TECHNOLOGY REPORT

Generation of a Mouse Model for a Conditional Inactivation of *Gtf2i* Allele

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Summary: The multifunctional transcription factor TFII-I encoded by the *Gtf2i* gene is expressed at the two-cell stage, inner cell mass, trophectoderm, and early gastrula stages of the mouse embryo. In embryonic stem cells, TFII-I colocalizes with bivalent domains and depletion of *Gtf2i* causes embryonic lethality, neural tube closure, and craniofacial defects. To gain insight into the function of TFII-I during late embryonic and postnatal stages, we have generated a conditional *Gtf2i* null allele by flanking exon 3 with loxP sites. Crossing the floxed line with the Hprt-Cre transgenic mice resulted in inactivation of *Gtf2i* in one-cell embryo. The Cre-mediated deletion of exon 3 recapitulates a genetic null phenotype, indicating that the conditional *Gtf2i* line is a valuable tool for studying TFII-I function during embryonic development. genesis 54:407–412, 2016. © 2016 Wiley Periodicals, Inc.

Key words: conditional; Gtf2i; TFII-I; mouse embryos; chromatin

TFII-I is a ubiquitously expressed transcriptional factor involved in both basal transcription and signal-induced transduction activation or repression (Roy, 2007, 2012). The *GTF2I* gene encoding TFII-I is the prime candidate responsible for cognitive abnormalities in the Williams-Beuren syndrome and autism spectrum disorders (Malenfant *et al*., 2012; Sakurai *et al*., 2011). In developing mouse embryos, TFII-I exhibits distinct and dynamic expression pattern (Enkhmandakh *et al*., 2004; Fijalkowska *et al*., 2010; Ohazama and Sharpe, 2007). *Gtf2i* is often subjected to alternative splicing, which generates TFII-I isoforms with different activities and distinct biological roles (Makeyev and Bayarsaihan, 2009).

Previously, we identified a set of developmental genes as direct TFII-I targets revealing the complexity of TFII-I-mediated processes associated with distinct regulatory networks (Chimge *et al*., 2008; Enkhmandakh *et al*., 2009; Makeyev and Bayarsaihan, 2011; Makeyev and Bayarsaihan, 2013). Genome-wide mapping studies revealed TFII-I occupancy at multiple genomic sites in mouse embryonic stem (ES) cells and embryonic tissues (Bayarsaihan *et al*., 2012; Makeyev *et al*., 2012). Moreover, many TFII-I-bound regions colocalize with H3K4me3/K27me3 bivalent chromatin in the promoters of lineage-specific genes (Adamo *et al*., 2015; Bayarsaihan, 2013; Makeyev *et al*., 2012). Interestingly, TFII-I appears to have specific roles in distinct phases of the mammalian cell cycle (Ashworth and Roy, 2009).

TFII-I plays an inhibitory role in regulating genes that are essential in osteogenesis and moreover, it has the ability to intersect with the bone-specific transcription factor Runx2 and the retinoblastoma protein Rb (Lazebnik *et al*., 2009). Activation of PI3K/Akt signaling, which is sufficient to maintain the pluripotency of mouse ES cells, results in down-regulation of *Gtf2i* (Chimge *et al*., 2012). Moreover upregulation of *Gtf2i* leads to activation of a specific group of developmental genes during ES cell differentiation.

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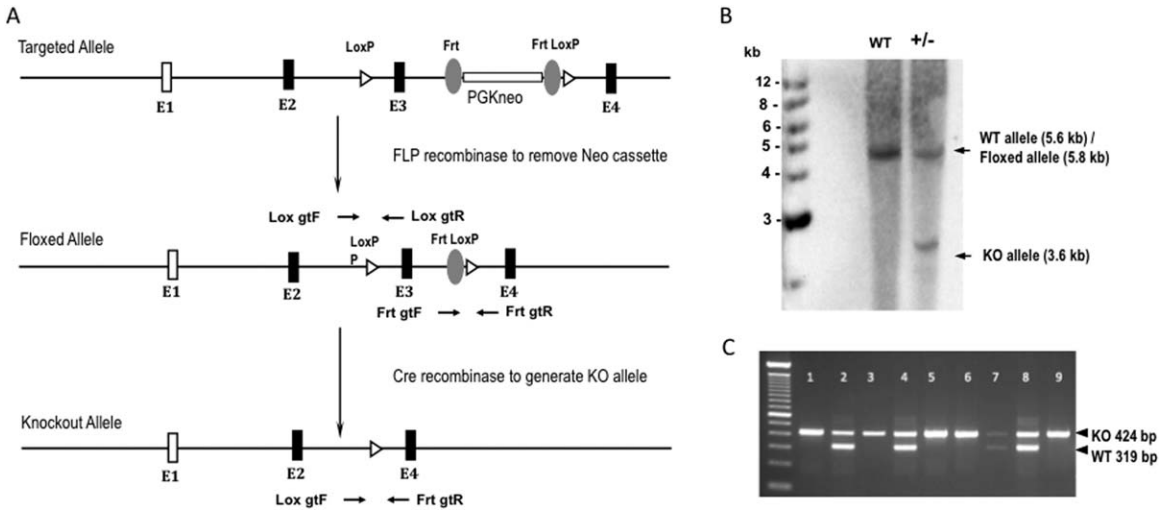

