Neil DNA glycosylases promote substrate turnover by Tdg during DNA demethylation

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DNA 5-methylcytosine is a dynamic epigenetic mark with important roles in development and disease. In the Tet-Tdg demethylation pathway, methylated cytosine is iteratively oxidized by Tet dioxygenases, and unmodified cytosine is restored via thymine DNA glycosylase (Tdg). Here we show that human NEIL1 and NEIL2 DNA glycosylases coordinate abasic-site processing during TET-TDG DNA demethylation. NEIL1 and NEIL2 cooperate with Tdg during base excision: Tdg occupies the abasic site and is displaced by NEILs, which further process the baseless sugar, thereby stimulating TDG-substrate turnover. In early Xenopus embryos, Neil2 cooperates with Tdg in removing oxidized methylcytosines and specifying neural-crest development together with Tet3. Thus, Neils function as AP lyases in the coordinated AP-site handover during oxidative DNA demethylation.

DNA 5-methylcytosine (5mC) is an epigenetic mark that has important regulatory roles in development and is involved in disease1,2. It has become clear that DNA methylation in animal cells is reversible by an enzymatic demethylation process involving oxidation of 5mC by the Ten-eleven translocation (Tet) family of dioxygenases3–7. Tet enzymes iteratively oxidize 5mC, thus forming 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). The oxidation products act as intermediates in DNA demethylation by both active and passive modes (reviewed in refs. 8,9). The active mode involves Tdg, which recognizes and excises higher-oxidation products and initiates downstream base excision repair (BER), thereby restoring unmodified cytosines5,10. This Tet-Tdg demethylation pathway is involved in gene-specific, rather than global, DNA demethylation. Tdg-null mice die around embryonic day 12.5 and show a modest 5mC increase at CpG-rich gene promoters11,12. Base-resolution sequencing of Tdg-knockdown around embryonic day 12.5 and show a modest 5mC increase at CpG-rich islands.

The early discovery of a role for Tdg in active DNA demethylation has suggested that the enzyme might promote DNA demethylation through direct excision of 5mC16. However, Tdg processes 5fC and 5caC, but not 5hmC or 5mC5,10,11. Tdg acts as a monofunctional DNA glycosylase, cleaving the N-glycosidic bond between the base and the sugar at 5fC and 5caC residues within double-stranded DNA and thus generating abasic (apryrimidinic, AP) sites. It has been assumed that the AP-site intermediate is subsequently processed by the canonical BER pathway: AP endonuclease 1 (Apex1) cleaves the phosphodiester bond, thus generating a 3′-OH and 5′-deoxyribose phosphate; DNA polymerase β removes the sugar phosphate moiety and incorporates an unmodified cytosine; and DNA ligase I or IIIα seals the nick17. However, determination of which BER components indeed function in the Tet-Tdg pathway has remained unexplored.

DNA-repair intermediates are often unstable and can cause genomic instability, cell-cycle arrest, cell death or cell transformation. DNA-repair pathways build, such that during each processing step the intermediates are sequenced and protected by the appropriate repair enzyme and are thereby passed along like a baton from one enzyme to the next in a coordinated, sequential fashion18,19. During BER, the AP site is a vulnerable intermediate, which if unprotected is unstable, mutagenic and cytotoxic. The cellular risks associated with AP-site intermediates are accentuated in Tet-Tdg demethylation, in which parallel processing of two abasic sites in a homomethylated mCpG dyad may generate double-strand breaks, and moreover demethylation may occur in 5mC tandem arrays in CpG-rich islands.

In addition to Tdg, the endonuclease VIII–like family of DNA glycosylases (Neil1–3) has been implicated in Tet-mediated DNA demethylation. Neils are bifunctional enzymes that excise the damaged base and introduce a DNA strand break via their AP lyase activity20,21. Neil1 and Neil3 were identified as potential binders for oxidized 5mC derivatives22. Overexpression experiments have suggested that NEILs function as alternative DNA glycosylases to TDG in the excision of 5fC and 5caC23, apparently in agreement with the reported preference of NEILs for repairing oxidized bases, including 5-hydroxymethylcytosine, thymine glycol and 8-oxoguanine20,21,24–29. However, their requirement for processing of Tet oxidation products and their mode of action in DNA demethylation are unknown.

We conducted a screen to search for new components of DNA demethylation and identified NEIL1 and NEIL2. Unexpectedly, our biochemical analysis revealed that NEIL enzymes neither bind nor process oxidized methylcytosines directly. Instead, they cooperate with Tdg in AP-site processing. NEIL1 and NEIL2 displace Tdg...
from the AP site and cleave the baseless sugar, thereby overcoming TDG product inhibition and accelerating 5fC and 5caC turnover. Knockdown experiments in early *Xenopus* embryos corroborated that Neil2, together with Tdg and Tet3, is required for the removal of genomic 5fC and 5caC and for neural-crest development. Neils are hence involved as AP lyases in the coordinated substrate handover during oxidative DNA demethylation.

**RESULTS**

**NEIL1 and NEIL2 are required for removal of 5fC and 5caC**

To identify new components of DNA demethylation, we first analyzed the ability of HeLa cells to demodify oxidized methylcytosines. When we incubated HeLa whole cell extracts with 160-bp oligonucleotides containing a single modified cytosine residue, we found that they replaced 5fC and 5caC efficiently with unmethylated cytosine. In a DNA demodification assay, we monitored replacement of cytosine derivatives by unmethylated cytosine according to the gain of HpaII sensitivity, which resulted in the presence of a 79-nt product (Fig. 1a,b). In contrast, oligonucleotides containing 5mC remained unprocessed, and 5hmC was only marginally converted. The demodification reaction was sequence-context independent and also proceeded with an oligonucleotide of unrelated sequence (**Supplementary Fig. 1a,b**). Whole cellular extracts might not mimic the *in vivo* repair scenario. Under these conditions, however, demodification of 5fC and 5caC oligonucleotides showed characteristics of short-patch BER: (i) the reaction without HpaII digestion (demodification intermediate assay) was accompanied by the occurrence of 79-nt and 80-nt products, corresponding to the expected cleaved AP site (79 nt) and incorporated single cytidine with unligated 3'-OH (80 nt) intermediates, respectively (Fig. 1c); (ii) the demodification reaction was insensitive to aphidicolin, an inhibitor of replicative DNA polymerases (**Fig. 1d,e**).

