Parp inhibition prevents ten eleven translocase enzyme activation and hyperglycemia induced DNA demethylation

Short Running Title: Mechanism of Hyperglycemia induced DNA demethylation

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Abstract

Studies from human cells, rats and zebrafish have documented that hyperglycemia induces the demethylation of specific cytosines throughout the genome. Previously, we documented that a subset of these changes become permanent and may provide in part a mechanism for the persistence of complications referred to as the metabolic memory phenomenon. In this report we present studies aimed at elucidating the molecular machinery that is responsible for the hyperglycemia induced DNA demethylation observed. To this end, RNA expression and enzymatic activity assays indicate that the ten-eleven translocation family of enzymes (Tet) were activated by hyperglycemia. Furthermore through the detection of intermediates generated via conversion of 5mC back to the unmethylated form the data were consistent with the use of the Tet-dependent iterative oxidation pathway. In addition, evidence is provided that the Poly-ADP ribose polymerase enzyme’s (Parp) activity is required for activation of Tet activity as the use of a Parp inhibitor prevented demethylation of specific loci and the accumulation of Tet induced intermediates. Remarkably, this inhibition was accompanied by a complete restoration of the tissue regeneration deficit that is also induced by hyperglycemia. The ultimate goal of this work is to provide potential new avenues for therapeutic discovery.
Introduction

Diabetes mellitus (DM) currently affects 285 million individuals world-wide and this is projected to increase to 439 million by 2030 (1). Evidence from both the laboratory (2-7) and large scale clinical trials (8-18) has revealed that diabetic complications progress unimpeded via the phenomenon of “metabolic memory” (MM) even when glycemic control is pharmaceutically reestablished (19;20). Epigenetic mechanisms are the primary method that confer the ability of cells/organs to “memorize” previous environmental conditions and hence are assumed to be significant mechanisms supporting MM. Variations in “normal” DNA methylation are correlated with many aspects of DM including: susceptibility (21-23), insulin resistance (24), diabetes complication development (25), and early detection (26-28). Very recently, a comprehensive genomic DNA methylation profiling of type 2 diabetic islets revealed that CpG loci displayed a significant hypomethylation phenotype and may provide insight on diabetic islets and disease pathogenesis (29). The first report demonstrating a cause and effect relationship between hyperglycemia (HG) and altered DNA methylation documented that genomic hypomethylation was induced within the liver of type 1 diabetic rats as early as 2 weeks post hyperglycemia onset (30). Pirola et al. examined human primary aortic endothelial cells exposed to high glucose (24 hr) in vitro and performed a more comprehensive analysis of both histone acetylation and DNA methylation (31). In this study they observed significant alterations in DNA methylation patterns and showed that induced methylation changes localized to regions within five kilobases of transcriptional start sites. They also observed broad changes to H3K9/K14 acetylation and reported that regionalized hyper-acetylation correlated well with DNA hypomethylation and hyperglycemia-induced gene induction. However these studies did not examine metabolic memory to determine the persistence of these changes.
Recently we have reported the use of a zebrafish model where an induced hyperglycemic state (DM) can be subsequently resolved such that the fish return to euglycemia (MM) (7). This model provides a unique opportunity to examine hyperglycemia-induced changes within a wide variety of tissues as the fish transverse through the normal, DM, and MM states. Importantly, this model is useful for the study of important regulatory systems underlying both the DM and MM states and defining the molecular relationship between the two. We have utilized this model to document that the complications of impaired limb (caudal fin) regeneration and impaired skin wound healing continued following restoration of euglycemia. Moreover, through methylated DNA immuno-precipitation followed by sequencing experiments, we documented that hyperglycemia induces specific CpG island demethylation which persists for a majority of loci in the MM state. When this data was viewed within the context of global gene expression a correlation of CpG island DNA demethylation changes and altered expression was observed. Therefore, the persistence of the HG-induce tissue regeneration capacity correlated directly with induced DNA demethylation and this correlated with persistent gene expression alterations in the MM state. From this we concluded that the epigenetic DNA methylation mechanism may be responsible, in part, for the metabolic memory phenomenon.

