylate p53 directly (Nyberg *et al*, 2002).

p53 is a tumour suppressor gene that regulates diverse process machinery involved in DNA repair, block of angiogenesis, apoptosis and cell cycle arrest or senescence. It forms a homotetramer which is usually rapidly turned over in the cell when not activated. MDM2 is a main inhibitor of p53 by binding to the N terminal domain, acting as an E3 ubiquitin ligase, targeting them for degradation by the proteasome. MDM2 is one of the transcriptional targets of p53 causing a negative feedback loop. Phosphorylation of p53 stabilizes it, and prevents MDM2 from binding. p53 can then act as a transcription factor activating genes involved in DNA repair, apoptosis, and cell cycle. p53 is non-functional in over half of all cancers (Riley *et al*, 2008). One of p53 transcriptional targets important in cell cycle regulation is p21, a general CDK inhibitor of CDK4, CDK6, CDK2 and CDK1 (Obya & Sedivy, 2002).

In G₁, there are two pathways that mediate the cell cycle arrest; a fast inhibition by targeting CDC25A and a slower one mediated by p53 – p21 that is required for prolonged arrest (Nyberg *et al*, 2002). In early and mid G₁, the levels of ATR are low and increase in late G₁ (Kastan & Bartek, 2004). CDC25A is phosphorylated by both CHK4 and CHK2, which results in its ubiquitination and degradation. As a result, CDK4 and CHK2 cannot be dephosphorylated, preventing phosphorylation of pRB, or preventing CyclinE-CDK2 initiating S phase (Kastan & Bartek, 2004). The second major pathway is through p53 transcription activity. ATM, ATR, CHK1 and CHK2 can all phosphorylate p53, stabilizing it and preventing degradation. ATM can also phosphorylate MDM2 to also

prevent p53 degradation. p21 has a major cell cycle regulatory role by inhibiting CDKs and Cyclins-CDK complexes (Kastan & Bartek, 2004; Nyberg *et al*, 2002).

S phase arrest can be due to either DNA damage and/ or a stalled replication fork. RPA to bind to stretches of single stranded DNA that forms around the stalled fork which causes an accumulation of these proteins. This accumulation recruits ATR-ATRIP resulting in ATRIP binding to RPA. ATR works to prevent fork collapse, triggers cell cycle arrest and promotes DNA repair (Flynn & Zou, 2011). ATM and ATR both respond to DNA damage and initiate the checkpoint but independently of p53 (Woo & Poon, 2003). CDC25A is targeted, inhibiting CDK2 activity, this also affects the binding of CDC45 onto chromatin, preventing initiation of replication (Kastan & Bartek, 2004).

The next checkpoint occurs in G₂ before the cell enters M phase. Unreplicated or damaged DNA will cause cell cycle arrest. ATM and ATR will phosphorylate Chk2 or Chk1 respectively, both phosphorylate CDC25C. Phosphorylation of CDC25C allows for one of the 14-3-3 proteins inhibiting its activity and is sequestered to the cytoplasm, preventing the dephosphorylation of CDK1, allowing for cell cycle arrest (Nyberg *et al*, 2002). p53 is also active at this stage, but not essential for arrest. p53 activity appears to be necessary for prolonged cell cycle arrest. The prolonged arrest is also associated with increased p21 (Bunz *et al*, 1998).

The M phase checkpoint or Spindle Assembly Checkpoint (SAC) occurs prior to Anaphase. If the spindle fibers are not attached to the kinetochores, the mitotic checkpoint complex (MCC) forms. This complex is composed of Mad2, BubR1, Bub3 and CDC20 (Lara-Gonzalez *et al*, 2012) and together inhibit the APC (Peters, 2002). The APC

is responsible for the degradation of cyclin B1 leading to mitotic exit. The APC also causes the degradation of securin allowing separase to cleave the cohesion ring allowing the sister chromatid to separate in anaphase (Peters, 2002).

1.5 p53 response to transcription stalling and inhibiting spliceosome components

p53 accumulates in the nucleus in response to RNA pol II stalling, even in the absence of DNA damage. When elongation is blocked, phosphorylation occurred at Ser-15 and acetylation of Lys-382 of p53. When the step between initiation and elongation is blocked, there is also an accumulation of p53, but no modifications to Ser-15 or Lys-382 (Reviewed Ljungman & Lane, 2004). There are a few reasons for the accumulation of p53. First, proteins with nuclear export signals (NES), such as p53, are linked with the export of mRNA. Blocking transcription results in fewer mRNA being exported and as a result fewer NES proteins are exported, resulting in an accumulation of proteins in the nucleus (Ljungman & Lane, 2004). In addition, ATR can phosphorylate p53 at Ser-15 in response to blocked transcription. It is suggested that ATR activation in response to transcription stalling may be similar to stalled replication. Transcription arrest could result in stretches of single stranded DNA around the stalled site due to DNA remodeling occurring before DNA transcription. These stretches could result in the recruitment of RPA which in turn could recruit ATR and result in the phosphorylation of p53. (Reviewed by Ljungman, 2007) This large accumulation of p53 can help increase the rate of DNA repair; however prolonged transcription stalling and stalling during S phase can induce apoptosis. Accumulation of NES proteins may promote apoptosis, as well a shift from

survival to pro-apoptotic genes. Survival genes are usually larger than apoptotic genes, and so have a greater chance of being affected by DNA damaging agents (Ljungman, 2007; McKay *et al*, 2004).

Inhibition of different splicing components (U1-70K, SF3B1, Ubiquitin specific peptidase 39 (USP39), Prpf31, Ubiquitin-like 5 (UBL5), Prpf8) through RNAi or by the small molecular inhibitor of CDC2 like kinase (responsible for phosphorylating SR proteins) TG003 causes a p53 response. There is an increase in p53 expression and transcriptional activity along with an increase in MDM2 and p21 and downregulation of MDMX in most cases. The stability of Mouse double minute 2 (MDM2) and MDMX also decreased (Allende-Vega *et al*, 2013). In HCT 116 cells, there was an increase in the G₁ cell population and in p53 null cell there was an increase at G₂/M. The resulting p53 response appears to occur globally in response to inhibition of any splicing component, and did not appear to be the result of DNA damage and these components does not cause transcription stalling (Allende-Vega *et al*, 2013). Cells treated with Spliceostatin A or Pladienolide B, two SF3B inhibitors, resulted in dephosphorylation of Ser2 at the CTD of RNA polymerase II, which does not occur with transcription inhibiting drugs. In addition, Spliceostatin A treatment resulted in the early disassociation of Pol II (Koga *et al*, 2015).

1.6 Rationale and Hypothesis

As detailed above, spliceosome inhibition can affect the p53 response and cell cycle progression yet the role of p53 in determining the outcome of spliceosome inhibition has not be examined in detail. Based on available evidence, we hypothesized

that inhibition of spliceosome assembly by isoginkgetin would activate a p53 response, leading to p53-dependent changes in gene expression and p53-dependent cell cycle checkpoints. As detailed in this thesis, isoginkgetin did not lead to a pronounced p53 response. Instead, alterations in cell cycle distribution are best explained by alternative splicing of cell cycle regulatory mRNAs.

2. Methods

2.1 Cell Culture

HCT 116 and an isogenic subline (HCT 116 p53^{-/-}) in which both alleles of p53 had been targeted by homologous recombination (Bunz *et al*, 1998) were used. Cells were grown in McCoy's Media (Hyclone, Logan, Utah) supplemented with a 10% mixture (1:3) of Fetal Bovine Serum (FBS) (Gibco, Mississauga, ON) and Newborn Calf Serum (NBCS) (Gibco, Mississauga, ON), 90 units/ml penicillin (Hyclone, Ottawa, ON) and 90 µg/ml of streptomycin (Hyclone, Ottawa, ON). Cells were split and evenly seeded into 6 cm tissue culture dishes 24 hours prior to treatment (Corning, Oneonta, NY). Cells were either treated with 3ml of media containing either; 15 µM or 30µM IGG (Calbiochem, Billerica, MA) suspended in Dimethyl Sulfoxide (DMSO) (Caledon Laboratories Limited, Georgetown, ON); an equivalent volume of DMSO as a vehicle control. Where indicated, no Drug control refers to growth medium with serum and antibiotics as described above.