To determine which of the 11 human DNA glycosylases that mediate BER are involved in 5fC and 5caC processing, we down-regulated their expression with short interfering RNAs (siRNAs) and carried out DNA glycosylase assays (Fig. 2a,b and **Supplementary Fig. 1c**). Knockdown of *TDG* robustly inhibited 5fC and 5caC removal (Fig. 2c and **Supplementary Fig. 2a**), in agreement with the notion that this DNA glycosylase has a central role in DNA demethylation. In addition, knockdown of the DNA glycosylases NEIL1 and NEIL2 inhibited 5fC and 5caC excision, notably after combined knockdown (Fig. 2d and **Supplementary Fig. 2b**).
NEIL3 was negative in this assay, and it differs from NEIL1 and NEIL2 in substrate specificity and structural features as well as by acting mainly as a monofunctional DNA glycosylase. Expectedly, TDG knockdown and combined NEIL1 and NEIL2 knockdown also inhibited the demethylation of 5fC and 5caC modified oligonucleotides (Fig. 2e and Supplementary Fig. 2c).

Tdg deficiency in mouse embryonic stem cells (mESCs) causes a five- to ten-fold increase in genomic 5fC and 5caC abundance. If NEIL1 and NEIL2 function in 5fC and 5caC removal, these bases should accumulate when NEILs are depleted. Indeed, in HeLa cells, knockdown of TDG, NEIL1 and NEIL2 increased the genomic levels of TET1-induced 5fC and, except in the case of NEIL2 depletion, also 5caC (Fig. 2f). Global 5hmC showed a moderate increase, whereas 5mC levels were unaffected, as expected, because TDG is involved in gene-specific rather than global DNA demethylation.

NEIL2 is required for TCF21 DNA demethylation

Recently, we have shown that transcriptional activation of the tumor suppressor TCF21 is directed by the long noncoding RNA TARID and is accompanied by TET-TDG–mediated DNA demethylation. TCF21 is silenced and hypermethylated in the cancer cell line HNO387 but can be demethylated and activated by ectopic TARID expression (ref. 32 and Fig. 3b,c). To determine whether NEIL1 and NEIL2 are required for gene-specific demethylation of the TCF21 locus, we monitored the expression levels and promoter methylation of TCF21 after depletion of NEIL1 and NEIL2 in HNO387 cells. NEIL2 showed strong expression, whereas NEIL1 was scarcely expressed in HNO387 cells (Fig. 3a). Interestingly, TARID-triggered TCF21 expression was blocked by knockdown of NEIL2 but not NEIL1 (Fig. 3b). Moreover, TARID-induced demethylation of the promoter CpGs was impaired when NEIL2 was downregulated (Fig. 3c). Again, knockdown of NEIL1 had no effect, as expected from its low expression level. Overexpression of TDG partially reversed the inhibition of TCF21 induction and the promoter hypermethylation resulting from NEIL2 knockdown (Fig. 3d,e). We conclude that NEIL2 is required for TET-TDG–mediated gene-specific demethylation of TCF21.

NEIL1 and NEIL2 promote TDG-mediated 5fC and 5caC excision

Because NEIL DNA glycosylases excise oxidized bases and have been reported to bind 5mC derivatives, our findings initially supported a model whereby NEILs act redundantly with TDG as DNA glycosylases, which directly excise 5fC and 5caC from DNA. To test this, we purified recombinant TDG, NEIL1 and NEIL2 (Supplementary Fig. 3a) and carried out in vitro DNA glycosylase assays. As previously...
reported\textsuperscript{5,10}, TDG but not the catalytically inactive point mutant TDG\textsuperscript{N140A} effectively excised 5fC and 5caC residues from oligonucleotides (Fig. 4a). Surprisingly, recombinant NEIL1 and NEIL2 showed no excision of 5fC and 5caC from double- or single-stranded DNA (Fig. 4b). However, they exhibited robust glycosylase activity toward their known substrate 5-hydroxycytosine\textsuperscript{21} and showed AP lyase activity (Supplementary Fig. 3b–d), thus indicating that the recombinant enzymes were active.

Furthermore, we carried out DNA binding assays with TDG, NEIL1 and NEIL2, using unmodified and hemimodified oligonucleotides. TDG bound its 5fC and 5caC substrates as well as its product, the AP site, with an affinity several fold higher than that for unmodified DNA (Fig. 4c). Low-affinity binding of TDG to unmodified DNA has previously been interpreted as a lesion-scanning mode\textsuperscript{33}. Interestingly, NEIL1 and NEIL2 did not discriminate between unmodified and 5fC- and 5caC-containing oligonucleotides (Fig. 4d.e). Interestingly, NEIL1 bound AP site–containing oligonucleotides with a three-fold-higher affinity than that for unmodified DNA, whereas NEIL2 exhibited an overall high DNA binding affinity (apparent \(K_d\) of 27 nM on unmodified DNA). Binding of NEILs was cooperative toward all ligands, a result indicative of a switch-like mode of binding and release. We conclude that purified NEIL1 and NEIL2 neither process nor specifically bind 5fC or 5caC.

It seems paradoxical that NEIL DNA glycosylases, given their known preference for oxidized lesions, do not directly bind or process 5fC or 5caC, either in double- or single-stranded DNA. How, then, do NEILs function in 5fC or 5caC, either in double- or single-stranded DNA? How, then, do NEILs function in 5fC or 5caC, either in double- or single-stranded DNA? How, then, do NEILs function in 5fC or 5caC, either in double- or single-stranded DNA? How, then, do NEILs function in 5fC or 5caC, either in double- or single-stranded DNA?