Until recently, CpG methylation has been viewed as a stable epigenetic modification that could only be reversed passively through DNA replication, presumably via a reduction of DNA methyltransferase activity. However, this idea has been challenged as active cytosine demethylation is associated with several stages of development, neuronal memory, differentiation of pluripotent stem cells, and several human disorders (32-35). A number of DNA
demethylation mechanisms, that share components, have been proposed and all require further modification of 5mC either at the amine group or the methyl group (Fig. 1) (33;34). In the first of these, the growth arrest and DNA damage inducible (Gadd45) family acts as an adapter to recruit the Aid/Apobec (activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme) complex which deaminates 5mC, converting it to thymine (Fig. 1). The other two proposed mechanisms are initiated by the ten–eleven translocation family (Tet1,2,3) of Fe(II)/2-oxoglutarate-dependent dioxygenases, which oxidize 5mC to produce 5 hydroxymethyl cytosine (5hmC) (36;37). Once 5hmC is formed, two separate pathways may be taken to convert 5hmC back into cytosine. In the first, iterative oxidation by the Tet family of enzymes leads to 5-formyl-cytosine (5fC) followed by 5-carboxy-cytosine (5caC) (Fig. 1). In the second, 5hmC is deaminated by the Gadd45/Aid/Apobec complex to form 5-hydroxymethyl-uracil (5hmU) (38).

In all the mentioned mechanisms, thymine DNA glycosylase (Tdg) [a base excision repair (BER) enzyme] removes the modified base leaving an apurinic/apyrimidinic (AP) site. Concomitantly, when either thymine or 5hmU are to be excised, it appears that Tdg acts in concert with Gadd45 and methyl CpG binding domain protein 4 (Mbd4) (35). Lastly, the AP site is recognized and repaired by either the base excision repair (BER) or nucleotide excision repair machinery resulting in the replacement of an unmodified cytidine (32;34). In this report we demonstrate that the Tet family of enzymes are activated by hyperglycemia which correlates with activated DNA demethylation. Additionally, we provide evidence that the poly ADP-ribose polymerase family of enzymes may initiate the demethylation cascade/s.
Materials and Methods

**Zebrafish Husbandry, Hyperglycemia induction, Fasting Blood Glucose Determination**

All procedures were performed following the guidelines described in “Principles of Laboratory Animal Care” (NIH publication no. 85-23, revised 2011) and the approved IACUC animal protocol 08-19. The maintenance of Zebrafish stocks (*Danio rerio*), the induction of hyperglycemia, and fasting blood glucose determinations (FBGLs) were performed as previously described (7;39;40). Fish were anesthetized by placing them in 1:1000 2-phenoxyethanol for 1-2 minutes. For intraperitoneal injection an insulin syringe with a 28.5 gauge needle was used to deliver 0.3% Streptozotocin (Sigma, S0130) solution in 5mM citrate buffer, pH 5.0 to a dose of 350 mg/kg (70 -150 microliters dependent on weight). Control fish were injected with a like volume of citrate buffer. Although hyperglycemia is detected within 24 hours of the first injection, in order to induce a prolonged state of very high hyperglycemia the zebrafish require a frequent injection induction phase followed by weekly maintenance injections as shown below. Week 1: 3 injections (Day 1,3,5), Week 2: 1 injection (Day 12), Week 3: 1 injection (Day 19), Week 4: (Day 21). Blood glucose levels were determined by fasting a subset of each group for 24 hours prior to blood collection. At this point, the fish were euthanized, and blood was collected following excision of the zebrafish head at the level of the anterior heart. One to two microliters of blood were collected from each fish and glucose levels were determined via the Quantichrom Glucose Assay (DIGL-200, Bioassay Systems) (40).
Parp inhibition protocol and fin regeneration experiments

Poly-ADP ribose polymerase (Parp) activity was inhibited by inter-peritoneal injection of 1,5-isoquinolinediol, (Sigma, I138) which has been previously utilized in DM rat studies (3mg/kg) (41). Several different injection regimens were employed (data not shown) and for the studies presented here it was determined that the best course was to administer the inhibitor (6mg/kg) 24 hours following STZ injection during the induction phase. As such, inhibitor injections were performed at days 2, 4 and 6 relative to the beginning STZ injection as day 1. During the maintenance phase of DM, two injections per week were given at days 10, 13, 17 and 20. The tissue regeneration capacity was determined at day 22 by the procedures that we have previously published (40).

