GLP is critical for oogenesis exhibiting a G9A-independent role in transcriptional repression

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Abstract

GLP (EHMT1) functions as an H3K9me1 and H3K9me2 methyltransferase through its reportedly obligatory dimerization with G9A (EHMT2). Here, we investigated the role of GLP in oocyte and embryo development in comparison to G9A using oocyte-specific conditional knockout mouse models (*G9a* cKO, *Glp* cKO, *G9a-Glp* cDKO). Loss of GLP in oogenesis severely impairs oocyte maturation, fertilization and embryo development, resulting in lethality before embryonic day E12.5. In contrast, loss of G9A has a milder effect with a proportion of embryos producing viable offspring. The *Glp* cKO also showed loss of G9A protein and, hence, was phenotypically very similar to the *G9a-Glp* cDKO. H3K9me2 was equally depleted in all cKO genotypes, whereas H3K9me1 was decreased only in *Glp* cKO and *G9a-Glp* cDKO oocytes. Furthermore, the transcriptome, DNA methylome and proteome were markedly more affected in *G9a-Glp* cDKO than *G9a* cKO oocytes, demonstrating that in the absence of GLP there are widespread epigenetic and gene expression changes in the oocyte independent of H3K9me2. Gene dysregulation with coupled changes in DNA methylation suggest localised loss of chromatin repression, resulting in upregulated protein expression. Together, our findings demonstrate that GLP can function independently of G9A in the oocyte and is required for oocyte developmental competence.

Keywords

Oocyte, epigenetics, histone modification, histone methyltransferase, H3K9 methylation, DNA methylation, GLP/EHMT1

Introduction

The mammalian germline is the context for widespread epigenetic reprogramming, in which the somatic epigenetic signature is erased and replaced by a germline signature that is distinct in sperm and oocyte. Prior to the onset of gametogenesis, primordial germ cells undergo complete erasure of epigenetic marks, such as DNA methylation (Guibert et al. 2012; Seisenberger et al. 2012) and histone-3 lysine-9 dimethylation (H3K9me2) (Seki et al. 2005). In the oocyte, reprogramming occurs postnatally during oocyte growth, resulting in a unique DNA methylation and histone modification landscape (Hanna et al. 2018a). As the oocyte does not divide during these processes, the oocyte can serve as a tractable system to study epigenetic regulation. Importantly, resetting of epigenetic marks is essential to support successful oogenesis, oocyte-to-embryo transition, as well as embryonic development (e.g. Kaneda et al. 2004; Andreu-Vieyra et al. 2010; Eymery et al. 2016; Kim et al. 2016; Xu et al. 2019).

G9A (EHMT2) and GLP (EHMT1) are best known as histone methyltransferases, although they modify non-histone targets as well. They preferentially function as heterodimers *in vivo* and comprise the main H3K9 mono- and di-methyltransferases in euchromatin (Tachibana et al. 2001; Peters et al. 2003; Rice et al. 2003; Tachibana et al. 2005; Tachibana et al. 2008). Both proteins contain a SET-domain, required for their catalytic activity as methyltransferases and heterodimer formation, as well as an ankyrin repeat domain that enables binding to H3K9me1 and H3K9me2 (Tachibana et al. 2001; Collins et al. 2008). They have been shown to be interdependent, as H3K9me1 and H3K9me2 levels are equally depleted in *G9a* KO and *Glp* KO embryonic stem cells (ESCs) (Tachibana et al. 2005). Moreover, *G9a* and *Glp* KO mouse models show very similar phenotypes with embryos displaying loss of H3K9me2, growth retardation and embryonic lethality between embryonic day (E)8.5 and E12.5 (Tachibana et al. 2002; Tachibana et al. 2005). As such, studies often do not distinguish between G9A and GLP, or solely consider G9A, resulting in limited knowledge of GLP function. However, recent

findings indicate that GLP can act independently of G9A, revealing a role for GLP in targeting H3K27me2 to the paternal pronucleus in the zygote (Meng et al. 2020).

In the oocyte, loss of G9A impairs maturation and meiosis, with consequences for preimplantation development, leading to partial embryonic lethality (Au Yeung et al. 2019). However, some embryos lacking oocyte-derived G9A develop to term and result in healthy pups. The role of GLP in the oocyte and its impact on embryo development remain unclear. To investigate the importance of maternal GLP in the oocyte and embryo and compare its function to G9A, we used cKO mice for *G9a* and *Glp*, as well as a *G9a-Glp* cDKO. We compared the progression of oogenesis and embryogenesis in each cKO, and evaluated changes in H3K9 methylation, gene expression, DNA methylation and protein abundance in cKO oocytes. Our findings demonstrate that GLP has a unique and essential function in the oocyte.

Results

GLP and G9a function during oocyte maturation and meiosis

To analyse the function of GLP in oocytes and compare it to that of G9A, we generated three cKO models. Mice carrying floxed alleles for *G9a* (*G9a* cKO) (Sampath et al. 2007), *Glp* (*Glp* cKO) (Schaefer et al. 2009) or both *G9a* and *Glp* (*G9a-Glp* cDKO) were crossed with a *Zp3*-Cre driver, which is expressed exclusively in growing oocytes after postnatal day 5 (Lan et al. 2004). During oocyte maturation in the ovary, the germinal vesicle (GV) oocyte undergoes a change in chromatin conformation from a non-surrounded nucleolus (NSN) to a surrounded nucleolus (SN) stage that coincides with global transcriptional silencing. In control animals, immunofluorescence (IF) analysis showed that G9A and GLP are detected throughout the nucleus of NSN oocytes, but were no longer detectable by the mature SN stage (**Supplemental Fig. S1A,B**). In *G9a* cKO oocytes, G9A protein was lost in NSN oocytes, while GLP remained detectable (**Fig. 1A**). In contrast, *Glp* cKO oocytes were

depleted for both G9A and GLP, similar to *G9a-Glp* cDKO oocytes, indicating that GLP is required for G9A stability but not *vice versa*, as was previously shown in mouse ESCs (**Fig. 1A**) (Tachibana et al. 2005).

Loss of G9A has been shown to affect oocyte maturation and meiosis (Au Yeung et al. 2019). To assess whether loss of GLP has additional effects on developmental capacity of the oocyte, we first analysed the NSN-to-SN maturation rate by staining fully-grown GV oocytes with DAPI and staging them according to chromatin conformation. In *Glp* cKO and *G9a-Glp* cDKO oocytes from 12-week old females the proportion of SN oocytes was significantly lower than in *G9a* cKO and control oocytes (**Fig. 1B**), indicating that loss of GLP has a stronger effect on oocyte maturation than loss of G9A alone.

To determine whether GLP is required for meiosis, we analysed spindle conformation and chromosome alignment of ovulated metaphase II (MII) oocytes after hormonal stimulation. In all three cKO models, an increase in abnormal chromatin configuration and spindle alignment was observed compared to controls, although variation between mice was high and differences did not reach statistical significance (**Fig. 1C**). Many meiotic abnormalities were observed, ranging from chromosomes that were located together but not aligned ("aggregates"), chromosome alignments where one or several chromosomes were misaligned, to chromosomes scattered throughout the nucleus (**Supplemental Fig. S1C,D**). Spindle abnormalities included collapsed, mono- or multipolar spindles (**Fig. 1C** and **Supplemental Fig. S1C**). Taken together, the results show that loss of G9A has mild effects on oocyte maturation compared to the significant impairment caused by loss of GLP, while effects on meiosis were similar.

