ChIP-Sequencing of TET Proteins

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i. Chapter title: ChIP-sequencing of TET proteins

ii. Abstract

TET proteins are methylcytosine dioxygenases that interact directly with chromatin to shape the DNA methylation landscape. To increase the understanding of TET protein function in a specific cellular context, it is important to be able to map the interactions between TET proteins and DNA. This ChIP-seq protocol details our procedure to analyse TET2 bound DNA in disuccinimidyl glutarate (DSG) and formaldehyde crosslinked chromatin, but can also be adapted to study other TET enzymes.

iii. Keywords: TET1, TET2, TET3, DNA methylation, DSG, ChIP-seq

1. Introduction

Chromatin immunoprecipitation (ChIP) coupled with high-throughput sequencing is a powerful technique to identify the pattern by which specific proteins associate with DNA in a genome-wide manner. It has been successfully applied to study transcription factors, DNA replication and repair proteins, histone modifications and chromatin-associated proteins. The Ten-Eleven-Translocation (TET) enzymes are a class of DNA-modifying enzymes capable of oxidizing 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxycytosine. All three TET enzymes (TET1-3) share a conserved catalytic domain in the C terminus, which has been shown to interact directly with DNA and flip out the target cytosine for modification [1, 2]. In addition to their catalytic domain, TET1 and TET3 also have a DNA binding CXXC zinc finger domain in the N terminus, which shows a preference for binding to CpG sites in DNA. These domains, as well as protein-protein interactors specific for each TET enzyme, are likely to play important roles to determine how the TET enzymes interact with chromatin in specific cell types.

In 2011, we and others reported the first genome-wide TET1 ChIP-seq dataset in embryonic stem cells [3–5]. These experiments were performed on formaldehyde-crosslinked chromatin using specific antibodies raised against TET1. As a control for specificity, depletion of TET1 by RNAi was shown to result in decreased signal on TET1 enriched sites. The first genome-wide TET2 ChIP-seq datasets based on formaldehyde-crosslinked chromatin from embryonic stem cells were published in 2013 [6, 7]. While these studies identified thousands of TET2-enriched chromatin regions, they did not quantify the unspecific background signal by assessing enrichment in TET2 knockout or RNAi depleted cell lines. The biological significance of these datasets is therefore still unclear. We and others have used CRISPR/Cas9 to introduce ChIP-grade epitope tags in the endogenous TET2 locus [8, 9]. In our experience, introduction of an epitope tag at the TET2 C-terminus results in stable TET2 expression

with no impact on global 5-hydroxymethylcytosine levels in embryonic stem cells. By using specific (and commercially available) antibodies against the tag these cells could subsequently be used to optimize ChIP conditions. We found that inclusion of the protein-protein crosslinker DSG as well as titration of antibody and input chromatin resulted in improved signal-to-noise ratio. Furthermore, we tested these optimized conditions using a polyclonal affinity-purified antibody raised against TET2 N-terminus ***(see note 1)*** and identified thousands of TET2 enriched genomic sites, which were absent in TET2 knockout control cells [9].

Here we describe our protocol to analyse chromatin occupancy of TET2 in mouse embryonic stem cells and hematopoietic suspension cells. However, this procedure has also previously been successfully applied to analysis of TET1 occupancy on formaldehyde crosslinked chromatin [3]. For the reasons described above, we highly recommend that experiments are designed to determine specificity of the ChIP signal by performing side-by-side analysis of proper negative control cells, such as a cell line without the epitope tag or a *TET2* knockout cell line (Figure 1).

2. Materials

2.1 Chromatin preparation and immunoprecipitation

2.1.1 General materials

DNA LoBind tubes

UltraPure Agarose for gel electrophoresis

Tris-acetate-EDTA (TAE) buffer

SYBR Safe DNA gel stain

6X loading buffer without dye (1X TAE in 60% glycerol)

Dimethyl Sulfoxide

DSG (disuccinimidyl glutarate) in lyophilized format (ThermoFisher Scientific #20593)

37% Formaldehyde solution in water with 10-15% methanol as stabilizer

2M Glycine solution in water

20% SDS in water

1M $NaHCO_3$ in water

PBS without Mg^{2+} and Ca^{2+}

Protein Assay Dye Reagent concentrate (BioRad #500-0006)

Bovine Serum Albumin (BSA) protein standard

cOmplete™, EDTA-Free protease inhibitor tablets

0.1M Phenylmethanesulfonyl fluoride (PMSF) solution in ethanol

Anti-FLAG® M2 Affinity Gel beads (Sigma-Aldrich #A2220)

