Trim33 regulates early maturation of mouse embryoid bodies in vitro

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1. Introduction

Embryonic stem cells (ESCs) have been used to investigate developmental processes, disease conditions, tissue regeneration and therapeutic targets. Previous studies have shown that tripartite motif-containing 33 protein (Trim33) functions as a chromatin reader during Nodal-induced mesoderm induction. Here we report that despite reduced proliferation, mouse ESCs deficient in Trim33 remained pluripotent when cultured under non-differentiating conditions. However, when induced to differentiate to embryoid bodies (EBs), the mutant cultures showed increased cell shedding and apoptosis at day 3 of differentiation. Gene set enrichment analysis (GSEA) indicated that several molecular functions associated with cell survival, transcriptional/translational activity and growth factor signaling were affected already by the second day of differentiation in Trim33-deficient EBs. Consistent with increased apoptosis, expression of Rac1, a critical factor for EB cell survival, was reduced in Trim33 mutant EBs. In addition, a set of genes involved in regulation of pluripotency was upregulated in mutant EBs. Our results suggest that Trim33 regulates early maturation of mouse embryoid bodies in vitro.

2. Materials and methods

2.1. Ethics statement

This research was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Experiments described are specifically approved by the University Committee on Use and Care of Animals at the University of Michigan-Ann Arbor (Protocol Number: #PRO00005004).

2.2. Establishment of embryonic stem cell lines and EB cultures

Mouse ES cells were derived from Trim33<sup>ff</sup>: UbcCre<sup>ERT2</sup> blastocysts [12] as described in [13]. ESCs were cultured on gelatin-coated culture dishes in the KSR-medium containing 15% of Knock Out Serum Replacement (Life Technologies, 10828-010), β-mercaptoethanol (100X, Life Technologies, 21985-023), GlutaMAX-I (100X, Life Technologies, 35050-061), Penicillin/Streptomycin (Life Technologies, 15140-122) in Knockout-DMEM:Ham's F12 (1:1). ESCs were cultured on gelatin-coated culture dishes in the KSR-medium containing 15% of Knock Out Serum Replacement (Life Technologies, 10828-010), β-mercaptoethanol (100X, Life Technologies, 21985-023), GlutaMAX-I (100X, Life Technologies, 35050-061), Penicillin/Streptomycin (Life Technologies, 15140-122) in Knockout-DMEM:Ham's F12 (1:1).
2.5. Proliferation assay

Forward Primer (tgacaggatgcagaaggaga), Reverse Primer (cgctcaggaggag-
Trim33 (Mm00658129_gH)

Taqman Assays

all targets. The reactions were quantified using Applied Biosystems ABI7300

Universal PCR 2X master mix; Applied Biosystems 4304437) were used for

synthesized using Omniscript RT (Qiagen 205111). Taqman Assay reagents

with methylene blue as shown above (see Section 2.5).

2.4. Real-time PCR (qRT-PCR)

Equivalent numbers of EBs were collected at day 2 and day 2.5 in triplicate mutant-control pairs in 100–200 μl commercially available
(Qiagen) RLT buffer. Total RNAs were isolated using (Qiagen RNeasy Mini Kit 741044). Messenger (m)RNAs and sequencing libraries were
prepared by the University of Michigan DNA Sequencing Core and reads generated on Illumina HiSeq. 2000. After quality assessment per
sample, single-end, 52 base pair reads were aligned to mm9 (Mus
musculus assembly July 2007) using STAR RNA Seq aligner [14]. Read
counts for differential expression were obtained using the HTSeq pro-
gram. Differential expression analyses were performed using the DESeq
program in R Statistical Package https://bioconductor.org/packages/3.3/bioc/vignettes/DESeq/inst/doc/DESeq.pdf. HTSeq-generated read
counts were separately normalized in R version 1.0.136, which uses the
Trimmed Mean of M values (TMM) normalization method [15]. GEO
submission GSE80166. Gene Set Enrichment Analysis (GSEA; http://
www.broadinstitute.org/gsea/index.jsp) was used to identify groups of
genes enriched in either control or mutant cells [16].

