NLRP7 affects trophoblast lineage differentiation, binds to overexpressed YY1 and alters CpG methylation

Sangeetha Mahadevan1,2,5,†, Shu Wen2,3,†, Ying-Wooi Wan2,5, Hsiu-Huei Peng2,‡, Subhendu Otta2,§, Zhandong Liu2,4,5, Michelina Iacovino6,§, Elisabeth M. Mahen6, Michael Kyba6, Bekim Sadikovic3,|| and Ignatia B. Van den Veyver1,2,5,3,∗

1Interdepartmental Program in Translational Biology and Molecular Medicine, 2Department of Obstetrics and Gynecology, 3Department of Molecular and Human Genetics and 4Department of Pediatrics (Neurology), Baylor College of Medicine, Houston, TX, USA, 5Jan and Dan Duncan Neurological Research Institute at Texas Children’s Hospital, Houston, TX, USA and 6Lillehei Heart Institute and Department of Pediatrics, University of Minnesota, Minneapolis, MN, USA

Received April 3, 2013; Revised August 12, 2013; Accepted September 13, 2013

Maternal-effect mutations in NLRP7 cause rare biparentally inherited hydatidiform moles (BiHMs), abnormal pregnancies containing hypertrophic vesicular trophoblast but no embryo. BiHM trophoblasts display abnormal DNA methylation patterns affecting maternally methylated germline differentially methylated regions (gDMRs), suggesting that NLRP7 plays an important role in reprogramming imprinted gDMRs. How NLRP7—a component of the CATERPILLAR family of proteins involved in innate immunity and apoptosis—causes these specific DNA methylation and trophoblast defects is unknown. Because rodents lack NLRP7, we used human embryonic stem cells to study its function and demonstrate that NLRP7 interacts with YY1, an important chromatin-binding factor. Reduced NLRP7 levels alter DNA methylation and accelerate trophoblast lineage differentiation. NLRP7 thus appears to function in chromatin reprogramming and DNA methylation in the germline or early embryonic development, functions not previously associated with members of the NLRP family.

INTRODUCTION

Autosomal recessive loss-of-function maternal-effect mutations in NLRP7 (HYDM1, OMIM#231090) (1–4) cause biparental hydatidiform moles (BiHMs), a rare gestational disease characterized by a hyperproliferative vesicular trophoblast and the absence of a developing embryo. Molecular studies on these BiHMs revealed a lack of maternally acquired DNA methylation at germ-line differentially methylated regions (gDMRs) in the imprinting control centers of some paternally expressed genes, including PEG3, SNRPN and KCNQ1OT1 (2,5,6). Where tested, the genes have been found to be aberrantly expressed (7). Interestingly, gDMRs that acquire their methylation during paternal germline transmission, such as the DMR upstream of H19, have been found to retain their imprinted methylation status in most studies (2,5), with a few exceptions (6). Only a few such genes have been examined, but thus far, no alterations have been found in repeated sequences, CpG islands of genes on the X chromosome that are subject to X-inactivation, or the methylation status of non-imprinted loci (8). Furthermore, women who carry these pregnancies have normal DNA methylation at imprinted and non-imprinted loci (9), which indicates a maternal effect-driven phenotype. These observations suggest that BiHMs result from a failure to reprogram maternally imprinted gDMR

†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors and are listed in alphabetical order.
‡Present address: Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Chang Gung University, Tao-Yuan, 333, Taiwan.
§Present address: Central Institute of Brackishwater Aquaculture, 75 Santhome High Road, RA Puram, Chennai 600028, India.
||Present address: Department of Pathology and Laboratory Medicine, McMaster University, Hamilton, ON, Canada.
∗To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, One Baylor Plaza, Mailstop BCM610, Baylor College of Medicine, Houston, TX 77030, USA. Tel: +1 8328248125; Fax: +1 8328259354; Email: iveyver@bcm.edu
#The Author 2013. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
methylation and that understanding the molecular pathogenesis of BiHMs will provide insight into fundamental mechanisms of genomic imprinting. While several mechanisms to elucidate how gDMRs within the developing oocyte are selected for targeted methylation have been proposed, the specific processes that govern this remain unknown (10–13).

NLRP7 encodes the NOD-like receptor pyrin domain (PYD)-containing 7 protein, which belongs to the CATERPILLER family of proteins, many of which are inflammasome components with roles in innate immunity, inflammation and apoptosis and contain several conserved domains (14–16) (Fig. 1A). The molecular mechanisms by which loss of function of NLRP7 causes women to have recurrent BiHMs are unclear. Although immune dysfunction in women with NLRP7 mutations has been proposed to contribute to BiHM formation (17–20), there is to date no evidence that this causes defective reprogramming of maternally acquired DNA methylation and consequently BiHMs. Furthermore, a maternal-effect mutation in NLRP2, which is highly homologous to NLRP7, is associated with Beckwith–Wiedemann syndrome (BWS) in offspring, who have hypomethylation in trans at KvDMR1 in the BWS region and also at the PEG1 DMR (21,22). NLRP7, NLRP2 and some other NLRPs are also highly expressed in human oocytes and the early embryo (15,16,23,24).

