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Maternal expression and early induction of histone gene transcription factor Hinfp sustains development in pre-implantation embryos

Prachi N. Ghule [a,b], Rong-Lin Xie [b], Jennifer L. Colby [b], Jaime A. Rivera-Pérez [b], Stephen N. Jones [b], Jane B. Lian [a,b], Janet L. Stein [a,b], Andre J. van Wijnen [b,c], Gary S. Stein [a,b,*]

[a] Department of Biochemistry and University of Vermont Cancer Center, University of Vermont College of Medicine, 89 Beaumont Avenue, Burlington, VT 05405, USA
[b] Department of Cell and Developmental Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655, USA
[c] Departments of Orthopedic Surgery and Biochemistry & Molecular Biology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

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ABSTRACT

Fidelity of histone gene expression is important for normal cell growth and differentiation that is stringently controlled during development but is compromised during tumorigenesis. Efficient production of histones for packaging newly replicated DNA is particularly important for proper cell division and epigenetic control during the initial pre-implantation stages of embryonic development. Here, we addressed the unresolved question of when the machinery for histone gene transcription is activated in the developing zygote to accommodate temporal demands for histone gene expression. We examined induction of Histone Nuclear Factor P (HINFP), the only known transcription factor required for histone H4 gene expression, that binds directly to a unique H4 promoter-specific element to regulate histone H4 transcription. We show that *Hinfp* gene transcripts are stored in oocytes and maternally transmitted to the zygote. Transcripts from the paternal *Hinfp* gene, which reflect induction of zygotic gene expression, are apparent at the 4- to 8-cell stage, when most maternal mRNA pools are depleted. Loss of *Hinfp* expression due to gene ablation reduces cell numbers in E3.5 stage embryos and compromises implantation. Reduced cell proliferation is attributable to severe reduction in histone mRNA levels accompanied by reduced cell survival and genomic damage as measured by cleaved Caspase 3 and phospho-H2AX staining, respectively. We conclude that transmission of maternal *Hinfp* transcripts and zygotic activation of the *Hinfp* gene together are necessary to control H4 gene expression in early pre-implantation embryos in order to support normal embryonic development.

1. Introduction

Early embryonic development from fertilization to the blastula stage requires carefully orchestrated cell proliferation. Duplicated genomes that are generated during each cell division must be tightly organized into nucleosomes to constrain the dimensions of the chromosomes and to support epigenetic control. The assembly of nucleosomes critically depends on production of histone H4 protein which represents the most highly conserved component of the histone octamer that packages DNA into chromatin during S-phase. Expression of human H4 mRNA requires HINFP and its CDK2-responsive co-factor NPAT (Holmes et al., 2005; Medina et al., 2006; Miele et al., 2005; Mitra et al., 2003; Zhao et al., 1998, 2000). Genetic loss of Hinfp in embryonic fibroblasts isolated from conditional Hinfp null mouse models generates genomic instability and compromised cell viability (Ghule et al., 2014). Our previous findings show that this cellular phenotype is caused by deficiency of histone H4 protein which accounts for increased nucleosomal spacing, replicative stress, impaired DNA synthesis, DNA damage and aneuploidy (Ghule et al., 2014).

Histone mRNAs are necessary for histone protein synthesis during the first cleavage division when cells proceed with the first zygotic cycle of DNA replication. Unlike non-mammalian vertebrates that store high levels of maternal mRNAs to accommodate multiple cell divisions, mammalian embryos have limited pools of histone mRNA that are degraded at the two-cell stage (reviewed by Graves et al. (1985), Wassarman (1988) and Zernicka-Goetz et al. (2009)). Histone mRNAs are presumably not transmitted

* Corresponding author at: Department of Biochemistry and University of Vermont Cancer Center, University of Vermont College of Medicine, 89 Beaumont Avenue, Burlington, VT 05405, USA.
E-mail addresses: prachi.ghule@uvm.edu (P.N. Ghule), ronglin.xie@yahoo.com (R.-L. Xie), jfaucette@gmail.com (J.L. Colby), jaime.rivera@umassmed.edu (J.A. Rivera-Pérez), stephen.jones@umassmed.edu (S.N. Jones), jane.lian@uvm.edu (J.B. Lian), janet.stein@uvm.edu (J.L. Stein), vanwijnen.andre@mayo.edu (A.J. van Wijnen), gary.stein@uvm.edu (G.S. Stein).

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during mitosis in the embryo, because they are rapidly degraded at the completion of S phase (Dominski and Marzluff, 2007; Marzluff et al., 2008; Muller et al., 2007; Stein et al., 2006). The selective degradation of histone mRNAs necessitates *de novo* synthesis during each division cycle. Therefore, a key question is when and how cells activate the expression of Hinfp to drive expression of histone H4 mRNAs during early embryogenesis.

HINFP is a unique zinc finger transcription factor that functions as the endpoint effector of the Cyclin E/CDK2/NPAT/HINFP signaling pathway which controls the cell cycle regulated transcriptional initiation of multiple H4 genes at the G1/S phase transition (Holmes et al., 2005; Medina et al., 2008, 2012, 2006; Miele et al., 2005; Mitra et al., 2003, 2009). The machinery for histone gene transcription (e.g., HINFP, NPAT) and processing of histone RNA transcripts (e.g., U7snRNP proteins Lsm10 and Lsm11, FLASH) co-localize at histone gene loci and are organized in the nucleus within specialized subnuclear domains referred to as histone locus bodies (HLBs) (Bongiorno-Borbone et al., 2008; Ghule et al., 2007, 2008; Ma et al., 2000; Ye et al., 2003; Zhao et al., 1998, 2000). Normal diploid cells have two or four HLBs that are formed in a cell cycle dependent manner; the HLBs at the Hist1 cluster on 6p are present throughout the cell cycle except mitosis and early G1, and the HLBs at the Hist2 cluster on 1q become visible as cells enter S-phase when NPAT is phosphorylated by cyclin E accompanied by increased histone expression. The HLB components HINFP, NPAT and FLASH are required to support synthesis of large quantities of histones necessary for packaging of newly replicated DNA in S phase (Barcaroli et al., 2006a; Bongiorno-Borbone et al., 2008; Gao et al., 2003; Ghule et al., 2014; Ye et al., 2003).

