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**אפיון מנגנון סינטזה דרך נזקי דנ"א בתאי גזע  
עובריים**

**Analysis of Translesion DNA Synthesis in  
Embryonic Stem Cells**

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## Table of contents

List of abbreviations .....	2
Summary .....	4
Summary in Hebrew (תקציר) .....	5
Introduction .....	6
Experimental methods .....	11
Results	
TLS is active in mES cells, and is reduced after differentiation .....	15
The Mutagenicity of TLS in mES cells changes upon differentiation .....	18
Naïve versus Primed mES .....	20
Involvement of p53 in TLS .....	22
Cell-Cycle Analysis .....	27
UV-C Survival of mES and RA-treated cells .....	31
PCNA Ubiquitination in mES versus differentiated cells .....	32
Characterization of free nucleotide Pools .....	33
Analysis of gene expression .....	35
Proteomic mass spectrometry analysis of mES and differentiated cells proteome .....	37
Discussion .....	41
UV sensitivity .....	43
The p53 protein and PCNA ubiquitination .....	43
Cell Cycle .....	44
Proteins & mRNA quantification .....	46
Free nucleotide pool .....	47
Appendix	
Base excision repair in mES .....	49
Reference .....	51
Acknowledgements .....	57

## List of abbreviations

BER	Base excision repair
BP-G	Benzo[ <i>a</i> ]pyrene-guanine adduct
BrdU	Bromodeoxyuridine
CisPt-GTG	Cisplatin-GTG
DDR	DNA damage response
DSB	Double strand break
DUB	Deubiquitinating enzyme
EB	Embryonic bodies
ES	Embryonic stem
ESI	Electrospray ionization
EpiSC	Epiblast stem cell
FACS	Flow cytometry
GG-NER	Global genome-nucleotide excision repair
GVHD	Graft versus host disease
hES	Human embryonic stem cell
HDR	Homology-dependent repair
HPLC	High performance liquid chromatography
HR	Homologous recombination
HRR	Homologous recombination repair
iPS	Induced Pluripotent Stem
KO	Knockout
LIF	Leukaemia inhibitory factor
MEF	Mouse embryonic fibroblasts
mES	Mouse embryonic stem cell
MMR	Mismatch repair
mUb-PCNA	Mono-ubiquitinated PCNA
NER	Nucleotide excision-repair
NHEJ	Non-homologues end joining
NPC	Neuronal precursor cells
NTC	Non-targeting control
NSG	No significant change
PI	Propidium iodide

pol $\eta$ , $\kappa$ , $\iota$ , $\nu$ , $\lambda$ , $\mu$	DNA polymerase $\eta$ , $\kappa$ , $\iota$ , $\nu$ , $\lambda$ , $\mu$
qPCR	Quantitative PCR
RA	Retinoic acid
ssDNA	Single strand DNA
TC-NER	Transcription coupled-nucleotide excision repair
TLS	Translesion DNA synthesis
UV	Ultra violet

## Summary

The work presented herein was initiated in order to examine the way ES (embryonic stem) cells cope with DNA lesions that are bypassed in an error-prone fashion when encountered during replication in other cell types. Based on the fact that ES cells are the cellular origin of the whole organism, we assumed that they might behave differently than differentiated cells under these situations.

Unexpectedly, mES (mouse embryonic stem) cells exhibited a higher extent of lesion bypass and higher mutagenicity compared to differentiated cells. This was confirmed using two independent mES cell lines and two independent differentiation methods. p53 was found to be a major effector in this process as its knockout shifted TLS (translesion DNA synthesis) efficiency from higher in mES to be higher in differentiated cells. Also, its ablation rendered the mutagenicity difference of a TLS event between mES and differentiated cells to be statistically insignificant, while it was highly significant in the WT cells. The knockout of p53 protein was found to also affect the mono-ubiquitination pattern of PCNA in response to UV irradiation. In WT mES UV irradiation led to PCNA mono-ubiquitination in differentiated cells but surprisingly not in pluripotent cells. In the knockout cells the pattern was reversed to be positive in pluripotent cells and negative in non-pluripotent cells, and in both cases (WT & knockout) it goes hand in hand with the differentiation state in which TLS is low.

Cell cycle differences between mES and differentiated cells were not correlated with the TLS outcome and thus were ruled out as the major contributor to the differences observed in TLS between mES and differentiated cells, and neither were the nucleotide pools. Analysis of mRNA expression of selected genes, and a global proteomic analysis detected generally elevated levels of DNA repair proteins in mES vs. differentiated cells. Of note, Npm1, previously found in our lab to be a positive regulator of TLS, decreased upon differentiation, and may be involved, at least in part, in the TLS difference. The elevated levels of repair proteins combined with the higher TLS extent in mES cells could potentially explain my finding of higher viability of mES under UV irradiation compared to differentiated cells.

In summary, the answer for the mechanism that stands behind TLS and mutagenicity rates that are high in mES WT cells but low in differentiated cells is still not fully understood. However, while trying to elucidate it, the critical role of p53 in this process was uncovered, along with other novel aspects of mouse pluripotent stem cells that were unknown before this work. Further studies will hopefully contribute to enable safe propagation of ES cells in vitro, for the benefit of safe therapeutic applications.

## תקציר

העבודה המוצגת בדו"ח המסכם הזה החלה על מנת לבחון את הדרך שבה תאי גזע עובריים מתמודדים עם נזקי דנ"א, אשר בהמצאם במזלג ההכפלה בתאים שאינם תאי גזע עובריים, ידוע כי המנגנון המטפל בהם, סינתזה דרך נזקי דנ"א (TLS - translesion DNA synthesis), נוטה לטעויות באופן מובנה. בהתבסס על העובדה כי תאי גזע עובריים הם המקור התאי של כלל האורגניזם, הנחנו כי תאים אלו עשויים להגיב בצורה שונה מתאים שאינם גזע עובריים בעת התמודדות עם נזקי דנ"א כאלה.

שלא כצפוי, בתאי הגזע העובריים נצפו הן אחוזי מעבר גבוהים של הנזקים והן רמות מוטגנזה גבוהות בהשוואה לתאים ממוינים. תצפית זו נתמכה ע"י הדירות התוצאות בשני קוי תאים בלתי תלויים של תאי גזע, ובשתי שיטות התמינות שונות. נמצא כי החלבון p53 הינו גורם משמעותי בתהליך זה, ונוקאאוט של חלבון זה מסיט את אחוזי TLS מגבוהים בתאי הגזע לגבוהים בתאים ממוינים. בנוסף, בעוד שבזן הבר המובהקות הסטטיסטית ברמות המוטגנזה בין תאי הגזע לתאים ממוינים היתה גבוהה, העדרו של חלבון זה הובילה לחוסר מובהקות הסטטיסטית.

הנוקאאוט של p53 נמצא כמשפיע גם על דפוס המונו-יוביקויטינציה של החלבון PCNA בעקבות הקרנת התאים ב – UV. בתאי זן הבר ההקרנה גרמה למונו-יוביקויטינציה של PCNA בתאים הממוינים, אך באופן מפתיע לא בתאי הגזע. בתאי הנוקאאוט הדפוס התהפך, ונעשה חיובי בתאים הפלוריפוטנטים ושילילי בתאים הממוינים. בשני סוגי התאים, זן הבר והנוקאאוט, הממצאים הם בהתאמה לפעילות TLS, הנמוכה יותר בתאים ממוינים מאשר בתאי גזע.

ההבדלים בשלבים השונים של מחזור התא בין תאים פלוריפוטנטיים וממוינים לא תאמו לתוצאות ה – TLS ולכן נשללו כגורם המרכזי להבדלים בתוצאות אלו בין תאים ממוינים ולא ממוינים. באופן דומה נשללו גם מאגרי הנוקלאוטידים בתאים כגורם המרכזי להבדלים אלו. אנליזת mRNA של גנים נבחרים ואנליזה פרוטאומית הראו בכללותן רמות גבוהות של חלבוני תיקון דנ"א בתאים פלוריפוטנטיים בהשוואה לתאים ממוינים. באופן בולט, החלבון Npm1 שנמצא בעבר במעבדתנו כרגולטור חיובי ל – TLS, הראה ירידה בתאים ממוינים ועשוי להיות מעורב, לפחות חלקית, בהבדל ב – TLS. הרמות הגבוהות של חלבוני תיקון דנ"א בשילוב עם פעילות TLS גבוהה יותר בתאים לא ממוינים עשויה להסביר את הממצאים המתוארים בעבודה זו ולפיהם תאים פלוריפוטנטיים מראים שרידות גבוהה יותר לאחר הקרנת UV בהשוואה לתאים ממוינים.

לסיכום, המנגנון הפועל מאחורי הקלעים של TLS ושיעורי המוטגנזה הגבוהים בתאים פלוריפוטנטיים ונמוכים בתאים ממוינים עדיין לא הובררו במלואם, אולם, במהלך עבודה זו נחשף תפקידו המהותי והחשוב של p53 בתהליכים אלה והיבטים חשובים נוספים של תאים פלוריפוטנטיים ממקור עכברי שלא היו ידועים עד כה. מחקרים נוספים צפויים לתרום לנושא ולאפשר גידול תאי גזע בתרבות באופן בטוח וחקר ממוטציות, לצורך שימוש ברפואה מתקדמת בעתיד הלא רחוק.

## Introduction

The maintenance of genetic information and genomic stability is crucial for all organisms and cell types. Indeed, genomic DNA replication has evolved to function at a very low mutation rate, estimated at less than one error for every  $10^9$  base pairs copied<sup>1</sup>. Additionally, to replication errors, genomic DNA is continuously damaged by a variety of factors which further exacerbate the genotoxic burden, originating internally as byproducts of metabolism, and externally to the body, such as sunlight, radiation, air pollution etc.

It has been estimated that each day genomic DNA is subject to roughly 50,000 hits per cell<sup>2</sup>, from both endogenous and exogenous factors to the cell. If not correctly repaired in a timely manner, these DNA lesions can at first stage hinder essential cellular processes such as transcription and replication, and may cause mutations and chromosomal aberrations, which at a later stage could possibly result in the development of cancer and other diseases or even death<sup>3</sup>.

In order to cope with this, all known living organisms have evolved multiple mechanisms of DNA repair, designated to cope with the large variety of DNA lesions<sup>4</sup>, such as: base excision repair (BER) and nucleotide excision repair (NER), in which a base or a segment carrying the lesion is removed and the resulting gap is filled in by a DNA polymerase that synthesizes the DNA according to the intact complementary strand<sup>5</sup>; mismatch repair (MMR) which mostly removes non-complementary base pairs by excising out the wrong base and incorporating the correct one through polymerization & ligation<sup>6</sup>; homologous recombination (HR) and non-homologous end joining (NHEJ), which repair double strand breaks (DSBs) by either homology and thus in an error free process, or by insertions or deletions of bases which renders it an error prone mechanism by default<sup>7</sup>; and direct damage reversal, which directly restores the original base(s)<sup>8</sup>. Despite the diversity and efficiency of the above mechanisms, some DNA lesions escape repair and persist in DNA during replication. The high-fidelity replication machinery is usually unable to continue synthesis through the lesions, leading to the formation of single stranded gaps, or stalled replication forks. If left unrepaired, these structures could easily break to form double strand breaks, which are highly deleterious. Replicative DNA polymerases, which had evolved for replicating intact DNA at an extremely low rate of errors, are unable to accommodate the damaged DNA base inside their catalytic pocket and therefore stall upon encountering a lesion. This may lead to two outcomes: collapse of the replication fork or re-initiation of

replication downstream to the lesion, leaving behind an ssDNA (single stranded DNA) gap opposite the lesion. This ssDNA gap that contains a lesion is not a substrate for excision repair which requires a DNA duplex substrate, and without its repair it will subsequently collapse and form a DSB, which in turn may lead to chromosomal aberrations and activation of apoptosis<sup>9</sup>.

In order to cope with this, special mechanisms have evolved early in evolution (as they are found in all known organisms), which fill-in the gap opposite the lesion and thus forming once again a substrate suitable for excision repair to work on. These mechanisms are thus named tolerance mechanisms rather than repair, as they do not repair the lesion *per se*. DNA damage tolerance mechanisms enable the completion of DNA replication without removing the lesion from the DNA, thereby preventing the formation of DSB. These damage tolerance mechanisms include homology-directed repair (HDR), which uses the newly synthesized sister chromatid as a homologous donor<sup>10–12</sup>, and translesion DNA synthesis (TLS), which utilizes specialized low-fidelity DNA polymerases with the ability to replicate across a variety of DNA lesions<sup>13–16</sup>. Due to the miscoding nature of most DNA lesions, TLS activity is inherently error-prone, and considered to be a major source of genomic point mutations.

While in prokaryotes<sup>17</sup> and in yeast TLS plays only a minor role compared to HDR in tolerating DNA damage during replication, it is not so in mammals which heavily rely on it when encountering lesions during DNA replication<sup>18</sup>. Consistently, while there are three TLS DNA polymerases in *S. cerevisiae*<sup>19</sup>, there are multiple specialized DNA polymerases in mammalian cells<sup>18</sup>. These include polη, polκ, polι and REV1 of the Y family of DNA polymerases, polζ of the B family, and most probably also polθ and polv of the A family<sup>20–22</sup>, polλ and polμ of the X family<sup>23–26</sup>, and the primase-polymerase PrimPol<sup>27</sup>. The importance of TLS in mammals is indicated by the embryonic lethality of polζ-deficient mice<sup>28,29</sup>, and the severe phenotype of a deficiency in human polη (elaborated below)<sup>30–32</sup>

TLS polymerases differ from replicative polymerases in their superior ability to bypass damaged nucleotides, and their much lower fidelity, which leads to the formation of 0.1%-30% errors per nucleotide in undamaged DNA templates<sup>1</sup>. This is due to their relatively large catalytic pocket which can accommodate damaged bases, as well as abnormal base pairing structures, and the lack of a 3'→5' exonuclease proofreading activity. Additional



unique characteristic of TLS polymerases is their mode of synthesis which is regarded as distributive rather than processive, namely, they carry out polymerization of a short patch, and then fall off the template<sup>5</sup>. This can be regarded as rather beneficial, given their low fidelity.

