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Regulation of TET Protein Stability by Calpains

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SUMMARY

DNA methylation at the fifth position of cytosine (5mC) is an important epigenetic modification that affects chromatin structure and gene expression. Recent studies have established a critical function of the Ten-eleven translocation (Tet) family of proteins in regulating DNA methylation dynamics. Three *Tet* genes have been identified in mammals, and they all encode for proteins capable of oxidizing 5mC as part of the DNA demethylation process. Although regulation of *Tet* expression at the transcriptional level is well documented, how TET proteins are regulated at posttranslational level is poorly understood. In this study, we report that all three TET proteins are direct substrates of calpains, a family of calcium-dependent proteases. Specifically, calpain1 mediates TET1 and TET2 turnover in mouse ESCs, and calpain2 regulates TET3 level during differentiation. This study provides evidence that TET proteins are subject to calpain-mediated degradation.

INTRODUCTION

The ten-eleven translocation (Tet) family of proteins was initially described when the gene encoding the founding member TET1 was identified as a fusion partner of the mixed lineage leukemia (MLL) gene in acute myeloid leukemia (Ono et al., 2002). However, TET proteins were not at a central stage until they were found to oxidize 5mC to 5-hydroymethylcytosine (5hmC) as part of the DNA demethylation process (Ito et al., 2010; Tahiliani et al., 2009). Subsequent studies demonstrated that TET proteins further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are removed through base excision repair, thus completing the demethylation process (He et al., 2011; Ito et al., 2011). Expressions of TET proteins are tightly regulated at the transcriptional level. For example, in mouse embryonic stem cells (mESCs) both *Tet1* and *Tet2* are positively regulated by Oct4, and their mRNA levels decrease dramatically upon mESC differentiation. In contrast, *Tet3* is significantly upregulated during differentiation (Koh et al., 2011). In addition to transcription, two recent studies reported that microRNA (miR-22) regulates Tet mRNA in leukemia and breast cancers (Song et al., 2013a, 2013b). However, regulation of TET proteins at the posttranslational level is less understood. One recent study suggests that IDAX and CXXC5 interact with TET2 and regulate its stability through caspase-dependent degradation (Ko et al., 2013). It is not clear whether TET1 and TET3 are subjected to a similar regulation.

Four major proteolytic systems mediate protein turnover: proteasome, lysosome, caspase, and calpain. Proteasomes are best known for degrading proteins that are modified by polyubiquitylation (Glickman and Ciechanover, 2002); Lysosomes mediate the bulk breakdown of proteins or organelles (Pan et al., 2008); caspases are a family of cysteine proteases involved in proteins cleavage during programmed cell death (Cohen, 1997). Finally, calpains are a family of calcium-dependent cysteine proteases with 14 members identified in human (Storr et al., 2011). So far, calpain1 and calpain2 (μ- and m-calpains, respectively) are the best characterized members. Known substrates for calpain include structural proteins, signaling molecules and transcriptional factors (Suzuki et al., 2004). Dysregulation of calpains have been linked to a number of human diseases such as muscular dystrophy, diabetes, and Alzheimer's disease (Zatz and Starling, 2005). Moreover, calpains have been implicated in stem cell maintenance and differentiation (Santos et al., 2012; Yajima and Kawashima, 2002). Because of the ubiquitous expression pattern and large number of family members, novel calpain substrates and biological functions of calpain-mediated protein cleavage have yet to be identified.

In this study, we took advantage of the various chemical inhibitors for different protein turnover pathways and identified calpains as major players that mediate TET protein turnover. We then use a well-established protocol to differentiate mESC toward neural progenitor cells (NPCs) to demonstrate that calpain1 and calpain2 are responsible for TET protein turnover in ESCs and NPCs, respectively.