Hinfp expression correlates with histone H4 gene expression throughout fetal mouse development (Liu et al., 2011). Complete genomic ablation of *Hinfp* in mice causes embryonic lethality before E6.5, while mice heterozygous for *Hinfp* are viable and survive to adulthood (Xie et al., 2009). Pre-implantation *Hinfp* null embryos (E3.5) exhibit abnormal growth and proliferation in outgrowth cultures (Xie et al., 2009). Thus, *Hinfp* is essential for early embryogenesis consistent with its crucial role in H4 gene expression. In this study, we addressed consequences of loss of *Hinfp* during early stages of embryogenesis (E0–E3.5) and whether *Hinfp* mRNA is generated by maternal and/or zygotic expression. We observed that depletion of *Hinfp* leads to decreased cell number and increased cell death in early stage embryos (E3.5). Our results indicate that maternally inherited pools of Hinfp RNA and/or protein sustain the earliest stages of embryogenesis in *Hinfp* null mice, and together with zygotic induction of *Hinfp* at the 4-cell stage support normal embryonic development.

2. Materials and methods

2.1. Mice and embryo isolation

Hinfp null ($Hinfp^{LacZ/LacZ}$; referred to as KO) mice were generated and offspring were subjected to genotyping by PCR as described earlier (Xie et al., 2009). Animals were maintained according to Institutional Animal Care and Use Committee (IACUC) guidelines. Isolation of early stage mouse embryos was done using timed pregnant females from either $Hinfp^{LacZ/+}$ (Het) crosses, or $Hinfp^{LacZ/+}$ male with wild-type (WT) C57BL/6 female crosses, or WT C57BL/6 male with $Hinfp^{LacZ/+}$ (Het) female crosses. Embryos were collected at E3.5 (blastocyst) by uterine flush, at E4.5 stage by uterine flush as well as surgical removal from uterus and at E2.5, E2 and E1.5 by flushing the oviduct with M2 medium (Invitrogen) (Nagy et al., 2003). Expression of the *lacZ* reporter gene under control of the endogenous *Hinfp* promoter was examined in embryos using β-galactosidase staining (Liu et al., 2011).

2.2. Ovary staining

Ovaries removed from *Hinfp* WT or Het animals were stained for β-galactosidase and processed to make frozen sections by standard histological preparations. Expression of *lacZ* driven by the endogenous *Hinfp* promoter was monitored using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining. Briefly, the tissues were fixed in 0.5% glutaraldehyde and stained with a solution containing 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$. The sections were counterstained with either Hematoxylin (H) or Hematoxylin and Eosin (H&E) (Leica) to visualize nuclei and cytoplasm. Slides were viewed under Zeiss Axioscope bright-field microscope and images were captured using Zen2011 software (Zeiss Inc.).

2.3. Outgrowth cultures

Embryos harvested at E3.5 from Heterozygous crosses between $Hinfp^{LacZ/+}$ mice were cultured in M15 media (Dulbecco's minimal essential medium, 15% fetal calf serum, 100 μM β-mercaptoethanol, 2 mM glutamine, and 1X penicillin-streptomycin) for up to 96 h. Embryo outgrowths were cultured on glass coverslips and immunofluorescence staining was performed for histone locus body marker protein, NPAT (BD Bioscience, mouse monoclonal). Briefly, the outgrowth cultures at day 4 were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.25% Triton-X-100 in 1X PBS for 30 min, stained with primary antibody and visualized using appropriate secondary antibody. The nuclei were stained with DAPI. The images were obtained using a Leica SP5 confocal microscope.

2.4. Immunofluorescence (IF) staining

E3.5 embryos from $Hinfp^{LacZ/+}$ crosses were isolated as described above. The blastocyst stage embryos were subjected to IF staining. Briefly, the embryos were washed with PBS and then fixed in 4% paraformaldehyde for 1 h at RT, washed 3 times in 1x PBS without Ca+ and Mg+, followed by a 20 min incubation in 1X PBS containing 1% BSA and 0.5% Triton-X-100 (PBT). The permeabilized embryos were then incubated in blocking solution (1% fetal bovine serum in PBT) overnight at 4°C. Next day, embryos were transferred to primary antibody solution and incubated either for 2 h at room temperature (RT) or overnight at 4 °C. Antibodies used: E-cadherin (Invitrogen, Rat polyclonal); phospho-H2AX (ser-139) (Millipore, mouse monoclonal); OCT4 (SantaCruz, Goat polyclonal); CDX2 (BioGenex, mouse monoclonal) (Strumpf et al., 2005); NPAT (BD Bioscience, mouse monoclonal); FLASH (gift from Dr. Zbig Dominski (UNC, Chapel Hill) (Yang et al., 2009), rabbit polyclonal); cleaved Caspase 3 (Cell Signaling, Rabbit polyclonal). Embryos were then washed 3 times in PBT and incubated in appropriate secondary antibodies for 1 h at RT. The nuclei were counterstained with DAPI. The images were obtained using either Leica SP5 confocal microscope or Zeiss LSM 510 META confocal microscope using 40x water immersion objective. Image analyses were performed either using Leica or Zeiss imaging software. Cell counting of embryos was performed microscopically as well as by analyzing Z-sections of embryos at 2 μm thickness. The E-cadherin staining was used to mark the cell boundaries. The embryos subjected to IF were genotyped retrospectively.

2.5. RNA isolation from E3.5 embryos

Total RNA was isolated from individual blastocysts (E3.5)

obtained from crosses between *Hinfp*$^{LacZ/+}$ mice. RNA was extracted using PicoPure RNA isolation kit (Applied Biosystems) and cDNA was prepared using SuperScript III First Strand Synthesis kit (Invitrogen/Life Technologies, Inc.) according to manufacturer's protocol. The transcript levels were determined by the ΔCt method using the cycle threshold (Ct) obtained in ViiA 7™ (Applied Biosystems by Life Technologies) and iTaq Fast SYBR Green Supermix with ROX (Bio-Rad Laboratories Cat# 172-5122). Primer sequences are listed here, mHinfp-exon56 Forward: cagacctcattggatcagca, mHinfp-exon7 Reverse: ggcaggtcatgtcacacaaa, rmGapdh Forward: aagggctcatgaccacagtc, rmGapdh Reverse: ggatgcagggatgatgttct, mHist2H4 Forward: tagccatgtctggcagagg, mHist2H4 Reverse: ctggatgttgtcacgcaaga, mHist1H3 Forward: ttctagtgtactgagatggctcgt, mHist1H3 reverse: gtagcggtgaggcttcttca.