The next general-purpose BER enzyme, APEX1, is in place to proceed with strand cleavage. APEX1 is thought to displace TDG from AP sites and thereby to enhance TDG turnover\textsuperscript{34,37}. Interestingly, NEIL1 and NEIL2 were able to substitute for APEX1 in this context: in the presence of NEIL1 or NEIL2, TDG processed 5fC and 5caC with enhanced steady-state turnover, notably toward 5caC (Fig. 5b and Table 1).

In agreement with the ability of NEIL1 and NEIL2 to replace APEX1, TDG stimulation by recombinant APEX1 was slightly lower than that by NEIL (Supplementary Fig. 4b compared to Fig. 5a). Moreover, APEX1 knockdown did not increase the genomic levels of TET1-induced 5fC and 5caC (Supplementary Fig. 4c), unlike knockdown of NEIL1 and NEIL2. Finally, APEX1 knockdown only marginally inhibited the excision of 5fC and 5caC in a glycosylase assay and the processing of 5fC and 5caC in a demodification assay (Supplementary Fig. 4d–f).

Recombinant TDG and NEIL proteins directly bound each other with moderate affinity in microscale thermophoresis assays (apparent \(K_d\) of 110–380 nM; Fig. 5c and Supplementary Fig. 5a,b). The affinity of NEIL1 and NEIL2 toward an AP-site oligonucleotide was one order of magnitude higher (apparent \(K_d\) of 18 nM and 24 nM, respectively; Fig. 3d,e) and was similar to that of TDG toward an AP site (apparent \(K_d\) 21 nM; Fig. 4c). The moderate NEIL-TDG interaction in combination with high-affinity AP-site binding may allow TDG to recruit NEILs, which then displace TDG from the AP site. Indeed, NEIL1 and NEIL2 effectively displaced TDG from an AP-site oligonucleotide, with near-equimolar stoichiometries (Fig. 5d).

Collectively, these results support a model wherein TDG and NEILs act in the coordinated AP-site handover during the processing of 5fC or 5caC (Fig. 5e). TDG hydrolyzes the modified base and becomes displaced by NEIL1 or NEIL2, whose lyase activity cleaves the DNA backbone, thus making the displacement irreversible. The resulting single-nucleotide gap, if bearing 3′- and 5′-phosphate termini, is probably processed by polynucleotide kinase, DNA polymerase \(\beta\) and DNA ligase I\(\mathrm{III}\)\(\alpha\) (ref. 38) to yield demethylated cytosine.

**Neil2 is required for neural-crest development in Xenopus**

Tet and Tdg are essential for vertebrate embryogenesis\textsuperscript{1,12,39,40}. To study the physiological relevance of Neil cooperation with Tdg in this context, we used *Xenopus laevis* embryos, in which Tet3 functions
Figure 4 NEIL1 and NEIL2 neither process nor specifically bind 5fC and 5caC in vitro. (a,b) Electropherograms of reaction products from DNA glycosylase assays with 20 nM double-stranded (5fC-G and 5caC-G) and single-stranded (5fC and 5caC) oligonucleotide substrates. Recombinant DNA glycosylase was in ten-fold excess over oligonucleotide substrate (single-turnover conditions). (a) Human TDG (reaction products highlighted in red in ten-fold excess over oligonucleotide substrates. Recombinant DNA glycosylase was single-stranded (5fC and 5caC) oligonucleotide from DNA glycosylase assays with 20 nM of [21x288]npg
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[42x572](with efficiencies shown as a percentage)
TDG (reaction products highlighted in red (single-turnover conditions). (b) Human NEIL1 and NEIL2. Data are representative of three independent experiments. (c–e) DNA binding assays with TDG (c), NEIL1 (d) and NEIL2 (e). Fitted curves, calculated dissociation constants ($K_d$) and Hill coefficients ($n_H$) derived from electrophoretic mobility shift assays toward indicated double-stranded oligonucleotides are shown. Binding assays are representative of two independent experiments with similar outcomes.

in early development, notably neural-crest specification, as shown by antisense morpholino (MO) oligonucleotide injection. In early Xenopus embryos neil1 expression was low during early development and increased during organogenesis, as assessed by quantitative PCR (qPCR) analysis (Supplementary Fig. 5a). Conversely, neil2 was expressed only during early development, maternally and in neurulae (Supplementary Fig. 6a). Injection of MO oligonucleotides against neil2, tdg or tet3 (ref. 40) induced a very similar phenotype, with malformed heads and several neural-crest derivatives (pharyngeal pouches, dorsal and tail fins) either reduced or missing (Fig. 6a–b). Control and neil1 morphants were normal, and co-injection of neil1 MO did not markedly enhance the neil2 MO phenotype (data not shown). neil2 morphants showed no enhanced apoptosis or reduced cell proliferation (Supplementary Fig. 6b–c). The neil2, tdg and tet3 MO phenotypes were specific because (i) they were rescued by mRNA co-injection of the respective human or Xenopus tropicalis homologs (Fig. 6a–b); (ii) a second MO against a nonoverlapping neil2 sequence (neil2 MO2) yielded the same neural-crest-deficiency phenotype as did neil2 MO (Supplementary Fig. 6d); (iii) in neil2 morphants, neil2 expression was induced over ten-fold (Supplementary Fig. 6e), thus suggesting that a specific cellular mechanism senses Neil2 protein reduction and attempts to compensate. The specificity of tet3 morphants has been established previously, and we were able to confirm the phenotype.

In agreement with a neural-crest defect, neil2, tdg and tet3 morphants showed downregulation of the neural-crest markers sox10, twist (official symbol twist1-a) and slug (official symbol snai2-a) on the injected side of unilaterally injected embryos, whereas en2 (midbrain; official symbol en2-a) and krox20 (hindbrain; official symbol egr2) were unaffected (Fig. 6c–d), with the exception of krox20, which was reduced by tet3 MO. The requirement of Neil2, Tdg and Tet3 for neural-crest specification was direct and not indirect via inducing mesoderm: neural crest can be directly formed from Xenopus animal caps without mesoderm by using combined injection of noggin (official symbol nog) and wnt8a mRNA injection. In this regime, neil2, tdg and tet3 MO inhibited expression of sox10, slug and twist but not N-cam (pan-neural; official symbol ncam1-a) (Supplementary Fig. 6e). We conclude that Neil2, Tdg and Tet3 are required for Xenopus neural-crest specification.

