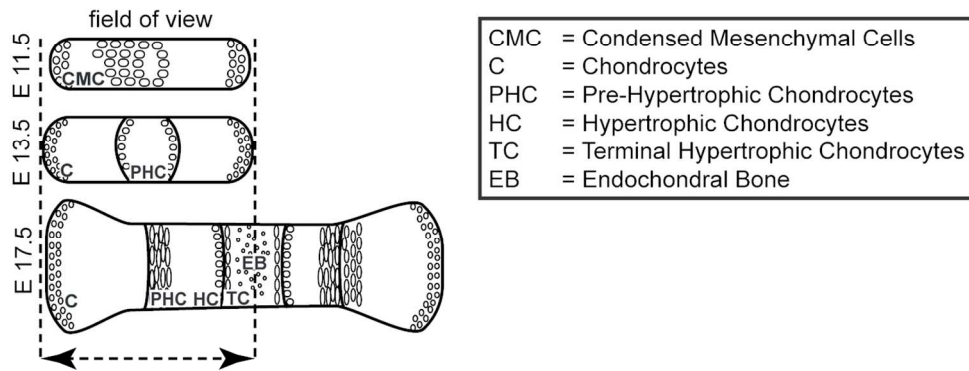


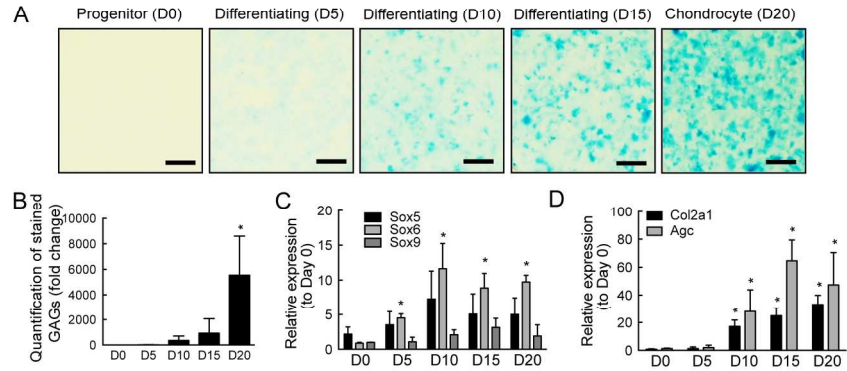
## Supplemental Figure 1



Supplemental Figure 1, related to Figure 1. Diagrammatic representation of embryonic growth plate development in the limb highlighting chondrocyte condensation, maturation and hypertrophy. The field of view for the images shown in B is outlined.

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Supplemental Figure 2



Supplemental Figure 2, related to Figure 2. Chondroprogenitor differentiation in ATDC5 in vitro.

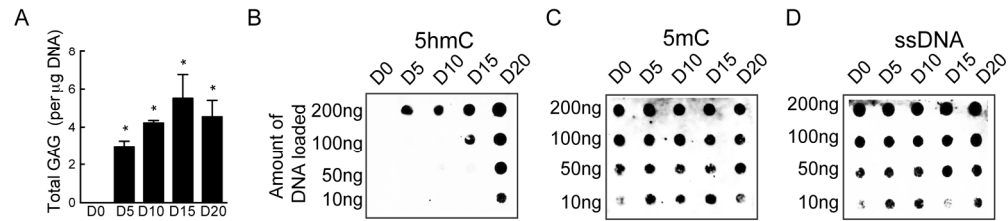
A. Glycosaminoglycan (GAG) staining of cells with Alcian blue over the course of chondrogenic differentiation from the progenitor cells (D0 = day 0) to mature, GAG secreting chondrocytes (D20 = day 20). Scale bar = 5mm.

B. Quantification of the relative GAG production by chondrocytes undergoing differentiation as represented in A. Data represented as mean  $\pm$  SD from three independent biological replicates (n = 3). \* denotes p < 0.05 compared to the control group at day 0.

C. Gene expression analysis for the chondrogenic transcription factors *Sox5*, *Sox6* and *Sox9* during chondrogenic differentiation. Expression was normalized to the housekeeping gene *Gapdh* and values presented are fold changes relative to day 0. Data represented as mean  $\pm$  SD from three independent biological replicates (n = 3). \* denotes p < 0.05 compared to the control group at day 0.

D. Gene expression analysis for the chondrocyte expressed extracellular matrix genes, type 2 collagen (*Col2a1*) and Aggrecan (*Acan*) during chondrogenic differentiation. Data represented as mean  $\pm$  SD from three independent biological replicates (n = 3). \* denotes p < 0.05 compared to the control group at day 0.

Supplemental Figure 3



Supplemental Figure 3, related to Figure 2. Global 5hmC levels.

A. Total glycosaminoglycan content (normalized to total cellular DNA) quantified with the DMMB assay over the course of chondrogenic differentiation. Data are mean  $\pm$ SD (n = 3). \* denotes  $p < 0.05$  compared to the control group at day 0.

