Supplemental Information for: "FGF maintains chondrogenic potential of limb bud mesenchymal cells by modulating DNMT3A recruitment"

by Deepak Kumar and Andrew B. Lassar

Supplemental Figures Legends

Supplemental Figure 1. (A) Wnt3A induces the expression of Twist2 in LBMCs, both in the absence or presence of FGF2 (relevant to Figures 1 and 2). LBMCs were isolated from day 4 chicken embryos (i.e., Hamburger-Hamilton stages 22-24) and cultured in either control medium or in medium containing either Wnt3A alone, or Wnt3A plus FGF2, as indicated. Cells were harvested after 0 or 4 days of culture as indicated, and gene expression was assayed by RT-qPCR and normalized to GAPDH levels. Significance was calculated using Student's *t*-test, and relative levels of Twist2 in lanes 2-4 were compared to that in lane 1. **P*<0.05, ***P*<0.01, ****P*<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean. **(B) Transient Wnt signals irreversibly block chondrogenesis only in the absence of FGF signals (relevant to Figures 1 and 2).** LBMCs were isolated from day 4 chicken embryos and cultured in medium containing either Wnt3A, FGF2, and the MEK1/2 antagonist, U0126 for the days indicated. Cells were harvested after 4 or 8 days of culture and chondrogenesis was assayed by alcian blue staining.

Supplemental Figure 2. (A-C) DNA replication is not necessary for FGF signals to induce reversible gene silencing (relevant to Figure 2). (A) Time course of experimental protocol. **(B, C)** LBMCs were isolated from day 4 chicken embryos and

cultured in medium containing either Wnt3A alone, or Wnt3A plus FGF2 for days 0-4. Subsequently, the explants were cultured in control medium in either the absence or presence of aphidicolin for days 5-8. (B) Cells were harvested after either 0 or 8 days of culture (as indicated) and gene expression was assayed by RT-qPCR and normalized to GAPDH levels. Significance was calculated using Student's *t*-test; lanes 4 and 6 were compared to lane 2, lanes 5 and 7 were compared to lane 3. **P*<0.05, ***P*<0.01, ****P*<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean. (C) Cells were administered 10μM BrdU at day 7, and harvested at day 8 to determine amount of BrdU incorporation relative to that in explants cultured in control medium in the absence of aphidicolin for days 5-8. Significance was calculated using Student's *t*-test; lanes 2, 4 and 6 were compared to lanes 1, 3 and 5, respectively. **P*<0.05, ***P*<0.01, ****P*<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean. **(D) Transient Wnt signals stably repress H3K9Ac modification of the Sox9 promoter only in the absence of FGF signals (relevant to Figure 2).** LBMCs were isolated from day 4 chicken embryos and cultured in either control medium or in medium containing either Wnt3A alone, or Wnt3A plus FGF2, as indicated. Cells were harvested after 4 or 8 days of culture and chromatin IP (ChIP) was performed to assay H3K9Ac modification over the Sox9 promoter (normalized to input DNA). Isotype control (IC) IgG IPs are also displayed. Significance was calculated using Student's *t*-test, and relative enrichment of H3K9Ac in b was compared to a; c was compared to b; e was compared to d; and f was compared to e. **P*<0.05, ***P*<0.01, ****P*<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean.

Supplemental Figure 3. (A) Transient Wnt signals induce CpG methylation of the second Sox9 CpG island only in the absence of FGF signals, as assayed by bisulfite sequencing (relevant to Figure 2). Bisulfite sequencing of the second Sox9 CpG island (CpG2) in genomic DNA isolated from LBMCs that had been cultured for 4 days in either control medium (blue), medium containing soluble Wnt3A (red), or medium containing both Wnt3A and FGF2 (green). % methylation of CpG residues (identified in Supplemental Table 1) in the second Sox9 CpG island (CpG2) is displayed. Below the graph is a diagram of the exon/intron structure of the Sox9 gene; coding region is highlighted in black. **(B-C) Levels of FGF2 that are sufficient to block Wnt-induced DNA methylation of the Sox9 promoter are necessary to maintain the competence to express Sox9 following transient exposure to Wnt signals (relevant to Figure 2).** **(B)** LBMCs were cultured in medium containing Wnt3A in either the absence or presence of increasing levels of FGF2 as indicated. Cells were harvested after 4 days of culture and methyl-DNA IP (MeDIP) performed to assay methyl CpG modification in the second Sox9 CpG island (CpG2). MeDIP was normalized to input DNA. Significance was calculated using Student's *t*-test; lane 2 was compared to lane 1, lanes 3-6 were compared to lane 2. **P*<0.05, ***P*<0.01, ****P*<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean. **(C)** LBMCs were cultured 4 days in medium containing Wnt3A in either the absence or presence of increasing levels of FGF2 as indicated. Explants were subsequently cultured in control medium for days 5-8. Cells were harvested after 8 days of culture and gene expression was assayed by RT-qPCR and normalized to GAPDH levels. Significance was calculated using Student's *t*-test; lane 3 was

compared to lane 2, lanes 4-7 were compared to lane 3. *P<0.05, **P<0.01, ***P<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean.

