

Supplementary Figure 1.

(A) Western blot analysis of GADD45a and TET1 catalytic domain (TET1_{CD}) co-immunoprecipitation (Co-IP). Protein lysates of HEK293T cells transiently transfected with Flag-TET1 catalytic domain (TET1_{CD}), myc-GADD45a and GFP as control were used for Flag-IP. Proteins were detected using antibodies against Flag and GADD45a. α -Tubulin and GFP were used as specificity controls. Input shows 5% of lysate used for IP.

(B) GADD45a oligomerization analysis by Microscale Thermophoresis. Binding of *in vitro* fluorescently labeled recombinant GADD45a to unlabeled recombinant GADD45a was assessed. Concentration of labeled GADD45a was kept constant at 50 nM, while increasing concentrations of unlabeled GADD45a were tested (0.1 nM to 5 μ M). The sigmoidal binding curve of GADD45a oligomerization was fitted according to the law of mass action using NTAnalysis software provided by NanoTemper technologies.

A

В

Suppl. Figure 2



Supplementary Figure 2.

(A-B) Western blot analysis using protein lysates of HEK293T cells transfected with empty vector (Ctrl), Flag-HA-TET1 and myc-GADD45a as indicated. For (B), cells were transfected with GADD45a specific (siG45a) or control (siCtrl) siRNA, 24h prior to DNA transfection. α -Tubulin was used as loading control.

(C) Relative knockdown efficiencies of the indicated genes assessed by qPCR in HEK293T cells, 72h upon transfection with the corresponding siRNA. Knockdown efficiency was calculated from the qPCR values after normalization to the housekeeping gene *GAPDH*. Bar graphs represent the mean of n=3 experiments with error bars as \pm SD.



Supplementary Figure 3. (A) *ANKRD1* and *WDR5* control gene expression is not affected by TET1 and GADD45a expression. Relative gene expression was monitored using qPCR in HEK293T cells, transfected with empty vector (Ctrl, control), GADD45a alone or along with increasing doses of TET1 full-length protein or its catalytic domain (TET1_{CD}) as indicated (see experiment Fig. 3A). Bar graphs represent the mean of n=4 experiments with error bars as \pm SD. (B) Western blot analysis using protein lysates of HEK293T cells transfected as in (A) and Fig. 3A. α -Tubulin served as loading control. (C) *TCEAL7* and *MAGEB2* target gene induction by TET1 and GADD45a is not affected by the O-linked N-acetylglucosamine transferase (OGT) inhibitor alloxan. Relative gene expression in HEK293T cells upon transfection of empty vector (Co, control), GADD45a alone or along with TET1 full-length protein or its catalytic inactive mutant (TET1_{CI}) as indicated was monitored using qPCR. Bar graphs represent the mean of n=3 experiments with error bars as \pm SD.(D) Kinetics of HpaII resistance changes in the *TCEAL7* locus at CpG -2648, -78, +34 and +457 relative to the transferitor start site (TSS) upon GADD45a and TET expression. Genomic DNA was harvested 14h or 24h after transfection and subjected to HpaII restriction digest and qPCR. HpaII resistance = fraction of modified C. Bar graphs represent the mean of biological triplicates with error bars as \pm SD.



Supplementary Figure 4. (A) HA-GADD45a specifically binds to the *TCEAL7* locus. HEK293T cells were transiently transfected with HA-GADD45a, chromatin harvested 48h after transfection and subjected to pulldown with a-HA antibody (as described in Schäfer et al. 2013). In qPCR analysis, GADD45a bound the *TCEAL7* promoter-, but not the negative control *GAPDH* promoter- region. Bar graphs represent the mean of biological triplicates wih error bars as \pm SD. (B) GADD45a and TET1 catalytically inactive pointmutant (TET_{CI}) alone do not induce global mC oxidation. Genomic DNA of HEK293T cells, 48h after transient transfection with empty vector (Ctrl), GADD45a (G45a), TET1 or TET_{CI} was analysed by SID-LC-MS/MS. Bar graphs represent the mean of biological triplicates wih error bars as \pm SD.

