Supplementary Information

Pharmacologic inhibition of the menin-MLL interaction leads to transcriptional repression of *PEG10* and blocks hepatocellular carcinoma

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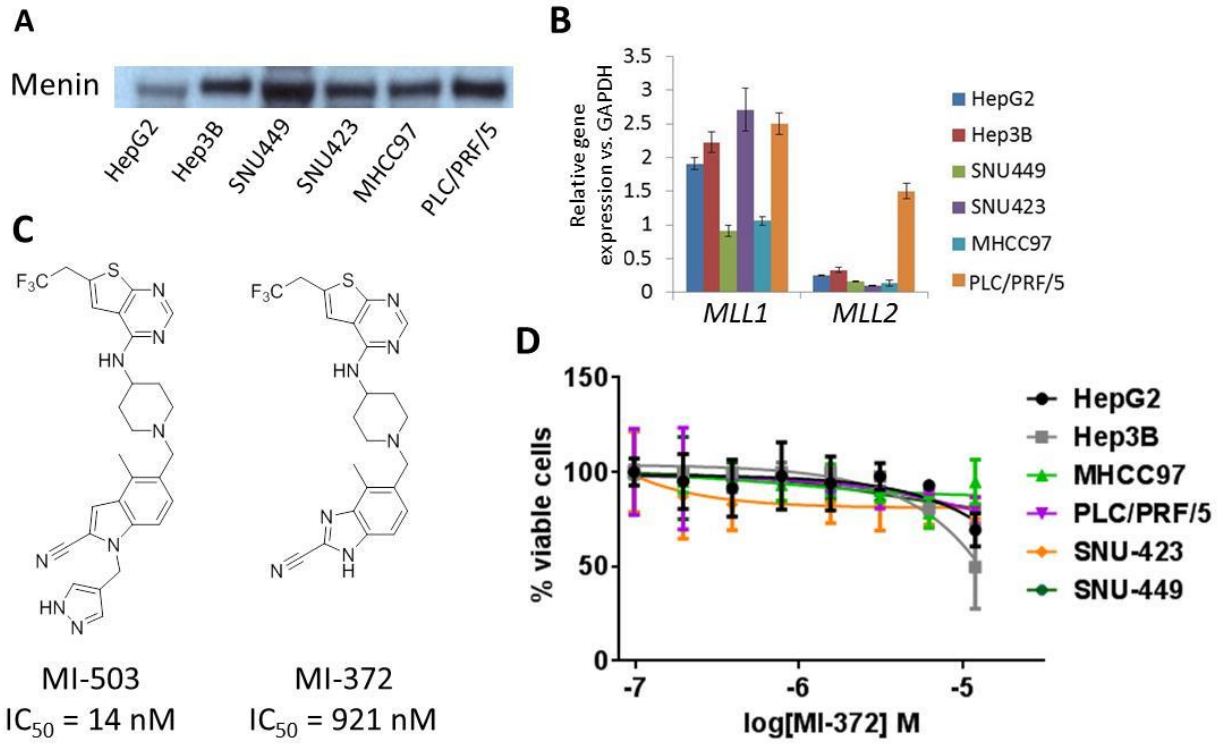
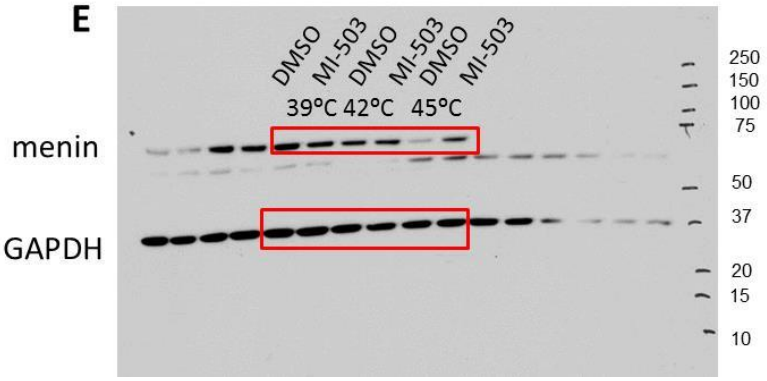
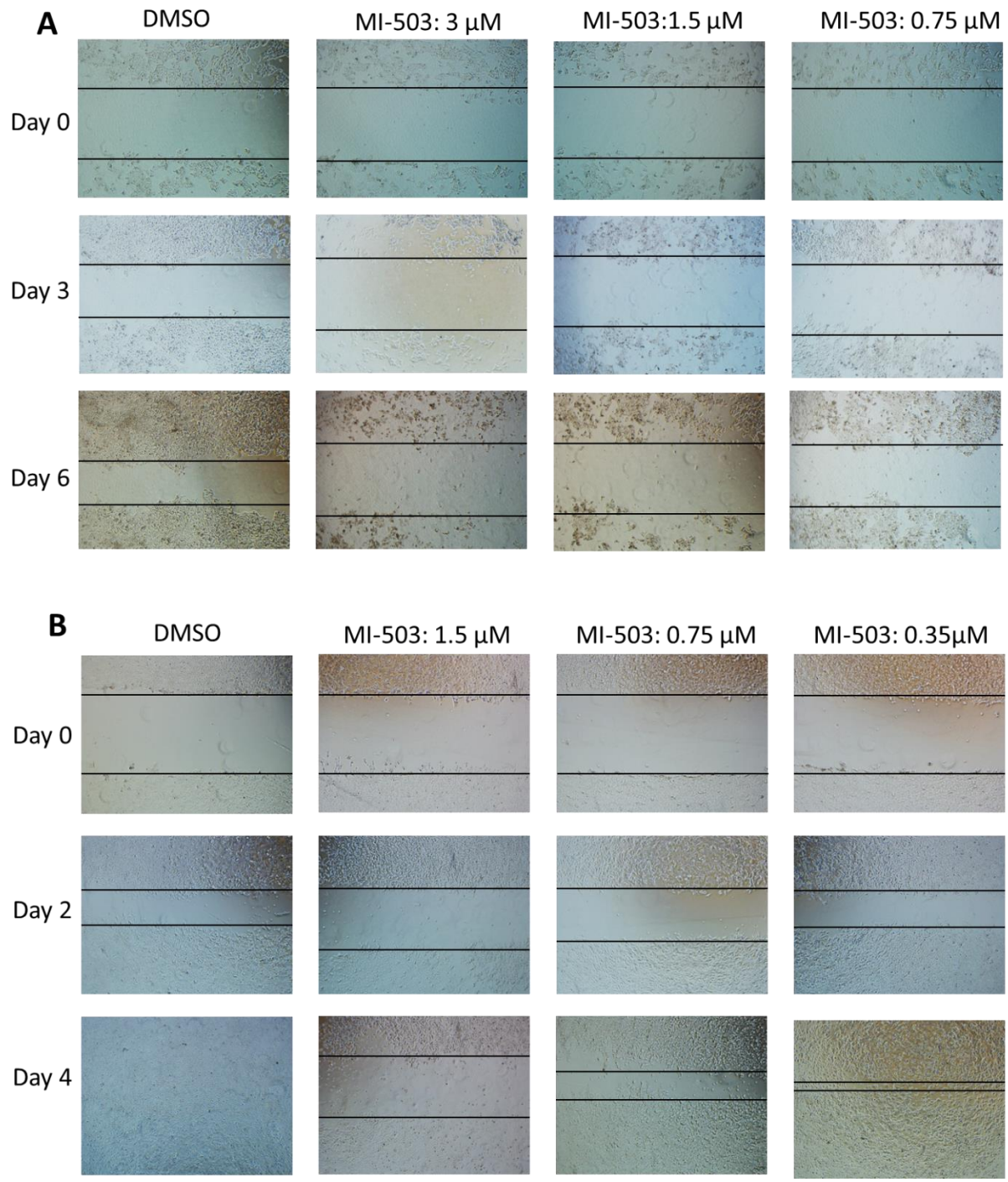
Supplementary Figures