Mammalian TLS polymerases are constitutively expressed, showing a wide tissue distribution, while being regulated mainly at the post-translational level<sup>3</sup>. The activity of Y-family polymerases is modulated via interactions with proliferating cell nuclear antigen (PCNA), a sliding DNA clamp, which is pivotal to eukaryotic DNA replication and repair. Following exposure of cells to damaging agents such as methyl methanesulfonate (MMS) or UV irradiation, PCNA is monoubiquitinated on lysine 164 by a complex of Rad6 and Rad18, which are E2 and E3 ubiquitin ligases, respectively. This modification is believed to switch the high fidelity replication into an error-prone mode, in which TLS polymerases are implicated<sup>33</sup>. PCNA is additionally subjected to deubiquitination by a deubiquitinating enzyme (DUB) termed USP1. Following UV irradiation, USP1 is deactivated through an autocleavage event, thereby enabling accumulation of mono-ubiquitinated PCNA to support TLS<sup>34</sup>. Mono-ubiquitination increases PCNA affinity to Y family TLS polymerases via interaction with ubiquitin binding domains in their C-terminus (i.e. UBM in the case of pol $\eta$  and Rev1, and UBZ for pol $\eta$  and pol $\kappa$ ). This interaction is further stabilized through PCNA Interacting Protein (PIP box) motifs<sup>35</sup> found on pol $\eta$ , pol $\iota$  and pol $\kappa$ , or a BRCT domain in the case of Rev1<sup>36</sup>.

Although PCNA ubiquitination is central to the regulation of TLS, there is growing evidence from chicken and mouse models that suggest the existence of a TLS pathway which is independent of PCNA ubiquitination<sup>36–39</sup>. This indicates the importance of additional regulatory pathways in vertebrates, to assure the effectiveness of TLS while minimizing its mutagenic outcome. Such regulation is not yet well understood, but might be achieved via processes such as protein shuttling, stability, and local concentration of TLS components, as indicated by a recent screen for mammalian regulatory proteins performed in our lab<sup>40</sup>. In this respect, pol $\eta$  was found to play a highly dynamic role at sites of DNA damage<sup>41,42</sup>, and to be subjected to proteasomal degradation following UV exposure<sup>43–46</sup>. These findings further broaden our understanding on how TLS polymerases are regulated, but many pieces in the puzzle are currently still missing. For example, we still lack sufficient mechanistic details to

explain what prevents the access of TLS polymerases to undamaged DNA, or what favors the activation of the most suitable polymerase for a certain TLS reaction<sup>11,47</sup>.

### **Genomic stability of embryonic stem cells**

Embryonic stem cells are the progenitor cells of all the cells in the mature organism. Thus, a mutagenic event in an ES cell may be passed on to the progeny of the cell and affect multiple lineages. Therefore, it is imperative that stem cells possess robust and stringent mechanisms for maintaining genomic stability. Such a need is further supported by the fact that mouse ES cells are globally more transcriptionally active than differentiated cells and their chromatin is less condensed, rendering it more vulnerable to DNA damage<sup>48,49</sup>.

Several differences regarding the DNA damage response between somatic cells and stem cells have been previously described, among these differences in the p53 protein is a marked example: DSB, which are the most toxic type of DNA-damage incurred in the dividing cell<sup>50</sup>, were shown to either lead to p53-independent apoptosis in mouse ES cells<sup>51</sup>, or promote cell differentiation by the direct repression of the *Nanog* pluripotency transcription factor by p53<sup>52</sup>. Following differentiation, p53 gains control over the cell cycle inhibition and apoptosis induction<sup>53</sup>. It does so by establishing the G<sub>1</sub>-S phase checkpoint, which is missing in pluripotent stem cells. This implies that in ES cells, one of the roles of p53 is to eliminate DNA damaged cells from the stem cell pool<sup>53,54</sup>.

Cell cycle arrest in response to DNA damage differs between embryonic stem cells and somatic differentiated cells. As mentioned above, mouse ES cells lack the G<sub>1</sub>-S checkpoint<sup>55-57</sup>, due to sequestration of the Chk2 kinase at the centromeres and lack of expression of the CDK inhibitor protein p21<sup>58</sup>. Arrest at the G<sub>1</sub> checkpoint can be restored by ectopic expression of Chk2, which protects cells from apoptosis, but does not activate p53<sup>59</sup>. It has been postulated that the p53-p21 pathway for activation of the G<sub>1</sub>-S checkpoint is completely inactive in ES cells, and gains function only upon differentiation<sup>60</sup>.

It was reported that mES (mouse embryonic stem) cells predominantly utilize the high fidelity HRR (Homologous recombination repair) rather than the more error-prone NHEJ mechanism for the repair of DSB, while somatic cells utilize mainly NHEJ<sup>61-63</sup>. Also, it was reported that BER activity is generally lower in differentiated cells compared to their progenitors, and appears to be dependent on cell cycle processes<sup>62,64</sup>. MMR mechanism was

as well found to be more robust in mES cells compared to MEF's (mouse embryonic fibroblast), with over 15-fold increase in repair efficiency compared to MEFs<sup>65</sup>.

Thus far, there hasn't been a known attempt to analyze TLS in ES cells. However, mutagen-induced mutations, which are formed by the mutagenic arm of TLS have been investigated. Analysis of mutations induced in the *Aprt* locus in mES cells and MEFs by EMS (Ethyl methanesulfonate), which forms O<sup>6</sup>-ethylguanine lesions that miscode for adenine, revealed that newly formed mutations in mES cells were two orders of magnitude lower than in isogenic MEFs<sup>66</sup>. A parallel analysis performed on the *Hprt* locus found the newly formed mutations to be lower by more than three orders of magnitude in mES cells compared to MEFs<sup>66,67</sup>. These cells differ also in mutation type – ES cells were more prone to chromosome loss, while MEFs were more prone to mitotic recombination<sup>66</sup>. When treated with the crosslinking agent mitomycin C (MMC), hESCs exhibited higher resistance to chromosomal breaks, fusions or translocations, and were less prone to telomere loss in comparison to somatic cells<sup>68,69</sup>. Despite this, mES cells are generally hypersensitive to various types of DNA damage, including MMC,  $\gamma$  radiation and UV radiation relative to MEFs<sup>70–72</sup>.

As mentioned above, very little is known about the DNA damage tolerance mechanisms TLS and HDR in embryonic stem cells. HDR requires the Rad51 protein, which is essential, as indicated by the early embryonic lethality in mice with disruptions of the *RAD51* gene<sup>73</sup>. However, this may be due to other deficiencies in HR (homologues recombination), not necessarily HDR. The function of TLS is somewhat enigmatic, since it is an error-prone mechanism that might endanger the genomic integrity of ES cells, with possible implications on the cells' progeny. However, there is evidence that TLS is important for embryonic development, since disruption of the *Rev3L* gene encoding the catalytic subunit of the error-prone DNA polymerase  $\zeta$  is embryonic lethal in mice<sup>28,29</sup>. Yet, the mechanism, regulation and function of these systems in any type of ES cells have not been elucidated so far.

## Experimental methods

**Tissue culture:** Embryonic stem cells were cultured in conditions maintaining the outmost naïve state on irradiated MEF cells and supplemented with medium based on DMEM (Gibco) supplemented with 2 mM Alanine-glutamine (Biological Industries), 100 units/mL of penicillin, 100 µg/mL of streptomycin (Biological Industries), 100 µM of Non-essential amino acids (Biological industries), 100µM of β-mercaptoethanol, 1µg/ml of LIF (Peprotech), 3µM of CHIRON99021(Axon medchem), 1µM of PD0325901 (Axon medchem) and 15% FBS (Biological industries). Retinoic acid differentiation to NPC's (neuronal precursor cells) of mES was performed without the presence of irradiated MEF cells, under same basic media composition, without LIF, CHIR & PD, and supplemented with 1µM retinoic acid for 7 days.

Prior to transfection of the mES cells, the irradiated MEF cells were excluded from the mixed culture by plating out on 0.1% gelatin in order to ensure pure mES culture.

Primed culture differentiation and maintenance was in the presence of medium containing 50%-50% of DMEM & Neuro-basal medium + 2 mM Alanine-glutamine (Biological Industries), 100 units/mL of penicillin, 100 µg/mL of streptomycin (Biological Industries), 100 µM of Non-essential amino acids (Biological industries), 100µM of β-mercaptoethanol, N2B27 (1% N2, 1% B27 v/v) (Invitrogen), 0.1% of BSA w/v (Invitrogen), 12ng/ml β-FGF + 20ng/ml Activin-A. Spontaneous differentiation was performed by the formation of EB's for 7 days in suspension culture, then the breakdown of the clumps by trypsinization and sub-culturing the cells for another 5 passages in adherent conditions. Medium was mES medium without presence of LIF. All cells were maintained at 37 °C in a 5% CO<sub>2</sub>, 4% O<sub>2</sub> atmosphere, and were periodically examined for Mycoplasma contaminations by EZ-PCR test kit (Biological Industries).

**UV sensitivity:** Culture plates were washed twice with PBS, PBS was aspirated and plates were irradiated with UV-C using a low-pressure mercury lamp (TUV 15w G15T8, Philips). UV dose rate was measured using a UVX Radiometer (UVP) equipped with a 254 nm detector. After irradiation fresh pre-warmed medium was added to the plates. Cell viability was determined using the CellTiter-Glo luminescent viability Assay (Promega) that measures the amount of cellular ATP present, which is rapidly depleted when cells undergo necrosis or apoptosis<sup>74</sup>.

**Colony-based plasmid TLS assay:** The assay and the construction of the gap lesion plasmids are described in details in Ziv et al, Methods Mol Biol, 2012<sup>75</sup>. Briefly, the culture of mES cells was sub-cultured until reaching the desired number of  $\sim 0.4 \times 10^6$  cells/well of a 6-well plate in addition to yielding a starter population for the differentiation procedure (induced by RA or spontaneously for terminal differentiations). On day 7 of RA differentiation, or the 5<sup>th</sup> passage of the spontaneous differentiation, or after 5 days of naïve/primed differentiations the culture was harvested, counted and seeded on 6-well plates with identical number of cells as done with the undifferentiated cells. After seeding, cells were incubated overnight to allow maximum adherence and were then co-transfected in identical triplicates with an equimolar mixture of a gap-plasmid carrying a site-specific DNA lesion ( $\text{kan}^R$ ), and a control gapped plasmid without a lesion ( $\text{cm}^R$ ), using Xfect transfection reagent (Clontech). The cells were incubated for 18 h to allow TLS, and plasmids were extracted under conditions in which only covalently closed plasmids remained non-denatured. The extracted plasmids were introduced by transformation into a TLS-defective *E. coli* strain, which was then plated in parallel on LB-kanamycin and LB-chloramphenicol plates. To obtain TLS extent values, the ratio of  $\text{kan}^R$  to  $\text{cm}^R$  colonies was calculated, and corrected by eliminating of the usually small fraction of non-TLS events (observed as large deletions or insertions) based on the DNA sequence analysis. For mutagenesis analysis, TLS products were amplified from  $\text{kan}^R$  colonies using the TempliPhi DNA Sequencing Template Amplification Kit (GE Healthcare) and the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Reactions were analyzed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

**Flow cytometry:** mES cells were grown under either conditions maintaining their pluripotency or promoting differentiation to NPC's (as described above under 'tissue culture'). BrdU (Sigma) was added to culture medium to reach 10 $\mu$ M and incubated 30 minutes. Then cells were harvested and the pellet was incubated in 70% EtOH for 30 minutes, washed and incubated 30 minutes in HCl-Triton (2N HCl + 0.5% Triton X-100) room-temperature. Then washed and incubated in BORAX ( $\text{Na}^+$  Tetraborate 0.1M, PH8.5). washed with Tween-BSA-PBS (1% BSA + 0.5% Tween20 in PBS) and incubated in 90% Tween-BSA-PBS +10% anti-BrdU Ab (BD Biosciences, Cat:MAB-34758), washed and incubated 30 minutes in propidium iodide (25 $\mu$ g/ml) (Sigma)+ RNase (50 $\mu$ g/ml) (Sigma), then analyzed using FACS Aria II Cell Sorter (BD Biosciences) and FlowJo software.

Calculations of the duration of different cell cycle phases was performed by the following set of equations:

1.  $T_S = T_C \frac{\log(\frac{S\%+G_2M\%}{100}+1)}{\log 2} - T_{G_2M}$
2.  $T_{G_2M} = T_C \frac{\log(\frac{G_2M\%}{100}+1)}{\log 2}$
3.  $T_{G_1} = T_C - T_{G_2M} - T_S$

$T_C$  is the duration of one complete cell cycle.

$T_{G_1}$ ,  $T_{G_2M}$ ,  $T_S$  are the durations of the cell cycle phases.

**Cell lysis & protein quantification:** Cells were lysed with RIPA buffer (Tris-HCl 50mM pH 7.4, NP-40 1% v/v, Na-deoxycholate 0.25% v/v, NaCl 150mM, EDTA 1mM, mammalian Protease Inhibitor [Sigma]). Total protein concentration in each extract was measured by BCA protein assay (Thermo scientific).

**Immunoblot analysis:** Extraction of Triton-soluble and insoluble fractions was done as described<sup>76</sup>. Cell lysates were fractionated by SDS-PAGE, after which they were transferred to a PVDF membrane and probed with 1:3000 mouse  $\alpha$  PCNA (PC10, SC-56, Santa Cruz). The secondary antibody used was Peroxidase-conjugated Goat anti-mouse IgG (Jackson). Proteins were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce).

**Nuclear fractionation:** Cells were harvested in 4500g, 15 minutes. Washed in PBS and gently lysed with lysis buffer (10 mM TRIS-HCl PH 7.5, 100mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.5% NP-40), vortexed and nuclei-containing pellet was harvested.

**Small molecules – mass spectrometry:** Cells were first harvested and their number was determined using a hemocytometer, afterwards they were resuspended in methanol/Tris-HCl buffer 70/30 (v/v) (Tris-HCl is: 50 mM, pH 5). Lysis was achieved by vortexing and cellular debris was removed by centrifugation. Supernatant was evaporated to dryness under nitrogen stream. Residue was dissolved in water and purified on polymeric weak anion columns Strata-XL-AW 100u (30mg/1ml, Phenomenex). Samples were later lyophilized, re-dissolved in water, and analyzed by LC-MS/MS three times. Standard mixtures of four NTPs and four dNTPs with equal concentrations of 0.1, 1 and 10  $\mu$ g/mL each were used for calibration LC-MS/MS device. Final concentrations of NTPs were determined as pmol per 1 million cells.

**RT PCR and Real time PCR:** Total RNA was extracted from the cells, using the Perfect-Pure RNA cultured cells kit (5- PRIME). 1000 ng RNA were taken for cDNA production using High capacity cDNA reverse transcription kit (Applied Biosystems). 5-25 ng cDNA were taken for each Real time PCR analysis using SYBR Green reagent (KAPA biosystems) and 7500 & Viia7 Real Time PCR Systems (Applied Biosystems). The data was normalized according to measurements HPRT and RPL19 genes using comparative  $C_T$  method<sup>77</sup>. The following primers were design using Primer3 web interface, and pre-examined for linearity and specificity.