Owing to the specificity of the defect observed in BiHM concept and the high expression of NLRP7 in oocytes and the early embryo, we hypothesized that NLRP7 is important for distinguishing which gDMRs should be targeted for DNA methylation in oocytes, or for the acquisition and maintenance of DNA methylation itself at these gDMRs. To this end, we sought to determine whether NLRP7 interacts with specific candidate proteins known to play a role in the imprinting process and whether it affects DNA methylation in human embryonic stem cell (hESC) cultures. The ability to induce trophoblast lineage

Figure 1. NLRP7 binds to YY1 in both nuclear and cytoplasmic fractions. (A) Map of the NLRP7 protein with different domains (PYD: pyrin domain; NACHT: nucleoside-triphosphatase domain named after proteins NAIP, CIITA, HET-E and TP1; NAD: NACHT-associated domain; LRR: leucine-rich repeat). (B) GST-tagged full-length NLRP7 (GST-FL-NLRP7) was co-expressed with Myc-tagged YY1. Immunoblotting was performed with an anti-myc antibody (αMyc) on the cell lysate (WB) on input and after pull-down on glutathione-sepharose (GST-PD). (C) Myc-tagged NLRP7 PYD, NACHT domain (NACHT) and LRR domain (LRR) were co-expressed with GST-tagged YY1 (GST-YY1 ‘+’ lanes) in HEK293T cells and pulled down with GST-PD. Precipitated material (top panel) or cell lysate (bottom panel; input) was immunoblotted with anti-Myc; the bottom panel shows the western analysis of unprecipitated cell lysates to confirm protein expression in each lane. (D) Same experiment as (C) but with tags reversed: NLRP7 domains are GST-tagged and YY1 is Myc-tagged. YY1 binds to the LRR, NACHT and PYD domain. (E) HEK293 cells were transfected with transfection agent alone (1), pDESTV5 (2), pDESTmyc (3), NLRP7-V5 (4), myc-YY1 (5) and NLRP7-V5 together with myc-YY1 (6). Whole-cell lysates show appropriate expression in all experimental groups (left panel). Endogenous YY1 (lanes 1–4) is at a lower molecular weight than myc-tagged YY1 (lanes 5 and 6). Subcellular fractionation followed by loading of 10% input from the IP experiment reveals presence of NLRP7 in cytoplasmic (lane 4) as well as nuclear fraction (lane 6). Additionally, there is no endogenous YY1 in the cytoplasmic fraction (lanes 1–4) suggesting an entirely nuclear localization of YY1 (middle panel). IP of NLRP7 followed by blotting with YY1 reveals an interaction of the two proteins in the cytoplasmic and nuclear fractions (right panels).
differentiation with BMP4 in hESCs also allowed us to examine how lack of NLRP7 affects trophoblast differentiation, a process that likely underlies the pathogenesis of the disease.

RESULTS

NLRP7 binds to YY1

We first asked whether NLRP7 interacts with proteins that play a role in chromatin modifications or DNA methylation acquisition and maintenance. We found that glutathione-S-transferase (GST)-tagged NLRP7 expressed in HEK293T cells pulls down myc-tagged Ying-yang 1 (YY1) (Fig. 1B), a factor that can bind to imprinted DMRs in a methylation-dependent manner to alter imprinted gene expression (25), and confirmed the YY1-NLRP7 interaction by co-immunoprecipitation (co-IP) of V5-NLRP7 with myc-YY1 (Supplementary Material, Fig. S1). YY1 binds to the PYD and LRR domains of NLRP7 (Fig. 1C); these binding patterns were confirmed when the tags were reversed (Fig. 1D). Thus, transiently overexpressed NLRP7 binds to YY1 via its LRR and PYD domains. Because endogenous expression of NLRP7 is very low in HEK293T cells (Supplementary Material, Fig. S2), we could not verify these findings in situ or with endogenously expressed proteins. To determine the subcellular compartments in which YY1 and NLRP7 interact, we co-transfected HEK293 cells with V5-tagged NLRP7 and Myc-tagged YY1 and compared their interactions in whole-cell lysates, and in nuclear and cytoplasmic fractions to appropriate controls, including transfection agent alone, pDESTV5, pDESTmyc and each tagged protein alone. We found that NLRP7 resides in the cytoplasm and in the nucleus and confirmed that YY1 is a mostly nuclear protein (Fig. 1E). IP of NLRP7 followed by immunoblotting with YY1 revealed interaction of the two proteins in the cytoplasmic and nuclear fractions (Fig. 1E), suggesting that at least part of the interaction occurs in the nucleus. We next attempted to verify binding of endogenously expressed NLRP7 and YY1 by performing co-IP in two cell lines (BeWo trophoblast cells and hESCs) with higher mRNA levels of NLRP7 with antibodies raised to these proteins. However, despite higher levels of mRNA in both cell types, we could not detect sufficiently high protein levels in western blot analysis with currently available antibodies to NLRP7 or after reciprocal immunoprecipitation experiments (data not shown).