Supplementary Information: GADD45a physically and functionally interacts with TET1

Supplementary Materials and Methods

Primer sequences and UPL monocolor hydrolysis probes (Roche) for expression analysis by qPCR

Designation	Primer Sequence	UPL No or SYBR
hGadd45a	f 5`- GCC AAG CTG CTC AAC GTC -3`	#40
	r 5`- CTC TGT CGT CGT CCT CGT C -3`	
hWDR5	f 5`- GCG TAT GAT GGG AAA TTT GAG -3`	#33
	r 5`- TGA CGA CCA GGC TAC ATC G -3`	
hGAPDH	f 5`- GCA TCC TGG GCT ACA CTG AG -3`	#82
	r 5`- AGG TGG AGG AGT GGG TGT C-3`	
hTCEAL7	f 5`- CAG ACA TCT GGG GGA AGA AA -3`	#15
	r 5`- TGC AGG GTT TTT GCA TGA T -3`	
hDHRS2	f 5`- TGA GAC TAT CAC CTA TCG CCA AG -3`	#18
	r 5`- CAG CAT AGT GGT TGG TGT CTG -3`	
hANKRD1	f 5`- GCG GAT CTC AAC ATC AAG AAC -3`	#90
	r 5`- TGC GAG AGG TCT TGT AGG AGT -3`	
hMAGEB2	f 5`- AGG ACC CGA GCG AGT GTA G -3`	SYBR
	r 5`- GCA CGG AGC TTA CTC TTC TGA -3`	
hXPA	f 5`- CGA GTA TCG AGC GGA AGC -3`	#71
	r 5`- TTT GGG GCT GCT TTT ACA TT -3`	
hXPB	f 5`- GTG GTG GCT GGG GAA TTT -3`	#20
	r 5`- CCC CAG ACA TAG AAC TCA TGG -3`	
hXPC	f 5`- CCG GCT GGT ATT GTC TCT ACA -3`	#6
	r 5`- TCT TTC CTT GGA AGG TTT CTT TC -3`	
hXPD	f 5`- TTG AGA CCC GGG AGG ATA TT -3`	#18
	r 5`- ATG CCA TCA GGG ACC ACA -3`	
hXPF	f 5`- CTG CAG ATG TTT CCA CTG ACA -3`	#72
	r 5`- CAC GCA TAT CCA CAA CTA TGC -3`	
hXPG	f 5`- TGA ATC TCT GTT TCC TGT ATT AAA GC -3`	#3
	r 5`- AAG GAA TCA ATT CGG AGC TG -3`	
hPOLe	f 5'- GTT CCC ACT TGC TGC TCA AT -3'	#37
	r 5`- CAC CTG GTT TGT GAT GTT GG -3`	
hPOLd	f 5`- CGG ACT GGG TGT CAG GTC -3`	#19
	r 5`- TGA TAA GCA GGT ATG GGA AGT AGA -3`	
hTET1	f 5'- TCT GTT GTT GTG CCT CTG GA -3'	#57
	r 5`- GCC TTT AAA ACT TTG GGC TTC -3`	
hTET2	f 5'- ACG CTT GGA AGC AGG AGA T -3'	#66
	r 5'- CAA GGC TGC CCT CTA GTT GA -3'	
hTET3	f 5`- CAC TCC GGA GAA GAT CAA GC -3`	#1
	r 5`- GGA CAA TCC ACC CTT CAG AG -3`	

Primer sequences and predesigned UPL monocolor hydrolysis probe for methylation- and modification sensitive PCR

Designation	Primer Sequence	UPL No
-		or SYBR
gn-hTCEAL7	f 5'- AAC TCC AGT AGA GAA ATG GTG AAA A -3'	#39
-2648	r 5`- TGA GTG GGC ACC ATC TAA CC -3`	
gn-hTCEAL7	f 5`- GAG AAA GGC AAA TTC TGA CCA -3`	#7
-78	r 5`- AGC GCT GCA GAA GTG GAC -3`	
gn-hTCEAL7	f 5`- ATC AGG AGC TGA CGT GAA CC -3`	#82
+34	r 5`- GTC TGC AGG GCA GTA AGG AG -3`	
gn-hTCEAL7	f 5'- TTT TAG GCC TGG GGT ATG TG -3'	SYBR
+457	r 5`- TTG AGT GAG GTG AAC TAT TTT CAA -3`	
poctTK-GFP	f 5'- ATA ACC AGC CAC CTT GAT CTG -3'	#39
-299	r 5`- ATT CGC CAA TGA CAA GAC G -3`	