FIG. 1. The generation of a conditional *Gtf2i* knockout mouse model. (**a**) The schematic representation of the targeting strategy for producing the *Gtf2i* conditional allele. The region including exon 3 of the wild-type *Gtf2i* locus is shown, and the approximate sites of the 5′ and 3′ flanking probes used for screening ES cell clones. The loxP sites and FRT sites are marked by triangles and gray ovals, respectively. (**b**) Southern blot analysis of targeted ES clones after SacI digestion was screened for homologous recombinants. The Southern probe hybridizes to the wild-type fragment of 5.6 kb, floxed fragment of 5.8 kb, and a fragment of 3.6 kb from the correctly targeted clone. (**c**) PCR genotyping of a litter of a correctly targeted ES clone from intercross of a pair of $Gtf2i^{\Delta E3/+}$ mice. A PCR product of 319 bp produces from wild-type allele, whereas a PCR product of 424 bp was amplified from the $Gtf2i^{\Delta E3/\Delta E}$ allele.

The mouse *Gtf2i* gene is composed of 34 exons spanning approximately 77 kb region on chromosome 5G2 (Bayarsaihan *et al*., 2002). A targeting vector for the generation of a *Gtf2i* conditional allele was constructed following a procedure described elsewhere (Liu *et al*., 2003). Briefly, one loxP site was inserted into intron 2, while a second loxP site along with the PGKneo selectable marker gene flanked by two Frt sites were introduced into intron 3 (Fig. 1a).

The final targeting vector has the 5′ end long homology arm of approximately 4.3 kb in size, spanning from intron 1 to exon 2, and a 3′ short arm of 3.3 kb, spanning from intron 3 to exon 4 including the HSV TK negative selectable marker (Fig. 1a). The removal of exon 3 will result in complete loss of *Gtf2i* allele that deletes the entire TFII-I protein because exon 3 is common to all known transcript variants (Makeyev and Bayarsaihan, 2009). The truncated protein product is very short and lacks important functional regions including nuclear localization signal and DNA-binding domains.

The purified vector was electroporated into the D2 embryonic stem (ES) cell line and colonies resistant to G418 and ganciclovir were screened by long-range PCR screening of the long and short arms to identify properly targeted clones (Fig. 1c). Ten correctly targeted clones were identified from several dozen G418-resistant colonies. Two-independent ES clones were used to generate mouse chimeras by morula aggregation with CD1 wild-type embryos. Chimeric males were bred with ROSA26-Flpe females to test for germline transmission and removal of the PGKneo cassette in the targeted allele. F1 mice were genotyped initially by PCR analysis using tail genomic DNA samples for the presence of the targeted $Gtf2i^{fl}$ allele. Southern blots containing DNA samples of positive clones were rehybridized with the probe from intron 1 to verify the expected integration of a loxP-neo-loxP cassette in the third intron (Fig. 1b). Heterozygous mice were intercrossed and genotyping of F2 progeny a week after birth detected homozygous $Gtf2i^{fl/fl}$ mice at the expected Mendelian ratio. The homozygous $Gtf2i^{fl/fl}$ mice exhibited a normal life span and fertility. The resultant $Gtf2i^{f/+}$ mice were intercrossed to generate homozygous $Gtf2i^{f/f}$ progeny with no detectable abnormalities. To test for Cre-mediated inactivation of the *Gtf2i* conditional allele in vivo, we crossed the $Gtf2i^{f/f}$ mice with Hprt-Cre mice, which express Cre in one-cell embryo causing deletion of loxP-flanked sequences in most tissues, including cells of the developing germ line. The resultant $Gtf2i^{\Delta E3/+}$ mice were normal, whereas analysis of embryos from $Gtf2i^{\Delta E3/+}$ heterozygous intercrosses revealed that the $Gtf2i^{\Delta E3/\Delta E3}$ mutant embryos died before birth. The $Gtf2i^{\Delta E3/\Delta E3}$ embryos exhibit the same phenotype as the previously reported *Gtf2i* null embryos (Enkhmandakh *et al*., 2009). Depletion of *Gtf2i* resulted in embryonic lethality before E10.5 (data not shown). Collectively, these results indicate that Cre-mediated deletion of exon 3 abolished the function of *Gtf2i* gene.

