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TARGETING DNA REPAIR PATHWAYS FOR CANCER THERAPY

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Cover: collaborative work of MTH1 inhibited by TH588 (in the front cover), OGG1 and MUTYH (in the back cover) to repair toxic DNA lesions, shown in red.

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Targeting DNA Repair Pathways for Cancer Therapy

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“All praise is for Allah for all his praiseworthy acts, for all his favors and blessings....His praise is evident through his generosity, whose bestowal stretches out liberally. His treasures never decrease, rather the frequency of his giving increases.” (Duaa Al-Iftitah)

*To Imam Mahdi,
my Parents and Family*

ABSTRACT

Accumulation of genomic mutations is the consequence of failure in DNA repair as well as increased exposure to endogenous/environmental mutagens. DNA repair pathways safeguard the human genome from such mutagens, and thereby suppress the multi-step process of carcinogenesis. DNA repair pathways that protect the genome from ROS (reactive oxygen species)-induced lesions are attractive anti-cancer targets, as their inhibition may render combinatorial sensitization of tumor cells to both DNA damage and oxidative stresses, known as non-oncogenic addictions of cancer. The aim of this thesis was to validate such DNA repair factors as anti-cancer targets and to develop their inhibitors for potential therapeutic applications.

In paper I, we assessed the addiction of cancer cells to MTH1, a nudix hydrolase eliminating oxidized purine nucleotides from the dNTP pool. MTH1 depletion resulted in exclusive accumulation of 8-oxo-dG lesions and cellular toxicity in transformed cells. *MTH1* suppression, impaired tumor growth in the xenografts of SW480 cells. We developed potent MTH1 inhibitors (TH278 and TH588), which exhibited target engagement and selective toxicity in transformed cells. Treatment with MTH1 inhibitors caused increased 8-oxo-dG levels in cancer cells, and inhibited the growth of xenografts *in vivo*. Taken together, our findings revealed the dependency of tumors to MTH1 that can be targeted for cancer therapy.

The study in paper II aimed to explore functional cooperation between MTH1 and MUTYH, a DNA glycosylase that removes deoxyadenines paired with 8-oxo-dG. Using stable cell lines expressing inducible shRNA constructs, we showed that combined depletion of MTH1 and MUTYH was more toxic to cells compared to individual knock-downs. In addition, overexpression of nuclear MUTYH could attenuate cell death induced by loss of MTH1. Collectively, this study provided supportive evidence for a protective role of MUTYH.

In paper III, we described TH5487 as a novel selective inhibitor of OGG1, a DNA glycosylase that excises 8-oxo-dG opposite deoxycytidine. TH5487 inhibited binding of OGG1 to its substrate and increased thermal stability of the purified protein through interactions with residues in the active site. Moreover, TH5487 engaged with its intended target, increased 8-oxo-dG level, and impaired recruitment of OGG1 to the damage site in cells. Treatment with TH5487 resulted in prolonged S phase, which was similar to the effect of OGG1 depletion using shRNAs. In addition, non-transformed cells could tolerate TH5487 treatment while cancer cells were more sensitive. In sum, this study highlighted the phenotypic lethality of OGG1 inhibition with tumors, by introducing TH5487 as a cell-active OGG1 inhibitor.

Overall, our results increased the knowledge about dependency of cancer cells to DNA repair pathways of ROS-induced lesions that can be employed for the development of promising anti-tumor therapies.

LIST OF SCIENTIFIC PAPERS

- I. Helge Gad*, Tobias Koolmeister*, Ann-Sofie Jemth*, **Saeed Eshtad***, Sylvain A. Jacques*, Cecilia E. Strom*, Linda M. Svensson, Niklas Schultz, Thomas Lundback, Berglind Osk Einarsdottir, Aljona Saleh, Camilla Gokturk, Pawel Baranczewski, Richard Svensson, Ronnie P.-A. Berntsson, Robert Gustafsson, Kia Stromberg, Kumar Sanjiv, Marie-Caroline Jacques-Cordonnier, Matthieu Desroses, Anna-Lena Gustavsson, Roger Olofsson, Fredrik Johansson, Evert J. Homan, Olga Loseva, Lars Brautigam, Lars Johansson, Andreas Hoglund, Anna Hagenkort, Therese Pham, Mikael Altun, Fabienne Z. Gaugaz, Svante Vikingsson, Bastiaan Evers, Martin Henriksson Karl S. A. Vallin, Olov A. Wallner, Lars G. J. Hammarstrom, Elisee Wiita, Ingrid Almlof, Christina Kalderen, Hanna Axelsson, Tatjana Djureinovic, Jordi Carreras Puigvert, Maria Haggblad, Fredrik Jeppsson, Ulf Martens, Cecilia Lundin¹, Bo Lundgren, Ingrid Granelli, Annika Jenmalm Jensen, Per Artursson, Jonas A. Nilsson, Pal Stenmark, Martin Scobie, Ulrika Warpman Berglund and Thomas Helleday. (2014) MTH1 Inhibition Eradicates Cancer by Preventing Sanitation of The dNTP Pool. *Nature* 508(7495):215-21 (* these authors contributed equally to this work.)
- II. **Saeed Eshtad**, Zahra Mavajian, Sean G. Rudd, Torkild Visnes, Johan Boström, Mikael Altun, and Thomas Helleday. (2016) hMYH and hMTH1 Cooperate for Survival in Mismatch Repair Defective T-cell Acute Lymphoblastic Leukemia. *Oncogenesis* 5(e275), doi:10.1038/oncsis.2016.72
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Paper not included in the thesis:

Thomas Helleday, **Saeed Eshtad**, and Serena Nik-Zainal. (2014) Mechanisms underlying mutational signatures in human cancers. *Nature Reviews Genetics* 15(9): 585-598.

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LIST OF ABBREVIATIONS

8-oxo-dG	8-oxo-2'-deoxyguanosine
APEX1	DNA-(apurinic or apyrimidinic site) lyase
BER	base excision repair
BRCA1	breast cancer type 1 susceptibility protein
CETSA	cellular thermal shift assay
DDR	DNA damage response
DKO	double knock out
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	deoxynucleoside triphosphate
DSBs	double strand breaks
DSF	differential scanning fluorimetry
EMSA	electrophoresis mobility shift assay
FAP	familial adenomatous polyposis
FC	flow cytometry
GG-NER	global genome NER
H/D	hydrogen/deuterium
HR	homologous recombination
IF	immune-florescent
IR	ionizing radiation
ITC	isothermal titration calorimetry
KD	knock-down
KO	knock-out
LP-BER	long-patch BER
MAP	MUTYH-associated polyposis
MGMT	O ⁶ -methylguanine DNA methyl transferase
MMEJ	micro-homology mediated end-joining
MMR	mismatch repair
MTH1	MutT Homolog 1
MUTYH	MutY homolog
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
NOA	non-oncogene addictions
OA	oncogene addictions
OGG1	8-oxoguanine DNA glycosylase 1
PARP1	poly (ADP-ribose) polymerase-1
POLs	(DNA) polymerases
RNA Pol II	RNA polymerase II
ROS	reactive oxygen species

RT	radiotherapy
shRNA	short-hairpin RNA
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SP-BER	short-patch BER
SSBs	single strand breaks
TC-NER	transcription-coupled NER
TLS	translesion synthesis
TMZ	temozolomide
UV	ultraviolet
WB	Western blotting
WT	wild type

1 INTRODUCTION

1.1 DNA Damage

It takes many years for cancer to develop in the human body through a multistep process in which mutations in genomic DNA are considered as an underlying cause [1-3]. Human cells gain growth advantage by acquiring certain driver mutations that result in oncogenic transformation and are positively selected during tumorigenesis. In contrast, passenger mutations do not necessarily bring survival benefits to cells but happened to exist in an ancestor of the cancer cells [3]. Spontaneous mutations are the outcome of multiple mutational processes that include exogenous/endogenous DNA damages and insufficient DNA repair, generating a pattern of mutations on the genome, termed mutational signatures [4].

1.1.1 Endogenous Mutagens

Endogenous and environmental mutagens render DNA damage which can be remained in the genome unless they get repaired (Figure 1) [5]. Spontaneous depurination/depyrimidination reactions frequently occur at a daily rate of 10^4 bases per cell [6, 7]. Natural deamination of 5-methylcytosine at CpG sites often results in mutations observed in various cancers [8]. Conversion of cytosine to uracil, reported in several cancers, is a deamination reaction mediated by the enzymes termed AICDA (activation induced cytosine deaminase) and APOBEC (Apolipoprotein B mRNA editing enzyme catalytic polypeptide) [9, 10]. On top of these, genomic DNA is subjected to constant attack by endogenous free radicals including ROS (reactive oxygen species) and RNS (reactive nitrogen species). Cellular aerobic respiration, programmed cell death and inflammatory responses contribute to ROS generation as a by-product [11]. Exposure of DNA to such reactive species create more than 30 different oxidized lesions [12]. Among all, 8-oxodG (8-oxo-2'-deoxyguanosine) is thought to be the most studied oxidized lesion which is able to pair with dA leading to G:C>>T:A mutation [13-15].

1.1.2 Exogenous Mutagens

In addition, DNA is vulnerable to exogenous mutagens of both physical and chemical types. UV (Ultraviolet) radiation can covalently link two adjacent pyrimidine nucleotides creating (6-4) PPs (6-4 pyrimidine photoproducts) and CPDs (cyclobutane pyrimidine dimers) [16, 17]. Accordingly, TT.AA>>CC.GG transversions have been reported in cancers originated from tissues exposed to UV [18]. On the other hand, ionizing radiation (IR) not only triggers ROS generation in cells by ionization of water molecules but also increases ROS release from biological sources in irradiated cells. Moreover, IR causes direct DNA damage by inducing formation of SSBs (single-strand breaks) as well as DSBs (double-strand breaks) [19-22].

Chemical carcinogens can induce mutations by either intercalation (*e.g.* proflavine and ethidium bromide) or covalent attachment to DNA [23]. For instance, TMZ (temozolomide), used for malignant glioma treatment, is an alkylating agent that mainly induces O6meG (O6-methylguanine) formation, leading to C.G >>T.A transitions [24-26]. The tobacco mutagen benzo[a]pyrene undergoes epoxidation reactions by cytochromes ultimately producing an extremely reactive electrophilic carcinogen which causes G.C>>T.A transversions. Thus, tobacco smoking can increase the risk of several human cancers by escalating somatic mutation rates [27-30]. Overall, the prevention of mutations by DNA repair pathways highlights their pivotal role to avoid tumor development (Figure 2).

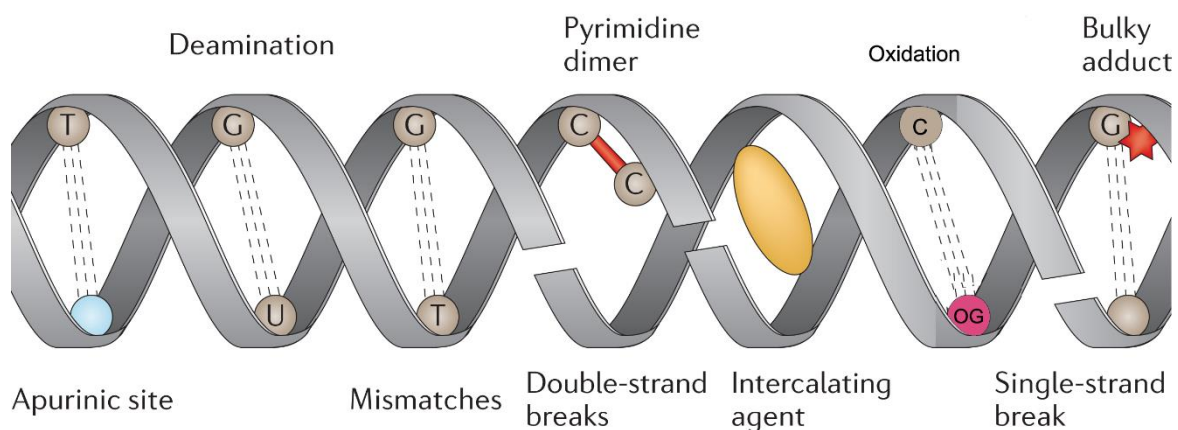


Figure 1: Different types of DNA damage. Endogenous and exogenous DNA damaging agents constantly attack DNA, modifying the genome in different ways. Exogenous sources of DNA damage include depurination, deamination, and oxidation. Environmental mutagens such as chemical agents, ionizing radiation, and UV can induce various DNA damages. OG: 8-oxo-dG. See text for details. Figure adapted from reference [4], printed with permission from Nature Publishing Group.