Target	Forward primer (5'→3')	Reverse primer (5'→3')
Oct4	CCTGGCCTGTCTGTCACTCA	TAAACAAGAATTTATTTCTGACCCTGTG
Nanog	CAGGAGTTTGAGGGTAGCTC	CGGTTTCATCATGGTACAGTC
Pol η	TGTCTACAAGAGCAACCACTCGAT	TTCGCTCTTGGCAGTTTTGA
Pol ι	CCGCATCTCTGCTGCAATC	CCCAGGCTTCAGCTGTGAAC
Pol κ	CTGCACGGACACCAAACCT	AAGGATAAACCCATTGCTGTAGGA
Rad18	AACAGGTGAGTCCGTGCAAAA	GTAACGGAGCCAGACCTGAGA
Rev1	CCAGCAGCTTCAAGTCGTCTT	AGGTTCACCCAGCCCAAAG
Rev3	CTGCAATGGTGAAAAGGATATGTG	TGAAGAGCTGTAGGAGGTGAGGAAT
Rev7	CCCCGCTTGATACCCCTAAA	TTCTTATGCGCTCGCTCTTCA
Pax6	CACACCTGTCTCCTCCTCACA	TTTGCCATGGTGAAGCTGG
Sox1	CCTGTGAAATCGAAACGTGCT	AAGTCACGGAAACGCTGCA
Nestin	GGTCACTGTGCGCGCTACTC	AAGCGGACGTGGAGCACTA
Ube2A	GTGTCTTCCATTTTAACGTCCAT	TATTCCCGCTTGTTCTCCTG
Ube2B	CCCACATATGATGTCTCTTCCA	GCTGGACTGTTTGGATTCTG
Klf2	CGCACCTAAAGGCGCATCTG	TTCGGTAGTGGCGGGTAAGC
Klf4	CTAACACAGGAGAGAAACCTTA	TTTCCACCCACAGCCGTC
Fgf5	GCGACGTTTTCTTCGTCTTC	ACAATCCCCTGAGACACAGC
RaRb2	GGCATACTGCTCAATCCATCG	TTGTCCTGGCAAACGAAGC
Atm	AACAAAGTCTTAGTGATACTGACCAGAGTTT	CACGCTCAGCTACTTTGTTGAAA
Atr	TGAAGGACATGTGCATTACCTCATA	ACCAAGGTACATCTGACAGAGTAAGTTT
Rpl19	ATCGCCAATGCCAACTCT	GAGAATCCGCTTGTTTTTGAA
Hprt1	GCAGTACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAAGCT

## Results

### TLS is active in mES cells, and is reduced after differentiation

One of the most common and effective methods to induce differentiation of mES cells is treatment with retinoic acid (RA)<sup>78,79</sup>. Our experimental protocol involved treating cultured mES cells (in the absence of the irradiated feeder MEFs) with retinoic acid (1 $\mu$ M) for 7 days. Every 24hr the RA-containing medium was replaced with fresh medium. At the end of the differentiation treatment the cells were harvested and seeded in a new plate, and assayed for TLS using the gap-lesion plasmid assay, which was extensively used in our lab. Briefly, the assay is based on a non-replicating plasmid, which carries a defined site-specific DNA lesion in a short ssDNA region (a structure of a gap opposite the lesion), and a kanamycin-resistance gene. Cells are transfected with the gap-lesion plasmid, and with a control gapped-plasmid (without a lesion) carrying a chloramphenicol resistance gene. TLS converts the gap-lesion plasmid into a fully double-stranded circular plasmid, which is resistant to denaturation by alkali. After allowing time for TLS to occur, the plasmid mixture is isolated under alkaline conditions, and used to transform indicator TLS-defective *E. coli recA*<sup>-</sup> cells. The cells are plates in parallel on LB plates containing kanamycin, to select for the filled gap-lesion plasmids that underwent TLS, and on LB plates containing chloramphenicol, to select for the filled control gapped plasmids. The ratio of the two is a measure of the extent of TLS (in % units). Colonies are then picked from the kanamycin plates and their plasmid content is isolated and subjected to DNA sequence analysis at the site opposite to where the damaged base was originally located, thereby providing sequence information on the accuracy and sequence specificity of the TLS reaction.

Each experiment included triplicate transfections for each cell type, and the transformed bacteria were plated in duplicates. TLS across three different DNA lesions was measured: an abasic site, a common lesion formed spontaneously and by oxidative agents, a cisplatin-GTG adduct (cisPt-GTG), a DNA lesion caused by the chemotherapeutic drug cisplatin, and a benzo[*a*]pyrene-guanine adduct (BP-G), a major tobacco smoke and combustion-induced DNA lesion. These lesions are bypassed by different TLS DNA polymerases and therefore enable the assessment of a broad range of TLS capabilities.



Figure 1 presents TLS extents obtained with the various DNA lesions. A typical example of the raw data that was used to draw Figure 1, including actual colony counts, is presented in Table 1. As can be seen in Figure 1, significant extents of TLS were obtained reaching 37%, 69% and 53% for the abasic site, cisPt-GTG and BP-G respectively. Surprisingly, the extent of TLS was reduced in RA-differentiated cells. This was found for each of the three types of DNA damage, with TLS extent of 22%, 35% and 35% for the abasic site, cisPt-GTG and BP-G respectively.

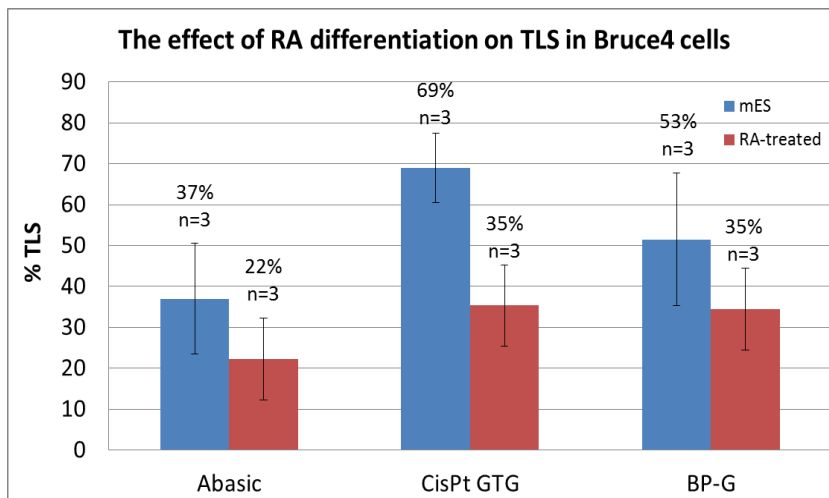


Figure 1. The effect of RA-differentiation on TLS in Bruce4 mES cells. Results are statistically significant

To examine whether the decrease in TLS extent upon differentiation was specific to the RA-treatment, a different differentiation protocol was used. It consists of spontaneous differentiation, occurring upon prolonged growth, up to 3 weeks under conditions that do not maintain the ‘stemness’ of the mES cells. Specifically, following removal of the irradiated MEFs from the mES cell culture, the cells were transferred to a petri dish to allow the formation of embryonic bodies (EBs). Once formed, the culture was trypsinized, seeded on regular tissue culture plates, and propagated for 5 passages for purposes of expansion and reaching homogeneity. The cells were then assayed for TLS using the gap-lesion plasmid assay. The results of the TLS assays performed with spontaneously differentiated cells are presented in Figure 2.

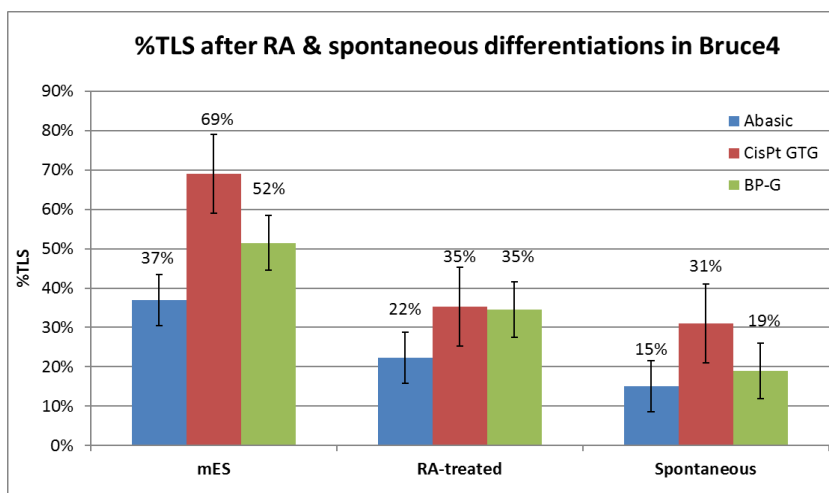


Figure 2. TLS extent in Bruce4 mES and RA-differentiated or spontaneously differentiated cells. The results of the mES & RA-differentiated cells were taken from Figure 1. Results are statistically significant

Table 1.

Bruce4: typical results of a TLS experiment						
		Kanamycin		Chloramphenicol		Average
					K/C	
mES		420	396	552	508	77%
		111	130	154	193	69%
		132	92	232	198	52%
CisPt GTG	RA treated	69	70	179	189	38%
		13	14	42	35	35%
		71	57	276	278	23%
	Spontan. diff.	44	44	134	134	33%
		60	65	218	214	29%
		41	44	124	136	33%

The experiment was performed as described above. Kanamycin-containing plates are seeded with 300µl of *E. coli* transformed with the plasmid descendants, whereas the chloramphenicol-containing plates are seeded with 100µl.

For each of the three DNA lesions, TLS was significantly lower in the spontaneously differentiated cells compared to the mES cells (Fig. 2), decreasing from TLS extents of 37%, 69% and 52%, to 15%, 31% and 19% for the abasic site, cisPt-GTG and BP-G, respectively. In fact, the down-regulation of TLS was more pronounced in spontaneously differentiated than in RA-treated cells (Fig. 2). Thus, the decrease in TLS upon differentiation is not limited to a specific differentiation path, consistent with the notion that it is a general fundamental difference between mES and differentiated cells.

## The Mutagenicity of TLS in mES cells changes upon differentiation

Since TLS is an inherently mutagenic mechanism, it was interesting to see the mutagenic signature in both cell types - mES and their differentiated counterparts. These data were obtained by sequencing the descendants of gap-lesion plasmids that were isolated from the indicator *E. coli* colonies obtained in the experiments described in Figure 1.

The sequencing results of these experiments are presented in Table 2 and in Figure 3.

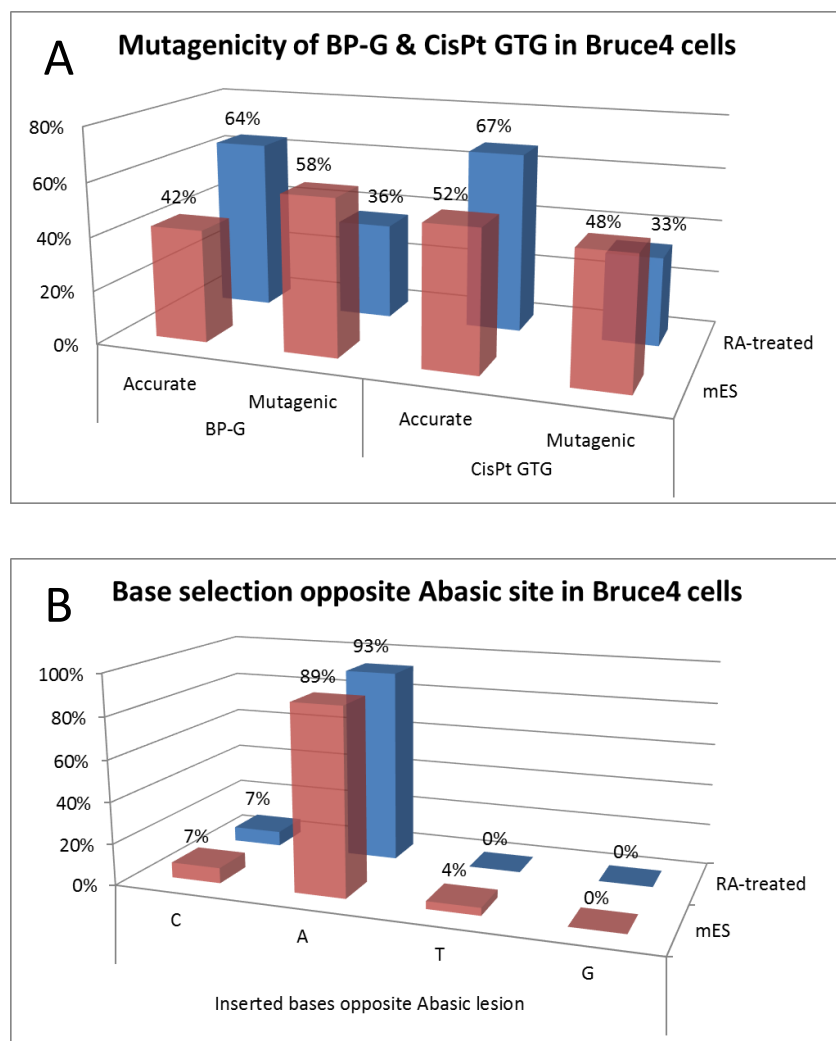


Figure 3. **A.** Mutagenic signature of lesion-bypass in Bruce4 mES and RA-differentiated cells. Chi-squared test for the difference in mutagenic TLS between ES and differentiated cells yielded p-values of 0.016 for BP-G, and 0.041 for cisPt-GTG. **B.** Base selection opposite abasic site in mES and RA-differentiated cells.

Table 2A

DNA sequence analysis of descendants of a gap-lesion plasmid carrying a site-specific BP-G after repair in mES and RA-treated Bruce4 cells				
DNA sequence opposite <b>BP-G</b>	mES		RA-treated	
	# events	percentage	# events	percentage
C (accurate TLS)	27	42%	41	64%
A	30	46%	20	31%
G	5	8%	-	
T	1	2%	3	5%
non TLS events	1	2%	-	
Total sequences	64		64	

Table 2B

DNA sequence analysis of descendants of a gap-lesion plasmid carrying a site-specific CisPt GTG after repair in mES and RA-treated Bruce4 cells				
DNA sequence opposite <b>CisPt GTG</b>	mES		RA-treated	
	# events	percentage	# events	percentage
CaC (accurate TLS)	50	52%	62	65%
AaC	30	31%	17	18%
CaA	12	13%	9	9%
AaA	1	1%	-	
Aa-	2	2%	1	1%
TaC	1	1%	1	1%
CaT	-		3	3%
non TLS events	-		3	3%
Total sequences	96		96	

While base selection opposite an abasic site is essentially unchanged in mES and differentiated cells, the mutagenicity of TLS across BP-G and cisPt-GTG became more accurate in differentiated cells compared to mES cells as seen in Figure 3. This shift is statistically significant with chi-test p-value=0.016 & 0.041 for BP-G & cisPt GTG lesions, respectively.

