Supplementary Information

Materials and Methods

Plasmids

For the expression of TET2 protein, TET2 ORF cDNA was cloned into pPB or pFAST expression vector (Invitrogen). TET2 mutants were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. The cDNA of 14-3-3Z/D and 14-3-3E were amplified and subcloned into pET28a (Novagene) and pCMV-tag2B (Agilent). The shRNAs used in current work were purchased from 3DMAX, AMPKα2shRNA-1: CGCAGTTTAGATGTTGTTGGA, AMPKα2shRNA-2: GTCATCCTCATATTATCAAAC,14-3-3Z/D shRNA: GCAGA GAGCAAAGTCTTCTAT, 14-3-3E shRNA: CGACGAAATGGTGGAGTCAAT.

Antibodies and Chemical reagents

Rabbit anti-TET2 antibodies were raised by immunizing rabbits with N-terminus GST-TET2 protein purified from *E.coli.* Anti-FLAG (M2) beads and antibody (F1804) were purchased from Sigma. Anti-HA beads (sc-7392ac) and antibody (2367S) were purchased from Santa Cruz and CST. Anti-14-3-3Z/D (14881-1-AP) and anti-14-3-3E (11648-2-AP) antibody were purchased from Proteintech. TET2pS99 phosphor antibodies were raised in rabbits, using the pre-phosphorylated peptide

(CGGIKRTVpSEPSLSGL-NH2) as an antigen and purified by positive and negative selection as previously described[1].

Chemical activators/inhibitors used in this study include: metformin hydrochloride (AMPK activator, PHR1084, Sigma), A769662 (AMPK activator, S2697, Selleckchem), calpeptin (Calpain inhibitor, Sigma, C8999), BV02 (14-3-3 inhibitor, Sigma, SML0140), R18 (14-3-3 inhibitor, Sigma, SML0108) and cycloheximide (Protein synthesis inhibitor, Sigma, C7698).

Cell Culture, Transfection and Inhibitor Treatment

Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% Pen-Strep unless otherwise specified. Cell transfection was performed using Lipofectamine 2000 (Invitrogen). Cells were harvested at 48 hours post-transfection for protein interaction analysis.

For the inhibitor treatment experiments in general, cells were treated with 2mM metformin hydrochloride for 8 hours; 5μM calpeptin for 16 hours; 1μM of BV02 or R18 for 16 hours; and 10μM A769662 for 4 hours.

TET2 Mutation Hotspot/cluster Analysis with the Modified MSEA Approach

TET2 missense mutation data was obtained from the COSMIC database (COSMIC v79). A total of 960 missense mutations are found in TET2, occurring at 467 amino

acid (AA) positions. There are 379 phosphorylation sites on TET2, out of which 85 correspond to 141 of the aforementioned mutation sites.

We split TET2 into two sections: TET2N (AA 1-1000) and TET2C (AA 1001-2002). To avoid the overshadow effect from the C-terminal mutation hotspots, we developed a modified mutation set enrichment analysis (MSEA) and focused the analysis on TET2N. The modified MSEA identified 290 missense mutations occurring at 206 AA positions on the TET2N. The TET2N region has 184 phosphorylation sites (SYT), out of which 41 correspond to 62 mutations (some mutations were recurrent).

Because most of the established methods do not consider phosphorylation sites[2], we developed a method that takes these sites into account. Specifically, we defined a region score: $S = \frac{\sum_i prop*weight}{\sqrt{\sum_i |i-peak|}}$, where i represents amino acid positions within the region, *prop* represents the proportion of mutations at the i^{th} position out of all mutations, $prop_i = \frac{\#\text{mutations at } i^{th}}{\sum mutations}$, weight indicates the phosphorylation sites, $weight = \begin{cases} 1, \text{non} - \text{STY sites} \\ 2, \text{STY sites} \end{cases}$, and peak is defined as the median position of the sites with the highest weighted proportion of mutations ($prop * weight$). This definition ensures that a region with more mutations occurring on phosphorylation sites (*weight*) or with a high frequency (*prop*) occurring within a converged area (controlled by the distance $\sqrt{\sum_i |i - peak|}$), will have a higher score. Given a defined region, we conducted randomization of the mutations to evaluate the significance of the region. In each randomization, the same load of mutations were randomly distributed along the

amino acid sequence and a random score for each region was calculated. An empirical p-value was calculated by the proportion of random scores exceeding the observed region score.

TAP Complex Purification and Proteomic Analysis of the TET2 Complex

The detailed purification procedure was performed as previously described[3]. In brief, the Flag–HA-tagged human TET2 was constructed into pPB vector and transfected into HEK293T cells. Stable cell lines were established through puromycin selection, and the resulting Flag–HA–TET2 stable cell lines were propagated. The TET2 complex was purified by using anti-FLAG M2 mAb-conjugated agarose beads (Sigma) followed by anti-HA 12CA5 mAb-conjugated agarose beads in buffer B (20 mM Tris-HCl, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1% NP-40, 10 mM 2-mercaptoethanol). Flag-HA double-purified material was separated by 4–20% gradient SDS–PAGE and stained with colloidal Coomassie. Protein bands were excised, digested with trypsin and analyzed by MS/MS mass spectrometry for peptide fragmentation at the Mass Spectrometry Center of Fudan University.

