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Crotonylation promotes human embryonic stem cell endodermal lineage differentiation and metabolic switch

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20

21 **Abstract**

22 Post-translational modifications of proteins are crucial to the regulation of their activity and function.
23 As a newly discovered acylation modification, crotonylation of non-histone proteins remains largely
24 unexplored, particularly in human embryonic stem cells (hESCs). Here we report the investigation of
25 induced crotonylation in hESCs, which resulted in hESCs of different pluripotency states
26 differentiating into the endodermal lineage. We showed that increased protein crotonylation in
27 hESCs was accompanied by transcriptomic shifts and decreased glycolysis. Through large-scale
28 profiling of non-histone protein crotonylation, we identified metabolic enzymes as major targets of
29 inducible crotonylation in hESCs. We further discovered GAPDH as a key glycolytic enzyme
30 regulated by crotonylation during endodermal differentiation from hESCs, where crotonylation of
31 GAPDH decreased its enzymatic activity thereby leading to reduced glycolysis. Our study
32 demonstrates that crotonylation of glycolytic enzymes may be crucial to metabolic switching and cell
33 fate determination in hESCs.

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Introduction

Embryonic stem cells (ESCs) hold great therapeutic potential thanks to their ability to self-renew and differentiate into all cell types¹⁻³. Pluripotency maintenance and germ layer-specific differentiation of human ESCs (hESCs) are complex processes that require crosstalk between diverse cellular pathways. During hESC differentiation, down-regulation of stem cell-specific markers and up-regulation of germ layer-specific genes depend on the coordinated action of networks of transcription factors and epigenetic regulators. For instance, histones and other chromatin-binding proteins help establish and maintain the unique chromatin states in hESCs, and post-translational modifications (PTMs) (e.g., methylation and acetylation) of these proteins in turn modulate and control their activities and functions. Additionally, hESCs also undergo germ layer-specific metabolic changes during differentiation⁴⁻⁷. While hESCs in pluripotency states rely largely on glycolysis for ATP production, reduced glycolysis and elevated oxidative phosphorylation have been detected during mesoderm and endoderm differentiation⁸⁻¹¹, whereas high glycolytic flux is maintained during ectoderm differentiation¹¹. Moreover, metabolic remodeling appears to be a key step in somatic cell reprogramming, where increased glycolysis can enhance reprogramming efficiency from human fibroblasts to inducible pluripotent stem cells¹²⁻¹⁵.

Small metabolic intermediates generated by metabolic enzymes can serve as PTM substrates¹⁶⁻²². For example, acetyl-CoA (Ac-CoA), the cornerstone of carbohydrate metabolism, is the substrate of protein acetylation. Histone acetylation plays a critical role in the maintenance of hESC open chromatin structure and pluripotency²³⁻²⁵. hESCs require high Ac-CoA levels for maintaining histone acetylation modifications as well as pluripotency and self-renewal capacity^{19,26}. During early differentiation of hESCs, the rapid decrease in glycolysis and Ac-CoA is associated with reduced histone acetylation^{19,26}. While inhibiting Ac-CoA synthesis can lead to the differentiation of hESCs^{19,26}, culturing these cells in the presence of the Ac-CoA precursor acetate can delay cell differentiation and block histone deacetylation in a dose-dependent manner¹⁹. More recently, crotonylation of histones has emerged as another important PTM linked to gene regulation²⁷⁻²⁹. The acetyltransferase

p300 was shown to also catalyze histone crotonylation, which could stimulate transcription to a greater degree than histone acetylation^{29,30}. In mouse ESCs (mESCs), crotonylation was found to be enriched on histones and decrotonylation resulted in their differentiation³¹. In hESCs, increased histone crotonylation promoted mesoendoderm differentiation³². These studies support the importance of histone crotonylation to stem cell fate regulation and underscore the intimate link between PTM and metabolic pathways.

In addition to histones, non-histone proteins involved in many cellular functions, such as cell cycle and nucleic acid metabolism, can be crotonylated on lysines in tumor cells^{33,34}. However, crotonylation of non-histone proteins remains poorly understood, particularly in cells such as hESCs where changes in metabolism and protein modifications can have profound effects on their pluripotency state and differentiation potential. In this study, we explored how changing the level of protein crotonylation in hESCs of different pluripotency states affected their transcriptomic profiles and induced their differentiation. Furthermore, by employing targeted metabolomic analysis we found that increased crotonylation also led to reduced glycolysis and enhanced TCA cycle. We went on to identify glycolytic enzymes such as GAPDH that could be crotonylated through high-resolution mass spectrometry and provide evidence that GAPDH might be a key player during hESC differentiation when cells shift away from relying on glycolysis. These findings demonstrate that crotonylation of glycolytic enzymes may be crucial to metabolic switching and cell fate determination in hESCs.

Results

Distinct transcriptomic changes occur in crotonate-treated primed hESCs. Fluctuations in the level of intermediate metabolites such as crotonyl-CoA can affect not only the equilibrium between different metabolic pathways but also the degree and types of PTM on proteins including those important for chromatin remodeling and gene expression^{27,28,35}. Crotonyl-CoA is generated from lysine/tryptophan metabolism and mitochondrial or peroxisomal fatty acid oxidation³⁶⁻⁴¹. Addition of the crotonyl-CoA precursor crotonate to cultures can increase the cellular level of crotonyl-CoA as well as protein crotonylation^{27,32}. To better understand the role crotonylation plays in cell fate determination and pluripotency maintenance in hESCs, we decided to take advantage of the H9 hESC line that expresses the LTR7/HERVH-driven GFP transgene⁴² and examine their transcriptional profiles following crotonate treatment by RNA-seq. LTR7/HERVH transcription is required for maintaining hESC pluripotency⁴³, where the expression of pluripotency genes directly correlates with HERVH levels. The GFP reporter therefore enables easy tracking of pluripotent hESCs during long-term culturing and upon differentiation induction. As shown before, the vast majority of the reporter cells had relatively low levels of GFP expression and normal expression of pluripotency markers such as NANOG and OCT4 (Fig. 1a, Supplementary Figs. 1a, b), indicating that they were in the primed pluripotency state. When we induced the reporter hESCs to differentiate into endodermal (with BMP4 and Activin A) (Supplementary Fig. 1c) or ectodermal cells (with BMP pathway inhibitors) (Supplementary Fig. 1d), we could observe GFP signal reduction that was accompanied by increased expression of germ layer-specific markers (Supplementary Figs. 1e-h).