3. Results

3.1. Hinfp null embryos exhibit delayed growth and compromised HLB organization

Recent work from our group has shown that genomic removal of histone H4 transcription factor *Hinfp* causes early embryonic lethality in mice between embryonic day (E) 5.5–6.5 (Xie et al., 2009). To establish when Hinfp becomes genetically essential prior to the blastocyst stage, we examined implantation stage embryos lacking *Hinfp*. Embryos from heterozygous *Hinfp*$^{LacZ/+}$ crosses were isolated at E4.5 and examined for *lacZ* expression under control of the native *Hinfp* promoter. Embryos from these crosses differ in size and appearance. Smaller, deformed embryos express *lacZ* (as measured by in situ β-galactosidase activity) at higher levels than embryos with a normal appearance (Fig. 1A). Retrospective genotyping confirmed that the smaller embryos consistently have a *Hinfp* null genotype (Supplementary Fig 1A). Both wild type (WT) and Heterozygous (Het) embryos can support implantation to the uterine wall while, *Hinfp* null embryos (Fig. 1A) with abnormal morphology do not survive. This developmental defect is consistent with peri-implantation stage embryonic lethality we observed previously (Xie et al., 2009).

HINFP interacts with the CDK2-responsive co-factor NPAT that resides at histone locus bodies (HLBs) (Ghule et al., 2014; Medina et al., 2012). To investigate if loss of *Hinfp* function alters the organization of HLBs, we first analyzed HLBs in outgrowth cultures of E3.5 stage embryos isolated from *Hinfp*$^{Lacz/+}$ crosses. Beyond the expected delay in hatching and abnormal growth of *Hinfp* null embryos (Xie et al., 2009), there appear to be fewer inner cell mass (ICM) cells in *Hinfp* null embryos compared to embryos with at least one functional *Hinfp* allele (Fig. 1B, left panels phase images). Staining of these cultures for the HLB marker protein *Npat* revealed that HLBs are present in embryos with all three genotypes (Fig. 1B). However, there are fewer and more diffused HLBs in the ICM cells of the null embryos compared to WT or Het embryos (Fig. 1B, lower panels, see arrows). There also appear to be subtle changes in nuclear morphology in Hinfp null embryos as visualized by DAPI staining.

To assess the HLB staining patterns observed in outgrowth cultures in detail, we directly stained embryos isolated at E3.5 from *Hinfp*$^{Lacz/+}$ crosses for *Npat* and an additional HLB marker protein; *Flash* (Fig. 1C). FLASH is a known component of HLBs and was first discovered as a FLICE-associated huge protein (FLASH) involved in apoptotic processes (Imai et al., 1999; Yang et al., 2009). FLASH is also involved in regulation of histone gene transcription and histone RNA processing, and loss of FLASH can disrupt punctate subnuclear organization of HLBs (Barcaroli et al., 2006b; Yang et al., 2014). As observed in outgrowth cultures, HLBs are present in E3.5 embryos of all genotypes. However, 5–10% (i.e., 1–3 cells per each null embryo) of cells in E3.5 Hinfp null embryos exhibit diffused staining for both *Flash* and *Npat* (Fig. 1C see arrow). Notably, in the null embryos we also observed intense cytoplasmic *Flash* staining that coincides with the diffuse nuclear staining for *Npat*. Although the intense staining observed with the *Flash* antibody could be a technical artifact, this intense cytoplasmic staining was not observed in any of the corresponding WT or Het embryos. Therefore, it is conceivable that in Hinfp null embryos, which display presence of apoptosis (see Fig. 3), the Flash protein is more readily detectable. Taken together, our results show that Hinfp is not required for formation of HLBs at this stage of embryogenesis.

3.2. Hinfp null mutation compromises cell survival and generates DNA damage in E3.5 stage embryos

Because WT and null embryo cultures exhibit differences in cell number, we used IF microscopy to quantify cell number in individual E3.5 embryos (from *Hinfp*$^{Lacz/+}$ crosses) in which cellular boundaries were demarcated by E-cadherin staining (Fig. 2A). The number of cells per embryo was counted using confocal microscopy imaging, while *Hinfp* status was established by retrospective genotyping (Supplementary Fig 1B) of all embryos ($n > 100$). We observed that *Hinfp* null embryos always have substantially fewer cells (25–35 cells) compared to WT (65–75 cells) or Het (55–65 cells) embryos (Fig. 2A). Thus, *Hinfp* null embryos exhibit a delayed growth such that E3.5 embryos morphologically resemble the morula stage rather than the blastocyst stage.

Our results from outgrowth cultures as well as the appearance of null embryos at E4.5 suggest a possible deregulation of cell proliferation in ICM cells. To assess whether there is any difference between the proportion of ICM and trophoblast cells in E3.5 null embryos, we directly stained embryos ($n=25$) with *Oct4* (pluripotency marker/ ICM marker) and *Cdx2* (trophoblast cell marker) (Strumpf et al., 2005). We observed that Oct4 staining was present in all cells in all three genotypes whereas Cdx2 staining was specific to trophoblast cells in all embryos. Therefore, we evaluated the proportion of cells staining for Oct4+/Cdx2+(presumably trophoblast cells) vs Oct4+/Cdx2- (presumably ICM cells). There are clear differences in the proportion of cells that are *Oct4* positive in null (KO) versus WT embryos (Fig. 2B). Interestingly, however, there is no difference in the ratio of *Cdx2* positive cells, as half the cells are *Cdx2* positive for each embryo. The null embryos are half the size of the WT embryos at E3.5 consistent with our observations from cell counting (see Fig. 2A). We also detected distinct differences in nuclear morphology of null embryos versus WT with presence of fragmented nuclei in null embryos. These results suggest that overall cell proliferation in null embryos is delayed.

Because Hinfp is critically required for histone gene transcription (Becker et al., 2007; Ghule et al., 2014; Medina et al., 2012; Xie et al., 2009), we examined histone H4 transcripts in E3.5 stage embryos obtained from *Hinfp*$^{Lacz/+}$ crosses by qRT-PCR. *Hinfp* null embryos do not express functional *Hinfp* mRNA, while Het embryos express half the amount of *Hinfp* RNA compared to WT (Fig. 2C). Null embryos have severely reduced expression of histone H4 RNAs compared to WT and Het embryos (Fig. 2C). However, there is no significant difference in expression of histone H3 RNAs (Fig. 2C) in null embryos compared to WT or heterozygous embryos.