Loss of maternal GLP but not G9A results in prenatal developmental arrest

To assess whether loss of GLP also compromises competence of oocytes, we examined developmental progression of embryogenesis. E3.5 embryos were collected from cKO females naturally mated with

wildtype (WT) C57Bl/6BabR males and scored according to developmental stage. The majority of embryos from control females had reached the blastocyst stage (**Fig. 1D**). In comparison, fewer embryos derived from cKO oocytes progressed to blastocysts, with the proportion of maternal *G9a-Glp* cDKO blastocysts significantly reduced, and the proportion of maternal *G9a-Glp* cDKO embryos arrested at the 1-cell stage significantly increased.

To determine the stage of embryo arrest, embryos were collected from superovulated, naturally mated cKO females at E1.5 and cultured *in vitro* for 3 days until day E4.5. Already at E1.5 a difference was observed, all three cKO models having a higher proportion of unfertilized oocytes and 1-cell embryos than controls. The *G9a-Glp* cDKO again showed the strongest phenotype, having significantly fewer 2-cell embryos than controls (**Supplemental Fig. S2A**). Fertilized embryos (1- and 2-cell) were selected for *in vitro* culture: none of the embryos from *Glp* cKO or *G9a-Glp* cDKO oocytes developed to blastocysts, arresting at earlier stages (1 to 4-cell stage) by E3.5 (**Supplemental Fig. S2B,C**). In contrast, 42.9% maternal *G9a* cKO embryos developed at least to morulae by E3.5 (**Supplemental Fig. S2B,C**). However, this was significantly less than controls where 91.1% of embryos reached morula or blastocyst stages by E3.5. These findings reveal that embryos derived from *G9a* cKO oocytes show reduced survival through preimplantation development, whereas preimplantation developmental competence of *Glp* cKO and *G9a-Glp* cDKO oocytes was severely impaired.

A small percentage of embryos from *Glp* cKO oocytes did progress *in vivo* to blastocysts, therefore we examined the implantation and development of embryos after natural mating. In line with findings above, there were significantly fewer implanted embryos at E6.5 from *Glp* cKO and *G9a-Glp* cDKO oocytes than *G9a* cKO and controls (**Fig. 1E**). At E8.5, 3 maternal *Glp* cKO and 6 *G9a-Glp* cDKO embryos were recovered: all were highly abnormal, with no clear tissue types or only extraembryonic tissue (**Supplemental Fig. S2D,E**). In contrast, although some abnormalities (predominantly developmental delay, but also abnormal morphology) were observed among E8.5 embryos from *G9a* cKO oocytes, most appeared normal (**Supplemental Fig. S2D,E**). By E12.5, 55% (11/25) embryos from *G9a* cKO

oocytes were grossly abnormal or exhibited developmental delay (**Supplemental Fig. S2F,G**). In *G9a-Glp* cDKO females, with one exception, all embryos had died and only resorption sites were observed (**Supplemental Fig. S2F**).

Finally, we analysed the number of live pups born after mating cKO females with WT males: no pups were born to *Glp* cKO or *G9a-Glp* cDKO females, but *G9a* cKO females did give birth to a mean of 3.46 healthy pups per litter, although litter size was significantly reduced compared to controls (**Fig. 1F**). These results confirm previous findings in showing that although loss of G9A in the oocyte affects developmental capacity of pre- and post-implantation embryos, some embryos develop normally resulting in birth of healthy pups (Au Yeung et al. 2019). Our results show loss of GLP in the oocyte severely impairs embryonic development and although a small proportion of embryos reach the blastocyst stage and implant they die *in utero* between E8.5 and E12.5.

Differential effects of loss of GLP or G9A on H3K9 methylation

GLP and G9A are H3K9 methyltransferases required for establishment of H3K9me1 and H3K9me2 in euchromatin (Tachibana et al. 2001; Tachibana et al. 2002; Tachibana et al. 2005). Therefore, we examined H3K9 methylation in NSN-stage GV oocytes from cKO females (12 weeks) by IF. Consistent with previous reports (Peters et al. 2003; Rice et al. 2003), H3K9me1 and H3K9me2 were localized mainly in euchromatic chromatin of NSN oocytes, whereas H3K9me3 was enriched in heterochromatic foci (**Fig. 2A**). Fluorescence intensity of H3K9me1 was significantly reduced in *Glp* cKO and *G9a-Glp* cDKO oocytes compared to controls, but not in *G9a* cKO oocytes; in contrast, H3K9me2 decreased significantly in all three cKOs (**Fig. 2A,B**). A small but significant loss of H3K9me3 was observed in *G9a-Glp* cDKO oocytes (**Fig. 2A,B**). Rather than a direct effect, this may result from loss of H3K9me1, as the H3K9me3 methyltransferase SUV39H requires H3K9me1 as a substrate (Pinheiro et al. 2012).

With H3K9me1 and H3K9me2 affected by loss of G9A and/or GLP, we sought to evaluate the genomic localisation of these marks by ultra low-input native ChIP-seq (ULI-nChIP-seq) in WT GV oocytes from 15 and 25-day old females. We were able to obtain quality ChIP-seq libraries for H3K9me2 (**Fig. 2C**), but not for H3K9me1. By quantifying 10kb running windows, we observed a reproducible H3K9me2 enrichment between replicates from d15 and d25 WT oocytes, with d25 showing greater enrichment than d15 GV oocytes (**Fig. 2C, Supplemental Fig. S3A**). H3K9me2-enriched domains in d25 GV oocytes were defined by merging consecutive 10kb windows with a $\text{Log}_2\text{RPKM} > 2.5$ (34,192 or 12.5% of total 10kb windows; 12,514 domains; **Fig. 2C, Supplemental Fig. S3B; Supplemental Table S1**). To link H3K9me2 enrichment to transcription levels, we used published data to define CpG-island (CGI) and non-CGI promoters of low ($\text{FPKM} < 0.1$), medium ($\text{FPKM} 0.1-1.0$) and high ($\text{FPKM} > 1.0$) expressed genes (Veselovska et al. 2015), and then compared the overlap of these promoters with H3K9me2-enriched or random domains (**Supplemental Fig. S3C**). There was no difference in transcription level between genes localised to H3K9me2-enriched domains or random domains, thus H3K9me2 does not appear to act as a transcriptional repressor in oocytes.

We then assayed H3K9me2 in replicates of *G9a* cKO, *G9a-Glp* cDKO, and littermate control d25 GV oocytes. Notably, H3K9me2 libraries from control oocytes were 4.94% of total chromatin, whereas H3K9me2 libraries from *G9a* cKO and *G9a-Glp* cDKO oocytes were only 0.58% and 0.33%, respectively (**Fig. 2D**), confirming that H3K9me2 is almost completely absent from cKO oocytes. Principal component analysis (PCA) of all biological replicates showed that *G9a* cKO and *G9a-Glp* cDKO H3K9me2 replicates clustered apart from control, d15 WT and d25 WT H3K9me2 replicates (**Fig. 2E**). When comparing enrichment across H3K9me2-enriched domains ($\pm 5\text{kb}$), *G9a* cKO and *G9a-Glp* cDKO showed significant loss of H3K9me2 compared to controls, with enrichment comparable to that in the IgG control (**Fig. 2F**). Furthermore, this effect was specific to H3K9me2-enriched domains, as there was no observable difference in enrichment between cKOs and controls across a set of random domains (**Fig. 2F**). There was a similar loss of H3K9me2 in *G9a* cKO and *G9a-Glp* cDKO oocytes, supporting the IF results that G9A and GLP are both required for H3K9me2 in oogenesis.