2.2 Flow Cytometry

2.2.1 One Parameter Flow Cytometric Analysis

For cell cycle analysis, only the adherent cells were collected at either 8 or 24 hours following treatment. Cell monolayers were rinsed with Phosphate Buffered Saline (PBS), detached with trypsin, resuspended in PBS in 15 mL conical tubes and collected by centrifugation (1200 RPM). For apoptosis, the floating were collected and later combined with adherent cells collected, as described above. Pelleted cells were rinsed

twice in PBS followed by centrifugation. The cell pellets were fixed in 70% Ethanol (Commercial Alcohol, Brampton, ON) at -20°C for a minimum of 1 hour, prior to being stained with 20 µM Propidium Iodide (PI) (Sigma, St. Louis, MO) with 73µM RNaseA (Thermo Fisher Scientific, Ottawa, ON). Samples were run through the BD Accuri C6 Flow Cytometer (BD Mississauga, ON) and analysed using BD Accuri C6 Software (BD Mississauga, ON) and ModFit LT 4.1 software (Verity Software House, Topsham, ME).

2.2.2 Two Parameter Flow Cytometric Analysis

Samples were prepared as previously described (Chang *et al*, 1999). After 23 hours of treatment, the media was replaced with fresh media containing 30 µM 5'Bromo-2'deoxyuridine (BrdU) (Sigma, St. Louis, MO) for 1 hour. Cells were washed with PBS then collected with trypsin. Cells were centrifuged for 7 min at 1200 rpm. Cells were resuspended in 70% Ethanol and fixed for a minimum of 1 hour at -20°C. Cells were centrifuged as before and resuspended PBS containing RNase A in for 15min at 37°C. 1 ml of PBS was then added and centrifuged as before. The samples were then resuspended in 0.1 M HCl containing 0.7% Triton X-100 (Calbiochem, Billerica, MA) and put on ice for 15 min. 1 ml of PBS was added and then spun down. Cells were then resuspended in dH₂O and boiled at 97°C for 15 min then immediately put on ice for 15 min. 1 ml of 0.5% Tween 20 (Bioshop Canada Inc, Burlington, ON) in PBS was added and spun down. Cells were resuspended in 1:20 Alexa Fluor anti-BrdU conjugated Antibody (BD Mississauga, ON) suspended in PBS, 5% FBS and 0.5% Tween (HBT) for 30 min. 1 ml of HBT was added and spun down at the same setting. The samples were then stained

with 20 μ M PI. BrdU incorporation and DNA content was analysed using BD Accuri C6 software (BD Mississauga, ON).

2.3 Immunoblotting

Cells were washed twice with PBS then collected using RIPA Buffer (Sigma, St.Louis, MO). Samples were sonicated and protein was quantified using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad). Equal concentrations were then run through NuPage 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked in 5% milk in TBST for 1 hour. A primary antibody: p21 (Calbiochem) or actin (Sigma) suspended in 0.5% milk-TBST for 1 to 2 hours. The membrane was washed (4 x 5 min) with TBST. Membranes were in secondary antibody (Goat anti-mouse with a conjugated Horse Radish Peroxidase) (Santa Cruz) for 30 min to 1 hour. The membrane was washed (4 x 5 min) with TBST. 1 ml of Super Signal Pico Chemiluminescent Substrate was added incubated for 5 min then imaged.

2.4 RNA collection and analysis

Cells were washed with PBS, and then RNA was collected using Qiagen RNeasy Kit (Qiagen, Valencia, CA) following manufacturer instructions. For microarray analysis 300 ng of total RNA was sent to the Stemcore Laboratories Microarray Facility (Ottawa Hospital Research Institute, Ottawa, ON) for labeling and hybridization to Affymetrix Human Transcriptome 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA). Changes in gene expression and pre-mRNA splicing was performed using the Affymetrix Transcriptome Analysis Console v3.0 software.

For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative Polymerase Chain Reaction was done using the Taqman® system, including Universal MasterMix II and Mouse double minute 2 (MDM2), Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A) and Glyceraldehyde 3-Phosphate dehydrogenase (GAPDH) primers (Applied Biosystems, Foster City, CA).

3. Results

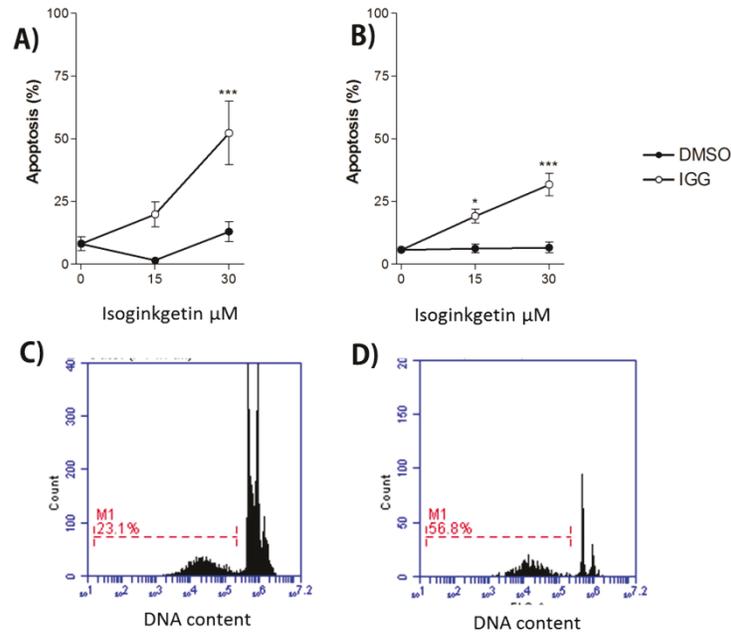


Figure 5 Sensitivity of colon cancer cells to isoginkgetin
HCT 116 (A) and HCT 116 p53^{-/-} (B) cells were incubated in the indicated concentration of IGG (D) in growth medium or an equivalent volume of DMSO (C), serving as a vehicle control. Apoptosis was assessed as the fraction of cells with subdiploid DNA content. The percent of subG₁ population is plotted in respect to dose. Each point (A) and (B) represents the mean (+/-Standard error of the mean (SEM)) determined from a minimum of 3 independent experiments (with exception of the HCT 116 IGG 15μM and corresponding vehicle controls with 2 experiments). IGG treatment led to significant differences in apoptosis compared to vehicle control. * and *** represent p<0.05 and p<0.001 respectively by two-way ANOVA and Bonferroni post-tests.

