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Maternal Vitamin C regulates DNA demethylation and development of the mouse embryonic germline

by

Stephanie L. Parker

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

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by

Stephanie L. Parker

// Acknowledgments

I would like to thank my graduate mentor, Miguel Ramalho-Santos, for his constant support and optimism. I would also like to thank fellow members of the Santos lab, past and present. In particular, I thank Steffen, Michelle, Aydan, and Trisha for providing an amazing community of people to work amongst and for their friendship. Our many conversations over really bad coffee made my time at UCSF truly unforgettable.

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Finally, I want to thank my Mom and Dad for constantly reminding me that they are proud. It means more than they know. I also thank my wonderful fiancé, Kevin DiTroia, for his unwavering support during the ups and downs of graduate school. Last but not least, I could not have completed this dissertation without Susie. Her companionship and playfulness were pivotal in the completion of this work.

Contributions

The research described in this dissertation was designed and performed by Stephanie Parker under the direct guidance and supervision of Professor Miguel Ramalho-Santos. Chapters 2 – 4 are modified from a manuscript currently under review at *Nature* for publication (Feb 2018).

Kathryn Mesh and Kevin Ebata set up the initial mouse colony for analysis of gestational Vitamin C deficiency on the developing germline. Marie-Justine Guerquin performed histology, immunohistochemistry and meiosis analysis on E14.5 fetal ovaries under the supervision of Gabriel Livera at the Laboratory of Development of the Gonads in Paris, France. Estelle Wall performed and analyzed whole-mount imaging on postnatal day 7 ovaries under the supervision of Diana Laird at UCSF's Center for Reproductive Sciences.

Vitamin C regulates DNA demethylation and development of the female embryonic germline

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Doctor of Philosophy

Department of Biomedical Sciences

University of California, San Francisco

2018

Abstract

There is a growing appreciation that environmental factors can impact embryonic development and program long-term physiology, but the underlying mechanisms remain largely unknown. The embryonic germline is of particular interest because of the potential for intergenerational epigenetic effects. The mammalian germline undergoes extensive DNA demethylation that is carried out in part by Tet enzymes, the activity of which has been shown to be modulated in vitro by nutrients and metabolites, including Vitamin C. Here we report that maternal Vitamin C is required in vivo for proper DNA demethylation and development of female fetal germ cells in a mouse model. Withdrawal of Vitamin C from the maternal diet does not affect overall embryonic development but leads to defects in the fetal germline, which persist well after Vitamin C re-supply during late gestation. These defects include reduced germ cell numbers in female embryos, delayed entry into meiosis, and reduced fecundity in adulthood with increased incidence of fetal loss. The

transcriptome of germ cells from Vitamin C-deficient embryos is remarkably similar to that of embryos carrying a null mutation in Tet1, an enzyme responsible for DNA demethylation and activation of regulators of meiosis. In agreement with these results, Vitamin C deficiency leads to an aberrant DNA methylation profile that includes incomplete demethylation of key regulators of meiosis and transposable elements. These findings reveal that deficiency in Vitamin C during gestation recapitulates mutation of Tet1 and disrupts germline reprogramming and development. Our work further indicates that the embryonic germline is sensitive to perturbations of the maternal diet, providing a potential intergenerational mechanism for adjusting fecundity to environmental conditions.

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Chapter 1: Introduction

Development is often assumed to be hardwired in the genome, but several lines of evidence indicate that it is susceptible to environmental modulation. Even in placental mammals, where the uterus and the placenta are thought to provide a privileged environment, factors such as nutritional deficits or exposure to toxins during gestation can affect development and long-term health. Very little is known about the mechanisms by which environmental inputs are interpreted during mammalian development, although epigenetic layers of gene regulation, such as DNA methylation, are thought to be involved. The mammalian germline is thought to be especially sensitive to environmental modulation because of its dependency on and requirement for genome-wide DNA methylation reprogramming. In this dissertation, I summarize our current understanding of mammalian germline reprogramming, review the established roles of Vitamin C in epigenetics, and report my findings on the consequences of maternal Vitamin C deficiency on germline development in female mouse embryos.

Part I: Reprogramming the mammalian germline

Germline development

The mammalian germline includes all cells with the potential to give rise to future generations. The first identifiable germline cells in mouse development are primordial germ cells (PGCs) and can be isolated from the allantois of an embryo by embryonic day (E) 7.5. These precursor germ cells begin their migratory journey from the allantois, through the hindgut, and finally settle in the genital ridge, where they will colonize the

developing gonad (Seki et al., 2007) (Figure 1-1). Once in the gonad, at approximately E12.5, PGCs begin to sexually differentiate into their male and female counterparts, preparing to become mature gametes.

The timeline to gamete production in mammals varies drastically between males and females. Males produce their gametes (spermatozoa) continuously throughout adulthood while females produce the extent of their gamete precursors (oocytes) in utero, which then are matured and ovulated cyclically during adulthood. Because of this, male germ cells mitotically arrest, or pause, in utero while female germ cells initiate meiosis. Female meiosis asynchronously begins between E13.5 and E15.5 in the mouse embryo (Hilscher et al., 1974; Speed, 1982). After birth and once sexually mature, male and female gametes can combine to form a zygote, which has the potential to form a new organism and start the developmental cycle once again.

Throughout development, transcription factors and epigenetic signals drive complex gene expression patterns which in turn determine cellular identity. Epigenetic signals, including DNA methylation, modify the transcriptional output of the genome without changing the underlying DNA sequence. DNA methylation is one of the best studied epigenetic modifications. It includes the addition of a methyl group at the 5' position on the nucleotide cytosine, named 5-methylcytosine (5mC). This epigenetic modification is most commonly found in the context of a symmetric cytosine followed by guanine (CpG). Its roles in transcription regulation are context-dependent, with promoter methylation often linked to the silencing of gene transcription. Interestingly, CpGs are found in dense clusters throughout the genome, named CpG islands (CGI), and are associated with gene promoters in approximately 70% of the annotated mammalian

genome (Deaton & Bird, 2011; Saxonov, Berg, & Brutlag, 2006).

Whether or not the 5mC modification could be actively removed remained a mystery until the discovery of DNA demethylase activity by ten-eleven translocation (Tet) proteins, Tet1, Tet2 and Tet3, in 2009 (Ito et al., 2010; Tahiliani et al., 2009). Tet proteins catalyze the iterative steps of converting 5mC to an un-modified cytosine. Tet hydroxylases first convert 5mC to 5-hydroxymetyl cytosine (5hmC), then 5-formylcytosine (5fC), and finally 5-carboxylcytosine (5caC) through sequential oxidation. 5caC can be excised or decarboxylated into an un-modified cytosine to complete the process known as active demethylation (Ito et al., 2011). The specialized role of each cytosine modification and intermediate is still an active area of research.

The mammalian germline undergoes epigenetic reprogramming, including genome-wide DNA demethylation, twice during early development. The two windows of DNA demethylation are conserved across mammalian development and occur immediately following fertilization and again specifically in the developing germline (Figure 1-1). During the second wave of germline reprogramming, DNA demethylation occurs exclusively in the developing germ cells while epigenetic information remains relatively stable across differentiated somatic cell types (Figure 1-1) (Meyenn & Reik, 2015; Sasaki & Matsui, 2008; Tang et al., 2015). This specialized wave of epigenetic reprogramming is thought to serve two important functions in the germline: re-acquisition of totipotent capability and prevention of inheritance of epigenetic mutations (Saitou & Yamaji, 2012).

Detailed DNA methylation analyses of developing PGCs revealed a global loss of

DNA methylation in migrating PGCs as well as locus-specific DNA demethylation at specific developmental stages (Seisenberger et al., 2012; Thienpont et al., 2012). This suggests that the process of DNA methylation reprogramming in PGCs after E7.5 is complex and finely regulated, involving both passive and active demethylation (Hackett et al., 2013; Seisenberger et al., 2012). Recent studies investigating the extent of active demethylation during development have found that Tets are required at various stages, including roles in demethylating imprint control regions and meiotic gene promoters. (Hackett et al., 2013; Hill, Amouroux, & Hajkova, 2014; Yamaguchi et al., 2012; Yamaguchi, Shen, Liu, Sendler, & Zhang, 2013).