Co-immunoprecipitation

Cells were lysed with the lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, protease inhibitors (including calpain inhibitors) and phosphatase inhibitor cocktail (Roche Applied Science)). The extract was spun at 14,000 rpm for 15

min at 4°C. Two percent of the extract was kept for input, while the rest was incubated with the appropriate antibody (1-2 μg) for 1 hr at 4°C. Protein A/G beads (Millipore) were then added for overnight incubation at 4°C. The beads were washed five times with lysis buffer, and the bound proteins were boiled in SDS sample buffer and Western blotted using indicated antibodies.

TET2 Recombinant Protein Expression and Purification

The TET2 CDS was subcloned into a modified pFastBac-HTA vector with a FLAG affinity tag fused to the N terminus. Bacmids were generated in DH10Bac cells following the instructions for the Bac-to-Bac baculovirus expression system (Invitrogen). Baculoviruses were generated and amplified in Sf-9 insect cells. For protein expression and purification, Sf-9 insect cells were grown in Sf-900™II medium supplemented with FBS. TET2 protein was expressed at 27 °C for 72 h using the previously generated viruses. Infected cells were harvested by centrifugation at 2,000g for 15 min and homogenized in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.9, 300 mM NaCl, 0.5% NP-40 and 0.5 mM phenylmethanesulfonyl-fluoride (PMSF)). The cells were disrupted using a cell homogenizer and the insoluble fraction was precipitated by ultracentrifugation (20,000g) for 30 min at 4 °C. FLAG M2 Agarose beads (Sigma-Aldrich) were added into supernatant, incubated 2 hours and washed four times with lysis buffer.

In Vitro Kinase Phosphorylation Assay and Interaction with 14-3-3Z/D

Active recombinant human AMPK (α2, β1, γ1) holoenzyme (14-902) was purchased from EMD Millipore. The purified FLAG-TET2 (WT/S99A) proteins(on M2 beads) were incubated with the recombinant AMPK holoenzyme in a 20 μl reaction containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT and 200 μM ATP. The reactions were incubated at 30°C for 1 hr. The phosphorylation reaction was stopped by washing three times with ice-cold binding buffer (25 mM Tris-HCl, pH 7.9, 150 mM NaCl, 0.1% NP-40 and 0.5 mM PMSF). Then recombinant 14-3-3Z/D proteins were added and the interaction assay was carried out at 4°C for another 4 hr. After the beads were washed five times by binding buffer, the samples were run under reducing conditions on SDS–PAGE gels and immunoblotted.

Isothermal Titration Calorimetry (ITC)

To obtain a direct binding affinity between 14-3-3 proteins and phosphorylated form TET2, TET2 peptides (LQNGGIKRTVpSEPSLSG) were titrated with 14-3-3Z/D using ITC-200 microcalorimeter (GE Healthcare) at 10°C. Unmodified peptides (LQNGGIKRTVSEPSLSG) were also titrated with 14-3-3Z/D to verify whether Ser99 phosphorylation is critical for binding affinity. RAF Ser259 phosphorylated peptides (RSRSTpSTP) were included as a positive control. All proteins and peptides were exchanged to a buffer containing 10 mM HEPES, pH 8.0 and 0.1 M NaCl by gel-filtration chromatography. ITC data were analyzed and fit using Origin 7.0 software (OriginLab Corporation).

Protein Half-life Assay

Analysis of TET2 protein stability by the cycloheximide(CHX) chase assay was carried out as following: cell lines expressing TET2 WT or mutant were seeded and cultured for 24 hours. Then the cells were pre-treated with calpeptin (Calpain inhibitor) for 4 hours. CHX (dissolved in DMSO) was added at final concentration of 100 μg/ml. The cells were harvested at the time points indicated and processed by Western blot.

Immunofluorescence staining

Cells were plated onto glass coverslips and cultured for 2 days. The fixation was carried out with 4% paraformaldehyde for 15 min, fixed cells were washed with cold PBS and permeabilized for 15 min with cold PBS containing 0.4% Triton X-100. Permeabilized cells were then washed and incubated for 1 hour in blocking buffer (10% donkey serum and 3% bovine serum albumin in PBS containing 0.1% Triton X-100). Cells were incubated with primary antibodies overnight in a humidified chamber at 4°C. After three consecutive 5-min PBS washes, cells were incubated with secondary antibodies and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 30 min. Cells were washed three times with PBS and mounted using fluorescent mounting medium (Dako). Images were acquired using Nikon Ti w/ Perfect Focus & Spinning Disk Confocal and analyzed with ImageJ.