Supplemental Figure 4. Wnt3A and/or FGF2 signals do not affect the expression levels of either exogenous or endogenous DNMT3A (relevant to Figure 3). LBMCs were infected with a retrovirus encoding either Flag/HA-hDNMT1, Flag/HA-hDNMT3A, or Flag/HA-hDNMT3B, and cultured for 4 days in either control medium or medium containing Wnt3A, in either the absence or presence of FGF2, as indicated. Cells were harvested after 4 days in culture and **(A)** gene expression was assayed by RT-qPCR (relative to GAPDH levels). Significance was calculated using Student's *t*-test. *P<0.05, **P<0.01, ***P<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean. **(B-C)** Western analysis was performed to assay expression of either exogenous Flag/HA-hDNMT3A (in infected cultures; B) or endogenous DNMT3A (in non-infected cultures; C).

Supplemental Figure 5. (A) FGF2 and FGF8, but not FGF10, block Wnt3A-induced recruitment of endogenous DNMT3A to the Sox9 promoter (relevant to Figure 3). LBMCs were cultured for 4 days in either control medium or medium containing Wnt3A, in either the absence or presence of FGF2, FGF4, or FGF8, as indicated. Cells were harvested after 4 days in culture and chromatin IP performed to assay the association of endogenous DNMT3A with the Sox9 promoter. ChIP was normalized to input DNA. Isotype control (IC) IgG IPs are also displayed. Significance

was calculated using Student's *t*-test. Relative enrichment of DNMT3A bound to Sox9 in b was compared to a; c-e were compared to b. *P<0.05, **P<0.01, ***P<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean. **(B) Wnt3A recruits endogenous DNMT3A to the Sox9 promoter, but not to other regions of the genome (relevant to Figure 3).** LBMCs were cultured for 4 days in either control medium or medium containing Wnt3A, in either the absence or presence of FGF2, as indicated. Cells were harvested after 4 days in culture and chromatin IP performed to assay the association of endogenous DNMT3A with either the Sox9 promoter, sequences -350 kb upstream of the Sox9 promoter, or the TATA Binding Protein (TBP) promoter. ChIP was normalized to input DNA. Isotype control (IC) IgG IPs are also displayed. Significance was calculated using Student's *t*-test. Relative enrichment of DNMT3A bound DNA in b and c was compared to a. *P<0.05, **P<0.01, ***P<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean. **(C) Administration of 5-Aza-2'-deoxycytidine blocks Wnt3A induced DNA methylation of the Sox9 promoter (relevant to Figure 3).** LBMCs were cultured in medium containing Wnt3A in either the absence or presence of 5-Aza-2'-deoxycytidine (DAzaC) as indicated. Cells were harvested after 4 days of culture and methyl-DNA IP (MeDIP) performed to assay methyl CpG modification in the CpG2 island of the Sox9 gene. MeDIP was normalized to input DNA. Significance was calculated using Student's *t*-test. Relative enrichment of methylated CpGs in lanes 2 and 3 was compared to lane 1. *P<0.05, **P<0.01, ***P<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean.

Supplemental Figure 6. (A-B) DNMT3A is directly phosphorylated by ERK2 (relevant to Figure 4). (A) Schematic diagram of murine DNMT3A depicting both the conserved ERK phosphorylation and docking sites, the PWWP domain, and a cysteine-rich region known as the ADD (ATRX-DNMT3-DNMT3L) domain. **(B)** In vitro kinase assay with either myelin basic protein (as a control) or bacterially produced and purified versions of murine DNMT3A and activated ERK2. **(C) Anti-phospho-S255-DNMT3A antibody specifically binds to DNMT3A that is phosphorylated at S255 (relevant to Figure 4).** Western analysis of in vitro ERK2-phosphorylated GST-hDNMT3A-(WT) employing either anti-phospho-S255-DNMT3A or anti-DNMT3A antibodies. The interaction between anti-phospho-S255-DNMT3A and phosphorylated GST-hDNMT3A-(WT) was competed with a hDNMT3A peptide (246a.a.-AVQQPTDPASPTVATTC-262a.a.) containing either phospho-S255 or non-phosphorylated S255. Note that interaction between anti-phospho-S255-DNMT3A and phosphorylated GST-hDNMT3A-(WT) was specifically competed by the hDNMT3A peptide containing phospho-S255 (middle blot). **(D) DNMT3A is phosphorylated at the ERK1/2 phosphorylation site in a proximal (low) to distal (high) gradient in the limb bud (relevant to Figure 4).** Limb buds (isolated from day 4 chicken embryos; H. H. stage 22-24) were separated into proximal (P), middle (M) and distal (D) regions. Equal protein amounts of each limb bud region were loaded onto SDS-PAGE, and Western analysis was performed with either anti-DNMT3A-(pS220) or anti-DNMT3A. Displayed are the original (non-cropped) images of the Western blots displayed in Figure 4E. The band identified by an arrow in the Anti-DNMT3A-p(S220) Western blot (left) exhibits an identical electrophoretic mobility as the prominent band (also designated by an arrow) observed in the total