Depletion of Hinfp and associated loss of histone H4 causes multiple cell autonomous defects in mouse embryonic fibroblasts (Ghule et al., 2014). Our results indicate that *Hinfp* null embryos show delayed growth and reduced cell number with atypical nuclear morphology (Figs. 1 and 2). To investigate whether the observed cellular differences are due to changes in capacity for cell

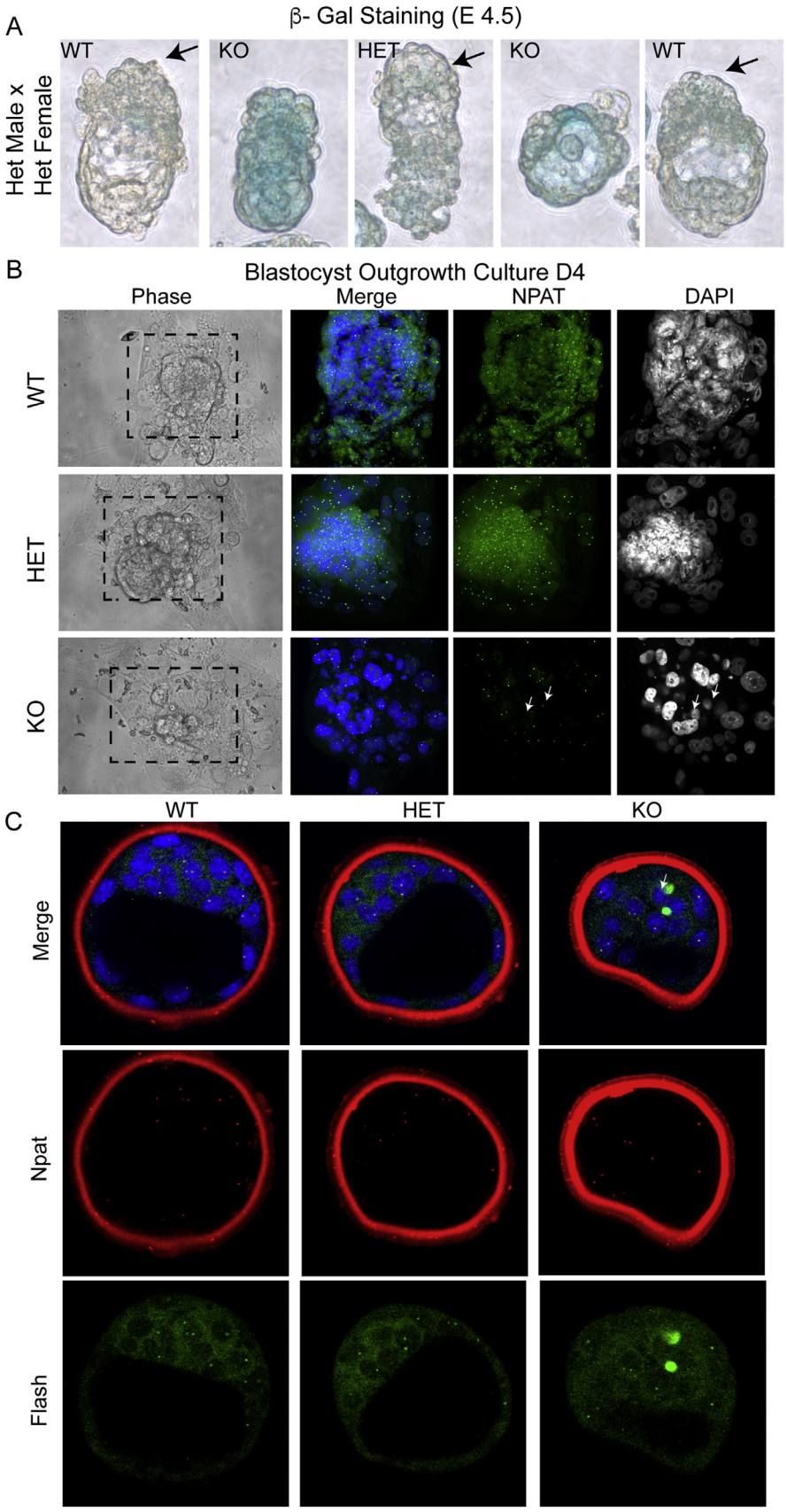

Fig. 1. Implantation stage *Hinfp* null embryos show delayed growth and compromised Histone Locus Bodies (HLBs). (A) Representative images of E4.5 embryos derived from $Hinfp^{LacZ/+}$ crosses are stained for β-galactosidase activity (blue). The arrows indicate the epiblast region in WT and Het embryos. The blue staining in WT embryos is remnants of maternally derived *lacZ*. (B) Outgrowth cultures of *Hinfp* null embryos exhibit delayed hatching and diminished HLBs. Representative images of outgrowth cultures of E3.5 embryos isolated from $Hinfp^{LacZ/+}$ crosses at day 4 and imaged by phase contrast microscopy (left panels). The cultures were then immuno-stained for Npat (green) and nuclei were stained with DAPI (pseudocolor white). The dotted squares correspond with IF images (cropped) for each embryo. Note the fewer number of ICM cells in the null embryos (left bottom panel). The white arrows in the bottom panels indicate ICM cells with diffused HLBs in null embryos. (C) E3.5 stage embryos isolated from $Hinfp^{LacZ/+}$ crosses were subjected to IF microscopy to detect HLB marker proteins Npat (red) and Flash (green). The nuclei were stained with DAPI (blue). The individual channel and merged images depict a single plane (slice) 2 μM thickness for each embryo (cropped). The zona pellucida shows nonspecific signal (red ring around each embryo) with Npat antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