Neil2 is required for 5fC and 5caC removal in Xenopus
We tested whether 5fC and 5caC residues accumulate concomitantly with Neil depletion in vivo. We first established quantitative monitoring of genomic 5mC, 5hmC, 5fC and 5caC by LC-MS/MS in Xenopus embryos. Tadpole-stage embryos had a high genomic level of 5mC (~6%), two-fold higher than the levels in HEK293T cells and mESCs (Supplementary Fig. 7a). 5mC levels were intermediate between those of HEK293T cells and mESCs, whereas 5fC and 5caC levels, which are characteristically elevated in mESCs, were both even higher in Xenopus embryos. Importantly, the levels of total genomic 5fC and 5caC in Xenopus animal caps were elevated by neil2 MO, were further increased by
combined neil1 and neil2 MOs, and were unaffected by neil1 MO alone (Fig. 7a). The levels of 5mC, 5hmC, 5fC-G (Fig. 7a) and the oxidative lesion 8-oxoguanine (8oxoG; Supplementary Fig. 7b) were unaffected, thus supporting the specificity of the MO effect. Moreover, the increase of 5fC and 5caC levels after injection with neil2 MO was effectively decreased by TDG mRNA co-injection (Fig. 7b). Conversely, knockdown of tdg expectedly increased 5fC and 5caC, and human NEIL2 mRNA co-injection was able to partially compensate for tdg knockdown, thus suggesting that residual Tdg activity is enhanced by excess NEIL2. In line with incomplete tdg knockdown, combined tdg and neil2 MOs further increased 5fC and 5caC (Fig. 7c). This functional cooperation between neil2 and tdg also manifested phenotypically: injection of subthreshold MO doses, which by themselves yielded normal embryos, induced malformations when neil2 was combined with tdg or tet3 MOs (Fig. 7d,e). This experimental system is analogous to synthetic lethality in combined mutants, an indicator of functional interaction. In summary, in Xenopus embryos Neil2 cooperates with Tdg in processing 5fC and 5caC residues and in specifying neural-crest development together with Tet3. These results, however, do not rule out a function of Tdg and Neil2 in two parallel pathways for 5fC and 5caC processing in vivo.

**DISCUSSION**

Neils function in the coordinated processing of 5fC and 5caC

Propositions of repair-based demethylation mechanisms have been controversial because of the perceived risk of genomic instability, notably for homomethylated mCpGs in tandem arrays. Our study reveals a cooperation of human NEIL1 and NEIL2 with TDG, which functions in the coordinated processing of 5fC and 5caC. TDG is a product-inhibited

**Table 1 Rate constants of TDG in the absence or presence of NEIL1 or NEIL2**

|                  | 5fC-G   | 5caC-G   |
|------------------|---------|---------|
| **5fC-G**        |         |         |
| Pre–steady state | 0.14 ± 0.04 | 0.0038 ± 0.0004 |
| steady-state     | 0.0038 ± 0.0004 | 0.0000 ± 0.0000 |
| base release     | 0.0038 ± 0.0004 | 0.0000 ± 0.0000 |
| min⁻¹             |         |         |
| **5caC-G**       |         |         |
| Pre–steady state | 0.10 ± 0.03 | 0.0252 ± 0.0011 |
| steady-state     | 0.0252 ± 0.0011 | 0.0000 ± 0.0000 |
| base release     | 0.0252 ± 0.0011 | 0.0000 ± 0.0000 |
| min⁻¹             |         |         |

Rate constants are calculated from multiple-turnover kinetics. Rate-constant errors are calculated technical errors derived from the curve fittings in Figure 5b.
enzyme whose turnover is slow. These features may protect against the mutagenic and cytotoxic properties of AP sites, which inhibit certain DNA polymerases, lack base-pairing information during replication and can lead to strand breaks42. APEX1 is thought to cooperate with TDG by stimulating its turnover for a variety of mismatches and lesions44,37. Here we show that NEIL1 and NEIL2 can also promote TDG product release. NEIL1 and NEIL2 bind with high affinity to DNA containing AP sites and therefore can compete with TDG for AP-site binding. Displacement of TDG by NEIL1 and NEIL2 gains directionality by the NEIL lyase activity. Thus, our study shows that Neil DNA glycosylases act in the coordinated substrate handover of the vulnerable AP-site intermediate from Tdg. A similar Apex1-bypass reaction has been demonstrated in cell free assays, in which NEIL1 enhances the activity of OGG1 DNA glycosylase in the removal of 8oxoG48,43. Our data do not exclude involvement of Apex1 in Tet-Tdg DNA demethylation; for example, Apex1 may process the intermediate of the Neil AP lyase reaction, the 3'-phospho-α,β-unsaturated aldehyde44.

What may be the relevance of the Apex1-bypass reaction? Neils may act preferentially on actively transcribed genes together with Tet and Tdg. NEIL2 associates with RNA polymerase II and binds to transcribed but not transcriptionally silent genes45. Hence, this preference may reflect the involvement of Neils in not only removing transcription-blocking lesions but also in processing AP sites during Tet-Tdg gene activation. Along these lines, NEILs bind to TET proteins23, in agreement with a recruitment of Neils to sites of DNA demethylation. Hence, Neils may act specifically in the context of epigenetic gene activation but not in universal lesion processing by TDG34,37. In Arabidopsis, for example, the Apex1 homolog APE11 functions in DNA demethylation downstream of the DNA glycosylases ROS1 and DME1,46,47.

TDG stalling at AP sites can also be overcome by SUMOylation, which potentiates the stimulatory effect of APEX1 (and presumably also NEIL1 and NEIL2) on TDG34,36. Further studies are needed to explore how these mechanisms are integrated in Tet-Tdg–mediated DNA demethylation.