Validation of the generality of the higher mutagenicity observed in Bruce4 ES cells was obtained by using another line of mES, termed WT-35. Sequencing results of WT-35 cells corroborate the transition from mutagenic to more accurate TLS upon differentiation observed in Bruce4 cells, and once again TLS was found to be more mutagenic in the embryonic stem state than in the differentiated state, with chi-squared test p-value=0.003 & 0.00006 for BP-G & cisPt-GTG lesions, respectively (see below Figure 7A & C).

In summary, using two completely independent lines of mES (Bruce4 & WT-35) I showed that a variety of TLS sub-pathways, despite being inherently mutagenic, are more active in mES than in differentiated cells. In addition, TLS is significantly more mutagenic in mES than in differentiated cells.

### **Naïve versus Primed mES**

When mES cells were first isolated 24 years ago<sup>80</sup> and up until very recently, they were considered to be a homogeneous population of pluripotent cells. This perception has been challenged in 2007 by two independent groups<sup>81,82</sup> with the identification of Epiblast stem cells (EpiSCs cells), derived from the epiblast of the mouse implanted embryo (post-implantation epiblast) at day 5.5, which resemble human ES cells more than mouse ES cells. These EpiS cell-like cells have a unique phenotype that is different from the “classical” mES cells, namely their chromatin is much more condensed, X-chromosome is already inactivated, genome-wide DNA methylation appears, along with the absence of Rex1, NrOb1, Fgf4 markers which are unique to the ground state, and the expression of Epiblast cell markers such as Blimp1 and Fgf5. Also, and similarly to hES, they are derived and maintained in the stabilizing presence of TGF $\beta$ /Activin-A and bFGF and are destabilized by the presence of LIF/Stat3. These EpiSCs cells were termed Primed ES cells, while the ground state ES cells was termed Naïve ESC's<sup>83</sup>.

Because the high level of TLS that we have observed in mES cells was unexpected given the mutagenic nature of this process, the discovery of the two mES cell types raised the question of whether the high TLS is a property of the ‘true’ naïve mES cells. To examine this possibility, we examined TLS separately in naïve and primed mES cells. Naïve cells were obtained by a 5-day exposure to LIF and 2i cocktail composed of CHIRON99021 (which increases WNT signaling) and PD0325901 (which inhibits ERK1/2). Primed cells were obtained using a similar methodology but with exposure to FGF2 and Activin instead of the 2i cocktail. A typical result of a TLS experiment in naïve and primed cells is presented in Table 3. Figure 5 presents the average results of the extent of TLS across three different DNA lesions in the various cell types.

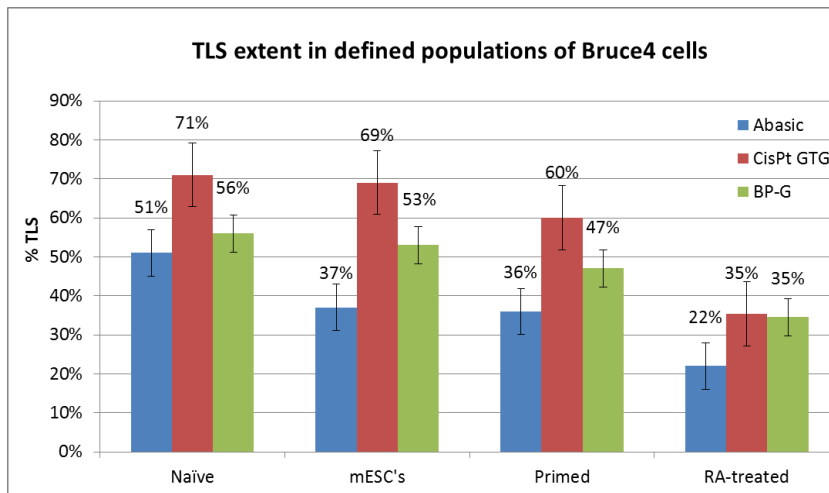


Figure 5. TLS extent in specific mouse cell populations. TLS is highest in naïve ES cells and lowest in the RA-treated cells, where it amounted to about 50% of the TLS values observed in the naïve.

Table 3.

Naïve & Primed: typical results of a TLS experiment							
		Kanamycin		Chloramphenicol		K/C	Average
CisPt GTG	Naïve	220	207	285	304	72%	71%
		333	376	503	468	73%	
		223	242	333	345	69%	
	Primed	56	64	96	107	59%	60%
		39	44	73	56	65%	
		40	34	53	78	56%	

From comparing TLS extent among these populations we learn that results are very close between Naïve and mES populations, which is consistent with the fact that most of the heterogeneous population is composed of naïve cells. As discussed above, TLS is lower in the differentiated population relative to ES cells (of any known pluripotency state), and the results from the primed cells are positioned somewhat in between pluripotent & differentiated cells. Yet, they do indicate a slight decrease, which can be explained by the fact that these cells are further down on the differentiation trend line.

In summary, these experiments suggest that TLS gradually decreases with the progression of the differentiation program.

## Involvement of p53 in TLS

It was previously found in our lab that the proteins p21 and p53 participate in the regulation of TLS<sup>47</sup>. This was attributed in part to the stimulation of PCNA mono-ubiquitination by p21 binding to PCNA, and the facilitation of a switch from the replicative to a TLS polymerase. It is also known that in ES cells the p53 protein is by large in the cytoplasm, and moves to the nucleus upon differentiation<sup>84</sup>. It was therefore interesting to test the involvement of p53 in this effect of high TLS in mES cells. To that end, we used p53 KO mES cells (obtained from Yael Aylon and Moshe Oren), to assay TLS before and after differentiation.

A typical result of a TLS experiment with WT-35 and p53 KO cells is presented in Table 4. Figure 6 presents the average results of TLS across all three types of DNA lesions previously mentioned (abasic site, cisPt-GTG & BP-G), in p53 KO and WT-35 cells before and after RA-induced differentiation. TLS in WT-35 mES cells was high in all three types of lesions, and decreased 38-53% upon RA treatment. In p53 KO cells the situation was nearly a mirror image of the wild type cells: TLS in the mES cells was low and upon RA treatment it increased by 47-51%, reaching values observed in WT-35 mES cells (Fig. 6).

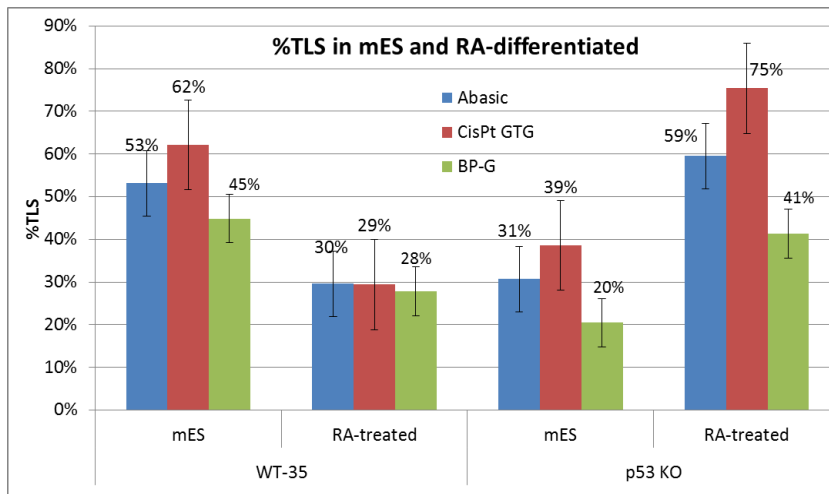


Figure 6. TLS in p53 KO mES cells. TLS across 3 lesions was measured in WT-35 and p53 KO mES and RA-treated cells.

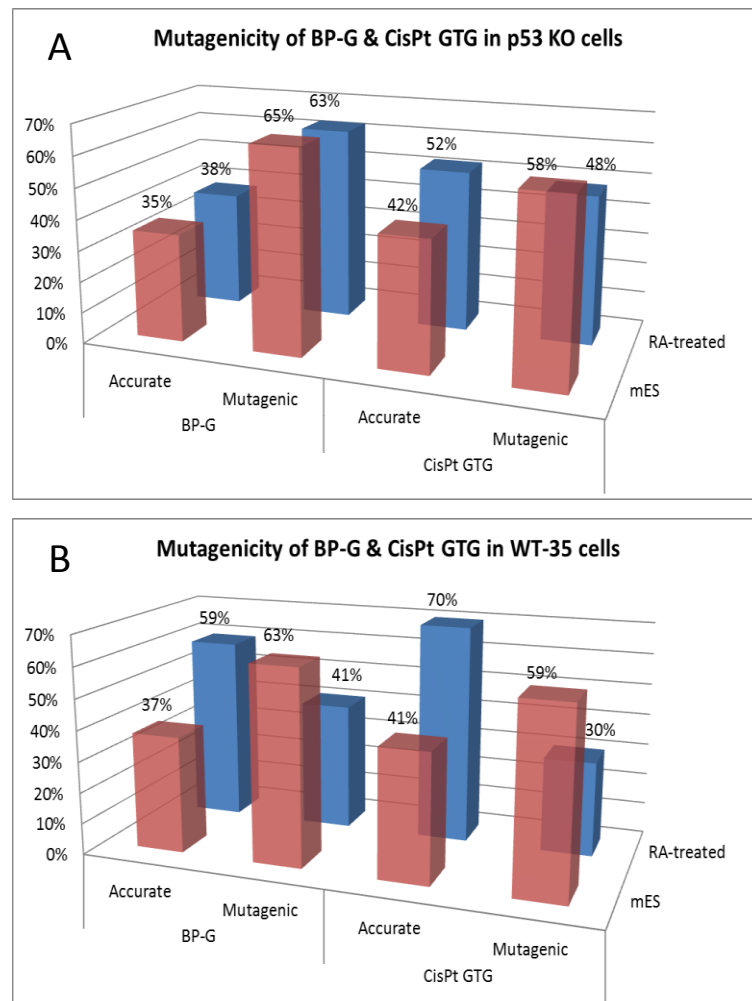
Table 4.

<b>WT-35: typical results of a TLS experiment</b>							
		Kanamycin		Chloramphenicol		K/C	Average
CisPt	mES	102	107	180	151	63%	62%
		234	243	398	382	61%	
GTG	RA treated	121	110	364	353	32%	29%
		133	117	461	477	27%	

<b>p53 KO: typical results of a TLS experiment</b>							
		Kanamycin		Chloramphenicol		K/C	Average
CisPt	mES	99	83	201	223	43%	39%
		138	148	423	410	34%	
GTG	RA treated	149	139	176	189	79%	75%
		201	189	261	282	72%	

In order to test the involvement of p53 in the base selection of TLS, I sequenced the colonies that originated from the WT-35 and p53 KO cells. These results are presented in Figure 7 in summary and in Tables 5 & 6 in details.





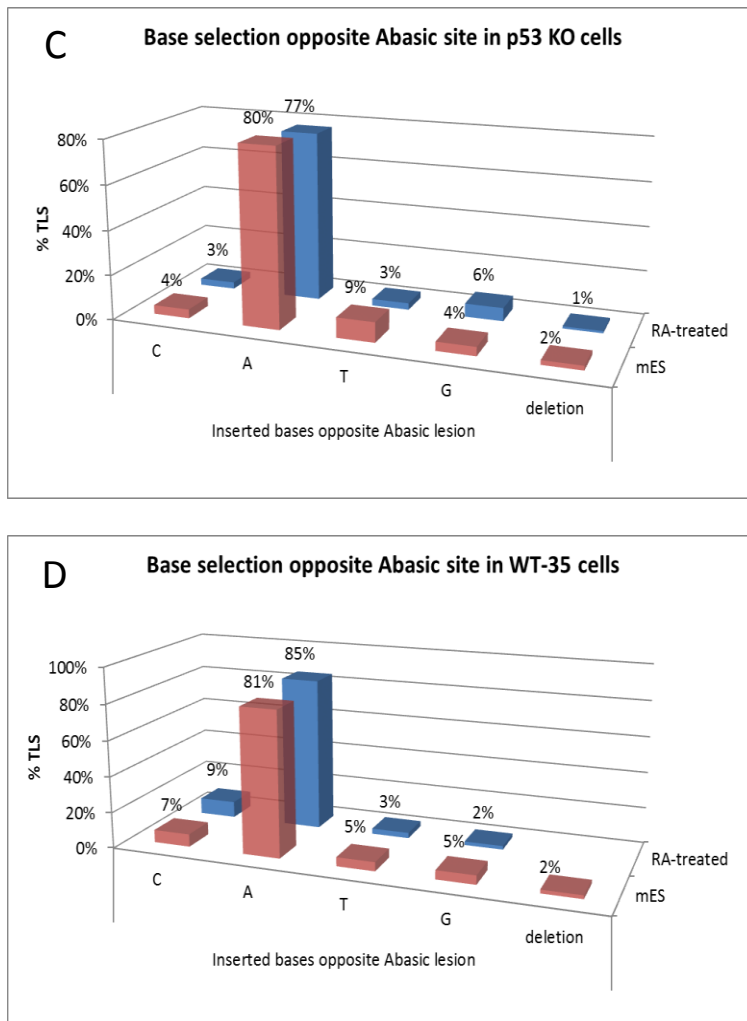


Figure 7. Comparison of DNA lesions bypass in WT and p53 KO, both mES and differentiated cells. A & B: mutagenicity of BP-G & cisPt-GTG lesions. C & D: base selection opposite an abasic site.

Table 5A.

DNA sequence analysis of descendants of a gap-lesion plasmid carrying a site-specific BP-G after repair in mES and RA-treated WT-35 cells				
DNA sequence opposite BP-G	mES		RA-treated	
	# events	percentage	# events	percentage
C (accurate TLS)	34	37%	55	59%
A	50	54%	31	33%
G	2	2%		
T	4	4%	7	7%
non TLS events	3	3%	2	2%
Total sequences	93		95	

Table 5B.

DNA sequence analysis of descendants of a gap-lesion plasmid carrying a site-specific CisPt GTG after repair in mES and RA-treated WT-35 cells				
DNA sequence opposite CisPt GTG	mES		RA-treated	
	# events	percentage	# events	percentage
CaC (accurate TLS)	39	41%	67	70%
Aa-	1	1%	-	
AaA	2	2%	3	3%
AaC	-		2	2%
AaG	-		2	2%
AaT	-		-	
AcC	32	33%	18	19%
-aA	-		-	
Ca-	1	1%	-	
CaA	11	11%	4	4%
CaT	6	6%	-	
CcC	-		-	
TaC	1	1%	-	
non TLS events	3	3%	-	
Total sequences	96		96	

Table 6A.