DNA, RNA, and Protein Extractions

In this study, samples were generated from 20 pooled fins and at least three independent samples were prepared for each condition. The DNA and RNA samples were generated as we have previously described (7). Nuclear extracts of proteins were prepared via the EpiQuik Nuclear Extraction Kit (Epigentek, Farmingdale, NY) following the manufacturers protocol without exception. For the initial homogenization step, the samples were placed in a 2 ml dounce homogenizer where 20 strokes with the A pestle was followed by 20 strokes with the B pestle before the remainder of the procedure was performed.

DNA, RNA and Protein assays.

We have previously described the techniques employed for both quantitative RT-PCR and genome wide DNA methylation analysis (7). As in our previous report the DNA methylation
sequencing procedure was performed by Array-Star (Rockville, MD). During preliminary foundation experiments suitable reference genes (Supplemental Table I) were identified and each oligomer pair was tested to ensure that the amplification efficiency approximated 100% (data not shown). In all cases, the three stable reference genes listed were utilized for normalization in each experiment. The ∆∆Ct method (42) was employed to determine the relative expression difference in the experimental samples as compared to controls (43). Each sample was assayed in triplicate (technical replicates) and an average was generated. These values from three samples were utilized to generate the mean for each gene, at each time point and comparison to control samples yielded the fold change reported. The sequence of the primers used for the RT-PCR studies can be seen in Supplemental Table 1. The MethylFlash Hydroxymethylated DNA Quantification Kit (Colorimetric) and MethylFlash 5-Formylcytosine (5-fC) DNA Quantification Kit (Colorimetric) (Epigentek, Farmingdale, NY) were utilized for the quantitation of 5–hydroxymethylcytosine and 5-formyl-cytosine respectively. For the detection of 5hmC, 200 ng of input DNA was used and 500ng of input DNA was used for the detection of 5-fC. As above, each sample was assayed in triplicate (at a minimum) and the 5mC derivative content reported is the average for at least three samples.

The effect of 1,5-isoquinolinediol on the methylation status of several loci we have previously reported (7) was examined via a methylated DNA immunoprecipitation followed by q-PCR (Me-Dip-qPCR). Briefly, triplicate genomic DNA samples of control, DM and DM + 1,5-isoquinolinediol were sonicated with a Branson Sonifier (Fisher Scientific, Pittsburgh, PA) such that greater than 90% of the fragments were < 500bp. The samples were normalized by performing Q-PCR (Syber Green, Life Technologies) with primer pairs to three different genes
(abhd12, uba2 and uhfr1). The samples were considered normalized once Ct values were within experimental error for each of the primer sets. Methylated DNA was then isolated from each normalized sample via immunoprecipitation utilizing the methylminer kit (Life Technologies Corporation, Carlsbad, CA). The capture reaction protocol for 1 ug was followed without exception and the methylated DNA was eluted via the single fraction elution procedure. The resultant DNA was precipitated overnight at -80°C and re-suspended in 60 μL of water. Two μL were used as template in triplicate Q-PCR reactions. The primers utilized are listed as Supplemental Table 2 and were generated from the Zv8 genome build. The reactions were normalized to an 18S reference sequence, the values obtained from triplicate samples were averaged, and the standard error was determined. Examination of Tet enzyme activity was performed by using the Epigenase 5mC-Hydroxylase TET Activity/Inhibition Assay Kit (Colorimetric) (Epigentek, Farmingdale, NY) with the nuclear extracts generated above. Again, each sample was assayed in triplicate at a minimum.