DNMT3A Western blot (right), that was run in parallel. We believe that the protein that is recognized by Anti-DNMT3A-p(S220) that runs at a smaller molecular weight than total DNMT3A does not represent ERK-phosphorylated DNMT3A, as this interaction was competed with a non-phosphorylated hDNMT3A peptide (246a.a.-AVQQPTDPASPTVATTC-262a.a.) (see Figure 4F and Supplemental Figure 7E). In Western blots of limb buds that were performed in the presence of this competing peptide, Anti-DNMT3A-p(S220) failed to recognize a band which runs at a smaller molecular weight than total DNMT3A (see Figure 4F and Supplemental Figure 7E). **(E) Immunoreactivity of anti-phospho-DNMT3A with chicken limb bud tissue is competed by a DNMT3A peptide containing phosphorylated serine (at the ERK phosphorylation site) (relevant to Figure 5).** Cryosections of limb buds (isolated from day 4 chicken embryos) were immunostained with anti-phospho-S220-DNMT3A; nuclei were visualized by DAPI staining. A DNMT3A peptide containing serine phosphorylation (at the ERK phosphorylation site) was added along with anti-phospho-S220-DNMT3A.

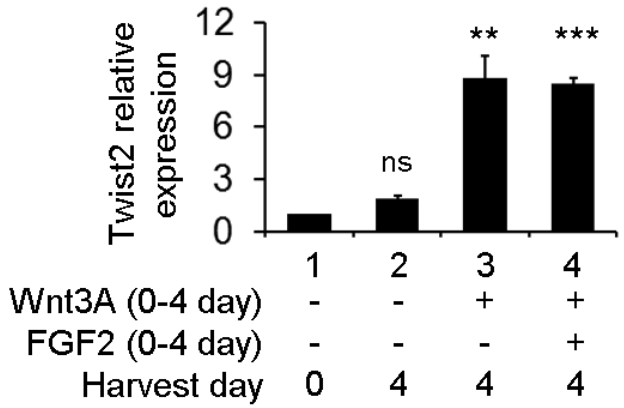
Supplemental Figure 7. (A-C) Wnt3A and/or FGF2 signals do not affect the expression levels of either exogenous DNMT3A or its mutants (relevant to Figure 6). LBMCs were infected with a retrovirus encoding either Flag/HA-hDNMT3A-(WT), Flag/HA-hDNMT3A-(L373,637A), Flag/HA-hDNMT3A-(S255A), Flag/HA-hDNMT3A-(S255A; L373,637A), Flag/HA-hDNMT3A-(S255D), or Flag/HA-hDNMT3A-(S255E) cultured for 4 days in either control medium or medium containing Wnt3A, in either the absence or presence of FGF2, as indicated. Cells were harvested after 4 days in culture

and **(A and B)** exogenous DNMT3A gene expression was assayed by RT-qPCR (relative to GAPDH levels). Significance was calculated using Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean. **(C)** Western analysis was performed to assay expression of either Flag/HA-hDNMT3A-(S255D) or Flag/HA-hDNMT3A-(S255E) in infected LBMCs, which were harvested after 4 days of culture. **(D-E) Activated ERK acts in both a catalytic and a stochiometric fashion to regulate DNMT3A binding to the Sox9 promoter (relevant to Figure 6).** **(D)** LBMCs were infected with a retrovirus encoding Flag/HA-hDNMT3A-(S255D) and cultured for 4 days in either control medium or medium containing Wnt3A, in either the absence or presence of FGF2, as indicated. In some cases either a MEK1/2 inhibitor (U0126) or an ERK inhibitor (FR180204) were added. Explants were cultured in control medium for an additional 4 days. Cells were harvested after 8 days in culture and gene expression of Sox9, Collagen II, Aggrecan and exogenous DNMT3A was assayed by RT-qPCR (relative to GAPDH levels). Significance was calculated using Student's *t*-test; for all transcripts, lane 2 was compared to lane 1; lanes 3-5 were compared to lane 2. **P*<0.05, ***P*<0.01, ****P*<0.001 and ns (non-significant difference) are indicated. Error bar indicates standard error of the mean. **(E)** Limb buds lacking ectoderm (isolated from day 4 chicken embryos) were cultured for 24 hours in either control medium, or medium containing either soluble Wnt3A, Wnt3A/FGF2, Wnt3A/FGF2/U0126, or Wnt3A/FGF2/FR180204 as indicated. Equal protein amounts of each explant culture were loaded onto SDS-PAGE, and Western analysis was performed with either anti-phospho-S220-DNMT3A (in the presence of competitor non-phosphorylated hDNMT3A