We thus cultured the H9-LTR7 GFP reporter hESCs in crotonate-containing (10 mM) maintenance medium for 12 days before harvesting the cells for RNA-seq. While the untreated hESCs were able to maintain GFP expression and the primed state morphology, crotonate-treated hESCs gradually lost their GFP signals (from 100% to 7.9%) although the edges of the colonies had not yet rounded up (Fig. 1a), indicating a more differentiated state. Coinciding with these changes was the up-regulation of genes involved in biological processes such as endoderm development, stem cell

differentiation, and embryonic organ development with molecular functions of receptor or growth factor binding, based on GO and KEGG databases (Fig. 1b, top). Meanwhile, genes implicated in nervous system development and synaptic signaling with functions of transmembrane transporter and ion channel activities were down-regulated (Fig. 1b, bottom). When we compared the transcriptomic profiles of the treated and untreated H9-LTR7 GFP reporter hESCs and with endodermal cells derived from the reporter cells, the Pearson correlation coefficients indicate that the crotonate-treated cells at day 12 resembled more closely the derived endodermal cells (Fig. 1c). These observations indicate that crotonate treatment led to the respective activation and inhibition of endodermal and ectodermal gene expression in hESCs. We next verified our RNA-seq results by RT-qPCR analysis. Indeed, endoderm markers such as *GATA4*, *GATA6*, *SOX17*, *LHX1*, and *HHEX* became highly expressed in crotonate-treated cells (Fig. 1d). These findings are supported by results from the recently published report that also examined crotonate-treated hESCs³². A closer look at gene expression during the two-week treatment period revealed that pluripotency marker genes (e.g., *OCT4* and *NANOG*) initially increased moderately and then decreased to fairly low levels (Fig. 1e, top). Endoderm markers such as *GATA6* and *SOX17* on the other hand exhibited a gradual and steady increase in the presence of crotonate (Fig. 1e, bottom). Taken together, our results suggest that crotonate treatment led to germ layer-specific differentiation of the primed hESCs with a bias towards the endodermal lineage.

Crotonate-treated hEPS cells exhibit transcriptomic changes similar to primed hESCs. Primed hESCs can be converted into extended pluripotent stem (EPS) cells that are capable of giving rise to both embryonic and extraembryonic tissues *in vivo*⁴⁴⁻⁴⁶. As expected, when the reporter hESCs were cultured in LCDM medium⁴⁴, they became more dome-shaped while maintaining similar levels of GFP signals and pluripotency marker expression (e.g., *NANOG* and *OCT4*) (Fig. 1f, Supplementary Figs. 1a-c), indicating a conversion to the more naïve-like EPS cell state. We then cultured these converted cells in the presence of crotonate and performed RNA-seq analysis as described above. Similar to the primed state reporter cells, the converted hEPS cells also lost LTR7-GFP signals with

crotonate treatment (Fig. 1f). In addition to the endodermal development pathway, RNA-seq results from the treated hEPS cells also found an enrichment of pathways that promote cell communication and signal transduction with molecular functions of protein binding and cytokine receptor binding in upregulated genes (Fig. 1g). At the same time, downregulated genes were enriched with those involved in ear development and neurogenesis. We again verified the RNA-seq results by RT-qPCR analysis (Fig. 1h) as well as immunofluorescence (IF) assays (Fig. 1i). Interestingly, compared to primed hESCs, where the expression of endoderm marker genes went up >1,000 fold (Fig. 1d), crotonate-treated EPS cells exhibited ~15-fold increase in endoderm marker gene expression (Fig. 1h). These subtle but noticeable differences suggest that lesser signals may be needed to induce hEPS cell differentiation and further highlight these cells as a more potent and naïve-like pluripotency state.

Increased protein crotonylation leads to ESC differentiation into the endodermal lineage.

Studies of crotonylation have largely focused on histones and the role of histone crotonylation in stimulating transcription. Here, both primed and extended pluripotency reporter hESCs displayed a dose-dependent increase in the crotonylation of histones and non-histone proteins with crotonate treatment (Fig. 2a). However, even though the expression of mesoendoderm marker genes (e.g., *GATA6*, *SOX17*, *MIXL1* and *TBXT*) was clearly induced in 5 mM crotonate (>1,000 fold) (Figs. 2b, c), significant loss of GFP signals in the primed reporter hESCs occurred with 10 mM crotonate treatment. In comparison, GFP signal loss in the converted reporter hEPS cells was already detectable in 5 mM crotonate, despite more modest induction of lineage marker expression at this concentration (Figs. 2d-f). When we examined the level of protein crotonylation in untreated primed vs. EPS reporter cells using a pan anti-crotonyl-lysine (Kcr) antibody, we saw clearly elevated histones and non-histone proteins crotonylation in primed hESCs compared to hEPS cells (Fig. 2g), another indication of the more naïve and potent pluripotency state of EPS cells. Notably, endoderm cells differentiated from primed hESCs displayed much higher crotonylation compared with primed hESCs, whereas the level of crotonylation in ectodermal cells differentiated from primed hESCs was

lower and more similar to the level in hEPS cells. These results are in line with our RNA-seq data linking increased protein crotonylation with germ layer-specific hESC differentiation. Next, we cultured the mouse ESC line E14 in different concentrations of crotonate and examined its ability to differentiate. Similar to hESCs, mESCs also exhibited a dose-dependent increase in protein crotonylation (Supplementary Fig. 2a) that correlated with increased percentages of differentiated clones and elevated expression of mesoendodermal markers (Supplementary Figs. 2b-f). Our findings combined indicate that crotonate treatment can induce stem cell differentiation towards endodermal cells and implicate crotonylation in differential cell fate determination.