Tet proteins are expressed across many stages and tissues during embryo reprogramming. Tet3 is abundant in oocytes and responsible for active demethylation during the first few cell divisions of the zygote (Gu et al., 2011). In contrast, Tet1 is highly expressed in PGCs and the developing germline. With generation of a genetically modified Tet1KO mouse, Tet1 was found to be essential for initiation of the meiosis transcriptional program. Loss of Tet1 leads to reduced fertility and aberrant imprints in progeny (Dawlaty et al., 2013; Yamaguchi et al., 2012; 2013). Interestingly, Tet1 does not impair genome-wide demethylation but only demethylation at specific sequences, including the promoters of key meiosis genes (Yamaguchi et al., 2012).

Germline sensitivity and the environment

It is understood that cells do not alter their underlying genetic code during development, but instead they re-distribute cell-type specific epigenetic profiles in order to perform the cellular functions required. In somatic cells, the epigenome is mitotically

inherited through division. This allows somatic epi-mutations to expand during the lifetime of the organism but expire when the organism dies. By contrast, epigenetic errors in the germline, be they advantageous or troublesome, contain the ability to survive the organism and be transmitted to future generations. Due to this potential for inheritance, epigenetic reprogramming of the developing germline is a finely-regulated process.

As described above, meiosis is a key feature of sexual reproduction whereby germ cells divide into gametes. It is not known if the male germline, which undergoes gamete production in adulthood, or female germline, which initiates meiosis in utero, is more susceptible to adverse environments. We hypothesize that the female germline is especially sensitive to environmental perturbation, including sensitivity to maternal diet during pregnancy. The timing of meiotic initiation in the female germline is well understood, yet the mechanisms that drive the switch from mitotic to meiotic division are not currently known. The breakthrough study from Yamaguchi et al. found that Tet1 is required for the activation of meiotic gene transcription in female germ cells (Yamaguchi et al., 2012), suggesting a key role for DNA demethylation of meiotic promoters in initiating the meiotic program. This study led us to hypothesize that environmental factors capable of influencing DNA methylation dynamics may disrupt the meiotic programming of female germ cells, thus interfering with their development.

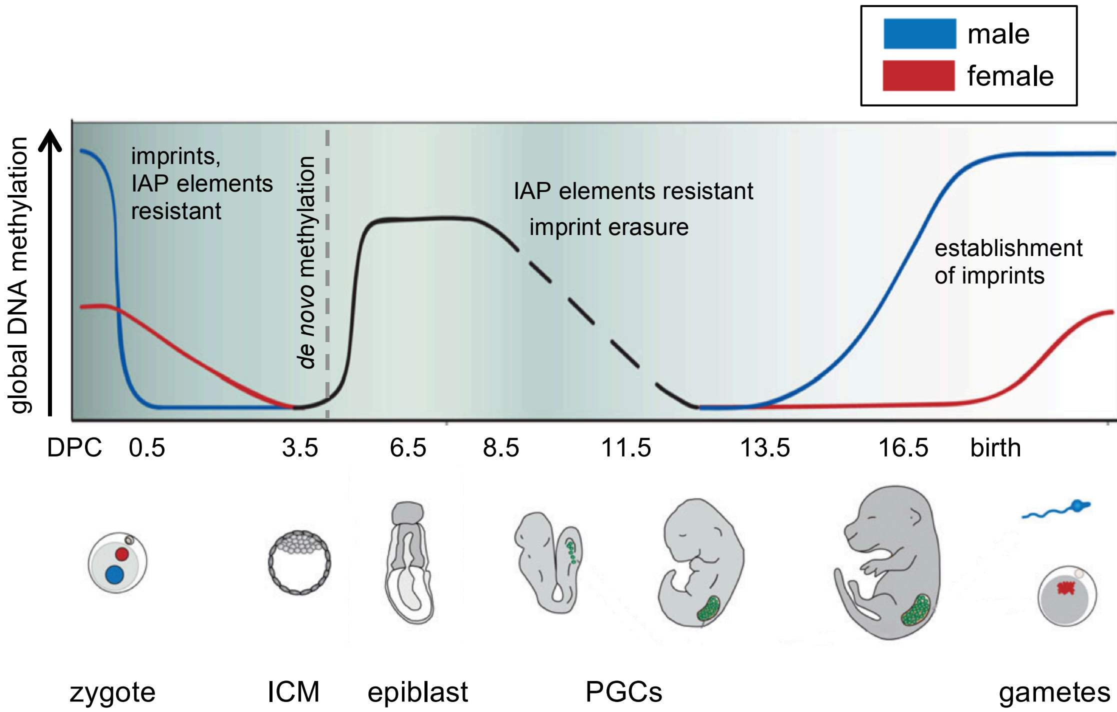


Figure 1-1: Development and DNA methylation reprogramming of the mouse germline.
The first identifiable germline cells in the developing mouse embryo arise around E7.5. These cells, named primordial germ cells (PGCs), will differentiate into gametes and hold the potential for giving rise to future generations. DNA methylation is highly dynamic during mouse embryo development. Genome wide demethylation occurs twice in utero: once in the zygote immediately following fertilization and second in maturing PGCs. Adapted from Seisenberger et al. 2013.

Part II: Vitamin C

Brief history of Vitamin C in medicine

The most common sign of long-term Vitamin C deficiency is scurvy. Scurvy was first described in humans as a combination of bleeding gums, swollen joints, skin blotches, fatigue, depression, and eventual death if left untreated. Scurvy was a common ailment in the 16th – 18th centuries when long sea voyages were common and travelers went months without Vitamin C intake (Magiorkinis, Beloukas, & Diamantis, 2011). James Lind, a British Naval Surgeon, was the first to report that a diet of lemons and oranges can cure scurvy. His publication *Treatise of the Scurvy* in 1753 was clear on the treatment, although the acting component within citrus fruits was unknown at the time. Centuries later, an Hungarian physiologist Albert Szent-Györgyi isolated ascorbic acid, also known as Vitamin C, in the adrenal gland of guinea pigs and documented its sufficiency in the treatment of scurvy (Szent-Györgyi, 1928). He later won the 1937 Nobel Prize in Physiology for his work.

Vitamin C across species

Intriguingly, most fungi, plants, and animals can synthesize Vitamin C. Only through evolution have few vertebrate species lost this ability, such as teleost fishes, bats, guinea pigs, anthropoid primates, and humans (Drouin, Godin, & Pagé, 2011) (Figure 1-2). In all cases this has occurred through accumulation of mutations in the gene coding for *L-Gulonolactone oxidase* (Gulo), the enzyme that carries out the last step in Vitamin C synthesis from glucose (Figure 1-2). Because of this, these species, including humans,

have evolved to become entirely dependent on dietary intake of Vitamin C for normal physiology. A common model for Vitamin C-deficiency studies is the *Gulo-/-* mouse, which is missing two exons from the *L-Gulonolactone oxidase* gene and begins to show signs of scurvy after several weeks without Vitamin C supplementation (Maeda et al., 2000).

In humans and other non-synthesizers, Vitamin C is absorbed through the intestine with excess amounts excreted into the urine. In synthesizers, Vitamin C is produced in the liver before being transported through the bloodstream to the rest of the body. In all animals, Vitamin C cellular uptake depends on sodium-dependent transporters, SVCT1 and SVCT2, while the oxidized form, DHA, can be transported through glucose transporters before recycling back to Vitamin C intracellularly (Goldenberg & Schweinzer, 1994).