Dysregulation of proteins associated with meiosis, fertilization and oocyte function in *G9a-Glp* cDKO oocytes

Beside their role as histone methyltransferases, G9A and GLP are known to methylate non-histone proteins and, by doing so, can potentially modulate their function, localisation or stability. To assess whether loss of GLP affects protein abundance in the oocyte, we performed low-input (200 oocytes) quantitative whole-proteome mass spectrometry isobaric labelling analysis of control, *G9a* cKO and *G9a-Glp* cDKO GV oocytes. In total, 21,358 peptides were detected at FDR<1% and 3,182 quantified proteins (**Supplemental Table S2**). Gene ontology (GO) analysis showed enrichment for processes involved in cellular localization and organization, as well as protein folding, metabolic processes and fertilization (**Fig. 3A**). Furthermore, among the top 50 most abundant proteins, we detected oocyte-specific proteins such as members of the subcortical maternal complex (PADI6, NLRP5, NLRP14, TLE6, KHDC3, NLRP4F), the zona pellucida (ZP1, ZP2, ZP3), as well as proteins known to be highly abundant in the oocyte (DNMT1, UHRF1).

Comparing *G9a-Glp* cDKO with control oocytes, we identified 187 proteins with a significant change in abundance ($P<0.05$ and $\text{Log}_2\text{FC}>0.3$; **Fig. 3B-D, Supplemental Table S2**). In contrast, there were only 38 differentially abundant proteins in *G9a* cKO oocytes, of which 21 overlapped with those in *G9a-Glp* cDKO oocytes. The majority of changing proteins increased in abundance in both *G9a* cKO (12 down, 26 up) and *G9a-Glp* cDKO oocytes (12 down, 175 up; **Fig. 3B,C**). Although few proteins were identified to have significant changes in abundance in *G9a* cKO oocytes, many proteins changing in *G9a-Glp* cDKO oocytes displayed the same directional trend in *G9a* cKO oocytes (**Fig. 3B**).

We detected five known G9A/GLP targets (ACIN1, DNMT1, DNMT3A, MTAL and RUVBL2): none of these were significantly regulated in cKO oocytes; this is not entirely unexpected as protein methylation does not necessarily influence abundance, but rather may affect activity. GO analysis did not detect any significant enrichment terms amongst the differentially abundant proteins. Even so,

we identified several proteins for which change in abundance may be related to the observed oocyte phenotypes. Among these, two were meiotic factors according to the MGI Gene Ontology Browser (CDC25B and SIRT1), while literature research linked a further seven (STAT3, TGON1, SET, IMPDH2, DSTN, SKA3, ROCK1) to meiosis in the oocyte (**Supplemental Table S2**). Furthermore, proteins involved in oocyte maturation (ERMP1) and fertilization (ASTL) showed significantly increased abundance, as well as oocyte transcription factor HMGB3. Increased abundance of these proteins may underlie the meiotic spindle abnormalities, impaired oocyte maturation and poor fertilization rates observed in *G9a-Glp* cKO oocytes. In line with observations in oocyte and embryo development, as well as H3K9 methylation, the more severe effect in *G9a-Glp* cKO oocytes suggests an additional role for GLP. The similar but lesser, non-significant changes in protein abundance observed in *G9a* cKO oocytes suggests that the function of GLP may be compensatory of G9A, rather than unique.

Loss of GLP results in local and distinct DNA methylation changes

G9A-mediated H3K9me2 has been linked to DNA methylation (Tachibana et al. 2008; Zeng et al. 2019), although genome-wide analysis only detected local DNA methylation changes in *G9a* cKO oocytes and *G9a* KO embryos (Auclair et al. 2016; Au Yeung et al. 2019). To examine whether loss of GLP affects DNA methylation establishment in the oocyte, we first analysed global DNA methylation (5mC and 5hmC) by IF. In NSN oocytes, 5mC was observed throughout the nucleus in both euchromatic and heterochromatic regions (**Fig. 4A**); 5hmC was enriched in heterochromatic foci but also present in euchromatin (**Fig. 4A**). No changes in localization of 5mC or 5hmC were observed in cKO oocytes. However, when assessing 5mC fluorescence intensity, there were significant decreases in the *Glp* cKO and *G9a-Glp* cKO compared to control oocytes, but not for *G9a* cKO oocytes (**Fig. 4B**). No significant changes in 5hmC levels were observed in cKO oocytes, although 5hmC levels also appear reduced in cKO oocytes. These data suggest that *de novo* DNA methylation may be impaired upon loss of GLP but not G9A in oocytes.

We then explored changes in DNA methylation in greater resolution by whole-genome bisulphite sequencing (BS-seq) of control, *G9a* cKO and *G9a-Glp* cDKO GV oocytes. In contrast to decreased 5mC seen by IF, BS-seq did not show significant global changes in DNA methylation (**Supplemental Fig. S4A**). An explanation for this apparent discrepancy is that the genomic regions assessed by the two methods differ: IF signal reports both euchromatic and heterochromatic fractions of the genome, while BS-seq data is enriched in euchromatic and non-repetitive regions that can be uniquely mapped and analysed. These differences highlight the value of using both methods to understand genome-wide patterns of DNA methylation and may explain previous contradictory findings (Au Yeung et al. 2019; Zeng et al. 2019).

Although global DNA methylation levels as detected by BS-seq did not differ, local changes in DNA methylation were analysed by binning the genome into consecutive windows of 100 CpG sites (100-CpG windows). Differential methylation analysis identified 9,187 DMRs in *G9a-Glp* cDKO oocytes (4.88% of all analysed 100-CpG windows), of which 4,184 (45.5%) were hypermethylated (mean methylation difference: 35.1%) and 5,003 (54.5%) hypomethylated (mean methylation difference: 25.6%) (**Fig. 4C; Supplemental Table S3**). Methylation was more affected in *G9a-Glp* cDKO than in *G9a* cKO oocytes, with >7 times more DMRs identified: there were 1,252 DMRs (0.67% of all analysed 100 CpG windows) in the *G9a* cKO, of which 432 (34.5%) were hypermethylated (mean methylation difference: 39.4%) and 820 (65.5%) hypomethylated (mean methylation difference: 33.8%) (**Supplemental Fig. S4B**). Importantly, the majority of *G9a* cKO DMRs overlapped *G9a-Glp* cDKO DMRs (**Supplemental Fig. 4C**). This indicates that while both G9A and GLP are required for normal DNA methylation establishment in the oocyte, GLP can partially compensate for loss of G9A, but it does not exclude the possibility that GLP has an additional G9A-independent role in DNA methylation.

To assess this possibility in more detail, we analysed the regions of the genome affected by DNA methylation changes. While hypermethylated DMRs show similar overlap with genic and intergenic regions, hypomethylated DMRs are enriched in genic regions (**Supplemental Fig. S4D**). This is

expected, as hypomethylated DMRs are found in regions that are methylated in control oocytes and it is well established that DNA methylation localises to transcribed genes in oocytes (Veselovska et al. 2015). To assess whether DNA methylation changes correlate with underlying histone modifications, we tested the overlap of DMRs with H3K9me2, H3K27me3 and H3K4me3 (**Fig. 4D**). The majority of DMRs did not overlap regions with H3K9me2 and there was no clear distinction between hyper- and hypomethylated DMRs, indicating that most DNA methylation changes in cKO oocytes are unlikely to be a direct consequence of loss of H3K9me2. Interestingly, hypermethylated DMRs were strongly enriched for H3K27me3, compared to hypomethylated DMRs. Importantly, this enrichment was not seen for H3K4me3, although both are enriched in regions of the genome lacking DNA methylation. Because H3K27me3 and DNA methylation are mutually exclusive, this result may indicate a localised redistribution of the two marks in cKOs.

