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The role of transcription elongation factor IIS in transcription-coupled nucleotide excision repair

**By:
Christine MacKinnon-Roy**

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Abstract

Transcription-coupled nucleotide excision repair (TC-NER) removes bulky DNA lesions from the template strand at actively transcribed genes. The RNA polymerase II (RNAPII) holoenzyme complex forms a stable ternary complex at the site of DNA damage which may block access of DNA repair proteins to the site of DNA lesions. Therefore, there is considerable interest in understanding how repair is coupled to transcription. Based on elegant *in vitro* studies, it has been hypothesized that transcription elongation factor IIS (TFIIS), by catalyzing the reverse translocation of RNAPII, may allow access of DNA repair proteins to sites of DNA damage. Here, we tested this hypothesis by assessing TC-NER capacity in cells in which TFIIS expression has been reduced by RNA interference. Surprisingly, we found that decreased TFIIS levels did not affect the repair of transcription-blocking DNA lesions and did not affect the sensitivity of targeted cells to UV light or cisplatin. These results do not support a role for TFIIS in TC-NER. We conclude conservatively that TFIIS levels are not limiting for TC-NER.

Table of Contents

1. Introduction.....	1
1.1. Nucleotide excision repair.....	2
1.2. Diseases associated with defects in nucleotide excision repair.....	8
1.3. Transcription-coupled nucleotide excision repair paradox.....	12
1.4. Transcription elongation factor IIS.....	16
1.5. Hypothetical role of TFIIS in TC-NER	20
1.6. Hypothesis.....	20
2. Materials and Methods.....	21
2.1. Cell Culture.....	21
2.2. RNA interference.....	21
2.3. Western Immunoblot analysis.....	22
2.4. Host Cell Reactivation.....	24
2.5. Recovery of mRNA synthesis.....	25
2.6. SubG1 assay.....	26
2.7. Real-time reverse transcription polymerase chain reaction.....	26
2.8. Growth assay.....	27
3. Results.....	28
3.1. Targeting TFIIS by RNA interference.....	28
3.2. Transcription elongation factor IIS is not limiting for the repair of a	

UV-damaged reporter gene.....	28
3.3. Transcription elongation factor IIS is not limiting for the recovery of RNA synthesis following UV exposure.....	40
3.4. Silencing of TFIIIS does not increase the sensitivity of colorectal carcinoma to UV-C light or cisplatin.....	40
3.5. Differences in cell proliferation following transfection of distinct siRNAs against TCEA1.....	43
4. Discussion.....	54
<hr/>	
4.1. Transcription elongation factor IIS is not limiting for transcription- coupled nucleotide excision repair.....	55
4.2. Does RNA interference against transcription elongation factor IIS affect cell growth?.....	56
4.3. How do DNA repair proteins gain access to DNA damaged lesion?.....	57
4.4. Conclusion.....	58
5. References.....	59

List of Figures

Figure 1 Schematic representation of nucleotide excision repair.

Figure 2. Transcription-coupled nucleotide excision repair complex.

Figure 3. Hypothetical RNAPII events upon encountering a damage DNA lesion.

Figure 4. A graphical representation of TFIIS found in *S. cerevisiae*.

Figure 5. Reduction of transcription elongation factor IIS in HCT116 colorectal carcinoma cells.

Figure 6. Relative gene expression of TFIIS isoforms in colorectal carcinoma cells.

Figure 7. Transcription-elongation factor-IIS is not required in the repair of UV-C induced cyclobutane pyrimidine dimers in a β -galactosidase reporter construct in colorectal carcinoma cells.

Figure 8. Protein levels of transcription elongation factor IIS in human fibroblasts.

Figure 9. Transcription elongation factor IIS is not required in the repair of UVC induced cyclobutane pyrimidine dimers in a β -galactosidase reporter construct in fibroblasts.

Figure 10. Transcription elongation factor IIS is not required for recovery of RNA synthesis.

Figure 11. Silencing TFIIS does not increase the sensitivity of colorectal carcinoma cells to UV-C irradiation.

Figure 12. Silencing of TFIIS does not increase the sensitivity of colorectal carcinoma cells to cisplatin.

Figure 13. Slight decrease in proliferation of colorectal carcinoma cells when TFIIS is targeted by siTFIIS#2.

Figure 14. Proliferation of breast adenocarcinoma cells is inhibited when TFIIIS is silenced by siTFIIIS#2.

Figure 15. Reduction in TFIIIS protein levels in human breast adenocarcinoma cells.

List of Tables

Table1. siRNA sequences and concentrations used in RNAi.

List of Abbreviations

6-4 PP: 6-4 pyrimidine pyrimidone photoproducts

CEN2: centrin 2

ChIP: chromatin immunoprecipitation

CPD: cyclobutane pyrimidine dimers

CS: Cockayne syndrome

CSA and CSB: Cockayne syndrome type A and B, respectively

DDB: damage DNA-binding protein

ddH₂O: double-distilled water

DHFR: dihydrofolate reductase gene

ERCC1: excision repair cross-complementing

EtOH: ethanol

GG-NER: global genome nucleotide excision repair

HCR: host cell reactivation

HMGN1: high mobility group nucleosome binding domain I

NaPPi: sodium pyrophosphate

NER: nucleotide excision repair

NT: non-targeting

PBS: phosphate buffered saline

PCNA: proliferating cell nuclear antigen

PI: propidium iodide

PIC: preinitiation complex

RFC: replication factor C

RNAi: RNA interference

RNAPII: RNA polymerase II

RPA: replication protein A

RRS: recovery of RNA synthesis

SDS: sodium dodecyl sulfate

siRNA: small-interfering RNA

TBST: tris-buffered saline Tween-20

TCA: trichloroacetic acid

TC-NER: transcription-coupled nucleotide excision repair

TFIIH: transcription factor IIH

TFIIS: transcription elongation factor IIS

UV: ultraviolet

XAB2: XPA binding protein 2

XP: xeroderma pigmentosum

XP-A to XP-G and XP-V: xeroderma pigmentosum complementation group A and

variant

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1. Introduction

DNA is continuously exposed to both endogenous and exogenous damaging agents, such as oxidative metabolism and the environment, respectively. In addition DNA lesions may in turn block biological processes such as DNA replication and transcription while indirectly modifying gene expression and leading to cell death.

Unrepaired DNA damage can be mutagenic and this can lead to the development of cancer. For example, exposure to UV light produces cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine pyrimidone-photoproducts (6,4-PPs) (Pfeifer, 1997). These covalent modifications cause structural distortions in DNA and the replicative bypass of these lesions results in mutations. The accumulation of mutations in tumour suppresser genes and oncogenes contributes to the development of skin cancers. Cigarette smoke represents another example of an environmental carcinogen. Cigarette smoke contains benzo[a]pyrene which is oxidized into a carcinogenic form by a series of metabolic reactions involving cytochrome P450 enzymes, ultimately resulting in the formation of DNA adducts (Nouspikel 2009). Again, fixation of these mutations can promote lung tumour development.

Ironically, DNA damaging agents can lead to neoplastic disease but they are also used to treat cancer. In fact, most conventional cancer therapeutics exert their anti-neoplastic activities through the induction of DNA damage. Cisplatin is one of the most common chemotherapeutic drugs used in clinical practice. It is used in the treatment of a

variety of cancers such as testicular and ovarian carcinomas. It interacts with DNA and results in the formation of intrastrand and interstrand DNA adducts. It is clear from the complex relationship between carcinogens and cancer therapy that understanding DNA damage and repair is important for our understanding of neoplastic diseases.

1.1) Nucleotide excision repair

Organisms have evolved many processes to repair DNA damage in order to prevent genomic instability and acute cell loss. One DNA repair pathway that has evolved is nucleotide excision repair (NER) and it recognizes structural distortions in DNA (Nospikel 2009). NER recognizes and repairs damaged DNA resulting from endogenous and exogenous agents.

Most of what is known about nucleotide excision repair stems from studies using UV-C light as a model DNA damaging agent. DNA damage resulting from UV irradiation is from short wavelength UV light, from either UV-B or UV-C (Pfeifer, 1997). UV-induced damage results mainly in the formation of cyclobutane pyrimidine dimers and 6,4-pyrimidine pyrimidone photoproducts (Pfeifer, 1997). A CPD is a dimer formed by the covalent bond between adjacent pyrimidines (Hanawalt *et al*, 2008). They can be thymine dimers, cytosine dimers, thymine-cytosine dimers and cytosine-thymine dimers, although the majority of CPDs are formed between adjacent thymines. The covalent bond between two adjacent pyrimidines is formed by two bonds from carbon-4 to carbon-4 and the other between carbon-5 and carbon-5, forming a ring and indirectly creating a

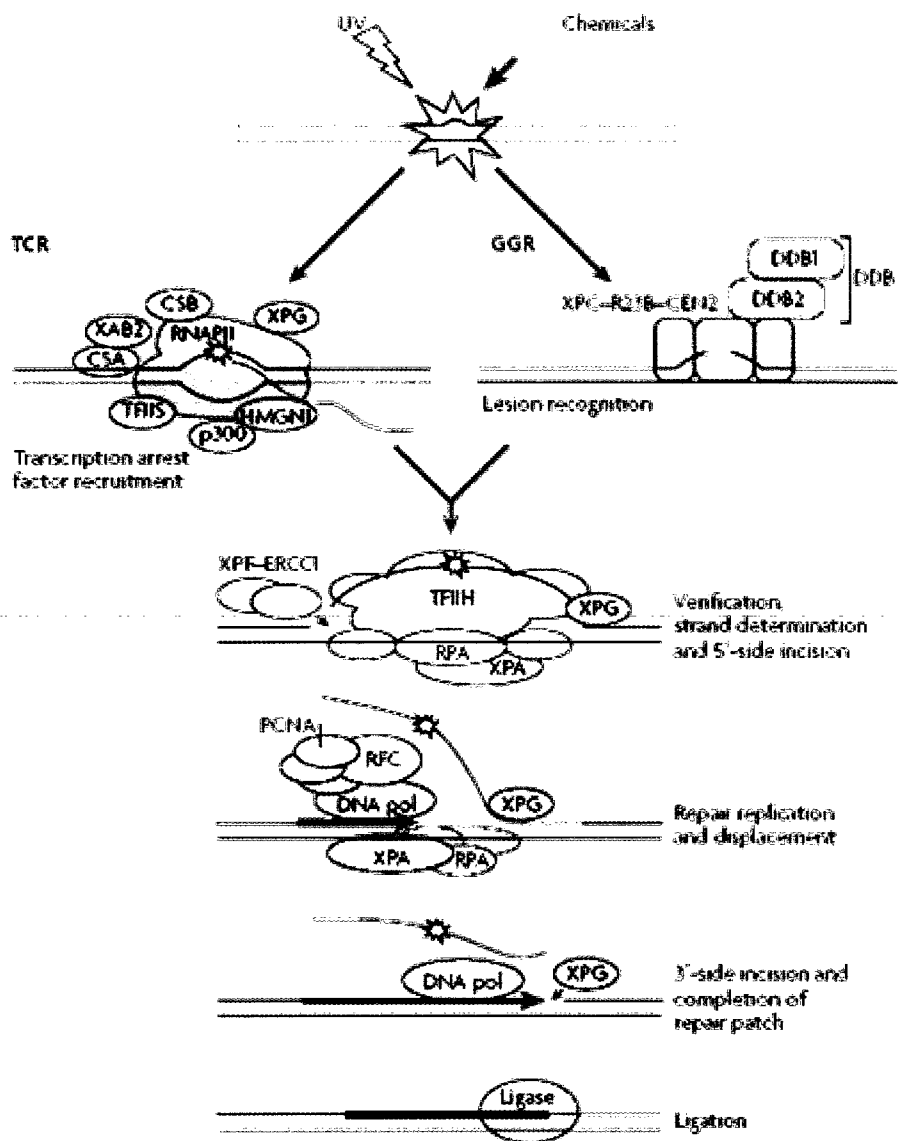
kink or a bend in the DNA (Pfeifer, 1997). 6,4-PPs result in the formation of one covalent bond between carbon-6 of one pyrimidine ring to carbon-4 of the other pyrimidine ring (Pfeifer, 1997). These lesions result in an even greater bend in DNA.