1.2 Pathways that Safeguard Genomic Integrity

1.2.1 Direct Repair and Mismatch Repair

Human cells have evolved various pathways to prevent accumulation of DNA damage introduced by either endogenous or environmental carcinogens. For instance, O⁶meG generated by TMZ can be directly repaired by MGMT (O⁶-methylguanine DNA methyl transferase), which transfers the methyl group to its own cysteine residue [31]. *MGMT* promoter hyper-methylation is now used as a predictive marker for survival in glioblastoma [32]. In a more complex manner, MMR (mismatch repair) factors scan DNA immediately after replication to detect incorrect nucleotides incorporated into the nascent strand. Using MMR, cells can reduce the errors of replicative DNA POLs (polymerases) by 100 times to 1 in 10⁻⁹ bases replicated [33]. Therefore, a significant rise in the spontaneous mutation rate occurs in MMR defective cells [34-36]. In MMR, the misincorporated nucleotide is recognized by MutS alpha or MutS beta which consist of MSH2-MSH6 and MSH2-MSH3 heterodimers, respectively. The heterodimer of MLH1-PMS2 is then recruited to the site. The endonuclease PMS2 makes a nick near the incorrect nucleotide introducing new entry points for the exonuclease EXO1. The strand with mismatch is then degraded by EXO1. Finally, the gap left in DNA is filled by Pol δ [37]. Failed MMR results in C>T mutation at NpCpG sequences (Signature 6) which largely contributes to substitutions and small indels (insertion/deletions), known as microsatellite instability. In addition, defects in MMR are considered as a very early somatic event in colorectal tumorigenesis [18, 38-40].

1.2.2 Base Excision Repair

Simple base modifications can also be corrected by BER (base excision repair) which facilitates elimination and replacement of a single base residue. BER has various substrates including DNA lesions caused by ROS, methylation, deamination and hydrolytic reactions [41]. A damaged base, for example 8-oxo-dG paired opposite dC (8-oxo-dG:dC) is detected and excised by a specific DNA glycosylase; OGG1 (8-oxoguanine DNA glycosylase). Hydrolysis of the *N*-glycosylic bond leaves an AP (apurinic/apyrimidinic) site, which is then incised by DNA APEX1 (apurinic or apyrimidinic site) lyase. DNA polymerase β (Pol β) can then remove the 5'-dRP (deoxyribosephosphate) moiety, as it possesses the dRP lyase activity. Pol β also incorporates a nucleotide in the resulting gap. Finally, the DNA ligase III-XRCC1 complex establishes a phosphodiester bond to fix the new nucleotide in DNA (extensively reviewed in [41-43]). Often, the dRP moiety may itself be damaged and resistant to removal by Pol β . Here, replicative polymerases in complex with PCNA can be recruited to insert several nucleotides and remove the offending dRP-moiety by strand-displacement synthesis (long patch BER). Although none of the mutational signatures are associated with BER failure, an increase in mutation rate and predisposition to cancer has been reported as a result of defective BER [4, 44]. For instance, unrepaired 8-oxo-dG:dC can lead to a G:C >> T:A mutation which is

avoided by OGG1 activity [45]. Furthermore, loss of OGG1 function predisposes to lung cancer [46-52].

1.2.3 Nucleotide Excision Repair

NER (nucleotide excision repair) can remove various DNA lesions, as it detects the bulky DNA lesions that distort the DNA helix. Substrates include bulky adducts caused by benzo[a]pyrene and UV radiation (that is, CPDs and (6-4) PPs), among others (reviewed in [53, 54]). Substrate recognition by NER is achieved through either GG-NER (global genome NER) or TC-NER (transcription-coupled NER). In GG-NER, the XPC (Xeroderma pigmentosum group C-complementing protein)-RAD23B complex constantly evaluates DNA integrity for various helix-distorting adducts, and UV-DDB (UV radiation-DNA damage-binding protein) facilitates the detection of lesions [53, 55-57]. On the other hand, in TC-NER, RNA Pol II (RNA polymerase II) stalls when it collides with a bulky lesion, indirectly facilitating DNA damage recognition. The DNA lesion then becomes accessible for repair when the CSB (Cockayne syndrome protein CSB)-CSA (Cockayne syndrome WD repeat protein CSA) complex binds to the halted RNA Pol II [53, 58-60]. After DNA lesion detection in both sub-pathways, the TFIIH (transcription initiation factor IIH) complex, which includes XPA, XPB, and XPD, is recruited to the bulky damage. One incision is then created by the XPF-ERCC1 endonuclease 5' to the lesion and another cut is made by XPG 3' to the damage, releasing a fragment of around 30 nucleotides [53]. The gap filling step is carried out by Pol δ , Pol ϵ , or Pol κ using the complementary strand as a template; and finally DNA ligases completes the NER process by ligating the newly synthesized fragment into the continuous strand [53, 61-65]. Nonfunctional GG-NER causes accumulation of adducts in the genome. The error-prone POLs may bypass such lesions to ensure cell survival that comes at the cost of higher mutation rate [53]. Therefore, patients with the xeroderma pigmentosum disorder, who carry mutations in GG-NER, are highly susceptible to UV-induced skin and mucous membrane cancers [66]. In addition, defective TC-NER is associated with mutational signature 7 and signature 4, observed predominantly in UV-induced skin cancer and tobacco smoking induced lung cancer, respectively. As a result, both signatures show strong transcriptional strand-bias [4, 18, 27].

1.2.4 Non-homologous End-joining Repair

Although IR is able to create DSBs, most of the endogenous DSBs in cells are generated by collision of DNA replication forks with unrepaired lesions, that results in replication fork collapse [67, 68]. DSBs are principally handled by two different repair pathways: NHEJ (non-homologous end-joining) and HR (homologous recombination). In NHEJ, DNA ends at DSBs are rapidly protected from exonucleases and held in close proximity by the Ku70-Ku80 heterodimer [69, 70]. End-joining is then mediated by involvement of DNA-PKcs (DNA-dependent protein kinase catalytic subunit), artemis and Pol λ [71, 72]. Finally, the

Ligase IV-XRCC4 complex ligates the damaged DNA strands [73-75]. The activity of NHEJ is independent of the cell cycle phases whereas HR only takes place after DNA replication as it requires sister chromatids as repair templates. Contrary to general expectations, NHEJ appears as the first choice for DSBs repair in G₂-phase cells, and is found to be an accurate process due to limited end-processing and fast kinetics [76-78]. However, if the DSB yields ends that cannot be ligated directly by NHEJ, especially in the G₁ phase where HR is not an option, re-joining will be mediated by microhomology. This process involves extensive end-trimming that results in a slower error-prone pathway called MMEJ (micro-homology mediated end-joining) [79-83]. During immunoglobulin gene rearrangement in B-cells, for generation of primary antibody repertoire, as well as T-cell receptor generation, DSB formation is programmed under physiological conditions which are repaired by MMEJ. Here, the re-joining process is mediated by RAG1 (V(D)J recombination-activating protein 1) and RAG2 [84-86]. Diversification of the primary antibody repertoire by immunoglobulin class switching is also facilitated through MMEJ initiated by AICDA [87]. Therefore, defects in NHEJ causes severe immunodeficiency associated with increased radiation sensitivity [88, 89].

1.2.5 Homologous Recombination Repair

Although HR is initiated by end-trimming like MMEJ, it is an error-free process where duplicated sister chromatids act as a template for DNA synthesis. During HR, the DSB is first detected by the MRN (MRE11-RAD50-NBS1) complex. End resection is then performed by DNA nucleases and helicase such as EXO1, CtIP (CtBP (C-terminal-binding protein)-interacting protein) and BLM (Bloom syndrome protein), resulting in formation of 3'-overhangs. RPA (replication protein A) protects the overhangs, and it is later displaced by RAD51. The replacement step is mediated by the BRCA1 (breast cancer type 1 susceptibility protein)-PALB2 (partner and localizer of BRCA2)-BRCA2 complex. RAD51 forms nucleoprotein filaments and invades the complementary DNA template. The invading strand primes new DNA synthesis and forms a structure named D-loop (displacement loop). After branch migration and DNA synthesis, resolution of Holliday junction intermediates (crossover recombinants) leads to accurate repair (extensively reviewed in [68, 90, 91]). Inherited predisposition to breast and ovarian cancer were found in females carrying mutations in *BRCA1* and *BRCA2* [92-94]. Moreover, inactivating germline mutations in *RAD51C*, which encodes RAD51, have been associated with concurrent breast and ovarian tumors [95]. Accordingly, signature 3, which includes large insertions and deletions, has been detected in breast and ovarian tumors with germline and somatic *BRCA1* and *BRCA2* mutations [18].

1.2.6 Replication Fidelity

Nucleotide selectivity and proofreading by replicative DNA POLs (*i.e.* Pol α , δ and ϵ), as well as post-replicative MMR are crucial factors determining the fidelity of replication [96]. Such DNA POLs are highly selective to ensure that new nucleotides are correctly incorporated into

the nascent strand and accurately paired with their complements on the template strand. However, DNA POLs do make errors with the rate of one in every 10,000 base pairs [97]. Considering the human genome size ($\sim 3 \times 10^9$ nucleotides), this small mutation rate can lead to 10^5 mistakes per division. However, replicative DNA POLs reduce the number of incorrectly incorporated nucleotides by approximately 100-fold using their 3'-exonuclease proofreading activity [98]. In addition, shortly after replication MMR removes the mismatches and improves replication fidelity by about 100- to 1,000-fold [96]. Thus, the mutation rate is estimated to be less than one error for every billion base pairs duplicated during DNA replication [99]. Active site mutation in Pol γ (Y955C) reduces the polymerase nucleotide selectivity by two-fold, increases mutations in mtDNA (mitochondrial DNA), and consequently causes progression of external ophthalmoplegia [100-102]. In addition, somatic and germline mutations in Pol ϵ impairing the proofreading activity, cause predisposition to colorectal and endometrial carcinomas with functional MMR [103-105]. These mutations in Pol ϵ are the underlying cause of the 'ultramutation' phenotype and associated with signature 10 [18, 106].