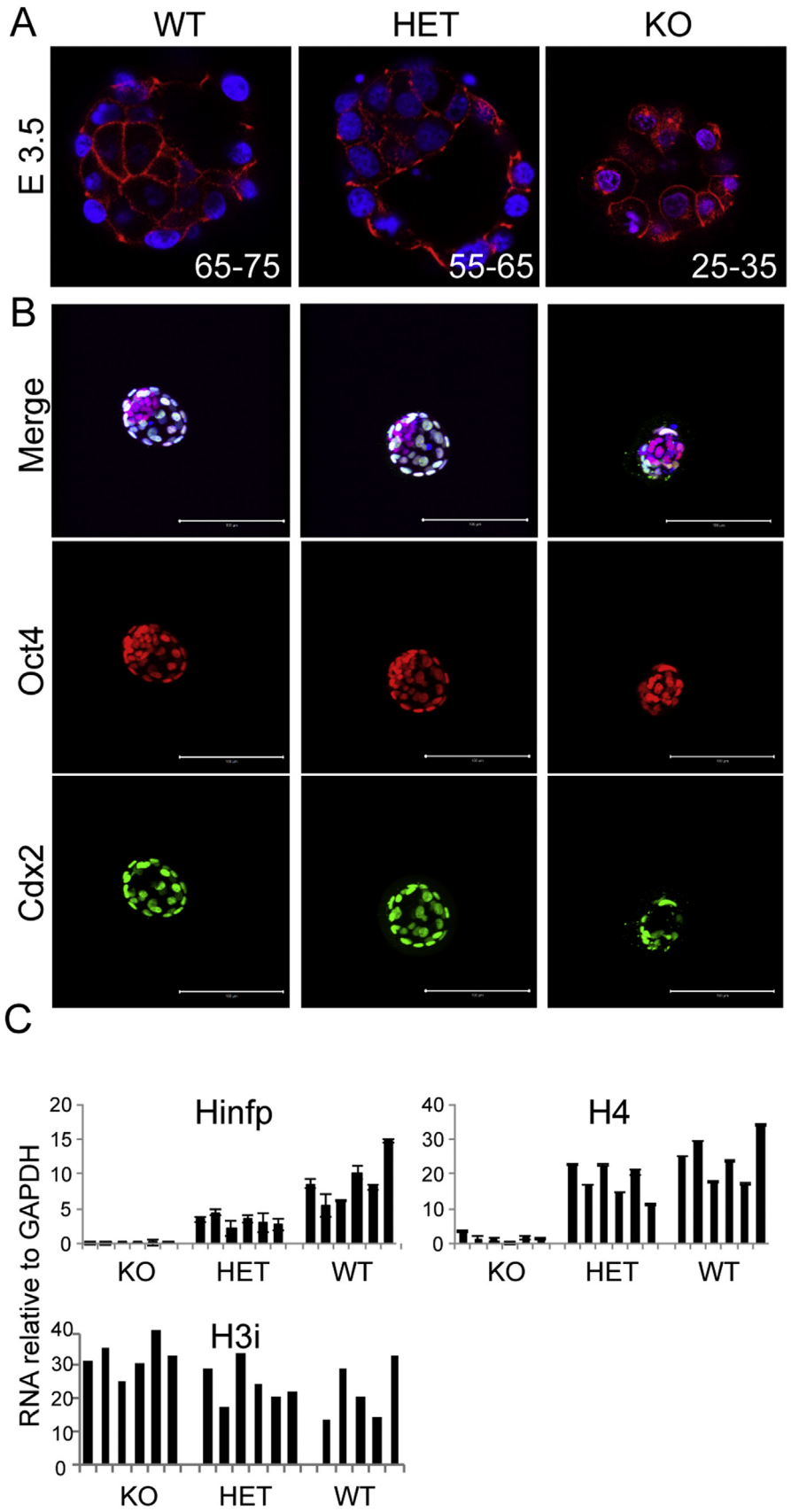

Fig. 2. E3.5 *Hinfp* null embryos are proliferation defective and show reduced histone H4 expression. E3.5 embryos isolated from *Hinfp*$^{LacZ/+}$ crosses were subjected to either IF microscopy or qRT-PCR. (A) Representative single plane confocal images (cropped) of WT, heterozygous and *Hinfp* null embryos stained for E-cadherin (red); nuclei were stained with DAPI (blue). The inset numbers indicate cell count for each genotype. (B) Representative individual or merged projection images at scale of WT, heterozygous and *Hinfp* null embryos stained for the ICM marker Oct4 (red), and the trophoblast marker Cdx2 (green); nuclei were stained with DAPI (blue). Scale bar: 100 μM Note the reduced number of ICM cells in null embryos. (C) qRT-PCR analysis of WT, heterozygous and *Hinfp* null embryos showing expression of *Hinfp, Histone H3i* and *Histone H4. Hinfp* null embryos exhibit severely reduced expression of *Histone H4* RNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