**Statistical Analysis**

Student’s T test and ANOVA analysis was employed where appropriate and indicated in the figure legends.
**Results**

**HG induces the expression of enzymes in the DNA demethylation pathways.**

This study was initiated by examining the HG effects on expression levels of several enzymes reported to play a role in DNA demethylation. Here HG was induced in a group of fish, and within 24 hours fasting blood glucose levels (FBGLs) increased from approximately 60 mg/dL to 120 mg/dL and by one week these levels increased to approximately 315 mg/dL which was maintained throughout the duration of the study. At 24 hours, one, two and three weeks post induction, triplicate samples were used for RNA isolation and gene expression analysis. In all cases where statistical changes existed between control and experiment groups, the p values were p < 0. 001. At 24 hours post induction there were no statistically significant changes in the expression of any of the genes assayed. In contrast the data indicate that HG induces the expression of the tet family and, gadd45 members by 1 week, and these levels remain elevated throughout the experimental time course (Fig.2). The expression of tdg is similar for weeks two and three but no increase was observed after the first week. In contrast, we could not detect transcriptional increases for the abopec family members or the T:G specific binding protein mbd4. To firmly establish a link between HG and tet expression the Tet enzyme activity was determined in protein extracts from control and week 2 DM fish. In control fish samples Tet activity was virtually undetectable (0.0023 +/- 0.0012 ng/min/mg) but this increased dramatically to 1.42 +/- 0.113 ng/min/mg in samples from week 2 DM fish (N = 8, p< 1 E-8). As a result of these data, we examined the contribution that the tet family of enzymes may play in HG induced demethylation.
HG induces 5mC demethylation intermediates consistent with Tet enzyme activity.

As seen in Fig 1 the initial steps of demethylation produce 5hmC if the pathway proceeds via the TET family of enzymes. Triplicate groups of control or DM fish were generated and genomic DNA was isolated from pooled fins at 24 hours, 1 week, 2 weeks or 3 weeks post induction (Fig.3A). These samples were then examined for the presence of 5hmC; at all time points control fish had low but detectable levels of 5hmC (0.106 +/- 0.023), and no significant differences were observed in DM fish after 24 hours (data not shown). However by one week, DNA exhibited significant (p < 0.0001) increases in the 5hmC content (1.13 +/- 0.19). These levels continued to increase until 2 weeks (3.58 +/- 0.19), at which time the levels were maintained but no further increase was observed at three weeks (3.64 +/- 0.26). In all cases the FBGLs were determined for these fish and as we previously reported, hyperglycemia was induced within the first 24 hours then maintained throughout the duration of the experiment (40).

Next, studies were performed to determine if 1) iterative oxidation, 2) deamination first, or 3) both pathways (Fig. 1) were subsequently utilized for demethylation. 5hmC is converted into 5-formyl-cytosine (5fC) in the next step of the iterative oxidation pathway and as such, the above samples were examined for the presence of 5fC. In parallel with the 5hmC data, there was no statistical difference in the 5fC content at any time point for control fish or for fish 24 hours post induction (data not shown). However, at the later time points, increases in 5fC content were observed within one week of HG (compare control 0.26 +/- 0.13, versus 1 week, 1.25 +/- 0.23) were further increased at two weeks (3.1 +/- 0.45), and maintained at 3 weeks (3.45 +/- 0.22) (Fig. 3B). The other pathway that 5hmC may follow is deamination by the Aid/Apobec enzymes yielding as an initial step 5-hydroxymethyl-uracil (5hmU) (Fig.1). Despite repeated attempts to detect 5hmU with antibodies from a variety of vendors that could detect
commercially purchased 5hmU we were not able to detect this derivative in either control or DM samples (data not shown).

**Poly ADP ribose polymerase inhibition prevents DNA demethylation**

Recently it was reported that poly ADP-ribose polymerase 1 (PARP1) activity is necessary for the up-regulation of Tet1 expression which in turn, is responsible for the initiation of active demethylation in mouse primordial germ cell DNA during development (44). We therefore determined the role that Parp plays in the HG-induction of DNA demethylation. We took advantage of the temporal window observed between HG induction and the induction of DNA demethylation by injecting control and DM fish with an inhibitor of the Parp enzymes. Subsequent to this, DNA was extracted and examined for its 5hmC content. As can be seen in figure 4A, DM induced the conversion of 5mC into 5hmC (compare C: 0.099 +/- 0.033 to DM: 3.51 +/- 0.32) which was completely prevented by the inclusion of the Parp inhibitor (1,5 isoquinolinediol) (DM+Parpi: 0.073 +/- 0.039). In addition, the 1,5 isoquinolinediol treatment had no effects on HG as the doubly treated group had similar FBGLs to DM fish (approximately 315 mg/dL for both groups) (data not shown).