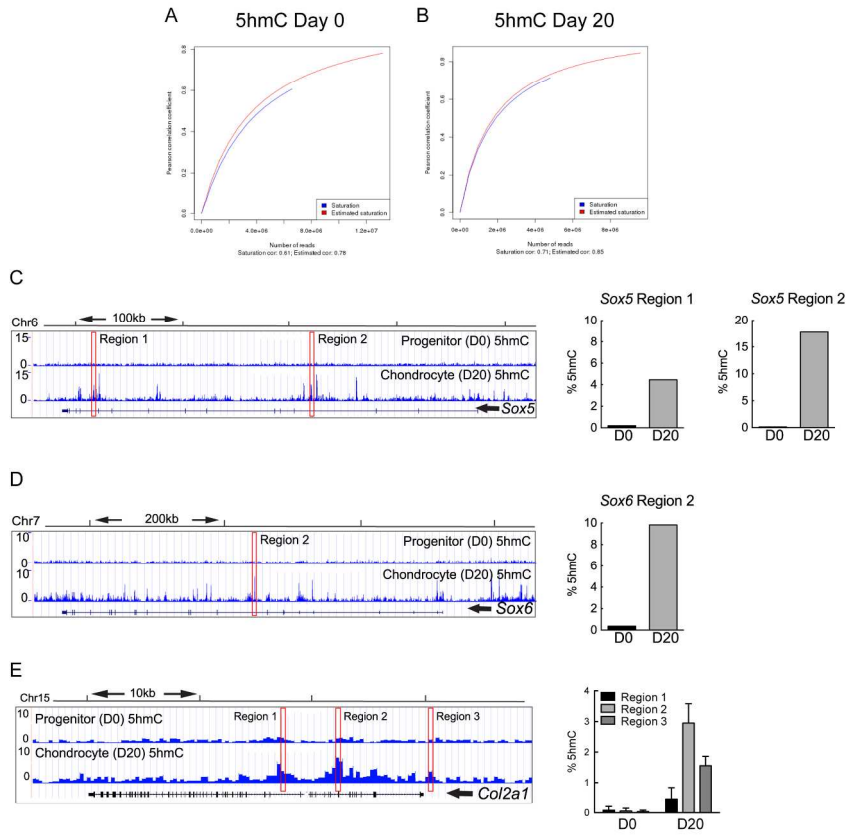
B - D. Levels of the modified cytosines 5hmC (B), 5mC (C) and loading control single stranded DNA (D) over the course of chondrogenesis in ATDC5 cells, assessed by dot blot using serial dilutions of DNA.

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Supplemental Figure 4



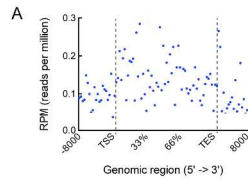
Supplemental Figure 4, related to Figure 4. Quality control and validation of sequencing data.

A and B. Saturation plots for the unique BWA aligned reads showed high correlation and good agreement between the estimated and actual saturation curves for all samples, indicating reproducibility of the data and adequate sequencing depth. Representative samples for 5hmC sequencing are shown. For total numbers of reads and details of PCC calculation see Supplemental Methods.

C - E. Validation of 5hmC peaks identified by hMe-Seal in *Sox5*, *Sox6* and *Col2a1* genes.

Snapshots of the 5hmC profile in the progenitor (D0) and mature chondrocytes (D20) in the genomic region encompassing the chondrogenic genes *Sox5*, *Sox6* and *Col2a1* are shown. The highlighted peaks were used for the validation shown alongside. Validation was performed using a restriction digest based approach (see Materials and Methods).

Supplemental Figure 5



B

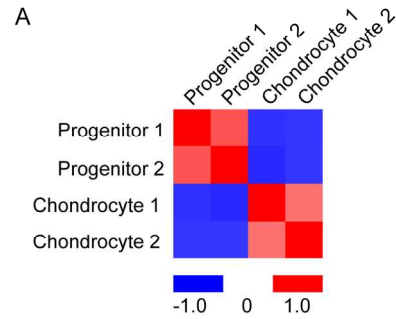
Top 25% of genes with a 10-fold or greater loss in 5hmC during chondrogenesis					
1110032F04Rik	Cdh23	Fbxw4	Mag2	Ppp2r2b	Tnfrsf1
2210408I2TRik	Cdk5rap2	Filip1l	Medf3l	Pvt1	Tmem170
2700054A10Rik	Ckai1	Gm13152	Micu1	Rsm2	Traf2
4930525F21Rik	Chf1	Gm4981	Mir101c	Rims2	Tug1
5031425F14Rik	Crebzf1	Izumo1	Myc	Rh45s	Ube2o
A530032D18Rik	Csda	Kcnc1	Nmi1	Rnz1b	Ubp3
Acacb	Csf2ra	Kctd16	Oas1	Sgpl1	Vwf
Aida	Csnk1d	Kpna1	Oxr1	Sfr1	Wdr27
Ank1	Deaf1	Lars2	Pdzrn3	Sp110	Zfp148
Anoz	Dnm3	Lipc	Phlpp2	Spag17	Zfp263
Apoc1	E030030I06Rik	Litar	Pisd-ps1	Stat2	Zfp600
Auis2	Eef2	Ltbp1	Pisd-ps2	Syn3	Zfp790
Bzw2	Eif4emif1	Mag2	Pice1	Syt12	Zfp821
Ccdc162	Etnk1	Mall1	Pfxdc2	Tet1	
Cdh13	Fan1	Mamdc2	Fplia2	Tiam2	