Bioinformatics Analysis

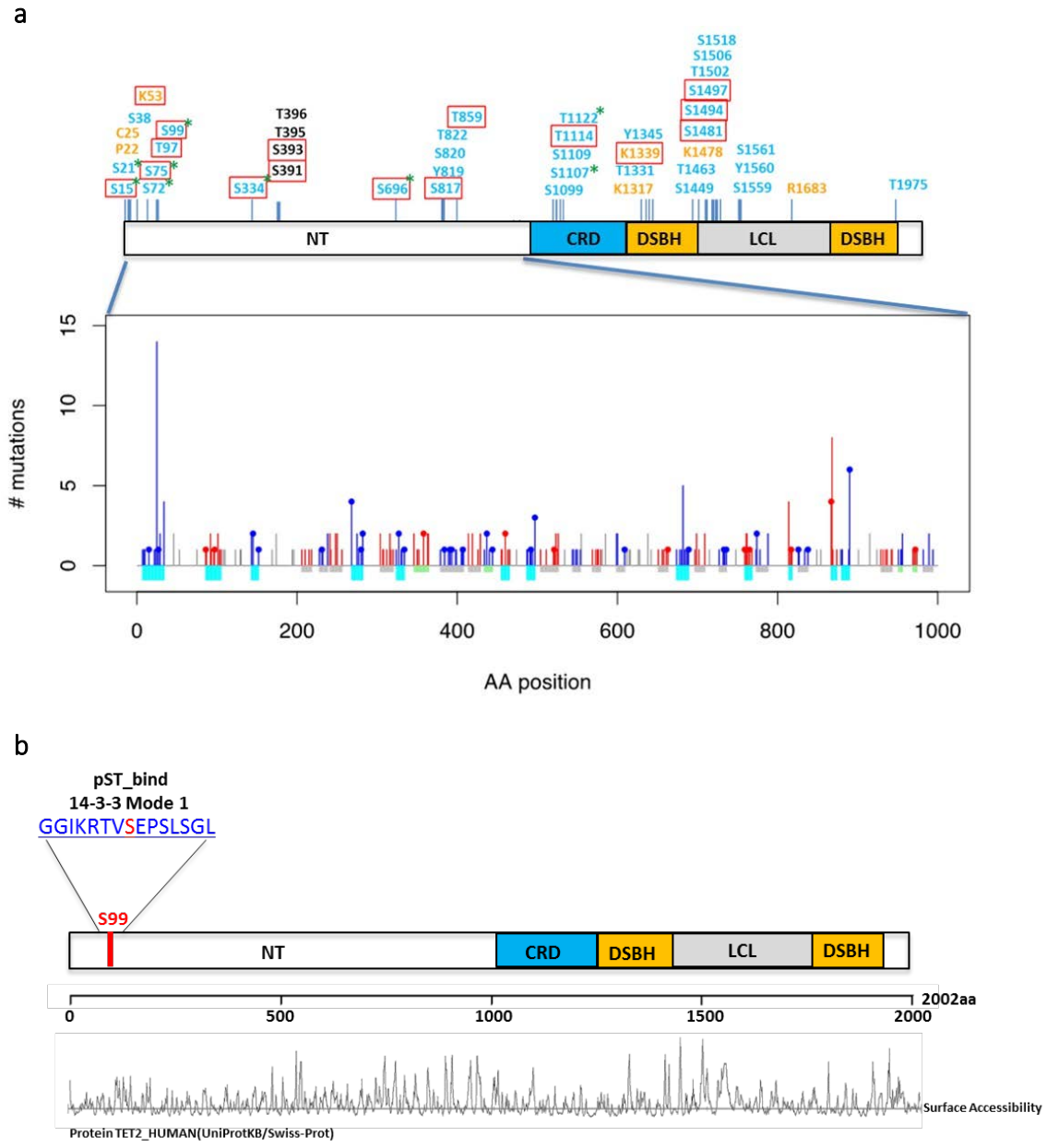
TET2 cancer mutations occurring on serine/threonine or residues that undergo post-translational modification were extracted from Catalogue Of Somatic Mutations In Cancer (COSMIC v79). Reported TET2 phosphorylation sites were obtained from PhosphoSitePlus under accession number Q6N021[4].These two datasets were compared and the overlapped sites were identified and further analyzed by conventional mutation/phosphorylation cluster scanning. Briefly, the mutation and phosphorylation co-occurrence status was evaluated in a 20aa window with 10aa step. In each window, the mutation or phosphorylation occurrence was quantified by accumulating all the records from the mutation or phosphorylation database that was located within the bin.

The prediction of 14-3-3 recognition sites was carried out by Scansite3[5]. Briefly, the sequence of human TET2 protein was used as the input sequence for 14-3-3 motif searching. In order to find the best matched motifs, high stringency was used.

References:

1 Ma H, Chen H, Guo X *et al.* M phase phosphorylation of the epigenetic regulator UHRF1 regulates its physical association with the deubiquitylase USP7 and stability. *Proceedings of the National Academy of Sciences of the United States of America* 2012; **109**:4828-4833.
2 Jia P, Wang Q, Chen Q, Hutchinson KE, Pao W, Zhao Z. MSEA: detection and quantification of mutation hotspots through mutation set enrichment analysis. *Genome biology* 2014; **15**:489.
3 Shi Y, Sawada J, Sui G *et al.* Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* 2003; **422**:735-738.
4 Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic acids research* 2015; **43**:D512-520.
5 Yaffe MB, Leparc GG, Lai J, Obata T, Volinia S, Cantley LC. A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nature biotechnology* 2001; **19**:348-353.