RESULTS

Posttranslational Regulation of TET Proteins

The three *Tet* genes have distinct expression profiles, whereas *Tet1* and *Tet2* are downregulated during ESC differentiation,

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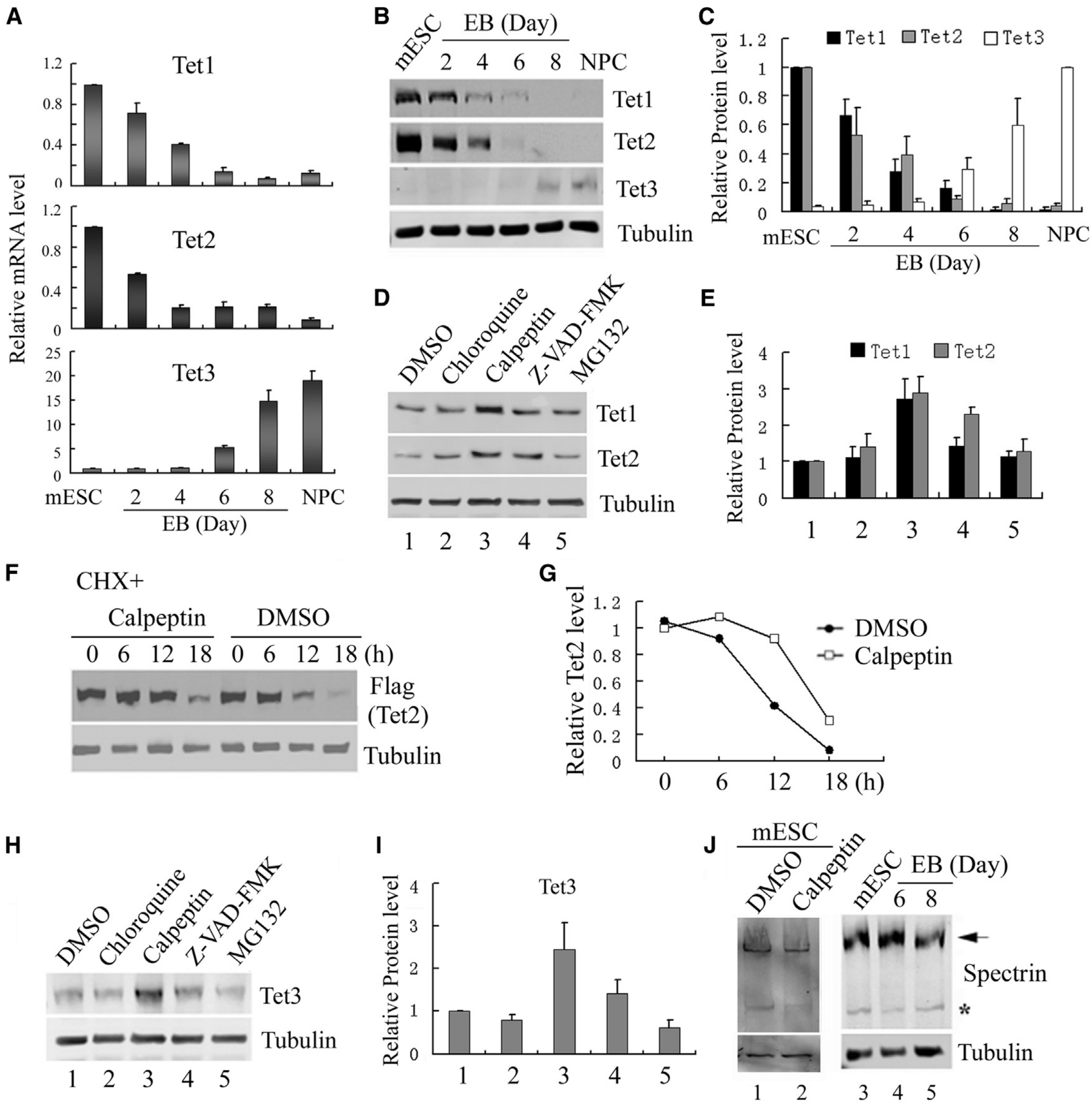

Figure 1. Regulation of TET Protein Levels by Transcription and Protein Stability

(A) qRT-PCR analysis of *Tet* mRNA levels during mESC to NPC differentiation. Although *Tet1* and *Tet2* levels decrease during differentiation, *Tet3* level is significantly upregulated. Data represent the mean of three independent experiments ±SD, and *Tet* levels in mESCs are set as 1.