Crotonate-treated hESCs show reduced glycolysis and enhanced TCA cycle. During aerobic respiration, glucose is converted through glycolysis into pyruvate, which delivers the acetyl group to the tricarboxylic acid (TCA) cycle for energy production⁴⁷. The pyruvate dehydrogenase reaction removes the carboxyl group to form acetyl-CoA, thus providing the primary link between glycolysis and the TCA cycle (Fig. 3a). A metabolic switch with decreased glycolysis and elevated oxidative phosphorylation can occur during hESC differentiation into endodermal cells^{10,11}. We therefore decided to investigate how crotonate treatment-induced crotonylation increase and differentiation might affect the glycolytic pathway in the reporter hESCs, and carried out targeted metabolomic studies by HPIC-MRM-MS/MS to measure the levels of key intermediate metabolites in glycolysis and the TCA cycle (Fig. 3a). For controls, we used untreated hESCs as well as endoderm cells derived from the reporter hESCs. Consistent with previous findings, the derived endodermal cells showed an accumulation of upstream glycolytic intermediates (e.g., glucose 6-phosphate and fructose 1,6-bisphosphate) and a corresponding decrease in downstream intermediates (e.g., 3-phosphoglycerate and phosphoenolpyruvate) as well as the end product pyruvate (Figs. 3b, c), suggesting reduced glycolysis. Crotonate treatment in hESCs led to similar shifts in the glycolytic pathway. When metabolites of the TCA cycle were determined, crotonate-treated hESCs and to a lesser extent the derived endodermal cells exhibited enhanced flux through the TCA cycle relative to

untreated hESCs (Figs. 3d, e), indicating more active TCA cycle in these cells. Together, these results demonstrate that a shift from glycolysis to oxidative phosphorylation occurred in hESCs induced to differentiate into the endodermal lineage with crotonate treatment, and support a role of protein crotonylation in regulating metabolic pathway switching during hESC differentiation.

Large-scale identification of lysine-crotonylated proteins in hESCs. The relative concentration of intermediate metabolites (e.g., acetyl-CoA vs. crotonyl-CoA) helps determine the rate and extent of protein acylation. Addition of acetate, the acetyl-CoA precursor, could increase glycolysis and global protein acetylation, accompanied by delayed differentiation of primed hESCs¹⁹. Similarly, adding crotonate to cells could increase global protein crotonylation^{29,33}. In hESCs, drastically increased crotonylation of non-histone proteins was clearly evident upon crotonate treatment (Fig. 2a). We speculated that crotonylation of non-histone proteins must be crucial to the differentiation induction process, given the markedly changed levels of metabolic intermediates upon crotonate addition. To uncover the identities of such proteins, we performed proteomic analysis by LC-MS/MS using crotonate-treated and untreated reporter hESCs. Crotonylated peptides/proteins were enriched using the pan anti-Kcr antibody following tryptic digestion (Fig. 4a). In the absence of crotonate, five proteins (TBA1C, FLNA, RYR2, APBP2, and RS24) were found to be crotonylated. That number increased to 53 with crotonate treatment where only TBA1C and APBP2 appeared in both (Supplementary Table 1). The list also includes known crotonylated proteins such as histone H2B1 and nucleophosmin 1 (NPM1)³³. It is clear that functionally diverse proteins undergo crotonylation in crotonate-treated primed hESCs, including those involved in nucleosome organization, chromatin assembly or disassembly, and DNA packaging (Fig. 4b).

Notably, metabolic enzymes, especially those participating in glycolysis and glucose catabolic processes, were highly enriched amongst the crotonylated candidates (Fig. 4b). This observation suggests that crotonylation of metabolic enzymes may help drive the metabolic shifts necessary for hESC differentiation. We decided to focus on two glycolytic enzymes -- GAPDH and ENOA -- and

determine whether they could indeed be crotonylated. First, we immunoprecipitated GAPDH from endodermal cells and untreated reporter hESCs for western blotting with the anti-Kcr antibody. As shown in Fig. 4c, we could readily detect crotonylated GAPDH in endodermal cells but the signal was nearly negligible in untreated hESCs. We reasoned that the difficulty in detecting endogenous GAPDH crotonylation in untreated hESCs might be because the level of endogenous GAPDH crotonylation in these cells was below the detection threshold of the anti-Kcr antibody. Additionally, although we tested several antibodies against ENOA, none worked well in immunoprecipitation assays. We therefore generated reporter hESCs that stably expressed HA-tagged GAPDH or ENOA and immunoprecipitated these tagged proteins using the anti-Kcr antibody. Even without crotonate treatment, the anti-Kcr antibody could bring down both HA-GAPDH and HA-ENOA, whose signals increased further with crotonate treatment (Fig. 4d). These results together support the idea that glycolytic enzymes such as GAPDH and ENOA could be crotonylated in cells and changes in their crotonylation levels may contribute to lineage-specific hESC differentiation.

Crotonylation regulates the enzyme activity of GAPDH. p300 has been identified as a crotonyltransferase that can catalyze *in vitro* histone crotonylation in the presence of crotonyl-CoA^{29,30}. We therefore examined whether p300 could similarly modify GAPDH in *in vitro* crotonylation reactions using recombinant GAPDH and p300. Indeed, in the presence of crotonyl-CoA, robust crotonylation of GAPDH could be detected (Fig. 5a). Next, we assessed the catalytic activity of *in vitro* modified GAPDH using NAD⁺ as a substrate. Compared to unmodified GAPDH, the ability of GAPDH to convert NAD⁺ significantly decreased in the presence of both p300 and crotonyl-CoA (Fig. 5b). We similarly analyzed ENOA and found that it too could be crotonylated *in vitro* (Supplementary Fig. 3a). Unlike GAPDH however, crotonylation appeared to have minimal effect on ENOA activity using D-2-phosphoglycerate as a substrate (Supplementary Fig. 3b). Next, we examined the enzymatic activities of *in vivo* crotonylated GAPDH and ENOA using crotonate-treated reporter hESCs. For comparison, untreated hESCs and derived endodermal cells were included as well. In the case of

239 ENOA, crotonate treatment led to a 50% increase in ENOA activity and differentiated cells exhibited
240 slightly higher activities than untreated hESCs (Supplementary Fig. 3c). In contrast, crotonate
241 treatment of hESCs lowered GAPDH activity by ~50% in hESCs, to levels similar to that found in the
242 differentiated endodermal cells (Fig. 5c). This decrease is consistent with the observed accumulation
243 of glycolytic metabolites glucose 6-phosphate and fructose 1,6-bisphosphate as well as the reduction
244 of pyruvate levels in crotonate-treated hESCs (Fig. 3c), and points to GAPDH as the major glycolytic
245 enzyme whose activity is tightly regulated by crotonylation during hESC differentiation to the
246 endoderm lineage.