Since G9A and GLP can interact with UHRF1 (Kim et al. 2009; Ferry et al. 2017), we also investigated the overlap of DMRs with regions that are hypomethylated in *Uhrf1* cKO oocytes (Maenohara et al. 2017). *Uhrf1* cKO oocytes show widespread hypomethylation, and we observed that both *G9a* cKO and *G9a-Glp* cDKO hypomethylated DMRs strongly overlap with *Uhrf1* hypomethylated regions compared to random and random methylated probes (**Fig. 4E**). These data support that loss of G9A and GLP may disturb a subset of UHFR1-mediated methylation.

Between 21 and 38% of 100-CpG windows identified as DMRs cluster and form larger regions that, in both genotypes, can span genes (**Fig. 4E, Supplemental Fig. S4E-G and Supplemental Table S3**). In total, there were 707 hypo- and 869 hyper-methylated domains in *G9a-Glp* cDKO oocytes, with fewer in the *G9a* cKO (140 hypo- and 66 hyper-methylated domains). To see whether the additional changes in the *G9a-Glp* cDKO are unique or whether similar (non-significant) trends are present in the *G9a* cKO, we performed unsupervised cluster analysis of DNA methylation of the cDKO domains (**Fig. 4F**). For both hypo- and hypermethylated domains, we identified domains common to both cKOs (523 hypo, 590 hyper) and domains unique to the *G9a-Glp* cDKO (229 hypo, 279 hyper; **Fig. 4F,G**;

Supplemental Fig. S4E-G). This indicates that the majority of effects seen in the *G9a-Glp* cDKO are also present in the *G9a* cKO, although to a lower, often non-significant magnitude. Furthermore, there appears to be a potentially unique role of GLP in oocyte DNA methylation, although this is likely to be minor compared to its function compensatory to loss of G9A.

Transcriptome changes direct DNA methylation and protein changes in *G9a-Glp* cDKO oocytes

We evaluated gene expression changes by RNA-seq in *G9a-Glp* cDKO, *G9a* cKO and matched control GV oocytes. Differential gene expression was determined by DESeq2 analysis followed by filtering for genes with Log₂FC>1.5. The vast majority of differentially expressed genes (DEGs) were upregulated in the *G9a-Glp* cDKO. Again, *G9a-Glp* cDKO oocytes were more severely affected than *G9a* cKO oocytes, with 330 DEGs (301 up, 29 down; **Fig. 5A, Supplemental Table S4**), in contrast to 79 DEGs in *G9a* cKO oocytes (64 up, 15 down; **Supplemental Fig. S5A**). Of the *G9a* cKO DEGs, 51 overlapped with *G9a-Glp* cDKO DEGs. Clustering analysis identified three clusters: 1) downregulated in both *G9a* cKO and *G9a-Glp* cDKO; 2) upregulated in both genotypes; and 3) uniquely upregulated in *G9a-Glp* cDKO (**Fig. 5B**). GO analysis did not show significant category enrichments, but amongst the deregulated transcripts we identified several transcription factors with a known function in the oocyte (*Prmt7*, *Etv5*), zygotic genome activation (*Zscan4d*) and embryo development (*Klf4*, *Hoxd1*, *Lmx1a*; **Supplemental Table S4**). Furthermore, several genes important for oocyte maturation, meiosis and fertilization were deregulated (*Atrx*, *Fgfr2*, *Prkcq*, *Ptgs2*, *Plac1*, *Mt1*), which may underlie some of the phenotypic effects we see.

Both our proteome and transcriptome analysis showed a preferential upregulation of protein and transcript abundance in *G9a-Glp* cDKO oocytes, respectively. To assess whether changes in transcript expression may be causative for changes in protein abundance, we used gene set enrichment analysis (**Fig. 5C**). Indeed, proteins with increased abundance were enriched for transcriptionally upregulated genes in *G9a-Glp* cDKO oocytes, whereas proteins with decreased abundance were enriched for

downregulated genes, although this may not account for all the variation in protein abundance. This indicates that loss of GLP results in deregulated gene expression, which in turn affects abundance of the corresponding proteins. Hence, although the majority of genes appear unaffected by loss of GLP, the transcriptional changes that do occur are likely to be of biological significance.

Using the DEG clusters, we assessed the overlap of DEGs with the distribution of histone modifications (**Supplemental Fig. S5B**). There was no significant enrichment for any of the clusters with H3K9me2, further supporting that H3K9me2 does not correlate with transcriptional repression in the oocyte (**Supplemental Fig. S3C**). Interestingly, similar to hypermethylated DMRs, upregulated DEGs (clusters 2 and 3) were enriched in H3K27me3. As transcription is linked to the deposition of gene body DNA methylation in the mouse oocyte, we analysed the correlation between expression and DNA methylation changes. Consistently, expression changes of DEGs positively correlated with gene-body DNA methylation changes in *G9a-Glp* cDKO oocytes (**Fig. 5D**). In contrast, when evaluating genes with differential methylation, the correlation was much weaker (**Supplemental Fig. S5C**). These trends were similar in *G9a* cKO oocytes (**Supplemental Fig. S5D,E**). This shows that the transcriptional changes observed in *G9a-Glp* cDKO oocytes impact the associated genic DNA methylation; however, this association does not explain the majority of DNA methylation changes seen in *G9a-Glp* cDKO oocytes, thus other mechanisms likely underlie the majority of changes observed.

Discussion

Our present study shows that GLP can partially compensate for loss of G9A and additionally has a G9A-independent role in oogenesis. We find that GLP is required for oocyte maturation, meiosis and developmental competence. Few embryos derived from *Glp* cKO oocytes implant and those that do die mid-gestation. In contrast, maternal *G9a* cKO embryos are less affected and some develop normally into healthy pups. This difference in severity is also reflected at a molecular level in gene expression, protein abundance and DNA methylation. Importantly, the differences between the

genotypes are independent of GLP's function as an H3K9me2 methyltransferase, as H3K9me2 was equally ablated in all. Therefore, we propose that the phenotypes arise from multiple mechanistic changes in histone modifications, transcriptional regulation and DNA methylation, summarized in **Fig. 6.**

To consider the molecular mechanisms underlying the observed phenotypes, it is important to appreciate that G9A and GLP are proteins with multiple functions. Although best known as histone methyltransferases, they can methylate and alter the function of non-histone proteins (Shinkai and Tachibana 2011; Scheer and Zaph 2017). Nevertheless, the strong correlation we observed between changes in transcript and protein abundance in *G9a* and *G9a/Glp* cKO oocytes supports that the changes in protein abundance are likely attributable to altered genomic regulation rather than G9A/GLP directly modulating protein stability. Loss of G9A and GLP resulted in transcriptional upregulation, supporting previous reports that G9A/GLP act as repressors (Tachibana et al. 2002; Tachibana et al. 2005). It is unlikely that this can be attributed mostly to loss of H3K9me2, because only a fraction of genes was upregulated despite almost complete ablation of H3K9me2, and because of the significant transcriptional differences between the *G9a* cKO and *G9a-Glp* cDKO despite similar deficits in H3K9me2. Alternatively, the effects may be mediated indirectly by G9A/GLP modulating activity of transcriptional repressors or through other chromatin changes. We also observed upregulation of some developmental transcription factors, suggesting that some gene expression changes reflect illegitimate expression of such factors in oocytes. The more extensive transcriptional deregulation in the *G9a-Glp* cDKO is associated with impaired developmental capacity of the oocyte, resulting in decreased oocyte maturation, meiotic spindle abnormalities, impaired fertilization and abnormal embryo development.