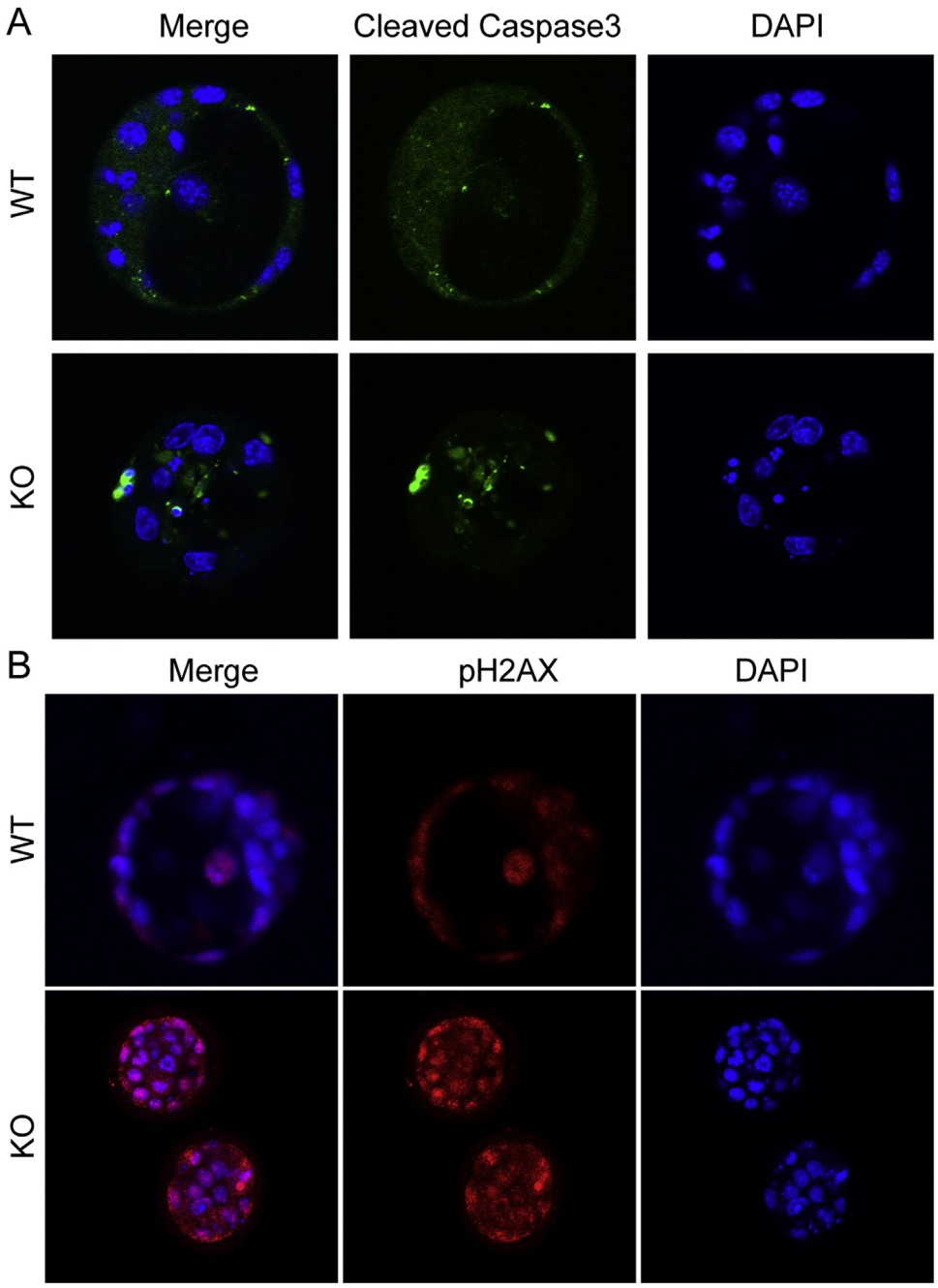

Fig. 3. E3.5 *Hinfp* null embryos show presence of cell death marker and exhibit DNA damage. (A) Representative individual and merged single plane images (cropped) of WT and *Hinfp* null embryos stained with cleaved Caspase 3 (green); nuclei were stained with DAPI (blue). 5–10% of cells in null embryo show bright activated Caspase staining. (B) Representative individual and merged single plane images (cropped) of WT and *Hinfp* null embryos stained with pH2AX (red); nuclei were stained with DAPI (blue). Note the accumulation of intense pH2AX staining in null embryos compared to WT. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

survival, we stained the E3.5 embryos for presence of cleaved *Caspase 3*, a molecular marker of apoptosis (reviewed in Budihardjo et al. (1999), Fig. 3A). We detected significantly higher staining for cleaved *Caspase 3* in *Hinfp* null embryos compared to WT embryos (Fig. 3A). These results indicate that there is increased apoptosis and suggest a possible mechanism for reduced cell number and delayed growth after removal of *Hinfp*.

Loss of Hinfp causes replicative stress and DNA damage (Ghule et al., 2014) and simultaneous loss of the tumor suppressor protein p53 exacerbates proliferative defects in cell culture models (Ghule et al., 2015). Therefore, we investigated whether loss of *Hinfp* provokes DNA damage in E3.5 stage embryos using accumulation of phospho-H2AX (Ser-139) foci as a marker for double-stranded DNA breaks. We observed intense phospho-H2AX staining in E3.5 *Hinfp* null embryos compared to their WT counterparts (Fig. 3B). These results indicate that DNA damage accumulates in embryos lacking *Hinfp*.

Taken together, loss of *Hinfp* generates defects in cell growth and cell survival, causes DNA damage and reduces histone H4 gene expression. Because the resulting depletion of histone H4 proteins is expected to reduce nucleosomal spacing, while invoking global replicative stress (Ghule et al., 2014), the reduction of cells in *Hinfp* null embryos is attributable to delays in cell cycle progression and cell proliferation.

3.3. *Hinfp expression is maternally inherited during early embryonic development*

Evaluation of E3.5 embryos revealed that a limited degree of cell proliferation is maintained for the first few days of embryogenesis (Figs. 1–3) in the absence of zygotic expression of functional *Hinfp* and severely reduced histone H4 mRNA levels. Therefore, we investigated how cell proliferation is sustained in Hinfp null embryos during early stages of development. *LacZ* expression driven by the native *Hinfp* promoter in both Het and null embryos was analyzed by β-galactosidase staining in E3.5 stage embryos isolated from $Hinfp^{Lacz/+}$ crosses. All embryonic offspring from these crosses, including wild type embryos, exhibit positive staining for β-galactosidase (Supplementary Fig 1D). Because oocytes can transmit cytoplasmic mRNAs, our results suggest that the maternal *Hinfp* allele is producing *lacZ* mRNAs during oogenesis in heterozygous females and that these transcripts as well as proteins are maternally inherited by the resulting zygote.

To validate the hypothesis that *Hinfp* driven *lacZ* RNA or protein (β-galactosidase) is maternally inherited, we monitored β-galactosidase activity in E3.5 embryos from either Het male with WT female crosses, or WT male with Het female crosses. Analysis of β-galactosidase activity revealed that 100% of embryos generated by $Hinfp^{LacZ/+}$ females stain positive (Fig. 4A, upper panels) although, WT embryos show less intense staining (see also Supplementary Fig 1D). In contrast, only ~50% of embryos generated by WT females exhibit β-galactosidase activity (Fig. 4A, lower panels). Because spermatids do not transmit mRNAs, only half the embryos are expected to inherit the paternal LacZ allele. Taken together, these results indicate that *Hinfp*-driven β-galactosidase activity observed in WT blastocysts generated by Het x Het and WT x Het crosses is inherited maternally.

We also analyzed Het x WT crosses to indirectly assess inheritance of maternal *Hinfp* during early embryonic development at the 2-cell, 4-cell and 8-cell stages (E1.5 to E2.5). All embryos generated by WT male with Het female crosses stained positive for β-galactosidase activity at any development stage (Fig. 4B, upper panels). In contrast, none of the embryos generated by Het male with WT female crosses stained positive for β-galactosidase at the 2-cell stage, while 50% of the 4-cell and 8-cell stage embryos showed β-galactosidase activity (Fig. 4B, lower panels). These findings demonstrate that zygotic expression of *Hinfp* (as reported by β-galactosidase activity) is initiated between the 2- and 4-cell stages of development.