Protein G Sepharose® 4 Fast Flow beads (GE Life Sciences #17061801)

Unspecific control IgG antibody

2X SYBR green qPCR master mix

FLAG peptide (DYKDDDDK)

Polystyrene tubes for sonication

Sonicator (e.g. Bioruptor® Plus sonication device)

End-over-end rotator

Spectrophotometer set to a wavelength of 595nm

PCR purification column (QIAgen or similar)

Qubit™ fluorometer

Qubit™ dsDNA HS assay Kit

Thermomixer

qPCR machine

Neon™ Transfection system (ThermoFisher Scientific #MPK5000)

UV transilluminator

Cell-specific qPCR primers targeting TET2-enriched chromatin in mouse embryonic stem cells

Target	Forward primer	Reverse primer
mm10 chr6:83422012-83422102	GGTCAATGCCCAAGAATCAT	CAGGCTCACCTGCTCCTTAT
mm10 chr3:135546453-435546536	CAAGCTGTCCAAGGATGACA	TACAGACACACCCGAGCAAG

2.1.2 Buffers

Prepare the following stock buffers:

1) SDS buffer: 50mM Tris-HCl pH 8.1, 100mM NaCl, 5mM EDTA, 0.5% SDS
2) Triton dilution buffer: 50mM Tris-HCl pH 8.6, 100mM NaCl, 5mM EDTA, 5% Triton X-100
3) SDS-free buffer: 50mM Tris-HCl pH 8.1, 100mM NaCl, 5mM EDTA

Mix the stock buffers in the described ratios to obtain the following:

1) IP buffer: *Mix stock buffers 1, 2, and 3 in ratio 1:2:3* to make buffer with 0.1% SDS and 1.66% Triton X-100
2) SDS-free sonication buffer: *Mix stock buffers 2 and 3 in ratio 1:2* to make buffer with 0% SDS and 1.66% Triton X-100
3) Sonication buffer: *Mix stock buffers 1 and 2 in ratio 1:0.5* to make buffer with 0.33% SDS and 1.66% Triton X-100

Wash and elution buffers:

1. 150mM Wash buffer: 20mM Tris-HCl pH 8.0, 150mM NaCl, 2mM EDTA, 0.1% SDS, 1% Triton X-100
2. 500mM Wash buffer: 20mM Tris-HCl pH 8.0, 500mM NaCl, 2mM EDTA, 0.1% SDS, 1% Triton X-100
3. FLAG elution buffer: 20mM Tris-HCl pH 8.0, 150mM NaCl, 2mM EDTA, 0.5mg/mL FLAG peptide

2.2 Library preparation

NEBNext® Ultra II DNA library preparation kit for Illumina (New England Biolabs #E7645)

NEBNext® Multiplex Oligos for Illumina (New England Biolabs #E7335)

PCR machine

AMPure XP beads (Beckman Coulter #10136224)

Magnetic rack for 0.2mL tubes

Agilent Bioanalyzer or Tapestation

3 Methods

3.1 Chromatin preparation and immunoprecipitation

3.1.1 Introduction of endogenous FLAG epitope tag on TET2 C terminus

1. Clone sgRNA to introduce cuts in the immediate vicinity of the TET2 C terminus and design a single-stranded DNA oligonucleotide repair template, which contains the tag of interest as well as 60bp homology arms. After successful homology-directed repair the insertion of the tag should destroy the sgRNA target site to avoid further cuts.
    a. sgRNA target site in TET2 C-terminus (PAM in bold): TTTGTATGACGCTGGCCATT**AGG**
    b. ssDNA repair template (2xFLAG tag in bold): ACCGTGACTACATCACCATATGCTTTCACTCAGGTCACAGGGCCTTACAACACATTTGTAGG CGCC**GACTACAAGGACGACGATGACAAGGACTACAAGGACGACGATGACAAG**CGTACCG GTTGACGCTGGCCATTAGGCCAGACCACCAAGGACGACCTGTGAGCAGTATGTCTTTCATG GCAT
2. Co-transfect sgRNA and repair template by lipofection. In hard-to-transfect cell lines and primary cells use Neon™ transfection system. Perform single-cell sort into 96-well plates and expand clonal cultures to screen for successful insertion of the tag.