DNA sequence analysis of descendants of a gap-lesion plasmid carrying a site-specific BP-G after repair in mES and RA-treated p53 KO cells				
DNA sequence opposite BP-G	mES		RA-treated	
	# events	percentage	# events	percentage
C (accurate TLS)	33	35%	36	38%
A	54	56%	51	53%
G	5	5%	5	5%
T	3	3%	4	4%
non TLS events	1	1%	-	
Total sequences	96		96	

Table 6B.

DNA sequence analysis of descendants of a gap-lesion plasmid carrying a site-specific CisPt GTG after repair in mES and RA-treated p53 KO cells				
DNA sequence opposite CisPt GTG	mES		RA-treated	
	# events	percentage	# events	percentage
CaC (accurate TLS)	40	42%	50	52%
Aa-	1	1%	-	
AaA	2	2%	1	1%
AaC	31	32%	26	27%
AaG	-		2	2%
AaT	6	6%	3	3%
AcC	1	1%	2	2%
-aA	1	1%	2	2%
Ca-	1	1%	-	
CaA	10	10%	7	7%
CaT	2	2%	1	1%
CcC	1	1%	-	
TaC	-		2	2%
non TLS events	-		-	
Total sequences	96		96	

Statistical analysis using the Chi-squared test of the differences in mutagenic TLS between the ES and differentiated cells, based on the data presented in Tables 5 and 6 yielded the numbers presented in Table 7.

Table 7.

P-value of BP-G & CisPt-GTG Mutagenicity		
Phenotype	DNA lesion	P-value
WT	BP-G	0.00312
	CisPt GTG	0.00006
p53 KO	BP-G	0.69101
	CisPt GTG	0.14812

P values, calculated by the chi-squared method, correspond to the difference in mutagenicity between the mES and the differentiated cells.

Thus, the differences in mutagenicity between mES and differentiated WT cells are significant both for BP-G and cisPt-GTG DNA lesions. In contrast, differentiation treatment of p53 KO cells was not associated with any significant change in mutagenicity. Therefore, we conclude that p53 is not only heavily involved in TLS but is also involved in the sub-pathway that controls the fidelity of the DNA lesion bypass.

## Cell-Cycle Analysis

One of the hallmarks of pluripotent stem cells is rapid cell division, and indeed, in my experiments, upon differentiation the culture tends to slow down the division rate.

Hence, one possible reason for the differences in TLS extent and mutagenicity that I am seeing in stem and differentiated cells could simply be the differential cell cycle which means that the TLS-assay plasmids might for example undergo longer S-phase in the ES cells than in the differentiated cells, and thus be repaired differently. The known involvement of p53 in cell cycle regulation and the faster division rate of RA-differentiated p53 KO cells compared to the WT further prompted us to examine this possibility.

To this end, I performed a flow cytometry (FACS) analysis of DNA content using propidium iodide (PI) staining following a BrdU incubation so to better define the S-phase. The results of this analysis are presented in Figures 8-10 and Table 8.

In the WT-35 culture, the proportion of pluripotent cells that are at S phase is significantly larger and of those that are at G2 phase is smaller than the non-pluripotent WT-35 cells. The proportion of cells in the G1 phase is similar between the pluripotent and non-pluripotent (Fig. 8). Thus, in these cells, a higher TLS extent and higher mutagenicity correlate with a more cells overall undergoing S phase. However, in the p53 KO cells, while the proportion of mES cells that are at S phase is still larger than in the non-pluripotent cells, TLS is higher in the latter, with no significant change in in TLS mutagenicity. In addition, it was previously shown in our lab that TLS occurs in both the S and G2 phases, and in fact peaks at the G2 phase, where it becomes also more mutagenic<sup>85</sup>. Thus, taken together, the proportion of cells being present at the various cell cycle stages cannot account for all the TLS effects that we have observed.

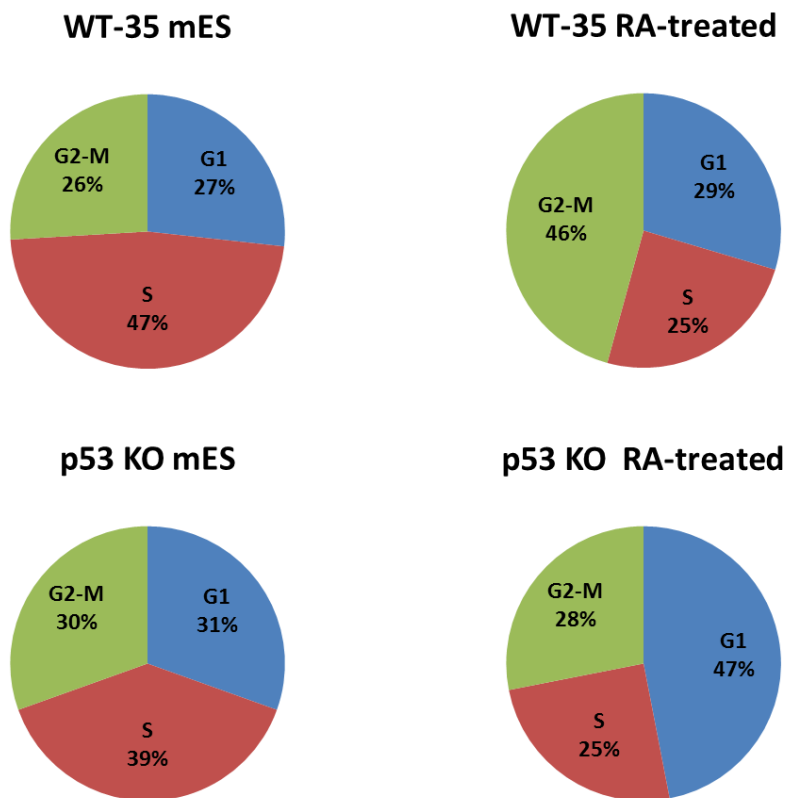


Figure 8. FACS cell cycle analysis of defined cell populations: WT-35: mES & RA-treated  
p53 KO: mES & RA-treated

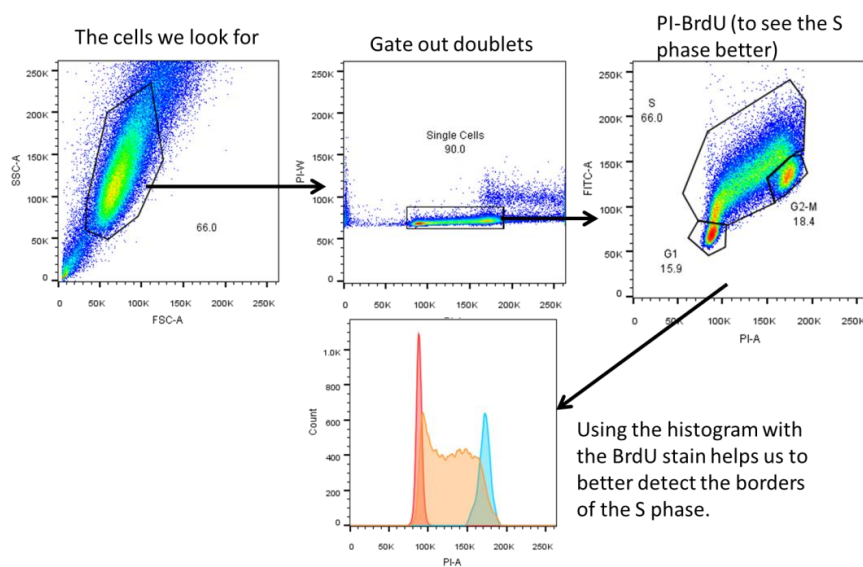


Figure 9. Gating setup for cell cycle analysis by FACS.

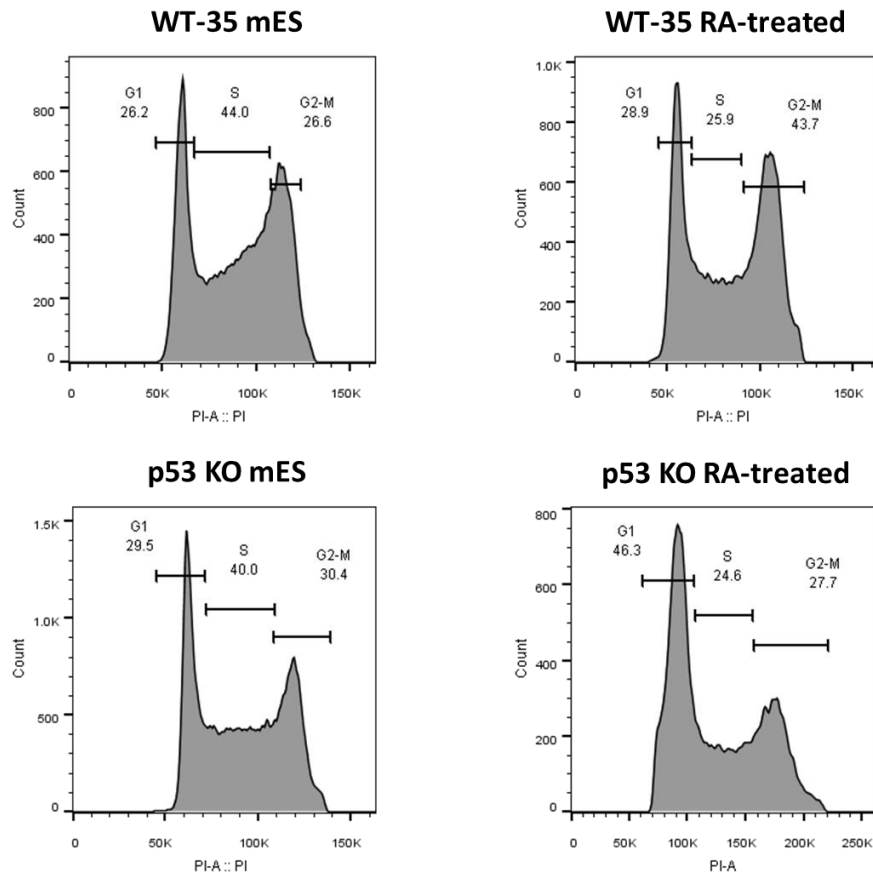


Figure 10. Histograms of PI uptake by the cells.

In addition to the proportion of the different cell cycle phases in the population which may greatly affect the outcome of TLS via over or under representation of phases in which TLS was shown to occur differentially, another crucial factor is the duration of each phase. This factor is somewhat inherently integrated in the proportion of phases as we would expect longer phases to be over represented and *vice-versa*. Nevertheless, the proportions map is affected by additional parameters such as the behavior of the various CDKs and more. To this end, I calculated the duration of each individual cell cycle phase of the various cell populations that I investigated, and the results are presented in Table 8. The calculations were done based on a set of 3 equations<sup>86</sup> that appear in the experimental methods section. Cell cycle Percentages were taken from Figure 8.

A quick inspection of Table 8 reveals that the proportions and the durations of the cell cycle phases are not linearly correlated, which accentuates the importance of this sort of analysis. As mentioned above, TLS takes place primarily in S & G2 phases and is mostly mutagenic in G2. Therefore, we would expect greater extent and mutagenicity of TLS in those populations with longer S & G2 phases. *Vise-versa*, we'd expect smaller extent and

mutagenicity rates in populations with longer G1. In contrast to that, this data in Table 8 does not correlate with greater TLS and mutagenicity rates in WT mES over differentiated cells, and the opposite picture for p53 KO cells which I showed at the beginning of this work. Taken together, these results suggest that the variations in cell cycle phases cannot solely explain the differences in the extent and mutagenicity of TLS.

If we are to find in the future a way to tweak these properties of TLS so to alleviate the mutagenic burden in cells growing in culture and most importantly in iPS cells, then this data presented herein is encouraging in that it can be done without disrupting the basic division cycle of the cells.

Table 8. Cell cycle analysis of WT and p53 KO mES and RA-treated cells

Duration of cell-cycle phases as measured by FACS								
		doubling time	G1 phase		S phase		G2-M phase	
		Tc	FG1	G1 duration	FS	S duration	FG2-M	G2-M duration
WT	mES	10	27%	2.1	47%	4.6	26%	3.3
	Differentiated	24	29%	5.4	25%	5.5	46%	13.1
p53 KO	mES	10	31%	2.4	39%	3.8	30%	3.8
	Differentiated	24	47%	9.3	25%	6.2	28%	8.5

Numbers represent time in hours (Tc & G1, S, G2-M duration), percentages represent the fraction of the population that is in this cell cycle phase (FG1, FS, FG2-M).

## UV-C Survival of mES and RA-treated cells

Following the results described above which focused p53 at the center of my interest as a TLS-effector candidate, and given its role as a major component in cell survival and its down-regulation of Nanog in mES upon extensive DNA damage<sup>52</sup>, it was obvious that a comparison of cell survival is needed to be done following exposure to genotoxic stress. Thus, I tested the viability of the four cell populations (WT, KO, mES, differentiated) 24 hours post UV-C irradiation (254nm). Viability was measured as a function of ATP concentration using CellTiter-Glo kit. The viability assay results are presented in Figure 11.

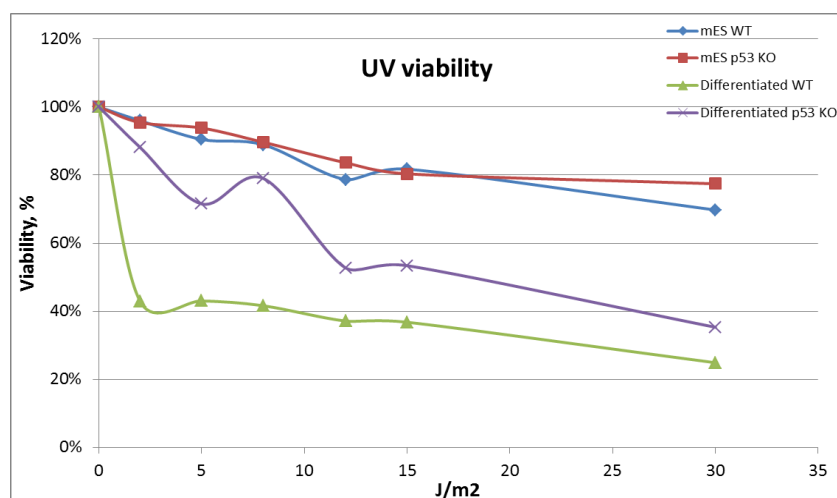


Figure 11. Cell viability following UV-C irradiation (254nm). The tested cell types and conditions are presented in the Figure. The experiment was repeated 3 times in triplicates.