Maternal expression of *Hinfp*-driven *lacZ* expression predicts that the *Hinfp* gene actively produces transcripts during oogenesis. Indeed, analysis of β-galactosidase activity in ovaries from both WT and Het animals reveals intense nuclear staining that reflects

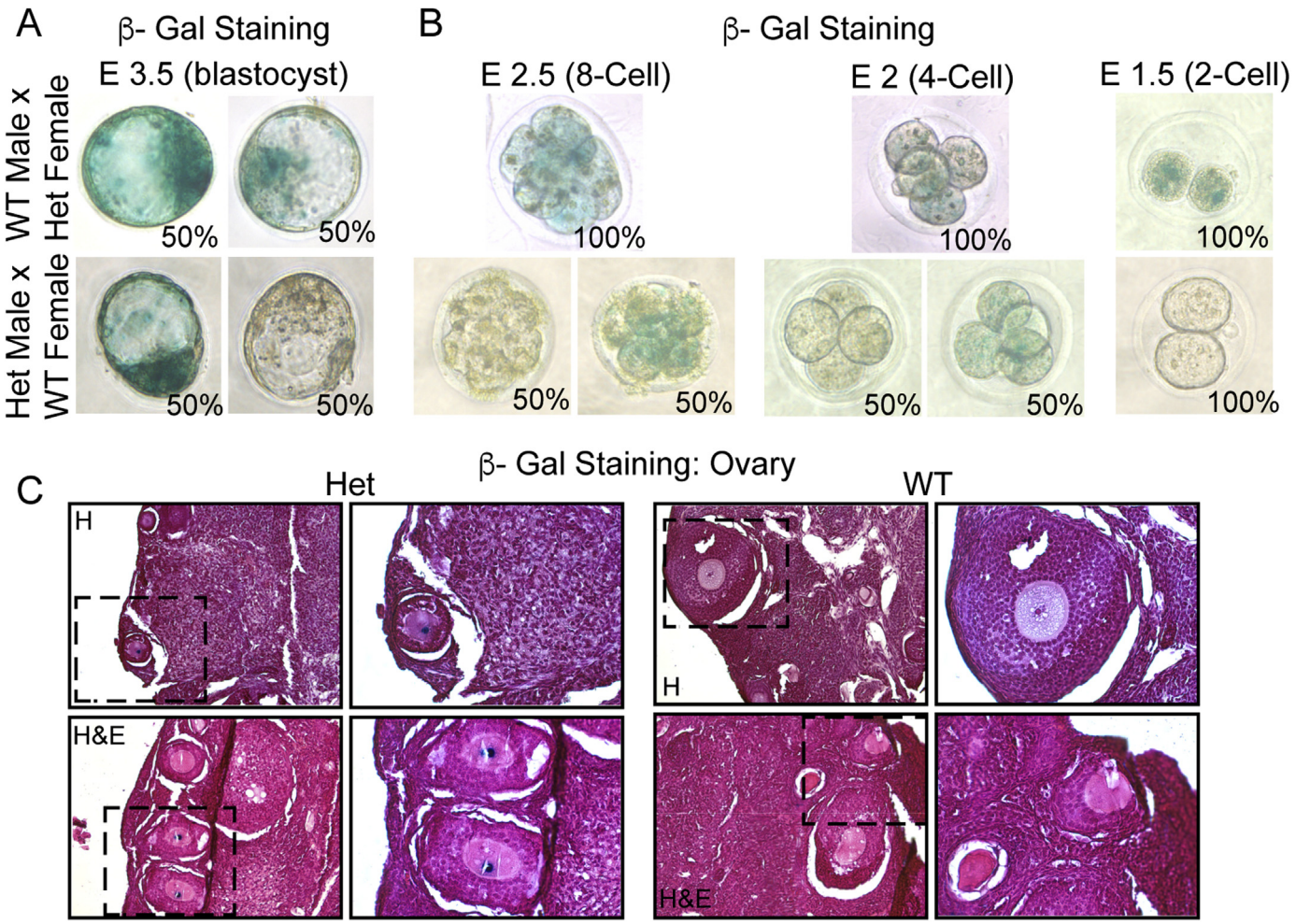

Fig. 4. Hinfp is maternally inherited during early embryonic development. (A) Representative images of E3.5 stage embryos isolated from WT X Het mating and stained for *lacZ* expression. All embryos isolated from Het female stained positive for β-galactosidase. (B) Representative images of early stage embryos (E2.5-E1.5) isolated from WT X Het mating showing β-galactosidase activity. Zygotic expression of *Hinfp*-driven *lacZ* is first detected at 4-cell stage. (C) Histological sections of WT and heterozygous ovaries are stained for β-galactosidase activity; sections were counterstained with either Hematoxylin (H) or Hematoxylin and Eosin (H&E).

lacZ expression in ova from Het but not WT ovaries (Fig. 4C). Collectively, our findings suggest that maternal Hinfp protein or Hinfp protein derived from maternally inherited Hinfp mRNA is present during the first few embryonic cell divisions, and is complemented by zygotic Hinfp expression. We conclude that the combination of maternal and zygotic expression of Hinfp protects embryos from histone deficiency and consequential effects on embryonic viability.

4. Discussion

4.1. Temporal onset of Hinfp and histone gene expression during early embryogenesis

This study examined the fundamental question how the essential histone H4 gene transcription factor Hinfp is regulated during the pre-blastocyst stage of mouse embryogenesis. This question is important because mammalian oocytes do not store histone proteins and contain sufficient mRNA to last for only one cell division (Graves et al., 1985; Wassarman, 1988; Zernicka-Goetz et al., 2009), yet histone H4 protein is essential for packaging DNA during the initial cleavage stages as well as throughout embryogenesis. We find that the *Hinfp* gene is active in oocytes (based on β-galactosidase staining) and that these maternal transcripts are transmitted to the zygote and remain detectable for several embryonic cell divisions. In contrast, transcripts from the paternal *Hinfp* gene are not detected until the 4-cell stage. Thus, zygotic expression of *Hinfp* from either the paternal or maternal allele is required at this very early stage of embryogenesis to support production of histone proteins to sustain embryonic viability. This finding is consistent with the well-established zygotic activation of genes, including histone genes, that occurs between the 2- and 8-cell stage (reviewed by Clarke (1992), Graves et al. (1985), Schier, (2007), Schultz (2002), Wassarman (1988) and Zernicka-Goetz et al. (2009).