(B and C) Representative western blot (B) and quantification of three repeats ±SD (C) demonstrate that TET protein levels generally follow mRNA levels during NPC differentiation.

(D and E) Representative western blot analysis of TET1 and TET2 levels in mESCs treated with chloroquine, calpeptin, Z-VAD-FAM, and MG132 for 24 hr. Quantification of three independent experiments ±SD was shown in (E).

(F and G) Calpeptin increases the half-life of FLAG-TET2 protein. Western blot (F) and quantification (G) of the FLAG-TET2 levels in the presence or absence of calpeptin upon inhibition of protein translation by cycloheximide.

(H and I) Representative western blot analysis of TET3 in day 7 embryoid body (EB) treated with chloroquine, calpeptin, Z-VAD-FAM, and MG132 for 24 hr, and the results were quantified in (I).

(J) Calpain activity is detectable in mESCs and during their differentiation. Western blot analysis of mESC lysate with a spectrin antibody identified both full-length (arrow) and cleaved spectrin (*), a marker for calpain activity. Spectrin cleavage is detectable during mESC differentiation (lanes 4 and 5) and was prevented by calpeptin treatment (compare lane 1 and 2).

Tet3 is upregulated in the same process (Koh et al., 2011). To systematically examine the relationship between TET mRNA and protein levels, we utilized an embryonic body (EB)-based protocol to differentiate mESC into NPCs (Figure S1A) (Bibel et al., 2007). Successful differentiation was verified by significant upregulation of the neural marker Nestin (Figure S1B). We then examined TET expression change during differentiation by quantitative RT-PCR (qRT-PCR) and western blot. We found that although both *Tet1* and *Tet2* are downregulated during mESC differentiation, *Tet3* is upregulated (Figure 1A). Western

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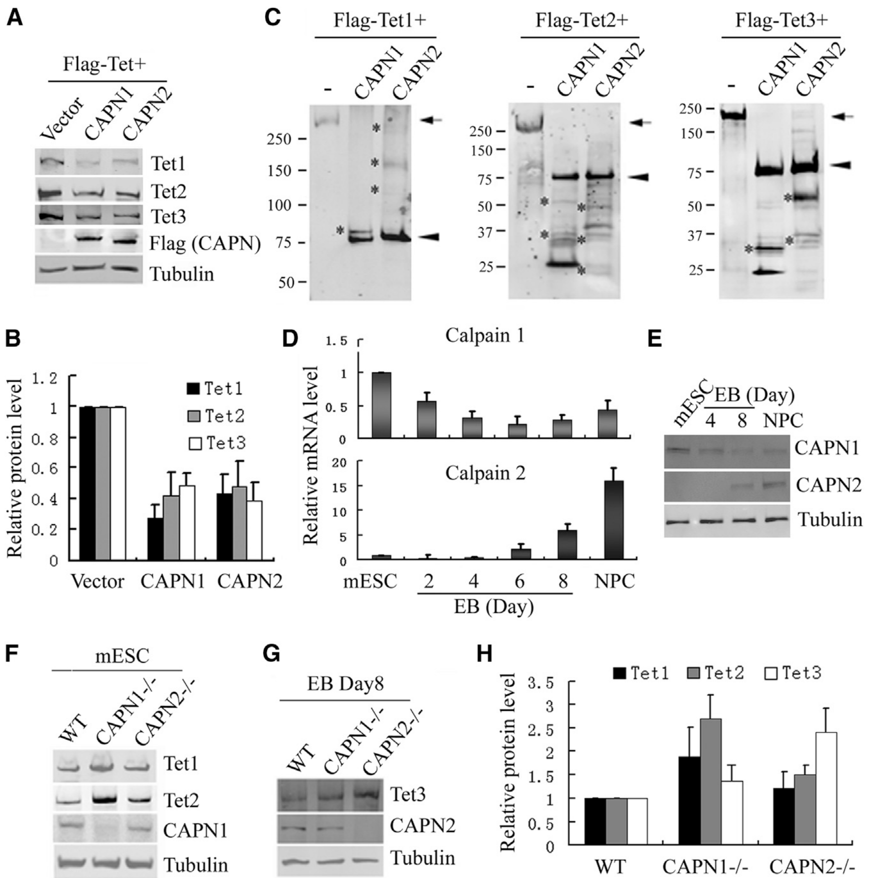