There are two related yet genetically separable pathways of NER, global genome nucleotide excision repair (GG-NER) and transcription-coupled nucleotide excision repair (TC-NER) (figure 1). The steps in each sub-pathway are identical excluding the initial recognition of the lesion. GG-NER repairs the majority of DNA lesions found within the genome in both active and inactive genes (van Hoffen *et al*, 2003). TC-NER repairs damaged DNA in the transcribed strand of active genes (Hanawalt *et al*, 1993). This sub-pathway removes DNA lesions with direct biological implications because bulky lesions like those induced by UV light and cisplatin, pose a block to elongating RNA polymerases. Sustained blockage of transcription constitutes a death signal leading to apoptosis (Ljungman *et al*, 1996, and Ljungman, 2005). Therefore, the repair of these lesions is crucial to the survival of the cell.

Nucleotide excision repair can be simplified into five steps: DNA damage recognition, 3' and 5' incision on either side of the damage site, removal of the damaged oligonucleotides, DNA repair synthesis, and ligation of the newly synthesized DNA to the existing strand (van Hoffen *et al*, 2003).

Global genome nucleotide excision repair repairs DNA throughout the genome, in both the transcribed and untranscribed strands of genes, whether active or inactive (van

Figure 1 Schematic representation of nucleotide excision repair. Bulky DNA lesions induced by agents such as UV-C irradiation and cisplatin are repaired by nucleotide excision repair. Two sub-pathways of NER differ in the DNA lesion recognition step of NER. GG-NER relies on the successive binding of DDB2, XP-C and XP-A to the DNA lesion. In contrast, the initiating step of TC-NER requires the assembly of repair proteins in a manner that depends on CS proteins. Subsequent steps, including incising the 5' and 3' ends of the lesion, DNA synthesis, and the ligation of the newly synthesized DNA are common to both sub-pathways of NER. Figure reproduced with permission from Hanawalt and Spivak, 2008.



Hoffen *et al*, 2003). The recognition of a distortion in the helix caused by the presence of a DNA lesion results in the sequential binding of specific protein complexes that eventually lead to the repair of the DNA lesion. It is thought that the binding of the damage DNA-binding (DDB) complex occurs first, followed by the binding of the XPC complex to the DNA lesion (Sugasawa, 2009). It has been found that 6,4-PP creates a distortion that is readily recognized by the XPC complex. However, a CPD does not create a large distortion in the DNA helix, therefore, the recruitment of the DDB complex is necessary, as it enhances the binding of the XPC complex, initiating DNA repair by NER (Sugasawa, 2009). The DDB complex consists of DDB1 and DDB2, where its role is to enhance the affinity of the XPC complex to the DNA damage, therefore intensifying the signal for NER (Sugasawa, 2009). The XPC complex contains XPC, HR23B, and centrin 2 (CEN2) (Araki *et al*, 2001). This complex allows XPC to bind tightly to the single stranded DNA opposite the lesion. (Maillard *et al*, 2007). Upon binding to the distortion XPC is ubiquitinated, however it does not undergo proteasome-mediated degradation (Sugasawa *et al*, 2005). Ubiquitylating XPC results in a higher affinity for the DNA damage site. HR23B and CEN2 are thought to stabilize the complex, allowing recruitment of subsequent NER proteins to the DNA lesion (Araki *et al*, 2001).

The subsequent steps in both GG-NER and TC-NER are identical. TFIIH binds to the DNA damage with the subsequent recruitment of XPA (Park *et al*, 2006). TFIIH consists of ten subunits of which two subunits, XPB and XPD, have ATPase and helicase properties respectively (Coin *et al*, 2007). TFIIH is thought to unwind the DNA surrounding the lesion. The resulting single stranded DNA is protected through the

binding of replication protein A (RPA), along with stabilizing the complex prior to excision of the DNA damage (Matsuda *et al*, 1995). 5' and 3' incisions are made on either end of the lesion by the structure-specific endonucleases XPF/ERCC1 (Mu *et al*, 1996) and XPG (O'Donovan *et al*, 1994), respectively. This liberates the oligonucleotides containing the DNA lesion allowing DNA synthesis to copy the intact template (Hanawalt *et al*, 2008). Proliferating cell nuclear antigen (PCNA), replication factor C (RFC), and DNA polymerases δ , ϵ and κ are required in the resynthesis of DNA following the removal of the DNA damage (Nichols *et al*, 1992). The newly synthesized 3' end of the DNA is ligated to the intact DNA strand to restore a functional DNA template.

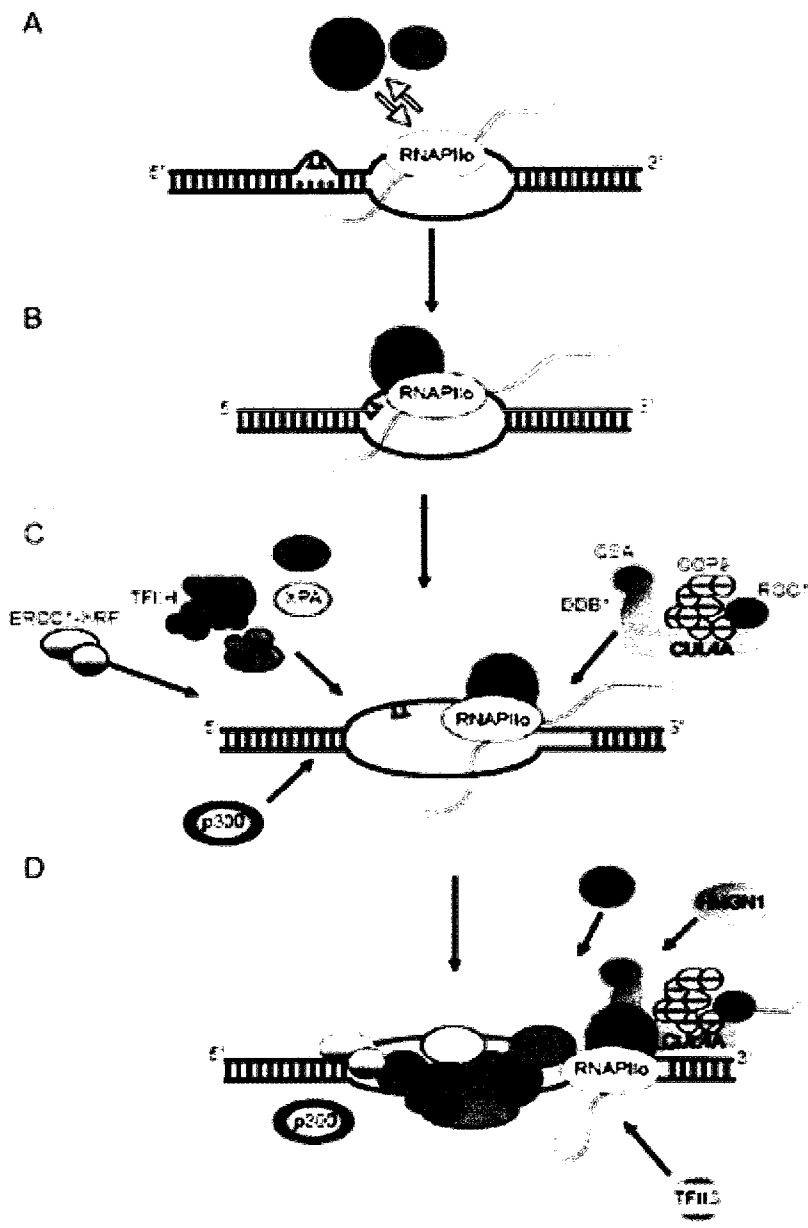
Transcription-coupled nucleotide excision repair was first identified in Chinese hamster ovary (CHO) cells in the 1980s by the preferential repair of UV-induced DNA damage of the dihydrofolate reductase gene (*DHFR*) compared to the whole genome (Bohr *et al*, 1985). Specifically, active genes were repaired with greater efficiency than inactive loci (Bohr *et al*, 1985, May *et al*, 1993, and Hanawalt *et al*, 1993). This was subsequently reproduced in human cells (Mellon *et al*, 1986). It was further demonstrated that repair was also faster on the transcribed strand compared to the non-transcribed strand of DNA (Mellon *et al*, 1987, and Christians, 1992). TC-NER is dependent on ongoing transcription but not translation, therefore it is thought to be initiated by a blocked RNA polymerase II (RNAPII) at a UV lesion, repairing both CPDs and 6,4-PPs with equal efficiency (van Hoffen *et al*, 1995).

There is no known *in vitro* system for TC-NER, and therefore the precise steps are less characterized. Recent work has deciphered the order of protein binding to chromatin at the site of stalled RNAPII following UV irradiation. CSB interacts loosely with an elongating RNAPII, and it has recently been proposed that XPG interacts dynamically with an elongating RNAPII (Sarker *et al*, 2005). RNAPII arrests upon encountering a DNA lesion increasing the association of CSB to RNAPII (Tantin *et al*, 1997, Fousteri *et al*, 2006). CSA also becomes more tightly associated with the stalled polymerase and this is CSB-dependent (Kamiuchi *et al*, 2002). CSA is a member of an E3-ubiquitin ligase complex containing DDB1-Cul4A-ROC1-Rbx1-CSN (Groisman *et al*, 2003). Core NER proteins are also recruited in a CSB-dependent but CSA-independent manner. Other putative TC-NER proteins like XPA binding protein 2 (XAB2) (Kuraoka *et al*, 2008), high-mobility group nucleosome binding domain 1 (HMGN1) and transcription elongation factor IIS (TFIIS) (Fousteri *et al*, 2006) bind in a CSA- and CSB- dependent manner. As discussed later (see section 1.4), transcription elongation factor IIS has been proposed to reverse translocate the stalled RNAPII complex allowing excision of the damaged DNA (figure 2).

1.2) Diseases associated with defects in nucleotide excision repair

There are several hereditary diseases associated with mutations in DNA repair genes including xeroderma pigmentosum and Cockayne syndrome. Much of the nomenclature of NER proteins come from their association with these disorders.

Figure 2. Transcription-coupled nucleotide excision repair complex. A) Elongating RNAPII transcribing DNA into RNA. B) RNAPII encounters the DNA damage lesion, stalls over the lesion, creating a stable ternary complex. CSB binds tightly to RNAPII. C) CSB acts as a coupling factor, recruiting factors such as the CSA complex, TFIIH, and ERCC1-XRF. D) Binding of the CSA complex recruits factors such as XAB2, HMG1, and TFIIIS to the site, loosening nucleosomes behind RNAPII, where it has been hypothesized it will reverse translocate due to TFIIIS, allowing the lesion to be repaired. Figure borrowed with permission from Fousteri and Mullenders, 2008.