*3.1.2 Cell culture **(see note 2)***

1. Thaw and expand experimental and control cells depending on the chosen strategy for immunoprecipitation. Experimental cells must express wildtype or an epitope-tagged TET protein, while control cells are either TET protein knockout or express TET proteins without the epitope tag.
2. Grow adherent or suspension cells in sufficient quantity (>20x10^6 cells per replicate). Use a minimum of two independent replicates per condition that are ***crosslinked and sonicated on different days*** to assess technical variation.
3. Obtain a single-cell solution by trypsinization of adherent cells (e.g. embryonic stem cells). Wash once in ice-cold PBS without Ca^{2+} and Mg^{2+}. Count cells and normalize the number of cells across experimental and control samples. Discard the leftover cells. NB: Different cell densities will affect sonication efficiency and should be avoided.

*3.1.3 Crosslinking **(See note 3)***

1. Remove a new vial of DSG (disuccinimidyl glutarate) from 4°C storage. When it is equilibrated to room temperature, resuspend the desiccated powder in DMSO to obtain a stock solution of 0.25M DSG. Immediately before adding to the cells, prepare the final crosslinking solution of 2mM DSG in ice-cold PBS (e.g. 80ul of 0.25M DSG in 10mL of PBS). Discard the unused

0.25M DSG stock solution as reconstituted DSG will tend to hydrolyse and become inactive during storage.

2. Resuspend cell pellet from each sample (20-100 x10^6 cells) in 10mL of freshly prepared, ice-cold 2mM DSG crosslinking solution. Place tubes on a roller mixer and allow crosslinking to proceed for 30 min while the solution is equilibrating to room temperature.
3. After the initial 30 min has passed, add formaldehyde to a final concentration of 1% (e.g. 270uL of a 37% stock solution in 10mL). Incubate on a roller mixer at room temperature for an additional 10 min.
4. Immediately after 10 min has passed, quench crosslinking reaction by adding 2M glycine to a final concentration of 0.125M (e.g. 625uL in 10mL). Incubate on a roller mixer at room temperature for an additional 5 min.
5. Spin cells (300 x g / 5 min / 4^{o}C) and wash twice with ice-cold PBS. Discard solution containing formaldehyde in the appropriate waste container. Proceed immediately to lysis and sonication.

3.1.4 Cell lysis and sonication of chromatin

1. Add PMSF protease inhibitor to SDS buffer to a concentration of 1mM. Keep this solution at room temperature to avoid precipitation of SDS. Resuspend crosslinked and washed cell pellets in 10mL of SDS buffer / 1mM PMSF and incubate 5 min at room temperature.
2. Spin cells (300 x g / 6 min / 20^{o}C) and discard supernatant. Resuspend pellets (often with a white and fluffy appearance) in sonication buffer containing protease inhibitors and store on ice. It is important to keep the chromatin concentrated for optimal sonication and immunoprecipitation. We recommend resuspending in 0.5 mL of sonication buffer per 20x10^6 cells in the starting material.
3. Transfer solution to polystyrene tubes for optimal sonication efficiency. Sonicate samples using your instrument of choice to obtain chromatin fragments of 200-600bp of DNA. It is necessary to optimize instrument settings for each cell type, cell density and crosslinking condition.
4. To check fragmentation, remove a 30uL aliquot and store the remaining sonicated chromatin until correct fragmentation has been verified. Sonicated chromatin can be frozen at -80^{o}C or stored for a few days at 4^{o}C. Spin the 30uL aliquot (20,000 x g / 30 min/ 4oC), transfer the supernatant to a fresh tube and add SDS to 1% and $NaHCO_3$ to 100mM and incubate (65^{o}C / 1,000 rpm) in a thermomixer for at least 3h or overnight to de-crosslink. Isolate DNA using a PCR purification column (QIAgen or similar) and load on 1% agarose gel to verify fragment sizes. Use a 6X loading buffer without dyes as these will often mask the DNA smear during gel

exposure under UV light. If needed, the stored chromatin can be retrieved from storage and sonicated further. Proceed to next step when optimal fragment size is observed.