G9A and GLP have been linked to DNA methylation in multiple studies. In the embryo, loss of G9A leads to hypomethylation of a subset of CGI promoters (Auclair et al. 2016). In the oocyte, we and others do not see widespread hypomethylation, but instead local sites of hypo- and hypermethylation

(Au Yeung et al. 2019). A variety of mechanisms could cause these changes. In the oocyte, *de novo* DNA methylation requires transcription (Kobayashi et al. 2012; Veselovska et al. 2015). The transcriptional changes in *G9a* cKO and *G9a-Glp* cDKO oocytes correlate with DNA methylation changes, indicating that upregulated gene expression is responsible for some of the hypermethylation observed. However, the significant localisation of *G9a* cKO and *G9a-Glp* cDKO hypermethylated regions with H3K27me3 suggests that the loss of G9A/GLP may impair the repressive chromatin landscape in the oocyte, either through H3K9me2 or modulation of a transcriptional repressor. Conversely, a large subset of hypomethylated DMRs overlapped with regions that are hypomethylated in *Uhrf1* cKO oocytes (Maenohara et al. 2017). UHRF1 function has been associated with G9A and GLP in several ways, either by direct interaction (Kim et al. 2009) or indirect recruitment through H3K9me2/3 or LIG1 (Rothbart et al. 2012; Rothbart et al. 2013; Ferry et al. 2017). However, it is not clear how these mechanisms apply in the oocyte because of the absence of DNA replication. Although UHRF1 appears to be required for some genomic DNA methylation, loss of DNMT1 has only minor effects, largely through a role in ensuring symmetric methylation of *de novo* methylated CpGs (Shirane et al. 2013; Maenohara et al. 2017). There are likely other mechanisms besides transcriptional regulation and UHRF1 interactions at play in the *G9a* cKO and *G9a-Glp* cDKO oocytes. These include the possibility of a direct interaction of G9A and GLP with DNMT proteins. For example, G9A and GLP can dimethylate DNMT3A at lysine 44. Methylated DNMT3A can be bound by MPP8, which in turn binds automethylated GLP, resulting in a DNMT3A-MPP8-GLP silencing complex (Chang et al. 2011).

In contrast to previous reports (Tachibana et al. 2005), we saw that while H3K9me2 depends on both G9A and GLP, the persistence of GLP in the *G9a* cKO was sufficient to establish H3K9me1. Notably, loss of G9A and GLP did not completely eliminate H3K9me1, indicating that other methyltransferases, such as PRDM3 and PRDM16, may be active in the oocyte. It remains unclear whether the decrease in H3K9me1 may be (partially) causative for the transcriptional and DNA methylation changes: so far, H3K9me1 has not been associated with either but rather is proposed to be predominantly a substrate for H3K9 di- and trimethylation (Pinheiro et al. 2012).

It is difficult to distinguish between a compensatory effect and a unique role for GLP in the oocyte. When comparing transcription, protein and DNA methylation data from *G9a* cKO and *G9a-Glp* cDKO oocytes, some genes/proteins/regions are progressively affected while others only affected in *G9a-Glp* cDKO oocytes. The possibility of a unique, G9A-independent role for GLP is supported by a recent study in zygotes, where GLP interacts with PRC2 to establish H3K27me2 in the paternal pronucleus (Meng et al. 2020). In our models, the unique function was especially apparent in the transcriptome, where genes with the greatest fold-change were unique to the *G9a-Glp* cDKO. The upregulation seen in most DEGs was reflected in an upregulation of protein abundance in *G9a-Glp* cDKO oocytes, indicating that although only a relatively small proportion of genes is derepressed upon loss of G9A and GLP, the transcriptional changes are likely to have a functional impact in the oocyte. Indeed, we saw changes in gene expression and protein abundance of several genes related to oocyte maturation, meiosis and fertilization. Taken together, our results highlight GLP as a multi-functional protein involved in different regulatory processes in the oocyte. They show that it has a unique role that is critical for the developmental capacity of the oocyte and independent of G9A and its role as H3K9me2 methyltransferase.

Materials and methods

Sample collections

All experimental procedures were performed under licences issued by the Home Office (UK) in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by the Animal Welfare and Ethical Review Body at the Babraham Institute.

Oocytes and embryos were collected in M2 medium (Sigma-Aldrich, M7167) unless stated otherwise. GV and MII oocytes for IF analysis were collected from adult mice aged ~12 weeks and fixed in 2% PFA for 15 minutes. MII oocytes were collected after superovulation. Oocytes used for ChIP-seq, BS-seq

and RNA-seq were collected from ovaries of 22-26 day-old mice using a collagenase/trypsin digest. For ChIP-seq, 300 GV oocytes were collected in nuclear lysis buffer and pooled from two to four mice for each replicate. For BS-seq and RNA-seq, replicates were stored in RLT+ buffer (Qiagen). Each replicate comprised all oocytes collected from one mouse (75-200 GV oocytes). For whole-proteome analysis oocytes were collected from ovaries of 22-26 day-old mice. To avoid contamination with proteins/peptides from the medium, oocytes were collected by manual dissection of ovaries in protein-free L15 medium (Thermo Fisher Scientific, 31415029). ~200 GV oocytes were collected from two mice in parallel, washed 3 times in PBS with 1x cOmplete Protease Inhibitor Cocktail (Merck, 11697498001).

Preimplantation embryos were collected after natural mating of control and cKO females with C57BL/6BabR WT males at E0.5 or E3.5. Implantation was determined by counting the number of implantation sites in uteri at E6.5 after timed mating. To score postimplantation development, embryos were collected after natural mating at E8.5 and E12.5. For embryo culture, female mice were superovulated and embryos dissected in M2 medium at E1.5 after natural mating with WT males. Fertilized embryos (1-cell and 2-cell stage) were selected for culture in M16 medium (Sigma-Aldrich, MR-016-D) under mineral oil (Sigma-Aldrich, M8410) at 37°C and 5% CO₂ for 3 days, and developmental progress recorded each day.

Immunofluorescence analysis

IF was performed after antibody staining as previously described (Santos et al. 2003). Primary antibodies are listed in **Supplemental Table S5**. Samples were analysed on a Zeiss LSM780 confocal microscope (63x oil-immersion objective). Single optical sections were captured and semi-quantification of fluorescence intensity was performed using Volocity 6.3 (Improvision).