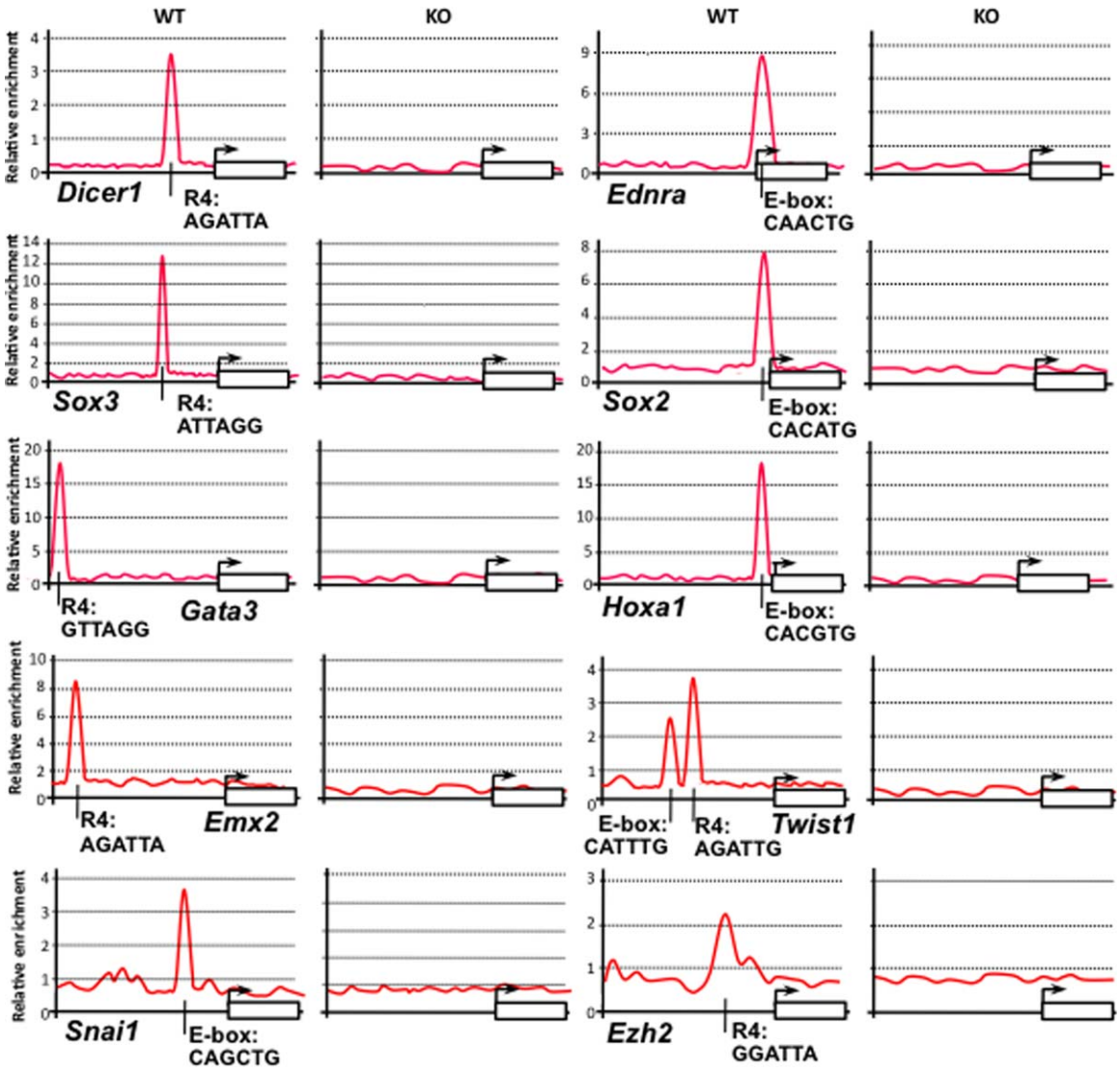

FIG. 2. ChIP-chip confirmed the TFII-I binding sites in wild-type ES cells and lack of promoter occupancy in *Gtf2i* depleted cells. The tiling array stretches genomic regions between 2 kb upstream and 500 bp downstream of transcription start sites. The TFII-I occupancy was confirmed for its cognate target genes in control ES cells, whereas no binding was observed in *Gtf2i*$^{\Delta E3/\Delta E}$ cells.