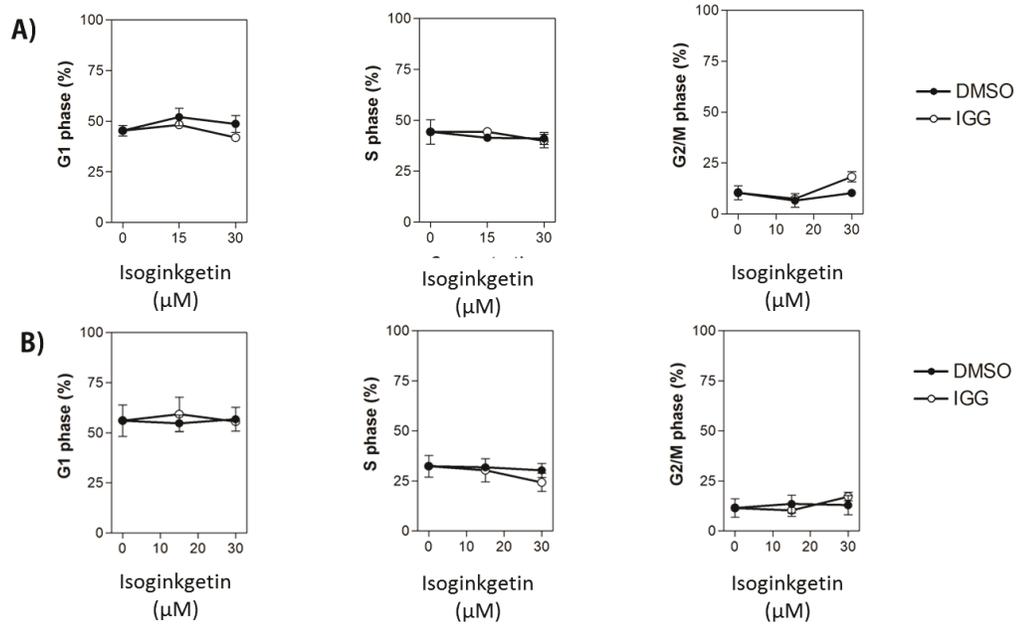


Figure 6 Isoginkgetin does not significantly alter cell cycle distribution in HCT 116 cells During the first 24 hours incubated in the indicated concentration in growth medium. An equivalent volume of DMSO was added as a control sample, serving as a vehicle control. HCT 116 cells were exposed to IGG for either 8 (A) or 24 (B) hours. Each point represents the mean (+/-SEM) determined from a minimum of 3 independent experiments. Cell cycle distribution was determined based on DNA content of propidium iodide stained cells. There were no significant changes in cell cycle distribution. Statistical analysis was performed by two-way ANOVA followed by Bonferroni post-tests.

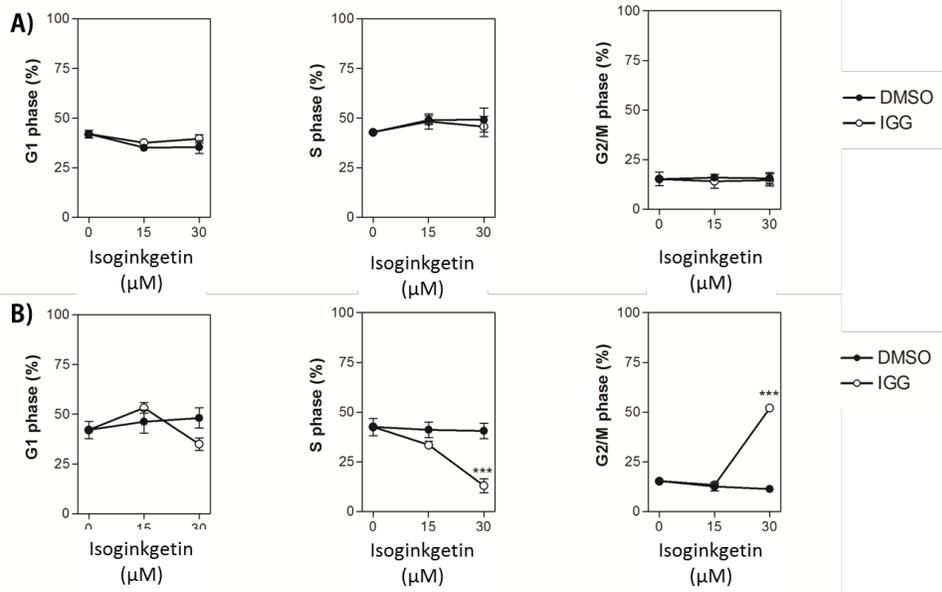


Figure 7 Isoginkgetin led to significant alteration in cell cycle distribution in HCT 116 p53 nullizygous cells.

HCT 116 p53^{-/-} cells were incubated in the indicated concentration in growth medium or an equivalent volume of DMSO as described in figure 6. IGG led to a significant decrease in S phase and a corresponding increase in the proportion of cells with 4C DNA content following a 24h exposure to 30 μM IGG. Each point represents the mean (+/-SEM) determined from a minimum of 3 independent experiments. *** indicate (p<0.001) and that the indicated dose is significantly different from the value of the corresponding vehicle control by Bonferroni post-tests.

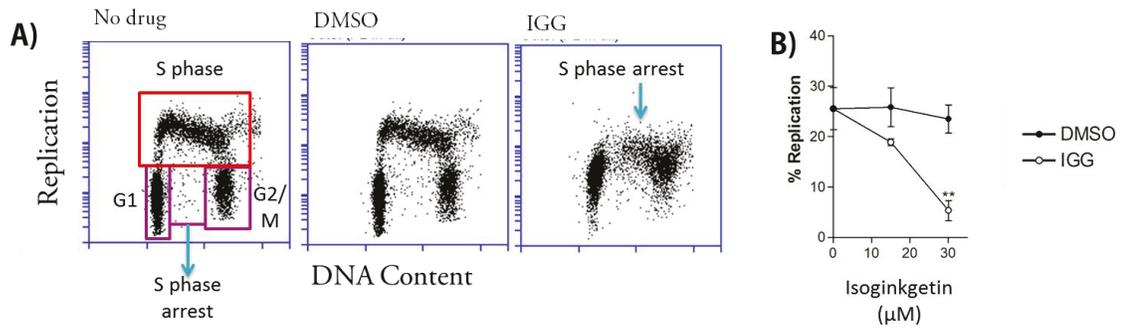


Figure 8 Isoginkgetin treatment led to decreased BrdU incorporation in HCT 116 cells. A) HCT 116 were treated with either IGG 30 μM or an equivalent volume of DMSO or left untreated. 23 hours following treatment bromodeoxyuridine was added for 1 hour to label nascent DNA. BrdU incorporation was measured with an FITC-conjugated anti-BrdU substituted DNA antibody. B) The percentage of cells incorporating BrdU is plotted with respect to dose. Each point represents the mean (+/-SEM) determined from a minimum of 3 independent experiments. ** indicates that the value is significantly different from in control ($p < 0.01$) by two-way ANOVA followed by Bonferroni post-tests.

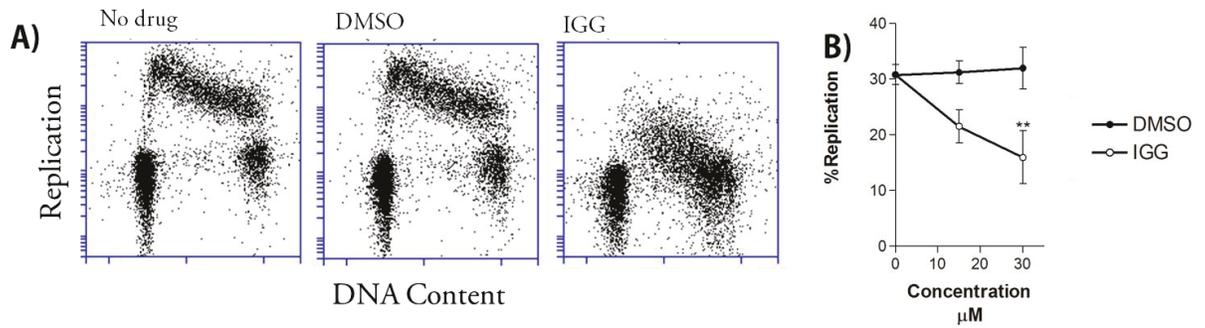


Figure 9 Isoginkgetin treatment cells led to decreased BrdU incorporation in HCT 116 p53^{-/-} cells.

A) HCT 116 p53^{-/-} were treated with either IGG 30μM or an equivalent volume of DMSO or left untreated. 23 hours following treatment bromodeoxyuridine was added for 1 hour to label nascent DNA. BrdU incorporation was measured with an FITC-conjugated anti-BrdU substituted DNA antibody. B) The percentage of cells incorporating BrdU is plotted with respect to dose. Each point represents the mean (+/-SEM) determined from a minimum of 3 independent experiments. ** indicates that the value is significantly different from in control (p<0.01) by two-way ANOVA followed by Bonferroni post-tests.