Figure 2. Tet Proteins Are Direct Substrates of calpain1 and calpain2

(A and B) Representative western blot analysis (A) and quantification of three independent repeats ±SD (B) demonstrate that exogenously expressed TET protein levels can be reduced by coexpression of calpain1 or calpain2 in 293T cells.

(C) Western blot analysis demonstrates that both calpain1 and calpain2 can cleave all three Tet proteins in vitro. Purified FLAG-Tet1, Tet2, and Tet3 were incubated with buffer alone or purified FLAG-calpain1 or calpain2 at room temperature for 30 min before western blot analysis with FLAG antibody. asterisk, cleaved products; arrow, full-length TET; arrowhead, calpains.

(D and E) qRT-PCR (D) and western blot (E) analysis demonstrate that *calpain1* and *calpain2* are reversely expressed in mESCs and NPCs. Data represent the mean of three independent experiments ±SD, and value from mESC is normalized as 1.

(F) Western blot analysis demonstrates that both TET1 and TET2 levels are increased in *calpain1* knockout mESCs, whereas *calpain2* knockout has little effect. *Calpain1* and *calpain2* knockout mESC were generated by CRISPR.

(G) Western blot analysis of the TET3 levels in day 8 EB demonstrates *calpain2* knockout increases TET3 levels, whereas the effect of *calpain1* knockout is modest.

(H) Quantification of three independent experiments (F and G), value in WT cells is set as 1, and error bars represent SD.

blot analysis revealed that TET protein levels correlate with mRNA levels (Figures 1B and 1C), suggesting TET expression is largely controlled at the transcription level. Nevertheless, the rapid protein turnover of TET1 and TET2 between EB days 2 and 6 suggests a possible posttranslational regulation. To explore this possibility, we analyzed the effect of various proteolytic pathways on TET protein turnover by focusing on ESCs for TET1 and TET2, and EB day 8 for TET3. We treated cells with inhibitors of the four major proteolytic pathways: proteasome (MG132), lysosome (chloroquine), calpain (calpeptin), and caspase (Z-VAD-FMK) and found that calpeptin treatment induced the most significant accumulation of TET1 and TET2 proteins, and a less prominent effect was observed by inhibiting caspase. However, no significant effect was observed when treated with lysosome or proteasome inhibitors (Figures 1D and 1E). We confirmed the effectiveness of MG132 as well as chloroquine (Figures S1C and S1D). Thus, lysosome and proteasome are not essential for TET protein turnover.

To further evaluate the role of calpeptin in stabilizing TET proteins, we attempted to determine the half-life of TET by cycloheximide treatment that blocks protein synthesis. Because mESCs are sensitive to cycloheximide, we expressed TET2 in 293T cells and then treated the cells with cycloheximide. We found that calpeptin extended TET2 half-life from 10 to 16 hr (Figures 1F and 1G), supporting a role of calpains in TET2 degradation. In addition to mESCs, we also analyzed the effect of the various proteolysis pathways on TET3 stability in EBs and observed a similar effect by calpeptin treatment (Figures 1H and 1I).

The above results suggest that calpains are likely responsible for TET turnover. Next, we examined calpain activity in mESC and EBs by monitoring the cleavage of spectrin, a well-characterized calpain substrate (Czogalla and Sikorski, 2005). Western blot analysis of EB day 6 and day 8 lysates clearly showed a lower band matching cleaved spectrin, which disappeared following calpeptin treatment (Figure 1J), suggesting calpain activity is present in both self-renewing and differentiated mESC. Collectively, the above results suggest that calpain-mediated proteolysis play a role in regulating TET protein stability, and caspases may also contribute to this process. Because the role of caspases has been recently reported (Ko et al., 2013), we focus our study on calpain-mediated regulation of TET proteins.