Chromatin immunoprecipitation tiling assays were used to confirm that ES cells derived from *Gtf2i*$^{\Delta E3/\Delta E3}$ embryos did not produce a functional TFII-I protein. Previously, we demonstrated that TFII-I binds to the regulatory elements containing either the core RGATTR motifs or the E-boxes within the promoters of key developmental genes (Makeyev *et al.*, 2012). While TFII-I occupies its cognate promoters in wild-type ES cells, no such binding was observed in the *Gtf2i* depleted cells (Fig. 2). Next, we investigated the expression pattern of a number of developmental genes affected by *Gtf2i* inactivation. Whereas some genes such as *Gata4* and *Esrrb* were expressed at significantly lower level, the expression of *Nodal* and *Pou5f1* was elevated in the *Gtf2i*$^{\Delta E3/\Delta E3}$ cells (Fig. 3). Therefore, the generated *Gtf2i* conditional line can be a valuable mouse model for dissecting the mechanisms by which TFII-I transcription factor regulates cell differentiation and tissue-specific gene expression.

MATERIALS AND METHODS

Generation of the *Gtf2i* Conditional Allele

After the sequence confirmation by NotI digestion, the purified vector was electroporated into the D2 embryonic stem (ES) cell line derived from F1 (C57BL/6jx129SV) embryos. Colonies resistant to G418 and ganciclovir were screened by long-range PCR screening

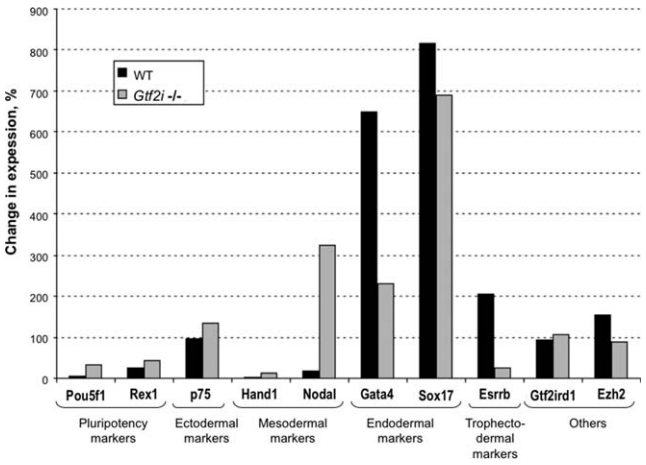

FIG. 3. RT-PCR analysis of genes from wild-type and mutant ES cells. The expression of several developmental genes was affected by *Gtf2i* deletion including mesodermal marker *Nodal*, endodermal marker *Gata4*, and trophectodermal marker *Essrb*.

of the long and short arms to identify properly targeted clones. The primer Gtf2iScrF1 lying outside of the 5′ homology arm and the primer PL452-loxp-sc1R specific to the 5′ loxP site were used to detect the proper integration of a long homology arm. The primer Gtf2i Scr1 resides outside of the 3′ homology arm and the primer PL452-3′-sc1F, which is specific to the 3′loxP site were used to detect the 3′arm. Ten correctly targeted clones were identified among 48 G418-resistant colonies. Two-independent ES clones were used to generate mouse chimeras by morula aggregation with CD1 wild-type embryos. Resulting chimeric males were bred with ROSA26-Flpe females (Jax stock no: 009086) to test for germline transmission and removal of the PGKneo cassette in the targeted allele. Both clones resulted in germline transmission. F1 mice were genotyped initially by PCR analysis using tail genomic DNA samples for the presence of the targeted $Gtf2i^{fl}$ allele. Southern blots containing DNA samples of positive clones were rehybridized with the probe from intron 1 to verify the expected integration of a loxP-neo-loxP cassette in the third intron. Heterozygous mice were intercrossed and genotyping of F2 progeny a week after birth detected homozygous $Gtf2i^{fl/fl}$ mice at the expected Mendelian ratio. The homozygous $Gtf2i^{fl/fl}$ mice exhibited a normal life span and fertility. The resultant $Gtf2i^{f/+}$ mice were intercrossed to generate homozygous $Gtf2i^{f/f}$ progeny with no detectable abnormalities. To test for Cre-mediated inactivation of the *Gtf2i* conditional allele in vivo, we crossed the $Gtf2i^{f/f}$ mice with Hprt-Cre mice (Jax stock no: 004302), which express Cre in one-cell embryo causing deletion of loxP-flanked sequences in most tissues, including cells of the developing germ line.