Generation of ¹⁵N labeled fC, hmC and caC standards

The ¹⁵N stable isotope labeled 5-hydroxymethyl2- deoxycytidine (hmdC), 5-formyl 2deoxycytidine (fdC) and 5-carboxy 2- deoxycytidine (cadC) were generated by a sequence of five enzymatic *in vitro* reactions: (i) PCR reaction was performed with ¹⁴N containing dTCP, dGTP, dATP (Thermo Fisher Scientific Inc) and ¹⁵N containing dCTPs (Silantes GmbH, Munich, Germany). PCR primers (forward : 5'-CTC CTC TGA CTG TAA CCA CG, reverse : 5'AGG CTT CTG GAC TAC CTA TGC) as well as the 83-mer PCR template (5' CTC CTC TGA CTG TAA CCA CGC CGG TAC GTT ACG ATA CGA TTA CGT AAT ACG ATT TCG AAC CGG CAT AGG TAG TCC AGA AGC CT) were custom synthesized by Sigma Aldrich (Germany). The PCR product, containing ¹⁵N-dC, was ethanol precipitated and the pellet dissolved in _{dd}H₂O. (ii) The primer regions, containing unlabelled cytidines, were removed via MspI (Promega GmbH., Mannheim, Germany) restriction digest, and the desired 43-mer product was PAGE purified on a non-denaturing 20% acrylamide gel. (iii) The cytidines in the oligo were in vitro methylated by M.SssI methyltransferase (New England BioLabs GmbH, Frankfurt Germany) using manufacturer's instructions, and after phenol-chloroform extraction ethanol precipitated. (iv) The methylated DNA was incubated with recombinant Naegleria NgTet-like dioxygenase for 30 minutes. Reaction conditions as well as ngTet-like protein purification were as described previously [1], (v) The 43-mer DNA containing a mixture of ¹⁵N labeled dC, mdC, hmdC, fdC and cadC was ethanol precipitated and degraded to nucleosides with nuclease P1 (Roche Diagnostics, Mannheim, Germany), snake venom phosphodiesterase (Worthington, Lakewood, USA) and alkaline phosphatase (Fermentas, St Leon-Roth, Germany). Individual nucleosides were separated on 15 cm ReproSil 100, 3µm C18 column (Jasco GmbH, Groß-Umstadt, Germany) maintained at 30 °C and using the gradient of (A) 5mM acetic acid and (B) Acetonitrile (LC/MS grade, both from Sigma Aldrich). The linear gradient was from 0% to 20% of solvent B in first 24 min, accompanied by a linear increase of a flow rate from 0.5 to 1 ml/min. Then, 20% of solvent B was kept for 6 min at a flow of 1 ml/min and finally the column was reconditioned for an additional 10 min at a flow rate of 1 ml/min with 100% A. During the last minute, the flow rate was linearly decreased to initial value of 0.5ml/min. The ¹⁵N labeled hmC, fC, and caC were identified by analytical HPLC in a tandem with triple quadruple mass spectrometer operated in a positive ion mode. The instrument settings and the monitored ion transitions of ¹⁵N labeled dC, mdC, hmdC, fdC as well as unlabeled compounds are listed in Table 1 and 2, respectively. The determined migration windows of three peaks were then used for fraction collections in preparative HPLC run. After collection, the buffer was evaporated using a SpeedVac, and the pellet was dissolved in ddH₂O.