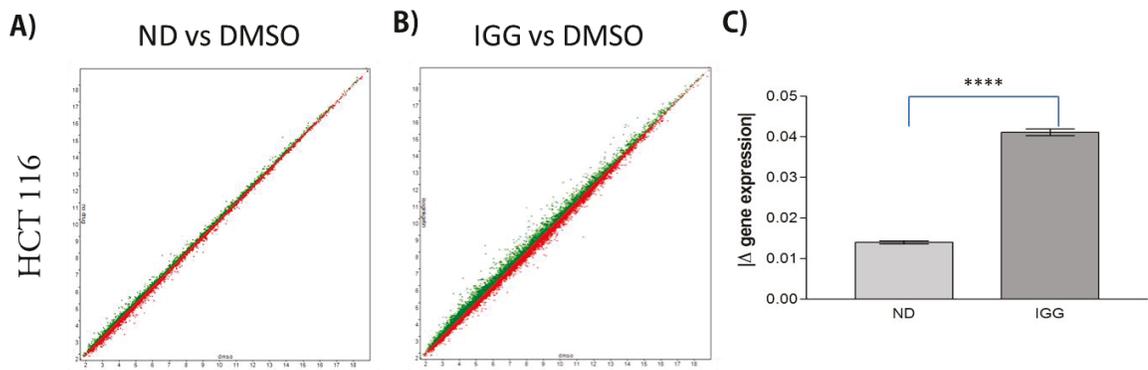


Figure 10 Microarray analysis of IGG - induced changes in gene expression in HCT 116 Cells were exposed to 30 μ M IGG, vehicle only (DMSO) or no drug (ND) for 8 hours and RNA was collected for oligonucleotide microarray analysis. Mean probeset intensity of ND (A) and IGG (B) are plotted with respect to vehicle control. The differences in probe intensity in A and B were determined and the mean absolute values of these differences (\pm SEM) are presented in C. There was a significant difference in the absolute value of differences in probeset intensities compared to the control $p < 0.0001$ (****) by student t-test.

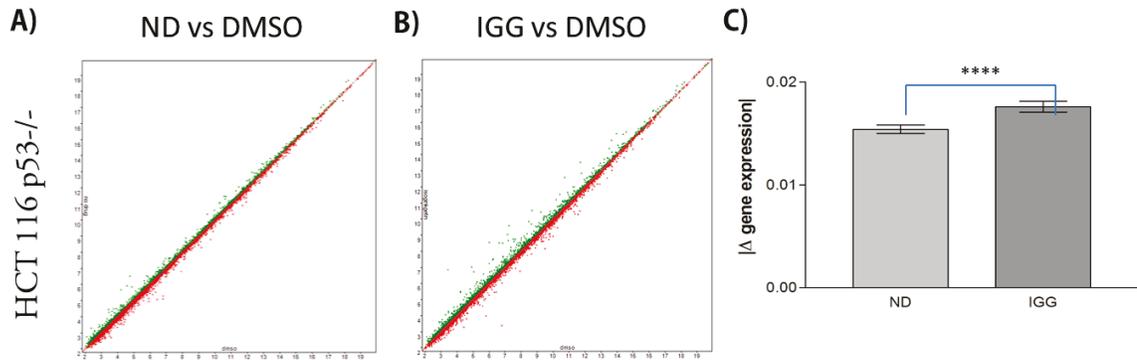


Figure 11 Microarray analysis of IGG - induced changes in gene expression in HCT 116 p53^{-/-} Cells were exposed to 30 μ M IGG, vehicle only (DMSO) or no drug (ND) for 8 hours and RNA was collected for oligonucleotide microarray analysis. Mean probeset intensity of ND (A) and IGG (B) are plotted with respect to vehicle control. The differences in probe intensity in (A) and (B) were determined and the mean absolute values of these differences (+/- SEM) are presented in (C). There was a significant difference in the absolute value of differences in probeset intensities compared to control $p < 0.0001$ (****) by student t-test.

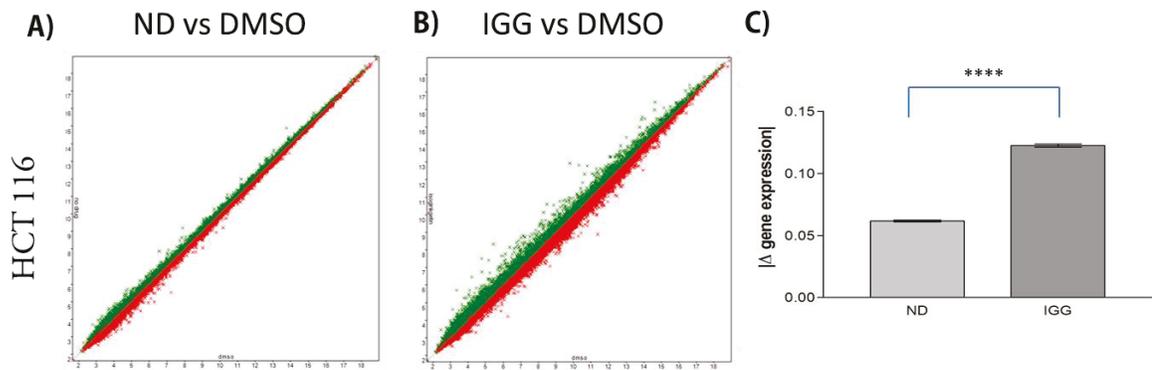


Figure 12 Microarray analysis of IGG - induced changes in pre-mRNA splicing in HCT 116 Cells were exposed to 30 μ M IGG, vehicle only (DMSO) or no drug (ND) for 8 hours and RNA was collected for oligonucleotide microarray analysis. Mean probeset intensity of ND (A) and IGG (B) are plotted with respect to vehicle control. The differences in probe intensity in A and B were determined and the mean absolute values of these differences (\pm SEM) are presented in (C). There was a significant difference in the absolute value of differences in probeset intensities compared to the control $p < 0.0001$ (****) by student t-test.

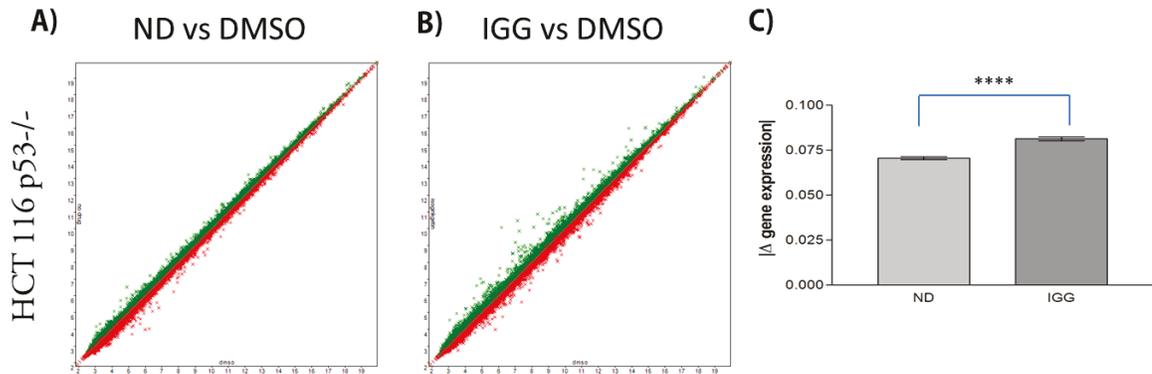


Figure 13 Microarray analysis of IGG - induced changes in pre-mRNA splicing in HCT 116 p53^{-/-}

Cells were exposed to 30 μ M IGG, vehicle only (DMSO) or no drug (ND) for 8 hours and RNA was collected for oligonucleotide microarray analysis. Mean probeset intensity of ND (A) and IGG (B) are plotted with respect to vehicle control. The differences in probe intensity in A and B were determined and the mean absolute values of these differences (+/- SEM) are presented in (C). There was a significant difference in the absolute value of differences in probeset intensities compared to the control $p < 0.0001$ (****) by student t-test.