The evolutionary reasons behind diet dependency of Vitamin C across diverse species are still the subject of debate (Drouin et al., 2011; Monfort & Wutz, 2013). It is frequently claimed that the loss of Vitamin C synthesis is a neutral evolutionary trait that happens at random due to the fact that every species gaining mutations live on diets with sufficient quantities of Vitamin C. However, more recent studies suggest diet dependency may provide an important interplay between environment, cellular physiology, and development (Monfort & Wutz, 2013). The thesis work described here gives further evidence supporting the idea that dietary restriction of Vitamin C during gestation allows the fetal germline to ‘sense’ its surroundings, selecting for higher fertility in female embryos developed in nutritionally rich environments.

Known mechanisms of action

A fundamental molecular requirement for Vitamin C is prolyl and lysyl hydroxylase activity (Magiorkinis et al., 2011; Peterkofsky, 1991). These two specific hydroxylases are involved in collagen production and when interrupted contribute to the symptoms of scurvy. Vitamin C can also act as a co-substrate for the entire family of Fe(II) 2-oxoglutarate (2-OG) dependent dioxygenases, the mechanism of focus in this thesis work (Arrigoni & De Tullio, 2002; Monfort & Wutz, 2013). Fe(II) 2- oxoglutarate (2-OG) dependent dioxygenases catalyze a variety of substrates requiring iron, 2-OG, and molecular oxygen. The reduced form of iron, Fe(II), is required for catalysis, but sometimes iron becomes oxidized due to an ineffective reaction occurring in the absence of a substrate. Vitamin C acts as an electron donor to reduce iron back to Fe(II) and recycle these enzymes back to an active form. Thus, Vitamin C greatly improves hydroxylase enzyme activity, but it is not absolutely required (Monfort & Wutz, 2013).

Other members of the Fe(II) 2-OG dioxygenase family include Tet enzymes and histone demethylases. Recently, Vitamin C was shown to increase the efficiency of cellular reprogramming through these epigenetic regulators (Blaschke et al., 2013; Chen et al., 2013; Ebata et al., 2017; Esteban et al., 2010; T. Wang et al., 2011). Importantly, these studies ruled out other common vitamins and antioxidants, showing that these were unable to substitute for Vitamin C in this molecular reaction, and concluded the mechanism of action by Vitamin C to be Tet activation. One study showed that Vitamin C increases the quality of reprogramming to induced pluripotency by preventing aberrant methylation on specific imprinting loci (Stadtfeld et al., 2012). Another revealed the ability of Vitamin C to establish a more blastocyst-like transcriptional/epigenetic state in cultured

mouse embryonic stem cells (ESCs) via Tet demethylation (Blaschke et al., 2013). However, whether or not this mechanism persists in the epigenetic reprogramming of both the zygote and germline in vivo remained unknown.

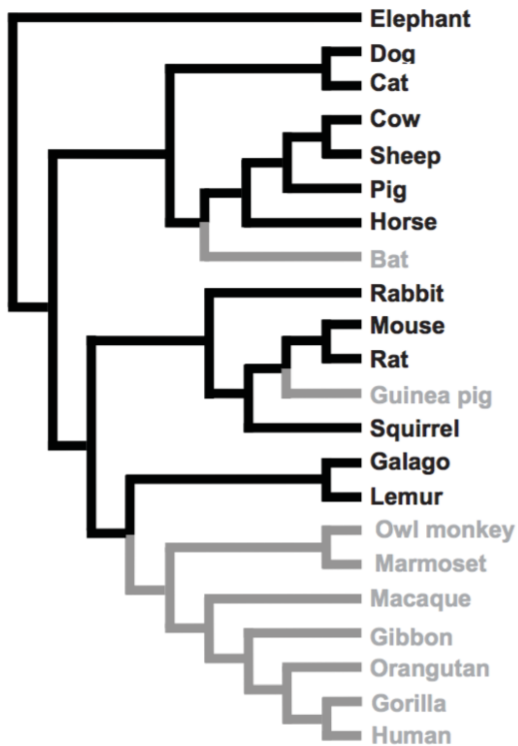
Vitamin C, Tet enzymes, and Cancer

There has been rising interest in the therapeutic potential of Vitamin C, specifically in cancers driven by aberrant methylation. Recent studies using adult *Gulo-/-* mice as a model system show that Vitamin C deficiency increases the frequency of hematopoietic stem cells and promotes leukemia (Agathocleous et al., 2017). Additionally, supra-physiological doses of Vitamin C in a mouse model reduces the leukemogenic behavior of hematopoietic progenitors, in a manner partially dependent on Tet2 (Cimmino et al., 2017). These recent studies have greatly advanced our knowledge of Vitamin C in biology and definitively proved that Vitamin C is directly involved in mechanisms involving DNA methylation and gene transcription in vivo.

Vitamin C and germline gene expression

As briefly mentioned, the Santos lab previously showed that Vitamin C plays a crucial role in enhancing Tet enzyme activity in mouse ESCs (Blaschke et al., 2013). Their study revealed that Vitamin C, but not other antioxidants, can increase the rate of 5hmC production through Tet mediated oxidation of 5mC. The dynamics of 5mC demethylation upon Vitamin C addition in mouse ESC culture is rapid, with signs of increased 5hmC at 12 hours after treatment. Interestingly, Vitamin C addition to mouse ESCs have a modest effect on the global transcriptome of these cells. Genes selected for up-regulation with treatment include germline genes, mirroring the gene expression changes in *Tet1* KO

mouse germ cells (Figure 1-3). Overall, the mechanism of Vitamin C activity in mouse ES cells suggests that maternal Vitamin C nutrition may play an important role in germ cell gene expression in developing embryos.



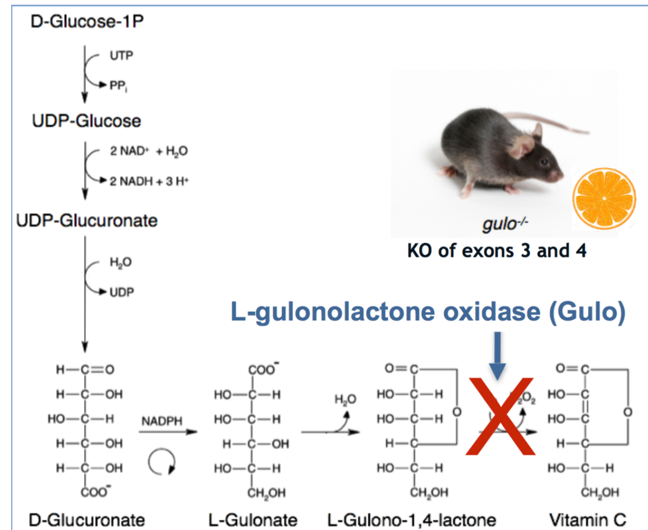


Figure 1-2: Evolution of diet dependency for Vitamin C and mouse model of Vitamin C deficiency.

Phylogenetic distribution of the ability to synthesize Vitamin C in mammals. Lineages able to synthesize vitamin C are in black and those incapable are in gray (adapted from Drouin 2011). Outlined to the right is the biosynthetic pathway of Vitamin C production from D-Glucose-1P to Vitamin C. The mutated gene causing inability to synthesize Vitamin C across many mammalian species is *L-gulonolactone oxidase (Gulo).* This gene is mutated in the mouse model for Vitamin C deficiency.