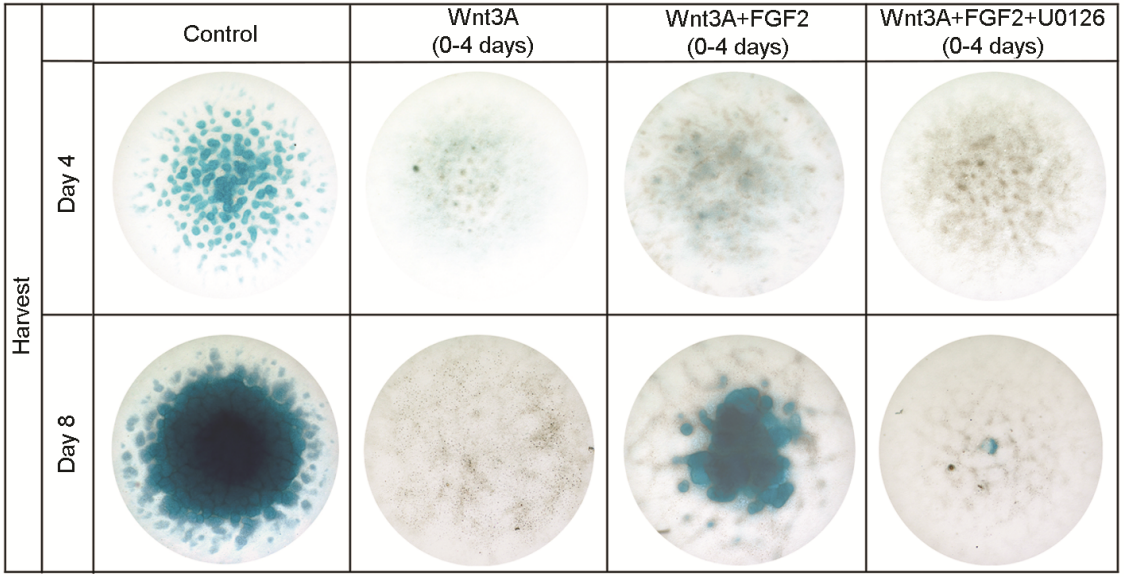
peptide (246a.a.-AVQQPTDPApSPTVATTC-262a.a.) at 5 mg/ml during primary antibody incubation), anti-DNMT3A, or anti–beta actin.