LC-MS proteome analysis

Oocytes were lysed in 20µl dissolution buffer containing 100mM triethylammonium bicarbonate (Sigma, T4708) and 0.1% Sodium Dodecyl Sulfate (SDS), followed by water bath sonication and boiling at 90°C for 5min. Proteins were reduced with tris-2-carboxyethyl phosphine (TCEP, Sigma) for 1h at 60°C at a final concentration of 5mM, followed by cysteine blocking for 10min at room temperature using methyl methanethiosulfonate (MMTS, Sigma) at final concentration of 10mM. Samples were digested overnight at 37°C with trypsin (Pierce #90058) and the next day peptides were labelled with TMT11plex reagents (0.4mg per sample) according to manufacturer's instructions (Thermo Scientific). To quench the reaction, 3µl of 5% hydroxylamine (Thermo Scientific) was added for 15min and samples combined and dried with centrifugal vacuum concentrator. The TMT mix was fractionated with Reversed-Phase spin columns at high pH (Pierce #84868). Nine fractions were collected using different elution solutions in the range of 5–50% ACN and were analysed on a Dionex UltiMate 3000 UHPLC system coupled with the nano-ESI Fusion-Lumos (Thermo Scientific) mass spectrometer. Samples were loaded on the Acclaim PepMap 100, 100µm × 2cm C18, 5µm, 100Å trapping column with the ulPickUp injection method at loading flow rate 5µl/min for 10 min. For peptide separation, the EASY-Spray analytical column 75µm × 25cm, C18, 2µm, 100 Å column was used for multi-step gradient elution. Full scans were performed in the Orbitrap in the range of 380-1500 m/z at 120K resolution and peptides isolated in the quadrupole with isolation window 1.2Th, HCD collision energy 38% and resolution 50K. Raw data were processed with the SequestHT search engine in Proteome Discoverer 2.1 software and searched against a Uniprot database containing mouse reviewed entries. The parameters for the SequestHT node were: Precursor Mass Tolerance 20ppm, Fragment Mass Tolerance 0.02Da, Dynamic Modifications were Oxidation of M (+15.995Da), Deamidation of N, Q (+0.984Da) and Static Modifications were TMT6plex at any N-Terminus, K (+229.163Da) and Methylthio at C (+45.988Da). The consensus workflow included TMT signal-to-noise (S/N) calculation and the level of confidence for peptide identifications was estimated using the Percolator node with decoy database search. Strict FDR was set at q-value<0.01. For downstream data analysis, the R package qPLEXanalyzer was used (Papachristou et al. 2018). The mass spectrometry proteomics data

have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al. 2019) with the dataset identifier PXD030265.

Preparation of sequencing libraries

ChIP-seq libraries were prepared using ULI-nChIP as previously described (Hanna et al. 2018b). Antibodies were added at 250ng/reaction for both anti-H3K9me2 (mouse monoclonal, Abcam, ab1220) and anti-IgG (rabbit polyclonal, Diagenode, EB-070-010). Library preparation was completed with a MicroPlex Library Preparation kit v2 (Diagenode) with Sanger 8-base indices for multiplexing. Relative enrichment over input was quantified using the library concentrations determined by Bioanalyzer High Sensitivity DNA Analysis (Agilent). Low input bisulphite (BS)-seq libraries were prepared by post-bisulphite adapter tagging as previously described (Hanna et al. 2018b). RNA-seq libraries were prepared as described (Hanna et al. 2019).

Sequencing and data processing

Libraries were sequenced on Illumina MiSeq, HiSeq2500 and NextSeq500 systems. ChIP-seq libraries were sequenced to an average of 57 million paired-end reads of 75 bp read-length (**Supplemental Table S5**). BS-seq libraries were sequenced to an average of 16 million paired-end reads for *G9a* cKO and 26 million reads for *G9a-Glp* cDKO oocytes of 100-125 bp read-length. RNA-seq libraries were sequenced to an average single read number of 1.9 million for *G9a* cKO and *G9a-Glp* cDKO oocytes of 50 bp read-length. Raw fastq files were processed with Trim Galore, then mapped to the mouse GRCm38 genome. Mapping of ChIP-seq data was done using Bowtie2, RNA-seq data by Hisat2 v2.1.0 guided by known splice sites, BS-seq data with Bismark v0.19.0.

Sequencing data analysis

Sequencing data analysis was conducted using SeqMonk (<https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/>). For ChIP-seq analysis, 10kb running windows ($N=272,566$) were quantified as reads per kilobase per million (RPKM). Windows

were filtered to exclude mapping artefacts, defined as RPKM>6 in at least one replicate set of 10% input libraries ($N=408$). H3K9me2 enrichment was defined as $\text{Log}_2\text{RPKM}>2.5$ in d25 GV oocytes ($N=34,192$). A set of random windows was sampled from all 10kb windows ($N=35,000$). H3K9me2-enriched and random windows were then merged with adjacent windows within 10kb, resulting in 12,154 H3K9me2-enriched domains and 26,077 random domains. Genic and intergenic regions were defined as overlapping or not overlapping oocyte transcripts, respectively, and promoters were defined as $\pm 500\text{bp}$ around transcription start sites of oocyte transcripts (Veselovska et al. 2015). CGIs were defined as previously described (Illingworth et al. 2008). Oocyte transcription levels were categorized into not expressed ($\text{FPKM}<0.1$), low expressed ($\text{FPKM } 0.1-1$) and high expressed ($\text{FPKM}>1$) using published data (Veselovska et al. 2015).

BS-seq data were analysed using a tile-based approach of 100 CpGs for each consecutive genome window, ensuring equal CpG content in all windows analysed. Methylation values were quantified using the bisulphite-sequencing pipeline quantification, which calculates per-base methylation percentages and averages these within each window. Filters were applied to ensure a minimum coverage of ≥ 10 observed cytosines per window. Only windows with this minimal coverage in all samples were taken into account, allowing assessment of 86.2% of 100 CpG windows ($N=188,433$). Differentially methylated regions (DMRs) were defined by statistical comparison of DNA methylation levels for each 100 CpG window between control and *G9a* cKO or control and *G9a-Glp* cDKO oocytes using the EdgeR function in SeqMonk. To assess overlap of DMRs with genomic features, CGI and oocyte gene annotations were used from (Veselovska et al. 2015). DMRs were merged to form differentially methylated domains (DMDs). Hierarchical clustering analysis of DMDs was performed in R using Euclidean distance and Ward's agglomeration method as implemented by the package pheatmap.

For analysis of RNA-seq data, expression of oocyte genes (Veselovska et al. 2015) was quantified using log transformed read count quantitation per million reads. Differential gene expression was analysed

using DESeq2 followed by filtering of genes with $\text{Log}_2\text{FC} > 1.5$. Clustering analysis of DEGs was performed in R using Euclidean distance and Ward's agglomeration method. Relative enrichment of differentially abundant proteins amongst DEGs was displayed using the barcode method from the Limma R package. The ranked list of expression changes only considered genes corresponding to proteins identified in the proteome. Spearman's rank correlation coefficient between DNA methylation difference and expression log_2FC was used to interrogate the relationship between DNA methylation and transcriptional changes.

Sequencing data were submitted to the GEO under accession number GSE191026. Publicly available ChIP-seq and BS-seq data under accessions GSE93941, DRA005849 were used to develop our studies.

Statistical analysis

Statistical analysis was conducted in Graphpad Prism, R and VassarStats. SN proportion was compared using two-way ANOVA (Stage x Genotype) with Šídák's multiple comparisons test. Proportion of embryo stage at E3.5, number of implantation sites at E6.5 and mean fluorescence intensity of confocal IF images were analysed using one-way ANOVA with Tukey's posthoc test. Litter sizes were compared using a nested one-way ANOVA with mouse as nested factor. A χ -square test was used to analyse frequency of overlap of genic/intergenic regions with DMRs, histone modifications with DMRs, Uhrf1 hypomethylated regions with DMRs and DEGs with histone modifications. *P*-value was adjusted using Bonferroni correction to control for multiple comparisons.

Figure legends

Figure 1. Developmental potential of *G9a* cKO, *Glp* cKO and *G9a-Glp* cDKO oocytes.