The influence of NLRP7 overexpression on YY1 levels and binding to imprinted gDMRs

We next explored whether the observed interaction between NLRP7 and YY1 affects YY1’s expression levels and binding to its recognition sites at imprinted gDMRs of PEG3 and SNRPN, which normally acquire methylation in the maternal germline. We first showed that overexpression of NLRP7 did not alter mRNA and protein expression levels of YY1 (Fig. 2A). We then performed chromatin-immunoprecipitation (ChiP) with anti-YY1 in HEK293T cells that transiently overexpress NLRP7 (NLRP7OE), followed by real-time quantitative PCR (qRT-PCR) amplification of known YY1-binding sites at the gDMRs of PEG3 and SNRPN, at sites flanking these regions and at non-imprinted YY1-binding sites. We did not find a significant change in the binding of YY1 at any of these regions (data not shown). Since these experiments were performed with overexpressed NLRP7 in a cell line with low endogenous NLRP7 expression (Supplementary Material, Fig. S2), we also investigated whether reducing its levels affects DNA-binding of YY1 to gDMRs. We transfected BeWo cells, a commonly used trophoblast cell line with high endogenous NLRP7 expression (Supplementary Material, Fig. S2), with a mixture of siRNAs that target NLRP7 to reduce NLRP7 mRNA levels by 85% (Supplementary Material, Fig. S3A) without affecting expression of the homologous NLRP2 gene (Supplementary Material, Fig. S3B). As expected, NLRP7 knockdown (siNLRP7KD), similar to overexpression of NLRP7, did not affect expression of YY1 (Fig. 2B). We also did not see changes in its binding to the gDMRs of PEG3 and SNRPN compared with mock-transfected BeWo cells or cells transfected with the negative control non-targeting siRNA pool (siNLRP7NT).

Modeling loss of NLRP7 in hESCs

The absence of NLRP7 from the rodent genome (15) precludes generation of mouse models for BiHMs. Instead, to investigate whether NLRP7 and its effect on YY1 function influence...
trophoblast differentiation, we studied hESCs, which have relatively high NLRP7 expression (Supplementary Material, Fig. S2) and can be induced by BMP4 to differentiate along trophoblast lineages (26). We used a retrovirus-based pSAM2 vector (27, 28) to stably express a short hairpin (sh) RNA (shNLRP7KD) that targets a different NLRP7 region than the siRNA pool used in BeWo cells (Fig. 3A–C). This reduced NLRP7 in hESCs by ≏80% without affecting NLRP2 expression (Fig. 3D). Despite reduced NLRP7 expression, the cells maintained their normal undifferentiated morphology and alkaline phosphatase expression, suggesting that NLRP7 is not necessary for hESC self-renewal or maintenance (Fig. 3D). We also observed no effect on YY1 expression levels (Fig. 2C).

**Figure 3. NLRP7 knockdown in H9 cells.** (A) Genomic region with exons of NLRP7. Position of shRNA sequences is indicated by numbers 1–4; number 1 (in red) was used for knockdown in hES cells. (B) Map of the pSAM2 vector containing the NLRP7 shRNA sequence (shNLRP7). Constitutive expression of the NLRP7 knockdown cassette is controlled by a U6 RNA promoter. The GFP cassette is separated from the shNLRP7 cassette by an IRES sequence and controlled by a doxycycline-inducible promoter (TRE, tetracycline response element; GFP, green fluorescent protein; IRES, internal ribosomal entry site; attB1 and attB2, cloning sites for Gateway cloning; U6, U6 promoter sequence for constitutive shRNA expression). (C) FACS results of hES cells transduced with the pSAM2 vector containing the shNLRP7 (upper panels; shNLRP7KD) and the vector containing the scrambled shRNA (lower panels; shNLRP7SC). C1 and C3 show the absence of GFP-labeled cells without doxycycline induction, and C2 and C4 show the presence of GFP-positive cells in gate P5 after doxycycline induction. GFP-positive cells were triple-sorted for purification. (D) NLRP7 mRNA expression (left graph) is reduced by 80% in shNLRP7KD hESCs that express shNLRP7KD compared with untransduced cells (H9 hESCs) and cells containing the scrambled shRNA (shNLRP7SC) (∗P ≤ 0.05), but NLRP2 expression (right graph) is unaffected. shNLRP7KD hESCs exhibit characteristic pluripotent colony morphology within 24 h of culture and high alkaline phosphatase (blue stain) activity prior to differentiation. (E) qRT-PCR results show significant reduction of POU5F1 mRNA expression to undetectable levels after 7 days of BMP4-induction of differentiation (****P ≤ 0.0001). (F) NLRP7 mRNA expression remains reduced by ≏50% after differentiation in NLRP7KD hESCs (left graph) that express shNLRP7KD compared with cells expressing scrambled shNLRP7SC (shNLRP7SC) (∗P ≤ 0.05), but NLRP2 expression (right graph) remains unaffected. Morphological changes after 24 h of BMP4-induced differentiation: hESCs adopt a more cuboidal shape. Alkaline phosphatase activity (blue stain) disappears after 7 days of differentiation with BMP4 when cells also adopt the larger, flatter more trophoblast-cell-like shape.
Reduced levels of NLRP7 accelerate trophoblast differentiation of hESCs