LC/MS analysis

Genomic DNA (gnDNA) of HEK293T cells was isolated using DNeasy blood & tissue kit (Qiagen), ethanol precipitated with ammonium acetate, pH 7.0 and dissolved in $_{dd}$ H₂O. 1µg gnDNA was first denatured by heating to 95°C for 5 min, rapidly cooled on ice and digested into nucleosides using a procedure adopted from M. Helm [2] with modifications. In brief, DNA was incubated with 0.003 U nuclease P1 (Roche Diagnostics, Mannheim, Germany), and 0.01 U snake venom phosphodiesterase (Worthington, Lakewood, USA) in nuclease P1 buffer (0.2 mM NH₄OAc, pH 5.3, ZnCl₂ 0.02 mM), in a total volume of 10-20 µL for 2 hours at 37 °C. After addition of 0.1 U of alkaline phosphatase in its 1x provided buffer (Fermentas, St Leon-Roth, Germany), the reaction was incubated at 37 °C for an additional hour. Finally, an equal volume of isotopic standard mixture was added to the DNA and about a 100 ng of total gDNA was injected for LC/MS analysis. The separation was performed on an Agilent 1290 UHPLC system equipped with ReproSil 100 C18 column (3 µm, 4.6 x 150 mm, Jasco GmbH, Groß-Umstadt, Germany) maintained at 30 °C. The running buffers were (A) 5mM ammonium acetate at pH 6.9 and (B) Acetonitrile, The separations were performed at a flow rate of 0.5 ml/min using the following gradients: 0% of solvent B from 0 min to 8 min, linear increase to 15% solvent B for next 16 min,

then to wash and recondition the column, the flow rate was switched to 1.0 ml/min, and washed with 15% solvent B for one minute and then for additional 5 min with 100 % buffer A. During the last minute, the flow rate was linearly decreased to initial value of 0.5 ml/min. The MS settings as well as the monitored ion transitions are listed in Supplementary Tables 1 and 2. Since the dynamic range of the instrument does not allow for simultaneous quantification of extremely low abundant modified and very high abundant non-modified nucleosides, the quantification of high abundant dC and 5mdC was performed from 100x diluted sample. The data was analyzed using Agilent Quantitative Analysis software Version B05.02, the isotopic standards were used to confirm the peak identity. The areas of the integrated peaks were exported to Microsoft Excel and normalized to the area of the corresponding isotopic standard. The absolute values for the nucleosides were calculated using a linear interpolation algorithm, where first the signal response in the analyte was compared to the signal response of the standard curve and the linear interpolation was performed using the two closely matching values from the standard curve. The FORECAST, in combination with OFFSET and MATCH functions of the Excel program were used to build the required logical algorithm. Using such an approach the accuracy is increasing with the frequency of the experimentally determined values of the standard curve. To generate standard curves, standards mixture (C, mC, hmC, fC and caC,) was four-fold serial diluted at high concentrations and two-fold serial diluted in low concentration ranges. In analogy to the sample preparation, the standards were always 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 the standard curve over that region was always monitored and confirmed to be in the range of R^2 values of 1 - 0.996 within a concentration range covering at least 2 orders of magnitude, and having five or more experimental data points. As experimentally determined the RSTD of the measurement was smaller than 7%. The standard curve for every nucleoside covered the amounts of corresponding nucleosides in all gDNA samples analyzed, and extrapolation was never used. The new standards were always prepared and run with every new dataset to avoid technical artifacts. The quantification of all samples was performed in a minimum of three biological replicas, and the average values of biological replicas with one standard deviation are shown.

Supplementary References

- Hashimoto H, Pais JE, Zhang X, Saleh L, Fu ZQ, Dai N, Correa IR, Jr., Zheng Y, Cheng X (2014) Structure of a Naegleria Tet-like dioxygenase in complex with 5-methylcytosine DNA. *Nature* 506: 391-395
- Kellner S, Ochel A, Thuring K, Spenkuch F, Neumann J, Sharma S, Entian KD, Schneider D, Helm M (2015) Absolute and relative quantification of RNA modifications via biosynthetic isotopomers. *Nucleic Acids Res* 42: e142

Supplementary list of P-values

Two-tailed, unpaired Student's t-test was used to calculate the level of significance. P-values ≤ 0.05 were considered significant and are marked in red. Shaded area marks the reference source.