Tet Proteins Are Direct Substrates of Calpains

To directly address the role of calpains in regulating TET stability, we asked whether exogenously expressed TET2 can be downregulated by coexpression of calpain1 or calpain2, two of the best characterized calpains. As shown in Figures 2A and 2B, TET levels are significantly decreased by coexpression of either calpain1 or calpain2. To examine if calpains directly cleave TET proteins, we performed calpain cleavage assays in vitro using purified calpain1, calpain2, and three TET proteins. Results shown in Figure 2C demonstrate that all three TET proteins are efficiently cleaved by both calpain1 and calpain2. The variable sizes of the cleavage products (Figures 2C and S2A) suggest multiple cleavages sites. The proteolytic activity of calpain1 and calpain2 is not due to contaminating proteases because neither calpain1 nor calpain2 cleaved RNF4 under the same conditions (Figure S2C).

To test if calpain1 and calpain2 regulate TET protein stability in vivo, we analyzed the expression profiles of calpain1 and calpain2 during mESC differentiation. qRT-PCR analysis indicated that calpain1 level is relatively high in mESCs, whereas calpain2 is mainly expressed in NPCs (Figures 2D and 2E). Considering Tet expression profiles (Figure 1A), we hypothesize that calpain1 mainly regulates TET1 and TET2 stability in mESCs, whereas calpain2 regulates TET3 during differentiation. To test this possibility, we utilized the CRISPR-based genome editing technology (Cong et al., 2013; Mali et al., 2013) and generated *calpain1* and *calpain2* knockout mESCs (Figure S2D). Targeting sequences were designed against exons of the N-terminal part of the transcript (Figure S2D), and no off-target was identified based on the established criteria (Hsu et al., 2013). The genotypes were determined by DNA sequencing. A clone with frameshifts on both alleles is chosen and further confirmed by western blot analysis (Figures 2F and 2G). As expected, both TET1 and TET2 levels are increased in *calpain1* knockout mESC compared to control (Figure 2F). Due to a low calpain2 level in mESCs, the effect of *calpain2* knockout is less apparent (Figure 2F). However, when the knockout mESCs are induced to differentiate, significant increase in TET3 levels is observed in *calpain2*$^{-/-}$ EBs, which is less apparent in *calpain1*$^{-/-}$ cells (Figure 2G). The observed effect is likely mediated at the protein level as Tet mRNA level is not significantly altered by calpain knockout (Figures S2F and S2G). These results strongly suggest that calpains regulate TET protein levels in vivo and the regulation exhibits isoform and cell differentiation state specificity.

Calpains Regulate TET Functions in mESC Maintenance and Differentiation

TET proteins play complicated roles in mESCs (Wu and Zhang, 2011). Although *Tet1* and *Tet2* double knockout results in a depletion of 5hmC and dysregulation of hundreds of genes, the mESCs remain pluripotent (Dawlaty et al., 2013). To understand the role of calpain-mediated TET cleavage in mESCs, we focused on some known functions of TET proteins. Because calpains functionally antagonize TET proteins, we anticipate that depletion of calpains and TET proteins result in opposite phenotypes. Similar to *Tet1/2* double knockout, *calpain1*$^{-/-}$ or *calpain2*$^{-/-}$ mESCs exhibit typical mESC morphology (Figure S2E), and no obvious defect in self-renewal was observed. Consistently, the levels of the key pluripotency factors, including *Oct4*, *Sox2*, and *Nanog*, are not significantly altered by calpain knockout (Figure 3A). Consistent with the report that 5hmC generation depends on TET1 and TET2 (Dawlaty et al., 2013), dot-blot analysis revealed a 2-fold increase in 5hmC levels in *calpain1*$^{-/-}$ mESCs, whereas *calpain2*$^{-/-}$ had little effect (Figures 3B and 3C). This result is consistent with the fact that *calpain1*, but not *calpain2*, is expressed in mESCs and regulates TET1/2 protein levels (Figure 2F). Although not affecting pluripotency, knockdown of Tet in mESCs does affect the expression of lineage-specific transcription factors. For example, trophectoderm marker *Cdx2* and *Eomes* are significantly upregulated in Tet1 knockdown cells, whereas expression of other markers such as *Lefty1* is decreased (Ito et al., 2010; Koh et al., 2011). We confirmed this observation and importantly obtained an opposite effect in *calpain1*$^{-/-}$ mESCs presumably due to the stabilization of TET1 proteins (Figure 3D). To rule out the possibility that the gene expression change in *calpain1*$^{-/-}$ cells is caused by other calpain1 substrates, we knocked down Tet1 in *calpain1*$^{-/-}$ mESCs, and the expression profiles of these genes were reversed (Figures 3D and S3D). These data suggest that although calpain1 knockout does not affect mESC maintenance, it affects 5hmC generation and lineage-specific gene expression in a way opposite to Tet1 knockdown, consistent with a role of calpain1 in regulating TET1 and TET2 stability.