3.1.5 Preclearing

1. Spin samples of sonicated chromatin (20,000 x g/ 20 min/ 4°C) and transfer the chromatin lysate to a fresh tube. Discard pellets containing insoluble material.
2. Determine protein concentration by Bradford (BioRad Protein assay or similar). Mix 2uL of chromatin lysate or BSA protein standard to 800uL of water. NB: do not add more than 2uL of chromatin lysate due to adverse effect of detergents in the assay. Add 200uL of 5X BioRad Protein assay and mix immediately. Transfer to cuvette and measure absorbance at 595nm.
3. Dilute the chromatin lysate so that 1mL will be sufficient for one IP sample (minimum 0.3mg chromatin in 1mL of IP buffer). The chromatin is initially diluted (3.3-fold) in SDS-free sonication buffer and then topped up with IP buffer to obtain a final concentration of 0.1% SDS.
4. Retrieve control affinity gel (e.g. protein G Sepharose 4 fast flow) from stock and pellet by centrifugation (1,800 x g / 1 min / 4°C). Wash three times with 1mL of IP buffer. Resuspend beads using a volume of IP buffer that corresponds approximately 1:1 to the bead packing volume after centrifugation. This beads solution is referred to as a 50/50 slurry.
5. Add 100ul of the 50/50 slurry to the chromatin lysates and incubate at rotating wheel for 3h at 4°C. Spin (1,800 x g / 1 min / 4°C) and transfer the precleared chromatin lysate to a fresh tube. Discard the beads.

3.1.6 Immunoprecipitation and washing steps ***(see note 4)***

1. Transfer 1mL each of the precleared chromatin lysate into two separate Eppendorf tubes (IP and IgG control). Transfer 10uL of the precleared chromatin lysate to an Eppendorf tube to be used as 1% input control. Keep the 1% input control at 4°C while performing the immunoprecipitation. Store the remaining precleared chromatin lysate at -80°C.
2. Retrieve FLAG M2 affinity gel and IgG control affinity gel (e.g. protein G Sepharose 4 fast flow) from stock and pellet by centrifugation (1,800 x g / 1 min / 4°C). Wash three times with 1mL of IP buffer. Resuspend beads in a volume of IP buffer that correspond approximately 1:1 to the bead packing volume after centrifugation to obtain a 50/50 slurry.
3. Add 1μg of unspecific control antibody to control affinity gel (to use in the IgG control immunoprecipitation) and incubate 3h on rotating wheel at 4°C to bind antibody. Wash and resuspend beads as described in previous step.

4. Add 20ul of the 50/50 slurry to the corresponding samples (FLAG M2 beads in IP sample and IgG control beads in IgG sample) and incubate at rotating wheel for 2-3h at 4°C.
5. Spin (1,800 x g / 1 min/ 4°C) beads and wash 3 times in 150mM Wash Buffer by incubating at rotating wheel for 5 minutes each time. Wash an additional two times in 500mM Wash buffer followed by a final wash step in IP buffer to reduce salt concentration.

3.1.7 Elution and decrosslinking of immunoprecipitated protein complexes

1. Prepare 10X stock solution (5 mg/mL) of FLAG peptide (DYKDDDDK) by dissolving lyophilized peptides in 20mM Tris-HCl pH 8.0. Prepare fresh FLAG elution buffer with 0.5mg/mL FLAG peptide.
2. Resuspend beads from the final wash step in 40uL of elution buffer and incubate for 30min at 20°C. Spin (1,800 x g / 1 min/ 4°C) and transfer supernatant to fresh tube. Repeat twice with new elution buffer and combine supernatants to obtain a final elution volume of 120uL.
3. Add SDS to 1% and $NaHCO_3$ to 100mM and incubate (65°C / 1,000 rpm) in a thermomixer for at least 3h or overnight to de-crosslink. Remember to also de-crosslink the 1% input control which was stored at 4°C at the beginning of the immunoprecipitation procedure. Isolate DNA using a PCR purification column (QIAgen or similar). Elute in 2x 40uL EB buffer in DNA LoBind Eppendorf tubes.

3.2. Quality control and DNA library preparation

3.2.1 Quality control

1. Measure DNA concentration in the DNA eluate using the Qubit® dsDNA HS assay Kit. Typical concentrations of immunoprecipitated DNA range from 30-100 pg/uL. It is therefore necessary to use 10uL of DNA eluate in 190uL Qubit® working solution to get a robust measurement.
2. Use DNA eluate for quantitative PCR (1-2ul per sample in a 10uL reaction volume) to estimate specific enrichment and genomic background signal in all samples (Input, IgG control, IP). Depending on the primer set used, it is desirable to observe a >8-fold enrichment over the off-target signal observed in cells without FLAG-TET2 before proceeding to library generation and next-generation sequencing. To design primer sets for qPCR analysis, it is necessary to have prior knowledge about cell-type specific TET binding sites.