Supplementary Figure 1. Expression of menin, *MLL1* and *MLL2* in HCC cell lines. A. Detection of menin expression in HCC cells by Western Blot analysis. Whole cell lysate from various HCC cell lines were immunoprecipitated against menin antibody (Bethyl). **B.** Quantitative RT-PCR analysis of *MLL1* and *MLL2* expression performed in a panel of HCC cell lines. Expression of *MLL1* and *MLL2* was normalized relative to GAPDH expression. qRT-PCR was performed using SYBR Green primers for *MLL1* and *MLL2* (see **Supplementary Methods** for sequences). C. Chemical structures and activity of MI-503 and MI-372 (negative control compound) for inhibition of the menin-MLL interaction (IC_{50} values were reported before by Borkin et al [1]). **D**. Results from the MTT cell viability assay after 7 days of treatment of HCC cell lines with MI-372 (negative control compound; GI_{50} > 12 μM in all cell lines). Data represents mean of quadruplicates ± SD. **E**. Uncropped scans of western blot for the Cellular Thermal Shift Assay (CETSA) performed in HepG2 cells. Molecular weight ladder is shown. Red boxes indicate the fragments of SDS-PAGE gel that is presented in Fig. 1E.

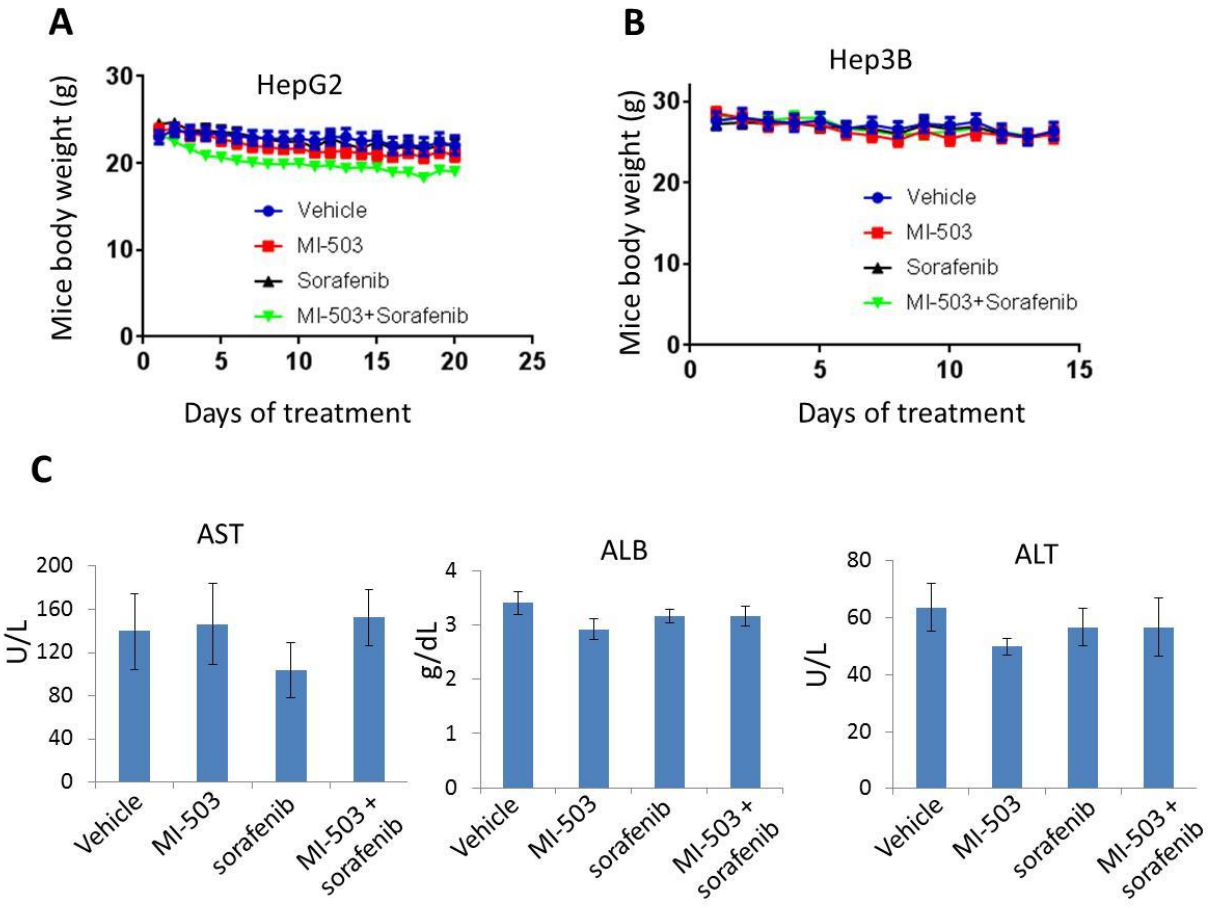

A
Menin
HepG2
Hep3B
SNU449
SNU423
MHCC97
PLC/PRF/5
B
Relative gene expression vs. GAPDH
3.5
3
2.5
2
1.5
1
0.5
0
MLL1
MLL2
HepG2
Hep3B
SNU449
SNU423
MHCC97
PLC/PRF/5
C
MI-503
IC50 = 14 nM
MI-372
IC50 = 921 nM
D
% viable cells
150
100
50
0
-7
-6
-5
log[MI-372] M
HepG2
Hep3B
MHCC97
PLC/PRF/5
SNU-423
SNU-449


E
DMSO
MI-503
DMSO
MI-503
DMSO
MI-503
39°C 42°C 45°C
menin
GAPDH
250
150
100
75
50
37
20
15
10

Supplementary Figure 2. MI-503 affects cell migration in HCC cell lines. A, **B**. Pictures demonstrating cell migration in HepG2 (**A**) or Hep3B (**B**) cells upon treatment with various concentrations of MI-503 or DMSO.