Supplemental Figure 1

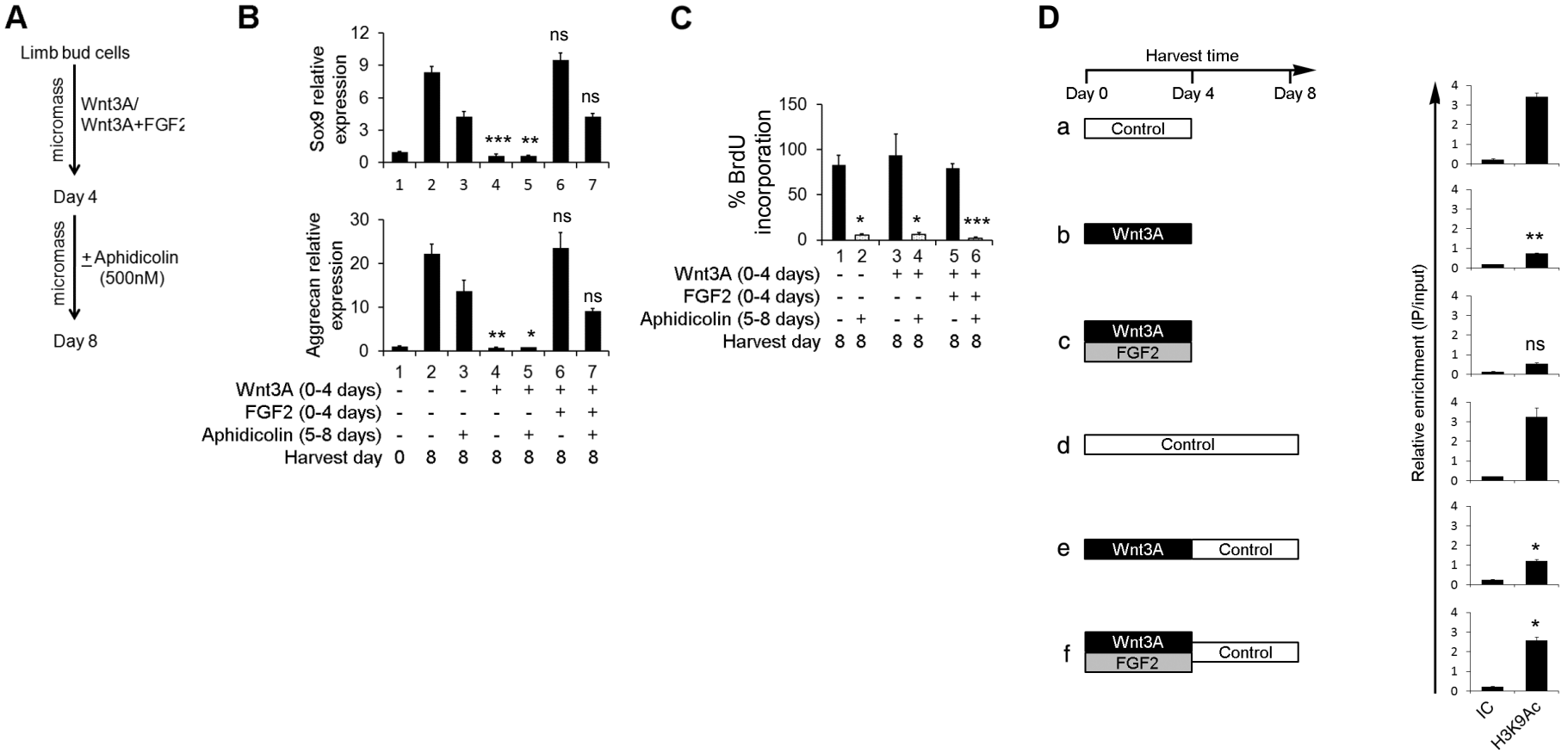
A



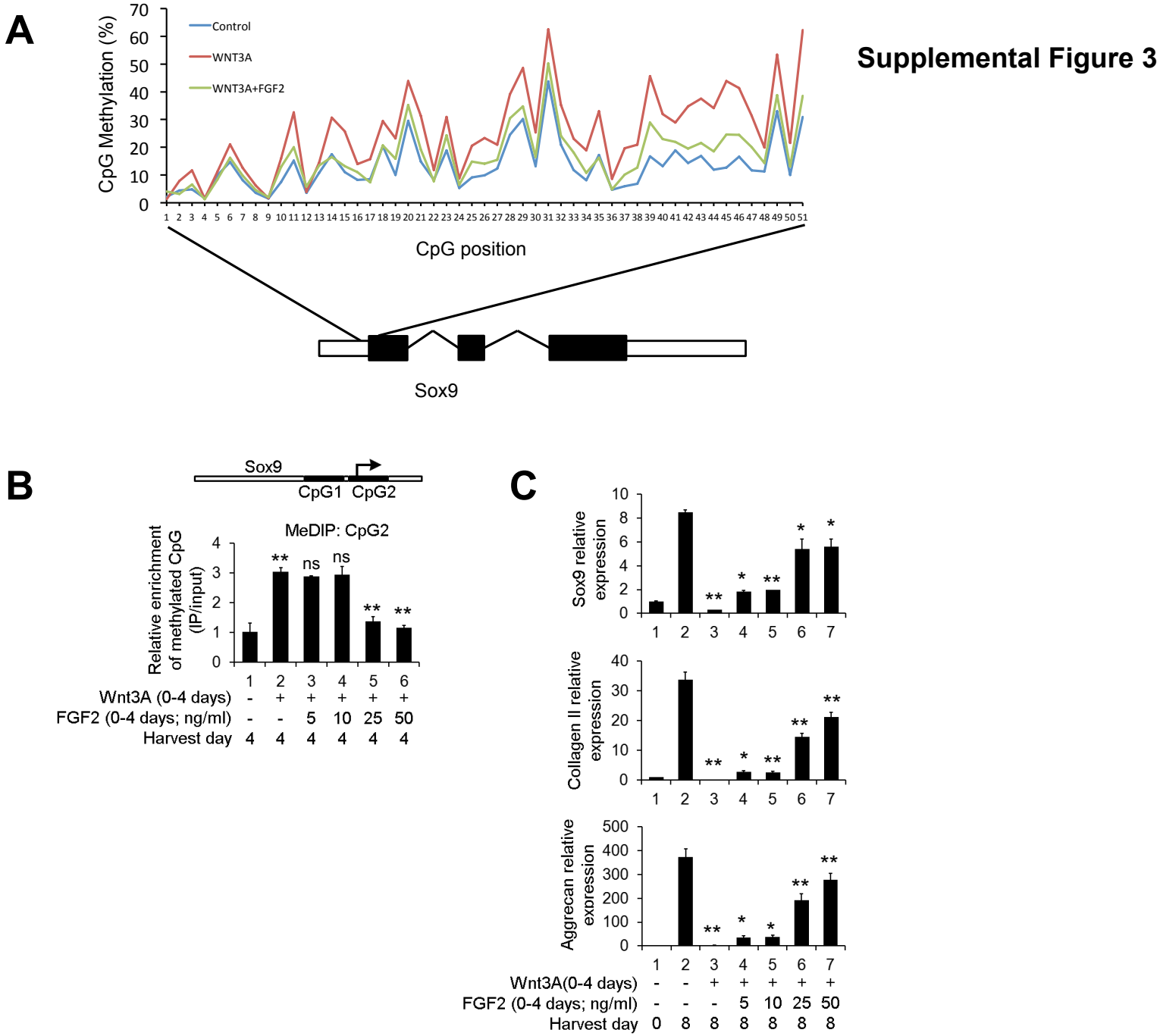
B



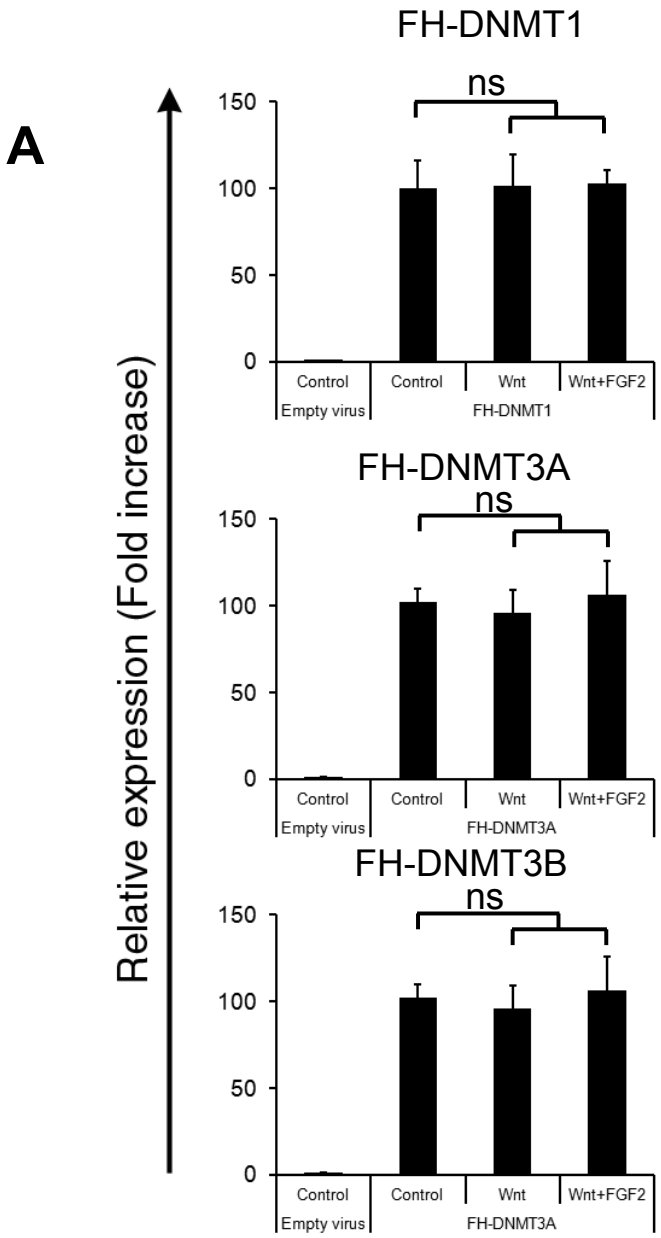