Because calpain2 regulates TET3 levels in EB differentiation (Figure 2G), we next analyzed the biological relevance of this enzyme-substrate pair during mESC differentiation. TET3 plays an important role in regulating expression of some neural transcription factors such as *Pax6* and *Ngn2* during neurogenesis in *Xenopus* (Xu et al., 2012). To test if this mechanism is conserved in mammals, we generated *Tet3*$^{-/-}$ mESC with a published CRISPR guiding sequence (Figure S3A) (Wang et al., 2013). Clones carrying frameshifts on both alleles were selected. Consistent with previous report (Wang et al., 2013), *Tet3* knockout does not affect mESC morphology or self-renewal (Figure S3B). EB-based differentiation followed by qRT-PCR analysis demonstrated that the expression levels of *Pax6* and *Ngn2* were significantly reduced in *Tet3* knockout mESCs (Figure 3E), suggesting a functional conservation of Tet3 between *Xenopus* and mammals. Importantly, both *Pax6* and *Ngn2* are upregulated in *calpain2*$^{-/-}$ EBs, and small hairpin RNA (shRNA)-mediated *Tet3* knockdown in *calpain2*$^{-/-}$ cells abolished this upregulation (Figures 3E and S3E). However, manipulation of calpain2 or Tet3 does not affect the expression of other neuronal marker genes, such as *β3-tubulin* (Figure 3E). This suggests that, although calpain2 and TET3 affect the expression of certain neural genes, they are not master regulators that drive differentiation from mESCs to NPCs.

EBs are composed of a mixed cell population that includes nonneural lineage cells. To study the effect of calpain2 and TET3 on differentiation efficiency from mESCs to NPCs, we disassociated EBs and switched to monolayer culture in chemically defined N2 medium, which enrich NPCs by eliminating none NPCs and intermediates. The surviving cells showed typical bipolar NPC morphology and were positive for *Nestin* and *Sox2* (Figure 3F). Although NPCs were successfully generated from all four groups of cells, the yield differs significantly (Figure 3G). The increased NPC differentiation efficiency in *calpain2*$^{-/-}$ mESCs is likely due to the increase in TET3 levels as Tet3 knockdown in *calpain2*$^{-/-}$ cells suppressed NPC generation (Figure 3G). This result suggests that calpain2-mediated degradation of TET3 modulate neuronal gene expression program and the efficiency of in vitro neural differentiation. Upregulation of calpain2 during NPC differentiation may be part of a negative feedback mechanism that prevents hyperactivation of Tet3.