To further support this data, we examined the effect that Parp inhibition had on loci that we utilized in our previous study (7) via a MeDip-qPCR approach. These included loci that are unaffected by HG remaining either fully methylated (abhd12, map1b) or fully unmethlyated (uba2, rac3a) and more importantly loci that are demethylated in response to HG (uhrf1, grtp1a, gcat, hnrnpa0). As expected, the loci that are not affected by HG exhibit equal amounts of methylation as indicated by equal qPCR Ct values irrespective of the sample origin (Table1). In
contrast, the Ct values for HG-affected loci are significantly lower in the DM samples than in all others, thus indicating that the inclusion of Parp inhibitor prevented DNA demethylation.

In our previous work we documented that HG causes an impairment of tissue regeneration and that impairment correlated with the induction of DNA demethylation (7). After observing that Parp inhibition appears to prevent the tet-dependent pathway/s of demethylation, we examined if this inhibition also restored regenerative capacity of the fin. As such, the rate of tissue regeneration was documented for the fish utilized in the DNA extraction experiment documented above. In these experiments the regeneration of control, Parp inhibitor injected, and DM plus Parp inhibitor injected fish all exhibited normal levels of regeneration (C: 100 %, PARP: 99.25 % DM+Parpi: 96.29) (Fig 4B and Supplemental Fig. 1). This is in contrast with DM fish which exhibited a reduced fin regeneration rate (62.5 %) similar to our previous reports (40).
Discussion

Epigenetic mechanisms are hypothesized to play a role in the metabolic memory phenomena as they provide a mechanism for continued altered gene expression without the presence of the initiating HG stimulus (45). We and others have reported that HG can induce site specific DNA methylation and we have also reported that these changes persist even after euglycemia is restored in a zebrafish model of type I diabetes mellitus (7). In this report we have revealed a role for the ten–eleven translocation family of enzymes (Tet) in HG induced DNA demethylation. More specifically, we provide evidence that HG induces the expression and activity of the Tet enzymes yielding known intermediates of the iterative oxidation pathway leading to the demethylation of 5mC. In addition, these studies have revealed that demethylation via this pathway can be prevented through inhibiting the poly ADP-ribose polymerases.

We initiated this study by examining the HG effects on expression of enzymes known to play key roles in each of the DNA demethylation pathways. In regard to the deamination first pathway where 5mC is converted to thymine, we observed increased expression for the adaptor Gadd45 protein but no increase in either the Aid/Apobec complex or the Mbd4 protein. Mbd4 appears to be critical for this pathway as it has been shown that it recognizes and excises mismatched bases paired with guanine (G:X), where X is uracil, thymine or 5-hydroxymethyluracil (5hmU) (46). On the surface this data might suggest that this pathway may not be induced by HG, however, we cannot make this conclusion without additional data regarding the presence of T:G mismatches. Unfortunately, we were unable to pursue this pathway further because there are no efficient means available for examination of these processes or the ability to examine Mbd4 activity specifically. The other two pathways are both initiated by the Tet family of enzymes and our data clearly revealed an increase in the expression
of these enzymes within two weeks of hyperglycemic onset. This expression increase was supported by the correlated increase in Tet enzymatic activity. In addition, the Tdg enzyme (a base excision repair enzyme) which is responsible for removal of the modified base in these pathways is also increased in its expression.

We next examined DNA from the control and DM groups for known intermediates of the demethylation pathways. The common intermediate for the Tet specific demethylation pathway/s is 5hmC and the data presented here reveals that by two weeks of hyperglycemia 5hmC levels are maximized and maintained at approximately 30 fold higher levels than in control fish. This data corresponds perfectly with the caudal fin regeneration deficit we have previously reported in that at 2 weeks, the HG induced deficit reaches its maximum and is maintained in subsequent weeks (40). Following 5hmC formation, cytosine demethylation can proceed via two routes, either through deamination or iterative oxidation and each of these pathways produces different intermediates. The next step in the iterative oxidation pathway is the production of 5fC. We were able to detect significant increases in this intermediate as expected due to the increase in Tet enzyme activity and as such our data is consistent with HG-induced DNA demethylation proceeding, at least in part, via the tet-dependent iterative oxidation pathway. Unfortunately, we were unable to detect 5hmU in any of our samples and cannot rule in/out the use of this pathway.