2.8. Immunohistochemistry

EBs were collected in sterile PBS and fixed for 15 min in freshly
prepared 4% paraformaldehyde in PBS at room temperature, embedded
in OCT compound and cryo-sectioned at 10 μm thickness. Primary anti-
bodies for SSEA1 (Developmental Studies Hybridoma Bank MC480),
cleaved caspase3 (Cell Signaling 9661), γ-H2AX (Millipore 05-635-61),
Tcf212 (R & D AF5726), Rac1 (Cytoskeleton ARCO3), Stat3 (Cell Signaling 9139) and phospho-Stat3 (Cell Signaling 9136).

2.9. Statistical analyses

For apoptosis and expression (qRT-PCR) assays, three or more
samples were analyzed. Averages, standard error and probability
(Student’s t-test) were calculated and p-values of less than or equal
to 0.05 were marked as significant.

3. Results and discussion

ESC lines were established from mouse embryos that were homo-
zygous for the floxed Trim33 allele and carried the UbcCreERT2 trans-
gene (Trim33FF, UbcCreERT2 ESCs). For feeder-less cultures, two separate
culture conditions were tested (‘KS medium with LIF + 2i’ and ‘2i medium
with LIF + 2i’; for details see Section 2). Colony morphology, marker
gene expression and growth/differentiation characteristics were
comparable in both media (Supplemental Figs. 1 and 2); ‘KS medium
with LIF + 2i’ was used in all subsequent experiments. When
Trim33FF, UbcCreERT2 ESCs were exposed to 4-hydroxytamoxifen (4-OHT),
sequences encoded by exons 2–4 were excised, and ESCs lacking the
functional Trim33 gene (Trim33KO) were generated (Fig. 1A, B). Trim33KO
ESCs were morphologically indistinguishable from control
cells when cultured under non-differentiating conditions in the
presence of LIF + 2i (Fig. 1 and Supplemental Fig. 1). They expressed a
stem cell marker SSEA-1, the key pluripotency factor Pou5f1 (Oct4) and
a naive pluripotency marker (Tcf212) [17], and showed characteristic
ESC colony morphology identical to that of control mESCs (Fig. 1C-H).
Trim33KO ESCs were propagated in culture over 20 days, corresponding
to 6 passages without detectable morphological changes. However, the
rate of proliferation was reduced in mutant ESCs when compared to

controls (Fig. 1K, L). When cultured without LIF + 2i, both control and mutant cells gradually lost expression of the pluripotency markers, as expected, and showed differentiated fibroblastoid-type phenotype (Fig. 1I, J).

The critical role of TGF-β/Nodal signaling in mesendoderm induction is well-established [18]. Moreover, previous studies have shown that the function of Trim33 as a chromatin reader is required for Nodal-triggered mesendoderm induction [19]. To complement and expand these findings, we tested whether deletion of Trim33 and inactivation of TGF-β/Nodal type I receptors (by a chemical inhibitor SB431542 [20]) would result in identical outcomes. To this end, ESCs were cultured without LIF + 2i in the presence of FCS on ultra-low attachment plates, i.e., under conditions that are permissive for ESCs to form EBs and differentiate [19]. Administration of 4-OHT at day 0 resulted in 80% reduction in Trim33 protein content at day 1, while less than 3% of Trim33 was detected at day 2 (Fig. 2A). Under these culture conditions, control ESCs (4-OHT-) formed well-defined, cystic EBs (Fig. 2B, E). In contrast, Trim33FF:UbcCreERT2 ESCs treated with 4-OHT formed EBs that showed a large number of shedding cells on the surface. These clusters gradually deteriorated and disappeared soon after the 5th day of differentiation (Fig. 2C, F). SB431542-treated cultures appeared to form differentiating EBs comparable to those in control cultures in earlier stages; however, after day 6 they lost the EB-like structure and assumed a flattened epithelium-like morphology. Clusters that survived remained attached even to low attachment dishes (Fig. 2D, G).

Since the control EBs and those cultured in the presence of 4-OHT or SB431542 appeared all morphologically different, we analyzed expression levels of well-established germ layer and pluripotency markers by using qRT-PCR. As expected, both SB431542-treated and 4-OHT-treated EBs failed to express the mesendoderm markers Gsc and Mixl1 (Fig. 2H). Expression levels of pluripotency markers Pou5f1 were markedly reduced in SB431542-treated cultures both at day 3 and day 7, when compared to control cultures while Pou5f1 expression was sustained in 4-OHT-treated Trim33KO cultures at identical time points (Fig. 2H). Nanog expression was lower both in Trim33KO and SB431542-treated cultures (Fig. 2H).