A role for the Tet-Tdg-Neil module in neural-crest formation

Our analysis in Xenopus embryos provides evidence that interplay between Tet, Tdg and Neil2 is of physiological relevance for neural-crest formation during early development. In addition, Tdg and Neil2 cooperate and are necessary to maintain normal levels of 5C and 5CtC in vivo. An unanswered question is whether these two biological roles of Tdg
Figure 7  Neil2 is required for 5fC and 5caC removal in *Xenopus* embryos and cooperates with Tet3 and Tdg. (a) LC-MS/MS analysis of genomic 5mC, 5hmC, 5fC and 5caC levels in *neil*-morphant animal-cap explants at stage 32. Error bars, s.d. (n = 4 explant batches, each consisting of 20 animal-cap explants). Gray, controls; black, morphants. (b) LC-MS/MS analysis as in a with control MO (blue)- and *neil2* MO (red)-injected animal caps with or without co-injection of human Tdg mRNA as indicated. Error bars, s.d. (n = 3 explant batches, each consisting of 20 animal-cap explants). (c) LC-MS/MS analysis as in a, but in control MO (green)- and *tdg* MO (orange)-injected animal caps (without or with co-injection of human *NEIL2* mRNA and *neil2* MO as indicated. Error bars, s.d. (n = 3 explant batches, each consisting of 20 animal-cap explants). (d) Functional cooperation of *neil2*, *tdg* and *tet3* in *X. laevis* embryonic development. Subthreshold doses of *neil2*, *tdg* or *tet3* morpholinos (10 ng/embryo) were singly injected with control MO (10 ng/embryo; top) or in combination (bottom) as indicated, to keep the total MO dose in all samples constant. Embryos were fixed at stage 34. Scale bars, 200 μm. (e) Quantification of embryo malformations shown in d (n > 30 embryos per group; details in source data). *P < 0.05; ***P < 0.005 by two-tailed unpaired Student’s t test.

and Neil2—neural-crest formation and 5fC and 5caC processing—are linked. It is generally difficult to pinpoint the role of genes with multiple functions and functional redundancies in vivo. For example, Tet proteins function not only enzymatically but also nonenzymatically in transcriptional regulation. Moreover, 5hmC, 5fC and 5caC may act not only as demethylation intermediates but also as epigenetic marks in their own right, because they are recognized by specific readers. Thus, it is unclear whether the Tet-mutant phenotypes are due to DNA hypermethylation, to failure to set oxidized-methylcytosine epigenetic marks or to defects in transcription. In addition, Tet1 and Tet2 single-mutant mice are viable, whereas a fraction of Tet1 and Tet2 double-knockout mice die perinatally with developmental abnormalities, results indicative of functional redundancy. Tet3 single mutants die perinatally, and triple mutants have not been reported. Similarly, Tdg functions in Tet–Tdg demethylation but is also a generic mismatch DNA repair enzyme. Moreover, like Tets, Tdg can also act nonenzymatically as a scaffold protein that recruits the transcriptional coactivator CBP (p300) to numerous transcription factors. Thus, although Tdg-knockout mice die in utero, the etiology is unclear.

Given the similar complexities in *neil2* morphants, it is difficult to know whether the observed neural-crest defects are due to reduced processing of 5fC and 5caC by Tdg. For example, we cannot exclude that Neil2 functions primarily as a lesion-repair DNA glycosylase and that the neural crest may be a particularly sensitive organ in this respect. However, the observations that (i) *tet3*, *tdg* and *neil2* morphants showed a similar phenotype affecting the neural crest, (ii) *neil2* morphants did not exhibit substantial cell-cycle arrest or apoptosis and (iii) combination of subthreshold MO doses cooperated in producing malformed embryos suggest an at least partially shared role for these enzymes in development, which is related to gene-specific 5fC and 5caC processing.

We note that in mice, *Neil1* and *Neil3* mutants also show neural defects, albeit in adults, whereas *Neil2* mutants appear overtly normal but are hyperresponsive to inflammation. The observed mouse phenotypes have been attributed to lesion-repair deficits. Our results provide a mechanistic and physiological framework for revisiting mouse Neil mutants to study the role of the enzymes in the context of DNA demethylation, with its many exciting biological facets ranging from ES-cell differentiation and reprogramming to cancer.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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ONLINE METHODS

Cell culture and transfection. HeLa, HEK293T (ATCC numbers CCL-2 and CRL-11268) and head and neck cancer (HNO387) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Lonza) supplemented with 10% Fetal Bovine Serum Gold (FBS, PAA), 2 mM l-glutamine (Lonza), and 100 U/ml penicillin-streptomycin (PEN-STREP, Lonza) at 37 °C in 5% CO2. All cell lines tested negative for mycoplasma contamination.

Cells were transiently transfected with siRNAs with Lipofectamine 2000 (Life Technologies) and with plasmid DNAs with Turbofect (Thermo Scientific) according to the manufacturer’s instructions. siRNA knockdown efficiencies were routinely assessed by qPCR and were at least 60%. Transfection of TDG shown in Figure 3d.e yielded approximately ten-fold overexpression, as tested by qPCR (data not shown).

Mouse E14TG2a (ATCC number CRL-1821) embryonic stem cells (mESCs) were cultured on plates coated with 0.1% gelatin (Millipore) in DMEM supplemented with 15% PANSera ES FBS (PAN Biotech), 2 mM l-glutamine, 100 μM nonessential amino acids (NEAA, Gibco), 1 mM sodium pyruvate (Gibco), 100 μM 2-mercaptoethanol (Sigma), 1,000 U/ml leukemia inhibitory factor (LIF, Milipore), and 100 U/ml PEN-STREP at 37 °C in 5% CO2 and 5% O2.

Reverse transcriptase–coupled quantitative real-time PCR (RT-qPCR). Total RNA was prepared with an RNeasy mini kit (Quagen) including an on-column DNase digestion according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized with SuperScript II reverse transcriptase (Life Technologies). Quantitative real-time PCR was performed on a LightCycler 480 (Roche) in technical duplicates with the Universal ProbeLibrary technology (Roche) including the supplier’s LightCycler 480 Probes Master. Quantitative analysis was performed with LightCycler 480 software (Roche). Primer sequences and hydrolysis probe numbers are listed in Supplementary Table 1.