Fig. S1 The clustered cancer mutations, enriched phosphorylation sites and putative 14-3-3 binding motif on TET2.

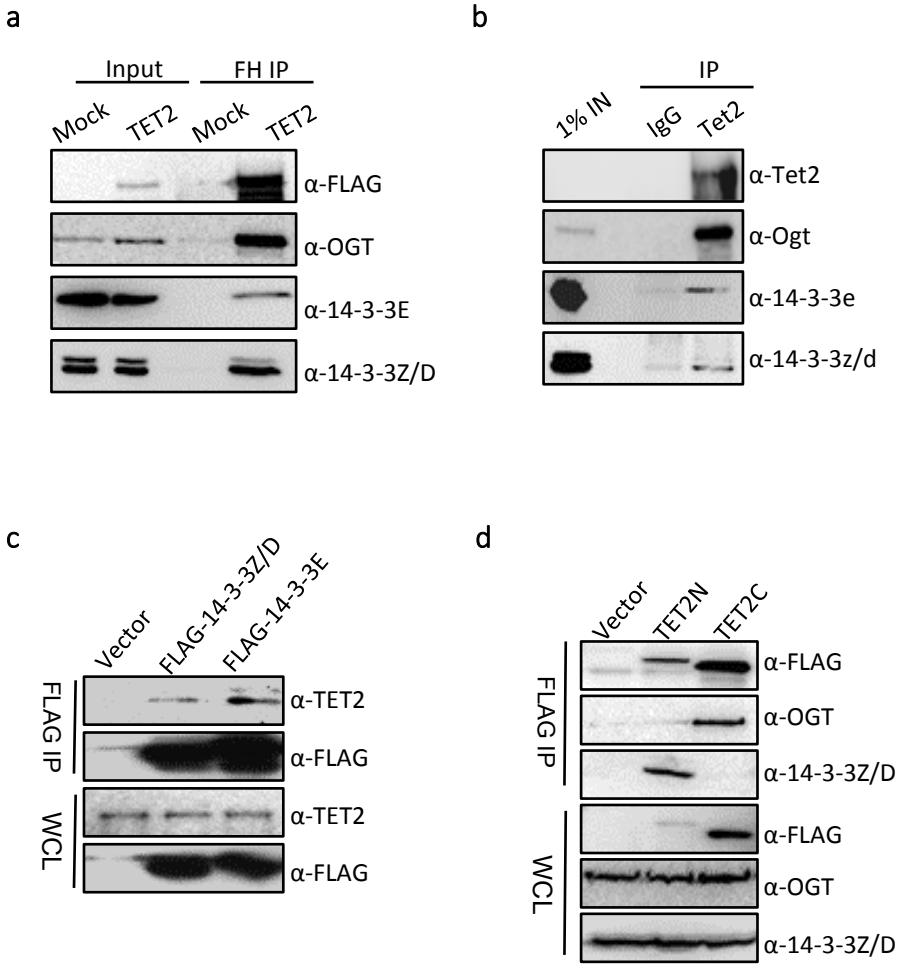


a MSEA analysis detects mutation clusters in the TET2 N-terminus (1-1000aa) (in red or blue). Potential phosphorylation-associated cancer mutations on TET2 are depicted on top. Those included in red boxes are phospho-proteomic-validated phosphorylated residues that are recurrent in somatic cancer mutations. Grey bars indicate mutations not forming clusters. Cyan bars indicate significant clusters ($p < 0.05$) and green bars

marginally significant clusters ($p < 0.1$). Cyan bars were determined by randomizing non-synonymous mutations.

b Scansite 3 Motif analysis identifies S99 as the only potential 14-3-3 binding site on TET2 under high-threshold settings.

Fig. S2 Biochemical purification of the TET2-associated cellular complex and proteomic determination of 14-3-3 proteins as new TET2 interacting partners.