A) Representative images showing IF for G9A and GLP in NSN oocytes. Scale bar: 20 μm .

B) Stacked bar chart showing percentage of NSN, intermediate and SN oocytes from mice aged 12 weeks. Number of mice: Control=5, *G9a* cKO=3, *Glp* cKO=2, *G9a-Glp* cDKO=3. Ctrl vs. *G9a* cKO:

$P=0.7667$; Ctrl vs *Glp* cKO: $P=0.0075$; Ctrl vs *G9a-Glp* cDKO: $P<0.0001$; *G9a* cKO vs *G9a-Glp* cDKO $P=0.0058$.

- C)** Bar charts showing percentage of chromosome misalignments and spindle abnormalities in MII oocytes. Dots represent datapoints from single mice. Examples of normal and abnormal spindles are shown in IF images below. *G9a* cKO: $P=0.7037$; *Glp* cKO: $P=0.0678$; *G9a-Glp* cDKO: $P=0.0670$.
- D)** Stacked bar chart showing developmental stage (%) of embryos from cKO females mated with WT males and collected on E3.5. Number of mice: Control=5, *G9a* cKO=5, *Glp* cKO=6, *G9a-Glp* cDKO=6. Blastocysts: Ctrl vs *G9a* cKO $P=0.9939$; Ctrl vs *Glp* cKO $P=0.0761$, Ctrl vs *G9a-Glp* cDKO $P=0.0073$; One-cell stage: Ctrl vs *G9a-Glp* cDKO $P=0.0406$.
- E)** Bar chart showing number of implantation sites scored at E6.5. Dots represent single mice. Ctrl vs *Glp* cKO $P=0.0093$; Ctrl vs *G9a-Glp* cDKO $P=0.0020$.
- F)** Boxplots showing average litter size of females with cKO oocytes mated with WT males. Number of mice: Control=9, *G9a* cKO=5, *Glp* cKO=3, *G9a-Glp* cDKO=3; Number of litters: Control=60, *G9a* cKO=26, *Glp* cKO=0, *G9a-Glp* cDKO=0; Ctrl vs *G9a* cKO: $P=0.0087$.

Figure 2. Analysis of H3K9 methylation in *G9a* cKO, *Glp* cKO and *G9a-Glp* cDKO oocytes.

- A)** Representative IF images of NSN oocytes for H3K9me1, H3K9me2 and H3K9me3 in control, *G9a* cKO, *Glp* cKO and *G9a-Glp* cDKO oocytes.
- B)** Boxplots of quantitation of IF images. Dots represent individual oocytes. Analysis is based on 2-5 mice per genotype and 3-4 replicate experiments for each antibody.
- C)** Genome screenshot showing H3K9me2 enrichment as determined by ChIP-seq in WT oocytes from mice aged 15 and 25 days. Y-axis scaling represents RPKM. Annotation tracks highlight oocyte transcripts, CpG islands, oocyte DNA methylated and unmethylated domains, and H3K9me2-enriched domains ($\text{Log}_2\text{RPKM}>2.5$ in d25 GV oocytes).
- D)** Boxplots showing relative enrichment over input of H3K9me2 ChIP-seq libraries. Dots represent individual samples.

E) PCA plot of H3K9me2 (blue shading), input (green shading) and IgG (yellow shading) ChIP-seq libraries, comparing WT (d15 and d25), control, *G9a* cKO and *G9a-Glp* cDKO samples.

F) Probe trend plot showing loss of H3K9me2-enrichment in *G9a* cKO and *G9a-Glp* cDKO oocytes compared to WT and control oocytes in H3K9me2-enriched domains but not random domains.

Figure 3. Proteome analysis of *G9a* cKO and *G9a-Glp* cDKO oocytes.

A) GO analysis of the oocyte proteome.

B) Volcano plot showing differential abundance of proteins in *G9a* cKO oocytes compared to controls. Significantly changing proteins ($P < 0.05$ and $\text{Log}_2\text{FC} > 0.3$) are highlighted in dark blue (decreased abundance) or dark red (increased abundance); light blue and light red dots indicate proteins changing significantly in cDKO oocytes.

C) Volcano plot showing differential abundance of proteins in *G9a-Glp* cDKO oocytes compared to controls. Significantly changing proteins ($P < 0.05$ and $\text{Log}_2\text{FC} > 0.3$) are highlighted in dark blue (decreased abundance) or dark red (increased abundance). Light blue and light red dots indicate proteins changing significantly in *G9a* cKO oocytes. Proteins changing significantly and with oocyte function of interest are labelled.

D) Venn diagram of significant changing proteins in *G9a* cKO and *G9a-Glp* cDKO oocytes.

Figure 4. DNA methylation changes in *G9a* cKO and *G9a-Glp* cDKO oocytes.

A) Representative IF images of NSN oocytes showing 5mC and 5hmC in Control, *G9a* cKO, *Glp* cKO and *G9a-Glp* cDKO oocytes.

B) Boxplots of quantitation of IF images. Dots represent individual oocytes. Analysis is based on 2 mice per genotype and 2 replicate experiments for each antibody.

C) Scatterplot showing methylation levels of 100-CpG windows in control vs *G9a-Glp* cDKO oocytes. Unique cDKO DMRs are highlighted in blue, DMRs shared with *G9a* cKO oocytes in red.

D) Barchart showing percentage overlap of DMRs and random probes with H3K9me2, H3K37me3 and H3K4me3. H3K9me2: *G9a* cKO hyper *adj. P*=0.0136, *G9a* cKO hypo *P*<0.0001, *G9a-Glp* cDKO hypo *P*<0.0001; H3K27me3: *G9a* cKO hyper *P*<0.0001, *G9a-Glp* cDKO hyper *P*<0.0001; H3K4me3: all genotypes *P*<0.0001.

E) Barchart showing percentage overlap of DMRs with 100-CpG windows that are hypomethylated in *Uhrf1* cKO oocytes (>20% methylation difference, (Maenohara et al. 2017)), random 100-CpG windows and 100-CpG windows that are highly methylated (>75%) in WT and *Uhrf1* cKO oocytes. *G9a* cKO hyper *adj. P*=0.0404, *G9a-Glp* cDKO hyper, *G9a* cKO hypo and *G9a-Glp* cDKO hyper *P*<0.0001.

F) Genome screenshot showing example of a region uniquely hypomethylated in *G9a-Glp* cDKO oocytes spanning the *Arhgef10l* transcript. Annotation tracks show position of H3K9me2-enriched domains and oocyte transcripts.

G) Heatmap showing clustering analysis of *G9a-Glp* cDKO hypo- and hypermethylated domains.

Figure 5. Transcription changes in *G9a-Glp* cDKO oocytes in relation to protein and DNA methylation changes.

A) Scatterplot showing expression levels in control vs. *G9a-Glp* cDKO oocytes. Unique cDKO DEGs are highlighted in blue, DEGs with Log₂FC>1.5 in red and DEGs shared with *G9a* cKO oocytes in green.

B) Heatmap showing relative expression levels (RPKM) of up- and down-regulated *G9a-Glp* cDKO DEGs that overlap with *G9a* cKO DEGs.