3.2.2 DNA Library preparation for Illumina sequencing

1. The immunoprecipitated DNA can be used as input in commercial DNA library preparation kits for Illumina sequencing. We routinely use NEBNext® Ultra II DNA library preparation kit (New England Biolabs) with an input of 1-3 ng of immunoprecipitated DNA (up to 50uL of DNA eluate). We generally dilute the adaptors 1:25-1:50 fold (0.6-0.3μM) to avoid formation of primer-dimers in the final library.
2. The adaptor-ligated DNA fragments are size-selected using AMPure XP beads to retain inserts of approximately 200bp according to instructions in the kit manual (1st bead addition: 0.4X, 2nd bead addition: 0.2X).
3. The adaptor-ligated and size-selected DNA fragments are amplified by PCR. It is important to use a low number of cycles to avoid over-amplification. We generally use 10-12 cycles to obtain sufficient material for quality control and illumina sequencing.
4. Purify amplified DNA library using AMPure XP beads and measure the size distribution of the library on an Agilent Bioanalyzer or Tapestation.

4 Notes

1. ChIP-seq analysis of endogenous TET2 protein (without an epitope tag) requires a ChIP-seq grade α-TET2 antibody. We successfully generated this by immunization of rabbits with an N-terminal fragment of mouse TET2 (aa1-300) coupled with GST. The antibodies were absorbed on GST-coupled cyanogen bromide-activated Sepharose and subsequently affinity-purified using Sepharose coupled with GST-TET2 (aa1-300). The specificity of the polyclonal antibody was validated by western blot and immunoprecipitation in wildtype and TET2 knockout cell lysates from murine cells. Our antibody does not detect human TET2, however a human specific α-TET2 antibody for ChIP-seq has been reported [10]. Further analysis is required to identify commercially available TET2 antibodies for ChIP-seq analysis in mouse and human cells.
2. We recommend a starting material of 20-100x10^6 cells per replicate sample. It is possible to perform the protocol starting with lower cell numbers, however this requires further detailed optimization of the ChIP procedure as described elsewhere [11]. The culture medium for embryonic stem cells (2i/LIF versus Serum/LIF) determines the level of DNA methylation. For TET protein ChIP studies, we would recommend that cells are grown in serum/LIF to ensure higher levels of DNA methylation.
3. We have found that combining DSG and formaldehyde crosslinking is essential to detect enrichment of TET2 on chromatin. In contrast, TET1 ChIP can be performed on formaldehyde

crosslinked chromatin without the use of DSG. This difference is likely a reflection of the mode in which the two enzymes interacts with chromatin as well as the direct contact of the CXXC domain in TET1 with DNA.

4. Immunoprecipitation with antibodies will enrich on-target (e.g. the TET2 protein) as well as off-target (background) chromatin. It is therefore important to titrate the ratio of chromatin and antibody concentration/bead volume to identify the experimental conditions offering the best on-target/off-target ratio. Off-target enrichment in a specific condition can be measured by ChIP-qPCR analysis in control cells that do not express the target (*TET2* knockout or TET2 without epitope tag). Off-target enrichment are often seen in regions of open chromatin and active promoters and can easily be mistaken for true occupancy [12]. We have found that an input of 0.3mg chromatin in 1mL of IP buffer and 20uL of a 50/50 slurry of FLAG M2 beads yield an optimal on-target/off-target ratio in embryonic stem cells. Similarly, for ChIP analysis with an antibody against endogenous TET2 we found that 0.3mg of chromatin in 1mL of IP buffer and 1ug of antibody yields an optimal on-target/off-target ratio. However, these parameters are dependent on the specific antibody used in the experiment and must be determined empirically.

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Figure 1. ChIP-sequencing of TET proteins