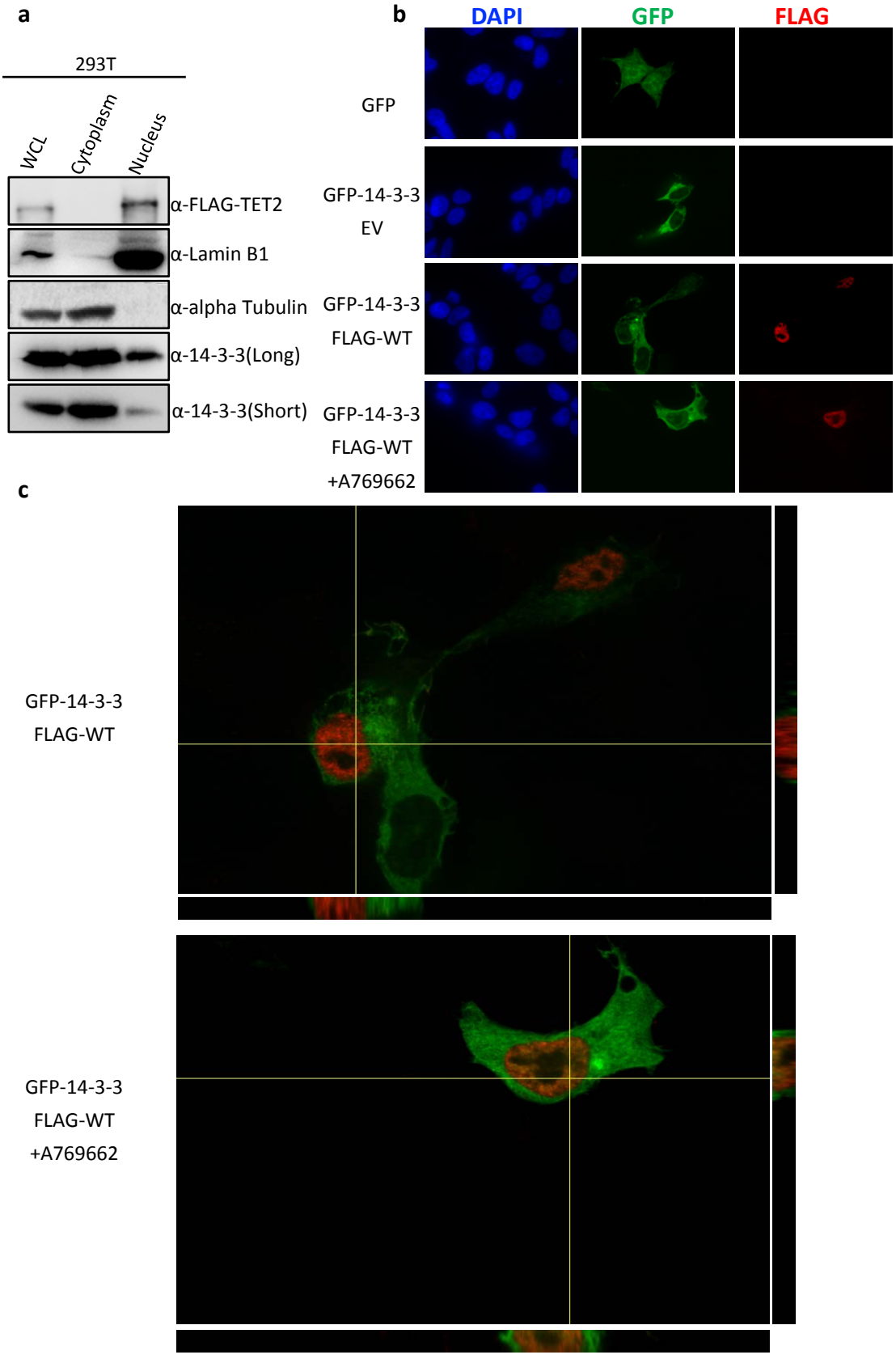
a Immunoblot validation of the major components of the TET2 complex using specific antibodies indicated.

b Endogenous TET2 specifically interacts with 14-3-3 family members in mouse ESC.

c Reciprocal Co-IP of TET2 by FLAG-tagged 14-3-3 proteins.

d Co-IP of 14-3-3 by the N- or C- terminus of TET2 shows that the N-terminus of TET2 specifically associates with 14-3-3.

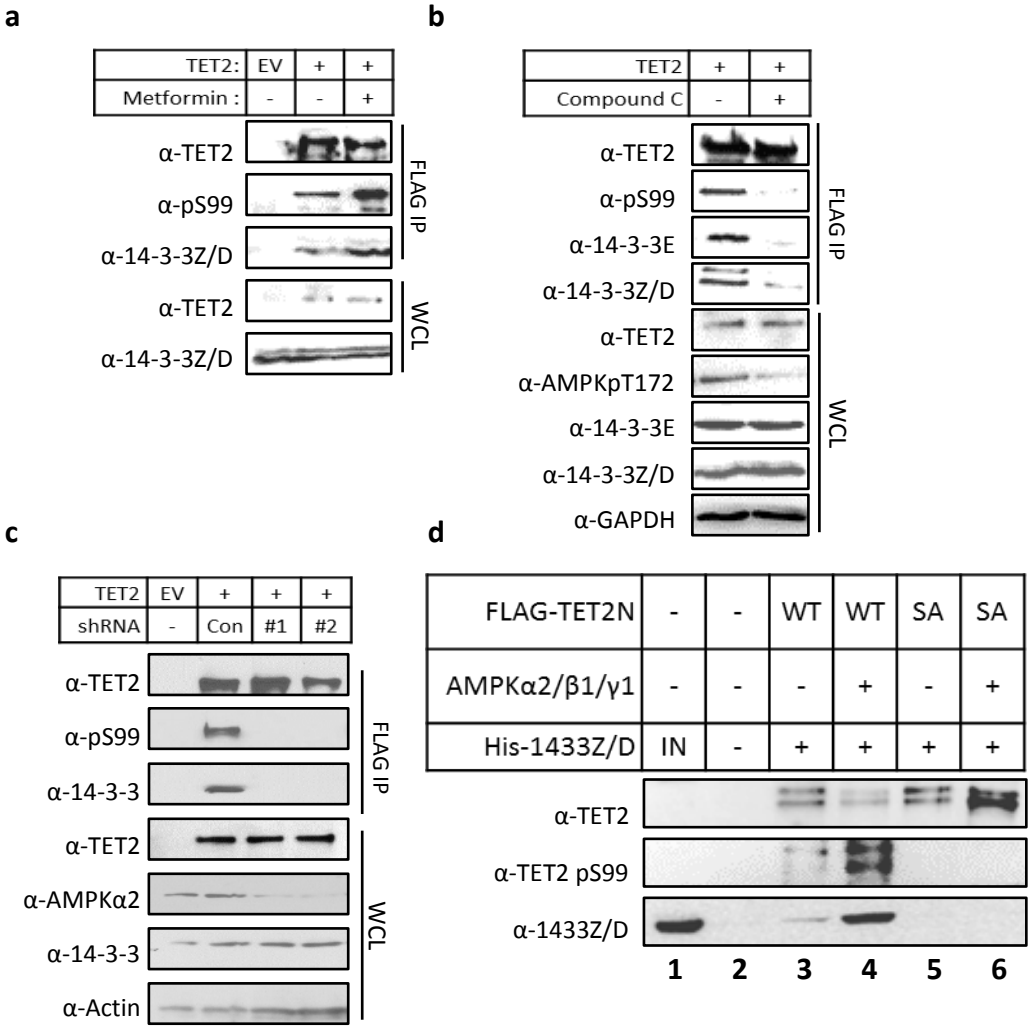
Fig. S3 A small portion of 14-3-3Z/D exists in nucleus and co-localizes with TET2.