To better understand the role of Trim33 in initial phases of EB maturation, we performed genome-wide transcriptomic analyses on
RNAs harvested from control and 4-OHT treated EBs (4-OHT treatment started at day 0 of differentiation). At day 2, of 56 differentially expressed genes (> 2-fold change; p ≤ 0.05, n = 3), 53 were upregulated and only three (including Trim33) were downregulated. Twelve hours later (day 2.5) 165 genes were downregulated and 750 genes were upregulated (Supplemental Table 1; GEO submission GSE80166). GSEA analysis revealed that gene sets associated to TAp63- and protein tyrosine phosphatase pathways, ECM-receptor interactions, apoptosis, second messenger-mediated signaling and protein kinase cascade are among the most significantly upregulated in Trim33KO EBs at day 2 (Fig. 3A and Supplemental Table 2). In contrast, gene sets associated to mRNA splicing, RNA processing, translation factor activity, structural constituents of ribosomes, RNA Polymerase II activity and RNA processing were downregulated in mutant EBs when compared to controls (Fig. 3B). At day 2.5, gene sets associated to tissue and ectoderm development, ECM-receptor interactions, transcription factor and repressor activity and negative regulation of cell proliferation were among the most significantly enriched in mutant EBs (Fig. 3C), while gene sets associated with DNA replication, RNA splicing and processing and regulation of mitotic cell cycle were among the downregulated sets in mutants (Fig. 3D). These findings suggest that overall transcriptional and translational activities and cell survival are reduced in Trim33KO EBs during the first three days of differentiation.

Since mutant EBs appeared non-compact and showed increased cell shedding (Fig. 2), and because GSEA assays suggested that gene sets associated with apoptosis were upregulated in Trim33KO EBs (Fig. 3), we analyzed whether mutant EBs would show detectable differences in programmed cell death. Our assays show that even the small mutant EBs showed cleaved-caspase-3-positive apoptotic cells in the core of EBs, which normally do not show apoptosis, while the shedding cells on the surface appeared viable (Fig. 4). While the large control EBs showed some apoptotic cells in the core, as expected, the cell boundaries in control EBs appeared largely intact as shown by staining for F-actin. In contrast, massive apoptosis was associated with the loss of F-actin staining in the Trim33 mutant EBs (Fig. 4A-D). The observed apoptosis was not caused by 4-OHT treatment or Cre-induction, since parallel
cultures, which were positive for the UbcCreERT2 transgene and wildtype for the Trim33 allele, were indistinguishable from the 4-OHT-negative controls (compare Fig. 4A, C and E, F). A recent study showed that Trim33 deficiency correlates with an enhanced sensitivity to DNA damage [21]. To examine whether DNA damage would contribute to the Trim33KO EB phenotype, we stained both control and Trim33KO EBs for the DNA damage marker γH2AX (Fig. 4H, I). These assays revealed that there are no detectable differences in γH2AX staining between controls and Trim33 mutants suggesting that in this context Trim33 is not involved in DNA damage response.

It was recently shown that the small Rho-related GTPase Rac1 is required for epiblast cell – basement membrane interactions in EBs and that Rac1 deficiency results in massive apoptosis [22]. Interestingly, Rac1 is strongly downregulated in Trim33 mutant EBs (Figs. 3A, C and 5A, B) suggesting that reduced Rac1 expression may contribute to the observed EB phenotype possibly by altering cell-basement membrane interactions.

As indicated above (Fig. 2), our findings are consistent with those of the previous report [19] indicating that Trim33 is required for mesoderm induction in vitro. To further explore the effect of Trim33 on expression of genes associated with mesoderm induction, we analyzed the RNA-Seq datasets for changes in established mesoderm markers. While at day 2 of differentiation, none of the mesoderm markers were differentially expressed between the controls and mutants, several of the early markers, e.g., Brachyury (T), Gsc and Eomes were upregulated in controls at day 2.5 of differentiation (Fig. 5). In contrast, Brachyury targets Snail-1 and Foxa2 did not show differential expression (not shown) consistent with the fact that EBs at day 2.5 of differentiation present very early stages of mesoderm induction.