In the context of DM, PARP1 senses HG-induced DNA damage and plays a pivotal role in stimulating the molecular pathways that underlie all diabetic complications (20). This fact coupled with the report linking poly ADP-ribose polymerase 1 (PARP1) activity and up-regulation of Tet1 expression led us to hypothesize that inhibition of the Parp enzymes may prevent Tet activity. When a known Parp enzyme inhibitor was included in our experiments, the levels of 5hmC returned to normal. In addition, enzyme inhibition also prevented the
demethylation of several specific loci examined. Several recent reports have documented that Parp inhibition can ameliorate renal hypertrophy, podocyte apoptosis, and peripheral neuropathy in animal models of DM (47-49). When we examined the effect Parp inhibition had on tissue regeneration we observed a complete restoration of the regenerative capacity in DM fish that were also treated with the Parp inhibitor. This data provides further evidence that Parp inhibition may provide a therapeutic avenue for the prevention or reversal of diabetic complications and also illustrates the usefulness of the zebrafish model for small molecule drug discovery pertaining to diabetes mellitus.

From a mechanistic perspective, our data is consistent with the HG induction of the Parp family of enzymes, which in turn stimulates the Tet enzymes leading to DNA demethylation and ultimately persistent diabetic complications.

Contributions

N.D. researched data, and discussed the data. M.S. Jr. contributed to discussion of the data and writing the manuscript. S.M. and E.L. provided technical support. R.I. researched data, discussed the data, contributed to writing and editing the manuscript.

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Figure Legends

Figure 1.
The pathways of active DNA demethylation. Cartoon representation of the known activated DNA demethylation pathways intermediates and their supporting enzymatic machinery. 5 methylcytosine (5mC), 5 hydroxymethyl cytosine (5hmC), formyl-cytosine (5fC), 5-carboxycytosine (5caC), 5-hydroxymethyl-uracil (5hmU), DNA damage inducible (Gadd45) (activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex (Aid/Apobec), ten–eleven translocation family (Tet 1,2,3), thymine DNA glycosylase (Tdg), methyl CpG binding domain protein 4 (Mbd4), base excision repair (BER).

Figure 2
Hyperglycemia induces gene expression changes in DNA demethylation machinery enzymes. Quantitative reverse transcriptase results (presented with standard error) are presented graphically as the fold increase when compared to controls. Gaa (gadd45 αα): Weeks 1-3: 2.01 +/- 0.25, 2.35 +/- 0.36, 2.12 +/- 0.34. Gab (gadd45 αβ): Weeks 1-3: 1.98 +/- 0.34, 4.82 +/- 0.29, 4.35 +/- 0.35. A2a (apobec2a): Weeks 1-3: 1.20 +/- 0.11, 0.98 +/- 0.23, 0.90 +/- 0.37. A2b (apobec2b): Weeks 1-3: 0.85 +/- 0.11, 1.13 +/- 0.12, 1.12 +/- 0.24. M4 (mbd4): Weeks 1-3: 0.94 +/- 0.26, 1.12 +/- 0.13, 0.98 +/- 0.11. T1 (tet 1): Weeks 1-3: 8.34 +/- 0.22, 5.34 +/- 0.41, 5.12 +/- 0.09. T2 (tet 2): Weeks 1-3: 5.87 +/- 0.34, 6.13 +/- 0.12, 6.23 +/- 0.08. T3 (tet 3): Weeks 1-3: 5.32 +/- 0.26, 5.32 +/- 0.36, 5.45 +/- 0.37. Tdg (tdg): Weeks 1-3: 1.01 +/- 0.27, 3.46 +/- 0.24, 3.23 +/- 0.34. Each gene at each time point was compared to the appropriate control and a
Students t-test was performed in order to determine statistical significance. Where statistically significant changes existed (indicated by an asterisk), p < 0.001.