1.2.7 Preventive Repair

Other factors that contribute to replication fidelity are balance and purity of the intracellular dNTP (deoxynucleoside triphosphate) pool [107]. Oncogene activation (*e.g.* cyclin E overexpression) enforces cell proliferation that leads to insufficiency in dNTP levels. This, in turn, can cause DNA replication stress and genome instability in the early onset of tumorigenesis. Such DNA perturbations were shown to be prevented by exogenously supplying nucleosides or expressing c-myc, which is a TF (transcription factor) that increases nucleotide biosynthesis [108]. In addition, it has been shown that the dNTP pool is much more vulnerable to damage by modifying reagents than their counterparts in the DNA duplex [109-111]. Accordingly, impurities in the dNTP pool can cause replication errors and mutations. For example, slight amounts of 8-oxodGTP in the mitochondrial dNTP pools are sufficient to negatively affect the fidelity of Pol γ , leading to A:T >> C:G transversions [112]. Therefore, cells have evolved a preventive DNA repair mechanism by which sanitizing enzymes prevent misincorporation of noncanonical or damaged nucleotides into DNA [113]. For instance, ITPA (Inosine triphosphate pyrophosphatase) removes noncanonical purine nucleotides from the pool and contributes to the maintenance of genome stability [102, 114]. Moreover, MTH1 (MutT Homolog 1) displays strong 8-oxo-dGTPase activity which plays a significant role in sanitation of the dNTP pool from oxidized purine nucleotides [115]. MTH1 overexpression suppresses DNA damages induced by the oncogene H-RAS and significantly minimizes the spontaneous mutation rates in cells with malfunctioned MMR, indicating the role of MTH1 in mutation avoidance [116, 117].

1.2.8 Translesion Synthesis

If DNA damage remains unrepaired prior to DNA replication, which often causes replication fork collapse, they will be bypassed by TLS (translesion synthesis) POLs to enhance cell survival. TLS is mediated mainly by the low-fidelity Y- family POLs, including Pol η , Pol κ , Pol ι , and REV1, which lack exonuclease activity. In fact, TLS allows damage to be tolerated until they can be later removed by DNA repair system, thus allowing DNA replication to be completed. TLS is initiated by stalling of the replicative DNA POLs at DNA lesions, followed by recruitment of a TLS polymerase to bypass the damage by incorporating nucleotides opposite the lesion. The replication machinery then switches the TLS polymerase back to the error-free replicative POLs [118]. Accordingly, Pol η activity during somatic hypermutation has been associated with Signature 9 found in CLL (chronic lymphocytic leukemia) and malignant B-cell lymphomas [18, 119].

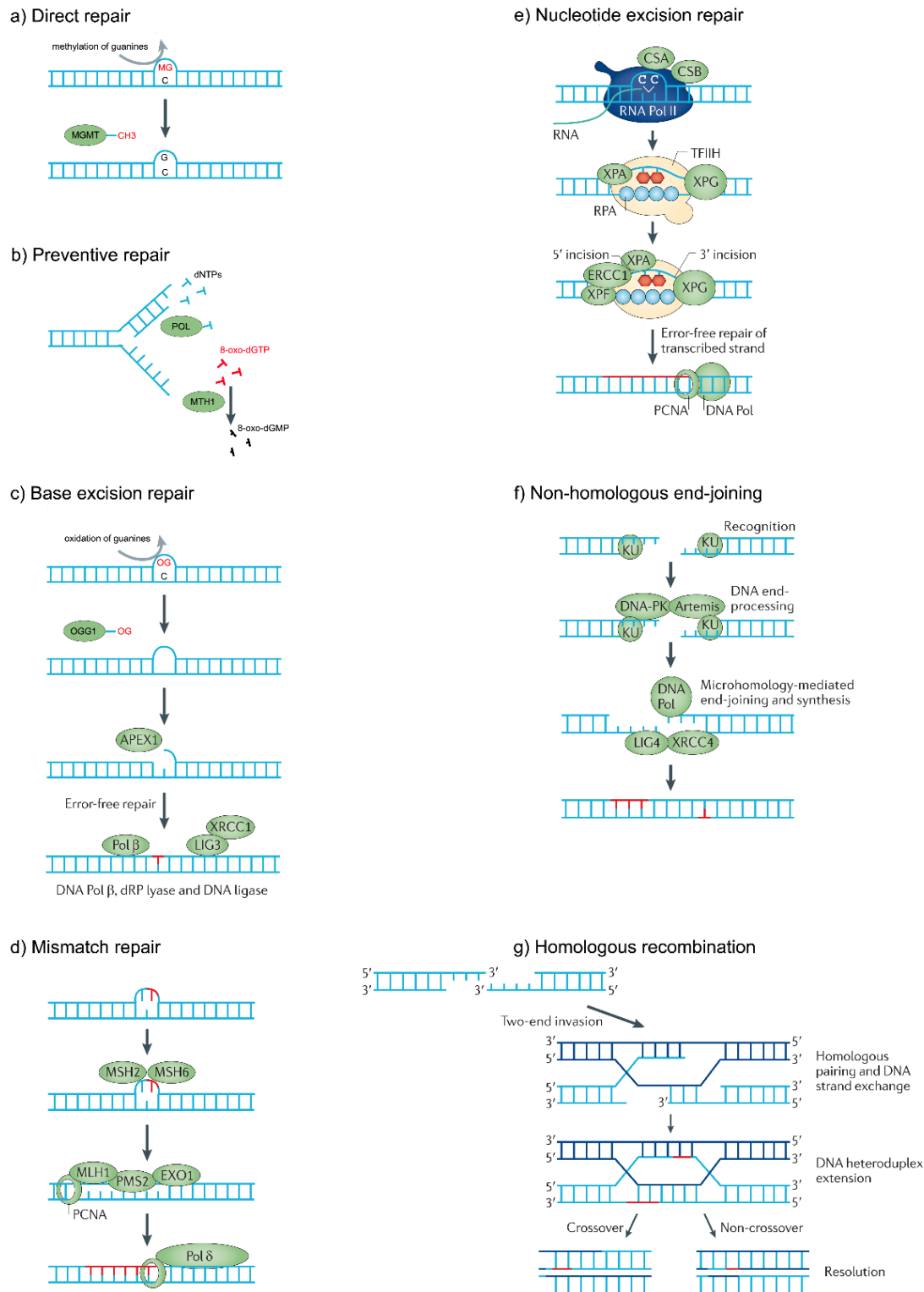


Figure 2: Pathways for maintenance of the genome integrity. a) Direct reversal of methylated lesions is carried out by direct repair pathway. b) Preventive repair does not permit incorporation of modified nucleotides into DNA. c) Base excision repair deals with simple base modifications. d) Mismatch repair recognizes and removes mispaired nucleotides. e) Bulky adducts and UV-induced lesions are fixed by nucleotide excision repair. f) No-homologues end-joining repairs double-strand breaks throughout the cell cycle. g) Homologous recombination uses the sister chromatids as template to repair double-strand breaks. OG: 8-oxo-dG, MG: O⁶mG. See text for details. Figure adapted from reference [4], printed with permission from Nature Publishing Group.

1.3 Repair of ROS Induced DNA Damage: Bringing 8-Oxo-dG into Focus

As discussed above, endogenous or exogenous ROS can induce a wide variety of damage to both DNA and its precursors, which are carcinogenic if not eliminated by repair pathways (reviewed extensively in [12]). The most prevalent and thoroughly examined oxidative DNA lesion is thought to be 8-oxo-dG which is estimated to reach 10^5 bases in genomic DNA per cell [13-15]. Perhaps, its abundance in the genome is due to the fact that guanine, compared to other bases, has the lowest oxidation potential which makes it the easiest nucleotide to get oxidized by ROS [13]. To restrict 8-oxo-dG accumulation in the genome, cells are principally armed with three complementary repair pathways for this simple base modification: preventive repair (*i.e.* MTH1), BER, and MMR (Figure 3).

1.3.1 Preventive repair: MTH1

MTH1, also known as nudix hydrolase 1 (NUDT1), is an antimutagenic enzyme that eliminates oxidized nucleotides from the dNTP pool by hydrolysis of 8-oxo-dGTP, 8-oxo-dATP and 2-OH-dATP [120, 121]. MTH1 belongs to the nudix (nucleoside diphosphate linked to another moiety x, NDP-X) hydrolase superfamily including a number of enzymes that catalyze the following reaction: $NDP-X \rightarrow NMP + P-X$. The nudix enzymes share a common 23-amino acid motif called the nudix box (GXXXXXXEXXXXXXREUXEEXGU), where U can be valine, leucine, or isoleucine, and X is any amino acid [122-125]. MTH1 activity prevents misincorporation of 8-oxo-dGTPs and other oxidized purine nucleotides into DNA, which would otherwise results in DNA damage and cellular senescence, as shown in human skin untransformed fibroblasts. This replicative senescence can be rescued when the cells are exposed to a low oxygen level [126]. Moreover, MTH1 activity appeared to be essential to avoid senescence, a critical step towards tumorigenesis, in RAS-transformed cells and its overexpression can prevent RAS associated DNA damage [116, 127, 128]. In line with these observations, Fouquerel *et al.* demonstrated that mis-insertion of 8-oxo-dGTP into telomere sequences upon MTH1 depletion leads to the premature stop of telomerase and induces death in cancer cells with shortened telomeres [129]. In addition, mice overexpressing human MTH1 exhibited increased longevity, owing to limited age-dependent accumulation of genomic 8-oxo-dG [130].

From another perspective, oxidative stress and DNA damage stress are considered to be non-oncogene addictions of cancer cells which may not drive tumorigenesis but are required for survival [131]. Thus, MTH1 is expected to play a vital role in cancer cells, mediating the adaptation to such persistent stress conditions. Indeed, lethality due to impaired MTH1 function as well as MTH1 overexpression in cancer cells has been reported in several independent studies. Loss of MTH1 activity has been shown to be toxic in various xenograft models using different shRNA (short-hairpin RNA) vectors [132-134]. *MTH1* suppression also prevented formation of tumor spheres and xenograft tumor growth of glioblastoma cells, where toxicity

could be enhanced by H₂O₂ treatment [135]. Using gRNA (guide RNA) and an artificial virus delivery system, the *MTH1* gene was disrupted leading to growth inhibition of subcutaneous xenograft tumors of SKOV3 cells [136]. Besides, comparison of healthy and tumors tissues revealed a significant correlation between MTH1 expression and disease progression in breast cancer [137, 138], colorectal cancer [139], gastric cancer [140], non-small cell lung carcinoma [141, 142], esophageal squamous cell carcinoma [143], renal-cell carcinoma [144], and multiple myeloma [145]. The MTH1 p26 isoform is a consequence of single nucleotide polymorphism (SNP) in exon 4, where the Val83 is replaced with Met83 [146, 147]. Although p26 has the same catalytic efficiency for oxidized nucleotides as other isoforms (p22, p21, and p18), it appeared to be less thermostable as well as less efficiently translocated into mitochondria [147, 148]. Consistent with these studies, Val83Met SNP in the *MTH1* gene was shown to significantly increase predisposition to small cell lung carcinoma (SCLC) [149].

1.3.2 *OGG1-mediated BER*

Misinsertion of 8-oxo-dGTP opposite dC, or direct oxidation of dG (deoxyguanine) in dG:dC base pairs, are mutagenic because in the next round of replication a dATP can be incorporated opposite 8-oxo-dG, causing G:C>>T:A transversions [150]. As mentioned earlier, BER-dependent removal of 8-oxo-dG:dC lesions is carried out by OGG1 [151, 152]. Both glycosylase activity and AP lyase activity are conducted by bifunctional glycosylases that can cleave the DNA strand after lesion excision without involvement of APEX1. At first, it was suggested that Asp268 was an essential residue responsible for the bifunctionality of human OGG1. In this model, Asp268 promotes the nucleophilic Lys249 to form a Schiff base enzyme-DNA covalent intermediate, which is then hydrolyzed with a water molecule to complete the β -elimination reaction [153, 154]. However, OGG1 was shown later to work in a monofunctional mode under physiological conditions. Based on this model, Asp268 initiates the catalysis whereas Lys249 was found to be indispensable for 8-oxo-dG recognition [155]. Consistent with this finding, OGG1 activity has been shown to significantly increase in the presence of APEX1; and the dRP lyase activity of Pol β appeared to be required for 8-oxo-dG:dC repair [156-158].