Supplementary Figure 3. MI-503 does not induce toxicity in mice. A, B. Body weight of mice treated with vehicle, MI-503, sorafenib and combination of both compounds in HepG2 (**A**) and Hep3B (**B**) xenograft models. n = 8-9 mice per group. C. Level of liver enzymes: AST (aspartate aminotransferase) and ALT (alanine transaminase) as well as ALB (albumin) in mice blood plasma upon treatment with vehicle, MI-503, sorafenib or combination of both agents in the blood samples isolated from HepG2 xenograft mice. n = 5 mice per group.



Supplementary Table 1. Inhibition of cell viability in HCC cell lines by menin-MLL inhibitors. GI_{50} values measured in the MTT cell viability assay after 7 or 12 days of treatment of the HCC cell lines or normal adipose-derived mesenchymal stem cells (ASC52, control cell line) with MI-503 or MI-372 are provided. ND – not determined.

Cell line	MI-503 7d (µM)	MI-503 12d (µM)	MI-372 7d (µM)	MI-372 12d (µM)
HepG2	**3.1**	**1.2**	**> 12**	**ND**
Hep3B	**1.3**	**0.56**	**12**	**ND**
SNU-423	**6.5**	**0.68**	**>12**	**>12**
MHCC97	**1.4**	**1.1**	**>12**	**ND**
SNU-449	**3.1**	**2.4**	**>12**	**>12**
PLC/PRF/5	**4.1**	**3.2**	**>12**	**>12**
ASC52	**>6**	**>6**	**ND**	**ND**

Supplementary Table 2. RNAseq data from MI-503 treatment in HepG2 cells. Results from global gene expression studies obtained from the RNAseq experiment performed in HepG2 cells upon 6 days of treatment with 3 μM MI-503 or DMSO. Supplementary Table 2 is provided as a separate Excel file.

Supplementary Table 3. Mutation status of *CTNNB1* in human HCC cell lines. Mutations or deletions in *CTNNB1* gene were found in HepG2 and SNU-449 cell lines. Other HCC cell lines harbor wild type *CTNNB1*.