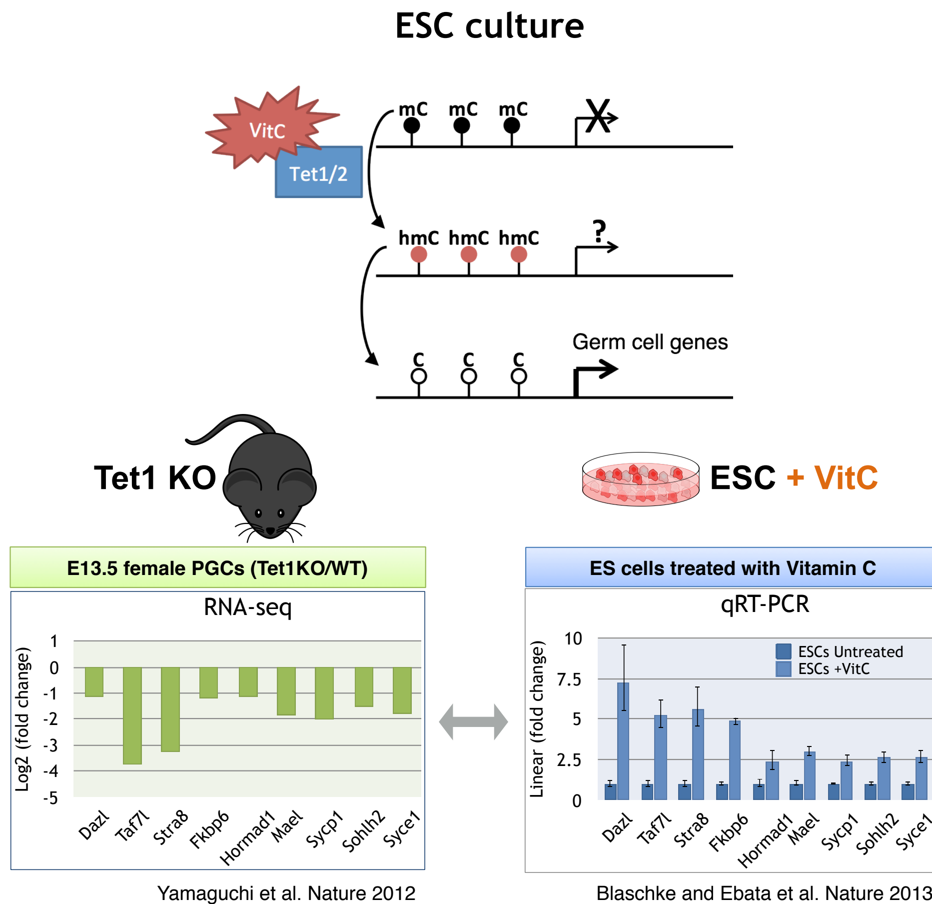


Figure 1-3: Model of Vitamin C induction of germline genes.
Model of Tet1/2-dependent demethylation in mouse ESCs as described in Blaschke et al. Figure also shows decreased expression of select germline genes in Tet1KO E13.5 germ cells (Yamaguchi et al., 2012) compared to increased expression of same germline genes upon Vitamin C addition to cultured mouse ES cells (Blaschke et al., 2013). Gene expression was measured by RNAseq in Yamaguchi et al. and qRT-PCR in Blaschke et al. Together these figures suggest Vitamin C may control germline gene expression in vivo, in a Tet-dependent manner.

Chapter 2: Maternal Vitamin C regulates reprogramming of DNA methylation and development of the mouse embryonic germline

Abstract

There is a growing appreciation that environmental factors can impact embryonic development and program long-term physiology, but the underlying mechanisms remain largely unknown. The embryonic germline is of particular interest because of the potential for intergenerational epigenetic effects. The mammalian germline undergoes extensive DNA demethylation that is carried out in part by Tet enzymes, the activity of which has been shown to be modulated in vitro by nutrients and metabolites, including Vitamin C. Here we report that maternal Vitamin C is required in vivo for proper DNA demethylation and development of female fetal germ cells in a mouse model. Withdrawal of Vitamin C from the maternal diet does not affect overall embryonic development but leads to defects in the fetal germline, which persist well after Vitamin C re-supply during late gestation. These defects include reduced germ cell numbers in female embryos, delayed entry into meiosis, and reduced fecundity in adulthood with increased incidence of fetal loss. The transcriptome of germ cells from Vitamin C-deficient embryos is remarkably similar to that of embryos carrying a null mutation in Tet1, an enzyme responsible for DNA demethylation and activation of regulators of meiosis. In agreement with these results, Vitamin C deficiency leads to an aberrant DNA methylation profile that includes incomplete demethylation of key regulators of meiosis and transposable elements. These findings reveal that deficiency in Vitamin C during gestation recapitulates mutation ofand

disrupts germline reprogramming and development. Our work further indicates that the embryonic germline is sensitive to perturbations of the maternal diet, providing a potential intergenerational mechanism for adjusting fecundity to environmental conditions.

Introduction

Development is often assumed to be hardwired in the genome, but several lines of evidence indicate that it is susceptible to environmental modulation. Examples include the induction of developmental pausing by unfavorable environmental conditions in a variety of metazoans, pheromone-induced metamorphosis in insects, or temperature-dependent sex determination in fish and reptiles (Schiesari & O'Connor, 2013; Smith-Gill, 1983). In placental mammals, the uterus and the placenta are thought to provide a privileged environment that is optimal for embryonic development. Despite this physical and chemical separation of the embryo from the external environment, factors such as nutritional deficits or exposure to toxins during gestation can affect developmental progression and long-term health (Barker, 2004; Boekelheide et al., 2012). In some cases, environmental factors have been reported to induce intergenerational effects [e.g., (Anway, Cupp, Uzumcu, & Skinner, 2005; Radford et al., 2014)], suggesting that the embryonic germline can be impacted. However, it is unclear whether the germline is directly sensitive to environmental factors at the time of insult, in utero, or whether intergenerational effects are secondary to effects on placental biology, maternal behavior or other confounding factors (Heard & Martienssen, 2014). Importantly, very little is known about the mechanisms by which environmental inputs are interpreted during mammalian

development, although epigenetic layers of gene regulation, such as DNA methylation, are thought to be involved (Boekelheide et al., 2012).

The mammalian embryonic germline undergoes genome-wide DNA demethylation, an event that has been proposed to promote the erasure of epialleles between generations (Gkountela et al., 2015; F. Guo et al., 2015; Hackett et al., 2013; Seisenberger et al., 2012; Tang et al., 2015). The loss of DNA methylation in Primordial Germ Cells (PGCs) is thought to occur in large part by passive dilution over successive cell divisions, accompanied by active DNA demethylation via Ten-eleven translocation (Tet) enzymes (Hajkova et al., 2008; Seisenberger et al., 2012). Tet enzymes convert 5-methylcytosine (5mC) to oxidized intermediates, triggering the ultimate full demethylation of cytosine (Wu & Zhang, 2014). Embryos deficient in Tet1, the Tet enzyme most abundantly expressed in PGCs, display incomplete DNA demethylation and down-regulation of germline genes, meiosis defects and decreased fecundity (Yamaguchi et al., 2012; 2013). These data demonstrate that Tet-mediated DNA demethylation in PGCs is important for germline development and fecundity.

Interestingly, the activity of Tet enzymes has been shown to be directly modulated in vitro by metabolites, such as the Krebs cycle intermediate, 2-oxoglutarate, and nutrients such as iron and Vitamin C (Blaschke et al., 2013; Carey, Finley, Cross, Allis, & Thompson, 2015; Wu & Zhang, 2011; Yin et al., 2013; Q. Zhang et al., 2014). Vitamin C is a co-factor for many enzymes of the 2-oxoglutarate-dependent dioxygenase family, which includes Tet enzymes, several histone demethylases, as well as collagen and hypoxia-inducible factor (HIF) prolyl hydroxylases (Salminen, Kauppinen, & Kaarniranta,

2015). Vitamin C is best known for its requirement to prevent scurvy in adults, but studies in guinea pigs and mice suggest that Vitamin C is important during embryonic development. Vitamin C deficiency during most of gestation in guinea pigs leads to a slight and reversible decrease in fetal weight, whereas deficiency in late gestation impairs postnatal hippocampal development (Schjoldager et al., 2015; Tveden-Nyborg et al., 2012). In mice, a homozygous null mutation in the major Vitamin C transporter Slc23a2 is compatible with development to term, but mutant pups die shortly after birth (Sotiriou et al., 2002). The underlying molecular mechanisms for the developmental phenotypes associated with Vitamin C deficiency were not investigated in these studies. We previously showed that exogenous Vitamin C induces Tet-mediated DNA demethylation and activation of germline genes in cultured mouse embryonic stem (mES) cells (Blaschke et al., 2013). However, it remained unknown whether Vitamin C has any essential role in vivo during development of the germline.