Xeroderma pigmentosum (XP) is an autosomal recessive disorder characterized by an array of symptoms including parchment like skin, photosensitivity (which may lead to discolouration of the skin), and a thousand fold increase in the risk of developing skin cancer, including basal cell carcinoma, squamous cell carcinoma and malignant melanoma (Nouspikel, 2008). Neurological symptoms have also been reported including microcephaly, decreased intelligence and a deficiency in the presence of neurological reflexes. The presence of progressive neurologic deterioration in XP patients may be further diagnosed with De Sanctis-Cacchione syndrome (DeSanctis *et al*, 1932). There are eight complementation groups of xeroderma pigmentosum, with each complementation group being clinically heterogeneous (Sugasawa, 2008). Each complementation group results from mutations found in a single gene, Xeroderma pigmentosum complementation group A to complementation group G, and Xeroderma pigmentosum complementation group variant. Complementation groups XPA to XPG have defects in nucleotide excision repair whereas XPV exhibits defects in translesion synthesis (Sugasawa 2008).

Another disease associated with defects in NER is Cockayne syndrome (CS). Patients suffering from Cockayne syndrome display an array of symptoms including short stature resulting from growth failure, a deficiency in neurological development, skin photosensitivity and atypical ocular diseases (Nance *et al*, 1992). Patients are classified into two groups, CS-I or CS-II, depending on the severity of their symptoms (Nouspikel, 2008). CS-I presents as the classical version of Cockayne syndrome displaying many of the mentioned symptoms with a life expectancy of twelve years

(Nance *et al*, 1992). CS-II presents with clinical overlap with xeroderma pigmentosum and results in an even shorter life expectancy (Nospikel, 2008). Cockayne syndrome can also be categorized into five complementation groups with each resulting from a mutation in a single gene. CS-A and CS-B (CS-I group), and XP-B, XP-D and XP-G (CS-II group) can all give rise to Cockayne syndrome (Nospikel, 2008). Cockayne syndrome presents as a deficiency in TC-NER, whereas the XP/CS group exhibits defects in both sub-pathways of NER. In all cases, the recovery of RNA synthesis is impaired and this has been used as a diagnostic tool. These TC-NER deficient cells have demonstrated an increased sensitivity to both UV light and cisplatin (McKay *et al*, 2001).

1.3) Transcription-coupled nucleotide excision repair paradox

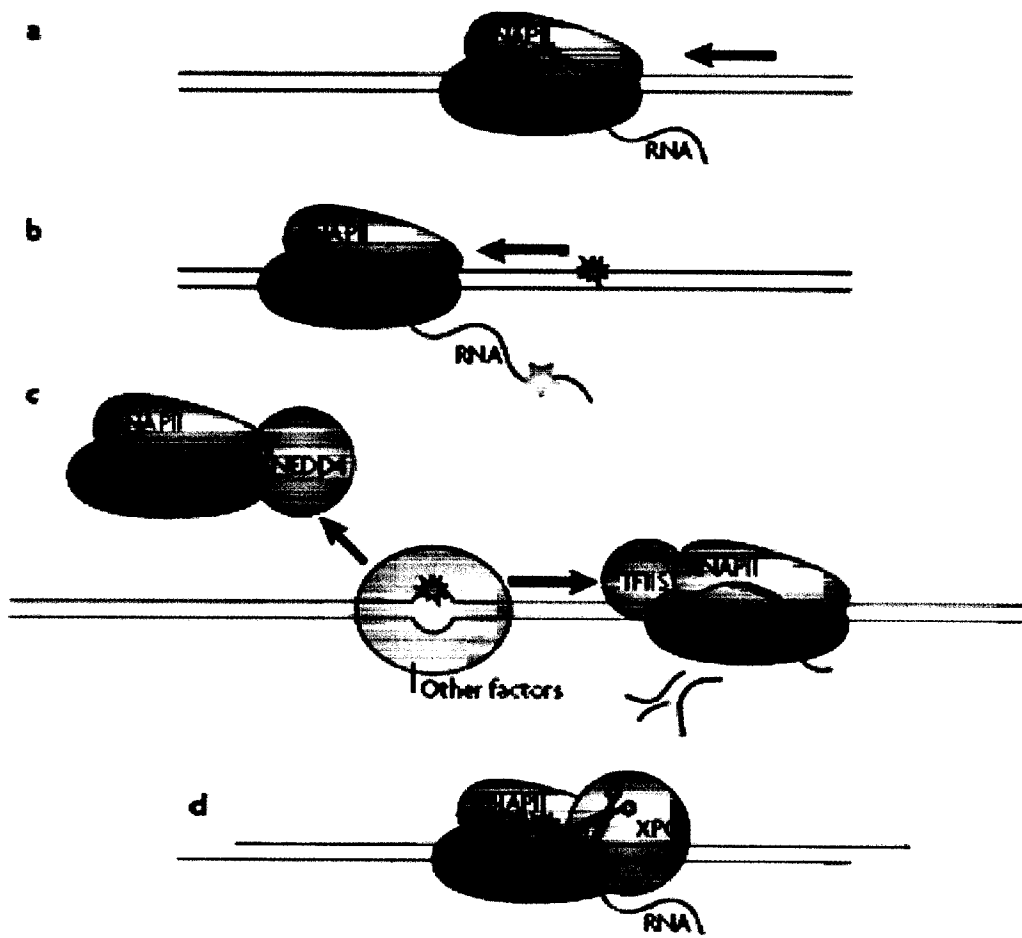
The RNAPII holoenzyme complex forms a stable ternary complex at the site of transcription blocking DNA lesions. This RNAPII-DNA-nascent RNA complex, has a half life of approximately 20 hours (Selby *et al*, 1997). Footprinting experiments indicate that RNAPII blocks access to DNA damage on either side of the DNA lesion extending over approximately fifty nucleotides (Selby *et al*, 1997, Tornaletti *et al*, 1999). Therefore, it is unclear how DNA repair proteins are accommodated at the site of DNA damage to permit TC-NER.

There are two hypotheses to explain how repair proteins gain access to the DNA damage. Both involve displacement of RNAPII. One hypothesis involves proteasome mediated ubiquitylation, while the other involves the reverse translocation of the

polymerase catalyzed by transcription elongation factor IIS (figure 3). In the former, it was reported that the large subunit is ubiquitylated following UV irradiation (Bregman *et al*, 1996, and Luo *et al*, 2001). This modification was dependent on CSA and CSB proteins consistent with a role in TC-NER. Ubiquitylation of RNAPII resulted in proteasome-mediated degradation of the polymerase (Ratner *et al*, 1998, and McKay *et al*, 2001). This is an attractive model in many ways but more recently it was reported that the effect of CSA and CSB on the ubiquitylation and degradation of RNAPII following UV irradiation was indirect through decreased transcription following DNA damage (Anindya *et al*, 2007). Targeting an E3-ligase, Nedd4, prevented the UV-induced ubiquitylation of RNAPII but did not appear to affect the sensitivity of targeted cells to UV exposure, suggesting that TC-NER was unaffected by loss of proteasome-mediated degradation of RNAPII (Anindya *et al*, 2007).

The second hypothesis involves removal from the DNA damage site by reverse translocation of RNAPII by TFIIS. It has been demonstrated that addition of high concentrations of human TFIIS to *in vitro* transcription reactions using templates with site specific CPD resulted in transcript shortening by activating the cryptic cleavage ability of RNAPII. This activity of TFIIS allows RNAPII to realign along the DNA template strand (Donahue *et al*, 1994). Recent chromatin immunoprecipitation experiments suggest that TFIIS becomes tightly associated with chromatin following UV irradiation along with a block in RNAPII. This also occurred in a CSA and a CSB dependent manner, consistent with a role in TC-NER (Fousteri *et al*, 2006). This is considered by many to be the preferred model because this model posits that the

Figure 3. Hypothetical RNAPII events upon encountering a damage DNA lesion. a) Prolonged arrested RNAPII triggers apoptosis. b) Translesion transcription over the DNA damage, resulting in transcriptional mutagenesis. c) Displacement of RNAPII through either ubiquitylation or reverse translocation due to TFIIS. d) Remodelling of RNAPII in a ATP-dependent manner with the recruitment of XPG and TFIIH. XPG and CSA recognize the arrested RNAPII and bind to the stalled RNAPII, allowing the recruitment of TFIIH and CSA. TFIIH is a helicase, unwinding the DNA around the lesion, causing the remodelling of RNAPII exposing the damaged DNA. This could allow XPG access to excise the 3' of the DNA damage without the removal of RNAPII from the site. Figure borrowed with permission from Hanawalt and Spivak, 2008.



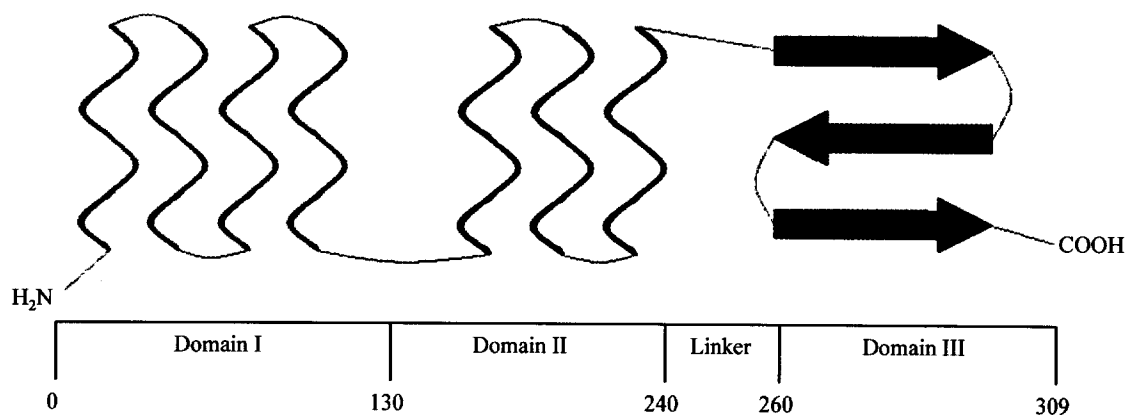
polymerase can resume RNA synthesis whereas proteasome-dependent degradation gives rise to RNAPII loss and requires re-initiation of transcription.

1.4) *Transcription elongation factor IIS*

TFIIS is a transcription elongation factor that associates with RNA polymerase II, and is approximately 38 kDa in size. It has three isoforms encoded by three distinct genes, *TCEA1*, *TCEA2*, and *TCEA3*. *TCEA1* is thought to be ubiquitously expressed, while both *TCEA2* and *TCEA3* are expressed in a tissue specific manner, being found in testes and ovaries, and heart, liver, skeletal muscle and kidney respectively (Wind *et al*, 2000). The predominant isoform expressed in the colorectal carcinoma cells used in the present thesis is generated from *TCEA1*.