Cell line	***CTNNB1* mutation status**
HepG2	deletion in exon-3 in *CTNNB1*[2]
Hep3B	wild type *CTNNB* [3]
SNU-423	wild type *CTNNB* [4]
SNU-449	point mutation in exon-3 of *CTNNB1*[2]
PLC/PRF/5	wild type *CTNNB* [5]

Supplementary Methods

Treatment with chemical compounds: Doses of compounds used in the cell-based studies were adjusted based on: (i) sensitivity of a specific cell line to these compounds; (ii) time course of the experiments; (iii) type of the experiment (e.g. to obtain sufficient amount of the material for analysis, if applicable).

Cell viability assay: Cells were plated in 24-well plates at a concentration of $3x10^4$ cells/ml and treated with the compounds or 0.25% DMSO followed by incubation at 37^0C for 7, 8 or 12 days. After 3 days of treatment cells in all wells were trypsinized and the DMSO treated cells were counted. The cell number was then adjusted to the original concentration in the DMSO treated samples and the same dilution factor was used to adjust cell number in the MI-503 treated cells. Compounds were re-supplied each time during media change. The same procedure was repeated at day 7 (or 8) for 12 days experiments. Before read-out, 100 µl of cell suspensions were transferred to the 96-well plates for each sample in quadruplicates. The MTT cell proliferation assay kit (Roche) was then applied. Plates were read for absorbance at 570 nm using a PHERAstar BMG microplate reader. The experiments were performed two to three times in quadruplicated with calculation of mean and standard deviation for each condition.

CETSA assay: Briefly, 5 x 10^6 HepG2 cells in EMEM medium with 10% FBS and 1% Pen Strep were seeded in T-25 cell culture flasks (BD Biosciences, San Jose, CA, USA) in appropriate volume and incubated overnight (37°C, 5% CO_2). On the following morning cells were exposed with either DMSO or 2 µM MI-503 for 3 hours at 37°C, 5% CO_2. Following the incubation the cells were harvested and washed with PBS. Cells were resuspended in PBS and equal amounts of cell suspensions were aliquoted into a 0.2 mL tubes. These 0.2 mL tubes were heated individually at different temperatures for 3 min followed by cooling for 3 min at room

temperature. Cells were lysed by addition of the lysis buffer (30 µL) and using 3 cycles of the freeze-thawing with liquid nitrogen. The lysates were centrifuged at 20,000 x g for 20 minutes at 4°C and supernatants were transferred to new tubes and analyzed by the SDS-PAGE followed by western blot analysis of menin using the anti-menin antibody (Bethyl) and GAPDH (Cell Signaling Technology) used as a loading control.

Real-time qPCR: Cells were plated at a concentration of $0.25x10^6$ cells/ml in the 12-well plate in duplicates and treated with MI-503 or DMSO for 6 and/or 13 days. After 3 days of treatment cells in all wells were trypsinized and the DMSO treated cells were counted. The cell number was then adjusted to the original concentration ($0.25x10^6$ cells/ml) in all samples. Media was changed and compound was re-supplied at that time. For the experiment with 13 days of treatment, media change was also performed at day 10 using the same procedure. 1µg RNA was then reverse transcribed to cDNA using HICAP RT kit from Applied Biosystems. Following cDNA synthesis, quantitative PCR was performed using SYBR Green Supermix with ROX (BioRad) using the primers for *YAP1, PEG10, SLC38A4, SEMA3C* and *GDF15* (see Supplementary Info for primer sequences). Similarly, RNA was isolated from the tumor tissues and cDNA was prepared and analyzed for expression of target genes. For the detection of MLL1 and MLL2, cells ($0.25x10^6$ cells/ml) were harvested from the HCC cell lines. RNA was isolated and reverse transcribed into cDNA. qPCR was performed using SYBR Green *MLL1* and *MLL2* primers (see Supplementary Information for primer sequences).

Sphere formation assay. HepG2 or Hep3B cells were plated at a density of 500 cells in 24-well ultra-low attachment plates (Corning) in X-VIVO 20 medium (Lonza) supplemented with 5 µg/mL insulin (Sigma-Aldrich) and 20 ng/mL human recombinant epidermal growth factor (Invitrogen).