a Different cellular fractions were prepared and blotted with indicated antibodies, 14-3-3 protein can be detected in both cytoplasm and nucleus.

b-c Immunofluorescent staining was carried out in 293T cells transfected with GFP-14-3-3Z/D and FLAG-TET2. While vast majority of GFP-14-3-3Z/D localized to cytoplasm, a small portion of GFP-14-3-3Z/D was shown localizing to nucleus. On the other hand, TET2 (red) is exclusively localized to nucleus. The co-localization analysis by immunofluorescent staining and the confocal microscopy (bottom two panels in b and 3D view of the localization in c) indicates that 14-3-3Z/D co-localizes with TET2 in nucleus and that is boosted by AMPK activator A789662 treatment.

Fig. S4 TET2 S99 phosphorylation by AMPK is required for the interaction between TET2 and 14-3-3 proteins.



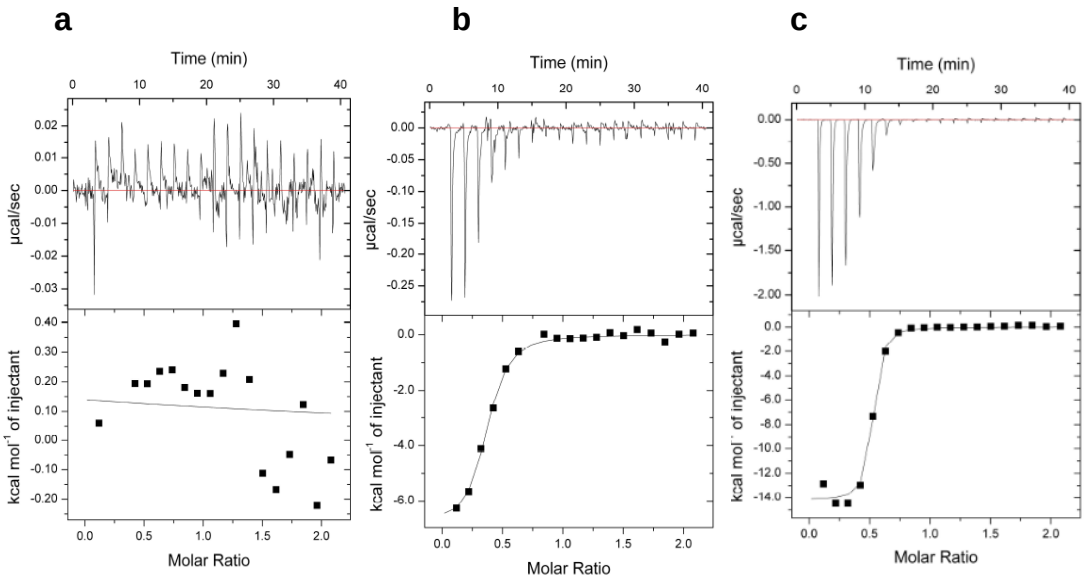
a The AMPK activator metformin increased TET2 S99 phosphorylation and enhanced the TET2/14-3-3 interaction.

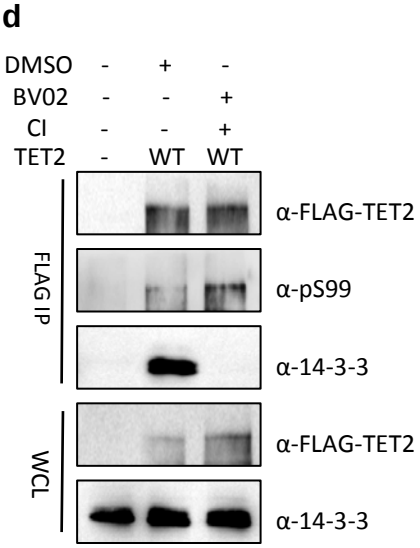
b The AMPK inhibitor, compound C, drastically decreased TET2 Ser99 phosphorylation and disrupted TET2/14-3-3 interaction.