The structure of mammalian TFIIS is still unknown, however the structure of yeast, *S. cerevisiae*, transcription elongation factor IIS homologue is known, and is composed of three domains, plus a linker region (Morin *et al*, 1996) (figure 4). Domain I has been found to be highly conserved among organisms and has subsequently been subdivided into two regions, the elongin domain and variable domain (Fish *et al*, 2006). It has been found that the elongin domain is highly conserved across species, and demonstrates sequence similarity to elongin A, whereas the variable domain, as the name suggests, differs amongst species. It has recently been reported that TFIIS is a component of preinitiation complex (PIC) formation, where domain II (Guglielmi *et al*, 2007), in conjunction with both domain I and the linker region, is important in the

Figure 4. A graphical representation of TFIIS found in *S. cerevisiae*. TFIIS is composed of three domains and an additional linker region between domains II and III. Domain I contains four helices within two sub-domains, and has been shown to be involved in preinitiation complex assembly. Domain II contains three helices and is required for RNAPII binding. The linker region has been shown to be flexible, joining domains II and III together. Domain III contains a zinc ribbon within three anti-parallel β -sheets. Its main functions include stimulating the intrinsic ability of RNAPII cleavage and TFIIS's read through abilities.



assembly of PICs (Kim *et al*, 2007). Interestingly, DST1, the yeast homologue of TFIIS, deletion strains have been found to be viable, suggesting that the requirement of TFIIS in PIC formation only when PIC formation is compromised due to varying conditions or when efficient PIC assembly is limiting (Kim *et al*, 2007). Both domains II and III are highly conserved across species (Wind *et al*, 2000). Domain II is required for binding of RNAPII, along with the linker region found between domains II and III (Awrey *et al*, 1998). Domain III contains a zinc ribbon and is found to be essential in enhancing the intrinsic cleavage ability of RNAPII (Awrey *et al*, 1998). The C-terminal domain of TFIIS, consisting of both domains II and III along with the linker region, is sufficient for RNAPII binding, stimulating RNAPII cleavage, and read-through of arrest sites (Fish *et al*, 2006).

TFIIS was first identified as a transcription elongation factor by stimulating blocked RNAPII to resume transcription (Reines *et al*, 1989). When RNAPII encounters a block such as complex intrinsic DNA sequences or DNA damage, it becomes either paused or arrested as it cannot re-initiate RNA synthesis from the pause site (Gnatt, 2002). A paused RNAPII retains an elongation competent conformation, backtracking by one to two nucleotides, while an arrested RNAPII adopts an elongation incompetent conformation, where it will reverse translocate several nucleotides causing the transcript to become dislodged from the active site, resulting in the cleavage of the nascent RNA stimulated by TFIIS (Kalogeraki *et al*, 2005). Once TFIIS has stimulated the intrinsic cleavage ability of RNAPII (Reines, 1992), the disengaged portion of the transcript is

cleaved. TFIIIS will then realign the new 3'OH end within the active site corresponding to the DNA strand, allowing RNAPII elongation to resume (Fish *et al*, 2002).

1.5) Hypothetical role of TFIIIS in TC-NER

As indicated above *in vitro* studies suggest that the reverse translocation of an arrested RNAPII stalled at a CPD can occur upon addition of TFIIIS (Donahue *et al*, 1994). In the chromatin of UV irradiated cells, an increase of TFIIIS binding to RNAPII was observed in a CS-dependent manner (Fousteri *et al*, 2006). These two observations suggest that TFIIIS could be involved in TC-NER, however, the hypothesized role of TFIIIS in TC-NER has never been tested *in vivo*.

1.6) Hypothesis

In the present work, we sought to test the hypothesis that TFIIIS is required for TC-NER of UV- and cisplatin-induced DNA damage.

Specific aims:

- 1- Identify siRNAs that efficiently decrease TFIIIS expression in tumour cells;
- 2- Determine the effect of these siRNAs on TC-NER;
- 3- Determine the effect of these siRNAs on the sensitivity of tumour cells to UV light and cisplatin.

2. Material and Methods

2.1) Cell Culture

Normal non-immortalized dermal fibroblasts, GM38, were grown in Dulbecco's modified Eagle's medium, DMEM, media (HyClone) supplemented with 10% fetal bovine serum (FBS; Multicell, Wisent). Non-immortalized dermal Xeroderma pigmentosum complementation group C fibroblasts (GM671) and non-immortalized dermal Cockayne syndrome type B fibroblasts (GM739) were grown in DMEM media supplemented with 15% FBS (Multicell, Wisent). Colorectal carcinoma cells (HCT116) were grown in McCoy's 5A media (Multicell, Wisent) supplemented with 10% FBS (Gibco) and with plasmocin prophylactic (InvivoGen, Invitrogen). Human breast adenocarcinoma cells (MCF7) were grown in DMEM (HyClone) supplemented with 10% FBS (Gibco) and with plasmocin prophylactic. All cell lines were grown at 37°C in 5% CO₂ in air.

2.2) RNA interference

Knockdown of mRNA was done using small interfering RNAs (siRNAs). All siRNAs were purchased from Dharmacon. Initially, a SMARTpool™ comprising 4 oligos was purchased and each individual siRNA was tested for its ability to target TFIIIS. The most effective siRNA from this pool was selected (TCEA1#6) for further study. A second siRNA reported in the literature (Hubbard *et al*, 2008) was also

synthesized and used. These siRNAs will be referred to as siTFIIS#1 and siTFIIS#2, respectively (table 1). siRNAs targeting CSB mRNA and a non-targeting control sequence (NT) were similarly obtained from Dharmacon (table 1). Twenty-four hours prior to transfection, cells were seeded into either 6-well or 10cm plates in their respective antibiotic free media. Cells at 50% confluence were rinsed twice with Opti-MEM I (+GlutaMAX, reduced-serum medium 1X, Gibco) and then transfected with siRNA targeting either TFIIS, CSB or NT using 1% oligofectamine reagent (Invitrogen) in Opti-MEM I. Four hours after addition of siRNA media, cells were supplemented with 30% FBS in appropriate medium. Twenty-four hours later, siTFIIS#1 and siTFIIS#2 cells were similarly transfected a second time with siRNA targeting TFIIS and refed with media four hours later.

2.3) Western Immunoblot analysis

Cells were washed with PBS, trypsinized (0.05%, HyClone), and collected by centrifugation. Cell pellets were lysed using 1% sodium dodecyl sulfate (SDS) and kept on ice for 10 minutes, and sonicated (sonicator 3000, Misonix Inc.) for 20 seconds. Quantification of protein was done using a Bio-Rad protein assay (Bio-Rad Laboratories). Equal amounts of protein were separated by gel electrophoresis (NuPAGE 4-12% Bis-Tris Gel, Invitrogen), with MOPS-SDS running buffer 1X (NuPAGE 20X, Invitrogen). Proteins were transferred onto a Hybond-C transfer membrane (Amersham Biosciences) using transfer buffer 1X (NuPAGE 20X, Invitrogen). Membranes were blocked overnight in 5% milk-TBST (tris-buffered saline Tween-20). Primary antibodies

Table1. siRNA sequences and concentrations used in RNAi.

siRNA	sense Sequence	Anti-sense sequence	Concentration
TFIIS#1	AGACUGACUUGUUCACAUGUU	PCAUGUGAACAAGUCAGUCUUU	200nM
TFIIS#2	ACAGGGGATGACTACATTCG	AGCAATGTAGTCATCCCCTG	100nM
CSB	GTGTCCCATGTGTCTTACGA	TCGTAAGACACATGCACAC	100nM
NT	AGCGACUAAACACAUCAAUU	UGAUGUGUUUAGUCGCUAAU	Either 100 or 200nM

include mouse anti-TFIIS (BD Biosciences), mouse anti-actin (Sigma), and goat anti-Ku86 (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated goat anti-mouse antibody was obtained from Calbiochem while similar rabbit anti-goat secondary antibodies were obtained from Santa Cruz Biotechnology, all at a dilution 1:1000 in 5% milk-TBST. A chemiluminescent substrate (Super Signal West Pico, Thermo Scientific) was used to visualize the proteins using X-OMAT LS film (Kodak Scientific Imaging Systems).

2.4) *Host Cell Reactivation*

Forty-eight hours following RNA interference (RNAi), either 1×10^4 HCT116 cells/well or 2×10^4 fibroblast cells/well were seeded into each well of a 96-well plate. *AdlacZ* virus was diluted in ice cold phosphate buffered saline (PBS; 1X, HyClone) in a 3cm plate, and placed on a magnetic stirrer. Virus was exposed to UV-C (Philips bulb) at a fluence rate of approximately $4.2 \text{ J/m}^2/\text{s}$ determined with a hand held UV meter (UVX radiometer). Aliquots were taken at the appropriate time to yield cumulative doses of UV-C between 0 and 5000 J/m^2 . Cells were infected with the irradiated *AdlacZ* at MOI (multiplicity of infection) of 50 and 75 for fibroblast and colorectal carcinoma cells respectively, in serum free media for approximately 1 hour. Growth media was added to a total volume of approximately $200 \mu\text{l}$, and kept at 37°C for 48 hours. Fifty microliters of $6.0 \times 10^{-4} \text{ g/ml}$ of chlorophenol-red- β -D-galactopyranoside (Fluka, Sigma) in a phosphate buffer (200ml 0.1M Na_2HPO_4 , 50ml 0.1M NaH_2PO_4 , 0.05g MgCl_2 , 0.01% Triton-X 100, pH 8.3) was added per well. β -galactosidase activity was assessed by chlorophenol red

conversion at 570nm, measured with a Multiskan ascent photometer (Thermo Labsystems).

2.5) Recovery of RNA synthesis

Cells were seeded at a density of 2×10^5 in 6-well plates 48 hours following transfection of siRNAs. Cells were labelled with $0.3 \mu\text{l/ml}$ [^{14}C]-thymidine ($0.05 \mu\text{Ci}/\mu\text{l}$, GE-Amersham) media for 24 hours prior to UV-C irradiation. Cells were exposed to $10\text{J}/\text{m}^2$ of UV-C irradiation (measured using UVX radiometer). Cells were allowed to recover for 0 to 24 hours. At each time point, $10 \mu\text{l/ml}$ [^3H]-uridine media ($1 \mu\text{Ci}/\mu\text{l}$, GE-Healthcare) was added one hour before collection. Cells were washed with PBS, trypsinized, and collected with PBS+1X sodium azide (10X, $0.2\text{mg}/\text{ml}$) into falcon tubes. PBS+1X NaN_3 was added to each to a final volume of 5ml. Samples were spun at 4°C at 1500 rpm for 2 minutes. Supernatant was discarded and cell pellet was frozen at -80°C until processed. Cell pellets were thawed rapidly and immediately placed on ice. Cells were lysed with the addition of $500 \mu\text{l}$ 1% SDS in double distilled H_2O (dd H_2O) to cell pellets, placed on ice for 30 minutes, followed by the addition of 1ml 10% trichloroacetic acid (TCA)/0.1M sodium pyrophosphate (NaPPi) to pellets, then placed on ice for 30 minutes. The lysate was transferred to GF/A filters (Whatman) under vacuum, and washed sequentially with 2ml 5% TCA/0.05 NaPPi, 5ml dd H_2O , and 2ml 100% ethanol (EtOH). Filters were dried for 1min on the vacuum, placed in scintillation vials with 5ml of scintillation fluid. [^{14}C] and [^3H] levels were assed using a scintillation counter

(1600TR Liquid Scintillation Counter, Packard). [³H] counts were normalized to [¹⁴C] counts.

2.6) *SubG1 assay*

Forty-eight hours following transfection of siRNAs, cells were seeded in 6-well dishes. Twenty-four hours later cells were exposed to either 10J/m² of UV-C irradiation or treated with 5, 10, or 15µM of cisplatin. Both floating and adherent cells were collected by centrifugation 48 hours following cell treatment. Cells were fixed overnight in 70% EtOH at -20°C. Cell pellets were washed with PBS, and re-suspended in propidium iodide (PI) stain. Apoptosis was estimated from the fraction of cells with less than 2N DNA content using COULTER Epics XL flow cytometer (Beckman Coulter).