247 MS analysis identified two potential *in vivo* crotonylation sites on GAPDH (K194 and K219),
248 both of which are located in the catalytic domain (Fig. 5d). We therefore generated a GAPDH mutant
249 in which both lysines were mutated to arginine (K194R/K219R). As shown in Fig. 5e, mutating the
250 two residues abolished the ability of p300 to crotonylated GAPDH *in vitro*. Next, we assessed the
251 catalytic activity of wildtype vs. mutant GAPDH (Fig. 5f). In the absence of p300, the double mutant
252 appeared to be slightly reduced in its ability to convert NAD⁺, perhaps a reflection of the importance of
253 these two lysine residues to GAPDH activity. Unlike wildtype GAPDH, which exhibited a marked
254 decrease in activity when crotonylated, the activity of the GAPDH K194R/K219R mutant remained
255 relatively constant regardless of the presence of p300 (Fig. 5f). Altogether, these results indicate that
256 the two lysine residues of GAPDH identified by MS analysis could indeed be crotonylated *in vitro* and
257 their crotonylation helped regulate GAPDH activity.

258 Given the changes that occurred in crotonate-treated hESCs including the heightened reliance on the
259 TCA cycle rather than glycolysis, we reasoned that modulating GAPDH activity might affect hESC
260 pluripotency. To test this idea, we examined reporter hESCs that had been cultured long term in the
261 presence of the alkylating agent 3-bromo pyruvate (3-BrPA), which can inactivate glycolytic enzymes
262 and counts GAPDH as its preferred and primary target⁴⁸⁻⁵⁰. In our study, 3-BrPA treatment was able to
263 diminish GAPDH activity by >50% (Fig. 5g). When we measured marker gene expression by
264 RT-qPCR, we found >30-fold increase in the expression of *GATA6* and *SOX17* in cells treated with

3-BrPA (Fig. 5h). These results suggest that modulating GAPDH activity can directly promote endoderm gene expression and provide further evidence that GAPDH crotonylation may be key to the metabolic switch during endodermal differentiation from hESCs.

Discussion

The heterogeneity of hESCs has long been a challenge in both developmental studies and clinical applications, where directional differentiation from homogenous cell populations is highly desirable but difficult to achieve. We found that during crotonate-induced hESC differentiation towards the endodermal lineage, cells exhibited highly elevated crotonylation of both histone and non-histone proteins, global gene expression changes, and metabolic pathway switching. Our study highlights the complex and interconnected events that have to occur upon crotonate treatment-induced differentiation of hESCs. The importance of maintaining the equilibrium between small-molecule metabolic intermediates is becoming increasingly appreciated. Often these metabolites can serve as PTM substrates and/or co-factors thereby providing pivotal links between metabolic processes in the cytoplasm and gene expression regulation in the nucleus. In this study, we focused on the role of non-histone protein crotonylation.

Since its discovery, studies of crotonylation have largely focused on histones^{27,33,34}. Abundant histone crotonylation in the human genome is generally associated with active chromatin^{27,51}, and plays an important role in spermatogenesis^{29,35}, kidney injury⁵², HIV latency⁵³, and ESC differentiation^{32,33}. In our study, metabolic enzymes appear to be major targets of induced crotonylation as well in hESCs. Interestingly, although the two glycolytic enzymes GAPDH and ENOA could both be crotonylated *in vivo* and exhibited increased crotonylation upon crotonate treatment, the activity of GAPDH, but not ENOA, was sensitive to levels of crotonylation. When we mutated the two potential crotonylation sites within the GAPDH catalytic domain, the activity of GAPDH became refractory to crotonate treatment. These results are supported by the increase/decrease in glycolytic intermediate metabolites up- and downstream of GAPDH from our

metabolomic analysis. Published reports have shown that GAPDH acetylation can enhance its activity in response to glucose^{34,54}. Here, we found that GAPDH crotonylation decreased its catalytic activity. Taken together, these observations point to GAPDH as a critical player in regulating hESC pluripotency and promoting endoderm differentiation, and in mediating metabolic switching in response to differentiation-inducing signals. Recently, another group showed that other glycolytic enzymes (e.g., hexokinase 2 and phosphoglycerate kinase 1) could also be crotonylated⁵⁵, adding further support to the role of crotonylation in regulating metabolic function in pluripotent stem cells.

Crotonate-treated hESCs resemble endodermal cells in terms of transcriptomic and metabolomic profiles, suggesting that elevating protein crotonylation inside cells may be employed in *in vitro* differentiation protocols using hESCs of different pluripotent states. This PTM manipulation may be done in conjunction with glycolytic inhibitors, which can further alter the activities of glycolytic enzymes and drive hESCs towards metabolic pathways conducive to germ layer-specific differentiation. Further studies in these areas promise to uncover additional parameters for establishing such protocols and prove beneficial to clinical applications that require pluripotent stem cells.

Methods

Embryonic stem cell lines and their maintenance and manipulation. All cell lines were routinely tested for mycoplasma and confirmed negative throughout the study. The H9 hESC line expressing the LTR7-GFP reporter was a gift from Dr. Jichang Wang at Sun Yat-sen University. The cells were cultured with daily media change in chemically-defined mTeSR medium (STEMCELL Technologies) on Matrigel (BD Biosciences)-coated plates under 20% O₂ and 5% CO₂ at 37°C, and passaged every 3-5 days with split ratios of 1:3 to 1:5 by adding 0.5 mM EDTA to 3-5 cell clusters⁴². The mouse ESC line E14 was maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (Hyclone, Logan, UT, US), 0.1 mM NEAA, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, LIF (1,000 U/ml, Millipore, Billerica, MA), and 2i (3 μM CHIR99021 and 1 μM PD0325901).