GSEA indicated several signaling pathways that were enriched in Trim33 mutant EBs at day 2 (Table 1). In three of them, i.e., protein tyrosine phosphatase (PTP1B), protein kinase and Jak/Stat pathways, Stat3 was upregulated. Stat3 has been shown to be a critical mediator of LIF-triggered Jak signaling, and several Stat3 transcriptional targets have been validated as mediators of pluripotency [23–27]. Deeper examination of our RNA-Seq datasets revealed that in addition to Stat3, many of its pluripotency-associated downstream targets e.g., Tfp21, Klf5, Klf4, Socs3 and Gbx2 [11,28] and other factors implicated in the regulatory network for pluripotency (Esrrb and Tbx3 [11]) were upregulated in Trim33KO EBs both at day 2 and day 2.5 of differentiation (Fig. 5).

To test whether cells of mutant EBs would be more potent in colony formation when returned to non-differentiation culture conditions, EBs were dissociated to single cells, and plated on gelatin-coated dishes in the ES culture medium supplemented with LIF and 2i (for details, see Section 2). Both control and mutant cultures established from day 1 EBs formed comparable numbers of ESC-like colonies. Consistent with the reduced proliferation rate of undifferentiated ESCs, the mutant colonies were smaller than control colonies. Both mutant and control cultures established from day 2 EBs failed to form characteristic ESC-like colonies; instead they showed attached fibroblastoid-type cells (Supplemental Fig. 3). To conclude, despite increased expression of
pluripotency associated genes in Trim33 KO EBs, their cells did not show increased or prolonged ability to form ESC-like colonies when returned to undifferentiating culture conditions suggesting that their ability of exiting the naïve state of pluripotency was not different than that of controls.

Our findings demonstrate that inducible loss of EBs deficient in Trim33 results in increased apoptosis, the mutant EBs are morphologically distinct with notable shedding of surface cells, and display changes in gene sets associated with multiple molecular and biological processes. Since these changes have not been reported in the previous study, which used systemic Trim33 null cells and showed that Trim33 is required for Nodal-induced mesoderm induction [19], we wondered whether the differences could be caused by different experimental strategies, i.e., systemic vs inducible gene inactivation. To this end, we induced Trim33 deletion by 4-OHT in undifferentiated ES cells and

### Table 1

Top ten pathways enriched in Trim33 mutant EBs at day 2.0 of differentiation.

| Pathway                  | Normalized enrichment score (NES) | Nominal p-value |
|--------------------------|-----------------------------------|-----------------|
| TAP63                    | 1.98                              | P < 0.001       |
| PTP1B                    | 1.97                              | P < 0.001       |
| cAMP/second messenger    | 1.90                              | P < 0.001       |
| Integrin1                | 1.84                              | P < 0.001       |
| TNF                      | 1.84                              | P = 0.0014      |
| Protein kinase           | 1.83                              | P = 0.0015      |
| IL2 receptor             | 1.83                              | P < 0.001       |
| Hedgehog                 | 1.79                              | P = 0.0015      |
| Jak/Stat                 | 1.66                              | P = 0.0097      |
| Map kinase               | 1.64                              | P = 0.007      |

* Stat3 enriched.
cultured them for 2 passages under standard ES culture conditions before starting the differentiation assay. In a parallel experiment recombination was induced at day 0 as described above. As shown in Supplemental Fig. 4, both mutant cultures showed similar phenotypes demonstrating that the observed differences between mutant and control cultures are not caused by induction of recombination simultaneously with early stages of differentiation.

Recent studies have shown that Trim33 plays a specific role in silencing transposable elements [29,30] and that at least in the mouse testis this is accomplished via specific binding to a subgroup of retrotransposons and subsequent ubiquitination and inactivation of the A-Myb transactivator [30]. Moreover, Trim33 has been shown to regulate differentiation of neural stem cells [31] and terminal differentiation of mammary epithelial cells [32]. In both contexts, Trim33 appears to control differentiation fates early on. Our present results show that Trim33 regulates early maturation events of mouse embryoid bodies. These results seem to be at odds with those of mouse embryogenesis, which showed that Trim33 is not required for early development or mesoderm induction [33,34]. It is conceivable that experiments with ESCs in vitro remove layers of redundancy in vivo allowing discovery of novel functions for Trim33. Future studies are needed to unravel the mechanisms by which Trim33 regulates gene expression programs required for normal embryoid body maturation.

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Author contributions

SR and VK designed the study, SR, CP, and VK performed the experiments, SR and VK analyzed the data and wrote the manuscript.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.10.002.

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