Cells were treated with MI-503 or DMSO and incubated at 37 °C with 5 % CO_2 for 2 weeks. The number of spheres with a diameter larger than 50 μm was counted after 2 weeks.

Cell migration assay. To analyze the wound healing capacity, we used an *in vitro* model of wound healing (CytoSelect™ 24-Well Wound Healing Assay) from Cell Biolabs (BIOCAT GmbH, Heidelberg, Germany). The CytoSelect™ 24-well Wound Healing Assay Kit consists of 2 × 24-well plates each containing 12 proprietary treated plastic inserts. The inserts create a wound field with a defined gap of 0.9 mm for measuring the migration and proliferation rate of cells. Hep3B and HepG2 cells suspension containing 1×10^5 cell/mL in Eagles minimum essential medium (EMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen Strep, Gibco) antibiotics were prepared. For optimal cell dispersion, 250 µL of cell suspension was added on each side of the insert by carefully inserting the pipette tip through the open end of the insert (0.5×10^5/well/500 µl). Cells were cultured until they formed a monolayer around the insert (48 h). Then, the inserts were removed from the wells, leaving a precise 0.9 mm open "wound field" between the cells. To assess how the "wound field" is affected by the cells itself, the wells were washed twice with PBS and then filled with 500 µL of prepared medium with compound or DMSO. To analyze migration capacity the first photographs of the wounded area were taken at days 0, 2 and 4 for Hep3B and after 3 and 6 days for HepG2 cells. Surface wound area was then set on the photographs and cell free region was measured at all time points.

Menin expression: The whole cell lysate was prepared from all the HCC cell lines (3-4 mln cells per cell line) and run onto the SDS-PAGE gel. Menin protein expression was detected using the anti-Menin antibody (Bethyl).

Primers for qRT-PCR (SYBR Green):

MLL1

Forward: GTGCTTTGTGGTCAGCGGAAGT

Reverse: TGTGAGACAGCAACCCACGGTG

MLL2

Forward: AGAATCTGCGTTCGGCTGGTTC

Reverse: TCTGGTTCCTGCTGTCTCCACT

SLC38A4

Forward: TGTATGCCACCCTGAGGTCCTT

Reverse: GCAGGTACATGACAAGCATCCC

GDF15:

Forward: CAACCAGAGCTGGGAAGATTCG

Reverse: CCCGAGAGATACGCAGGTGCA

SEMA3C:

Forward: ACCCACTGACTCAATGCAGAGG

Reverse: CAGCCACTTGATAGATGCCTGC

PEG10:

Forward: ACCACCAGGTAGATCCAACCGA

Reverse: TGTCAGCGTAGTGACCTCCTGT

GAPDH

Forward: GTCTCCTCTGACTTCAACAGCG

Reverse: ACCACCCTGTTGCTGTAGCCAA

Primers for Chromatin Immunoprecipitation

Primers for PEG10 Promoter and Downstream Regions	**Genomic coordinates**
PEG10 P-A forward: 5'-CAACAGATTGTCAGTTTCCCAAGC-3'	chr7:94,654,908-94,654,931
PEG10 P-A reverse: 5'-CGATTACGAGGTTTACACAGAGACC-3'	chr7:94,655,191-94,655,215
PEG10 P-B forward: 5'-CTCTGTTTTCTTGGAGCAGGACC-3'	chr7:94,657,370-94,657,392
PEG10 P-B reverse: 5'-ACCACAACTACACCGCCACTTC-3'	chr7:94,657,617-94,657,638
PEG10 P-C forward: 5'-CTCCCTAAGCCTGCCTCTGC-3'	chr7:94,658,482-94,658,501
PEG10 P-C reverse: 5'-GGGCTCAGGCAAGGAAGGTG-3'	chr7:94,658,603-94,658,622
PEG10 P-D forward: 5'-TAACTTGTGCTGCCTCAGTCGC-3'	chr7:94,660,520-94,660,541
PEG10 P-D reverse: 5'-CCTGCCACCAGACATTTCATTC-3'	chr7:94,660,783-94,660,804

Supplementary References

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