The human extended pluripotency stem (hEPS) cells were derived as described previously⁴⁴. Briefly, single-cell suspensions (in LCDM medium) of the H9 LTR7-GFP hESC line were seeded on mitomycin C-inactivated MEF feeder cells in LCDM medium ($\sim 2 \times 10^5$ cells) with medium change daily. After 6-7 days, cells were passaged at ratios of 1:3 to 1:10 every 3-4 days by digestion with 0.05% Trypsin-EDTA and cultured under 20% O₂ and 5% CO₂ at 37°C. The derived hEPS cells were removed from feeder cells based on their adherence to the dish bottom and collected for subsequent experiments.

HA-tagged GAPDH and ENOA were cloned into the pET28a vector. hESCs stably expressing HA-tagged proteins were generated by lentiviral infection. Fluorescence microscopy was done on a Zeiss Imager Z1 microscope. Flow cytometry analysis was performed using Beckman CytoFLEX (C01158) and the CytExpert software (Beckman). For drug treatment, cells were cultured in maintenance media containing the appropriate amount of sodium crotonate (Sigma, 391719), sodium acetate (Sigma, S2889), or 3-BrPA (Sigma, 16490) for the indicated amount of time. LCDM medium (50ml) contains equal parts DMEM/F12 (Thermo, 11330-032) and Neurobasal (Thermo, 21103049)(24 ml each), 250 µl N2 supplement (Thermo, 17502-048), 500 µl B27 supplement (Thermo, 12587-010), 1 mM glutamine (Thermo, 35050-061), 1% non-essential amino acids (Thermo, 11140-050), 0.1 mM β-mercaptoethanol (Thermo, 21985-023), penicillin-streptomycin (Thermo, 15140-122), 5% KSR (Thermo, A3181502), 10 ng/ml recombinant human LIF (Peprotech, 300-05), 1 µM CHIR99021 (Tocris, 4423), 2 µM (S)-(+)-Dimethindenemaleate (Tocris, 1425), 2 µM Minocycline hydrochloride (Sigma, M9511), 1 µM IWR-endo-1(Selleckchem, S7086), and 2 µM Y-27632 (Selleckchem, S1049).

***In vitro* differentiation assays.** Cells were seeded onto matrigel-coated plates at a density of $1-2 \times 10^4$ cells/cm² and cultured in differentiation media to derive endoderm or ectoderm cells as previously described^{56,57}. For endodermal cells, hESCs were cultured in E8 medium containing 5 ng/ml BMP4 and 25 ng/ml Activin A. CHIR99021 (1 µM) was added for the first 2 days only, and the cells were

subsequently maintained with medium change daily. For ectodermal cells, hESCs were cultured in MEF-CM medium (Knock-out DMEM containing 20% KSR, 1 mM glutamine, 1% nonessential amino acids, 0.1 mM β -mercaptoethanol, and 1% penicillin-streptomycin) supplemented with 0.1 μ M LDN193189 (Selleckchem, S2618) and 10 μ M SB431542 (Selleckchem, S1067) with medium change daily.

RNA-seq and data analysis. RNA-seq analysis was performed by Berry Genomics (Beijing, China). Briefly, total RNA was extracted using the RNeasy Mini Kit (Qiagen) and enriched for mRNA with Oligo (dT), followed by mRNA fragmentation, cDNA synthesis, and PCR amplification. The Agilent 2100 Bioanalyzer and ABI StepOnePlus real-time PCR system were used to analyze sample libraries, which were sequenced using the Illumina HiSeq2500 system in a mode of 150-bp paired-end read. For differential analysis, clean reads of each sample that mapped onto human genome (hg38) with clean ratios above 95.06% were obtained using Tophat (version 1.3.2) and Cufflinks (V1.1.0). The HTSeq (version 0.6.0) was used to quantify mapped counts of human genes. Sequencing read counts were calculated using StringTie (v1.3.0). The expression levels of different samples were normalized by the Trimmed Mean of M values (TMM) and converted into FPKM. The edgeR package of R was used to analyze differential gene expression between samples. Significantly different genes were defined as those with ratios of FPKM greater than 1.5 and p-values less than 0.05. Functional annotation was performed using the David bioinformatics resource (<https://david.ncifcrf.gov/>). The functional annotation term categories of biological process (BP), molecular function (MF) and cellular component (CC) were analyzed.

Targeted metabolomic analysis using HPIC-MRM-MS. Targeted metabolomic analysis was performed by Shanghai BIOTREE Biological Technology Co., Ltd. (Shanghai, China). Briefly, cell samples (1×10^7 cells) were homogenized in 500 μ L precooled Chloroform/MeOH (1/3, v/v) for 4 min at 35 Hz and sonicated for 10 min in ice-water. The entire process was repeated three times

before the samples were mixed at 4 °C for 15 min, incubated at -80 °C for 1 h, and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was dried under gentle nitrogen flow and resuspended in 150 µL ultrapure water for filtration and analysis by high-performance ion chromatogram-multiple reaction monitoring-mass spectrometry/mass spectrometry (HPIC-MS/MS) (Thermo Scientific Dionex ICS-6000 HPIC System). Each target peak was detected and different metabolites were left after relative standard deviation de-noising. The missing values were filled up by the median value. The final dataset containing the information of peak number, sample name, and normalized peak area was imported to the SIMCA16.0.2 software package (Sartorius Stedim Data Analytics AB, Umea, Sweden) for multivariate analysis. Data were scaled and logarithmic transformed to minimize the impact of both noise and high variance of the variables. After these transformations, the unsupervised analysis PCA (principle component analysis) that reduces the dimension of the data was carried out to visualize the distribution and grouping of samples. The 95% confidence interval in the PCA score plot was used as the threshold to identify potential outliers in the dataset. Data acquisition occurred on the mass spectrometer operating in negative polarity multiple reaction monitoring (MRM) mode. MRM data were acquired using the AB SCIEX Analyst Work Station Software (1.6.3 AB SCIEX) and analyzed using MultiQuant 3.0.3. The ThermoFisher Chromeleon processing of targeted metabolites included automated peak detection and integration using the default method and all data were processed by BIOTREE Biological Technology Co., Ltd. (Shanghai, China).