2.7) *Real-time reverse transcription polymerase chain reaction*

RNA was isolated using RNeasy Mini kit (Qiagen) according to the manufacturer's recommendations. Total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's recommendations. Real-Time PCR was used to quantify cDNA. Primers specific for *TCEA1*, *TCEA2*, *TCEA3*, and *ACTB* were purchased from Applied Biosystems (assay ID's: Hs01896387_g1, Hs00371401_m1, Hs00957469_m1 and Hs99999903_m1, respectively). The expression of *TCEA1*, *TCEA2* and *TCEA3* was normalized to *ACTB*

mRNA levels. The relative gene expression was quantified using a 7500 Real-Time PCR System (Applied Biosystems).

2.8) Growth assay

Cells were seeded at $1-2 \times 10^5$ cells per well of 6-well dishes. Cells at the various times were detached and collected by centrifugation. Viability was assessed by trypan blue exclusion using a Vi-Cell XR cell viability analyzer (Beckman Coulter).

3. Results

3.1) Targeting TFIIS by RNA interference

Three isoforms of TFIIS are encoded by three distinct genes, *TCEA1*, *TCEA2* and *TCEA3* (Wind *et al*, 2000). As a means of testing the role of TFIIS in TC-NER, synthetic siRNAs directed against *TCEA1* mRNA encoding the ubiquitous form of TFIIS were tested as described in Materials and Methods. One siRNA (duplex #6) from the Dharmacon TCEA1 smartpool, referred to here as siTFIIS#1, was very efficient in reducing TFIIS protein levels. A second previously reported siRNA (Hubbard *et al*, 2008) was similarly effective at reducing TFIIS expression (figure 5A). The effectiveness of the knockdown of TFIIS was determined by diluting the non-targeting control sample up to eight fold. The intensity of the TFIIS band following RNAi suggested that the knockdown was approximately seven fold (figure 5B). Therefore, there is a significant reduction in TFIIS protein following transfection of these siRNAs in HCT116 colorectal carcinoma cells. Importantly, the expression of *TCEA2* and *TCEA3* was lower than *TCEA1* in HCT116 cells (figure 6A), and the siRNA against *TCEA1* only affected the intended target and not *TCEA2* or *TCEA3* (figure 6B).

3.2) Transcription elongation factor IIS is not limiting for the repair of a UV-damaged reporter gene

Figure 5. Reduction of transcription elongation factor IIS in HCT116 colorectal carcinoma cells. A) A western blot depicting reduced TFIIS levels when targeted with siTFIIS#1 and siTFIIS#2. B) A western blot depicting an approximate 7-fold reduction of TFIIS protein levels as compared to protein dilutions of the non-targeting siRNA. The 1:1 ratio contains 10 μ g of protein. Monoclonal antibodies against TFIIS, β -actin and Ku86 were used.

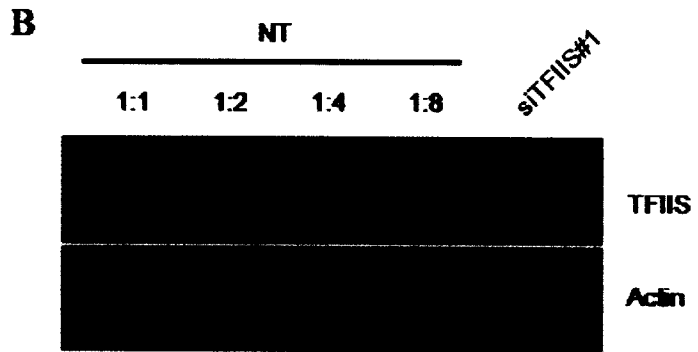
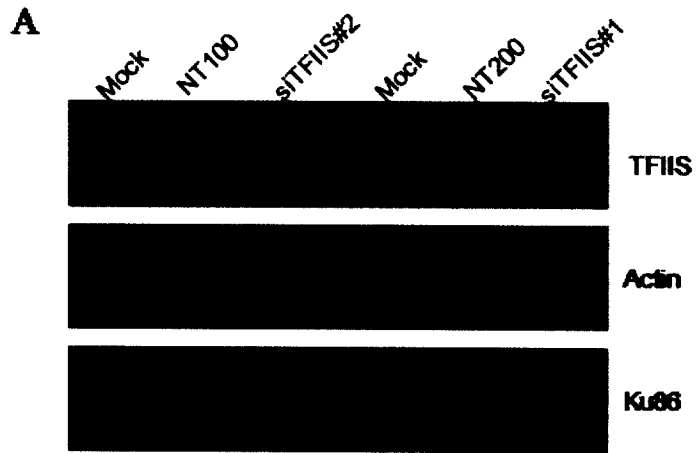
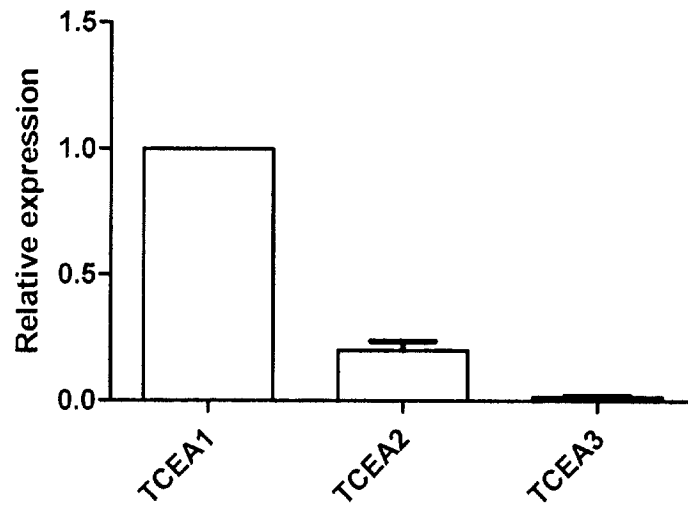
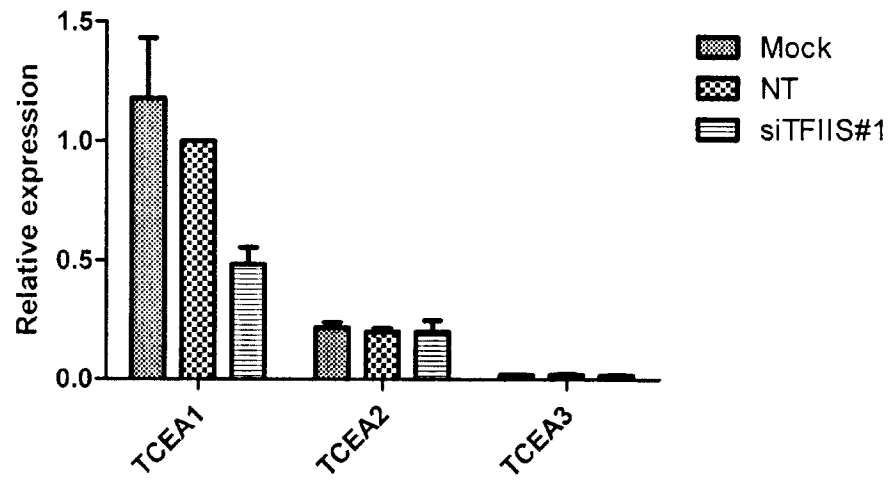


Figure 6. Relative gene expression of TFIIIS isoforms in colorectal carcinoma cells. A) The relative expression of each TFIIIS isoform in HCT116 cells as compared to mock. B) RT-PCR of each TFIIIS isoform in HCT116 cells when *TCEA1* has been targeted using siTFIIIS#1.

A



B



A single lesion in the template strand of active genes is sufficient to block gene expression (Sauerbier *et al*, 1978). This concept forms the conceptual basis of a host cell reactivation assay (HCR) that can be used to measure TC-NER (Francis *et al*, 1999). Specifically, UV-irradiation of an adenovirus expressing the bacterial *lacZ* gene inhibits transgene expression in a dose-dependent manner. The ability of cells to express *lacZ*, as assessed by β -galactosidase activity, reflects the capacity of cells to repair transcription blocking DNA lesions (Rainbow *et al*, 2000). Here, HCR was used to assess the affect of decreased TFIIIS expression on the repair of transcription blocking lesions.

As expected, UV irradiation inhibited β -galactosidase activity in a dose-dependent manner in HCT116 colorectal carcinoma cells (figure 7A and B). Transfection of CSB siRNA served as a positive control and reduced HCR of the UV-damaged reporter gene (figure 7C). In contrast, targeting TFIIIS by RNAi did not affect the host cell reactivation of β -galactosidase activity (figure 7A and B). Therefore, decreased expression of TFIIIS did not inhibit the repair of UV lesions in the bacterial *lacZ* gene.

Similar experiments were performed in a series of primary fibroblast cells. Normal, XP-C and CS-B fibroblasts were transiently transfected with siTFIIIS#1. TFIIIS levels were decreased in all cases (figure 8A-C). RNAi against *TCEA1* has no effect on HCR of the UV-damaged reporter gene, even in the absence of GG-NER in XP-C fibroblasts (figure 9A-C). These results indicate that TFIIIS is not limiting for TC-NER of the UV-damaged reporter gene.

Figure 7. Transcription elongation factor IIS is not required in the repair of UV-C induced cyclobutane pyrimidine dimers in a β -galactosidase reporter construct in colorectal carcinoma cells. An AdlacZ virus was irradiated with indicated doses of UV-C light (0-5000 J/m²) and infected at MOI 75 into HCT116 cells where TFIIS has been targeted by RNAi using A) siTFIIS#1, and B) siTFIIS#2, and compared to a knockdown of a known TC-NER factor CS-B, using C) siCSB, (all n=3). Targeting TFIIS using either siTFIIS#1 or siTFIIS#2 did not decrease the activity of β -galactosidase compared to the positive control, siCS-B. The * denotes that the indicated mean is significantly different than the mean of mock and NT ($P \leq 0.05$, single factor ANOVA).