We report here that maternal Vitamin C is required in vivo for proper DNA demethylation and development of the mouse embryonic germline. Vitamin C-deficient embryos develop normally with regards to overall growth, stage and expression of markers of the somatic cells of the gonad, but have defects in germ cell number and entry into meiosis. Females deprived of Vitamin C while in utero, but maintained on a Vitamin C-containing diet from late gestation onwards, display reduced fecundity with increased incidence of failed pregnancies and embryo resorptions. We show that Vitamin C deficiency transcriptionally recapitulates the Tet1 mutation in the developing female germline, with corresponding defects in DNA demethylation. These findings reveal a novel role for Vitamin C in germline reprogramming and development, and raise the

possibility that the germline has mechanisms to sense environmental quality and adjust fecundity across generations.

Results

Gestational Vitamin C deficiency is compatible with embryogenesis but disrupts germ cell development

To study the role of Vitamin C in germline development, we generated a homozygous mouse colony deficient in Vitamin C synthesis and carrying a transgene for PGC identification ($Gulo^{-/-}$;Oct4/EGFP) (Maeda et al., 2000; Szabó, Hübner, Schöler, & Mann, 2002). Like humans, $Gulo^{-/-}$ mice are fully dependent on diet for Vitamin C due to a mutation in the L-gulonolactone oxidase gene. With proper Vitamin C supplementation in the drinking water, these mice are viable, fertile and indistinguishable from their heterozygous or wildtype littermates (H. Kim et al., 2012; Maeda et al., 2000). In agreement with previous reports (H. Kim et al., 2012), we found that it takes about 7 days of withdrawal for Vitamin C to become essentially undetectable in the blood plasma of pregnant $Gulo^{-/-}$ mice (Figure 2-1). To ensure minimal contribution of circulating maternal Vitamin C to developing embryos, $Gulo^{-/-}$;Oct4/EGFP females were removed from Vitamin C 3-7 days before mating with $Gulo^{-/-}$ males. Vitamin C supplementation was returned to the drinking water of pregnant females at the developmental stage corresponding to Embryonic day (E) 13.5 (Figure 2-2A). We chose E13.5 because it represents the lowest point of global DNA methylation during germline reprogramming (Seisenberger et al., 2012). We focused on female germ cells because they enter meiosis at E13.5, whereas in males this only occurs postnatally. In all our analyses, we compared the female progeny

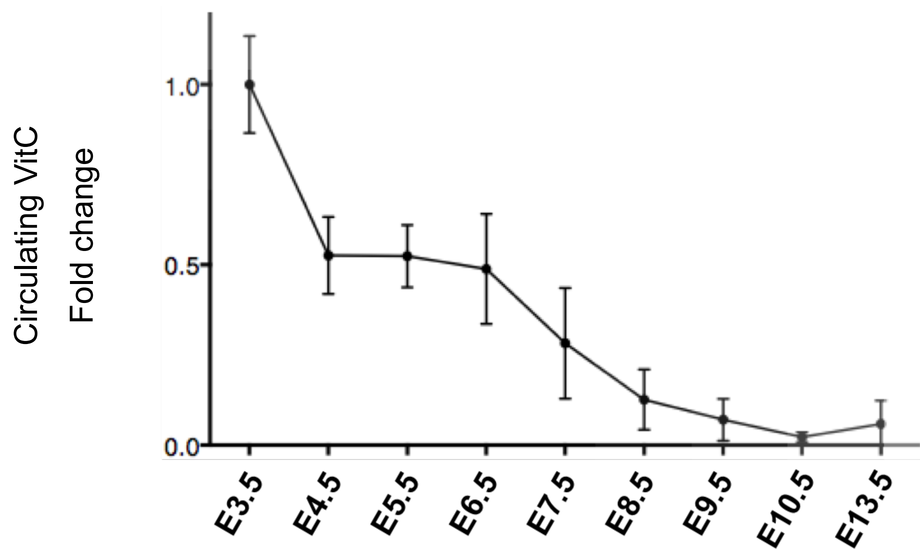
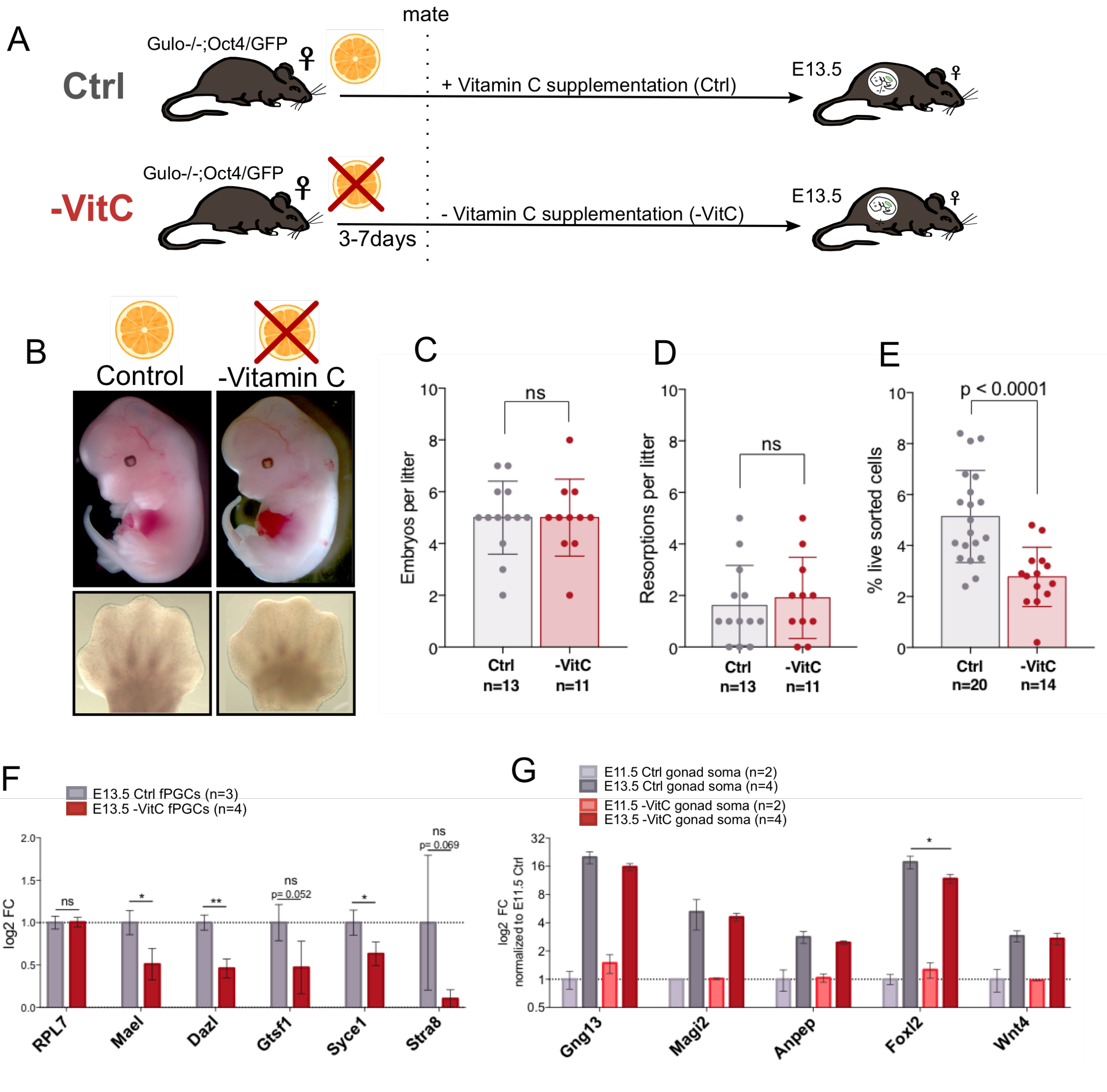