Figure 2A

	vs Co	vs G45a	w/o vs w/G45a
Co			///////
G45a	2,11E-03		2,11E-03
TET1	3,61E-03	9,59E-01	
TET1 + G45a	1,59E-04	6,13E-04	1,91E-04
TET1CI	5,81E-02	5,12E-06	
TET1CI + G45a	1,23E-01	4,15E-04	5,56E-05

Figure 2B-D

		Figure 2B, Hpa	II resistance	
		vs Co	vsG45a	vs TET1
Co	12h	///////////////////////////////////////		
	18h			
	24h			
	30h			
	48h			
G45a	12h	3,47E-01		
	18h	7,37E-01		2
	24h	4,69E-03		1
	30h	4,30E-02		
	48h	3,10E-02		1
TET1	12h	2,75E-01		
	18h	6,81E-01		
	24h	3,07E-02		
	30h	1,94E-02		
	48h	1,18E-01		,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,
TET1+G45a	12h	6,58E-01	6,84E-01	6,02E-01
	18h	5,86E-01	7,92E-01	4,77E-01
	24h	6,39E-03	7,27E-01	2,55E-01
	30h	6,09E-03	1,12E-02	6,61E-03
	48h	5 71E-03	5 13E-03	1.57E-03





Figure 2E

	w/o vs w/G45a	siCo vs siTET1
Mock	///////////////////////////////////////	
Mock + G45a	4,08E-02	
siCo		
siCo + G45a	1,41E-02	
siTET1		8,58E-02
siTET1 + G45a	1,06E-02	3,24E-04

Figure 2F

	siCo vs siG45a	w/o vs w/TET1
Mock]	
Mock + TET1		6,31E-04
siCo		
siCo + TET1		2,85E-03
siG45a	1,05E-01	
siG45a + TET1	1,44E-02	1,78E-01

Figure 2G

siRNA	vs siCo	vs mock
Mock	5,03E-02	
Co		5,03E-02
G45a	1,93E-03	2,85E-03
TET1	1,37E-02	7,02E-03
TET2	3,39E-02	7,29E-01
TET3	4,56E-02	8,75E-01

igure 3A										
		TCEAL7			DHRS2			MAGEB2		
		vs Co	vs G45a	w/ vs w/o G45a	vs Co	vs G45a	w/ vs w/o G45a	vs Co	vs G45a	w/ vs w/o G45a
Co	Co	///////////////////////////////////////								
	G45a	7,64E-03			2,80E-02	////////		5,10E-03		
	TET1	8,11E-03			9,78E-01			3,94E-02		V///////
	TET1 +	1,35E-05			1,73E-03			1,52E-03		
	TET1 ++	1,56E-06			1,76E-05			3,79E-03		
	TET1 CD	5,66E-01			8,01E-02			3,85E-01		
	TET1 CD +	2,31E-01			1,64E-02		7777777	8,86E-03		1//////////////////////////////////////
	TET1 CD ++	2,17E-01	1		9,31E-03			4,96E-02		
45a	TET1	2,20E-04	3,68E-04	1,80E-03	1,22E-02	1,23E-02	3,93E-01	1,17E-03	1,65E-03	1,74E-02
	TET1 +	7,48E-09	7,22E-08	5,34E-08	2,17E-05	4,59E-03	2,42E-04	2,20E-04	5,30E-04	4,97E-04
	TET1 ++	1,16E-05	8,76E-05	2,06E-05	5,26E-07	8,51E-05	3,09E-06	9,19E-04	3,47E-03	1,73E-03
	TET1 CD	3,52E-03	4,64E-03	1,51E-01	1,37E-01	4,62E-02	1,45E-01	3,95E-02	4,24E-02	5,28E-01
	TET1 CD +	2,13E-03	2,98E-03	3,36E-02	6,95E-01	2,74E-02	3,70E-02	1,44E-03	2,07E-03	2,01E-02
	TET1 CD ++	1,11E-03	1,42E-03	2,77E-02	3,28E-01	1,40E-01	2,06E-02	1,02E-02	1,31E-02	4,94E-02