Adding BMP4 to the hESC cultures induced trophoblast lineage differentiation as evidenced by loss of expression of pluripotency marker POU5F1 (Fig. 3E), morphological changes and loss of alkaline phosphatase activity (Fig. 3F). Differentiated shNLRP7KD cells maintained 50% reduced NLRP7 expression without alterations in NLRP2 expression (Fig. 3F). Expression of trophoblast markers GCM1 and INSL4, but not PAPPE, was significantly increased in shNLRP7KD cells 7 days after the induction of differentiation (Fig. 4A). Furthermore, the amount of human chorionic gonadotropin (hCG) secreted in the media increased by day 5 in all hESC cultures, but levels were significantly higher in shNLRP7KD hESC cultures than in shNLRP7SC or untransduced hESCs (Fig. 4B). These findings are striking, considering that molar pregnancies are characterized by excessive trophoblast proliferation and high hCG production.

Altered methylation of CpG islands in shNLRP7KD hESCs

Although hESCs do not completely recapitulate the epigenetic states of germ cells or embryos and have relatively stable epigenetic marks at imprinted genes, they can yield useful data on molecular mechanisms that underlie chromatin modifications and DNA methylation in undifferentiated and differentiated states (29,30). Considering recent data supporting widespread gDMR methylation reprogramming in mouse female germ cells (31–33), we reasoned that DNA methylation profiling of shNLRP7KD hESCs could uncover a subset of the CpGs that require NLRP7 for normal methylation. We hybridized three replicates each of undifferentiated and differentiated shNLRP7KD, shNLRP7SC and untransduced hESCs to a Human-Methylation450 BeadChip array and analyzed the data using the following filters: ANOVA P-value, mean methylation difference and F-value (indicating signal-to-noise ratio) in three consecutive probes within a single region. As shown in the volcano plot (Fig. 5A) and Supplementary Material, Table S1, we found significantly altered DNA methylation at 864 probes (0.18% of all probes on the array) in shNLRP7KD cells at a signal-to-noise ratio of F > 50, with more hypermethylation (788 probes) than hypomethylation (76 probes), the majority of which are in CpG islands (Supplementary Material, Table S2). There were 234 distinct genes with altered methylation at F > 5 (Supplementary Material, Table S3).

To follow up on the observed interactions between YY1 and NLRP7, we next searched for loci that had altered methylation patterns at YY1-binding sites by first overlapping sequences within 5 kb of probes on the array with those of the 11608 YY1-bound sites identified by ChIP-Seq on hESCs [Gene Expression Omnibus (GEO) ID: GSM803513]. This revealed potential YY1-binding sites for 21242 genes, most significant change in methylation. FBXO4 was of interest because it is a ‘reprogramming DMR’ in human iPSCs resistant to general demethylation during reprogramming (34). ZFP42 is a pluripotency marker specific to the eutherian lineage thought to have arisen through a duplication of the ancestral YY1 gene (35). ZFP42 is highly expressed in undifferentiated ESCs, where it interacts with POU5F1, NANOG and SOX2 (36,37), and loss of its murine ortholog, Rex1, causes hypermethylation in blastocysts at DMRs of maternally imprinted genes, Peg3 and Gnas (38). Hypermethylation of FBXO4 and ZFP42 was associated with decreased expression (Fig. 5E).
Because BiHM pregnancies caused by maternal-effect mutations in NLRP7 show loss of methylation at maternally imprinted gDMRs, we also searched for DNA methylation changes of probes within 5 kb of known imprinted gDMRs upon knockdown of NLRP7 by overlapping them with lists of imprinted genes (www.geneimprint.com and ige.otago.ac.nz), but not surprisingly considering the epigenetic state of hESCs (29,30), we did not see any significant changes in the shNLRP7

DISCUSSION

Two key features of BiHMs, the lack of methylation at maternally imprinted (methylated) gDMRs and the hyperplastic vesicular trophoblast with no normal embryonic development, point toward an important role of NLRP7 in germ cells and early development. In support of this prediction, our data demonstrate that NLRP7 interacts with the important chromatin regulator YY1. We found that its knockdown in hESCs has a widespread impact on DNA methylation and trophoblast differentiation. Differential CpG methylation at gDMRs is the most widely studied imprinting mark, and controls mono-allelic expression of single or clustered imprinted genes. In addition to DNA methylation, histone modifications and DNA-binding proteins have been implicated in the establishment and read-out of imprinting marks. Among the studied DNA-binding proteins, YY1 can bind to imprinted gDMRs in a methylation-dependent manner to help coordinate imprinted gene expression (25). It remains unclear whether NLRP7 participates directly in the binding of YY1 to DNA or can affect YY1’s affinity for DNA through another mechanism.