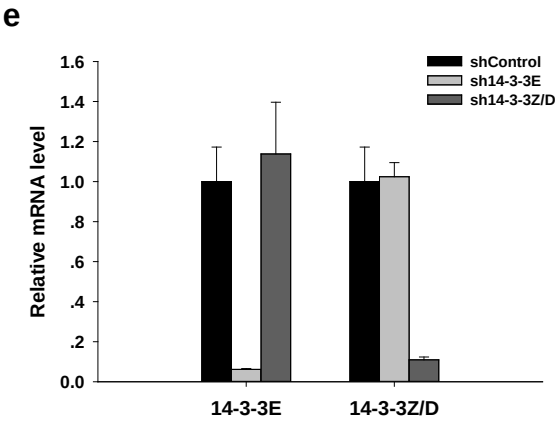
c Downregulation of AMPK by AMPKα2 shRNA greatly reduced TET2pS99 levels and consequently diminished the TET2/14-3-3 interaction.

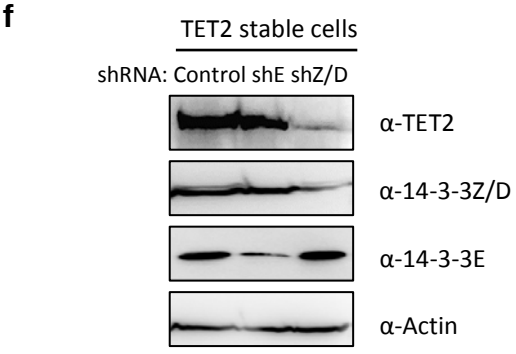
d Direct interaction between TET2 and 14-3-3 requires TET2pS99 in an *in vitro* binding assay. The TET2 S99A mutation abolishes the direct TET2/14-3-3 interaction *in vitro*. **Note:** 1) In lane 3, recombinant WT TET2 protein purified from insect cells had residual S99 phosphorylation, therefore showing weak interaction with 14-3-3 even without AMPK treatment; 2) In lane 4, although loading of total WT TET2 recombinant protein in this lane is less visible than that in the lane 3, the S99 phosphorylation level after active AMPK treatment showing on this lane is much higher than that in lane 3. Accordingly, the interaction between 14-3-3 and TET2 appears to be the strongest. Please also see the technical note in the main Figure legend.

Fig. S5 14-3-3Z/D specifically binds to TET2pS99 peptide in ITC and is involved in TET2 stability regulation.









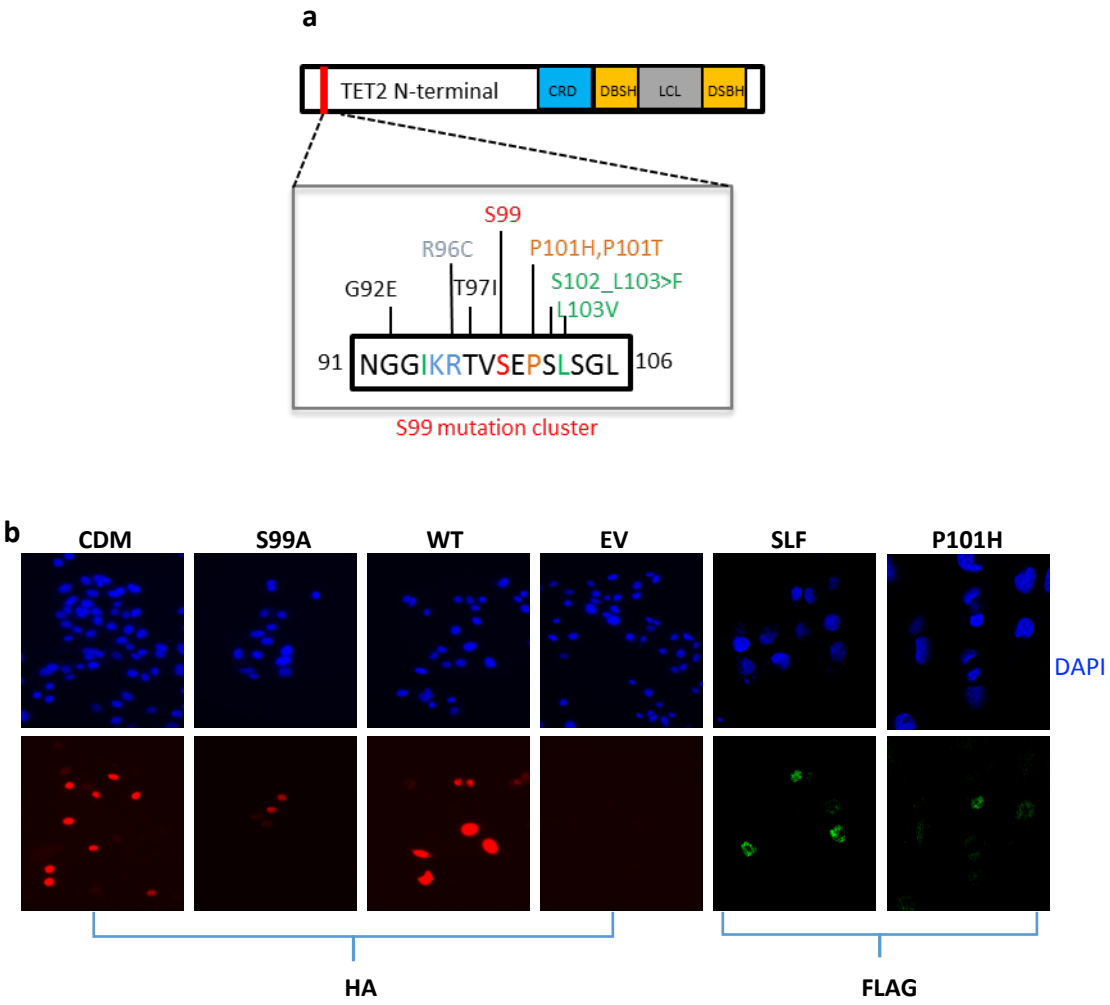
a-c Kinetic analysis of the interactions between 14-3-3Z/D and the unphosphorylated TET2Ser99 peptide **(a)**, phosphorylated TET2pSer99 peptide **(b)** or a phosphorylated RAF kinase peptide, RAFpSer259 **(c)**. The kinetic assays were performed using isothermal titration calorimetry (ITC). RAFpSer259, a well-known interacting partner of 14-3-3Z/D, is a positive control. ITC measurements for a fit curve to a standard single-site binding model were carried out following the standard protocol. The fit curve data shows that 14-3-3Z/D binds to TET2pSer99 (Kd = 810 nM) in a similar magnitude of strength as that of RAFpSer259 (Kd = 230 nM), whereas the unmodified peptides do not show any noticeable interaction (Kd is not determinable).