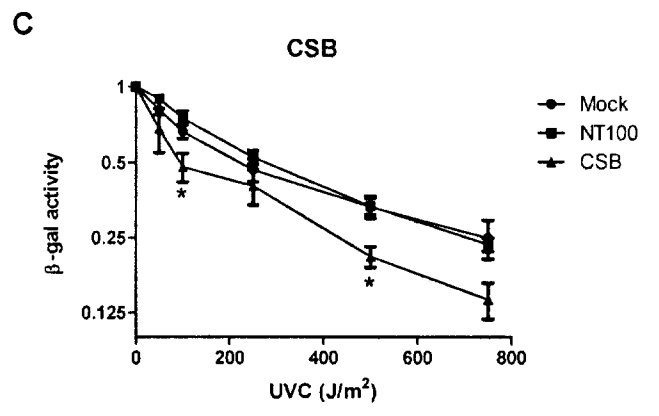
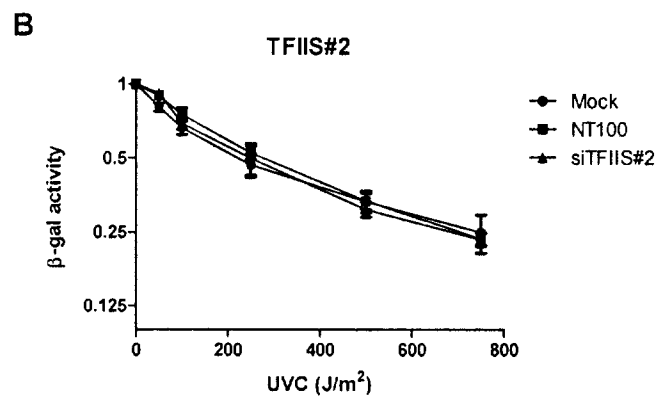
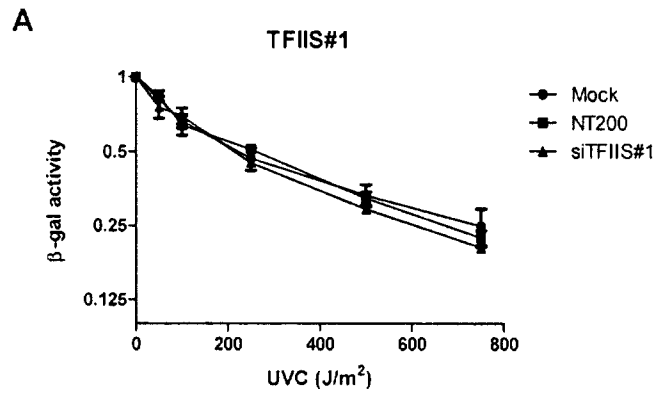


Figure 8. Protein levels of transcription elongation factor IIS in human fibroblasts.

Westerns depicting reduction of TFIIS in A) normal fibroblasts (GM38), proficient in both TC-NER and GG-NER, B) fibroblasts from a patient with Xeroderma Pigmentosa complementation group C (GM671), proficient in TC-NER, and C) fibroblasts from a patient with Cockayne syndrome type B (GM739), proficient in GG-NER, where TFIIS has been targeted using siTFIIS#1. Monoclonal antibodies against TFIIS and β -actin were used.

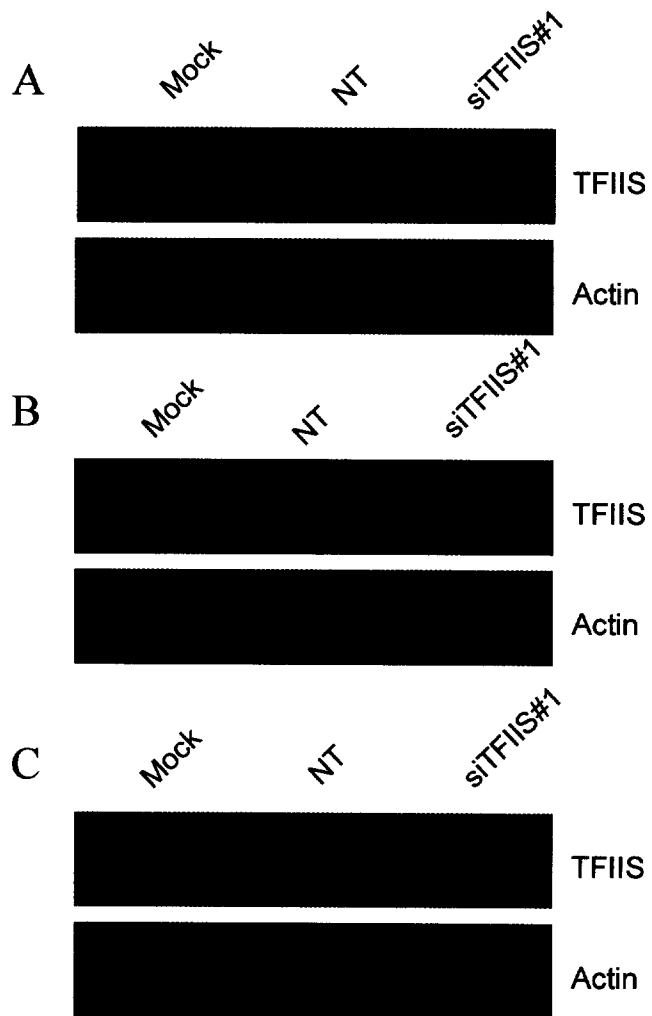
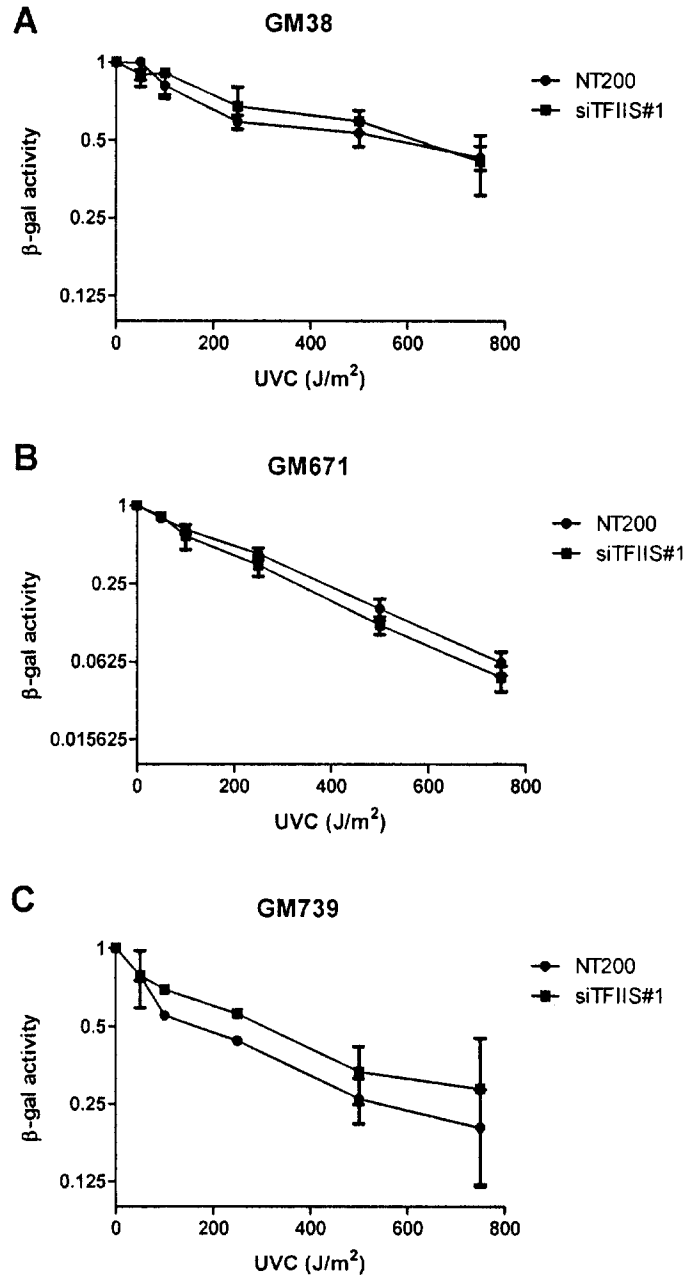


Figure 9. Transcription elongation factor IIS is not required in the repair of UVC induced cyclobutane pyrimidine dimers in a β -galactosidase reporter construct in fibroblasts. An AdlacZ virus was irradiated with indicated doses of UV-C light (0-5000 J/m²) and infected at MOI 50 into normal, XP-C and CS-B fibroblasts. TFIIS was targeted by RNAi using siTFIIS#1 in A) normal (GM38, n=3), B) XP-C (GM671, n=3), and C) CS-B (GM739, n=2). Silencing TFIIS did not inhibit CPD repair in the *lacZ* gene, as similar β -galactosidase levels were observed between the cells with TFIIS targeting and the control cells, mock (not shown) and the non-targeting siRNA (NT200).



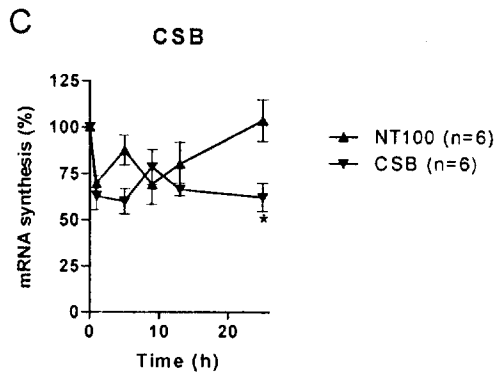
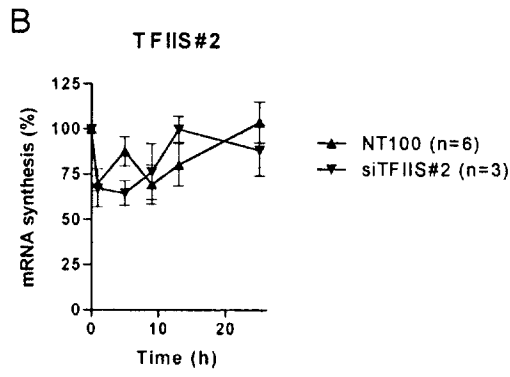
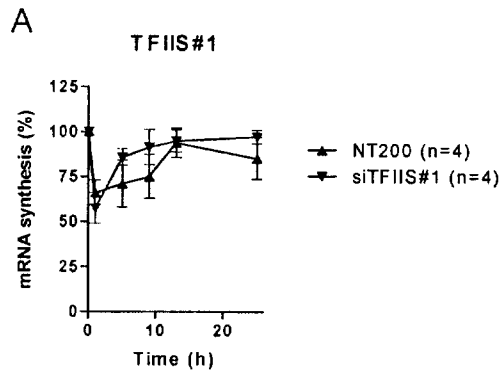
3.3) Transcription elongation factor IIS is not limiting for the recovery of RNA synthesis following UV exposure

The host cell reactivation assay measures the repair of a transgene and may not always reflect repair of endogenous genes. Therefore, the recovery of nascent RNA synthesis was assessed as an additional measure of TC-NER. Again, UV-induced DNA lesions block transcription so the incorporation of [³H]-uridine is decreased following UV exposure. The resumption of RNA synthesis requires the repair of transcription blocking lesions. TFIIS was targeted by RNAi using either siTFIIS#1 or siTFIIS#2 in HCT116 colorectal carcinoma cells. As expected, nascent RNA synthesis was decreased in all samples immediately following UV exposure. The recovery of nascent RNA was unaffected by the RNAi against TFIIS (figure 10A and B) but was greatly inhibited by siRNA against CSB (figure 10C). Consistent with our HCR data, we find no evidence for a role of TFIIS in TC-NER. We conclude that TFIIS is not limiting for TC-NER.

3.4) Silencing of TFIIS does not increase the sensitivity of colorectal carcinoma to UV-C light or cisplatin

It has been found that TC-NER deficient fibroblasts display an increased sensitivity to a variety of DNA damaging agents including UV-C and cisplatin (McKay *et al*, 2001). Similarly, RNAi against CSB, XPA and XAB2 increase the sensitivity of a variety of tumour cells to UV and cisplatin (Stubbert L, Hamill J, and McKay BC, unpublished). Therefore, a TC-NER deficiency resulting from decreased TFIIS would be

Figure 10. Transcription elongation factor IIS is not required for recovery of RNA synthesis. A time course of 24 hours was used to assess the recovery of nascent RNA synthesis following 10 J/m^2 of UV-C irradiation in HCT116 cells where TFIIS has been silenced using A) siTFIIS#1 and B) siTFIIS#2, and compared to a known TC-NER factor CS-B using C) siCSB. Targeting TFIIS using either siRNA, nascent RNA synthesis was completely recovered after 24 hours. The siRNA against CS-B served as a positive control. The * denotes that the indicated mean is significantly different then the mean of NT ($P \leq 0.05$, two sample T-test).