The human *OGG1* gene resides in chromosome 3p25 and encodes two main isoforms: α -OGG1 (Type 1a, 39 kDa) and β -OGG1 (Type 2a, 47 kDa) [151, 159, 160]. α -OGG1 is translocated to both mitochondria and nuclei whereas β -OGG1 is exclusively mitochondrial [160]. β -OGG1 (also named OGG1-2a) was shown to be catalytically inactive for the repair of 8-oxo-dG:dC lesions [161]. However, another isoform, OGG1-1b, has recently been reported to mediate BER-dependent repair of such lesions in mitochondria, similar to that of the nuclear α -OGG1 (OGG1-1a) isoform [162, 163]. During interphase, OGG1 is associated with chromatin and the nuclear matrix while it is co-localized to condensed chromosomes in mitosis [164]. Particularly during S-phase, OGG1 was found in nucleoli [165]. Following treatment with potassium bromate (KBrO₃) which induces 8-oxo-dG formation [146], OGG1 was preferentially recruited to euchromatic domains and co-localized with RNA polymerase II [166].

Enhanced expression of OGG1 in the nucleus and mitochondria protects DNA and consequently improves cell survival upon exposure to oxidative damage. However, over-expression of R229Q OGG1 mutant failed to exhibit such effects [167]. In fact, although R229Q mutation does not affect the catalytic activity of OGG1, it renders it thermolabile at physiological temperature [168]. In addition, mitochondrial targeted over-expression of α -OGG1 in the PyMT transgenic mouse model of mammary tumorigenesis resulted in decreased mtDNA damage, improved mitochondrial function, and attenuated breast cancer progression and lung metastasis [169]. Deletion at the *OGG1* locus or inactivating mutations, with the Ser326Cys polymorphism being the most frequent, have been associated with increased risk of cancer in lung squamous cells [46, 48-52], kidney [52, 170-172], oropharynx [173], esophagus [174], and stomach [175]. It has been found that the Ser326Cys OGG1 has almost equal catalytic activity to the WT (wild type) protein for 8-oxo-dG:dC excision [176-178]. However, this polymorphic OGG1 is excluded from the nucleoli where the WT protein preferentially resides during S-phase. In contrast to the WT OGG1, the Ser326Cys mutant protein displayed defective associations with chromatin as well as nuclear matrix. The disrupted localization of Ser326Cys OGG1 was suggested to be the consequence of altered phosphorylation at Ser326 [164, 165]. On the other hand, it has been suggested that oxidation of OGG1 at Cys326 can negatively affect its repair efficiency and causes dimerization of OGG1 which leads to anomalous DNA binding and lack of stimulation by APEX1 [179-181]. In addition, novel SNPs of *OGG1* in 5'-UTR (5'-untranslated regions) that reduce *OGG1* expression levels have been associated with breast cancer [182]. Consistent with this observation, another SNP in 3'-UTR, causing lower *OGG1* expression, has been linked to increased risk of cancer in the people with inherited *BRCA1/BRCA2* mutations, exhibiting shortened telomeres [183-185].

OGG1-dependent recognition of 8-oxo-dGs in promoter regions has been reported to promote transcription by several TFs such as Hif-1 (hypoxia inducible factor-1), MYC, NF- κ B, and estrogen receptor. [186-190]. Particularly in estrogen-induced transcription, demethylation of H3 lysine 9 at promoter sites is catalyzed by an epigenetic modulator enzyme called LSD1 (lysine-specific demethylase 1) [191, 192]. LSD1 activity results in H₂O₂ production that in turns results 8-oxo-dG formation in the surrounding DNA strands, recruiting OGG1 [190]. In a similar manner, LSD1-mediated recruitment of OGG1 has been found for Myc-dependent transcription [189]. Thus, OGG1 not only is required for repair of oxidized lesions but also exhibits an important role in ROS-associated transcriptional regulation (reviewed in [193]).

1.3.3 *MUTYH*-dependent BER

The replicative POLs can misincorporate dATP opposite the already existing 8-oxo-dG in template DNA, with *syn* conformation, to form dA:8-oxo-dG pairs [194, 195]. Such lesions, if left unrepaired, can lead to C:G>>A:T transversion mutations. Another DNA glycosylase called MUTYH (MutY homolog) initiates repair of dA:8-oxo-dG mispairs by monofunctional excision of the incorrectly paired adenine from the DNA strand. MUTYH is also known as

adenine DNA glycosylase and it has the capacity to remove both dA and 2-OH-dA when paired with dG or 8-oxo-dG [196-199]. In case of dA:8-oxo-dG mispairs, since MUTYH removes the undamaged base, the enzyme needs to distinguish the nascent strand from the template strand, otherwise activity of MUTYH on these lesions where dA is in the template strand can give rise to mutations [200]. In fact, MUTYH has been shown to possess a replication-associated function. Accordingly, MUTYH interacts with several proteins involved in DNA replication, co-localizes with PCNA (proliferating cell nuclear antigen), and is up-regulated during S-phase [201, 202]. In addition, MUTYH-dependent repair of dA:8-oxo-dG lesions was enhanced by DNA replication where expression of a mutant form of MUTYH lacking its PCNA binding motif could not increase the repair efficiency [203]. On the other hand, MUTYH-initiated SP-BER (short-patch BER) appeared to be futile since it only generates dA:8-oxo-dG mispairs [204]. Instead, it has been shown by several studies that MUTYH dependent repair includes a replication-coupled LP-BER (long-patch BER) pathway [202, 205, 206]. It has been demonstrated that Pol λ is involved in MUTYH-initiated LP-BER where it can accurately incorporate dCTP opposite 8-oxo-dG [207, 208]. RPA and PCNA collaboratively function as a molecular switch to activate Pol λ dependent LP-BER and suppress Pol β activity [209]. The 8-oxo-dG apposite the AP site, which is generated by MUTYH activity, can be recognized by OGG1. However, it was shown that murine Mutyh can inhibit Ogg1 in this context to prevent mutation [210]. In sum, MUTYH removes adenine from dA:8-oxo-dG mispairs, where dA is in the newly synthesized strand, and represses OGG1 activity. Then, the APEX1 makes an incision at the AP site. Afterwards, RPA and PCNA promotes Pol λ to incorporate dCTP and extend the primer by one additional nucleotide, while Pol β activity is inhibited. The one-nucleotide 5' overhang is then processed by FEN1 (flap endonuclease 1), and subsequently the DNA ligase I seals the nick. Thus, the MUTYH repair pathway creates substrates (*i.e.* dC:8-oxo-dG mispairs) for OGG1-mediated SP-BER where Pol β is involved [208].

The human *MUTYH* gene is mapped to chromosome 1p34.1 and encodes three main transcripts: α , β , and γ generated from three different exon 1 sequences. These transcripts generate at least 9 different isoforms of MUTYH protein among which type 1 and type 2 are the major mitochondrial (60 kDa) and nuclear (57 kDa) variants, respectively [196, 198, 211] [159, 201, 212-214]. Since the mitochondrial targeting sequence is located at the N-terminus of MUTYH, missense mutations in this region can disrupt the protein localization. Accordingly, Pro18Leu and Gly25Asp MUTYH variants have been associated with increased susceptibility to colorectal and gastric cancer [215, 216]. It has been shown that depletion of MUTYH sensitizes the cells to oxidative DNA damage [217]. Moreover, in LCLs (lymphoblastoid cell lines), derived from patients harboring loss of function mutations in the *MUTYH* gene, accumulated 8-oxo-dG lesions and KBrO₃-induced hypermutability has been observed, indicating the protective role of MUTYH against oxidatively induced DNA damages [218, 219]. Consistent with these reports, *AluYb8MUTYH* SNP, insertion of a mobile element in the intron 15, causes substantial decrease in type 1 mitochondrial MUTYH, leading to increased mtDNA damages. This SNP has been associated with increase susceptibility to

age-related diseases as well as type 2 diabetes [220-224]. Reduced *MUTYH* level has also been reported in prostate adenocarcinoma and it is also associated with a poor prognosis in gastric cancer [225, 226].

Inherited mutation in the *APC* (adenomatous polyposis coli) gene is known as the underlying cause of an autosomal dominant disease called FAP (Familial adenomatous polyposis). The *APC* gene is also the most frequently mutated gene in sporadic colorectal cancer. Formation of numerous adenomatous polyps in the gastrointestinal tract is a FAP associated characteristic (extensively reviewed in [227-230]). Al-Tassan and coworkers identified a British family with multiple colorectal adenomas and carcinoma that were not carrying inherited inactivating mutations in the *APC* gene. Instead, the *APC* gene contained a high level of somatic G:C>>T:A transversions, which could be a signature of defective OGG1/*MUTYH*-mediated repair. The patients appeared to harbor biallelic driving mutations in *MUTYH*, which was the cause of their predisposition to MAP (*MUTYH*-associated polyposis), an autosomal recessive disease [231-235]. Spontaneous G:C>>T:A transversions in *APC* or *KRAS*, as a result of failed *MUTYH*-dependent repair, can trigger tumorigenesis in MAP patients [235-238]. In fact, MAP is not restricted to gastric tumors and thus increased risk of extraintestinal malignancies including ovarian, bladder, skin, and breast cancers has been reported in MAP patients [239-241].

Pathogenic *APC* mutations mostly generate a truncated protein. However, the majority of pathogenic *MUTYH* variants (>50) are missense mutations. The most prevalent missense mutations observed in MAP patients (~80%) are Tyr165Cys and Gly382Asp [214]. The *MUTYH* Tyr165Cys variant, in which substitution occurs at the DNA minor groove binding motif, displayed severe impaired glycosylase activity and substrate binding capacity [242-247]. The Gly382Asp mutation is localized in the C-terminal domain of *MUTYH* where the nudix domain exists, which is critical for 8-oxodG recognition [248-250]. The *MUTYH* Gly382Asp variant showed reduced glycosylase activity and partial suppression of mutation frequency compared to the WT protein [242-244, 246]. Consistent with these observations, phenotypic effects of Gly382Asp mutation appeared to be relatively milder than that of Tyr165Cys variant in MAP patients [251].

1.3.4 Role of MMR and NER in 8-oxo-dG Removal

If the POLs misinsert 8-oxo-dGTP opposite the already existing dA in DNA, the newly formed 8-oxo-dG:dA mispair can be detected by MMR [200]. Activity of MutS alpha can be stimulated by binding to 8-oxo-dG:dA and 8-oxo-dG:dT lesions, but not by 8-oxo-dG:dC mispairs [252]. In addition, both baseline and H₂O₂ induced 8-oxo-dG levels were significantly higher in MMR-defective cells [253]. MSH2- deficient cells were also shown to be highly sensitive to H₂O₂ and methotrexate treatments [254]. Collectively, these studies indicate the important contribution of MMR in removal of oxidatively induced DNA damages.

Although 8-oxo-dG lesions are not considered as substrates for NER, TC-NER appears to have a regulatory role on the BER-mediated repair of simple oxidative DNA damage [255]. CSB-deficient cells showed a considerably reduced capacity for repair of 8-oxo-dG lesions owing to decreased expression of OGG1, suggesting a regulatory function on OGG1-dependent repair [256-259]. In fact, CSB is recruited to oxidative DNA lesions but it does not initiate NER [260].

1.3.5 Mouse Models of the 8-oxo-dG Repair Pathways

Mth1 deficiency did not lead to elevated genomic 8-oxo-dGs [261]. However, the *Mth1*-KO (knock-out) mice exhibited a significantly higher number of spontaneous tumors in the lung, liver and stomach in comparison with their WT littermates [262, 263]. On the other hand, overexpression of human MTH1 in mice prevented age-associated accumulation of genomic 8-oxo-dGs and improved longevity compared to WT mice [130].