d Treatment of 14-3-3 inhibitor BV02 abolished interaction between 14-3-3 protein and TET2, however, it did not reduce TET2 S99 phosphorylation. Calpain inhibitor (CI) was added to ensure the TET2 protein levels are similar under different conditions.

e RT-qPCR measurement to determine the knockdown efficiency of shRNAs.

f 14-3-3Z/D but not 14-3-3E depletion induces significant decrease of TET2 protein levels. We should note that knockdown of 14-3-3E, another 14-3-3 isoform identified in TET2 complex, alone doesn't have noticeable effect on TET2 stability. While the nature of the distinct effect of the different 14-3-3 isoforms (14-3-3Z/D vs. 14-3-3E) on TET2 protein stability is still unclear, we speculate that 14-3-3Z/D is the major player contributing to stabilizing TET2. It is possible that 14-3-3E is indirectly or redundantly involved in regulation of TET2 stability or plays other roles by reading TET2Ser99 phosphorylation, as it has been demonstrated that different member of 14-3-3 family proteins plays different roles under various cellular context.

Fig. S6 The mutation cluster around TET2 Ser99 don't affect TET2 localization.



a Diagram of TET2 S99 mutation cluster on TET2 N-terminal portion.

b Nuclear localization of TET2 WT and mutants. The cells expressing WT and various forms of TET2 mutant proteins were processed according to the same protocol, and the images were captured under identical microscopic settings. The representative IF staining images indicate similar nuclear localization of WT and mutants of TET2. Red color: HA epitope-tagged TET2; Green color: FLAG epitope-tagged TET2.

Table S1 The list of major components of TET2 complex identified by proteomic analysis.

Protein Name	Protein Information	Hits(Unique peptides)
TET2	Methylcytosine dioxygenase TET2	668(71)
OGT1	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase	239(38)
SIN3A	Paired amphipathic helix protein Sin3a	53(15)
14-3-3E	14-3-3 protein epsilon	36(8)
RBM14	RNA-binding protein 14	12(5)
14-3-3Z/D	14-3-3 protein zeta/delta	12(4)
HDAC1	Histone deacetylase 1	14(4)
SAP25	Histone deacetylase complex subunit SAP25	14(3)
NONO	Non-POU domain-containing octamer-binding protein	6(2)
EWS	RNA-binding protein EWS	3(2)
RBBP4	Histone-binding protein RBBP4	3(2)
ZN281	Zinc finger protein 281	2(2)
MSH6	DNA mismatch repair protein Msh6	8(1)
ING2	Inhibitor of growth protein 2	7(1)
DPY30	Protein dpy-30 homolog	2(1)
KPYM	Pyruvate kinase isozymes M1/M2	2(1)

Table S2 Parameters of the ITC measurement

Peptides	N(Sites)	K (M^{-1})	ΔH(cal/mol)	ΔS(cal/mol/deg)
pSer99	0.343 ±0.00741	1.18E6 ±1.97E5	-6939 ±210.1	3.96
Ser99	0.00167 ±14.4	7.43 ±8.74E3	-2.486E8 ±2.214E12	8.54E+05
RAFpSer259	0.485 ±0.00530	4.26E6±1.33E6	-1.420E4 ±253.7	-18.4