Figure 4-1: Kinetics of Vitamin C depletion from maternal blood serum.
Kinetics of Vitamin C depletion from the serum of pregnant Gulo$^{-/-}$ mice after withdrawal from their drinking water. Pregnant females were removed from Vitamin C supplementation at E3.5 and circulating blood serum was tested over the time-course indicated. It takes 5 days of withdrawal for the circulating Vitamin C levels to be <25%, and 7 days to be essentially undetectable.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D
ns
Resorptions per litter
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-VitC n=11
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F
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E11.5 Ctrl gonad soma (n=2)
E13.5 Ctrl gonad soma (n=4)
E11.5 -VitC gonad soma (n=2)
E13.5 -VitC gonad soma (n=4)
log2 FC normalized to E11.5 Ctrl
*
Gng13
Magi2
Anpep
Foxl2
Wnt4

Figure 2-2: Gestational Vitamin C deficiency disrupts female germ cell development. a, Diagram of Vitamin C withdrawal from the maternal diet during early gestation. Control (Ctrl) litters are genetically identical to Vitamin C deficient litters (-VitC). Ctrl females are provided with physiological levels of Vitamin C in the drinking water throughout their lifespan. -VitC females are removed of Vitamin C supplementation 3 to 7 days before mating and throughout early gestation (E0 – E13.5). b, Vitamin C deficiency does not affect embryo development to E13.5, as determined by morphological assessment of the embryos and hand plate staging. c, Gestational Vitamin C deficiency does not affect litter size. d, Gestational Vitamin C deficiency does not affect resorption rate. e, Reduction in the number of Oct4/EGFP+ germ cells upon Vitamin C deficiency in E13.5 female embryos. Statistical significance assessed by Welch's t-test. g, Overall we see a normal induction of key markers of developmental progression of somatic cells of the ovaries between E11.5 and E13.5 in Vitamin C-deficient embryos, as measured by qRT-PCR. E13.5 somatic ovary cells are matched with PGCs in (F). Foxl2 expression is strongly induced upon Vitamin C deficiency, but to a somewhat lower extent than in control samples. Error bars depict mean ± SD of 4 biological replicates. Statistical significance assessed by Student's t-tests. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

of Vitamin C-depleted mothers to genetically identical controls supplied with physiological Vitamin C levels throughout development.

Withdrawal of Vitamin C from the maternal diet before mating and during early gestation is compatible with development through E13.5, as evidenced by normal embryo morphology and hand plate staging (Figure 2-2B). Depletion of Vitamin C does not alter average litter size or percentage of embryo resorptions per litter (Figure 2-2C&D). However, there is a significant reduction in the numbers of Oct4/EGFP+ germ cells in Vitamin C-deficient female embryos, compared to controls (Figure 2-2E). This finding was confirmed using independent staining for the germ cell surface marker Stage-specific embryonic antigen-1 (SSEA1) (Figure 2-3).

When added to ES cells in culture, Vitamin C induces the expression of a set of key germline genes in a Tet1/2-dependent manner (Blaschke et al., 2013), and these genes also depend on Tet1 for expression in PGCs in vivo (Yamaguchi et al., 2012). Interestingly, we found that those same germline genes are consistently down-regulation in female PGCs developed under Vitamin C-deficient conditions (Figure 2-2F). To investigate if this reduction in germline gene expression in Vitamin C-depleted embryos is a secondary effect of delayed or disrupted gonad development, we transcriptionally staged female gonad somatic cells using markers that are sharply induced between E11.5 and E13.5 (Jameson et al., 2012). We found that Vitamin C deficiency has no overall impact on the developmental progression of fetal gonads, with the exception of a slight reduction in *Foxl2* induction (Figure 2-2G). Together, these data indicate that Vitamin C deficiency during gestation up to E13.5 is compatible with embryonic and gonadal

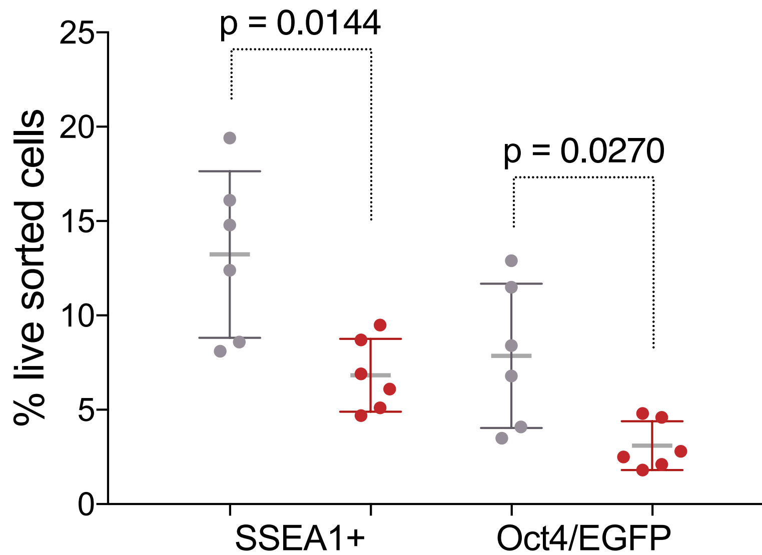


Figure 2-3: The reduction in E13.5 female germ cells numbers upon Vitamin C deficiency is confirmed using both Oct4/EGFP and SSEA1 positivity.

development but leads to reduced germ cell numbers and defective expression of Tet1-dependent germline regulators.

Maternal Vitamin C is required for normal meiosis in fetal female germ cells

Several of the germline genes identified as Vitamin C-responsive in vivo are important regulators of meiotic initiation and progression, including *Stra8, Sycp1, Syce1, Mael, Dazl* and *Gtsf1*. For example, *Stra8* is a critical mediator of entry into meiosis (Baltus et al., 2006). Therefore, we evaluated whether meiosis is affected by gestational Vitamin C deficiency. We observed a decrease in STRA8 protein levels in fetal germ cells of Vitamin C-deficient embryos at E14.5 relative to controls (Figure 2-4A), in agreement with the reduction at the mRNA level at E13.5 (Figure 2-2F). A decrease in germ cells positive for SYCP3, an essential component of the synaptonemal complex, was also detected in Vitamin C-deficient germ cells (Figure 2-4B), suggestive of a delay in meiotic progression in Vitamin C-deficient female embryos.

Quantification of meiotic stages in E14.5 and E18.5 ovaries confirmed a delay in meiosis initiation and progression. Specifically, E14.5 Vitamin C-deficient ovaries display a significant enrichment in the proportion of germ cells in meiotic S phase (also called preleptotene) accompanied by a decrease of leptotene (earlier) and zygotene (later) germ cells, compared to controls (Figure 2-5A-C). This delay in meiotic progression persists at E18.5, when Vitamin C-deficient ovaries have a significant enrichment in germ cells at the zygotene (earlier) stage accompanied by a decrease in germ cells at the pachytene (later) stage, relative to controls (Figure 2-5D).