All members of the NLRP family of proteins were until now considered to be cytoplasmic components of the inflammasome with a role in innate immunity and apoptosis (39,40). Although NLRP7 had not been previously shown to localize to the nucleus (23), we found that NLRP7, when overexpressed, can be found in the nucleus and can interact with YY1 in both the nuclear and cytoplasmic compartments. We speculate that low endogenous protein levels have thus far prevented detecting the presence of a small fraction of the total cellular NLRP7 within the nucleus. Furthermore, the process of germline reprogramming of maternal imprinting marks occurs in the growing oocyte during prophase I of meiosis, which arrests at diplotene stage until the time of ovulation when meiosis resumes and the nuclear envelope disappears (32,41). It is thus possible that persistence of maternally transcribed genes such as NLRP7 and NLRP2 is required to protect imprinting marks at gDMRs once meiotic division progresses, which is consistent with the imprinting disease phenotypes caused by maternal-effect mutations in these genes and with their expression patterns (1–3,21). We speculate that rodents, which have shorter intervals between early embryonic development and reproductive maturity, can achieve this with a single gene (Nlrp2), whereas the longer mammalian intervals between early development and reproductive maturity benefit from the evolutionary duplication event that generated NLRP7 from this single ancestral gene. This is consistent with the recent discovery that maternal-effect mutations in KHDC3L, which is likewise not present in rodents, also cause BiHMs (42).

We examined the function of NLRP7 in hESCs for three important reasons. First, because there is no rodent NLRP7 ortholog, in vivo studies in an animal model are not possible. Second, hESCs are pluripotent cells that can be differentiated into trophoblast lineages (26,43), the primary diseased tissue in BiHMs. Third, although hESCs are derived from the inner cell mass at a stage when imprinted loci have relatively stable epigenetic marks (30,44), and thus do not fully recapitulate the epigenetic states of germ cells and their expression patterns (1–3,21). We speculated that rodents, which have shorter intervals between early embryonic development and reproductive maturity, can achieve this with a single gene (Nlrp2), whereas the longer mammalian intervals between early development and reproductive maturity benefit from the evolutionary duplication event that generated NLRP7 from this single ancestral gene. This is consistent with the recent discovery that maternal-effect mutations in KHDC3L, which is likewise not present in rodents, also cause BiHMs (42).

Another intriguing finding was that DNA methylation changed significantly with the knockdown of NLRP7 at multiple loci that were different from those with altered methylation because of induced differentiation. Although initially surprising, the absence of DNA methylation changes at imprinted gDMRs upon NLRP7 knockdown can be explained by the previously reported high degree of epigenetic stability and resistance to perturbation at imprinted loci in hESC lines (30). Nevertheless, the combined observations that NLRP7 affects methylation at imprinted loci in the human disorder (BiHMs) and at non-imprinted loci in this study are in line with new data in mice.
which indicate that hundreds of germline DMRs become methylated in growing oocytes (31–33), but that the majority are transiently imprinted DMRs—only those that are protected from acquiring methylation on the paternally inherited allele in the early embryo become permanently imprinted DMRs (33). It remains to be seen whether this requires interaction with YY1 and whether downregulation of ZFP42 contributes to this process.

In conclusion, we show that NLRP7, an NLRP protein with high expression in germ cells, has unanticipated important non-inflammasome functions affecting DNA methylation. This has wider implications for the function of other NLRP family members, especially those that are expressed at early developmental stages (24).

MATERIALS AND METHODS

Cell culture

HEK293T cells and BeWo cells were from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C and 5% CO2 to 80% confluence. H9 hESCs were obtained from the Baylor College of Medicine (BCM) Stem Cells and Regenerative Medicine Center (StaR) stem cell core and maintained in mTeSR1 medium (Stemcell Sciences). For differentiation into trophoblast, 100 ng/ml recombinant human BMP4 (R&D Systems, 314BP) was added daily to standardized volumes of fresh culture medium for 7 days starting on day 0. H9 hESCs were obtained from the Baylor College of Medicine (BCM) Stem Cells and Regenerative Medicine Center (StaR) stem cell core and maintained in mTeSR1 medium (Stemcell Technologies) in six-well matrigel-coated plates (BD Biosciences). For differentiation into trophoblast, 100 ng/ml recombinant human BMP4 (R&D Systems, 314BP) was added daily to standardized volumes of fresh culture medium for 7 days starting on day 0. hCG kit from Abbott). Cells were harvested for analysis on day 7.