**Figure 3**

HG induces the formation of Tet enzyme activity intermediates. A. Graphic representation of the 5hmC genomic content (ng/0.2mg) C: 0.106 +/- 0.023, W1 (week 1): 1.13 +/- 0.19, W2 (week 2): 3.58 +/- 0.19, W3 (W3): 3.64 +/- 0.26. n = 18 for C, W1 and W2 and 16 for W3. B. Graphic representation showing the time course of 5-formyl-cytosine (5fC) formation (ng/0.5mg) induced in fin tissue DNA. Control: 0.26 +/- 0.13, W1: 1.25 +/- 0.23, W2: 3.1 +/- 0.45 and W3 3.45 +/- 0.22. N = 18 for C, W1 and W2 and 16 for W3 and p < 0.0001. In both studies, a Students t-test was performed in order to determine statistical significance. Where statistically significant changes existed (indicated by an asterisk), p < 0.0001. In addition, a one way ANOVA analysis revealed that week 1 was statistically different than weeks 2 and 3 p< 1.0 E-5 for both assays.

**Figure 4**

Parp inhibition prevents both the accumulation of 5hmC and the HG-induced fin regeneration deficit. A. The administration of a Parp inhibitor prevents 5hmC formation. C: 0.099 +/- 0.033, DM: 3.51 +/- 0.32, Parpi (I = inhibitor) 0.007 +/- 0.003, DM+Parpi 00.073 +/- 0.039). N = 8. A one way ANOVA analysis revealed that only DM samples were statistically different from the other samples (p< 1.0 E-6) indicated by an asterisk. B. Parp inhibition prevents the HG-induced impairment in fin regeneration. C: 100+/-1.3 % , DM: 62.5 +/-4.7 %, Parpi: 99.3 +/-5.2 %, DM+Parpi: 96.3 +/- 6.3 %. N = 18 for all groups. A one way ANOVA analysis revealed that the regeneration of only DM fish were statistically different from the other samples (p< 5.0 E-5).
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Fig. 1
Fig. 3

A. Sample Type

B. Sample Type

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Fig. 4

A. 5-hmC (ng/0.2mg)

| Treatment | C | DM | Parpi | DM+Parpi |
|-----------|---|----|-------|----------|
| 5-hmC (ng/0.2mg) | 0 | 3.5 | 0 | 0 |

B. % Regeneration

| Treatment | C | DM | Parpi | DM +Parpi |
|-----------|---|----|-------|-----------|
| % Regeneration | 100 | 60 | 100 | 100 |
Table 1: Parp inhibition prevents DNA de-methylation as determined by Quantitative-PCR results. The gene loci are indicated and the average Ct values and their associated standard errors are reported for control (C), DM, DM plus Parp inhibitor (DM+I) and Parp inhibitor (I) injected fish. Statistical significance was determined by a one-way ANOVA analysis. Where statistically significant differences existed (indicated by an asterisk) $p < 0.01$.

| Locus  | C       | DM     | DM+I   | I       |
|--------|---------|--------|--------|---------|
| abhd12 | 21.0 +/- 0.21 | 20.8 +/- 0.34 | 21.6 +/- 0.56 | 20.7 +/- 0.76 |
| map1b  | 20.4 +/- 0.44  | 20.1 +/- 0.36  | 19.7 +/- 0.66  | 20.4 +/- 0.66  |
| uba2   | 36.8 +/- 2.69  | 37.9 +/- 3.75  | 36.3 +/- 2.66  | 39.4 +/- 4.78  |
| rac3af | 38.9 +/- 4.20  | 40.9 +/- 2.33  | 40.3 +/- 3.56  | 40.6 +/- 4.14  |
| uhrf1  | 21.0 +/- 0.41  | 36.3 +/- 2.45* | 22.7 +/- 1.98  | 20.9 +/- 1.65  |
| grtp1a | 25.6 +/- 0.18  | 30.1 +/- 0.20* | 24.9 +/- 0.38  | 25.2 +/- 0.65  |
| gcat   | 24.8 +/- 0.21  | 29.0 +/- 0.17* | 24.4 +/- 0.21  | 23.9 +/- 0.41  |
| hnrnpa0| 26.4 +/- 0.67  | 36.0 +/- 0.89* | 27.4 +/- 0.67  | 27.6 +/- 0.97  |
Supplemental Table 1: Primers used for RNA expression Analysis