Although accumulated nuclear 8-oxo-dGs and slightly higher mutation rate were observed in the liver of *Ogg1*^{-/-} mice, they did not exhibit elevated carcinogenesis [264, 265]. This indicates that back-up repair pathways could contribute to avoid tumorigenesis in the absence of Ogg1 activity. In addition, it confirms that Ogg1-dependent repair is not necessarily coupled to replication since liver is a non-proliferative tissue. In another study, Ogg1 deficiency resulted in remarkably higher 8-oxo-dG accumulations as well as mutagenicity in the liver of the KO animals compared to their WT counterparts. However, similar to the previous study, these animals did not show elevated tumor formation [266]. When these animals were exposed to KBrO₃, a substantial increase of 8-oxo-dG lesions were observed in both liver and kidney, without any escalated tumorigenesis [267-269]. Contrary to these studies, Sakumi *et al.* created *Ogg1*^{-/-} mice in which 8-oxo-dG content of their genome was considerably high, leading to increased spontaneous tumors in lung [261]. DKO (double knock-out) of *Ogg1/Mth1* in these mice led to a moderate increase in genomic 8-oxo-dG lesions while suppressed tumor formation in the lung, suggesting that *Mth1* deficiency might trigger tumor cell death under these conditions.

Similar to *Ogg1*, age-dependent 8-oxo-dG accumulation appeared only in the liver of *Mutyh*-null mice. However, concurrent deletion of *Ogg1* and *Mutyh* caused a significantly greater level of 8-oxo-dG lesions in lung and small intestine, in addition to liver [270]. Moreover, mice with combined deficiency in *Ogg1* and *Mutyh*, were more prone to development of lymphomas, lung and ovarian tumors compared to WT mice [271]. In an independent study, Sakamoto and colleagues showed increased intestinal tumorigenesis in *Mutyh*-KO mice that could be enhanced by KBrO₃ treatment [272]. Simultaneous deletion of *Mth1*, *Ogg1*, and *Mutyh* could dramatically increase the mutation rate where somatic and germ line mutations appeared to be G:C >> T:A transversions. Such TKO (triple knock-out) mice developed various types of tumors in several organs and had a shorter lifespan [273].

Msh2-deficient mice displayed a strong mutator phenotype and were considerably predisposed to lymphoma [274, 275]. Deletion of *Mth1* in *Msh2*^{-/-} background led to a remarkable increase in spontaneous G:C>>T:A transversions compared to *Msh2*-null mice [276]. Furthermore, when both *Msh2* and *Mutyh* were deleted, higher levels of 8-oxo-dG lesions were observed in several tissues compared to WT and single KO mice. However, these DKO mice displayed a strong delay in lymphoma development, suggesting a protective role for MUTYH against MSH2-associated tumorigenesis [277]. On the other hand, KO of the *Csb* gene did not affect the level of 8-oxo-dG lesions in the liver of mice. However, concurrent inactivation of *Csb* and *Ogg1* led to higher accumulation of oxidative DNA damage in the liver, kidney and spleen compared to single KO mice, indicating an alternative role for CSB protein [278].

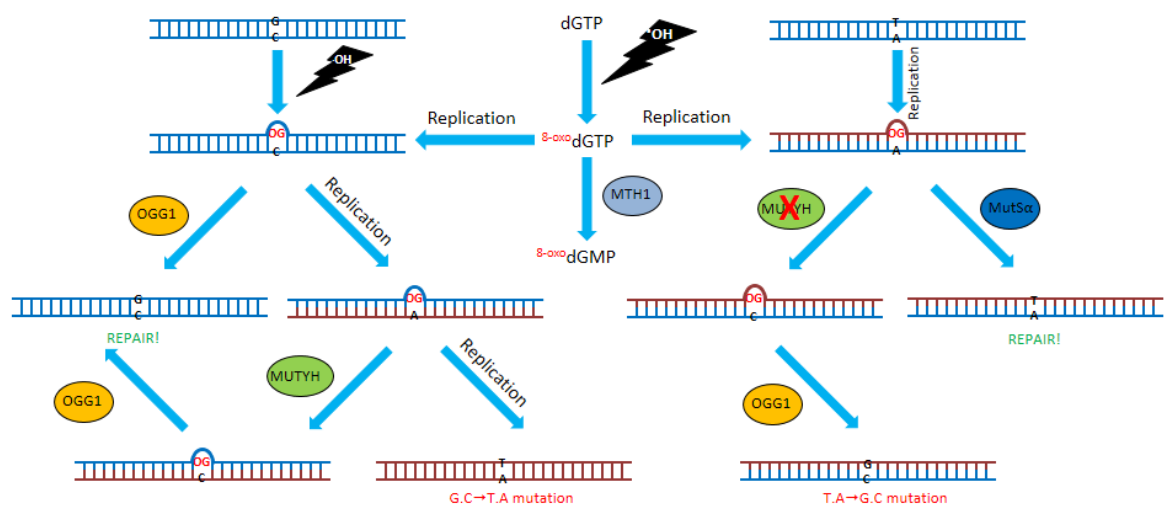


Figure 3. Main pathways responsible for the repair of 8-oxo-dG. MTH1 eliminates 8-oxo-dGTP from the dNTP pool. OGG1 excises the 8-oxo-dG when paired with dC. MUTYH recognizes dA:8-oxo-dG mispairs and removes the dA from the nascent strand. MMR (MutSα) detects dA:8-oxo-dG mispairs and removes the 8-oxo-dG from the newly synthesized strand. The nascent strand and the template strand are shown in red and blue, respectively. See text for details. Figure reprinted from paper II, with permission from Nature Publishing Group.

1.4 Anti-Cancer Targets within DNA Repair Pathways

1.4.1 Oncogene Addictions

During carcinogenesis, cells gain a common set of features referred to as the hallmarks of cancer. Such properties are acquired through a multi-step process that includes gain-of-function mutations/ overexpression of oncogenes along with inactivating mutations/deletion of tumor suppressors [131, 279]. These genetic alterations are considered as driver mutations which lead tumor development [280]. Therefore, targeting these OA (oncogene addictions) is an established strategy to treat cancer (reviewed in [281]). For instance, inhibitors of EGFR (epidermal growth factor receptor) are developed to disturb sustainable proliferative signaling, which is a known hallmark of cancer cells. Overexpression of EGFR is reported in at least 62% of NSCLC (non-small-cell lung cancer) and its expression confers an adverse prognosis [282-286]. Elevated level of EGFR can constantly trigger the SOS-Ras-Raf-MAPK cascade that rewires the signaling pathway to promote cell proliferation [287]. Inhibitors of the tyrosine kinase activity of EGFR, such as gefitinib and erlotinib, are clinically used for treatment of NSCLC patients with activating EGFR mutations [288-293]. These drugs compete with ATP molecules for binding to the EGFR active site [294, 295].

1.4.2 Non-oncogene Addictions

On the other hand, cancer cells display additional common properties that are not responsible for the tumorigenic process but are critical for enhancing their cellular adaptations and viability. These NOA (non-oncogene addictions) include various stress phenotypes of cancers such as DNA damage stress, oxidative stress, and mitotic stress, among others. In other words, the genes or pathways contributing to NOA are required to favor cancer stress phenotypes but are not essential for survival in normal cells. Accordingly, application of additional stress to tumor cells can selectively kill them while exerting minor effects on normal cells. In addition, sensitization of cancer cell to their existing stress phenotypes could be another approach to trigger death exclusively in tumors [296]. To achieve stress sensitization, the pathways should be targeted whose inhibition renders synthetic lethality with genotype or phenotype of tumors [131, 296, 297]. Based on these principles, several targets within DNA repair pathways have been identified [298, 299] (Figure 4).

1.4.3 Stress Overload

Approximately, half of cancer patients are currently treated with RT (radiotherapy) [300]. In fact, this well-established treatment overloads cells with DNA damage stress and oxidative stress. However, since tumor cells exhibit elevated stress phenotypes, they are more vulnerable to IR treatment. It has been shown that inhibiting certain proteins in DNA repair pathways can make cancer cells sensitive to IR and reduce its toxic effects on normal cells. For instance, DNA-PK defective cells were found to be highly sensitive to IR [301-304]. Several attempts

have been made to develop small molecules inhibiting the kinase activity of DNA-PKcs. NU7026 and NU7441 are potent and selective inhibitors of DNA-PKcs which can considerably sensitize the cells to IR as well as DSB inducing agents (*e.g.* topoisomerase inhibitors) [305-309]. However, due to poor pharmacokinetic parameters, their clinical applications are restricted [308, 310]. Currently, MSC2490484A is being assessed in phase I clinical trials, either as monotherapy or in combination with RT, for the treatment of advanced solid tumors or CLL (chronic lymphocytic leukemia) (ClinicalTrials.gov Identifier: NCT02316197, NCT02516813). Furthermore, CC-122 and CC-115 (dual inhibitors of DNA-PK and mTOR) are also being investigated in phase I clinical trials for treatment of hematologic malignancies or advanced solid tumors (NCT01421524, NCT01353625).

Frontline chemotherapeutic alkylating agents can also be considered as examples of the stress overload strategy for cancer treatment (reviewed in reference [26]). For instance, FDA (Food and Drug Administration) granted accelerated approval for the TMZ treatment of patients with resistant anaplastic astrocytoma, a rare malignant brain tumor, in 1999. TMZ treatment was combined with RT to improve the efficacy in tumors, leading to a full FDA approval for treatment of GBM (glioblastoma multiform) in 2005 [311]. In addition, patients with inactivated MGMT appeared to benefit from TMZ treatment [312, 313]. Thus, blocking the direct repair pathway using small molecule inhibitors of MGMT can further sensitize tumor cells to alkylating agents [314-316]. Many of the chemotherapeutic drugs as well as RT, create DNA damage that BER contributes to their repair process. Therefore, combination of BER inhibitors with DNA damaging agents is another example of the stress sensitization approach [317, 318]. TRC102 is the most advanced inhibitor of APEX1 which is currently in phase I clinical trials. Combination of TMZ with TRC102 is being evaluated for treatment of relapsed solid tumors and lymphomas (NCT01851369). In another trial, TRC102 has been combined with pemetrexed in patients with advanced solid tumors [319].

1.4.4 Stress Sensitization and Synthetic Lethality

Exploiting the synthetic lethality concept for tumor treatment can be considered as the stress sensitization approach where rational targeting of pathways synergizes with the cancer genotype or phenotype to induce cell death. For instance, BRCA1/2-inactivated cells were reported to be highly addicted to the activity of an enzyme called PARP1 (Poly (ADP-ribose) polymerase-1) [320]. Inhibition, PARP1 activity has been shown to be synthetically lethal with *BRCA* deficiency, which is found in inherited breast and ovarian cancers [92-94, 321-323]. PARP1 binds to SSBs and promotes their efficient repair [324]. Strom *et al.* suggested a model for this synthetic lethality in which the PARP1 inhibitor traps the protein onto SSB intermediates, creating an obstacle to replication fork progression that would require HR to bypass [325]. Alternatively, it is shown that replication restart at stalled forks is dependent on HR and PARP1. Therefore, preventing PARP1 activity in a BRCA-null background causes inefficiency in replication restart [296, 326-328].