Transient transfections, co-affinity purification assays

cDNA clones for full-length NLRP7 (transcript variant 3; NM_001127255.1) and tested candidate interactors were obtained from Open Biosystems (Thermo Scientific Molecular Biology). They were first cloned into pDONR223 and then shuttled by Gateway® cloning (Invitrogen) into mammalian expression vectors 5′-pDESTMyc (gift from Dr Marc Vidal, Dana Farber Cancer Institute) and 5′-GST-pDEST27 (Invitrogen). HEK293T cells were transfected with expression vectors using Lipofectamine 2000 (Invitrogen) at baseline and at 24 h. NLRP7-knockdown HEK293T cells were transfected with expression vectors using Lipofectamine 2000 (Invitrogen) and collected after 48 h for co-affinity purification assays. Protein lysates were prepared in lysis buffer containing 20 mM Tris, 180 mM NaCl, 1 mM EDTA, 0.5% NP-40 and protease inhibitor cocktail complete ULTRA Tablet, Mini, EASYpack (Roche Applied Science). Glutathione Sepharose-4B Media (GE Healthcare Life Sciences) was used to perform the GST pull-down as per the manufacturer’s protocol. Mouse anti-Myc (EMD Millipore, 05-724; 1:1000 dilution) and horse anti-mouse secondary antibody (Cell Signaling Technologies, 7076S; 1:5000 dilution) were used for western blotting and detection.

Subcellular fractionation and co-IP assays

Gateway®-cloned V5-NLRP7 and Myc-YY1 were overexpressed for 48 h in HEK293T cells, and protein lysates were prepared as described above. Protein A Dynabeads (Life Technologies, 10001D) were conjugated with 5 μg of anti-NLRP7 (Imgenex, IMG-6357A), anti-V5 (Life Technologies, R960-25), anti-YY1 (Santa Cruz Biotechnology, sc-1703X) or rabbit anti-IgG (Abcam, ab46540). Subcellular fractions were prepared using the Active Motif Nuclear Extract Kit (Cat no. 40010). Following preparation of the cytoplasmic fraction, the nuclear pellet was washed twice in cold 1 × PBS containing protease inhibitors to eliminate residual cytoplasmic protein. An amount of 100 μl of cytoplasmic and nuclear extracts were then added to Dynabeads conjugated with anti-NLRP7 antibody and allowed to immunoprecipitate overnight at 4C. Ten microliters (10%) was reserved as input. Following overnight immunoprecipitation, the beads were washed four times in cold lysis buffer. Protein was eluted from the beads by boiling at 100°C with 50 mM glycine, pH 2.8. The immunoprecipitated protein and inputs were run on a 4–12% Bis-Tris gel and then transferred to a nitrocellulose membrane. The inputs and immunoprecipitated blot was probed with anti-YY1 and anti-NLRP7 at a 1:1000 dilution. Beta-actin (ab8227) and TATA-binding protein (sc-273) served as purity controls for the cytoplasmic and nuclear fractions, respectively. Anti-mouse and anti-rabbit secondary antibodies from Cell Signaling Technologies (7067S and 7074S, respectively; 1:5000 dilution) were used for detection with Western Light- ing™ Ultra chemiluminescence substrate kit (Perkin Elmer, NEL10001EA) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34077).

NLRP7 knockdown

BeWo cells

At ~60% confluence, 20 pmol of Dharmacon siRNA ON-TAR-GETplus SMARTpool NALP7 (old nomenclature for NLRP7) (Thermo Fisher Scientific, L-016880-01-0005), containing a mixture of siRNAs that target exons and the 3′ untranslated region of NLRP7, was double-transfected with Lipofectamine RNAiMax (Invitrogen, 13778-075) at baseline and at 24 h. Cells were collected 24 h after the second transfection.

hES cells

Four shRNA oligos, targeting different regions of NLRP7 (Fig. 3A), and a scrambled oligo (negative control) were first inserted into the BLOCK-it U6 RNAi Entry Vector according to the manufacturer’s protocol (Invitrogen BLOCK-it™ U6 RNAi Entry Vector Kit, K4945-00). The U6 promoter and sh-oligos were subsequently shuttled into the retrovirus-based pSAM2 lentiviral vector, containing a doxycycline-inducible GFP cassette to identify transduced cells. The different constructed pSAM2 vectors were electroporated into H9 hESCs and pulsed with 1 μg/ml of doxycycline for 24 h to induce GFP expression. H9 hESCs expressing rtTA and GFP-positive cells were triple-sorted by fluorescence-activated cell sorting (FACS) after pulsing the cultures with doxycycline to induce GFP expression (Fig. 3C). The hESC line with the most efficient
knockdown was used for further experiments. Sequences of the NLRP7 sh-oligos are NLRP7-KD-F (CACCAGAAGCTGCGCA GAAATTCGTTCAAGACAGAATTTCTGCAGTT TCTT) and NLRP7-KD-R (AAAAAGAAACTGGCGGAG AAATTTCTGTTCTTAGAACAGAATTTCTGCAGTT TCTT) (bold indicates nucleotides targeting NLRP7). The scrambled sh-oligos have the order of six pairs of dinucleotides (bold, underlined and italic) in the targeting regions reversed so as to no longer target NLRP7 while maintaining similar GC content. Sequences are NLRP7-SC-F: CACCAGAAGCTGCGGAG AAAATTTCTGTTCTTAGAACAGAATTTCTGCAGTT TCTT; NLRP7-SC-R: AAAAAGAAACTGGCGGAG AAAATTTCTGTTCTTAGAACAGAATTTCTGCAGTT TCTT.