4.2. Hinfp-dependent cell autonomous defects during early embryogenesis

We have previously shown that conditional loss of Hinfp in mouse embryonic fibroblasts has acute effects on chromatin integrity and cell viability, because Hinfp depletion causes H4 deficiency (Ghule et al., 2014). The immediate cellular consequences of H4 deficiency are structural changes in chromatin organization that predispose to DNA damage and aneuploidy (Ghule et al., 2015, 2014). These genomic changes ultimately generate cell cycle arrest and/or cell death. Our observation that *Hinfp* expression is induced at the 4-cell stage during early mouse embryonic development supports the importance of Hinfp as a developmentally critical protein and may explain the combined cellular effects of Hinfp loss. Mammalian cells apparently store enough histone mRNA for the first cell division (Graves et al., 1985; Schier, 2007; Schultz, 2002; Wassarman, 1988). We have previously shown that loss of Hinfp also affects some non-histone target genes involved in DNA damage response pathway (e.g. ATM, PRKDC and CKS2) (Medina et al., 2007) although most Hinfp target genes are histone H4 (Medina et al., 2012). Our findings collectively show that the combined effects of loss of Hinfp and failure to make new histone H4 RNAs result in increased phospho-H2AX staining as well as increased cleaved Caspase 3 activity in Hinfp null embryos. Our current study also supports the concept that zygotic expression of *Hinfp* mRNA from either the maternal or paternal allele is functionally required to promote production of new histone mRNAs and to avert catastrophic genomic defects when maternal pools of histone mRNA, *Hinfp* mRNA and Hinfp protein are depleted.

4.3. Comparisons of the null phenotypes for histone gene regulatory proteins

This study presents a detailed analysis of the early embryonic defects of *Hinfp* null mice. Based on the combined results from present and previous studies (Ghule et al., 2014; Liu et al., 2011; Xie et al., 2009), the embryonic phenotype of Hinfp deficiency can now be depicted as follows. The first noticeable defect in *Hinfp* null embryos is a delay in the development of blastocysts (E3.5) which exhibit a reduced cell number. This finding indicates that Hinfp deficiency compromises early cell proliferation. Null embryos exhibit alterations in the nuclear organization of Npat and Flash, display presence of activated Caspase 3 and accumulate DNA damage. E3.5 stage *Hinfp* null embryos have a hatching defect *in vitro* and in utero, and have compromised ability to implant to the uterine wall, although they are somehow able to initiate the decidual reaction. The latter defect clarifies the presence of uterine resorption pits and disappearance of *Hinfp* null embryos by E6.5 in dams carrying litters from heterozygous parents (Xie et al., 2009).

From a molecular perspective, production of mature histone H4 mRNAs requires a molecular complex in which promoter-bound Hinfp interacts with its co-activator NPAT and the latter interacts with the histone RNA processing co-factor FLASH. The established functions of these proteins and their co-localization at HLBs together indicate that they form a single dedicated subnuclear complex that generates and processes primary histone transcripts to produce mature non-polyadenyated histone mRNAs. Consistent with the importance of all three proteins as integral components of the same histone mRNA production unit, null mutations in *Hinfp*, *Npat* and *Flash* cause similar but subtly different defects in early embryogenesis. Null mutation of *Npat* causes embryonic lethality by arresting embryos at the uncompacted eight-cell stage (Di Fruscio et al., 1997). Hence, NPAT function in localizing the histone mRNA production machinery and bridging transcriptional components (Hinfp) with 3′ end RNA processing factors (FLASH) is indispensable when maternal storage pools of *Npat* mRNA and/or protein are depleted. Maternal storage of another regulatory protein involved in histone mRNA processing, stem-loop binding protein (SLBP), is also critically important for accumulation of histone H3 and H4 protein in oocytes (Allard et al., 2005; Arnold et al., 2008), emphasizing the importance of maternal pools of these key histone regulatory proteins.

For comparison, mice with null mutations in either *Flash*/*Casp8ap2* (De Cola et al., 2012; Minamida et al., 2014) or *Hinfp* (Xie et al., 2009) are also embryonic lethal, but have slightly delayed pre-implantation defects compared to *Npat* mutants. Null embryos for *Flash* or Hinfp can be recovered at E3.5, but perish soon thereafter (De Cola et al., 2012; Xie et al., 2009). Null mutants for *Flash* have embryonic defects in the morula stage (De Cola et al., 2012), and our present work reveals that *Hinfp* null embryos at E3.5 stage resemble morula stage embryos, which indicates delayed development and reduced cell number. There are multiple scenarios that could account for the delayed phenotypic manifestation of null mutations in the *Hinfp* and *Flash* genes compared to the *Npat* null mouse. First, the half-lives of the maternally produced mRNAs or the two proteins themselves could be much longer compared to *Npat*. The protein half-life for *Hinfp* has been measured in somatic cells and it is fairly long for a transcription factor (~8 h) (Medina et al., 2006). A similar half-life has been determined for NPAT provided that HINFP is present (Medina et al., 2006). Yet, these half-lives are short enough that protein levels would be reduced by at least 3–4 fold per cell division cycle. Therefore, other compensatory mechanisms may be operative. One plausible model is that functional compensation in histone protein synthesis is partially achieved by production of histone variant proteins that, first, are produced from genes that are

controlled independently of Hinfp, and second, that are translated from polyadenylated mRNAs and thus do not require the FLASH-containing histone RNA processing machinery.

5. Conclusion

This study shows that the histone gene transcription factor Hinfp is an essential developmental regulator of the earliest stages of embryogenesis. Importantly, we find that transcripts derived from the *Hinfp* gene are expressed in oocytes and maternally transmitted to the zygote to regulate histone gene expression and maintain the integrity of chromatin. Beyond this conceptual finding, Hinfp depleted embryos, which have severely decreased H4 mRNA levels, may represent a versatile experimental model for future testing early developmental consequences of histone H4 mutations that affect post-translational modifications.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.09.003.

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