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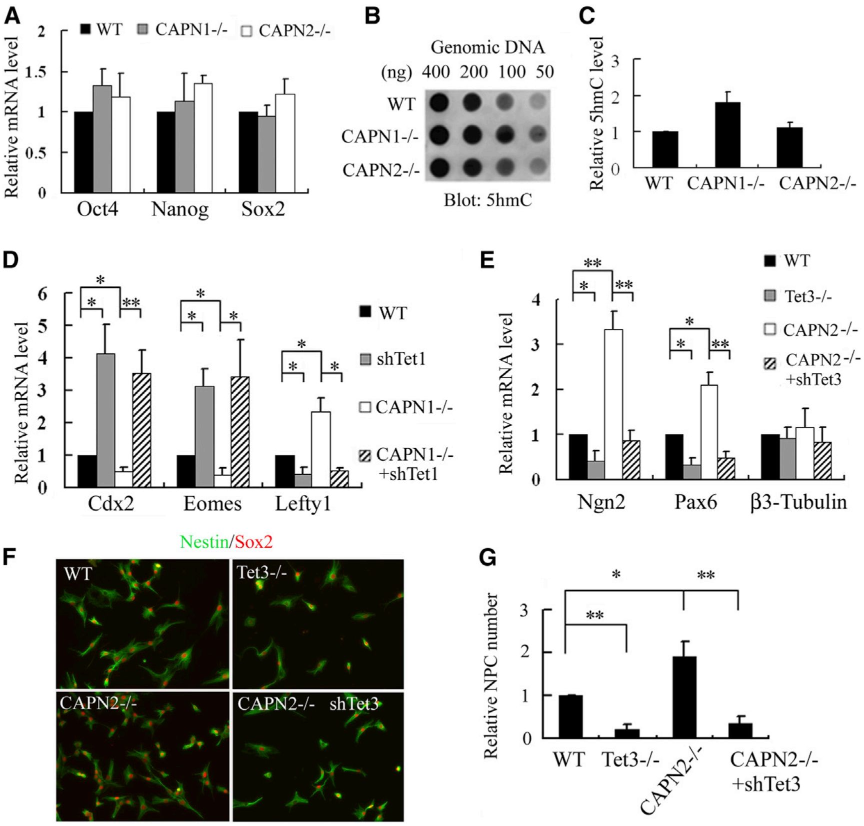

Figure 3. Effects of Calpain-Mediated TET Cleavage on Gene Expression and NPC Differentiation

(A) qRT-PCR analysis demonstrates that *calpain1* or *calpain2* knockout in mESCs does not affect pluripotent gene expression. Data represent the mean of three independent experiments ±SD, and value in WT mESC is set as 1.

(B and C) Dot-blot analysis (B) and densitometry quantification of three repeats (C) demonstrate that *calpain1* knockout, but not *calpain2*, increased 5hmC levels in mESCs.

(D) qRT-PCR analysis demonstrates that *Tet1* knockdown in mESC enhances trophectoderm lineage genes (*Cdx2* and *Eomes*) expression and inhibits *Lefty1*. Knockout of *calpain1* opposes this tendency, which is rescued by *Tet1* knockdown. Data represent the mean of three independent experiments ±SD, and value in WT mESC is set as 1.

(E) qRT-PCR analysis demonstrates that during differentiation to NPC (EB day 8), *Tet3* knockout significantly reduces the expression of neuronal markers *Ngn2* and *Pax6*, whereas *calpain2* knockout enhanced their expression, which is reversed by *Tet3* knockdown. In contrast, *β3-tubulin* expression is not affected by either Tet3 or calpain2. Value in WT EB is set as 1, and error bars represent SD.

(F) Immunostaining demonstrates generation of *Nestin* and *Sox2* double-positive NPCs. After EB disassociation and a 48 hr adherent culture, *Nestin*- and *Sox2*-positive NPCs were successfully generated from all WT and knockout cells.

(G) TET3 and calpain2 have opposite effect on mESC differentiation to NPC. Although $Tet3^{-/-}$ significantly reduced NPC generation, $CAPN2^{-/-}$ enhanced the differentiation efficiency, which is abolished by Tet3 knockdown. Number from WT cells is normalized to 1. Error bars represent SD; *p < 0.05; **p < 0.01.

DISCUSSION

In this study, we provide evidence that TET proteins are direct substrates of calpains. Specifically, calpain1 modulates TET1 and TET2 levels in mESCs, whereas calpain2 promotes TET3 turnover during neural differentiation. Calpain-mediated regulation of TET proteins is physiologically relevant, given that it affects global 5hmC level and expression of certain lineage-specific genes in mESCs, as well as mESC differentiation.