ChIP assay

Biological triplicates of HEK293T cells with NLRP7 overexpression, BeWo cells with transient NLRP7 knockdown and H9 hESCs with stable NLRP7 knockdown were cross-linked with 37% formaldehyde to a final concentration of 1%. ChIP was then performed using the SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling, 9003S). Micrococcal nuclease-digested chromatin was further sonicated using Diagenode Bioruptor® with 10 pulses of 20 s on and 30 s off at high power to yield sheared chromatin of 150–900 bp. Ten micrograms of chromatin was used per IP with 5 μg of ChIP-grade anti-IgG (Santa Cruz Biotechnology, sc-1703X), ChIP-grade normal rabbit IgG #2729 and Histone H3 (D2B12) XP® Rabbit mAb (ChIP formulated) from Cell Signaling Technology were used as negative and positive controls, respectively. Two percent input was reserved for qPCR quantification. qPCR of YY1-binding sites at PEG3 and SNRPN was performed using the PerfeCTa® SYBR® Green FastMix (Quanta Biosciences) on a StepOnePlus real-time instrument (Applied Biosystems). Primers for PEG3 were 5′-CAAGGAGCGCGCACCTCCAC-3′ and 5′-CATAGGTGTGGGTTGTCCA-3′ and for SNRPN were 5′-CTCTCGTTGGGAAACCGCTTC-3′ and 5′-CATTCTGGAAGGAGTGCAC A-3′. Fold-enrichment was calculated by the percent input method.

DNA methylation profiling

Total genomic DNA was prepared using a Gentra Puregene DNA extraction kit (Qiagen) from three biological replicates of each shNLRP7KD, shNLRP7SC and untransduced H9 hESCs cultures that were undifferentiated or at 7 days post-BMP4-induction. DNA was hybridized to an Infinium HumanMethylation450 BeadChip array (Illumina) using standard protocols. Methylation data were imported into the Illumina Genomic Studio software and converted to a Partek project file (.ppj) with the Partek Methylation Module. This file contained the processed beta-values (quantitative methylation levels for each probe) and was imported into the Partek Genomics Suite software (Partek) using the Illumina Methylation Workflow tool. Data were log-transformed and analyzed using the Partek ANOVA tool to generate probe-level statistics that included P-value (t-test), F-value (signal-to-noise ratio), mean methylation levels and group methylation differences. These values were also determined for genomic regions by using the Partek’s Region Detection tool with following parameters: minimum three consecutive probes with significance of P < 0.01, mean methylation difference of 0.2 (20%) and mean F-value across the region of F = 50. To identify methylation differences that were the result of NLRP7 knockdown in differentiated and undifferentiated hESCs, above statistics were used to compare all six shNLRP7KD data sets (undifferentiated and differentiated combined) as the test group to all other samples (H9 hESCs and shNLRP7SC) as the control group. Significant differences between groups meeting these criteria at the probe level are presented in Supplementary Material, Table S1, and the volcano plot in Figure 5A, and at the gene region level in Supplementary Material, Table S3. We also calculated descriptive statistics for probes with altered methylation in shNLRP7KD vis-à-vis CpG islands, gene bodies and intergenic regions (Supplementary Material, Table S2). Array-data analysis was repeated for differentiated and undifferentiated hESCs separately, but no regions met the above outlined significance criterion for DNA methylation level changes in shNLRP7KD compared with other hESCs (H9 hESCs and shNLRP7SC). We used the Partek Genomics Suite software Genome Viewer Tool for all data visualizations.

Bisulphite sequencing

Bisulphite conversion was carried out on 1 μg of DNA from shNLRP7KD, shNLRP7SC and untransduced undifferentiated and BMP4-differentiated H9 hESCs using the EZ DNA Methylation Kit (Zymo Research, D5001) or as previously described (45). Primers for the amplification of bisulphite-converted DNA were 5′-GAGATTTAGTGGATGAGGGGG-3′ and 5′-CTCGAA AACACTCTTACTGACTAA-3′ for SUCLG2, 5′-GATGTT AGTGAATTAGAGAGAGG-3′ and 5′-AATCCACAAAAAATA CTTCCCT-3′ for FBXO4, 5′-AATTGGYGGGTTGGTGA TCGGTTGTTGAG-3′ and 5′-CTTCCACACTACAC-3′ for ZFP42. Amplicons were cloned into pcDNA3.3 Topo TA vector (Invitrogen, K8300-01) and transformed into One Shot® Mach1™ T1 Phage-Resistant Chemically Competent E. coli (Invitrogen, C8620-03). Ten to 16 clones for each gene were sequenced in forward and reverse orientation at Beckman Coulter Genomics (Danvers, MA, USA). Electropherograms were analyzed using the Sequencher® version 5.1 software (Gene Codes Corporation).