WT and p53 KO mES cells exhibited very similar viability, which is consistent with an attenuated activity of p53 in mES cells, as discussed above. In contrast, upon RA-treatment the p53 KO cells became slightly more resistant to UV. Interestingly, for both WT and p53 KO cells, the differentiation treatment caused UV sensitization, which differs from the current view that ES cells are more sensitive to DNA damaging agents compared to differentiated cells<sup>62,66,67</sup>. Interestingly, the higher UV viability of mES cells correlates with the higher TLS compared to the differentiated cells.



### PCNA Ubiquitination in mES versus differentiated cells

One of the events that are at the heart of DNA damage tolerance is the ubiquitination of PCNA. The current dogma is that PCNA poly-ubiquitination is a signal for the error-free branch of DNA damage tolerance (template switch or HDR), while mono-ubiquitination signals for the error-prone branch, namely TLS. Monoubiquitination is a critical step in TLS, mediating between stalled PCNA and TLS polymerases containing a UBD (ubiquitin binding domain) that substitute the replicative polymerase at the time of need<sup>36,87</sup>.

Monoubiquitination occurs on Lys164 of PCNA, and is carried out by a reaction of two enzymes: Rad6 & Rad18 (E2 and E3 ligases respectively). Additionally, it was previously reported by our lab that this monoubiquitinated PCNA (mUb-PCNA) increases TLS, but is not essential for it. Indeed, experiments using cells with a mutant Arg164 PCNA, which cannot undergo monoubiquitination, showed that TLS does occur, but with reduced activity and altered mutagenicity<sup>36</sup>. Moreover, previous work from our group also showed that p53 is required for efficient monoubiquitination of PCNA<sup>47</sup>. I examined this ubiquitination in response to UV-C irradiation in WT mES & RA-differentiated cells, as well as the mutant p53 KO mES & RA-differentiated cells. Figure 12 shows the monoubiquitination of PCNA in these cells (254nm) after irradiation at 20-50 J/m<sup>2</sup>.

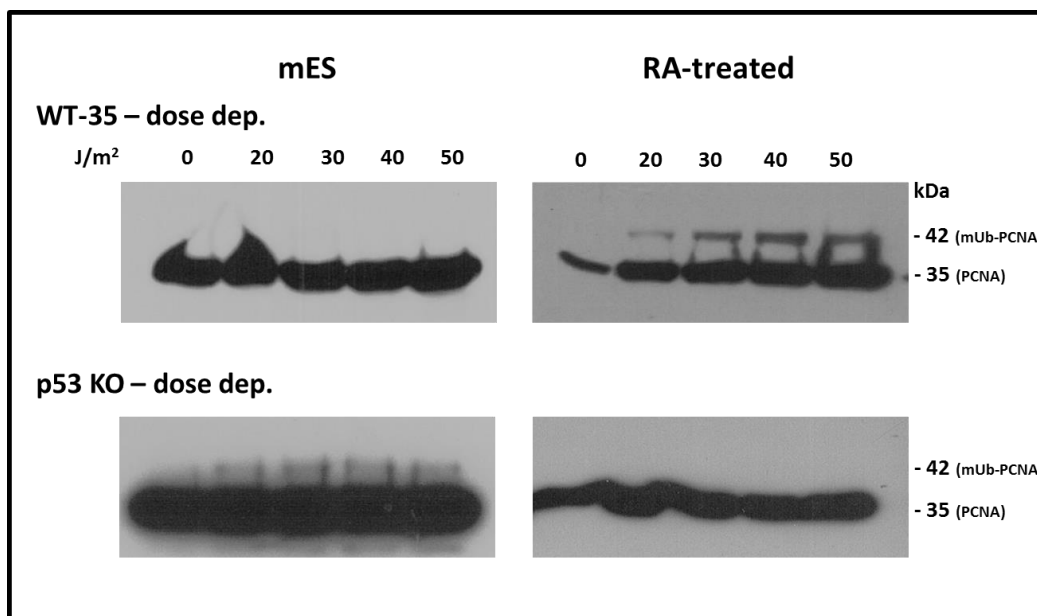


Figure 12. PCNA mono-ubiquitination in mES and RA-treated cells. WT or p53 KO cells (mES or RA-treated) were UV irradiated at the indicated doses, and 4 hours later they were extracted using a triton based buffer. The triton insoluble fraction was later fractionated by SDS-PAGE and immunoblotted with anti-PCNA antibody.

Strikingly, monoubiquitination of PCNA was not detected after UV irradiation of WT mES (Fig. 12). This does not appear to be a technical problem as the RA-treated WT mES cells did show a clear dose dependent increase in mUb-PCNA (Fig. 12). This is surprising, because TLS was actually higher in the mES cells, as shown above. In contrast, monoubiquitination seems to be weak, if any, in the p53 KO cells both before and after RA-treatment (Fig. 12). Thus, a mUb-PCNA-independent TLS pathway may be responsible for the increased TLS in mES.

### **Characterization of free nucleotide Pools**

Perturbed size of DNA precursor pools has been long known to have a detrimental impact on genomic integrity<sup>88,89</sup>. For example, starvation for pyrimidine nucleotides was shown to cause DNA damage as the cell attempted to progress through S phase, which in turn leads to the phosphorylation and activation of p53 through the ATR–CHK1 pathway<sup>90</sup>. Furthermore, exhausted dNTP pools lead to the formation of multiple stalled replication forks and consequently to their collapse into genotoxic DSB's structures<sup>91</sup>. HPV-16, or in its more common name – the human papilloma virus activates the Rb-E2F pathway which is a regulator of S phase entry and thus regulates the cell proliferation. Aberrant activation of this pathway leads to the depletion of nucleotide pool which promotes genomic instability and cancer development<sup>92</sup>. The infected cells can be salvaged by the introduction of exogenic nucleosides into the cells. On the other hand, the expansion of dNTP pools in yeast and the yeast model of colon-cancer in humans was also shown to lead to an increased rate of mutations<sup>93–95</sup>.

Commonly, dNTP pools are quantified using HPLC<sup>96</sup>, but with the progression in recent years in mass-spectrometry of small molecules, quantification systems of HPLC followed by tandem MS devices are now the state of the art. Since this work describes primarily the difference in activity and mutagenicity of a DNA repair mechanism in different cell populations, quantifications of the available building blocks for this repair process is an important step in elucidating the mechanism causing the phenotype I am seeing. Table 9 presents measurements of the NTP's and dNTP's concentrations in the indicated cells. We observed a 2-3 fold increase in the dNTPs concentrations upon differentiation of WT mES cells, and a pronounced 10-20 fold increase in p53 KO cells. NTPs concentrations were also increased upon differentiation, but by a significantly higher magnitude. Thus, NTPs

concentrations increased 2.5-8.2 fold in WT mES cells, and 9.4-56 fold in p53 KO mES cells (Table 9). In general, these results do not appear to explain the increased TLS in mES cells compared to differentiated cells, because it is expected that higher dNTP concentrations will facilitate TLS (see Discussion section).

Table 9. Quantification of NTP's & dNTP's in 1 million cells using HPLC-MS/MS.

<b>dNTPs in 1 million cells extracts (pmol)</b>				
	<b>dATP</b>	<b>dCTP</b>	<b>dTTP</b>	<b>dGTP</b>
<b>WT mES</b>	4.1 ± 1.0	1.0 ± 0.2	16.1 ± 4.0	1.6 ± 0.2
<b>WT RA-treated</b>	11.2 ± 4.6	3.4 ± 0.6	31.2 ± 8.9	3.4 ± 0.6
<b>p53 KO mES</b>	2.4 ± 0.5	0.7 ± 0.2	15.0 ± 2.4	1.2 ± 0.2
<b>p53 KO RA-treated</b>	43.8 ± 10.2	15.7 ± 4.0	170.1 ± 52.6	17.5 ± 2.2

<b>NTPs in 1 million cells extracts (pmol)</b>				
	<b>ATP</b>	<b>CTP</b>	<b>UTP</b>	<b>GTP</b>
<b>WT mES</b>	775.7 ± 66.1	20.3 ± 2.5	28.8 ± 2.3	273.2 ± 46.7
<b>WT RA-treated</b>	5702.3 ± 1042.0	115.3 ± 11.0	236.0 ± 14.8	675.6 ± 25.8
<b>p53 KO mES</b>	1033.9 ± 126.9	17.7 ± 0.7	14.7 ± 3.3	191.8 ± 30.9
<b>p53 KO RA-treated</b>	18630 ± 4022.8	434.8 ± 66.1	824.8 ± 93.8	1795.6 ± 259.2

## **Changes in the landscape of DNA repair and TLS proteins in mES cells upon differentiation**

### **Analysis of gene expression**

In an effort to map significant changes between ES and RA-treated cells in components of DNA damage tolerance, we measured their gene expression by Real-Time PCR (qPCR) using comparative  $C_T$  method<sup>77</sup>. The results are presented in Table 10.

From studying Table 10, firstly it is clear that the cells are responsive to either of the differentiation treatments. In RA-differentiations, *Rarb2* rises high as the cells are exposed to RA and differentiated, and *Oct4* and *Nanog* strongly decrease. In primed differentiation, *Oct4* does not change since the cells are still in the stem state and could in principle even be reverted all the way back to naïve state. In contrary, *Nanog* does decrease as expected, that is in agreement with the literature<sup>97</sup>.

When considering the genes involved in TLS, it appears that in mES WT cells there is a modest increase of 2-4 fold in expression upon RA-treatment. This effect was observed for both Bruce4 and WT-35, and included *Rev3L* and *Rev7*, the two key subunits of DNA polymerase  $\zeta$ , *Rev1*, which is a TLS scaffold protein, and *Ube2A* and *Ube2B*, the two Rad6 homologs, which are E2 ubiquitin-conjugating enzymes involved in TLS (Table 10). This is unexpected, because TLS was lower in RA-treated cells compared to mES cells. The TLS DNA polymerases  $\eta$  and  $\kappa$ , and the Rad18 E3 ligase that ubiquitinates PCNA (along with *Ube2A* and *Ube2B*) show different behavior in the two WT mES cells, excluding *pol1*, which shows no change upon RA-treatment, and might therefore not have a general significance. The expression patterns in the p53 KO mES cells show a mixed response upon RA-treatment compared to the WT mES cells, some respond similarly to the WT mES cells (e.g., *Rev1*, *Ube2A*, *Ube2B*), whereas others show an opposite response (e.g., *Rev3*, *Rev7*).

Table 10.

qPCR: RNA fold change in treated Vs. non-treated cells					
Gene	Bruce4		WT-35	p53 KO	Role
	Primed/Naïve	RA/mES	RA/mES	RA/mES	
Oct4	NSG	↓ 21	↓ 15	↓ 70	pluripotency markers
Nanog	↓ 7	↓ 7	↓ 26	↓ 999	
Klf2	↓ 3	–	–	–	Naïve pluripotency markers
Klf4	↓ 2	–	–	–	
FGF5	↑ 33	–	–	–	differentiation marker
RaRb2	–	↑ 221	↑ 194	↑ 353	RA receptor
Sox1	–	↑ 2	NSG	↑ 21	Neuroectoderm markers
Pax6	–	↑ 3	↑ 20	↑ 3	
Nestin	↑ 2	↑ 7	↑ 17	↓ 2	Neuronal stem cells marker
Pol η	NSG	↑ 2	NSG	↓ 2	TLS core polymerases
Pol ι	↓ 2	NSG	NSG	↓ 6	
Pol κ	↑ 2	NSG	↑ 6	↑ 2	
UBE2A	↑ 2	↑ 2	↑ 2	↑ 2	Ub-E2 Rad6 isoforms
UBE2B	↑ 3	↑ 3	↑ 3	↑ 6	
Rad18	NSG	↑ 4	NSG	↓ 2	E3 ligase
Rev1	↑ 2	↑ 2	↑ 4	↑ 3	TLS core protein
Rev3	NSG	↑ 4	↑ 2	↓ 5	polymerase ζ
Rev7	↓ 2	↑ 2	↑ 3	↓ 2	
ATM	NSG	NSG	NSG	↓ 4	DNA repair kinases
ATR	NSG	NSG	NSG	↓ 5	

\* NSG – No Significant Change

## **Proteomic mass spectrometry analysis of mES and differentiated cells proteome**

Thus far, the reported results for the higher activity and expression levels of most key proteins participating in DNA-repair mechanisms in stem & differentiated cells are based on low-throughput methods such as Western-blot analysis and qPCR. But, as this seems to be a much more global and systemic rather than local phenomenon, there is a growing need to globally look at the cell's DNA-repair machinery from a bird's-eye view. Several reports have described in the past the proteomic analysis of ES cells or differentiated cells, but no one to date has yet to perform a full proteomic comparison between the two types of cells, let alone, a full proteome comparison between mES progenitors and their differentiated progeny. Thus, it was very interesting to study the differential expression levels of the cellular proteome, with the aim of identifying the main DNA-repair pathways and to elucidate the mechanism governing the reported high repair capacity and high TLS and mutagenicity in stem cells compared to differentiated cells.

To this end we have performed a full proteome comparison analysis of stem and differentiated-progeny cells, of both- whole cell & nuclear fraction proteins.

The analysis was iterated 4 times for the whole cell, and twice for the nuclear proteome.

Table 11 presents a comparison of key proteins of the canonical DNA-repair mechanisms. The MS analysis did detect proteins belonging to essentially all known DNA repair pathways, although not all proteins involved in these pathways were detected. Yet the data presented in Table 11 does provide some general insights into the differences that occur in the DNA repair pathways upon differentiation by RA-treatment. Unfortunately, no TLS DNA polymerase was detected. However, upon differentiation there was a decrease in the nuclear fraction of Rad6A and the overall amount of Rad18, proteins needed for monoubiquitination of PCNA, which is required for effective TLS. This is consistent with the lower TLS in RA-treated cells, although it should be pointed out that TLS in mES cells might be Ub-PCNA-independent, as discussed above. The amount of Usp1, a negative regulator of TLS decreased, which is inconsistent with the decreased TLS, but Usp1 is involved in process other than TLS, e.g. double-strand break repair. Interestingly, the MS analysis detected 7 out of 17 new TLS genes identified in our lab by a siRNA screen and functional validation<sup>40</sup>. One of these, Npm1 (nucleophosmin) is a chaperon required for TLS, and its lower amount in nuclei of RA-treated cells may explain, at least in part, the lower TLS.

Table 11. Mass spectrometry results of selected DNA-repair key identified proteins. “TLS related proteins” are those identified in the work of Ziv *et al.*<sup>40</sup>.