Supplemental Figure 2

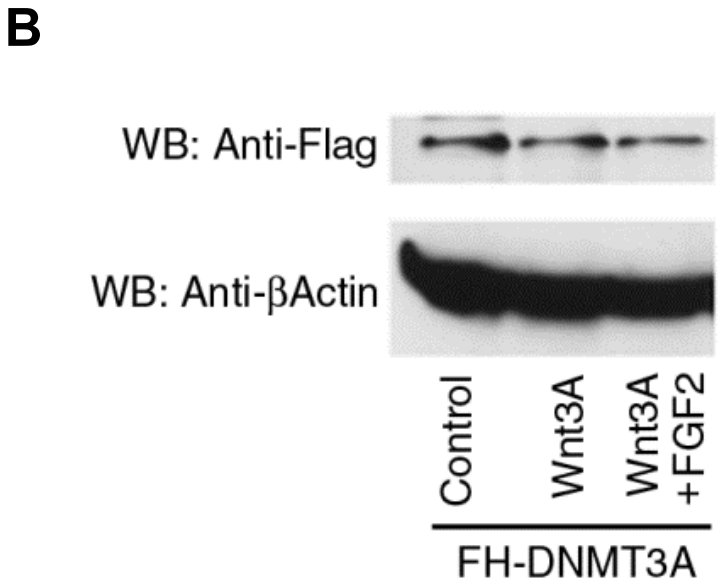


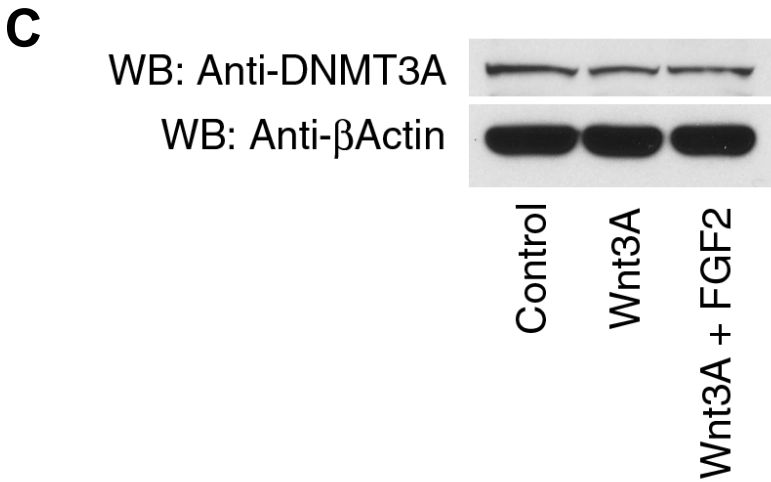
Supplemental Figure 3



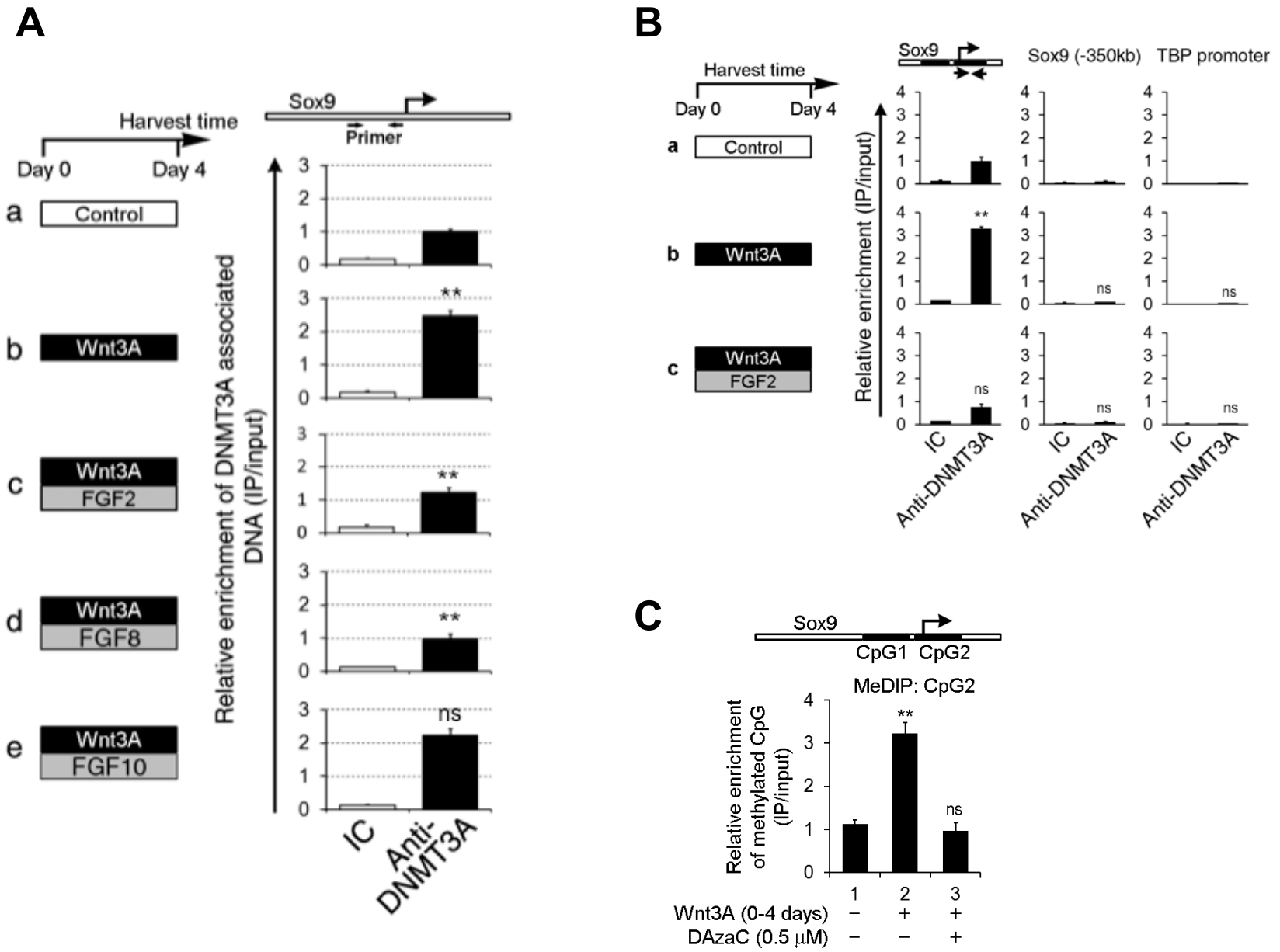