Gene expression analysis by qRT-PCR

One microgram of total RNA was reverse-transcribed into cDNA using the qScript cDNA SuperMix (Quanta Biosciences). qRT-PCR was performed using PerfeCTa® SYBR® Green FastMix on the Applied Biosystems StepOnePlus Real-Time instrument. Analysis was by the ΔΔCt method, with GAPDH as the housekeeping gene. Primer sequences for qRT-PCR are provided in the following table.
Statistical analysis
Unpaired Student’s t-test was used for statistical analysis of RT-PCR results from ChIP and gene expression experiments, with P < 0.05 considered significant. Significance levels for each result are provided in the main text and figures where applicable. Statistical analysis for methylation array data is outlined above.

Accession numbers
The Illumina Infinium HumanMethylation450 BeadChip array data are available from the GEO database under accession number GSE45727.

AUTHORS’ CONTRIBUTIONS
S.M. and S.W. designed and performed most of the experiments and data analysis, prepared figures and wrote initial drafts of the paper. H.-H.P. and S.O. performed initial experiments for NLRP7 interactome identification. B.S. performed the initial methylation array data analysis and presentation. Y.-W.W. and Z.L. analyzed methylation array data for overlap with YY1-binding sites. M.K. designed the shNLRP7 KD vector, generated knockdown ES cell lines and supervised M.I. and E.M.M., who conducted the interpretation, final manuscript and figure preparation. H.-H.P. and S.O. performed initial experiments for NLRP7 interactor identification. B.S. performed the initial methylation array data analysis and presentation. Y.-W.W. and Z.L. analyzed methylation array data for overlap with YY1-binding sites. M.K. designed the pSAM2 shNLRP7 KD vector, generated knockdown ES cell lines and supervised M.I. and E.M.M., who conducted and confirmed the shNLRP7 KD vector. I.B.V. was responsible for project design, oversight of all experiments and interpretation, final manuscript and figure preparation.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
We thank Dr M. Goodell for critical reading of the manuscript and Robert Milczawrek and the BCM Human ESC Core for help initiating the human ESC cell work. We also thank Vicky Brandt for editorial help.

Conflict of Interest statement. None declared.

FUNDING
This work was supported in part by NIH grants R21HD058081, R01HD045970, 5P01GM081627 and by grant 6250-51000-055 from the USDA/ARS to I.B.V. The project was also supported by the BCM IDDRC grant number 5P30HD024064 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development and grant number C06RR029965 from the National Center for Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Eunice Kennedy Shriver National Institute of Child Health and Human Development or the National Institutes of Health.

REFERENCES

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5′-CTCCCCGTTGGCCTCTG-3′</td>
<td>5′-AGTTAAAAAGCAGCCTG-3′</td>
</tr>
<tr>
<td>NLRP7</td>
<td>5′-GCCCAAGTTGATGTGGTTGAA-3′</td>
<td>5′-TTCTTCTTCTACCTCTGCAA-3′</td>
</tr>
<tr>
<td>NLRP2</td>
<td>5′-CCCTGACCTCTCCT-3′</td>
<td>5′-CCAGACCTTTATTGAGTCT-3′</td>
</tr>
<tr>
<td>POU5F1</td>
<td>5′-GCGGTACAGAACCACACT-3′</td>
<td>5′-AGTGAGAGGCAACACCCTGGGA-3′</td>
</tr>
<tr>
<td>PPAP1</td>
<td>5′-CCAAGCCCAAGAGATCAATAC-3′</td>
<td>5′-TCTGGGAGCTTTGCTGGTT-3′</td>
</tr>
<tr>
<td>GMC1</td>
<td>5′-TTGAGGGGAGTCAAAGTG-3′</td>
<td>5′-TAGAAGATCGGACACTCTG-3′</td>
</tr>
<tr>
<td>FBXO4</td>
<td>5′-GCATTTTTTGGACTACATGGCA-3′</td>
<td>5′-CGGCTGAAATTGGAAGCT-3′</td>
</tr>
<tr>
<td>INSL4</td>
<td>5′-GCCATTGCTGAGAAGACATT-3′</td>
<td>5′-GGTTGTTGAGGTTGACACCA-3′</td>
</tr>
<tr>
<td>ZFP42</td>
<td>5′-GAGGCTGGACCTGTGGT-3′</td>
<td>5′-TGTCGCCCTCTTGAAAGGTTT-3′</td>
</tr>
<tr>
<td>SUCGL2</td>
<td>5′-TGATGTCTGACAAAGGGAGTA-3′</td>
<td>5′-GCTGCCCAACAACTTAGGG-3′</td>
</tr>
</tbody>
</table>