Pathway	Protein name	Protein Description	Change ratio of differentiated / mES	
			Whole cell	Nucleus
HDR	SHPRH	E3 ubiquitin-protein ligase SHPRH		0.24
Direct Damage Reversal	MGMT	Methylated-DNA--protein-cysteine methyltransferase	2.5	0.27
SSBR	TDP1	Tyrosyl-DNA phosphodiesterase 1	0.4	0.74
	PARP1	Poly (ADP-ribose) polymerase family, member 1		0.52
	PARP2	Poly [ADP-ribose] polymerase 2		0.06
NHEJ	KU70	X-ray repair cross-complementing protein 6 (Fragment)	0.3	0.36
	KU80	X-ray repair cross-complementing protein 5	1.6	0.54
	MRE11A	Double-strand break repair protein MRE11A	0.7	0.36
		DNA repair protein RAD50 (Fragment)	0.5	
	RAD50	DNA repair protein RAD50	0.8	0.56
		Isoform 2 of DNA repair protein RAD50	1.1	
		Isoform 3 of DNA repair protein RAD50	0.6	
	XRCC4	Isoform 2 of DNA repair protein XRCC4	4.0	
MMR	MLH1	DNA mismatch repair protein Mlh1	0.6	
		DNA ligase 1	0.6	0.14
	LIG1	DNA ligase 1	1.6	
		DNA ligase 1 (Fragment)	0.6	
		DNA ligase 1	0.3	
	MSH2	DNA mismatch repair protein Msh2	0.7	0.34
	MSH6	DNA mismatch repair protein Msh6	0.5	0.09
	MSH3	DNA mismatch repair protein Msh3	0.4	

HR	RAD51	DNA repair protein RAD51 homolog 1	0.9	0.95
		DNA repair protein RAD51 homolog 1	0.4	
	BRCA1	Breast cancer type 1 susceptibility protein homolog	0.3	0.10
	RECQL4	ATP-dependent DNA helicase Q4	0.5	
		Isoform 2 of ATP-dependent DNA helicase Q4	1.4	
	RAD50	DNA repair protein RAD50		0.56
	RAD54L	DNA repair and recombination protein RAD54-like		0.24
	BLM	Bloom syndrome protein homolog	0.3	
DDR	ATM	Serine-protein kinase ATM	0.8	
		Serine-protein kinase ATM (Fragment)	0.9	
	MDC1	Mediator of DNA damage checkpoint protein 1	0.8	0.38
		Mediator of DNA damage checkpoint protein 1	0.2	
	CHEK2	Serine/threonine-protein kinase Chk2	0.6	0.51
	CHEK1	Serine/threonine-protein kinase Chk1	1.2	0.18
		Serine/threonine-protein kinase Chk1	0.4	
	TP53BP1	Isoform 2 of Tumor suppressor p53-binding protein 1	1.0	
		Isoform 3 of Tumor suppressor p53-binding protein 1	1.0	
	ATR	Latrophilin-1		0.11
TLS related proteins	FANCI	Fanconi anemia group I protein homolog	0.2	
	RUVBL2	RuvB-like 2	0.6	0.92
	NPM1	Nucleophosmin	0.4	0.29
		Nucleophosmin	0.6	
	RPN1	Glycosyltransferase subunit 1	2.3	0.64
	TRIP11	Protein Trip11	0.9	1.67
		Protein Trip11 (Fragment)	1.2	
	VCPIP1	Deubiquitinating protein VCIP135	1.1	
		Isoform 2 of Deubiquitinating protein VCIP135	0.7	
	MCM3	DNA replication licensing factor MCM3	1.2	1.05
	UBE2G2	Ubiquitin-conjugating enzyme E2 G2 (Fragment)	25.0	



TLS	RAD6A	Ubiquitin-conjugating enzyme E2 A	0.9	0.05
	RAD6B	Ubiquitin-conjugating enzyme E2 B	2.6	
	RAD18	E3 ubiquitin-protein ligase RAD18 (Fragment)	0.3	0.69
	USP1	Ubiquitin carboxyl-terminal hydrolase 1		0.42
BER	XRCC1	DNA repair protein XRCC1	1.0	0.52
	UNG	Uracil-DNA glycosylase	0.4	0.27
	APEX1	DNA-(apurinic or apyrimidinic site) lyase	0.7	0.66
		DNA-(apurinic or apyrimidinic site) lyase (Fragment)	1.0	
	FEN1	Flap endonuclease 1	0.7	1.04
	SMUG1	Single-strand selective monofunctional uracil DNA glycosylase		3.73
NER	XAB2	Pre-mRNA-splicing factor SYF1 (Fragment)	1.6	
		Pre-mRNA-splicing factor SYF1	1.0	0.41
	RAD23B	UV excision repair protein RAD23 homolog B	1.2	2.46
	DDB1	DNA damage-binding protein 1	1.5	1.68
	CCNH	Cyclin-H	0.7	0.21
		Cyclin-H (Fragment)	1.1	
	ERCC5	DNA repair protein-complementing XP-G cells homolog	3.6	5.88
		DNA repair protein complementing XP-G cells homolog	0.8	
	GTF2H1	General transcription factor IIH subunit 1	0.8	0.64
	GTF2H5	General transcription factor IIH subunit 5	0.7	
	ERCC2	TFIIF basal transcription factor complex helicase XPD subunit		0.23
	ERCC3	TFIIF basal transcription factor complex helicase XPB subunit	0.7	0.88
	ERCC6	DNA excision repair protein ERCC-6-like	0.7	0.27
	ERCC4	DNA repair endonuclease XPF		0.02
	GTF2H2	General transcription factor IIH subunit 2	1.2	
	XPA	Digestive organ expansion factor homolog		0.74
	XPC	DNA repair protein complementing XP-C cells homolog		0.59
	ERCC1	DNA excision repair protein ERCC-1		0.51

Of note, given the putative number of ~25,000 genes in a mouse genome<sup>98</sup>, in this proteome analysis an impressive number of 7363 individual identifications were identified (although a fraction of these are putative, fragments or isoforms of proteins) in the whole cell analysis alone, and an additional number of proteins in the nucleus.

## Discussion

My work on TLS in embryonic stem cells was initiated in order to examine how stem cells process DNA lesions that are only substrates for TLS and no other repair mechanism. Given the nature of TLS that can lead to a mutagenic outcome, the answer to this is important in two aspects: 1. As the body of the mature organism is all made of cells that originate from the embryonic stem cells, and given that the number of these ES cells is rather small within the inner cell mass of the blastocyst (20-40 cells), any genetic aberration in the DNA of these ES cells will be present in large portions of the adult organism. This brings about a possible beneficial effect as random mutation is one of the basic principles of evolution, but in greater mutation frequencies it is most likely to compromise the genomic integrity which may lead to the onset of disease and congenital abnormalities. 2. While from the embryology point of view ES cells exist only for a short time, in today's labs ES cells are being maintained *ex-vivo* in tissue culture for time durations ranging from days to years under conditions that support their self-renewal and the expansion of the culture. Additionally, induced pluripotent stem cells are not only stem cells in the sense of culture maintenance and expansion, but also have undergone a long road of sheer cellular stress in order to reach this iPS state, and therefore harbor yet a greater mutational potential. And while in the petri dish it is important to a certain degree that the culture will be free of genomic stress, in the developing field of regenerative medicine and organ transplantation it is imperative that the genomic integrity of the cells will be kept intact to the utmost degree.

The extent of TLS and the outcome of its activity in stem cells were unknown before the initiation of this work, and being inherently mutagenic by nature, it was not clear if TLS operates in stem cells at all. In this work, I show that not only TLS can be active and that the necessary machinery is present in mES, but also that it is in fact even more active and most surprisingly also more mutagenic than in the stem cells' - differentiated progeny cells. This difference is underscored by the finding that TLS in primed mES cells, which start their differentiation path, appears to be higher than in naïve mES cells, yet lower than in RA-differentiated cells. The higher TLS in mES cells compared to differentiated cells was shown in two independently derived mES cell lines, suggesting that it is not cell line specific. It was also observed using two independent differentiation methods, namely RA-treatment and spontaneous differentiation, indicating that it is not specific to a particular differentiation pathway.

Several approaches were taken in an attempt to elucidate the mechanism underlying the higher and more mutagenic TLS in mES cells. These included: (1) analysis of the cell cycle and (2) the free nucleotide pools, which might affect TLS, (3) UV survival, (4) targeted expression analysis of TLS and related genes, and (5) comprehensive proteome analysis. In addition, we analyzed (6) the possible involvement of p53, previously shown in our lab to modulate TLS, and (7) monoubiquitination of PCNA, a key event in TLS.

Our results indicate the following:

- (1) Cell cycle difference cannot solely explain the differences in TLS.
- (2) All 4 dNTPs and 4 NTPs increase in amount/cell upon RA-differentiation, however, this increase cannot explain the TLS changes observed.
- (3) Analysis of the mRNA expression of 11 TLS and related genes did not show any explanatory correlation.
- (4) Proteome analyses revealed that generally there was an increase in DNA repair proteins from all DNA repair pathways, consistent with previous reports for some of them. Unfortunately, TLS DNA polymerases were not detected, but some TLS regulatory proteins were identified. Of note – We observed that upon RA-induced differentiation, the amount of nucleophosmin (Npm1) decreased 3.4-fold in the nuclear protein extract, and 2-fold in the whole cell protein extract. This protein was recently identified in our lab as a positive regulator of TLS.
- (5) mES cells were more resistant to UV light than RA-treated cells, consistent with the higher amount of repair proteins in mES cells, and in disagreement with previous reports.
- (6) The p53 protein was required for the high level of TLS observed in mES cells. In p53 KO mES cells, the extent of TLS was similar to differentiated WT mES cells.
- (7) When differentiated WT mES cells were compared to differentiated p53 KO cells – TLS in the latter was higher, similar to the result previously obtained in our lab with MEF.
- (8) Preliminary results show that UV irradiation of mES cells did not cause PCNA ubiquitination, while after induction of differentiation a clear UV-dose dependent PCNA ubiquitination was observed. This is an unexpected result, because the canonical TLS pathway requires PCNA ubiquitination. Thus, it is possible that in mES cells TLS operates via a mechanism that does not require PCNA ubiquitination, however exploring this exciting possibility will require additional experimentation.

## UV sensitivity

The findings presented above suggest that cell survival post UV-C irradiation was higher in mES cells than in differentiated cells, which is inconsistent with the notion that mES cells prefer death over mutagenic-compromised survival<sup>53,62,68</sup>. In WT differentiated and mES cells, UV-induced DNA lesions are repaired mainly by the NER mechanism. Several reports, including my own work of proteomic quantification presented herein report the up-regulation of NER proteins<sup>62,68</sup> in ES cells relative to differentiated cells. Furthermore, this work herein is to my knowledge the first to compare UV-survival of pluripotent cells to primary cells (achieved by RA-differentiated progeny of the stem cells) and not to immortalized or cancerous cell lines such as MEF or HeLa cells respectively<sup>99</sup>. A key difference in the assays performed here compared to the previously reported assays is that we present viability assays performed 24 hours post-irradiation, whereas previous studies used the colony formation assay that detects the outcome after about 3 weeks, which may account for the different outcomes. Interestingly, the UV viability of WT mES and p53 KO mES cells was similar, which can be explained by the fact that p53 is localized mostly in the cytoplasm<sup>100</sup>. This result is in striking contrast to the work of Han Qin *et al.*<sup>101</sup> on hES cells, showing that a reduction of 15-fold in p53 expression level using siRNA was sufficient to cause the elimination of only ~30% of the culture compared to ~70% of the WT culture after UV irradiation of 20 J/m<sup>2</sup>, through the induction of apoptosis by p53-caspase 9 pathway. Interestingly, Li Z. Luo *et al.*<sup>102</sup> have found less DNA damage induced by UV-C and ROS in human pluripotent cells than in fibroblasts. Additionally, they found that global genome (GG-NER) and transcription coupled-nucleotide excision repair (TC-NER) pathways of UV-C-induced damage are faster in pluripotent than in non-pluripotent cells. In conclusion, it remains to be established whether the differences in the UV survival is due to the difference in the assays, or represents other differences such as the exact state of pluripotency.

## The p53 protein and PCNA ubiquitination

Upon the encounter of the replication complex with a DNA lesion it needs to signal and summon the relevant proteins for the repair. A critical step in this process is the ubiquitination of PCNA Lys164 which is known to undergo polyubiquitination as a signal for error-free repair, or the conjugation to a single molecule of ubiquitin as a signal of error-prone DNA repair. This monoubiquitination was previously reported to be important yet not essential for repair activity<sup>36</sup>. Additionally, p53 was shown to take part in this monoubiquitination process<sup>47</sup> through an interaction with p21 and the facilitation of a switch

from the replicative to a TLS polymerase. Combined with the mentioned above regarding p53 being by large present in the cytoplasm of stem cells and shuttles to the nucleus upon differentiation, it was therefore interesting to test the involvement of p53 in this observation of high TLS in mES cells. The finding that UV irradiation caused PCNA (mUb-PCNA) in differentiated cells, but not in mES cells, was surprising because TLS was higher in mES cells than in differentiated cells. It was previously shown in our lab that p53 is required for efficient monoubiquitination of PCNA, although it is not absolutely required. This can explain the lack of mUb-PCNA in UV-irradiated p53 KO cells, either in the mES or differentiated state – although here the dependence on p53 is stronger than previously observed in human cells. As for the WT cells, it could be that following DNA damage p53 is not yet in the nucleus and thus cannot induce mUb-PCNA, while in the differentiated state it had already shuttled to the nucleus and thus can in fact induce the mUb-PCNA. As mentioned earlier, other signaling pathways also contribute to the mobilization and activation of TLS proteins to the lesion site in addition to mUb-PCNA<sup>36</sup>, and thus it might be that these pathways are more pronounced in those differentiation states where PCNA monoubiquitination is below detection level but TLS is in fact high.

## Cell Cycle

As the cell prepares to divide it replicates its genome. This means that while in other stages of the cell cycle it might be tolerable that the quality of DNA had been compromised especially in non-transcribing parts of the genome, at this stage of DNA replication the cell cannot allow any unwanted obstacles that might hinder the replication and therefore TLS, which serves as the last resort before replication fork arrest and ultimately – its collapse, must be “on the watch” and ready to react. Thus, it is no surprise that TLS was recently found to operate mainly in S-G2 phases<sup>85</sup>.

Because of that, the effect that I am seeing of high TLS and mutagenicity in WT mES compared to differentiated cells could be simply a matter of a more rapid cell cycle, regardless of the opposite TLS phenotype in p53 KO cells. The TLS experiments that I performed were for the duration of 18 hours from transfection until harvesting, and during this time the mES cells had undergone through roughly 2 cell cycles contrary to the roughly 1 cell cycle of the differentiated cells. Therefore, I compared the proportion of the different cell cycle phases in the un-synchronized population of WT & p53 KO, mES & differentiated cells by FACS, using PI to determine the quantity of the DNA in the cell which is indicative of the

cell cycle, and BrdU to better detect the cells that are in the S phase and thus had incorporated it into their DNA during replication.