Expression constructs. Human NEIL1, NEIL2 and APEXI cDNAs (BC010876.1, BC019364.2 and BC008145.1, respectively) were from the ORFeome clone collection. Human TDG and mutant TDG140A cDNAs (plasmids pRS210 and pRS211, respectively, encoding both N-terminally HA-tagged proteins) were a kind gift from P. Schar, Department of Biomedicine, University of Basel, Basel, Switzerland. TDG, TDG140A, NEIL1, NEIL2 and APEXI cDNAs were inserted into pET-24a(+) and/or pET-28a(+) (both from Novagen) encoding C-terminally (TDG, TDG140A, NEIL1 and NEIL2) and N-terminally (NEIL1, NEIL2 and APEXI) hexahistidine-tagged proteins for gene expression in Escherichia coli. TDG, NEIL1 and NEIL2 cDNAs were inserted into pCS2FLAG (Addgene plasmid 16331) encoding N-terminally (TDG) and C-terminally (NEIL1 and NEIL2) 2×FLAG-tagged proteins. Additionally, NEIL1 and NEIL2 cDNAs were inserted into pCS2HA (Addgene 16330) as C-terminal HA-tag expression constructs. Plasmid pXC1010 containing N-terminally hexahistidine-SUMO-tagged N. gruberi Tdt1 (ref. 58) was a kind gift of Y. Zheng (New England BioLabs, Ipswich, Massachusetts, USA). Human TET1 catalytic domain (aa 1418–2136) was cloned from FH-TET1-pEF4 containing full-length human TET1 cDNA (a kind gift from A. Rao, Division of Signaling and Gene Expression, La Jolla Institute for Allergy and Immunology, La Jolla, California, USA, Addgene plasmid 49792) into pCS2FLAG as an N-terminal 2×FLAG-tag expression construct. X. tropicalis tet3 cDNA was from Open Biosystems (MXT1765-99235490).

Protein expression and purification. E. coli BL21-CodonPlus(DE3)-RIL cells (Strategene) were transformed with the respective expression construct. Cultures were grown at 37 °C to an OD600 of 0.6, induced with 1 mM isopropyl β-D- thiogalactopyranoside (IPTG), and cultivated at 16 °C overnight. Pelleted cells were resuspended in lysis buffer (25 mM HEPES-KOH, pH 7.6, 0.5 M NaCl, and 40 mM imidazole) and lysed by passage through a Constant Systems LTD cell disruptor (1.8 kbar, constant run). Cell debris was pelleted by centrifugation at 38,400g for 30 min, and the supernatant was subjected to a Ni2+-charged chelating Sepharose Fast Flow column (GE Healthcare). Bound proteins were eluted by a stepwise gradient of imidazole (60–500 mM) in lysis buffer. Fractions containing enriched target proteins were united, desalted with HP buffer (20 mM HEPES-KOH, pH 7.6, and 5 mM β-mercaptoethanol) and applied to a HiTrap Heparin HP column (GE Healthcare). Bound proteins were eluted with a linear gradient of 0–1.5 M NaCl in HP buffer. Fractions containing pure protein were concentrated with Vivaspin ultracentrifugation devices (GE Healthcare). Concentrations of proteins were determined by Bradford assay (Bio-Rad) with BSA as standard.

DNA substrates. 160-bp double-stranded oligonucleotides, in which one strand contained both a single modified CpG dinucleotide and a 5′-end fluorescent label, were individually prepared, purified and annealed. 160-nucleotide single-stranded oligonucleotides were prepared by ligation of three oligonucleotides (73-mer, 12-mer and 75-mer; sequences in Supplementary Table 1) with T4 DNA ligase (NEB), according to the manufacturer’s instructions, in the presence of a complementary splint oligonucleotide. Ligation products were PAGE purified on a 14% denaturing polyacrylamide gel (7 M urea and 1× TBE) and subjected to ethanol precipitation. Purified DNA strands were hybridized to 160-bp substrates with 500 nM fluorescently labeled strand and 750 nM complementary strand in 1× SSC. Substrates for multiple-turnover kinetics were hybridized with 10 μM fluorescently labeled 20-mer and 15 μM complementary strand in 1× SSC. Oligonucleotide sequences are shown in Supplementary Table 1.

Activity assays. Demodification assay. HeLa whole cell extracts were prepared by lysis in 10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, and 1% Nonidet P 40 (Sigma), and cleared at 600g for 5 min. Concentrations of cell extracts were determined by Bradford assay (Bio-Rad) with BSA as standard. Demodification assays were performed in 50-μl reactions containing 50 μg cell extract and 20 nM double-stranded 160-bp modified oligonucleotide substrate in 40 mM HEPES-KOH, pH 7.8, 70 mM KCl, 5 mM MgCl2, 10 μM ZnCl2, 0.5 mM DTT, 2 mM ATP, 20 mM dNTPs, 20 mM phosphocreatine, 2.5 μg creatine phosphokinase (type I, Sigma C7357), and 5 μg salmon sperm DNA for 3 h at 37 °C. Reactions were stopped by phenol/chloroform extraction and ethanol precipitation of the DNA. Reaction products were in incubated with 5 U HpaII (Promega) in 7.5 μl for 1 h at 37 °C, or were mock treated (demodification intermediate assay). Reactions were stopped by addition of 2.5 μl loading dye (95% formamide, 20 mM EDTA, pH 8.0, and 10 mg/ml dextran blue), and reaction products were heat denatured and subjected to denaturing 4% PAGE analysis with an Applied Biosystems ABI Prism 377 DNA Sequencer according to the manufacturer’s instructions. Peak areas were quantified with GeneScan Analysis (Applied Biosystems).