Supplemental Figure 4



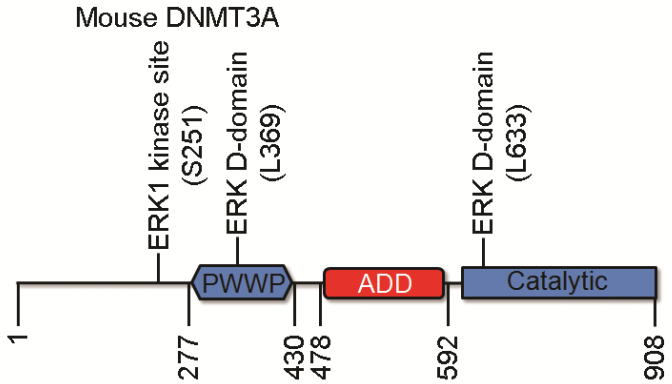


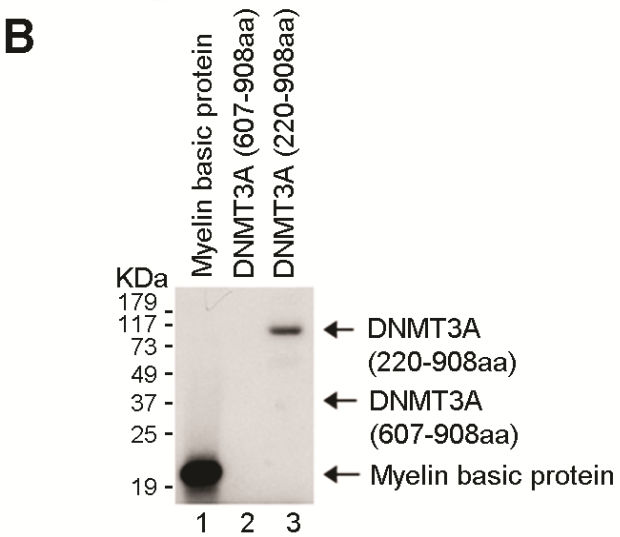


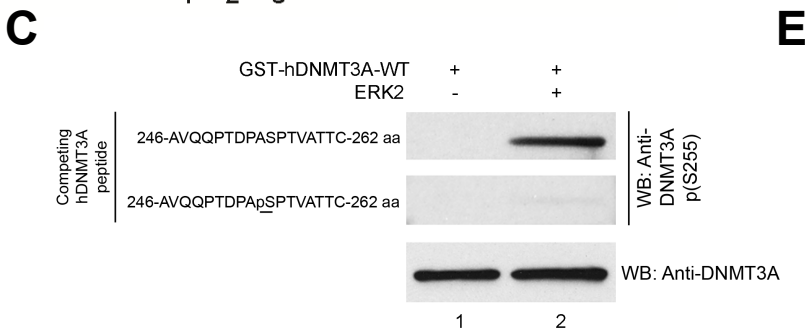