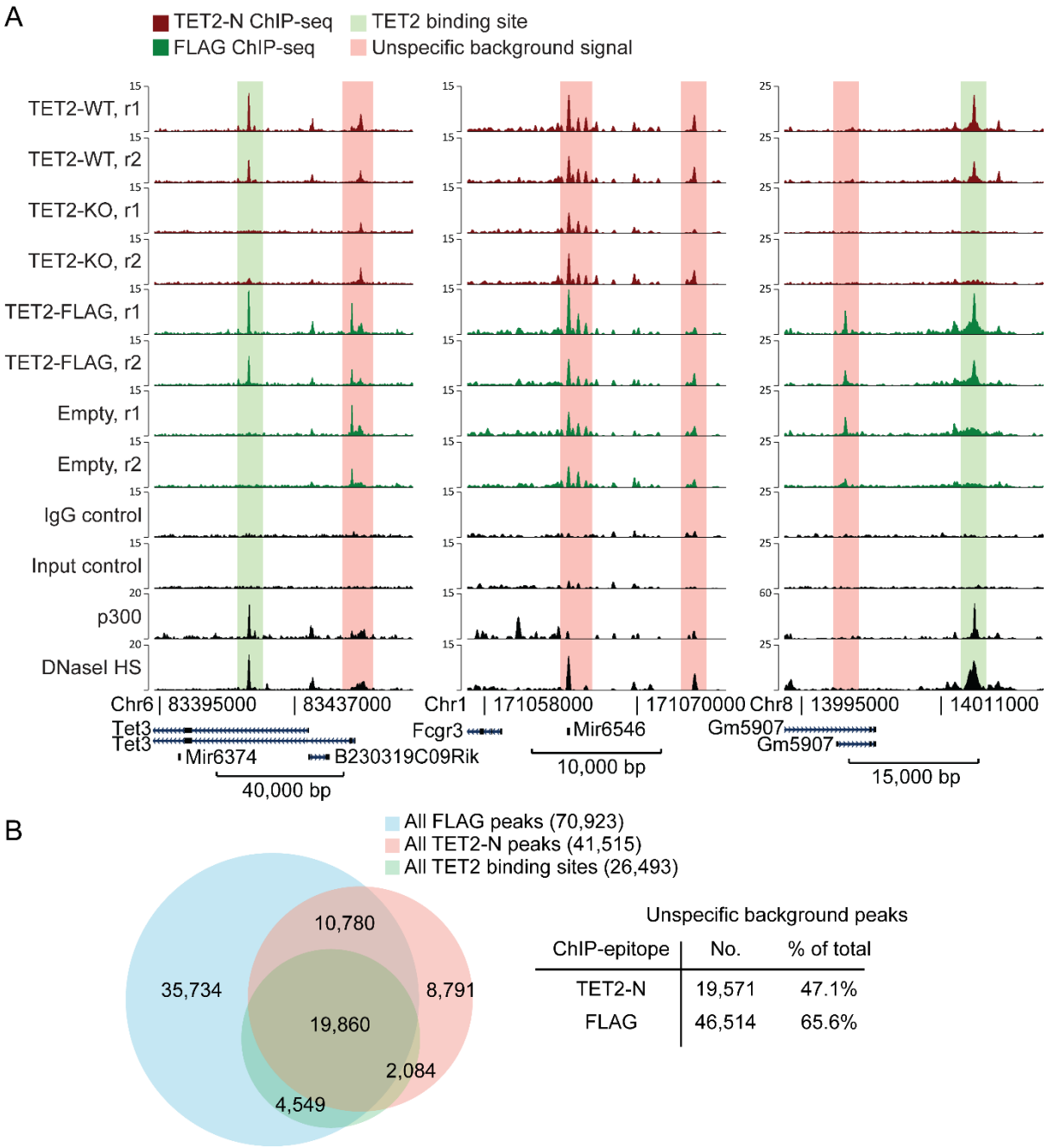

Figure captions

Figure 1: (A) ChIP-seq tracks (mm10) of 3 representative loci showing TET2 binding sites in DSG and formaldehyde-crosslinked chromatin from embryonic stem cells. The chromatin immunoprecipitation and sequencing were done as described in this protocol. Biological duplicate experiments from wildtype and *TET2* knockout cells or cells expressing FLAG-tagged TET2 (TET2-FLAG) or parental cells without the endogenous tag (Empty) are shown. ChIP-seq tracks of IgG control and input control from the same cells are also shown, as well as datasets of p300 occupancy (ENCFF358NVC) and DNaseI

hypersensitivity (ENCFF754ILF) publicly available from the ENCODE consortium. Bona fide TET2 binding sites (green) as well as unspecific background peaks (red) are highlighted. Note that IgG and Input controls are insufficient to identify the regions with increased background signal that are unrelated to TET2 chromatin binding. ChIP-seq tracks were generated using Easeq [13]. **(B)** Venn diagram showing overlap of consensus peaks (relative to IgG control) derived from biological duplicate TET2-N or FLAG ChIP-seq experiments as well as *all* TET2 bindings sites including both high- and low-confidence sites as defined in [9]. The total number of peaks in each section is indicated. A large fraction of peaks (47.1% in TET2-N and 65.6% in FLAG) remain strongly enriched in the negative control cells (*TET2* knockout or empty cells) and can be defined as unspecific background peaks.