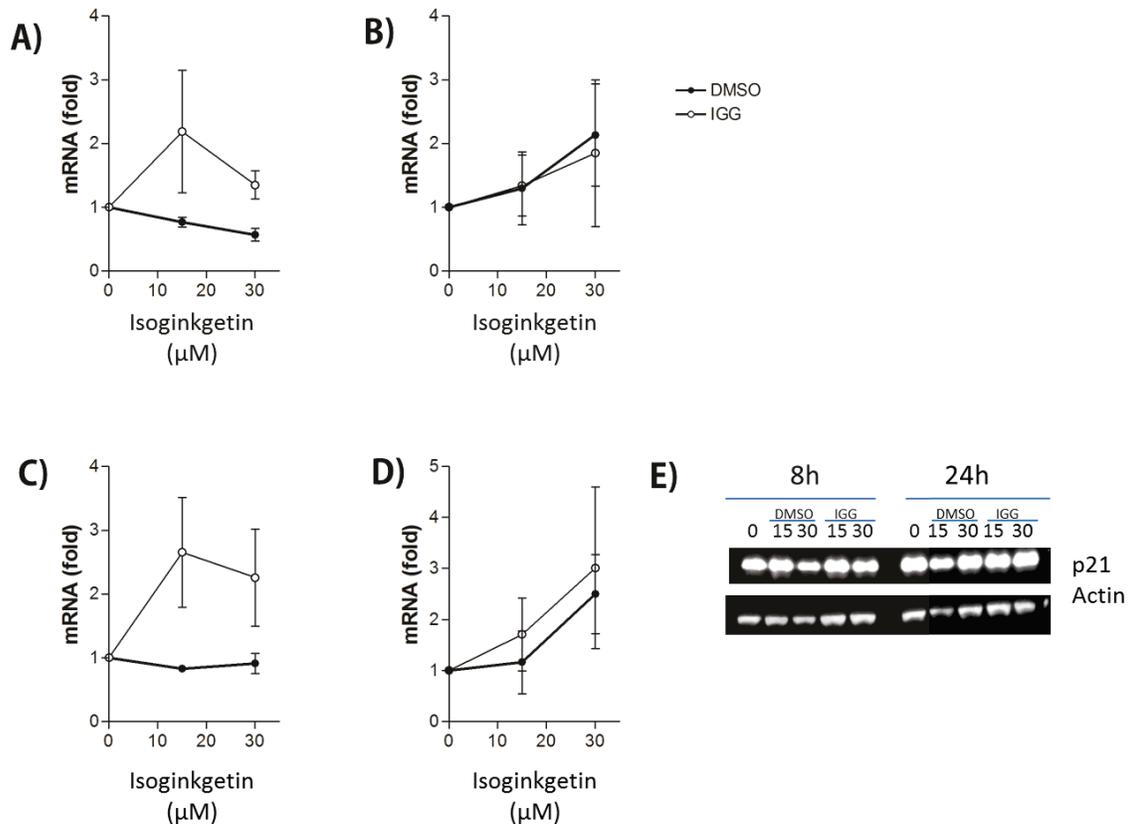


Figure 14 Isoginkgetin did not lead to a pronounced p53 response. RNA samples were collected following 8 (A and C) or 24 (B and D) hour exposure to the indicated concentration of IGG. The expression of p53 regulated transcripts: CDKN1A (A and B) and MDM2 (C and D) were assessed by qRT-PCR. Expression was normalized to GAPDH and is expressed in relation to untreated control. Each point (A-D) represents the mean (+/-SEM) determined from a min of 3 independent experiments (with exception of the HCT 116 DMSO 30 volume with only 2). Statistics were performed using a T test. (E) Protein samples were collected under similar conditions and p21 (encoded by CDKN1A) was visualized by immunoblot analysis.

3.1 Isoginkgetin affects cell viability/apoptosis

IGG is reported to have anti-cancer activity (Yoon *et al*, 2006). Here we sought to determine the dose response to HCT 116 using apoptosis as an endpoint. Apoptosis was assessed as the proportion of cells with sub diploid DNA content after 48h of drug treatment. A significant increase in the proportion of cells undergoing apoptosis between 15 and 30 μ M (Figure 5A) correlated well with the IC₅₀ of 30 μ M of IGG in splicing assays (O'Brien *et al*, 2008). The p53 tumour suppressor is an important determinant of sensitivity of many cells to apoptosis, so an isogenic p53 null cell line was included for comparison. As expected, there was significantly more cell death in the wild type p53 expressing cells, but apoptosis was still detected in the p53 null subline (Figure 5B). These results establish a relevant dose range to study the effects of IGG on these cells and suggests that p53 contributes to apoptosis following IGG treatment.

3.2 The effect of isoginkgetin on cell cycle distribution

Inhibition of pre-mRNA splicing reportedly led to mitotic defects in other systems. siRNAs against SON DNA binding protein (SON) (Huen *et al*, 2010), CDC5L (Mu *et al*, 2014), as well as yeast strains lacking Synthetic lethal with CDC40 1 (Syf1) (Russell *et al*, 2000), or CDC40 (Ben-Yehuda *et al*, 2000; Dahan & Kupiec, 2004) arrested in G₂ and/or M phase. A small molecular inhibitor of SF3b (FR901464) has been shown to arrest in G₁ and G₂/M (Nakajima *et al*, 1996). The effect of IGG on the cell cycle distribution has never been established. Therefore, we assessed cell cycle by 1 parameter flow cytometry of PI stained cells. Surprisingly, there was very little change in cell cycle distribution at either 8 or 24 hours in HCT 116 (Figure 6A and 6B). In contrast,

HCT 116 p53^{-/-} cells exhibited a large increase in the proportion of cells with 4C content with a corresponding large decrease in S phase (Figure 7B). It appears that loss of p53 in HCT 116 cells affects the response to IGG resulting in a large accumulation of cells with 4C DNA content at the expense of the S phase population. This is consistent with the onset of G₁ and either G₂ and/or M cell cycle arrests in the p53 null cell line but not the parental cell line.

One parameter flow cytometric analysis of cell cycle distribution is a useful technique, however, resolving early and late S phase populations requires predictive software (Modfit, Verity Software House) as these populations cannot be readily distinguished from G₀/G₁ or G₂/M populations, respectively. To address this, two parameter flow cytometric analysis of BrdU labelled cells was performed. This synthetic analog of thymidine can be visualized using a fluorescein isothiocyanate (FITC) conjugated anti-BrdU antibody. This method was used to simultaneously assess DNA replication (BrdU incorporation) and DNA content (PI staining) on a single cell basis. Treatment of HCT116 cells with IGG led to a dramatic cell cycle change that was not apparent by 1 parametric flow cytometric analysis. IGG led to a large decrease in replication, estimated by BrdU incorporation (Figure 8). Quantification of this decrease as a percentage of replicating cells confirmed that replication was greatly impeded in these cells (~3 fold). There was no large alteration in DNA content suggesting that passage of cells through all phases of the cell cycle was similarly decreased.

Two parameter flow cytometric analysis of the p53 nullizygous cells (Figure 9) indicated that there was also impaired S phase progression but that it appeared to be

less severe (~2 fold). Consistent with 1 parameter flow cytometric analysis, cells appeared to accumulate with 4C DNA content. Therefore, these p53 null cells may accumulate at either G₂ and or M phase because they progress through S phase and could reach the subsequent arrest more readily.

3.3 Preliminary Microarray Analysis

RNA was collected from HCT116 and HCT116 p53^{-/-} cells after 8 hours of treatment with IGG, ND or with a vehicle control (DMSO). This was used for oligonucleotide microarray analysis with Affymetrix human transcriptome 2.0, which allows exon level analysis of gene expression and pre-mRNA splicing. The mean probe intensity was determined for ND and IGG with respect to the DMSO control (Figures 10A, 10B, 11A and 11B). There was significantly more variation in probeset intensities ($p < 0.0001$) in IGG treated cells than ND cells (Figure 10C and 11C). In both gene expression and splice site expression, this effect was greater in wildtype expressing HCT 116 compared to p53-null cells (Figures 12 and 13).