Protein crotonylation identification using liquid-chromatography fractionation and high-resolution tandem mass spectrometry (LC-MS/MS) and data analysis. Protein crotonylation identification was performed as previously described with minor modifications³³. Briefly, to enrich for lysine crotonylated peptides, tryptic peptides dissolved in Binding Buffer (20 mM HEPES, pH 8.0, 150 mM KCl, 1 mM DTT, 10% glycerol, 0.1% NP-40, 100×Cocktail) were incubated at 4°C overnight with the pan anti-crotonyllysine antibody (4 µg/4 mg peptides) (PTM biolab,

PTM-501) that had been pre-coupled to protein A beads (Invitrogen). The bound peptides were washed four times with Wash Buffer (20 mM HEPES, pH 8.0, 150 mM KCl, 1 mM DTT, 0.1% NP-40, 100×Cocktail) and twice with ultrapure water before being eluted in buffer containing 0.15% TFA and 20% ACN. The eluted peptides were vacuum-dried, cleaned using C18 Spin Columns (10 ng to 30 µg protein) (Thermo, 89870), re-suspended in ddH₂O containing 0.1% formic acid, and loaded into a nanoViper C18 (Acclaim PepMap 100, 75 µm×2 cm) trap column in 4 µL/min aliquots. Following trapping and desalting (in 20 µL volume of 100% solvent A (0.1% formic acid)), the peptides were eluted in 60 min on an analytical column (Acclaim PepMap RSLC, 75 µm×25 cm C18-2 µm 100 Å) with a gradient of 5-38% solvent B (80% acetonitrile, 0.1% formic acid). Tandem MS data were acquired on a Thermo Fisher Q Exactive mass spectrometer (Thermo Fisher, USA) fitted with a Nano Flex ion source using DDA (data-dependent acquisition) mass spectrum techniques. For full mass spectrometry survey scan, the target value was 3×10^6 and the scan ranged from 350 to 2,000 m/z at a resolution of 70,000 and a maximum injection time of 100 ms.

For protein identification and quantification, the MS/MS data were analyzed using PEAKS Studio 8.5. The local false discovery rate at PSM was 1.0% after searching against Homo sapiens database with a maximum of two missed cleavages. Precursor and fragment mass tolerance were set to 10 ppm and 0.05 Da, respectively. All data were then searched against with the UniProt Human database (88817 sequences) including variable modification of crotonylation (lysine, C₄H₄O, + 68.03). Crotonylated proteins were analyzed by Kobas based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

***In vitro* crotonylation and enzymatic activity assays.** *In vitro* crotonylation assays were performed as described previously²⁹. Briefly, 0.5 µg of recombinant GAPDH (Ab82633, Abcam) or ENOA (Ab89248, Abcam) proteins were incubated with 0.5 µg of p300 proteins (Active Motif, 81158) in a 30 µL reaction containing 50 mM Tris pH 8.0, 1 mM TCEP, 0.1 mM EDTA, 2 mM MgCl₂, and 20 µM Crotonyl-CoA (Sigma, 28007) for 1 h at 30°C. The reactions mixtures were then used for activity

assays using the GAPDH and Enolase Activity Assay Kits (Sigma, MAK277 and MAK178) following the manufacturer's instructions. Alternatively, cells were lysed and incubated with Assay Buffer before enzymatic activity assays.

Immunofluorescence. Immunofluorescence assay was performed according to standard procedures. Cells were fixed with freshly prepared 4% paraformaldehyde in PBS at 4°C for 15 min, permeabilized in 0.1% Triton X-100 in PBS at room temperature for 30 min, washed three times, and left in blocking solution (4% goat serum in PBS) for 1 h. Samples were incubated with primary antibody dissolved in blocking solution at 4°C overnight, washed three times and incubated for 1 h with secondary antibodies at room temperature. Samples were washed and the nuclei were stained with DAPI in Vectashield mounting medium. Fluorescence was detected and imaged using a Zeiss Imager Z1 microscope. The anti-NANOG (ab80892, Abcam), anti-OCT4 (ab181557, Abcam) and anti-GATA6 (5851, Cell signaling technology) antibodies were used at 1:200 dilution. Secondary antibodies including AlexaFluor 488 anti-rabbit IgG (A-21206, 1:1000), and AlexaFluor 555 anti-Mouse IgG (A-31570, 1:1000) were obtained from Invitrogen.

Protein immunoprecipitation and western blotting. A total of 1×10^7 cells were lysed in lysis buffer (40 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40) containing protease inhibitors. Protein concentration was measured using BCA protein assay (Thermo Fisher) and normalized across samples prior to immunoprecipitation. For Kcr immunoprecipitation, 5 µg of antibody and 50 µL of protein A beads were added to lysates overnight at 4 °C. Lysates were centrifuged for 3 min at 4 °C and the beads were washed three times with ice-cold lysis buffer and resuspended in lysis buffer and boiled for 5 min.

Western blotting was performed according to standard procedures. Cells were resuspended in RIPA Buffer containing 50mM Tris (pH 7.4), 150mM NaCl, 1mM EDTA, 1% Triton X-100, 10% glycerol, 0.25% deoxycholate, and 0.1% SDS supplemented with protease inhibitor cocktail (Sigma)

on ice for 20 min. Lysates were resolved by SDS–PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% nonfat milk/TBST and incubated with primary antibodies at 4°C overnight. Antibodies used in the study include: Anti-crotonyllysine (PTM Biolab, PTM-501, 1:1,000), anti-β-actin (Abclonal, AC026, 1:5,000), anti-histone H3 (Abcam, ab1791, 1:5,000), anti-GAPDH (Proteintech, 60004-1, 1:5,000), anti-HA (Sigma, H9658, 1:5,000). Odyssey IRDye 680CW anti-mouse (926-32220) and IRDye 800CW anti-rabbit (926-32211) secondary antibodies were used at 1:5,000 dilution.