The *Gtf2i* conditional allele will be available to the research community upon acceptance of the manuscript.

Generation of $Gtf2i^{\Delta E3/\Delta E}$ ES Cells

Mouse blastocysts were obtained by superovulation and breeding of $Gtf2i^{f/+}$ mice. Blastocysts were flushed 3.5 dpc and plated on mouse embryo fibroblast (MEF) cells. Blastocysts were cultured in ES media with the addition of a Mek1/2 inhibitor (product U0126 from Cell Signaling Technologies). The blastocysts were allowed to hatch and attach with feeders replenished after the fourth day of attachment. Inner cell masses were allowed to grow and were trypsinized and plated on feeder layers until the ES cell lines were established. These lines were cultured in media without MEK1/2 inhibitor until ES cells were grown to sufficient density in a large enough plate to freeze down vials. Aliquots of cell suspension were transferred into precooled cryovials for storage in liquid nitrogen. The remaining ES cells were washed from the tube that contained the cells with some ES cell media and plated on a gelatin-coated plate. Each grown colony was plated on the new gelatin plates and cultured for a few passages to obtain pure ES cells without the presence of any MEF cells.

Southern Blot and PCR Genotyping

The 1084 bp repeat-free fragment from intron 1 was selected as a probe for a Southern blot and amplified by PCR from wild-type genomic DNA using primers 5′-GCGTGCCCCGGGACTAGAGT-3′ (forward) and 5′-GGCGTTACTGTGGCCGAGCC-3′ (reverse). Fifteen micrograms of SacI-digested genomic DNA was run using agarose gel electrophoresis and after the transfer to a positively charged nylon membrane BrightStar-Plus (Ambion), DNA was hybridized with the biotinylated probe (NEBlot Phototope Kit, NEB) according to the manufacturer's instructions. The hybridization signals were registered using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) and ChemiDoc MP gel imaging system (Bio-Rad) equipped with CCD camera.

For the PCR genotyping, we used Lox gtF (5′-CCA AGG TTT CAG CTT TGG AG-3′) and Frt gtR (5′-AGC CAC TCC AAC AGT TAC CG-3′) primers. The PCR product of 319 bp was detected in wild-type cells, while in $Gtf2i^{\Delta E3/\Delta E3}$ cells a 424 bp fragment was amplified as expected. Additional primers used in the study were: Gtf2iScrF1 (5′-CTCAGAAATTCACCTGCCTC-3′), PL452-loxp-sc1R (5′-GAGGGACCTAATAACTTCGT-3′), Gtf2i Scr1 (5′-GCAGGAGAGAATGTACTGGCTC-3′), PL452-3′-sc1F (5′-CTTCTGAGGCGGAAAGAACCA-3′).

qRT-PCR Profiling

Change in the expression of selected genes derived *Gtf2i*$^{\Delta E3/\Delta E}$ (TFII-I KO) and normal (WT) mouse ES cells at early stage of differentiation (3 d after LIF withdrawal) was analyzed by qRT-PCR using Mouse Stem Cell RT2 Profiler PCR Array PAMM-405A (Qiagen) containing ESC differentiation markers (Fig. 3). Total RNA was isolated from cells using RNeasy Kit (Qiagen) and cDNA was prepared using RT2 First Strand Kit (Qiagen). Real-time measurements were performed using ABI 7900HT instrument (Applied Biosystems) and RT2 SYBR Green ROX qPCR Mastermix (Qiagen).

Chromatin Immunoprecipitation (ChIP)

ChIP experiments were performed essentially as it was described in Makeyev et al. (2012) using ChIP-IT kit (Active Motif, CA). Chromatin was immunoprecipitated from 2×10^6 ES cells with rabbit anti-TFII-I polyclonal antibodies 4562 (Cell Signaling Technology, MA). After reverse cross-linking and purification on QIAquick spin columns (Quagen, CA), immunoaffinity-enriched DNA fragments (IP) and the input samples were amplified using whole-genome GenomePlex Complete WGA kit (Sigma-Aldrich, MO). IP and input samples were labeled in separate reactions with Cy5 and Cy3, respectively, and then were cohybridized to Mouse 385K RefSeq Promoter Arrays (Roche NimbleGen, WI). The data were extracted using NimbleScan software. Peaks were detected by searching for four or more probes with a signal above a cut-off value using a 500 bp sliding window. A $\log_2$ ratio of the IP versus input samples was calculated and the transcription start site mapping was performed using SignalMap software (Roche NimbleGen, WI).

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