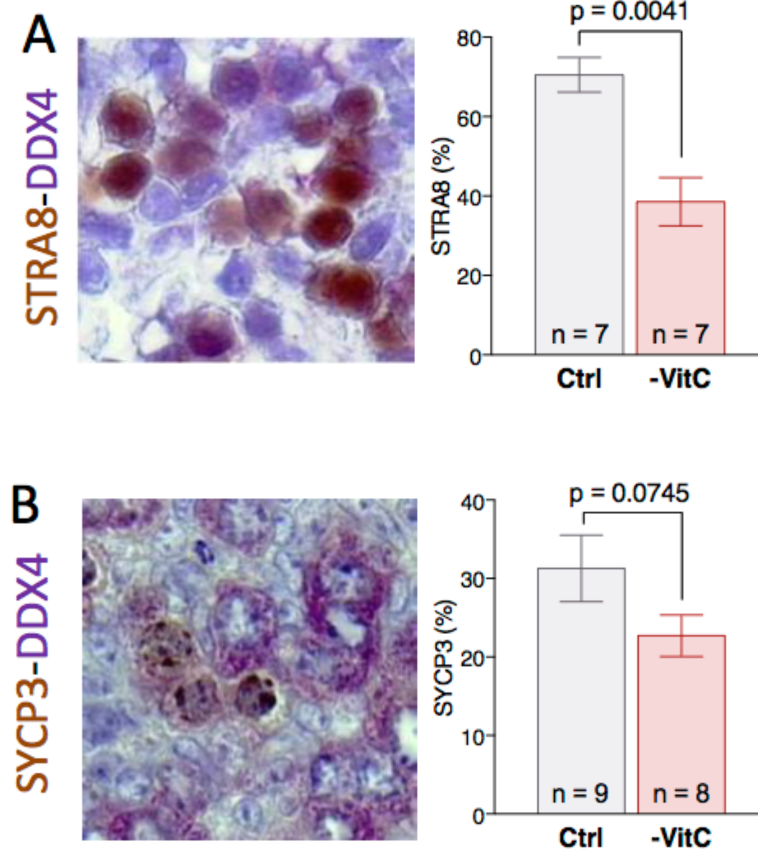
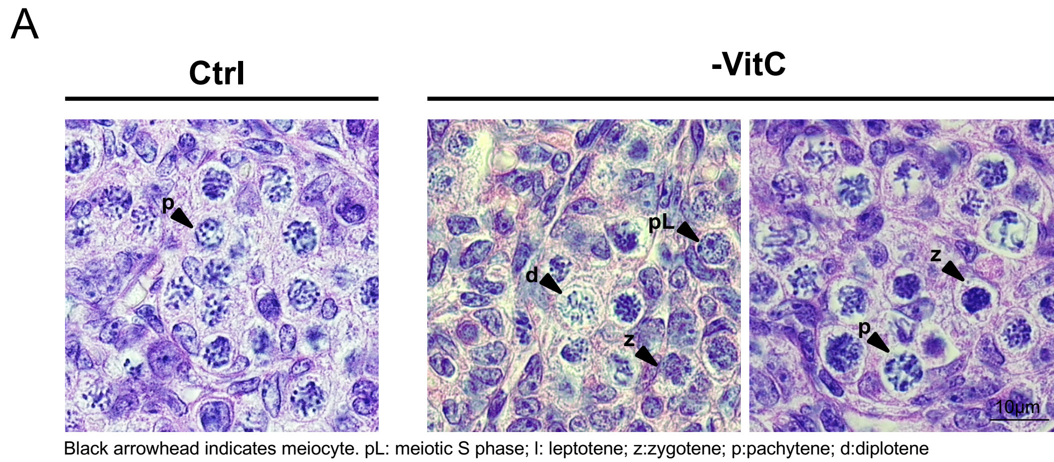


Figure 2-4: Detailed analyses of meiotic staging in germ cells of E14.5 Vitamin C-deficient ovaries.

a, Significant reduction in the percentage of Ddx4+ germ cells that are Stra8+ upon Vitamin C deficiency. Error bars depict mean ± SEM. Statistical significance assessed by Mann-Whitney test. b, Vitamin C-deficient E14.5 female germ cells display a trend towards reduction in the percentage of Ddx4+ germ cells that are Sycp3+. Error bars depict mean ± SEM. Statistical significance assessed by Mann-Whitney test.



Black arrowhead indicates meiocyte. pL: meiotic S phase; l: leptotene; z:zygotene; p:pachytene; d:diplotene

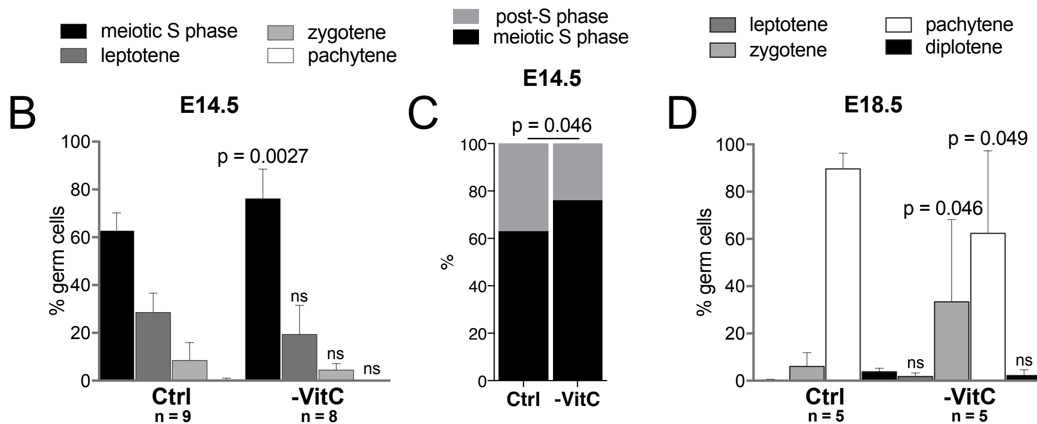


Figure 2-5: Vitamin C deficiency disrupts Meiotic staging in embryonic ovaries.
a, Representative haematoxilin/eosin staining of E14.5 embryonic ovaries for meiotic staging. b&d, Staging of meiosis progression in E14.5 and E18.5 female ovaries reveals a delay in meiosis progression in -VitC ovaries. Error bars depict mean ± SD. Statistical significance assessed by Sidak's multiple comparison test. c, The percentage of germ cells in meiotic S phase vs post-S phase is significantly higher in -VitC E14.5 females, relative to controls, assessed by the Chi-square test.

As an alternative test for meiotic progression, we stained for centromeres in E18.5 female germ cells. The successful pairing of chromosomes during meiosis in mouse germ cells leads to approximately 20 centromeric foci per cells; however, Vitamin C-deficient embryos have a significant increase in germ cells with >25 centromere foci per cell (Figure 2-6). This increase in centromere foci signifies a notable delay or defect in synapsis during meiotic progression.

Interestingly, fetal male testes, where meiosis does not take place until puberty, display no apparent defects in number, cell morphology, nor differentiation (as defined by analysis of mitotic arrest) with Vitamin C deficiency (data not shown). Taken together, these data document that maternal Vitamin C deficiency induces significant deficits in meiotic initiation and progression in fetal female germ cells.

Gestational Vitamin C deficiency leads to reduced fecundity in female progeny

Given the defects in germline gene transcription, meiosis, and variability in germ cell abundance observed in Vitamin C-deficient female embryos, we investigated potential postnatal effects on ovarian reserve and fecundity. Pregnant females (F0) were depleted of Vitamin C as before, returned to a Vitamin C-containing diet at E13.5 and allowed to give birth (Figure 2-7A). Whole-ovary imaging at postnatal day 7 revealed an abnormally variable ovarian reserve in pups that had been depleted of Vitamin C in utero (-VitC F1), with several of them having very low numbers of developing oocytes relative to controls (Ctrl F1) (Figure 2-7B&C). These defects occurred in the absence of significant changes in ovary volume (Figure 2-7D). These results corroborate our findings that the germline compartment of the gonads is particularly sensitive to Vitamin C levels in utero.