Olaparib (AZD2281) is a potent PARP1 inhibitor that was evaluated, for the first time in a phase I study, as a single agent in tumors from *BRCA* mutation carriers [329]. In this study, antitumor activity of olaparib was observed in patients with inherited *BRCA*-mutated breast, ovarian, or prostate tumors [329]. The efficacy of olaparib was confirmed in a proof-of-concept phase II trial where approximately one-third of the *BRCA*-defective patients responded to the therapy without severe toxicities [330, 331]. Finally, olaparib received FDA approval in 2014 based on a clinical trial in which good responses were observed across various tumor types associated with inherited *BRCA* mutations [332]. The successful story of olaparib prompted the development of several PARP1 inhibitors that are currently in clinical trials, such as rucaparib (AG014688) and veliparib (ABT-888) (extensively reviewed in reference [333]). Since PARP1 contributes to the repair of SSBs, applications of PARP1 inhibitors are not restricted to *BRCA* deficiency and can be used for chemopotentiation of DNA damaging agents [333-335]. In addition, PARP1 inhibitors would be considered as radiosensitizers (NCT01264432, NCT01460888) [336-338].

NOA of ATM-(ataxia telangiectasia mutated)deficient cells to DNA-PK activity has revealed another synthetic lethality interaction [339]. ATM is a kinase that initiates the DDR (DNA damage response) upon formation of DSBs and mediates their HR-dependent repair [340-344]. Thus, deficiency in ATM-mediated DDR attenuates HR, leading to increased radiosensitivity [345]. *Atm*-null mice are sterile with increased incidence of lymphomas, while DNA-PKcs KD mice are fertile with severe immunodeficiency [346-348]. However, deleting *Prkdc* (the gene encodes DNA-PKcs) in *Atm*^{-/-} background leads to early embryonic lethality [349]. Consistent with this, toxicity of DNA-PKcs inhibitors in ATM-defective lymphomas has been reported [350]. Dependency of ATM-mutated tumors on NHEJ for DSB repair accounts for this synthetic lethality [351]. In addition, APEX1 inhibitors can be used as monotherapy in HR-defective tumors due to synthetic lethal interactions with inactivating mutations in *BRCA* and *ATM* [352]. TRC102 is currently in clinical trials and might be assessed for synthetic lethal strategies.

Regardless of the tumor genetic background, exploiting synthetic lethality with cancer phenotypes could be a more general therapeutic approach. For example, the inhibitors of heat shock protein 90 (HSP90) are able to sensitize tumors to proteotoxic stress, which is an NOA [353]. Here, we present targeting DNA repair pathways of oxidatively induced lesions as a phenotypic lethality approach for cancer therapy.

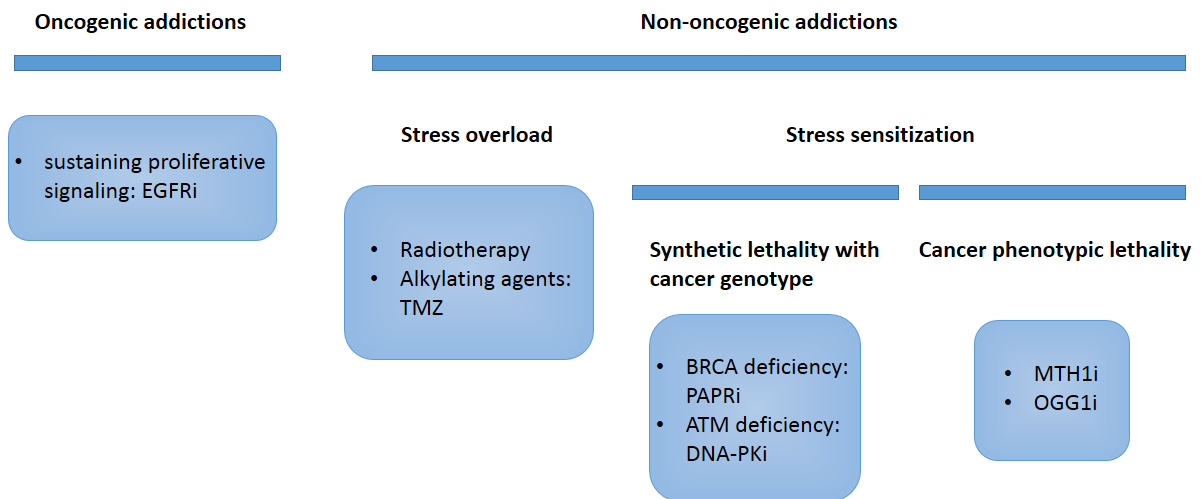


Figure 4. Different Therapeutic approaches for cancer treatment. Targeting oncogenic addictions is a simple and widely used anti-cancer strategy. However, non-oncogenic dependencies of cancer cells can be targeted by application of more stress or sensitization to their stress phenotypes. Rational targeting of DNA repair pathways can lead to synthetic lethality with the genotype or phenotype of tumors. Examples of these strategies are listed in the boxes, which are discussed in details in the text. EGFRi: EGFR inhibitors, TMZ: temozolomide.

2 AIM OF THE THESIS

DNA repair proteins that maintain genomic integrity against oxidative damage pose attractive anti-cancer targets, as inhibiting these pathways can principally sensitize tumors to both DNA damage stress and oxidative stress. Accordingly, the overall aim of this thesis is to validate such enzymes as targets for inducing phenotypic lethality in cancer. Moreover, considerable efforts were made to develop novel small molecule inhibitors of MTH1 and OGG1, followed by evaluation of the compounds in various assays.

In paper I, we aimed to assess MTH1 as a novel therapeutic target within DNA repair. We showed the NOA of cancer cells to MTH1 activity and subsequently developed a potent MTH1 inhibitor (TH588). TH588 was validated in this study and appeared to inhibit tumor growth *in vivo*.

The aim of paper II, as a follow-up study to paper I, was investigation of functional cooperation between MTH1 and MUTYH. The study tried to provide supportive evidence for a protective role of MUTYH in the absence of MTH1. This study revealed a toxic synergism upon concurrent loss of both MTH1 and MUTYH.

In paper III, we aimed to examine the dependency of cancer cells on OGG1 activity. The second goal was to generate selective and potent small molecules inhibitors of OGG1. TH5487 was described and validated as a novel OGG1 inhibitor with selective anti-tumor effects.

3 METHODOLOGY

3.1 Target Validation

Target validation is a process in which a molecular target is meticulously investigated to ensure that the target deserves drug development. Several techniques can be exploited to validate a target ranging from *in vitro* methods to animal models. Simply, target validation can be carried out by depletion of the target in cells and subsequent analysis of observed phenotypes. Genetic techniques are used to knock down a target, such as RNAi (RNA interference) or CRSPR/Cas9 gene perturbation [354].

3.1.1 Silencing of Target Genes by RNA Interference

siRNAs (small interfering RNAs) are introduced to cells by transient transfection or invading virus particles that contains shRNAs (small hairpin RNAs). An enzyme called Dicer mediates the cleavage of shRNAs, converting them into siRNAs. One strand of the resulting siRNA, called guide strand, triggers silencing of the target gene by binding to the RISC (RNA-induced silencing complex) complex, which mediates fragmentation of the complementary mRNA to the guide strand [355]. We suppressed expression of several target genes in our studies using transient transfection of siRNAs or by establishment of stable cells lines expressing lentiviral shRNAs (paper I: Figure 1, 2, Extended Data Figure 1; paper II: Figure 1; paper III: Figure 4, 5).

3.1.2 Assessment of Target Knock-down

When a target is silenced by siRNAs, the efficiency of KD (knock-down) needs to be assessed at both mRNA and protein levels. qRT-PCR (quantitative real-time polymerase chain reaction) is a widely used method to analyze the efficiency of KD at mRNA level and it can also be used for gene expression analysis. In principle, total RNA is extracted from samples and then cDNA is synthesized by reverse transcription using random hexamers and oligo (dT) primers. The resulting cDNA sample is mixed with the qRT-PCR reaction buffer which contains SYBR Green, a hot-start DNA polymerase, dNTPs, and primer pairs. The design of primer pairs is a critical step because they should be specific to the target gene and preferentially be inefficient to amplify the genomic DNA contaminations. SYBR Green is a DNA stain that emits fluorescence more strongly when bound to double stranded DNA, and thus its signal correlates with the amount of DNA amplified in qRT-PCR reaction. The qRT-PCR instrument collects the intensity of fluorescence after each cycle and the results are normalized to multiple housekeeping genes. The comparison of normalized expression between control samples and KD-samples determines the efficiency of suppression [356]. We assessed the efficiency of KD

for several targets using qRT-PCR. In addition, qRT-PCR was used for gene expression analysis in our studies (paper I: Extended Data Figure 7, 8; paper II: Figure 1, 5)

In addition, WB (Western blotting) can be used to evaluate the target KD at protein level. Briefly, after preparation of cell extract, the sample is heated to denature proteins in the presence of reducing agents. After electrophoresis of the sample, the proteins are transferred from SDS-PAGE into an adsorbent membrane. The membrane is then blocked for unspecific bindings and then probed with specific antibodies detecting their targets [357]. We frequently used WB to study the efficiency of KD, change in protein levels, and cellular signaling such as protein phosphorylation during DDR (paper I: Figure 2, Extended Data Figure 1, 2, 6, 7, 8; paper II: Figure 1, 3, 4; paper III: Figure 5, 6).

3.1.3 Analysis of Cell Cycle Distribution

Depletion/inhibition of an anti-cancer target often leads to a change in normal cell cycle distribution of transformed cells. FC (flow cytometry) provides robust analysis of the cell cycle distribution. In univariate analysis, cells are simply fixed and stained with DNA specific dyes such as DAPI (4',6-diamidino-2-phenylindole), Hoechst 33342, or PI (propidium iodide). The DNA content frequency histograms display cell populations in G₁, S and G₂/M phases as the intensity of dye correlates with amount of DNA in each cell cycle phase of individual cells. In bivariate analysis, cells are pulsed with EdU (5-ethynyl-2'-deoxyuridine). The active replicating cells incorporate EdU into their nuclear DNA which facilitates discrimination of cells in S phase from others. Cells are then stained with a DNA specific dye as well as a clickable fluorescent marker for conjugation to EdU. The scatter plots, in which DNA content is plotted against EdU incorporation, display three distinct populations representing G₁, S, and G₂/M cells [358]. We analyzed the effect of silencing our molecular targets, by shRNA-mediated KD or compound treatment, on the cell cycle distribution using FC (paper II: Figure 2, 4; paper III: Figure 5).

3.2 Cell Death Assays

Cell death is examined after depletion or inhibition of a potential target to validate the dependency of cancer cells to the target. Since cell death may be triggered by various pathways such as apoptosis, necrosis, autophagy, and mitotic catastrophe, it is essential to distinguish such death modes and to use appropriate cell death assays [359].

3.2.1 Vital Stains and Dye Exclusion Method

A very simple assay of cell death assessment is dye exclusion method in which a vital dye selectively stain dead cells by penetrating their compromised plasma membrane. For instance,

Trypan blue and DAPI are vital dyes which have been used frequently in our studies [360]. Cellular proliferation upon depletion/inhibition of a target can be monitored using this method (paper III: Figure 4)

3.2.2 Colony Formation Assay

Colony formation assay, also known as clonogenic survival assay, measures the capability of an individual cell to grow into a colony that may consist of approximately >50 cells. Before or after treatment, cells are plated in appropriate densities to grow at least seven days. Cells are then stained with a solution containing methanol and methylene blue, and subsequently colonies are counted using colony counters [361]. The toxicity of MTH1 depletion/inhibition was assessed using this method (paper I: Figure 1, 4, Extended Data Figure 1, 5)

3.2.3 Resazurin Viability Test

The measurement of metabolism is a common way of examining cellular viability. Tetrazolium dyes, such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and resazurin, are absorbed by living cells and converted from colorless salts into colored substances. The conversion is catalyzed by mitochondrial reductases using NAD(P)H. Therefore, any other factor that affects this conversion can also influence the read-out in this assay, suggesting that the MTT test is not a stand-alone method for measuring viability [362] [363]. In our studies, the viability of cells were monitored using resazurin dye after treatment with MTH1 or OGG1 inhibitors (paper I: Extended Data Figure 5; paper III: Figure 3, 4, and 6).