Small-scale siRNA screen. HeLa cells were harvested 48 h after siRNA transfection, and whole cell extracts were prepared as described above. DNA glycosylase assays were performed with 50 μg cell extract and 20 nM 160-bp DNA substrate in 10 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10 mM EDTA, and 0.5 mM DTT in a total volume of 50 μl for 60 min at 37 °C. Reactions were stopped, and DNA was isolated as described above. To generate strand breaks at AP sites, reaction products were incubated with 5 U recombinant APEXI (M0282, NEB) for 30 min at 37 °C in a total volume of 10 μl. Enzymatic strand cleavage by APEXI instead of alkali treatment of the reaction products proved necessary because DNA substrates containing 5C or 5CaC residues showed substantial strand cleavage after being boiled in 150 mM NaOH even in the absence of any prior enzymatic incubation (data not shown). After strand cleavage, 2.5 μl loading dye was added, and reaction products were heat denatured and subjected to electrophoresis as described above. Reactions were stopped by addition of 2.5 μl loading dye, and reaction products were heat denatured and subjected to gel electrophoresis as described above.

Glycosylase assays with purified proteins. Single-turnover reactions with TDG, NEIL1 and NEIL2 were performed with 200 nM enzyme and 20 nM 160-bp substrate in glycosylase buffer (10 mM HEPES-KOH, pH 7.4, 1 M trimethyramine-N-oxide52, 100 mM KCl, 10 mM EDTA, 0.5 mM DTT, and 100 μg/ml BSA) for 20 min at 37 °C. Stimulation of TDG excision was carried out with 6 mM TDG alone or in combination with 30 nM NEIL1 or NEIL2 and 100 nM of 160-bp substrate in glycosylase buffer for 2 h at 37 °C. Control reactions with mutant TDG140A, SMUG1 and APEXI were performed essentially as described above. Multiple-turnover kinetics were conducted with 150 nM TDG alone or in combination with 20 nM NEIL1 or 75 nM NEIL2 and 1 μM 20-bp DNA substrate (sequences in Supplementary Table 1) in glycosylase buffer at 37 °C for up to 3 h. For stimulation assays, the active enzyme fraction was determined and used. The TDG active fraction was derived from multiple-turnover kinetics41, and NEIL1 and NEIL2 active fractions were determined by AP trapping assay with cyanoborohydride28 (data not shown). On the basis of this assay, only a minor fraction of NEIL1 and NEIL2 protein appeared to be active.
and therefore some contribution of the ‘inactive’ Neil fraction in the TDG stimulation assay cannot be ruled out. Downstream processing of reaction products and analysis was essentially as described above. Data for multiple-turnover kinetics were fitted by nonlinear regression to the following equation with GraFit 7 (Erithacus Software):

\[ A_t = A_{\infty} \left(1 - e^{-kt}\right) + vt \]

where \( A_t \) is amplitude of the burst, \( k \) is the first-order rate constant, \( t \) is time, and \( v \) is the reaction rate in the linear phase.

AP assay. To test the ability of NEIL1 and NEIL2 to incise at AP sites, 20 nM of a 160-bp substrate bearing a single T-G mismatch was incubated with 200 nM TDG in glycosylase buffer for 20 min at 37 °C; this was followed by phenol/chloroform extraction and ethanol precipitation of the DNA. After resuspension, DNA was incubated with 200 nM NEIL1 or NEIL2 (or N-terminally hexahistidine tagged) or 20 nM APEX1 in a total volume of 10 µl for 20 min at 37 °C. Reactions were stopped by addition of 2.5 µl loading dye, and reaction products were heat denatured and subjected to gel electrophoresis as described above.

Electrophoretic mobility shift assays (EMSAs). End-labeling of DNA was performed with 200 nM unmodified 20-mer oligonucleotide with [γ-32P]ATP (PerkinElmer) and T4 polynucleotide kinase (NEB) according to the manufacturer’s instructions. 100 nM labeled DNA was hybridized in SSC buffer (150 mM NaCl and 15 mM trisodium citrate) with 100 nM complementary oligonucleotides bearing either no modification or a single 5fC, 5caC, or a tetrahydrofuran (PerkinElmer) and T4 polynucleotide kinase (NEB) according to the manufacturer. Binding reactions were composed of 50 nM labeled protein and increasing amounts of enzyme in binding buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 100 µg/ml BSA) for 20 min at 4 °C or at room temperature. AP site binding competition assays were performed with 20 pM of an AP site containing double-stranded oligonucleotide, preincubated with 50 µg/ml BSA (control) or 20 nM TDG in binding buffer for 10 min at room temperature before addition of 500 µg/ml BSA (control), 250 nM NEIL1 or 750 nM NEIL2 for another 10 min. PAGE was performed as described previously with 10 % native gel (GE Healthcare), and quantification was performed with ImageQuant TL software (GE Healthcare). For K2 calculation and AP-site competition, the active enzyme fractions were used (described above).

Microscale thermophoresis. For protein-protein interactions, purified TDG, NEIL1 or NEIL2 were labeled with Monolith NT Protein labeling Kit RED-NHS (NanoTemper Technologies), essentially as described by the manufacturer. Binding reactions were composed of 50 nM labeled protein and increasing amounts of unlabeled recombinant NEIL1, NEIL2, TDG or SMUG1 (NEB) in MBT buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM MgCl2, and 0.05% Tween 20) and incubated for 5 min at room temperature (RT). Binding was monitored in hydrophilic capillaries on a Monolith NT.115 (NanoTemper Technologies) with 80% LED power, 60% MST power, 30-s laser-on time and 5-s laser-off time. For microscale thermophoresis experiments to determine high-affinity binding, 30 nM purified EGFP (STA-201, Cell Biolabs) was incubated with increasing amounts of anti-GFP antibody (DLN-07227, Dianova, validated for specificity before microscale thermophoresis) in MBT buffer for 5 min at RT. Binding was conducted as described above with the exception of standard treated capillaries and 100% LED power (Supplementary Fig. 4b). Curve fitings and calculation of the dissociation constants (\( K_d \)) were performed with NT Analysis software (NanoTemper Technologies).