3.4 Isoginkgetin does not cause a p53 response

Due to the differences observed between the two cell lines, the expression of two p53 regulated transcripts, among the most common indicators of p53 activity, Cyclin-dependent kinase inhibitor 1A (CDKN1A) (encodes p21) (Figure 14 A and B) and MDM2 (Figure 14 C and D) were analysed at 8 and 24 hours by qRT-PCR. There was no prominent change in expression of these mRNA, indicating that p53 was not very active. Protein samples were collected under similar conditions and p21 (encoded by CDKN1A) was visualized by immunoblot analysis (Fig 14 E) consistent with mRNA, no significant

change in p21 protein was observed. Microarray analysis did not show a change in p53 regulated gene expression either. It does not appear that there is a prominent p53 response to IGG in these cells.

4. Discussion

4.1 Isoginkgetin induces apoptosis

IGG is a natural biflavonoid that inhibits the spliceosome prior to the formation of or assembly of B complex (O'Brien *et al*, 2008). The cellular consequences of IGG on cell viability and death remains unclear therefore we performed a dose response using apoptosis as an endpoint to determine an appropriate dose range for further study. IGG caused a significant increase in apoptosis within 48 hours of treatment in HCT 116 and HCT 116 p53^{-/-} cells. p53 is an important regulator of DNA damage induced apoptosis because this protein can increase the expression of specific pro-apoptotic proteins like p53 upregulated modulator of apoptosis (PUMA), Phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1 also known as NOXA) and B-cell lymphoma 2 (Bcl-2) associated X (BAX) (Fridman & Lowe, 2003), however p53 can also induce apoptosis through transcription independent mechanisms (Moll *et al*, 2005). As expected, the cells with functioning p53 were more sensitive than the p53 nullizygous cells but with cell death detected in both cell lines at 30 μ M IGG. It is noteworthy, that we didn't detect a typical p53 transcriptional response by qRT-PCR, Western blotting or microarray analysis so this effect on apoptosis may be through the transactivation-independent pathway but that was beyond the scope of the present thesis work.

There have been few studies that have looked at the effect of IGG on apoptosis. Yoon *et al*, 2006, looked at the cytotoxicity of IGG in HT1080 fibrosarcoma cells that express wildtype p53 (Tarunina & Jenkins, 1993) and did not find a significant increase in cell death. This study only looked at cell death after 24 hours and at doses between 5-20

μM (Yoon *et al*, 2006). We assessed IGG induced apoptosis up to 30 μM , the reported IC_{50} for spliceosome assembly inhibition (O'Brien *et al*, 2008). It is conceivable that inhibition of pre-mRNA splicing could activate DNA damage response due to DNA damage, however, in a single test experiment, we were unable to detect γH2AX immunoreactivity by flow cytometry (McKay Lab, unpublished data).

In another study, IGG has been shown to cause cell death. Heinhuis *et al*, 2016, were studying the role of alternative splicing of the interleukin 32 (IL-32) mRNA and its consequences on apoptosis. IL-32 can be alternatively spliced into: IL-32 α , IL-32 β , and IL-32 γ . Overexpression of IL-32 β , and IL-32 γ triggers apoptosis by inducing caspase-8 pathway (Heinhuis *et al*, 2016). IGG treatment of (10 and 30 μM) was used to prevent alternative splicing of IL-32 in 2 thyroid cancer lines, FTC-133 (p53 mutation at codon 273) (Wright *et al*, 1991) and BCPAP (p53 mutation at codon 259) (Forbes *et al*, 2006). IL-32 γ was the predominant form of IL-32 under these conditions and this led to a significant increase in cell death in both cell lines. There was significantly more cell death in FTC-133 compared to BCPAP cells. This difference is thought to reflect differences in IL-8 signalling (Heinhuis *et al*, 2016).

4.1.1 The connection between splicing and apoptosis

The effect of splicing inhibition on cell death is not unique to IGG. Inhibition of pre-mRNA splicing led to apoptosis in a variety of systems (Hsu *et al*, 2015; Kurokawa *et al*, 2014; Mu *et al*, 2014). There appear to be target specific differences. For example, inhibition of CDC5L, a component of the NTC (involved in the spliceosome from the B complex until the end of splicing), caused cell death through mitotic catastrophe (Mu *et*

al, 2014). siRNA against CDC5L in HeLa cells caused mitotic arrest due to chromosome misalignment, reduced mitotic attachment to kinetochores and spindles that were much larger than the control (Mu *et al*, 2014). Microarray analysis showed that the expression of genes involved in cell death, cell survival and DNA damage response were altered. Cells spent a prolonged period in prometaphase which led to mitotic catastrophe (Mu *et al*, 2014).

Loss of SRSF1 in chicken B cell line DT40, led to the induction of apoptosis. SRSF1 is involved in splice site selection (Li *et al*, 2005). siRNA against SRSF3 led to apoptosis in HCT 116 colorectal cancer cells. Alternative splicing by skipping exon 8 of Homeodomain Interacting Protein Kinase-2 (HIPK2) was detected without a corresponding change in the expression of the protein (Kurokawa *et al*, 2014). This shorter isoform lacks a binding site for the E3 Ubiquitin ligase Seven in Absentia Homolog 1 (Siah-1) (Winter *et al*, 2008). Cells with lower levels of the full length transcript had an increase in apoptosis and caspase-3 activity (Kurokawa *et al*, 2014). Downregulation of anti-apoptotic BCL2 gene and pro-apoptotic gene BCL2A1 were also detected suggesting that loss of SRSF3 may affect a variety of proteins involved in apoptotic response (Kurokawa *et al*, 2014). Furthermore, inhibition of several components of the spliceosome including BUD31 in MYC hyperactive cells led to apoptosis (Hsu *et al*, 2015). Taken together, inhibition of pre-mRNA splicing through a variety of mechanisms led to distinct apoptotic responses.

It is noteworthy that apoptosis can itself be regulated by alternative pre-mRNA splicing. Splicing factors can be regulated by phosphorylation and dephosphorylation and this may contribute to changes in apoptotic sensitivity. Components throughout the

pathway can be alternatively spliced including: pro-apoptotic death receptor proteins (Fas); adaptor proteins (Tumor necrosis receptor associated factor (TNRAF) 2 and TNRAF3); members of the Bcl-2 family, (Bcl-x (anti-apoptotic), Bak (pro-apoptotic), Bim (pro-apoptotic)); Caspases (pro-apoptotic Caspase 2,9,10) and others (Schwerk & Schulze-Osthoff, 2005). Often, these alternative isoforms have antagonistic functions. For example, caspase 2 can be spliced to include or exclude exon 9. If retained, exon 9 has an early stop codon leading to Caspase-2S isoform which is anti-apoptotic instead of pro-apoptotic (Schwerk & Schulze-Osthoff, 2005). Therefore, splicing inhibitors have the potential to alter apoptotic responses through changes in alternative pre-mRNA splicing.

4.2 The Effect of isoginkgetin on the Cell cycle

The most remarkable cellular phenotypes identified in the present work were cell cycle related. Previous studies in yeast have identified arrests at G₂ and/or M phase with the inhibition of pre-mRNA splicing (Ben-Yehuda *et al*, 2000; Dahan & Kupiec, 2002; Russell *et al*, 2000). It has also been reported that IGG led to decreased proliferation (O'Brien *et al*, 2008), but cell cycle analysis was not assessed. In the present work, one parameter flow cytometric analysis of PI stained cells was performed to determine the cell cycle distribution based on DNA content. There was no significant change in the proportion of cells in G₁, S, or G₂/M within 24 hours (Figure 5B) in response to IGG using this method in HCT 116. However, similar experiments in the p53 null cell line led to remarkable changes in S and G₂/M phases. Specifically, IGG led to a dose-dependent decrease in S phase and a corresponding increase in the proportion of cells with 4C DNA

content (G₂ and M phase combined) (Figure 6B). Therefore we detected a large cell cycle alteration primarily in the absence of p53.