Reverse transcription quantitative real-time PCR (RT-qPCR). RT-qPCR was performed as previously described. Briefly, total RNA was isolated using the RNeasy Mini Kit (Qiagen) and reversely transcribed using reverse transcriptase (Vazyme, R223). Real-time quantitative PCR reactions were conducted using Power SYBR Green PCR Master Mix (Vazyme, Q321) on an ABI Prism 7300 Sequence Detection System. The data were analyzed by the delta-delta CT method. The expression level of each gene was normalized with actin RNA. Primers are listed in Supplemental Table S2.

Protein extraction for mass spectrometry analysis. Protein extraction was done essentially as described previously. Cells were resuspended in 400 µl of lysis buffer (8 M urea and cocktail protease inhibitors (Sigma), 20 mM HEPES, pH 8.0). The samples were sonicated at 20% power in a tip sonicator and incubated on ice for 20 min. The supernatant was collected after 15 min of centrifugation at 20,000g at 4 °C, and the protein concentration was determined by BCA assay (Thermo Fisher). For digestion, 2-4 mg proteins were reduced in 500 mM DTT for 1h at 60°C and alkylated with 1 M iodoacetamide at room temperature for 30 min protected from light. For trypsin

digestion, the protein samples were diluted by adding 50 mM NH_4HCO_3 pH 8.0 to reduce the urea concentration below 2 M. Trypsin was added at 1:100 trypsin-to-protein mass ratio for an overnight digestion. The samples were desalted by C18 Column (Maximum 500 mg protein) (Waters, 186004619) and the peptides were dried by vacuum centrifugation.

Mutagenesis and GAPDH mutant protein purification. The GAPDH double lysine mutant was generated using the Quickchange II site-directed mutagenesis kit (Agilent Technologies). pET28a plasmids containing His-tagged wild-type GAPDH or GAPDH mutant were expressed in BL21 *Escherichia coli*. HisSep Ni-NTA Agarose Resin (YEASEN, 20503ES1) was used for protein purification. Cells were cultured at 30°C for 6 h, harvested and resuspended in lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8.0). Following sonication and centrifugation, the supernatant was loaded onto a nickel agarose resin column pre-equilibrated with lysis buffer. The column was washed with 5 column volumes of wash buffer (lysis buffer with 20 mM imidazole) and the proteins eluted with elution buffer (lysis buffer with 250 mM imidazole). After purification, proteins were dialyzed at 4°C overnight.

Statistical analysis. Statistical tests were performed by GraphPad prism. A value of $p < 0.05$ was considered statistically significant.

Data availability

The authors declare that all other data supporting the findings of this study are within the manuscript and its supplementary files or are available from the corresponding authors upon request.

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625

626 **Author contributions**

627 Z.S. and G.S. designed the experiments. J.Z., and G.S. performed the experiments and analyzed the
628 data. J.Z., G.S. and Z.S. wrote the manuscript and supervised the research. All authors discussed the
629 results and commented on the manuscript.

630

631 **Competing interests**

632 The authors declare no competing interests.

633

634

Figure Legends

Fig. 1 Crotonate-treated hESCs display transcriptomic changes associated with endodermal development. **a** H9-LTR7 GFP reporter hESCs were cultured in crotonate (Cr)-containing (10 mM) maintenance medium for 12 days before phase-contrast and fluorescence microscopy. Scale bar, 100 μ m. **b** Cells from (a) were harvested after 12 days of Cr treatment for RNA-seq. Untreated H9-LTR7 GFP reporter hESCs served as controls. Results from gene ontology analysis of genes up- or down-regulated with treatment was plotted as shown. **c** Results from (b) were compared to RNA-seq data using endodermal cells derived from H9-LTR7 GFP reporter hESCs to calculate the Pearson correlation coefficients. **d** Cells from (a) were harvested after 12 days of Cr treatment for RT-qPCR analysis of the indicated endodermal development genes. Untreated cells served as controls. Error bars represent mean $\pm$ S.D. (n=3 independent experiments). **e** H9-LTR7 GFP reporter hESCs were cultured in crotonate (Cr)-containing (10 mM) maintenance medium and harvested for RT-qPCR analysis at the indicated time points for pluripotency (top) or endoderm (bottom) markers. Error bars represent mean $\pm$ S.D. (n=3 independent experiments). **f** H9-LTR7 GFP reporter hESCs were cultured first in LCDM medium for 15 passages to derive hEPS cells, which were then cultured in crotonate (Cr)-containing (10 mM) maintenance medium for 12 days before phase-contrast and fluorescence microscopy. Scale bar, 100 μ m. **g** Cells from f were harvested after 12 days of Cr treatment for RNA-seq and gene ontology analysis. **h** RT-qPCR for the indicated marker genes. Error bars represent mean $\pm$ S.D. (n=3 independent experiments). **i** H9-LTR7 GFP hESCs ($\pm$ 10mM Cr for 12 days), converted EPS cells ($\pm$ 10mM Cr for 12 days), and endodermal cells derived from the reporter hESCs were immunostained with an anti-GATA6 antibody. DAPI was used to stain the nuclei. Scale bars, 10 μ m.