Supplemental Figure 5, related to Figure 5. 5hmC distribution during chondrogenic differentiation.

A. Composite profile of the 5hmC pattern in top 25% of the subset of genes with a ten-fold or greater 5hmC loss in the differentiated chondrocytes as compared to progenitor cells.

B. The 88 genes which fall into the top 25% of the subset of genes with a ten-fold or greater 5hmC loss in the differentiated chondrocytes as compared to progenitor cells are listed.

Supplemental Figure 6



B

Sample	Progenitor 1	Progenitor 2	Chondrocyte 1	Chondrocyte 2
Progenitor 1	1	0.67	-0.81	-0.79
Progenitor 2	0.67	1	-0.84	-0.79
Chondrocyte 1	-0.81	-0.84	1	0.56
Chondrocyte 2	-0.79	-0.79	0.56	1

Supplemental Figure 6, related to Figure 6. Correlation of clustered genes in replicate array samples for the microarray.

A. A heatmap depicting the r-values for a representative probe set in the samples examined. The representative probe set was extracted based on consistent intensity in at least two arrays and a minimum intensity of 150.

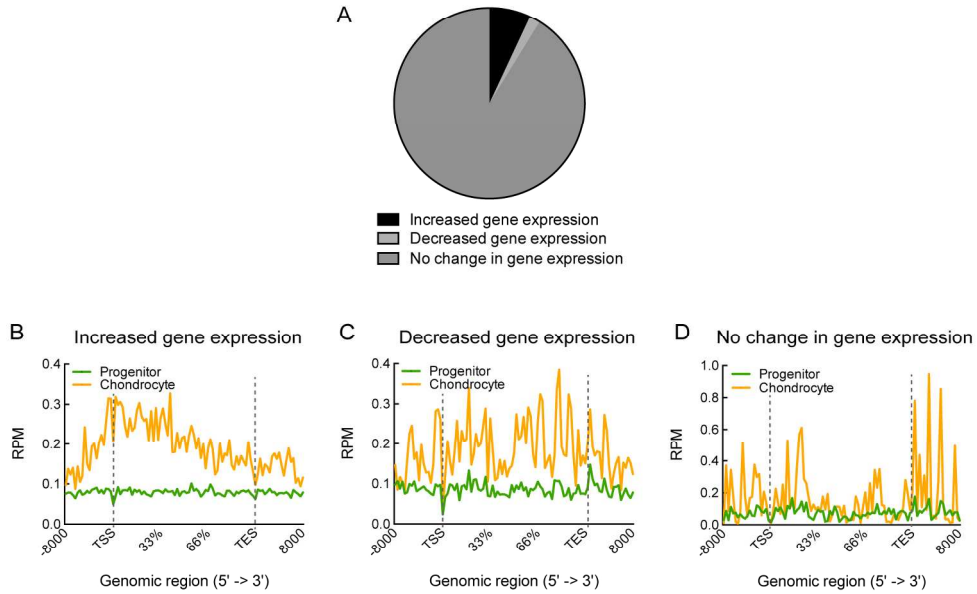
B. Absolute r-values used to produce the heatmap for the sample correlation from the representative probe set in A.

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A. A heatmap depicting the r-values for a representative probe set in the samples examined. The representative probe set was extracted based on consistent intensity in at least two arrays and a minimum intensity of 150.

B. Absolute r-values used to produce the heatmap for the sample correlation from the representative probe set in A.

Supplemental Figure 7



Supplemental Figure 7, related to Figure 6. Analysis of the gene expression of the top 25% of genes associated with 10x or greater increases in 5hmC in the chondrocyte (day 20) when compared to the progenitor (day 0).

A. Changing gene expression over the course of chondrogenesis in the top 25% of genes with a ten-fold increase in 5hmC. Total number of genes which there is gene expression data for = 3276, number of genes with; increased gene expression = 274 (8%), decreased gene expression = 66 (2%) and no change = 2936 (90%).

B. Genomic distribution of 5hmC in the subset of genes with increased gene expression.

C. Genomic distribution of 5hmC in the subset of genes with decreased gene expression.

D. Genomic distribution of 5hmC in the subset of genes with no change in gene expression.

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