3.2.4 Measurement of Caspase Activity

Caspase proteins are well-studied factors that play central roles in programmed cell death. Although they appear to have additional inflammatory functions, analysis of their activity still remains essential for apoptosis detection [359, 364]. Measurement of cleaved/activated the executioner caspase-3 level is a widely used method for assessment of apoptosis induction. There are several decent antibodies for detecting cleaved caspase-3 which can be used in WB, FC or IF (immune-florescent) microscopy (paper I: Figure 2; paper II: Figure 3).

3.2.5 Assessment of DNA Fragmentation (sub-G₁ fraction)

Internucleosomal DNA fragmentation by endonucleases is a well-known hallmark of cell death [365, 366]. Thus, as a result of DNA degradation, apoptotic cells exhibit fractional DNA

content when stained with DNA specific dyes. Such cell population constitute a typical sub-G1 peak on the histograms of DNA content [367] [368]. We measured the level of sub-G1 populations in our studies using DAPI staining (paper II: Figure 2, 4; paper III: Figure 4).

3.2.6 Redistribution of Phosphatidylserine

PS (phosphatidylserine) is normally located in the inner plasma membrane leaflet. Redistribution of this phospholipid to the outer leaflet occurs as an early event in apoptosis. Using a PS-binding protein called annexin V, it is possible to detect PS exposure during apoptosis [369]. When annexin V is combined with a cell impermeable DNA-binding dye such as DAPI or PI, it would be more convenient to distinguish apoptosis from necrosis [370, 371]. In paper II, apoptosis induction was detected using annexin V-DAPI staining (paper II: Figure 3).

3.3 Assessment of DNA Damage

Since we have focused on targeting DNA repair pathways for cancer treatment, detection of DNA damage is critical for our studies. Generally, the methods for detecting DNA damage can be classified into two groups: indirect and direct detection methods. DNA damage induces formation of nuclear repair foci and modifications in chromatin. Therefore, monitoring these damage-induced consequences are considered as indirect detection methods. On the other hand, DNA damage can be analyzed by direct quantifications of the lesions [372].

3.3.1 Detection of DSBs

Upon induction of DSBs, repair factors are recruited to the site of damage. Several repair proteins, including RAD51 and 53BP1 (p53-binding protein 1), exhibit diffuse nuclear staining in normal conditions but they form nuclear foci upon DSBs generation. These foci can be detected by specific antibody staining and subsequent IF microscopy [372]. We showed induction of 53BP1 foci after depletion/inhibition of MTH1 using IF microscopy (paper I: Figure 1, 2, 3, Extended Data Figure 1, 6). Another method for indirect measurement of DSBs is quantification of histone H2AX phosphorylation at Ser 139 (γ H2AX). Immediately after DSB formation, γ H2AX is formed by ATM kinase activity, and thus the number of γ H2AX foci directly correlates with the number of induced DSBs. Using antibodies against γ H2AX, the induction of DSBs can be detected by WB, FC or IF microscopy [372]. We measured the γ H2AX level after depletion/inhibition of OGG1 using WB analysis (paper III: Figure 5).

3.3.2 Live Cell Imaging

DNA repair factors can be tagged with a fluorescent protein (*e.g.* GFP) and expressed in cells. Therefore, foci formation or recruitment of the GFP-tagged protein can be analyzed after DNA damage induction in live cells. FRAP (fluorescence recovery after photobleaching) is a technique in which a defined region of the cells expressing a GFP-tagged protein is bleached by laser and immediately the recovery of the GFP signal in the region is monitored. The recovery of the signal indicates the kinetics of diffusion for the GFP-tagged protein from the undamaged sites to the region [372]. Using FRAP analysis, we showed that treatment of cells with TH5487 can reduce the nuclear mobility of OGG1, indicating the engagement of the compound with OGG1 (paper III: Figure 3).

3.3.3 Comet Assay

DNA strand breaks in individual cells can be visualized by comet assay, also known as single cell gel electrophoresis assay. For the comet assay, cells are harvested after treatment to prepare single cell suspension. The cells are then embedded in a low-melting temperature agarose gel covering microscopic slides. Next, the cells are subjected to in-gel lysis, flowed by alkaline DNA unwinding and electrophoresis. Afterwards, the samples are neutralized, stained with a specific DNA dye (*e.g.* YOYO-1 and SybrGold), and finally the strand breaks are visualized as comet tails under a microscope. Although the comet assay is a sensitive method, technical variability and small sample size are considered as drawbacks of this technique [373]. We used a modified comet assay in our studies that is described in the next section.

3.3.4 Quantification of 8-oxo-dG

Considering the low background level of 8-oxo-dG in normal conditions, a highly sensitive method with minimal variability is required to quantify such lesions. Antibodies are developed to detect 8-oxo-dG which can be used in IF microscopy. However, these antibodies are not very specific and the results require a secondary assay for confirmation. Avidin appeared to have a capability of 8-oxo-dG recognition [374]. The K_d (μ M) values for 8-oxod-G and 8-oxo-dA were found to be approximately 117 and 24, respectively [375]. Fluorophore conjugated avidins can be used in IF microscopy or FC analysis [376]. Similar to the antibodies, avidin is not highly specific to 8-oxo-dG and avidin-based measurements were not found to be sensitive. Chromatographic methods such as HPLC-ECD and LC-MS/MS have been employed to measure 8-oxo-dG. However, these methods are troubled by spurious dG oxidation during sample preparation steps [377, 378]. To detect 8-oxo-dG lesions with high sensitivity and specificity, a modified version of comet assay has been developed where cells are treated with purified OGG1 after lysis [379]. The technical variability is known to be the major downside of this assay [373, 377]. Overall, the 8-oxo-dG detection assays require further improvements and standardization. In paper II, we detected 8-oxo-dG levels using avidin-based IF

microscopy as well as modified comet assay (paper I: Figure 1, 4, Extended Data Figure 5). In paper III, we used LC-MS/MS to measure 8-oxo-dGs (paper III: Figure 3).

3.4 Evaluation of Drug Target Interactions

3.4.1 Gel Electrophoresis Mobility Shift Assay

The interactions of proteins with DNA can be examined using EMSA (gel electrophoresis mobility shift assay). In principle, the mixture of protein with labeled DNA fragments prepared in an appropriate binding buffer. Then, the mixture is loaded on a polyacrylamide gel for electrophoresis under native conditions. After electrophoresis, migration of the mixture is visualized in which the protein-nucleic acid complexes principally migrate more slowly compared to the free oligonucleotide probes [380]. In paper III, we showed the binding of OGG1 to a DNA fragment containing 8-oxo-dG:dC in the presence or absence of TH5487, the OGG1 inhibitor (paper III: Figure 2) .

3.4.2 Differential Scanning Fluorimetry

DSF (differential scanning fluorimetry) is a method to monitor shift in T_m (melting temperature) of a purified protein when interacts with its ligands. DSF is based on a fact that the binding of the ligand increases the thermal stability (and therefore the T_m) of the recombinant protein. The protein with or without the ligand is mixed with an environmentally sensitive dye (*e.g.* SYPRO Orange) and the mixture is then loaded on a real-time PCR machine. The PCR instrument measures the fluorescent intensity of SYPRO Orange as the temperature gradually goes up to denature the protein. The SYPRO Orange dye emits more strongly when bound to hydrophobic regions of the protein, which are exposed upon protein unfolding [381]. Using DSF, we displayed the increased thermal stability of OGG1 in the presence of TH5487, conforming their interactions (paper III, Figure 2).

3.4.3 Isothermal Titration Calorimetry

ITC (isothermal titration calorimetry) is a biophysical method to measure the in-solution thermodynamic parameters of interactions between a purified protein and its ligand. ITC is based on a fact that the binding of ligands to a recombinant protein cause heat release or absorption in the interactive system. ITC uses heat exchange as signal to determine the ligand affinity (K_a) and enthalpy change (ΔH) upon binding to the protein [382]. We confirmed binding of MTH1 inhibitors to the protein and calculated the corresponding K_a and ΔH values using ITC (paper I: Extended Data Figure 3).

3.4.4 Hydrogen/Deuterium Exchange Mass Spectrometry

MS-(mass spectrometry)based measurement of H/D (hydrogen/deuterium) exchange is an analytical method to investigate protein dynamics during ligand binding. When a protein is placed in a D₂O solution, the backbone hydrogens can be exchanged with deuterium atoms in the solution. Deuterium is a heavy stable isotope of hydrogen with a mass twice that of light hydrogen. Thus, in the presence of a ligand, establishment of interactions between the protein and its ligand can limit H/D exchange which can be mapped to protein residues using MS [383]. The peptides containing the OGG1 residues interacting with TH5487 have been identified using this technique (paper III: Figure 2).

3.4.5 Cellular Thermal Shift Assay

Thermal stability of a protein when bound to its ligand can be assessed in cells by CETSA (cellular thermal shift assay). The method relies on the fact that engagement of a ligand/compound with its target can increase the thermal stability of the protein in cells. In principle, cells are treated with a compound of interest and then heated to thermally denature proteins. The cell lysate can be subjected to WB analysis of individual proteins or MS-based analysis at proteome level. For high throughput assays, the protein stability can be measured with two antibodies using the AlphaScreen (amplified luminescent proximity homogeneous assay screen) technology [384] [385]. In our studies, we showed target engagement of MTH1 and OGG1 inhibitors in cells using CETSA (paper I: Figure 4; paper III: Figure 3).

4 RESULTS AND DISCUSSION

4.1 Paper I: MTH1 Inhibition Eradicates Cancer by Preventing Sanitation of The dNTP Pool

Depletion of MTH1 using siRNA caused reduced clonogenic survival in transformed cell lines, which was associated with increased DNA damage (53BP1, pATM, pDNA-PK foci) and elevated incorporation of 8-oxo-dGTP and 2-OH-dATP into genomic DNA (Figure 1,2, Extended Data Figure 1,2). However, MTH1 appeared to be a non-essential enzyme in non-transformed cells (*e.g.* VH10 and BJ-hTERT) as its loss was much less toxic (Figure 1, Extended Data Figure 1). Moreover, expression of the WT protein rescued survival and suppressed the 53BP1 foci formation in U2OS cells. In contrast, the catalytic inactive variant (E56A) failed to prevent the DNA damage and enhance cell viability (Figure 1), indicating the NOA of cancer cells to MTH1 sanitation role. Using additional MTH1 mutants D119A and W117Y, with hydrolytic deficiency of 2-OH-dATP and 8-oxo-dGTP respectively, it was shown that both of these oxidized purine nucleotides contribute to cell death (Extended Data Figure 2). Analysis of the DDR revealed that ATM becomes activated upon MTH1 depletion to mediate phosphorylation of p53 that in turn triggers cell cycle arrest (p21 induction) and apoptosis (c-caspase3 increase) (Figure 2, Extended Data Figure 2). Importantly, inducible shRNA-mediated KD of *MTH1* in SW480 xenografts could effectively stop the tumor growth *in vivo*, confirming the addiction of the tumor cells to MTH1 activity (Figure 2, Extended Data Figure 2).