X. laevis embryo manipulation and staining. Animal experiments with X. laevis were approved by state authorities (Landesuntersuchungsamt Rheinland-Pfalz). No blinding or randomization was performed. No statistical method was used to predetermined sample size. Embryos were obtained by in vitro fertilization as previously described. Human and X. tropicalis expression constructs as described above were used as templates to generate mRNAs with the MEGAscript SP6 Transcription kit (Life Technologies) according to the manufacturer’s instructions. Morpholino antisense oligonucleotides (sequences in Supplementary Table 1) were designed to target the start codon of the respective genes. Morpholinos and mRNAs were injected 4 times into animal blastomeres at two- to four-cell stage with a total volume of 10 nl per embryo. Total amounts of single morpholinos injected per embryo were as follows: neil1 MO, 60 ng; neil2 MO and neil2 MO2, 40 ng; tdg MO, 20 ng; and tet3 MO, 60 ng (30 ng per isoform-specific MO). The synergy experiment was performed with reduced doses of morpholinos per embryo: neil2 MO, 10 ng; tdg MO, 10 ng; and tet3 MO, 20 ng. For neil1 and neil2 double knockdown, embryos were injected with a mixture of 20 ng neil1 MO and 40 ng neil2 MO. The control mRNA used for injections was bovine preprolactin. Animal-cap assays were performed as previously described. In brief, the indicated MOs or mRNAs were animalinjected in both blastomeres of two-cell-stage embryos, and the animal caps were dissected from the embryos at stage 8.5, cultivated further and harvested at the equivalent of stage 16 for qPCR analysis or stage 32 for MS analysis. Whole-mount in situ hybridization was performed as previously described. In situ—hybridization probes were generated from cDNAs for X. laevis neuronal marker genes with the Dig RNA labeling kit (Roche). For lineage tracing, lacZ mRNA was co-injected (250 pg/blastomere), and β-gal staining was performed as previously described. X-gal as substrate. TUNEL assays and whole-mount immunostaining of phospho—histone H3 were carried out as previously described.

Mass spectrometry analysis of genomic DNA. Generation and purification of 15N-labeled 5hmC, 5fC and 5caC. Stable isotope labeling of DNA modifications was performed by a series of enzymatic reactions. First, a DNA strand of 83 bp in length was PCR amplified with 15N-labeled dCTP (Silantes) instead of unlabeled dCTP in the reaction mixture. Forward primer, CTCCTCTGACTGAAACCAG; reverse primer, AGGGTCTTGAGCATCCTATGTC; template, CTCCTCTGAG TGAACTACGCGCGTACGTTCAGATCGATTACGTTAATACGATTT CGAACCCGATAGTTGGCAGAGC. The purified PCR product was digested with MspI (NEB) to remove unlabeled primer sequences and was PAGE purified on a nondenaturing 20% polyacrylamide gel. Subsequently, DNA was in vitro methylated with M.SssI (NEB), phenol/chloroform-purified and incubated with purified NgTet1 for 30 min as described previously. Finally, DNA was degraded to nucleosides with nuclease P1 (Roche), snake venom phosphodiesterase ( Worthington) and alkaline phosphatase (Fermentas), essentially as previously described. Individual nucleosides were separated on an Agilent 1290 Infinity Binary LC system (Agilent Technologies) with a ReproSil 100 C18 column (Jasco); Isotopically labeled 5hmC, 5fC and 5caC were identified by analytical HPLC in tandem with triple quadrupole mass spectrometry (Agilent 6490, Agilent Technologies) and purified by preparative HPLC. An aliquot was mixed with known concentrations of corresponding unlabeled 5hmC, 5fC and 5caC nucleosides (Berry & Associates), and the concentrations of 15N-labeled nucleosides were determined after LC-MS/MS by comparing the areas of the labeled and unlabeled compounds.

Genomic-DNA preparation and LC-MS/MS analysis. Genomic DNA was isolated after RNase A (Fermentas) treatment of lysed cells with a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer’s instructions; this was followed by ethanol precipitation with ammonium acetate as salt. For the assessment of genomic 8oxoG levels, DNA was isolated in the presence of desferrioxamine, essentially as previously described. Approximately 1 µg of DNA was degraded to nucleosides with nuclease P1 (Roche), snake venom phosphodiesterase (Worthington) and alkaline phosphatase (Fermentas). An equal volume of isotopic standard mixture ([15N3]5hmC, [15N3]5fC, and [15N3]5caC [TRC], and in house—synthesized [13N]5hmC, [13N]5fC and [13N]5caC, (described above) was added to the DNA, and approximately 100 ng of total DNA was injected for LC-MS/MS analysis. Quantitative analysis was performed on an Agilent 1290 Infinity Binary LC system (Agilent Technologies) with a ReproSil 100 C18 column (Jasco) coupled to an Agilent 6490 triple quadrupole mass spectrometer (Agilent Technologies). Running buffers were 5 mM ammonium acetate, pH 6.9 (A) and acetonitrile (B). Separations were performed at a flow rate of 0.5 ml/min with the following gradient: 0% of solvent B from 0 min to 8 min and linear increase to 15% solvent B for the next 16 min. Washing and reconditioning of the column was performed with a flow rate of 1.0 ml/min with 15% solvent B for one minute and 100% buffer A for additional 5 min. During the last minute, the
flow rate was linearly decreased to the initial value of 0.5 ml/min. The detailed mass spectrometer settings as well as the multiple reaction monitoring (MRM) transitions are listed in Supplementary Table 2.

Quantification of highly abundant C and 5mC was performed with 100×-diluted samples. The data were analyzed with Agilent MassHunter Quantitative Analysis, version B.05.02 (Agilent Technologies) using isotopic standards to confirm the peak identity. Areas of the integrated peaks were exported into Microsoft Excel, with which the areas were normalized to the area of the corresponding isotopic standard. Absolute values for the nucleosides were calculated with linear interpolation from a standard curve. Linear interpolation was performed with the two closely matching data points from the standard curve. Standards were spiked into the mixture of isotopic standards to normalize for ionization variability. The standard curve for every nucleoside was prepared to cover the amount of the corresponding nucleoside in the DNA sample analyzed. The linearity of standard curves over each region was monitored after every run and confirmed to be between 1 and 0.996 (R²-values) within a concentration range of at least two orders of magnitude. The technical s.d. was <7%. Standard curves were newly prepared with every new data set.

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