Supplemental Figure 5

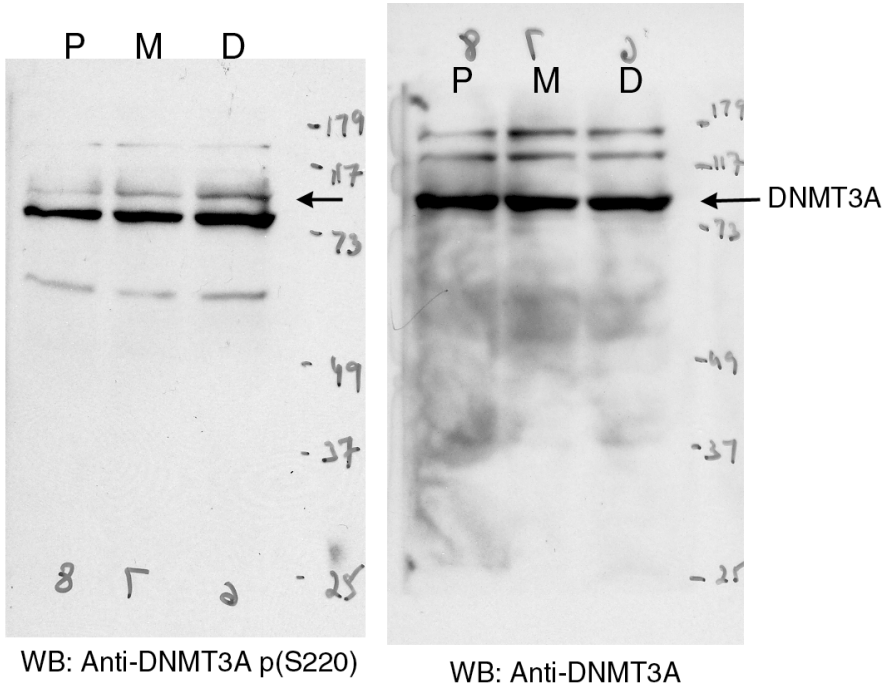


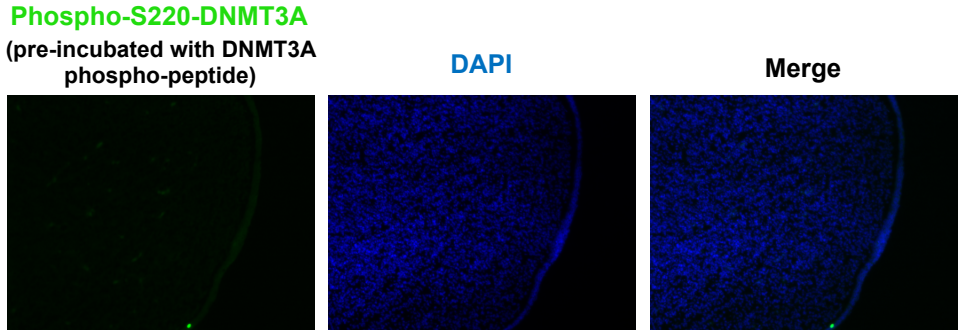
Supplemental Figure 6











Supplemental Figure 7