Early studies looking into the connection between cell cycle progression and splicing was first done in yeast. Cell division cycle proteins (CDC) proteins like CDC40 were first identified for their role in cell cycle progression. CDC40, SYF1 and SYF3 depletion led to G₂/M arrest in yeast cells (Ben-Yehuda *et al*, 2000; Dahan & Kupiec, 2004; Russell *et al*, 2000). Double mutants of Interactor of Syf1p (ISY1) and SYF2 caused spindle checkpoint arrest due to accumulation of unspliced Tubulin 1 (TUB1) and TUB3 leading to a decrease in α -tubulin (Dahan & Kupiec, 2002).

The human ortholog of SYF1 is XPA binding protein 2 (XAB2), and these proteins are present in similar splicing complex. This protein is involved in DNA repair, transcription and pre-mRNA splicing (Nakatsu *et al*, 2000). Initially, XAB2 was studied in our lab due to its role in transcription coupled nucleotide excision repair (TC-NER). In an effort to facilitate knockdown, an adenovirus vector was used to express a shRNA directed against XAB2 (AdshXAB2). Infection of tumor cells with AdshXAB2 induced a variety of cellular phenotypes in a cell specific manner. For example, there was a large increase in apoptosis in A2780CP ovarian cell cancer lines. In contrast, PC3 (prostate cancer cell line) cells underwent endoreduplication, and to a lesser extent, apoptosis. In HCT 116 and HCT 116 p53^{-/-} there was an arrest in G₂/M and some endoreduplication. Importantly, knockdown of XAB2 in primary human fibroblast cells did not alter the cell cycle distribution or undergo apoptosis (Stubbert *et al.*, in preparation). Therefore

there is some similarity between XAB2 knockdown in human cells and SYF1 loss in yeast (Ben-Yehuda et al., 2000)(Russell *et al*, 2000).

Other studies have noted a link between splicing inhibition and cell cycle regulation in yeast, humans and other species, not all have shown that the arrest is due to the effect on splicing products. The NTC complex has been demonstrated *in vitro* in *Xenopus* eggs to be involved in the spindle assembly independently of its role in splicing (phenotype not observed when treated with spliceostatin A or actinomycin D) (Hofmann *et al*, 2013). Microtubule kinetochore attachment was affected as well as a decrease in bipolar spindles (Hofmann *et al*, 2013) However, as mentioned previously, inhibition of CDC5L, a component of the NTC resulted in splicing defects in genes important in cell death, cell survival, cell cycle and mitotic progression. The HeLa cells arrested in M phase with misaligned chromosomes, kinetochore microtubule attachment were observed and splicing was at least partially responsible for this phenotype (Mu *et al*, 2014).

Downregulation of SRSF3 causes G₁ arrest with decreased levels of cyclin D1, D3, E2F1, and E2F7, while pRb remained hypophosphorylated. Treatment with FR901464, a small molecular inhibitor of SF3b resulted in a G₁ and G₂/M arrest (Kaida *et al*, 2007; Nakajima *et al*, 1996). This molecule in addition to its methylated derivative caused a C-terminal truncated p27, a CDK inhibitor. This isoform was resistant to proteasome degradation and interacted with and inhibited activity of CDK2 (Kaida *et al*, 2007). SON, a SR associated protein, arrest cells in M phase around prometaphase (Ahn *et al*, 2011; Huen *et al*, 2010), and resulted in abnormal nuclei, aneuploidy and death (Huen *et al*,

2010). SON is involved in splicing of weak splice sites, involved in splicing of genes involved in cell cycle (Ahn *et al*, 2011). Microarray analysis showed a decrease in cell cycle, DNA recombination, DNA replication and DNA repair, G₂/M checkpoint, and others (Ahn *et al*, 2011). Finally, cell cycle arrest has been shown to change in response to differences in the efficiency of splicing factor knockdown. siRNA against snRNP polypeptide B and B1 (SmB/B') (SNRPB) caused a decrease in APC2, Mitotic arrest deficient 2 (Mad2), Bub1 Related Kinase (BubR1) and γ -tubulin and the decrease levels correlated with the decrease in SNRPB. siRNA against SNRPB caused G₂ arrest but with increasing concentrations caused earlier G₁ arrest (Karamysheva *et al*, 2015). It appears that the effect on cell cycle depends on the component(s) being inhibited.

To gain additional insight into cell cycle alterations associated with IGG treatment, the S phase population was visualized more clearly using a two parameter flow cytometric analysis of DNA replication (measured by BrdU incorporation) and DNA content (PI staining). This method unmasked a clear IGG dependent change in cell cycle distribution in both cell lines. DNA replication was significantly reduced in HCT116 and HCT116 p53^{-/-} cells. Therefore, IGG led to an S phase arrest in these cell lines, albeit to a lesser extent in p53 nulls. The overall distribution of the cells based on DNA content was similar in both cases to changes identified by PI staining alone. The proportion of cells in any given phase of the cell cycle is affected by changes in the other phases. The proportion of cells is dependent on the transit time in the specific phase in question as well as the rate of entry into and exit from this phase of the cell cycle. For the S phase population, the proportion of cells entering S phase from G₁, completing S phase and

the proportion of cells undergoing apoptosis or other forms of cell death from S phase will contribute to this fraction. We did not detect significant apoptosis within 24 hours, the time used to assess the effects of IGG on cell cycle distribution. Therefore, the proportion of cells in S phase here is dictated primarily by the rate of entry and time to complete S phase.

In HCT116 cells, there was a very pronounced S phase arrest as indicated by a 3 fold decrease in the rate of DNA replication. The corresponding increase in transit time should have increased the proportion of cells in S phase if there was no corresponding decrease in the rate of S phase entry. However, the proportion of cells in S phase did not increase so the proportion of cells entering S decreased equally. Through similar logic, it is clear that there were roughly equivalent delays in all phases of the cell cycle in these cells.

In the p53 null cells, the slowdown of S phase was associated with a decrease in the S phase population. This counterintuitive pattern is best explained by a more pronounced decrease in S phase entry (G_1 arrest) than S phase progression. The cells that transited S phase accumulated in G_2 and/or M phase accumulation. Alternatively stated, blocks in G_1 , G_2 and/or M phase were greater than the S phase arrest in the p53 null cell line.

The only other reported change in S phase associated with inhibition of pre-mRNA splicing was observed with knockdown of Crooked Neck Pre-mRNA Splicing Factor 1 (CRNKL1), a spliceosome associated protein (Karamysheva *et al*, 2015). However, this was only studied in a one parameter flow cytometric analysis.

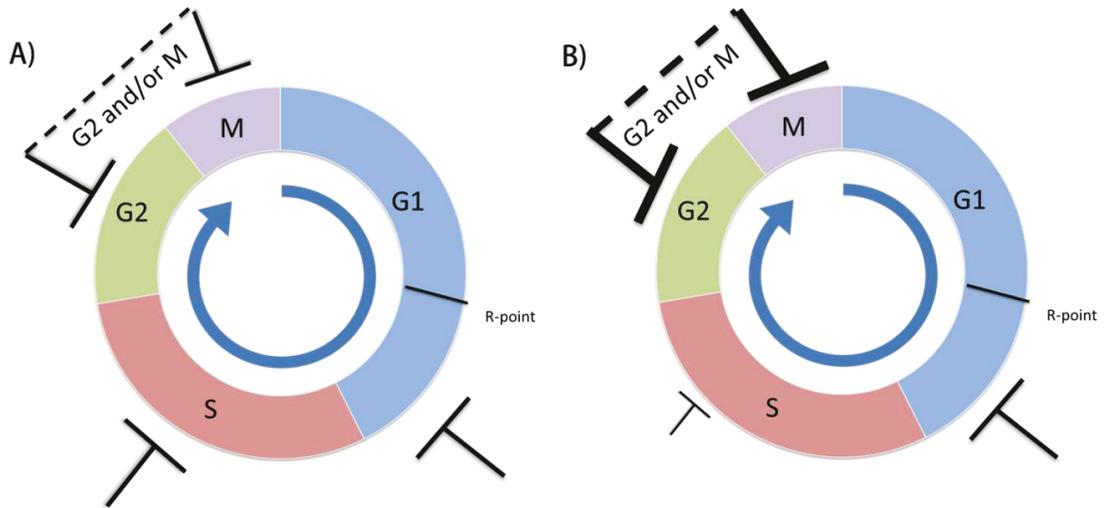


Figure 15 Cell cycle arrest due to isoginkgetin treatment

The potential checkpoints affected by treatment with isoginkgetin in A) HCT 116 and B) 116 $p53^{-/-}$. The study was not sensitive enough to differentiate between G_2 and/or M phase arrest(s) so dashed lines are used to denote the possible arrest site(s).