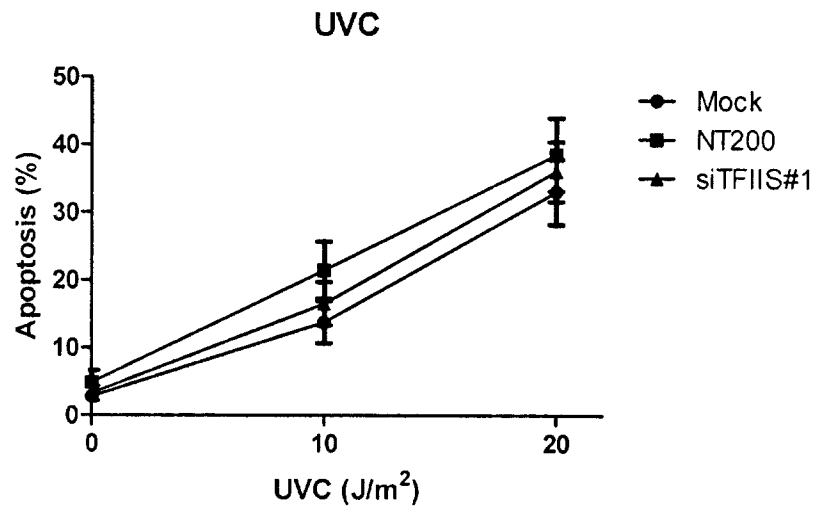
expected to increase the sensitivity to these agents. Consistent with the results in figures 7, 9 and 10, RNAi against TFIIIS did not affect the sensitivity of HCT116 colorectal carcinoma cells to UV- (figure 11) or cisplatin-induced (figure 12) apoptosis. Therefore, a reduction in TFIIIS protein levels does not increase the sensitivity of tumour cells to either of these agents. Again, these results fail to support a role for TFIIIS in TC-NER. We conclude that TFIIIS is not limiting for this repair process.

3.5) Differences in cell proliferation following transfection of distinct siRNAs against TCEA1

Hubbard et al (2008) reported that targeting TFIIIS by RNAi decreased the rate of proliferation of cancer cells. In that report, RNAi against TFIIIS decreased the growth of three different tumour cell lines, MCF7, A549, and PL45. Curiously we had not detected growth inhibition in HCT116 colorectal carcinoma cells (figure 13A). RNAi against TFIIIS using the siRNA designed by Hubbard and coworkers (siTFIIIS#2) in HCT116 cells had relatively little effect on growth (figure 13B). In contrast to HCT116 cells, the proliferation of MCF7 cells was dramatically reduced in response to siTFIIIS#2 but not siTFIIIS#1 (figure 14), despite the fact that both siRNAs were very effective at reducing TFIIIS protein levels (figure 15). In fact siTFIIIS#1 tended to be more efficient (figures 5 and 15). The simplest explanation for the disparity is a secondary off-target effect of siTFIIIS#2. Notwithstanding this difference in behaviour of the siRNAs, neither siRNA affected TC-NER, or its sensitivity to UV-C irradiation or cisplatin.

Figure 11. Silencing TFIIIS does not increase the sensitivity of colorectal carcinoma cells to UV-C irradiation. TFIIIS levels were decreased in HCT116 cells with two different siRNAs, A) siTFIIIS#2 and B) siTFIIIS#2. Targeted cells were treated with the indicated dose of UV light and apoptosis was assessed 48 hours later by sub-diploid DNA content.

A



B

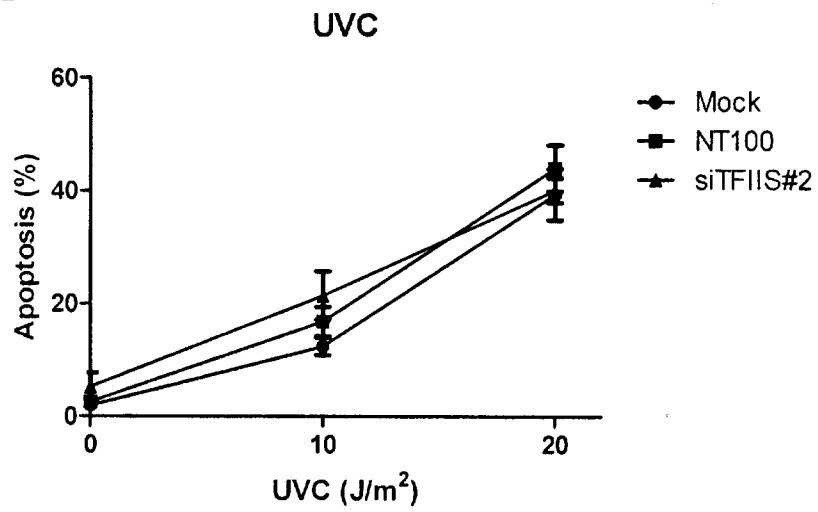


Figure 12. Silencing of TFIIIS does not increase the sensitivity of colorectal carcinoma cells to cisplatin. TFIIIS levels were decreased in HCT116 cells with two different siRNAs, A) siTFIIIS#1 and B) siTFIIIS#2. Targeted cells were treated with the indicated concentration of cisplatin and apoptosis was assessed 48 hours later by sub-diploid DNA content.

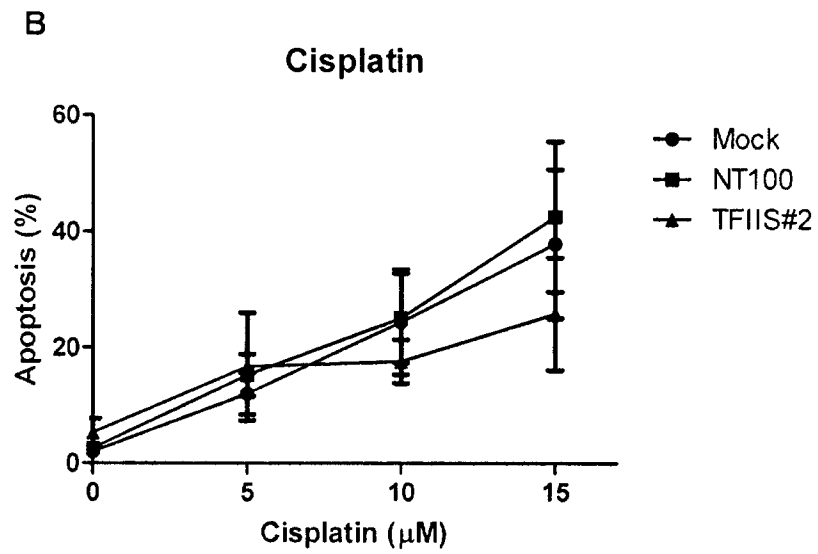
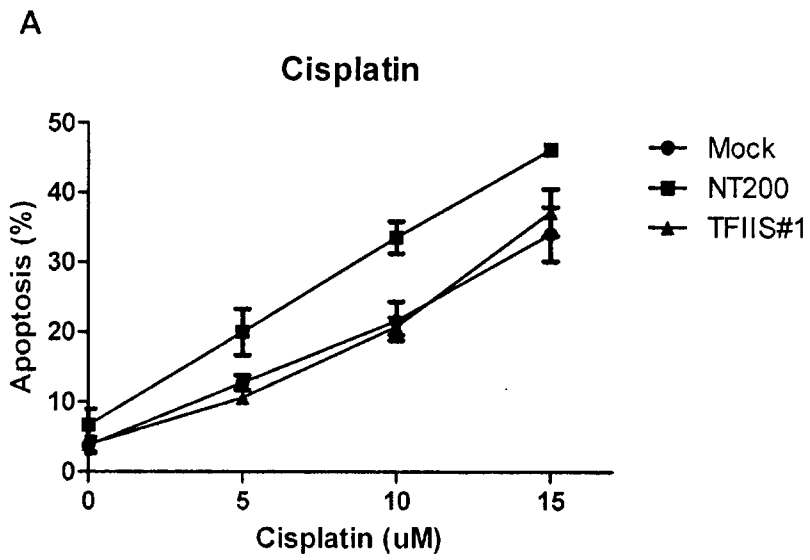


Figure 13. Slight decrease in proliferation of colorectal carcinoma cells when TFIIS is targeted by siTFIIS#2. TFIIS was targeted by RNAi using either siTFIIS#1 or siTFIIS#2 in HCT116 cells. A) siTFIIS#1 did not affect the growth rate of HCT116 in comparison to mock or the non-targeting samples. B) siTFIIS#2 produced a slight decrease in proliferation in HCT116 cells.

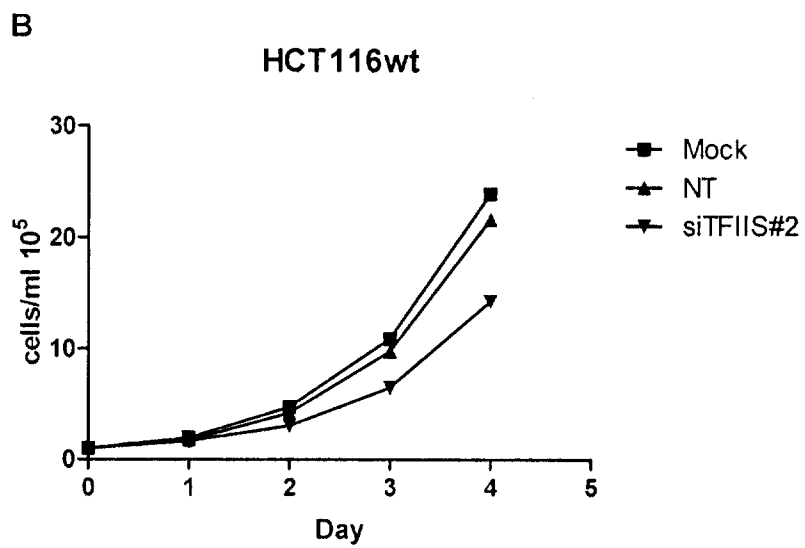
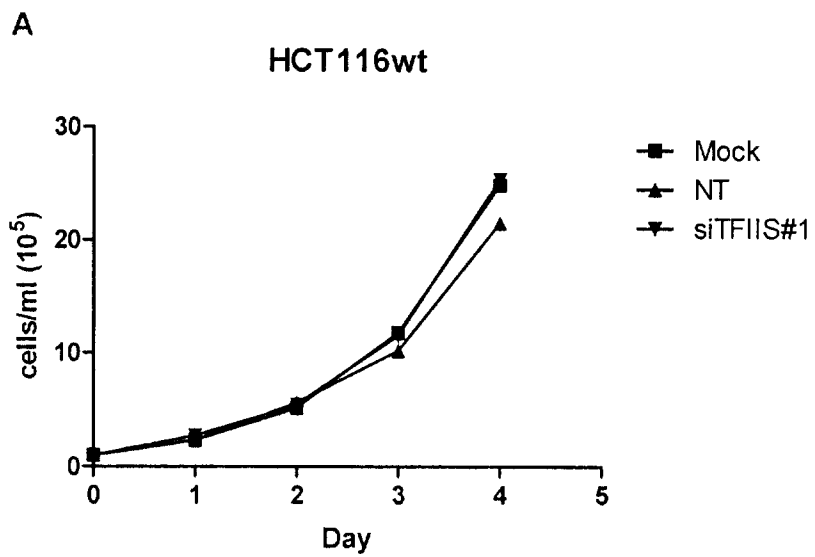


Figure 14. Proliferation of breast adenocarcinoma cells is inhibited when TFIIIS is silenced by siTFIIIS#2. TFIIIS was targeted in MCF7 cells by RNAi using either siTFIIIS#1 or siTFIIIS#2. A) siTFIIIS#1 did not affect the growth rate of MCF7 in comparison to mock or the non-targeting samples. B) siTFIIIS#2, however, inhibited proliferation in MCF7 cells.