| Gene Name        | Primer Pairs                  |
|------------------|-------------------------------|
| gadd45aa         | f-GAAAGGATGGACTCGGTGATTAA     |
| gadd45aa         | r-GGGACTTTTCAGCCTCGTAA        |
| gadd45ab         | f-GGCCACAGATGGAGGAGATGT       |
| gadd45ab         | r-CACTCGCAAGATATTGATGTCGTT    |
| apobec2a         | f-GAGGCTCAGAGATGGGCCGATAGA    |
| apobec2a         | r-TCCTCTTTTCTTGGCATCATTTTC    |
| apobec2b         | f-CAGGCTCAGATGGGCCGAGAAGA     |
| apobec2b         | r-CAAGGGCTGGAGCAGATGTGA       |
| mbd4             | f-GAGCCAGGCGTGATAATCGT        |
| mbd4             | r- CGGCAAATACGGCAATGACT       |
| tet1             | f-CACGCCCACCTCTTGCACTTT      |
| tet1             | r-GACACCCCCCCAATTTTGGTT      |
| tet2             | f-TGATCGCTCTCTACATGGAACTAAGTG|
| tet2             | r-CCCTGACAGCAGCATTTCT        |
| tet3             | f-CCCAGACTGGCCCTGATTACC      |
| tet3             | r-TTGAGCCGTACCCGAAAGAT       |
| tdg              | f-AAATACGGCATCGGCTTCAC       |
| tdg              | r-CCACTAAGATTTTACGGCGCTTCTC  |
| 18S ref gene     | f-TCGCTAGTTGCGATCGTTATG      |
| 18S              | r-CGGAGGGTTGCGAGAATTCA       |
| elfa ref gene    | f-CTTCTCAGGCTGACTGTCG        |
| elfa             | r-CCGCTAGCATTACCCCTCC        |
| Rnap ref gene    | f-CCAGATTCAGGCCGCTTCGAGG     |
| map              | r-CAAACGGAATGAGGGCTC         |
Supplemental Table 2. Primer sequences for q-PCR

| Gene Name | Chromosomal Location of CGI | Primer Pairs |
|-----------|-----------------------------|--------------|
| gcat      | chr3:23085523-23085903      | f-TACGTGTCAGCCTTGTTTCTAGT  
|           |                             | r-TCAGGCGACGCACCGAAAC     |
| grtp1a    | chr17:320095-320443         | f-TCATCGCGACCGACGAAC      । |
| hnrnpa0   | chr14:40707997-40708674     | f-TCTTTCTTAGCAGCGAGCTAGCTCT  
|           |                             | r-GACGTCTAGACCGCCAACGA     |
| uhrf1     | chr22:5550020-5550274       | f-AGCAATGAGCGGCGTTATTC    । |
| abhd12    | chr17:20988536-21030458     | f-CACACACTCCGCGCTGAAC     । |
| map1B     | chr5:33728247-33728561      | f-TCCACAGACAGCTACTTCA      । |
|           |                             | r-GTACTGACGTCTCGCATTAT    । |
| uba2      | chr25:12017457-12017689     | f-CAGAGGTGCGCGATTTAAACCT  
| rac3a     | chr3:33491203-33491596      | f-TGGGGGAAATCAGAGATATTC  
|           |                             | r-ACCTACGACAATTTTACGCT    |
Supplemental Figure 1. Parp inhibition prevents the HG-induced regeneration defect. Representative images of regenerating fins (72 hours post amputation) from control (A), DM (B), DM + Parp Inhibitor (C) and Parp inhibitor alone (D). The amputations were performed 22 days post DM induction and the transection line is indicated by the black dotted line. All tissue above this line represents the new tissue outgrowth.