Figure 3C hmC													
			v	s Co			vs T	TET1			vs	G45a	
		-2648	-78	+34	+457	-2648	-78	+34	+457	-2648	-78	+34	+457
14h	Co	///////////////////////////////////////	<i>X//////</i>	X/////////////////////////////////////	<i>\////////////////////////////////////</i>								
	G45a	3,51E-01	4,54E-02	6,35E-01	2,13E-01	1				7///////	X////////	X////////	<i>\////////////////////////////////////</i>
	TET1	3,25E-02	8,17E-03	2,65E-02	1,77E-05	///////////////////////////////////////	<i></i>	X///////					
	TET1 + G45a	2,52E-01	2,96E-03	1,63E-04	1,09E-04	5,68E-01	2,93E-02	1,70E-02	4,03E-01	7,98E-01	1,30E-02	2,35E-03	5,19E-03
24h	Co	///////////////////////////////////////		X/////////////////////////////////////									
	G45a	8,92E-01	8,84E-01	9,19E-03	4,07E-01								
	TET1	5,15E-04	4,57E-03	1,47E-03	1,19E-04	///////////////////////////////////////	X/////////////////////////////////////	X////////	<i>\////////////////////////////////////</i>				
	TET1 + G45a	2,53E-03	4,81E-04	1,66E-04	1,87E-04	1,67E-01	6,76E-03	4,65E-02	3,84E-02	8,97E-04	1,41E-04	2,85E-04	1,82E-04

Figure 3D													
fC/caC			vs	Co			vs T	ET1		vs G45a			
		-2648	-78	+34	+457	-2648	-78	+34	+457	-2648	-78	+34	+457
14h	Co					4							
	G45a	1,99E-02	5,95E-01	1,53E-01	6,35E-01					///////////////////////////////////////			<i></i>
	TET1	3,75E-01	4,57E-01	9,14E-03	8,80E-01								
	TET1+G45a	7,25E-01	4,56E-01	7,70E-01	6,24E-01	5,32E-01	2,12E-01	3,31E-02	5,81E-01	2,59E-02	2,43E-01	6,97E-01	3,73E-01
24h	Co			X////////	<i></i>								
	G45a	7,32E-02	8,47E-01	4,38E-01	9,48E-01								
	TET1	5,83E-03	6,89E-04	2,81E-04	2,50E-04		X///////		X///////				
	TET1+G45a	1.08E-01	5.72E-03	1.69E-05	3.83E-01	2.79E-03	2.07E-02	9.10E-03	1.03E-03	1.33E-02	2.21E-02	1.93E-05	4.96E-01

Figure 4 A-D

Figure 4 A-D					
		vs Co			
		A: mC	B: hmC	C: fC	D: caC
Co	Co				<i></i>
	TET1	8,16E-01	9,81E-04	1,95E-03	
	TET1 +	1,99E-01	2,12E-03	1,83E-03	
	TET1 CD	4,90E-01	1,03E-02	1,97E-02	
	TET1 CD +	1,56E-02	8,93E-05	1,01E-03	
G45a	Co	1,88E-01	1,96E-01	3,77E-01	
	TET1	1,03E-01	2,61E-03	8,07E-04	
	TET1 +	1,54E-01	3,81E-04	6,70E-05	
	TET1 CD	5,76E-02	1,09E-03	2,74E-03	
	TET1 CD +	4 93E-02	6 97E-04	2 75E-03	

w/ vs w/o G45	5a		
mC	hmC	fC	caC
	///////////////////////////////////////	X/////////////////////////////////////	×//////
	<u> </u>		
	<u> </u>	<u> </u>	<u>,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,</u>
1,75E-01	1,80E-01	3,50E-01	N/D
7,27E-02	4,90E-03	1,01E-02	1,16E-02
9,22E-01	2,80E-02	4,13E-03	4,25E-05
4,72E-01	6,55E-02	8,28E-01	1,43E-01
7 37E-01	5.06E-04	7.61E-03	6.71E-03

Figure 4E

	siCo vs siG45a			
	mC	hmC	fC	caC
Co	9,18E-01	6,57E-01	1,55E-01	N/D
TET1	7,98E-01	8,87E-03	7,07E-03	9,39E-03