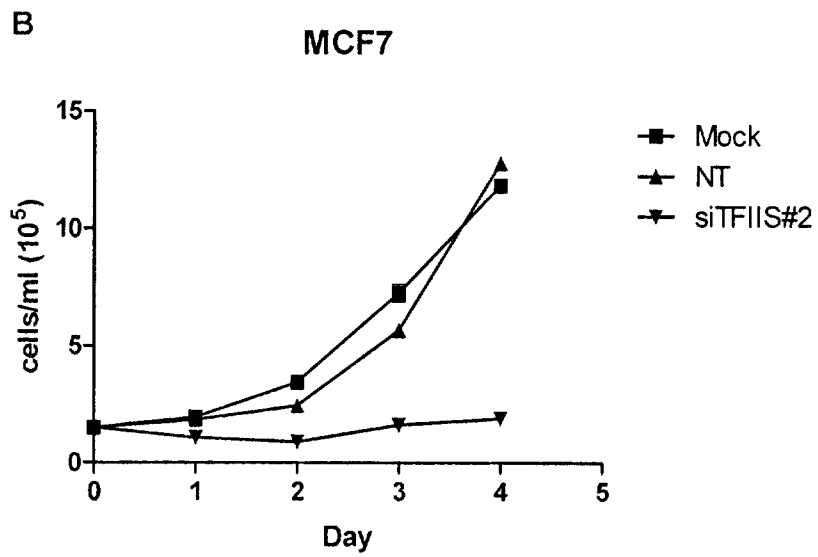
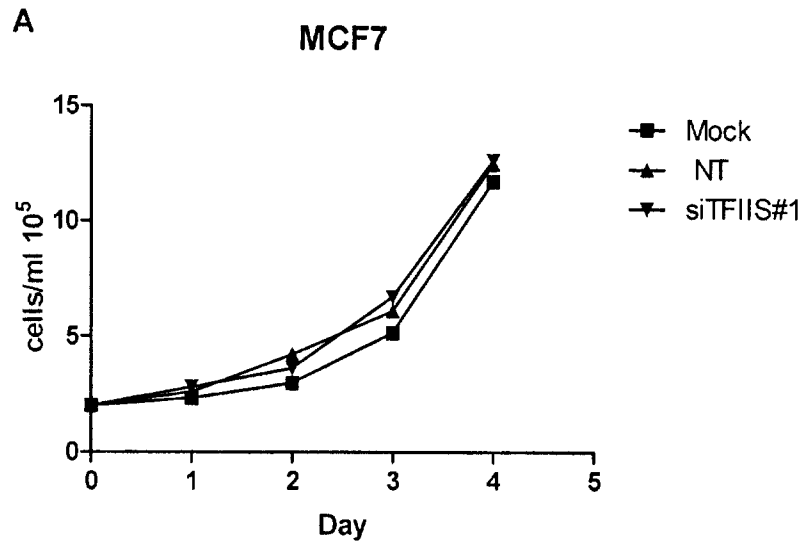
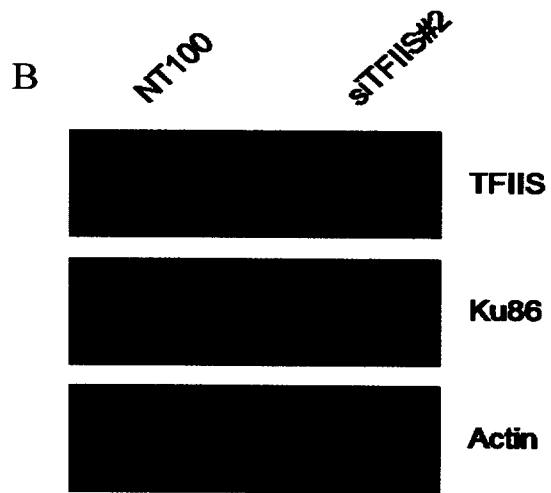


Figure 15. Reduction in TFIIIS protein levels in human breast adenocarcinoma cells. siRNA targeted to TFIIIS using siTFIIIS#1 and siTFIIIS#2 in MCF7 cells, demonstrate a reduction of TFIIIS protein levels. Monoclonal antibodies against TFIIIS, Ku86 and β -actin were used in these western blot analyses.



4. Discussion

During transcription, TFIIS aids RNAPII to bypass arrest sites created by DNA-bound protein, small DNA-bound drugs and tracts of thymidines in DNA (Gnatt, 2002). TFIIS will enter the catalytic core of an arrested RNAPII where it will stimulate the intrinsic cleavage ability of RNAPII, cleaving the dislodged nascent RNA strand (Reines, 1992). This causes the polymerase to backup and realign the mRNA with the template strand permitting transcription to resume (Fish *et al*, 2002). Donahue and coworkers originally proposed a role for TFIIS in TC-NER (Donahue *et al*, 1994). They used synthetic templates with site-specific DNA lesions. Under these conditions, the arrested RNAPII formed a stable complex that prevented access of T4 endonuclease V protein to the DNA lesion (Donahue *et al*, 1994). Addition of TFIIS to the reconstituted system allowed the cleavage of nascent transcripts up to approximately 25 nucleotides (Tornaletti *et al*, 1999). T4 endonuclease V, a bacteriophage encoded repair protein, was able to excise the DNA damage (McMillan *et al*, 1981). RNAPII was then able to elongate past the original site of the CPD (Donahue *et al*, 1994). This has not been demonstrated *in vitro* with fully reconstituted mammalian NER components.

Chromatin immunoprecipitation experiments conducted by Fousteri and coworkers have identified a number of key interactions. Briefly, they found that UV-irradiation led to an increase in the association of CSB with RNAPII. They subsequently found that all other TC-NER proteins were recruited in a CSB-dependent manner. Consistent with a role for TFIIS in TC-NER, TFIIS could be chromatin

immunoprecipitated with RNAPII following UV irradiation, in a CSA- and CSB-dependent manner. This suggests that TFIIS may be recruited to UV-damaged chromatin during TC-NER (Fousteri *et al*, 2006). However, a role for TFIIS in this repair process has not been demonstrated. In fact, despite the conservation of mechanism, TFIIS is not required for TC-NER in yeast (Verhage *et al*, 1997). Therefore, we sought to test the role of TFIIS in transcription-coupled nucleotide excision repair.

4.1) Transcription elongation factor IIS is not limiting for transcription-coupled nucleotide excision repair

In the present work, the *TCEA1* transcript was targeted with two distinct siRNAs. It was found that the relative *TCEA2* and *TCEA3* levels were negligible in comparison to *TCEA1*. Therefore, it is unlikely that the increased expression of *TCEA2* and *TCEA3* compensate for the loss of *TCEA1* in our experiments. On a western blot, a seven fold reduction in TFIIS protein levels was found in HCT116 colorectal carcinoma cells. Despite this large decrease in TFIIS expression, we did not detect a decrease in the ability of targeted cells to repair transcription blocking UV lesions assessed by two independent methods, HCR and RRS. Consistent with these results, we did not detect an increase in the sensitivity to UV- or cisplatin-induced apoptosis. Collectively, these results fail to support a role for TFIIS in TC-NER. We must conclude that TFIIS does not appear to be limiting for TC-NER.

4.2) Does RNA interference against transcription elongation factor IIS affect cell growth?

The absence of a cellular phenotype following the knockdown of TFIIS raised the question of whether the knockdown was biologically sufficient. When these experiments were initiated, there was no known biological phenotype of TFIIS loss in mammalian cells. Hubbard and coworkers recently reported that siRNAs targeting TFIIS inhibited the growth of tumour cells (Hubbard *et al*, 2008). We had not detected an anti-proliferative effect in HCT116 colorectal carcinoma cells with siTFIIS#1. Therefore, we selected an siRNA sequence reported by these authors, siTFIIS#2. In our hands, this siRNA inhibited the growth of HCT116 cells mildly but had a very dramatic growth inhibitory effect in MCF7 breast adenocarcinoma cells, a cell line used in the Hubbard paper. Importantly, siTFIIS#1 did not inhibit growth in MCF7 cells.

The difference in proliferation between TFIIS#1 and TFIIS#2 siRNA transfected cells does not reflect the relative effectiveness of these siRNAs, because they were similarly effective at reducing TFIIS protein levels in both cell lines. Intriguingly, the siRNA reported by Hubbard and coworkers consistently led to a reduction in actin protein levels forcing us to use a second loading control (Ku86) to ensure equal loading in all western experiments (figures 5A and 15). One preliminary experiment suggests that cells transfected with siTFIIS#2 have disorganized actin stress fibres (data not shown). The reported effect of TFIIS on proliferation may reflect off target effects of this siRNA. Nonetheless, none of the siRNAs tested affected TC-NER, or the sensitivity of

cells to UV light or cisplatin. Again, the present work fails to support a role of TFIIIS in TC-NER and clearly indicates that TFIIIS is not limiting for this repair process.

4.3) How do DNA repair proteins gain access to DNA damaged lesion?

Although there is compelling evidence from *in vitro* assays that suggest that TFIIIS could permit access of RNA repair proteins to DNA lesions (Donahue *et al*, 1994, Tornaletti *et al*, 1996), there is presently no strong evidence to support this model *in vivo*. At present, the only evidence to support a role for TFIIIS in TC-NER is the fact that TFIIIS binds chromatin more tightly following UV exposure, and this does not occur in TC-NER deficient CS-A and CS-B cells (Fousteri *et al*, 2006).

Similar circumstantial evidence supports an alternate model as well. UV light and cisplatin lead to ubiquitylation of the largest subunit of RNAPII in a CSA- and CSB-dependent manner. This modification promotes the proteasome-mediated degradation of the largest subunit of RNAPII (RNAPII LS). The degradation of RNAPII LS was delayed or absent in Cockayne syndrome fibroblasts (Bregman *et al*, 1996, McKay *et al*, 2001, and Luo *et al*, 2001). Proteasome-mediated degradation of RNAPII LS could hypothetically allow access of DNA repair proteins to DNA lesions. However, recent evidence suggests that this effect is indirect as well (Anindya *et al*, 2007). UV irradiation reduced the number of elongating polymerases following UV, so there were fewer polymerase modifications by ubiquitylation and proteasome-mediated degradation

(Anindya *et al*, 2007). Perhaps a similar indirect mechanism could explain the decreased association of TFIIS with UV-damaged chromatin in CS cells.

4.4) Conclusion

The evidence presented in this study does not support a role of TFIIS in transcription-coupled nucleotide excision repair. TFIIS was not found to be limiting in TC-NER nor did it increase the sensitivity of tumour cells to UV irradiation or cisplatin treatment. The lack of a biological phenotype associated with targeting TC-NER force us to conservatively interpret the data to suggest that TFIIS is not limiting for TC-NER.

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