657

658 **Fig. 2** Increased protein crotonylation upon crotonate treatment promotes hESC differentiation into
659 the endodermal lineage. **a** H9-LTR7 GFP hESCs were cultured in different concentrations of
660 crotonate for 24 hours before being harvested for western blotting as indicated. **b** H9-LTR7 GFP
661 hESCs were cultured in different concentrations of crotonate for 12 days before being analyzed by
662 microscopy (left) and flow cytometry (right). Percentages of GFP-expressing cells were plotted
663 below. Cells cultured without crotonate or with 10 mM acetate (Ac) for 12 days served as controls.
664 Scale bar, 100 μ m. Error bars represent mean $\pm$ S.D. (n=3 independent experiments). Significance
665 was calculated using unpaired t-test. ****p** < 0.01. **c** Cells from (b) were analyzed by RT-qPCR for
666 marker gene expression. *OCT4* and *NANOG*, pluripotency markers. *GATA6* and *SOX17*, endoderm
667 markers. *MIXL1* and *TBXT*, mesoderm markers. *SOX1* and *NESTIN*, ectoderm markers. Error bars
668 represent mean $\pm$ S.D. (n=3 independent experiments). **d** hEPS cells derived from reporter hESCs
669 were cultured and western blotted as described in (a). **e, f** hEPS cells derived from reporter hESCs
670 were treated as described in (b) and similarly harvested for microscopy and flow cytometry (e) and
671 RT-qPCR analysis (f). Scale bar, 100 μ m. Error bars represent mean $\pm$ S.D. (n=3 independent
672 experiments). Significance was calculated using unpaired t-test. ****p** < 0.01. **g** H9-LTR7 GFP hESCs
673 and the converted EPS cells were western blotted with the indicated antibodies, along with ectoderm
674 and endoderm cells derived from the reporter hESCs.

675

676 **Fig. 3** Crotonate treatment leads to reduced glycolysis and enhanced TCA cycle. **a** Key metabolic
677 intermediates in glycolysis and the TCA cycle are shown. Those in red were measured by
678 HPIC-MRM-MS/MS. **b, c** Reporter hESCs were cultured in crotonate-containing media (10 mM) for

679 12 days before being harvested for targeted metabolomic analysis. Untreated cells and endodermal
680 cells differentiated from the reporter hESCs were used as controls. Levels of the measured
681 intermediates were normalized to standards. The results for several glycolytic intermediates and the
682 end product pyruvate were compared across samples in a heatmap (b) or individually plotted (c). Error
683 bars represent mean $\pm$ S.D. (n=3 independent experiments). Significance was calculated using
684 unpaired t-test. *p < 0.05; **p < 0.01. **d, e** Based on metabolomics data, results for several TCA
685 cycle intermediates were compared across samples in a heatmap (d) or individually plotted (e). Error
686 bars represent mean $\pm$ S.D. (n=3 independent experiments). Significance was calculated using
687 unpaired t-test. *p < 0.05; **p < 0.01.

688

689 **Fig. 4** Large-scale analysis of lysine-crotonylated proteins in crotonate-treated hESCs identifies
690 glycolytic enzymes as major targets. **a** Reporter hESCs were cultured in crotonate-containing media
691 (10 mM) for 12 days before being harvested along with untreated hESCs for protein extraction and
692 peptic digest. Crotonylated peptides were enriched with an anti-Kcr antibody before LC/MS-MS
693 analysis. **b** Gene ontology analysis of crotonylated proteins in response to crotonate treatment that
694 were identified by LC/MS-MS. **c** H9-LTR7 GFP reporter hESCs and endodermal cells derived from
695 the reporter cells were immunoprecipitated (IP) with an anti-GAPDH antibody. The
696 immunoprecipitates were then western blotted as indicated. IgG served as a negative control. **d**
697 H9-LTR7 GFP hESCs stably expressing HA-tagged GAPDH (left) or ENOA (right) were cultured in
698 crotonate-containing media (10 mM) for 24 hours before being harvested for IP with the anti-Kcr
699 antibody and western blotting with an anti-HA antibody. Parental (Mock) and untreated cells served
700 as controls.

701

702 **Fig. 5** Crotonylation of GAPDH reduces its catalytic activity. **a, b** Recombinant GAPDH was
703 incubated with crotonyl-CoA and recombinant p300 and analyzed by western blotting with the
704 indicated antibodies (a) or assessed for GAPDH activity (b). Coomassie blue staining served as
705 loading control. Error bars represent mean $\pm$ S.D. (n=3 independent experiments). Significance was
706 calculated using unpaired t-test. ****p** < 0.01. **c** H9-LTR7 GFP hESCs were treated with 10 mM
707 crotonate for 12 days and then harvested to assess the activity of *in vivo* crotonylated GAPDH.
708 Untreated cells and endodermal cells derived from the reporter cells served as controls. Error bars
709 represent mean $\pm$ S.D. (n=3 independent experiments). Significance was calculated using unpaired
710 t-test. ****p** < 0.01. **d** LC-MS/MS identified two potential crotonylation sites on GAPDH, both of
711 which reside in the catalytic domain. Their respective MS/MS spectra are shown as well. **e, f**
712 Recombinant His-tagged wild-type and mutant GAPDH proteins were purified from *E.coli* (BL21)
713 and incubated with crotonyl-CoA and recombinant p300. The reaction products were then resolved by
714 SDS-PAGE and probed with the indicated antibodies (e) and assessed for GAPDH activity (f).
715 Coomassie blue staining served as loading control. Error bars represent mean $\pm$ S.D. (n=3
716 independent experiments). Significance was calculated using unpaired t-test. *p < 0.05; ****p** < 0.01.
717 NS, not significant. **g, h** H9-LTR7 GFP hESCs were cultured with or without 5 μ M 3-BrPA for 16
718 days before being collected for assessing the activity of GAPDH (g) or expression of marker genes by
719 RT-qPCR (h). Error bars represent mean $\pm$ S.D. (n=3 independent experiments). Significance was
720 calculated using unpaired t-test. ****p** < 0.01.

721

Fig.1 a, b

a

Untreated

+10 mM Cr

b

Up-regulated genes

Down-regulated genes

Fig.1 c-e

c

e

d

Fig.1 f, g

Fig.1 h, i

h

i

Fig.2 a-c**a** **b** **c**

Fig.2 d-g**d** **e** **f** **g**

Fig.3 a, b

a

b

Fig.3 c-e

Fig.4 a-d

Fig.5 a-d**a** **b** **c**

Relative GAPDH expression level

d

y9y8y7y6y5y4 y3y2y1

TV D G P S G K194(Cr) L W R

b2 b3

y8y7y6 y4y3y2y1

AV G K219(Cr) V I P E L N G K

b2 b4 b5

Fig.5 e-h

Supplementary Files

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- [Supplementarydata.pdf](#)