C) Plot showing link between changes in transcript and protein abundance. The X-axis shows Log₂FC (Control vs. *G9a-Glp* cDKO) of genes present in both transcriptome and proteome datasets (*N*=2933). Blue and red boxes represent down- and up-regulated transcripts, respectively, with a FC>1.5 (Log₂FC>0.585). Vertical blue and red lines represent differentially abundant proteins (*P*<0.05 and Log₂FC>0.3; ten proteins not detected in the RNA-seq data are not represented). Enrichment scores, shown above and below, show that downregulated proteins are enriched

among downregulated transcripts and upregulated proteins enriched among upregulated transcripts (Spearman correlation $R=0.43$; $P=2.2 \times 10^{-9}$).

D) Plot showing correlation between methylation changes (% methylation difference) and expression changes (Log_2FC) of DEGs in *G9a-Glp* cDKO oocytes. Relative expression levels (RPKM) of each gene are indicated by the colour scale. Spearman correlation is shown.

Figure 6. The distinct roles of GLP and G9A in the oocyte.

A) Summary of the developmental and molecular phenotypes of *G9a* cKO, *Glp* cKO and *G9a-Glp* cDKO oocytes.

B) Proposed activities of GLP homodimers and G9A/GLP heterodimers on H3K9 methylation.

C) Interaction of GLP and/or G9A/GLP with UHRF1 could explain DNA hypomethylation in cKO and cDKO oocytes.

D) Alternative chromatin changes that could contribute to gene up-regulation in *G9a* cKO and *G9a-Glp* cDKO oocytes.

Competing interest statement

The authors declare no competing interests.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Distinct roles for G9A and GLP in the oocyte

Control	<i>G9a</i> cKO	<i>Glp</i> cKO	<i>G9a-Glp</i> cDKO
<p>G9a-GLP heterodimer as predominant form</p>	<p>GLP homodimer</p>	<p>G9a homodimer not stable</p>	<p>No G9a nor GLP</p>
	Reduced litter size	Embryonic lethal	Embryonic lethal
<p>Direct regulation:</p> <ul style="list-style-type: none"> - H3K9me2 - H3K9me1? - Non-histone targets? <p>Direct or indirect regulation:</p> <ul style="list-style-type: none"> - Transcription - Protein abundance - DNA methylation - Oocyte maturation - Meiosis 	<p>Compensation or direct function of GLP:</p> <ul style="list-style-type: none"> - H3K9me1 - Proteome - Oocyte maturation <p>Deregulated processes:</p> <ul style="list-style-type: none"> - H3K9me2 depletion - Small transcriptional changes - Regional changes in DNA methylation - Abnormal meiosis 	<p>Deregulated processes:</p> <ul style="list-style-type: none"> - H3K9me2 depletion and decreased H3K9me1 - Reduced oocyte maturation - Abnormal meiosis <p>Not investigated:</p> <ul style="list-style-type: none"> - Transcription - Protein abundance - DNA methylation 	<p>Deregulated processes:</p> <ul style="list-style-type: none"> - H3K9me2 depletion and decreased H3K9me1 - More transcriptional changes - More and larger changes in DNA methylation - Changes in protein abundance - Reduced oocyte maturation - Abnormal meiosis

B

H3K9 methylation

C

UHRF1-dependent hypomethylation

D

Supplemental Figures

Figure S1: G9A and GLP expression in the oocyte and their effects on meiosis

A, B) Representative images showing DNA stained with DAPI, G9a expression (**A**) and GLP expression (**B**) in NSN and SN control oocytes. The scale bar represents 20µm. **C**) Example images showing meiotic abnormalities observed in MII oocytes. The spindle is stained with an anti- α -tubulin antibody (green) and the chromatin with DAPI (blue) and anti-pan-histone (red). **D**) Stacked bar charts showing the percentage of types of meiotic abnormalities (chromosome alignment and spindle conformation) observed in oocytes.

Figure S2: *In vitro* preimplantation and *in vivo* postimplantation development of embryos lacking maternal G9A and/or GLP.

A) Stacked barchart showing developmental stage of embryos collected at E1.5 after superovulation and natural mating with C57Bl6/BabR WT males. One-way ANOVA with Tukey's posthoc test: 2-cell stage Control vs G9a-Glp cDKO $P = 0.0068^{**}$. **B)** Stacked barcharts showing developmental stage of embryos cultured in vitro after natural mating on E1.5, E2.5, E3.5 and E4.5. One-way ANOVA with Tukey's posthoc test: morula stage Control vs G9a cKO $P = 0.0006^{***}$. **C)** Images showing examples of embryos cultured in vitro on E3.5. **D, E)** Table showing embryo development at E8.5 (**D**) and E12.5 (**E**) after natural mating of conditional KO females with WT males. Each line represents one female mouse. Indicated are the number of normal embryos, abnormal embryos and resorption sides for each female.

Figure S3: ChIP-seq analysis showing H3K9me2 distribution in control, *G9a* cKO and *G9a-Glp* cDKO oocytes.

A) Screenshot showing H3K9me2 enrichment and reproducibility of replicates. **B)** Histogram showing number of 10 kb windows with a certain enrichment level in d25 WT oocytes. The red line indicates the threshold set to define H3K9me2 enrichment (RPKM > 2.5). **C)** Pie charts showing overlap of H3K9me2 enriched and random probes with untranscribed (FPKM < 0.1), lowly transcribed (FPKM 0.1-1) and highly transcribed genes (FPKM > 1).

Figure S4: DNA methylation analysis of *G9a* cKO and *G9a-Glp* cDKO oocytes with corresponding littermate controls.

A) Box-whisker plots showing global DNA methylation levels using average methylation levels of 100 CpG windows. Ctrl vs *G9a* cKO $P = 0.5378$; Ctrl vs *G9a-Glp* cDKO $P = 0.6077$. **B)** Scatterplot showing methylation levels of control vs *G9a* cKO oocytes. Unique *G9a* cKO DMRs are highlighted in blue and DMRs shared with *G9a-Glp* cDKO oocytes in red. **C)** Venn diagrams, showing overlap of hypermethylated and hypomethylated DMRs between *G9a* cKO and *G9a-Glp* cDKO oocytes. **D)** Bar chart showing percentage of DMRs and random probes overlapping CGIs, genic and intergenic regions. Qi-square comparing genic and intergenic DMRs: *G9a* cKO $adj. P = 0.026^*$; all others $P < 0.0001^{***}$. **E-G)** Genome screenshots showing examples differentially methylated domains. Shown are a region that is hypomethylated in both *G9a* cKO and *G9a-Glp* cDKO oocytes (**E**), a region that is hypermethylated in both *G9a* cKO and *G9a-Glp* cDKO oocytes (**F**) and a region that is uniquely hypermethylated in *G9a-Glp* cDKO oocytes (**G**).

Figure S5: RNA-seq analysis showing transcriptome changes in *G9a* cKO and *G9a* cDKO oocytes and their link with DNA methylation.

A) Scatterplot showing expression levels of control vs. *G9a* cKO oocytes. Unique *G9a* cKO DEGs are highlighted in blue, DEGs with $\text{Log}_2\text{FC} > 1.5$ in red and DEGs shared with *G9a-Glp* cDKO oocytes in green. **B)** Bar chart showing proportion of DEGs overlapping with histone modifications. DEGs are split according to the clustering analysis of **Figure 5B**. Qi-square analysis comparing DEGs to random probes: $P < 0.0001^{***}$. **C)** Plot showing link between methylation and expression changes of differentially methylated genes in *G9a-Glp* cDKO oocytes. **D, E)** Plot showing link between methylation and expression changes of differentially expressed genes (C) and differentially methylated genes (D) in *G9a* cKO oocytes. **C-E)** Expression levels are indicated by the colour scale. Spearman correlation indicated in plot.