The target evaluation was followed by a high-throughput malachite green-base screening assay for small molecule inhibitors of MTH1 using dGTP as a substrate. The hits were validated in clonogenic survival assays to identify potent cell-active compounds, named TH287 and TH588 (Figure 4). The inhibitory functions of these compounds were validated for the specific MTH1 substrates (8-oxo-dGTP and 2-OH-dATP) *in vitro* (Extended Data Figure 3). The specificity of these inhibitors were examined against a panel of nudix enzymes as well as other nucleotide hydrolyzing enzymes, demonstrating their selectivity for MTH1 inhibition (Figure 5, Extended Data Figure 8). Using CETSA, the inhibitors were shown to engage with MTH1 in the cell (Figure 4). MTH1 inhibitors exhibited selective toxicity to cancer cells by inducing DNA damage and increasing the accumulation of oxidized nucleotides in the genome, which was consistent with RNAi-mediated KD of *MTH1*. In contrast, MTH1 inhibitors were tolerated by non-transformed cells in clonogenic and viability assays, and did not cause induction of DNA damage (Figure 4, Extended Data Figure 5, 6). Moreover, TH650, a structurally similar but inactive analogue of TH588, failed to inhibit MTH1 potently in the biochemical assay, engage with the target in the cell, and induce DNA damage (Figure 4, Extended Data Figure 5, 6, 8). Above all, the growth of therapy-refractory melanoma patient-derived xenografts were significantly limited by TH588 treatment (Figure 4). Additional xenografts (SW480 and MCF-7) were also used to assess the cytotoxic effects of TH588 (Extended Data Figure 4). Overall,

the study introduced MTH1 as a promising anti-cancer target and described the activity of TH588 against human tumor cells.

In line with this study, several independent reports have shown the NOA of tumors to MTH1 activity. MTH1 depletion inhibited tumor growth in various xenograft models [132, 133, 136] [135]. Moreover, loss of MTH1 induced telomere oxidation and cell death in cancer cells with shortened telomeres [129]. In contrast, Kettle *et al.* showed that siRNA-mediated KD of *MTH1* did not affect the DDR and cell viability in their experiments [386]. MTH1 depletion could not limit cancer cell growth in another study, but the *MTH1* siRNA sequence was not provided by the authors [387]. On the other hand, Warpman-Berglund and coworkers have performed a detailed comparison of several *MTH1* siRNAs and validated the previous reports showing the anti-tumor effect of *MTH1* suppression [132].

Apart from paper I, several attempts were made to develop specific MTH1 inhibitors. Huber *et al.* used a proteomic approach to identify cellular targets of a drug called SCH51344, which is a potent inhibitor of RAS transformation [388]. These authors fished MTH1 out from the cell lysate using an SCH51344 affinity matrix. Subsequently, they screened a library of kinase inhibitors and identified a chiral drug called Critozinib as a potent MTH1 inhibitor. In fact, *S*-Critozinib, not the *R*-enantiomer, showed an inhibitory effect on MTH1 *in vitro*, induced DNA damage and cell death in RAS mutated cancer cells. Although MTH1 overexpression suppressed the *S*-Critozinib induced SSBs formation, it could not improve cell survival. Importantly, the growth of SW480 xenografts were significantly restricted by *S*-Critozinib treatment [133]. Moreover, a high-throughput screen of natural products led to the discovery of echinacoside as an inhibitor of MTH1. Treatment of cells with echinacoside resulted in increased 8-oxodG accumulation, DNA damage and apoptosis [389]. Ji and colleagues described a novel close-to-target assay in which an ARGO (ATP-linked chimeric nucleotide) was used to generate luminescence signal upon MTH1 reaction. Using ARGO, they screened a library of kinase inhibitors and identified NVP-AEW541 as a potent MTH1 inhibitor [390]. NVP-AEW541 is a known inhibitor of insulin-like growth factor-1 receptor (IGF-1R) with established antitumor activity *in vivo* [391]. Petrocchi *et al.* adopted a structure based design approach to discover novel MTH1 inhibitors, leading to a sub-nanomolar MTH1 inhibitor called IACS-4759. This compound could potentially inhibit MTH1 activity in cell lysate but did not show toxicity in a panel of cancer cell lines [392]. Although the authors did not provide information about target engagement of the compound in cells, they concluded that MTH1 inhibition by IACS-4759 is not sufficient to confer anti-proliferative phenotype in tumors. In addition, Kettle and coworkers developed three distinct chemical series of MTH1 inhibitors with high potency and verified target engagement in cells. However, comparison of MTH1-null SW480 cells with parental cell lines showed that toxicity of their compounds were not dependent on MTH1 activity [386]. In fact, treatment of cells with these compounds did not affect genomic 8-oxodG content and tail moment in the modified comet assay [132]. Purine-based MTH1 inhibitors (NPD7155 and NPD9948) have also been described which showed less potency and cellular toxicity compared to TH287 and *S*-Critozinib. Using proteomic profiling, it was revealed that purine-based MTH1

inhibitors, SCH51344 and S-Critozinib had a similar mode of action to camptothecin. In contrast, TH287 was clustered with tubulin poisons such as nocodazole, vinblastine, and paclitaxel. TH287 and TH588 appeared to prevent tubulin polymerization *in vitro* and induced a G2/M block in cell cycle analysis, proposing a possible additional mechanism of action for these compounds [387, 393]. In an independent study, a correlation between endogenous ROS production and sensitivity of melanoma cells to TH588 was found, and the TH588 toxicity was not rescued by MTH1 overexpression [394]. Finally, Warpman-Berglund *et al.* described TH1579, an optimized analogue of TH588, as a clinical candidate that showed selectivity for MTH1 in thermal proteome profiling analysis. TH1579 showed anti-tumor activity in xenografts that was accompanied by increased genomic 8-oxodGs in tumor samples [132]. In conclusion, further investigations are required to understand the mechanisms of action for the MTH1 inhibitors as well as their potential off-target effects.

4.2 Paper II: hMYH and hMTH1 Cooperate for Survival in Mismatch Repair Defective T-Cell Acute Lymphoblastic Leukemia.

In order to investigate the interplay between *MTH1* and *MUTYH*, these genes were silenced individually or simultaneously using inducible shRNA vectors. Sorting the shRNA-bearing cells using FACS helped to achieve efficient KD at mRNA as well as protein level (Figure 1). Sub-G1 populations, as a cell death marker, were measured using FC and indicated synergistic lethality upon concurrent depletion of both *MTH1* and *MUTYH* (Figure 2). Analysis of Annexin V and cleaved caspase 3 revealed that apoptosis induced by loss of *MTH1* is dependent on *MUTYH* level, again confirming the protective role of *MUTYH* (Figure 3). On top of that, overexpression of the nuclear *MUTYH* isoform could partially attenuate the cell cycle arrest and cell death associated with *MTH1* loss (Figure 4). Gene expression analysis of DNA glycosylases suggested *NEIL1* down-regulation as a survival mechanism to avoid toxic repair intermediates when *MTH1* and *MUTYH* are depleted together (Figure 5). Collectively, this study provides supportive evidence for functional cooperation between *MTH1* and *MUTYH* to sustain viability in tumor cells.

Cumulative evidence suggests a protective role for *MUTYH* against oxidative DNA damages. For instance, *MUTYH* loss was shown to increase the sensitivity of cells to oxidative stress [217-219]. Furthermore, siRNA-mediated KD of *MUTYH* decreased the proliferation of PC (pancreatic ductal adenocarcinoma) cells, which could be further reduced by induction of oxidative stress or treatment with chemotherapeutic drugs. *MUTYH* depletion impaired PC cell metastatic potential, and importantly limited PC tumor growth in mouse xenografts *in vivo* [395]. Consistent with these reports, Xie *et al.* showed that *Mutyh* and *Ogg1* DKO mouse fibroblasts were more sensitive than WT and individual KO cells to oxidative stress, strongly indicating the functional cooperation between these two repair proteins occurs to sustain viability [396]. In contrast, Sheng and coworkers proposed that *Mutyh*-mediated BER augments cell death and neurodegeneration in *Ogg1*-null mice and *Mutyh* suppression protects the brain against oxidative stress [397]. However, *Mutyh*^{-/-} mice in an independent study, exhibited better learning and memory performance compared to *Mutyh*^{-/-}*Ogg1*^{-/-} DKO mice [398].

4.3 Paper III: Development of a Potent OGG1 Inhibitor for Cancer Treatment

OGG1 overexpression protects genomic DNA from oxidative stress in cultured cells and impairs cancer progression *in vivo* [167, 169]. Therefore, we aimed to develop potent and selective small molecule inhibitors of OGG1 for cancer therapy. In order to screen for OGG1 inhibitors, we established a cleavage assay using a duplex oligonucleotide with a quenched fluorophore (Figure 1, Supplementary Table 1). To exclude inhibitors of APEX1, a counter screen was carried out and subsequent chemical optimization work led to identification of TH5487 as a potent OGG1 inhibitor. Selectivity of TH5487 for OGG1 was compared to additional glycosylases as well as a panel of nucleotide processing enzymes, confirming the specificity of the compound (Supplementary Table 2, 3). TH5487 impairs OGG1 binding to its substrate in EMSA which is not due to intercalation of DNA (Figure 2, Supplementary Figure 1). TH5487 increases the stability of the recombinant protein in a DSF assay through interactions with residues in the active site, which was further confirmed through (H/D) exchange (Figure 2). Importantly, TH5487 engages its intended target in cells, increasing the thermostability of OGG1 (Figure 3). This inhibitor induces oxidative DNA damage and reduces the nuclear mobility of OGG1-GFP, which mimics the phenotype of a mutant OGG1 variant (K249Q) (Figure 3). Treatment with TH5487 exhibited toxicity in Jurkat A3 cells, while an inactive similar compound (TH2840) was well tolerated in the cells (Figure 3). The observed toxicity cannot be the consequence of microtubule dynamic perturbations since TH5487 does not affect it (Supplementary Figure 2). The compound was assessed in viability assay and showed toxicity across various transformed cell lines while being less toxic in non-transformed and *Ogg1*-deficient mouse fibroblasts (Figure 4).

To compare the cellular effects of OGG1 depletion with those from TH5487 treatment, we established cells containing inducible *OGG1*-shRNA. Loss of OGG1 inhibited proliferation of Jurkat A3 cells with a similar trend to that of TH5487 (Figure 4). Induction of shRNA or TH5787 treatment similarly resulted in elevated sub-G1 population accompanied by reduced EdU incorporation (Figure 4). Intriguingly, prolonged S phase was observed upon OGG1 depletion/inhibition (Figure 4). In addition, drug combination screenings revealed synergistic effects when TH5487 was used together with taxol or karonudib (MTH1 inhibitor) (Figure 5). Overall, this study describes TH5487 as a novel OGG1 inhibitor which can exemplify the phenotypic lethality in cancer.

So far, two independent research teams have presented small molecule inhibitors of OGG1 [399, 400]. However, their work is limited to biochemical assays and the compounds have not been examined in cell lysates or in cells. Mahajan *et al.* synthesized 9-alkylated-8-oxoguanines but the most potent compounds could inhibit OGG1 activity by approximately 30% in the cleavage assay, indicating lack of potency [399]. In an independent study, Donley *et al.* screened for OGG1 inhibitors using a fluorescence-based assay. They assessed the top five compounds in a gel-based cleavage assay and confirmed their inhibitory activity. However, none of the compounds disturbed the interaction of OGG1 with the specific substrate

in an EMSA. The major drawback of this study is the screening assay in which OGG1 was used as a bifunctional DNA glycosylase without adding APEX1 into the reaction mix. In fact, we assessed the most potent compound (O8) in APEX1 cleavage assay (Supplementary Figure 3). Accordingly, it was revealed that O8 prevents APEX1-mediated digestion of AP-site substrates, suggesting a different mechanisms of action for the compounds described by Donley and coworkers.

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