Mitosis		Other cell cycle transcripts	
transcript	complex OR function	transcript	complex or function
CDC16	Anaphase promoting complex	CDKL3	cyclin-dependent kinase
CDK11B	Centromere (CDK)	CDKN2AIP	CDKN2A interacting protein
CENPQ	Centromere	COP55	COP9 signalosome, interacts with SKP1-CUL1-F-box complexes
CEP290	Centrosome	CUL1	SKP1-CUL1-F-box complex
CEP97	Centrosome	MCM8	pre-replication complex
NUF2	Centromere/kinetochore complex	ORC2	origin recognition complex
PCNT	Centrosome		
SEPT7	Centromere/kinetochore/Cytokinesis		

Table 2 Changes in splicing index (probe set signal intensity) in response to isoginkgetin 30 μ M after 8h (McKay Lab, unpublished)

4.3 Preliminary Microarray analysis

Microarray analysis of cells treated with IGG for 8 hours was performed to identify the changes in gene expression that may lead to the cell cycle phenotypes observed (Figure 9 and 10). Affymetrix Human Transcriptome 2.0 oligonucleotide microarrays were used. These microarrays allow exon level analysis of gene expression and pre-mRNA splicing. In general there was a greater tendency to be upregulated rather than downregulated in response to IGG. This bias may reflect the fact that stable transcripts require time to decay so at 8 hours down-regulated transcripts may not yet be detectably decreased due to the stability of pre-existing mRNAs. The most prominent group of induced transcripts were those encoding components of the unfolded protein response (DNA damage-inducible transcript 3 + 4 (DDIT3 and DDIT4 respectively), Glutathione-specific gamma-glutamylcyclotransferase 1 (CHAC1) suggesting that this stress response is activated by IGG-mediated inhibition of spliceosome assembly.

We also detected alternations in pre-mRNA splicing (Table 2) Gene ontology analysis based on changes in splicing index in HCT 116 cells included MCM8 and ORC2, both of which are required for DNA synthesis and S phase (Woo & Poon, 2003). MCM8 is involved in elongation but not initiation of replication (Maiorano *et al*, 2005). Additional cell cycle related genes that were affected included Cullin1 (CUL1), a component of the SCF complex and Constitutive photomorphogenesis 9 (COP9) signalosome subunit 5 (COPS5) (Table 2), which interacts with SCF (Vodermaier, 2004). The SCF is important in the degradation of components of the cell cycle from late G₁ to early G₂ (Obaya & Sedivy, 2002). The splice variant of CUL1 could result in the

dysregulation of components of cell cycle regulatory proteins normally targeted for proteasome-mediated degradation in a CUL1-dependent manner.

In addition, many genes involved in mitosis were alternatively spliced in response to IGG including: centromere, centrosome, microtubule and kinetochore components. In addition, CDC16, a component of the APC was alternatively spliced (Table 2). Based on the altered gene expression of so many mitotic genes, it is likely that the accumulation of cells with 4C DNA content was due to M phase arrest and not G₂ arrest. However, future experiments will be required to distinguish these alternatives because our cell cycle analysis could not distinguish between G₂ and / or M phase arrest.

4.4 Isoginkgetin had an unexpected p53 response

As there was a distinct differences between HCT 116 and HCT 116 p53^{-/-} cells in the cell cycle distribution, apoptosis and microarray studies, we looked at the changes in gene expression in 2 genes regulated by p53 that are the most common indicators of p53 activity. MDM2, an inhibitor of p53 (Vogelstein *et al*, 2000) and p21, a CDK inhibitor that is important in the G₁ cell cycle arrest and maintenance of the G₂ arrest (Bunz *et al*, 1998). Surprisingly, analysis with qRT-PCR resulted in very little change in gene expression in HCT 116 cells (Figure 13 A-D), which was similar to the p53 null cells (Data not shown). Western blot analysis of p21 in HCT 116 cells failed to identify difference in the expression of p21 protein (Figure 13E).

Previous studies looking at the p53 response to splicing inhibition in HCT 116 and HCT 116 p53^{-/-} have found different responses. As previously mentioned Allende-Vega *et al*, 2013 inhibited multiple components including siRNA against; U1-70K, USP39, Prpf31

UBL5, Prpf8 as well as a small molecular inhibitor against CDC2 like kinase, TG003. A p53 response was observed in all as well as an increase in p53 transcriptional response including MDM2 and p21 (Allende-Vega *et al*, 2013; Kurokawa *et al*, 2014). In most cases there was a decrease in MDMX (independent of p53) and a decrease in MDM2 and MDMX stability. Inhibition of prp8f, UBL5 and TG003 caused a G₁ arrest in HCT 116. In HCT 116 p53^{-/-}, there wasn't a G₁ arrest but an accumulation of cells at G₂/M was observed in response to decrease Prp8f and TG003 (Allende-Vega *et al*, 2013; Kurokawa *et al*, 2014). In contrast, downregulation of SRSF3 caused G₁ arrest and apoptosis in HCT 116 and HCT 116 p53^{-/-} cells and there was no change in p53 phosphorylation or levels. SRSF3 knockdown resulted in alternative splicing of HPK2 and the increase of the proteins lacking exon 8 caused phosphorylation at Ser46, but this alone was not enough for the caspase-3 activity initiation (Kurokawa *et al*, 2014). We used an adenovirus expressing an shRNA targeting XAB2 and similarly failed to identify a prominent p53 response despite large scale differences in cell cycle distribution (Stubbert *et al*, in preparation). Therefore, distinct RNAi strategies to inhibit pre-mRNA splicing affect whether p53 is activated or not.

Use of FR901464, an inhibitor of SF3b, resulted in G₁ and G₂/M arrest in MCF-7 breast cancer cells, resulted in a decrease in p53, c-Myc and E2F (Nakajima *et al*, 1996). Meayamycin, an analogue of FR901464, had antiproliferative effects in cancer cells but not in healthy cells. Meayamycin had an increase in cell death without going through apoptosis or mitotic arrest in A549 lung cancer cells (control lung fibroblasts, IMR-90 had lower levels of death). p53 was activated in both cell lines, but it was greater in

A549. Despite this, HCT 116 and HCT 116 p53^{-/-} proliferation was equally inhibited in meayamycin indicating p53 was not involved in the antiproliferative effects (Albert *et al*, 2009). Therefore, drugs that inhibit pre-mRNA splicing may induce a p53 response in some but not all cell lines.

We conclude that IGG does not induce a p53 dependent G₁ or G₂ arrest in these cell lines, yet we detected p53-dependent differences in apoptosis and cell cycle regulation. The precise mechanism will require further study.

Conclusions

Isoginkgetin causes cell cycle arrest in multiple phases of the cell cycle. There is precedent for G₁, G₂ and M phase arrest from various spliceosome targeting knockdowns, but we have identified a novel S phase arrest resulting from spliceosome inhibition that is much more prominent in the p53-expressing cells. In the absence of p53, IGG resulted in a more prominent arrest that is most likely in M phase. Taken together, we identified a novel and unexpected pattern of p53-dependent cell cycle regulation in IGG-treated cells that could be important in determining the efficacy of this putative anti-neoplastic agent.

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