Cell differentiation is a highly orchestrated process with dynamic proteomic changes as unwanted proteins are degraded. The importance of major proteolytic systems including proteasome, caspase, calpain, and lysosome has been implicated in cell differentiation (Buckley et al., 2012; Fujita et al., 2008; Guan et al., 2013). Utilizing inhibitors against these proteolytic systems, we identified calpains as important regulators of TET protein turnover (Figures 1D–1I). We also observed a modest effect by inhibiting caspase (Figures 1D and 1E), which is consistent with a recent report (Ko et al., 2013). In fact, calpain and caspase are proteases that share many properties and substrates (Wang, 2000). Although we focused on calpains in this study, the relative contribution of calpain and caspase in regulating TET protein turnover remains to be determined. It worth noting that whereas we observed an effect of calpains and caspases on TET turnover, no obvious effect was detected by inhibiting proteasomes, indicating that the ubiquitylation pathway does not play a major role in regulating TET protein turnover.

It is well known that calpain-mediated cleavage can either result in protein turnover or generate functional truncated proteins. Calpain-mediated TET cleavage likely results in turnover because TET degradation products observed in vitro (Figure S2A) were undetectable in mESCs or 293T cells cotransfected with TET2 and calpains, suggesting that the cleaved TET fragments are unstable and are quickly turned over in vivo. Moreover, the wide spectrum of TET degradation products suggests many cleavage sites, making it difficult to generate mutant TET proteins that are resistant to calpains, which would otherwise be useful tools in functional studies. However, the fact that knocking down Tet in $calpain^{-/-}$ cells can rescue the $calpain^{-/-}$ phenotypes strongly supports the biological relevance of this enzyme-substrate pair (Figures 3D, 3E, and 3G). In this study, we have tested only two of the bested characterized calpains, and the role of the other 12 calpains in regulating TET stability remains unknown.

Tet protein levels are consistent with their mRNA levels, suggesting a dominant regulation at the transcriptional level (Figures 1A and 1B), yet posttranslational mechanism may be required to fine-tune TET protein level and function. Considering the large numbers of calpain substrates, and the difficulty in generating calpain-resistant TET mutants, we choose to study the function of calpain-mediated TET degradation by focusing on some known TET functions, such as 5hmC generation and expression of some lineage-specific genes. The opposite effects from depletion of Tet and calpain, and the observation that Tet knockdown reverses the phenotypes of calpain knockout (Figures 3B–3G) strongly support a role of calpain-mediated TET protein degradation. Given that calpains are calcium-dependent proteases, studying calpain-Tet in physiological contexts such as neuron activation is of great relevance. In addition, cancer cells may prove to be another useful model in understanding calpain-mediated TET degradation as calpain levels are frequently elevated, whereas TET are downregulated in cancer cells (Storr et al., 2011; Yang et al., 2013). Our findings provide mechanistic basis for these future studies.

EXPERIMENTAL PROCEDURES

Differentiation of Neural Progenitors

Experiment was performed as described (Bibel et al., 2007). ESCs (4×10^6) were plated into nonadherent dish in differentiation medium (ES medium with 10% fetal bovine serum and no leukemia inhibitory factor) to form embryoid body. On day 4, 5 μM retinoic acid was applied. On day 8, embryoid bodies were disassociated and cultured in N2 medium in PORN/laminin-coated plates.

Knockout Calpains by CRISPR

Design of targeting constructs was described in Hsu et al. (2013). To knock out *calpains*, CRISPR constructs were cotransfected with a puromycin resistant vector. After puromycin selection, single clones were picked, and the genotypes were determined by sequencing. Clones with frameshifts on both alleles were selected for further analysis.

In Vitro Calpain Assay

Proteins were exogenously expressed and purified from 293T cells. TET proteins were incubated with calpain1, calpain2, or elution buffer as control. $CaCl_2$ (1 mM) was added and the reaction was performed at room temperature for 30 min before being stopped by adding Laemmli buffer.

More details are available at Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.12.031.

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