The results of the cell cycle analysis indicate that the profile of the WT and the p53 KO is similar, with ~30% at G1, ~43% at S phase and ~30% at G2-M phase on mES cell cycle<sup>103–107</sup>. Expectedly, after differentiation the profile of cell cycle changed and the WT cells exhibit greater proportion as well as longer G2-M at the expense of a reduction in these two parameters of the S phase, while the p53 KO cells exhibit increased duration and proportion of the G1 over a reduction at the S-phase.

Having started profiling the propensity of cell cycle phases in my cells in order to find if indeed this could explain the results of the TLS experiments I report herein, I found that this does not correlate in any way with the results of the TLS extent and mutagenicity in mES and differentiated cells. While the cell cycle profile of mES WT & KO is practically identical in respect to the two cell cycle phases, the results of the TLS show rather an identical profile between the KO mES and differentiated WT. Furthermore, the change in TLS that the WT cells present before and after differentiation is identical but inverted to the one of the KO cells. This also stands in contrast to the cell cycle profile of the cells as the differentiated KO cells are the only ones of the 4 populations that exhibits a greater G1-phase propensity on the expense of S-phase which means that based on the work of Diamant *et al*<sup>85</sup>, showing that TLS operates in S & G2-phases, this should lessen the extent of TLS and not enhance it to the levels of WT mES. Additionally mutagenicity-wise, while the WT cells show high mutagenicity of mES which lowers after differentiation, the KO cells show a rate of mutagenicity that is somewhat in between, and that also does not correlate in any way with the cells' cell cycle profile.

Thus in conclusion, the cell cycle analysis shows a profile that is rather expected at least in respect to the WT cells, and that the profiles of the WT and the KO cells cannot explain the high TLS in WT mES & differentiated KO and lower TLS in WT differentiated & KO mES. Neither can it explain the mutagenicity signature of any comparison between these 4 cell populations. Therefore, the results of the TLS in this work are not as a result of cell cycle differences between the 4 populations of the cells.

## Proteins & mRNA quantification

In my work presented here I am measuring the changes in DNA repair between mES and differentiated cells. Apart from these changes it's been long known that during differentiation the cells undergo a whole transformation to a completely different cell type. In this process the machinery of the cell undergoes major changes of differential expression levels, restart and & shutoff of different genes. Thus, I quantified the changes in the whole proteome of mouse pluripotent stem cells differentiating to NPCs by retinoic acid. Since unfortunately none of the TLS DNA polymerases was detected in this proteomic analysis (presumably due to a very low abundance), I performed a quantification of the mRNA level of these polymerases and other selected TLS proteins using qPCR, which is more sensitive than mass spectrometry, considering of course that it quantifies RNA levels which do not always stand in precise correlation with protein levels.

Both in Bruce4 and WT-35, most of the TLS genes detected show only a modest increase as a response to differentiation (Table 10). As mentioned in the results section, the up-regulation of Rad6 homolog genes detected by qPCR is unexpected as they encode the E2 ubiquitin-conjugating enzymes involved in TLS, which work along with Rad18 to form mUb-PCNA, and TLS was found to be lower in differentiated cells where these Rad6 homologs were found to be higher. Rad18 on the other hand shows differential expression only in Bruce4 mES, but not in WT-35 mES. Also, the expression pattern in the p53 KO cells showed no distinctive trend, which may correlate with the high TLS in the differentiated cells but equal mutagenicity between mES and differentiated cells.

As for other pathways quantified by mass spectrometry, in general it appears that the amount of DNA repair proteins is higher in mES compared with RA-treated cells (Table 11). This includes mismatch repair (MMR), with a decrease in the nuclear amount of Msh2 (about 3-fold) and Msh6 (about 11-fold) in RA-treated cells, consistent with a report using a functional mismatch repair assay to compare mES and fully differentiated cells<sup>65</sup>. Similarly, I observed a decrease in homologous recombination (HR) repair proteins, noticeable in the critical Brca1 protein, which was reduced in the nuclear extract 10-fold upon RA-treatment, consistent with the reported higher HR observed in mES cells compared to MEF<sup>108</sup>. In that study it was reported that non-homologous end joining proteins (NHEJ) is higher in MEF compared to mES cells. Our results show moderate decrease in several NHEJ proteins, including Ku70, Ku80, Mre11A and Rad50. However, the rate limiting XRCC4 protein, which forms a complex with DNA ligase 4 and is essential of ligation of the DSB, exhibited a 4-fold increase in the whole cell extract. As for nucleotide excision repair (NER), the biggest

effect is a 50-fold decrease in XPF nuclease (ERCC4), and many other NER proteins decrease as well. Base excision repair (BER) proteins are generally slightly reduced, consistent with functional BER assays that we have conducted (see appendix), and with reports in the literature<sup>62,65</sup>. The interesting exception is the enzyme SMUG1, which was 3.7-fold higher in RA-treated cells compared to mES cells. This enzyme removes the oxidative lesion 5-hydroxymethyl uracil from DNA, and may also be involved in epigenetic reprogramming, which might be the reason for its increase upon differentiation.

### **Free nucleotide pool**

Up until recently, NMR was the analytical method of choice, superseding GC/MS during the 90's. With the invention and development of the electrospray ionization mass spectrometry (ESI-MS) in the 80's it gradually became the predominant method for detection of small molecules and metabolites<sup>109</sup>. Unsurprisingly, most of the quantifications of nucleotide pools were done solely by liquid chromatography (HPLC) and only in very recent years the preference has been shifting towards HPLC followed by ESI-MS/MS (LC-MS/MS)<sup>110</sup>.

As mentioned in the results, the free nucleotides pool size is tightly regulated<sup>111</sup> since too small or too large pools can induce mutagenesis and chromosomal aberration<sup>88-95</sup>. Thus, imbalanced pools could potentially lead to the phenotype of TLS in the cells at the scope of this work<sup>112</sup>. To this end I performed a quantification of free nucleotides, both NTPs & dNTPs.

There appears a 2-3 fold increase in the dNTPs concentrations upon differentiation of either WT or p53 KO cells (Table 9), and yet a greater increase in NTPs concentrations. This may be explained by the larger volume of RA-differentiated cells, compared to mES cells. Also, dNTPs & NTPs concentrations seem to be similar when comparing the same state of the cells (i.e. ES or differentiated). For BP-G and cisPt-GG, accurate TLS involves insertion of dCMP opposite the lesions, whereas mutagenic TLS in these cases involves usually insertion of dAMP. Thus, higher dCTP concentrations may drive higher TLS, whereas higher dATP to dCTP ratios may facilitate mutagenicity of TLS. This does not correlate with what is actually seen, because dNTPs concentrations were higher in the differentiated cells whereas TLS was lower. The dATP:dCTP ratio was 4.1 in mES cells, and only 3.3 in RA-treated cells (Table 12), which is consistent with the lower mutagenicity in RA-treated cells. However, it is questionable whether such a small difference in the average dNTPs concentrations can account for the observed effect, particularly since in p53 KO RA-treated cells have a



mutagenic TLS similar to WT mES cells, but the dATP:dCTP ratio is in fact lower (2.8 compared to 4.1, Table 12). Thus, it appears that changes in the free dNTP pools are unlikely to be the main explanation for the changes observed in the mutagenicity of TLS. As for the insertion of dAMP opposite the abasic site under all conditions examined, this is consistent with previous results showing that essentially all DNA polymerases tend to insert dAMP opposite an abasic site (the so-called A-rule<sup>113</sup>). This correlates with the FACS analysis of cell cycle (Fig. 8 & Table 8) that shows a shared propensity and a very similar duration of S-phase between the two differentiation states.

Table 12.

<b>dNTP pmol concentrations relative to dCTP in mES and RA-differentiated cells</b>				
	<b>dATP</b>	<b>dCTP</b>	<b>dUTP</b>	<b>dGTP</b>
<b>WT mES</b>	4.3	1.0	16.8	1.7
<b>WT RA-treated</b>	3.3	1.0	9.1	1.0
<b>p53 KO mES</b>	3.5	1.0	22.0	1.8
<b>p53 KO RA-treated</b>	2.8	1.0	10.8	1.1

To summarize the main findings of this work, pluripotency of cells is accompanied by high TLS and its outcome mutagenesis rates. As the cell exits pluripotency, the extents of TLS and mutagenicity lower. This process is also accompanied by PCNA monoubiquitination as a response to UV-C irradiation. p53 appears to be a major player in this phenotype as cells lacking it present an opposite pattern of TLS, mutagenesis and also PCNA monoubiquitination. In addition to activity levels of TLS, other components of the cell's barrier against DNA lesions are also over expressed in mES compared to differentiated cells.

With the fascinating new therapeutic field that was opened by the findings of Yamanaka as for how to establish a defined iPS population<sup>114</sup>, the sky's the limit in respect to regenerative medicine. But, this might be a double-edged sword, with the therapeutic potential at one edge and the results I bring here in my work on the other. With prolonged *in vitro* culturing of cells being an integral part of this regenerative field, while present in stem cell-like state the cells harbor a much greater mutagenic potential which obviously can lead to a neoplastic outcome<sup>115–117</sup>.

## **Appendix**

### **Base excision repair in mES**

As discussed in the introduction, in recent years there is ever growing evidence that DNA repair in stem cells is much more active than in differentiated cell. And so, in addition to TLS activity, it was interesting to see the activity of base excision repair enzymes, namely OGG1 (8-oxoguanine DNA glycosylase 1) APE1 (AP endonuclease 1) and MPG (methylpurine DNA glycosylase) which act on oxidative and methylation DNA damage. To do so, we used an assay developed in our lab that measures the activity of these three enzymes in mammalian cells. The way this assay is performed in short is: protein extracts of the cells are prepared and incubated with a fragment of DNA containing the DNA lesion that acts as a substrate for the enzymatic activity. Given that these enzymes are DNA glycosylases, the output of the assay, after alkali treatment, is the cleavage of the DNA fragment where the lesion is present and thus shorter DNA fragments are formed. This assay was performed on mES and RA-differentiated cells as a comparison between the two. The results are presented in Figure 14.

In agreement with previous reports (discussed in the Introduction), the activity of each of the BER DNA glycosylases that were assayed, MPG, OGG1 and APE1, was reduced upon differentiation. This further supports the growing evidence that DNA repair mechanisms are much more active in ES than in differentiated cells<sup>65</sup>. This higher level of these enzyme activities provide an additional protection barrier against the accumulation of oxidative DNA damage, with the first line of defense being the Warburg effect which reduced the formation of ROS in ES cells due to much more pronounced glycolysis over mitochondrial ATP production.

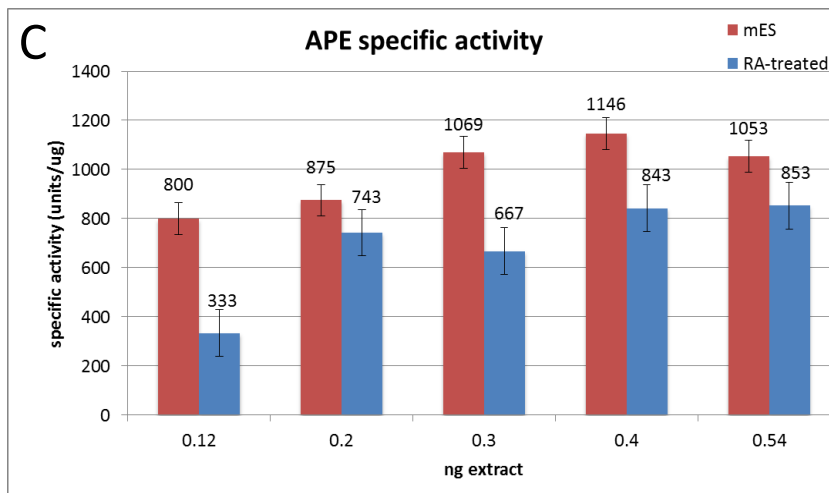
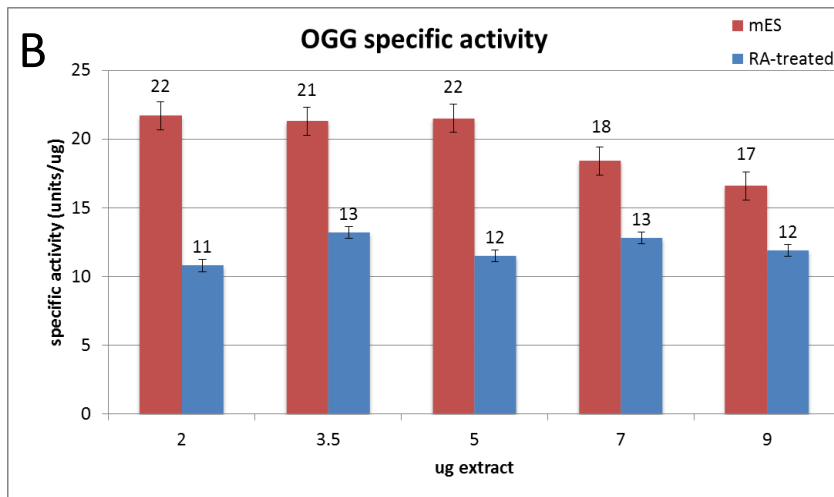
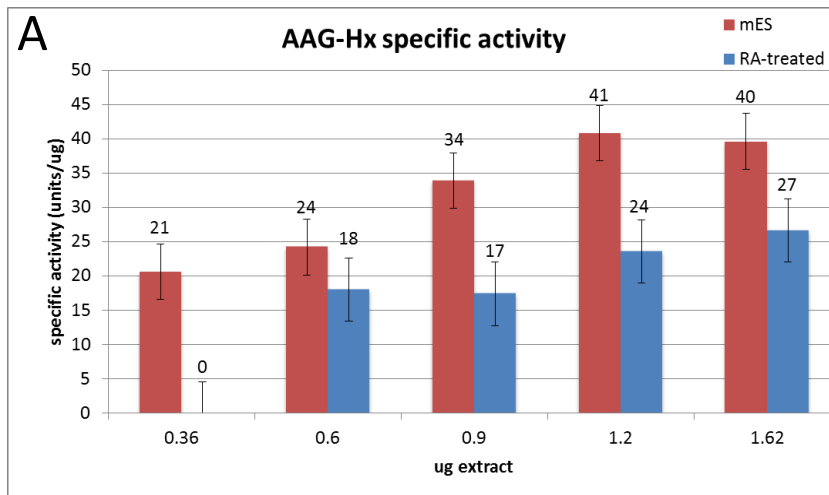


Figure 13. Specific activity of three BER enzymes (MPG, OGG, APE) between RA-differentiated and mES cells. The activity of all three enzymes is greatly reduced upon RA-differentiation.

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**This work is dedicated to Sagi Barzilai & Dalia Elinger, two dear and true friends,  
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