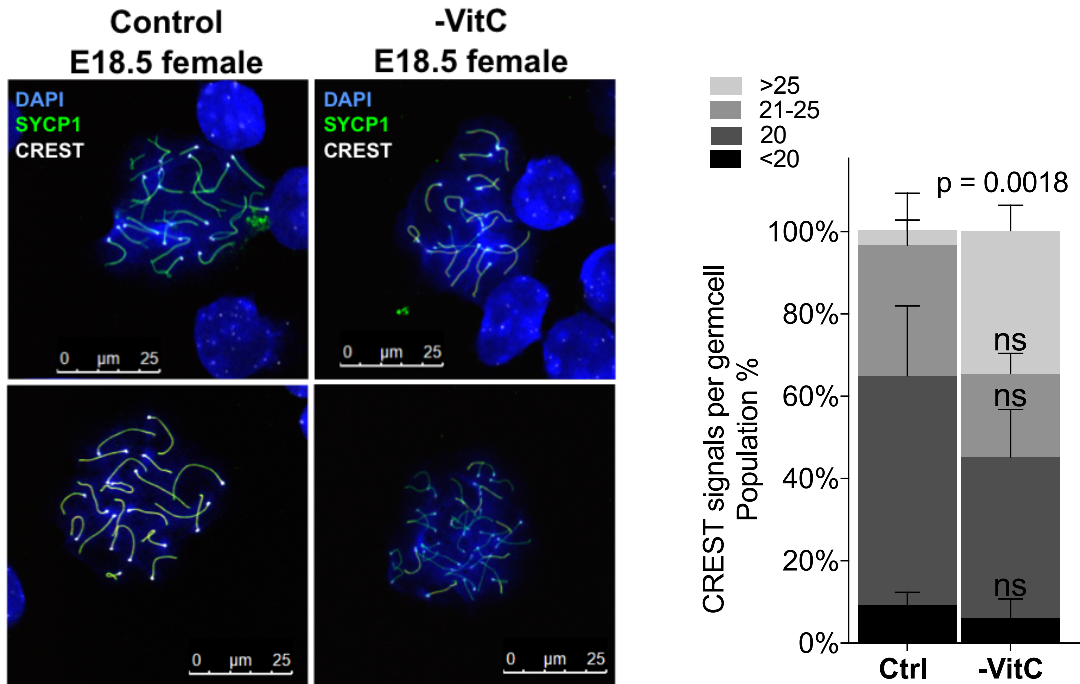


Figure 2-6: High numbers of centromere foci observed in E18.5 female germ cells from Vitamin C-deficient embryos.

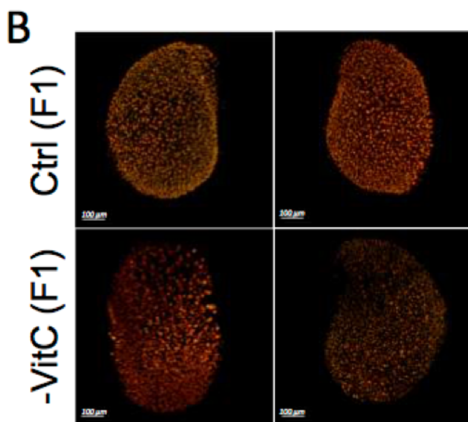
Centromere number per E18.5 female germ cell was determined by quantification of CREST staining of SYCP1+ germ cells. Error bars depict mean ± SEM. Statistical significance assessed by Welch's t-test.

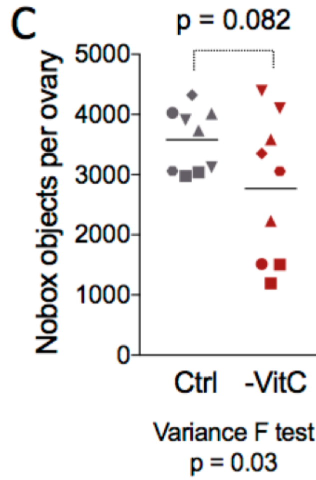
To determine whether potential defects in meiosis and ovarian reserve in -VitC females affect their fecundity, we crossed -VitC and Ctrl F1 females at 20-25 weeks of age to wildtype males, as outlined in Figure 2-7A. Interestingly, -VitC F1 females have a significantly reduced number of implanted embryos per mating, with a high incidence of entirely failed pregnancies (lacking implantation sites) relative to Ctrl F1 (Figure 2-7E&F). Even within the successful pregnancies of -VitC F1 females, there is an abnormally high frequency of embryo resorption, both in quantity and percentage of litters containing resorption of an implanted embryo (Figure 2-7G&H). Thus, while -VitC F1 females can be fertile, they have significantly reduced fecundity. These results document an intergenerational impact of Vitamin C deficiency on female reproductive potential.

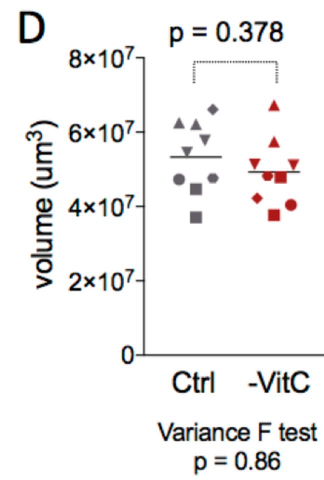
Vitamin C deficiency recapitulates transcriptional defects induced by Tet1 loss in the embryonic germline

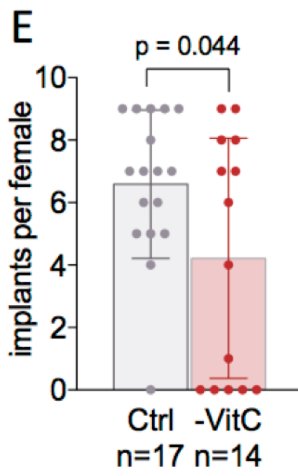
Our results indicate that resupply of Vitamin C to the maternal diet at E13.5 does not erase the defects in germline function induced by Vitamin C deficiency earlier in gestation. In order to understand the full transcriptional impact of Vitamin C deficiency in the germline, we characterized the transcriptome of E13.5 germ cells from single embryos across multiple -VitC and Ctrl litters using RNA-seq (Table 2-1, Figure 2-8). Principal component analysis and hierarchical clustering revealed that -VitC germ cells are overall transcriptionally distinguishable from controls (Figure 2-9A&B). 511 genes were identified as differentially expressed in germ cells upon Vitamin C deficiency during gestation (FDR < 0.05, Log2FC $> |0.7|$), with a preponderance of down-regulated genes (390 genes down-regulated versus 121 up-regulated, Figure 2-9B&C). Several key regulators of meiosis are among the top down-regulated genes in -VitC germ cells (Figure 2-9C).

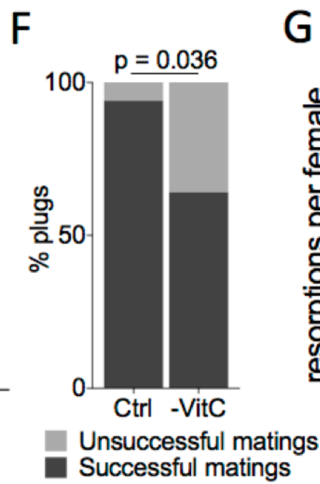
A
F1
♀F0
♀F1
E0 – E13.5
Postnatal day 7: Count developing oocytes
20 - 25 weeks: Test fecundity

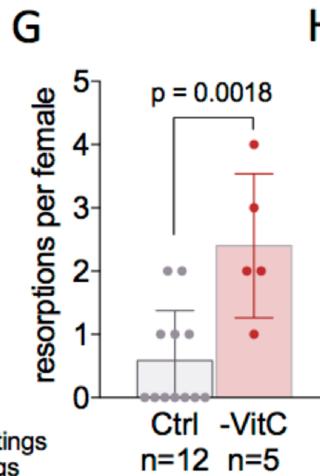

B
Ctrl (F1)
-VitC (F1)


C
p = 0.082
Nobox objects per ovary
5000
4000
3000
2000
1000
0
Ctrl
-VitC
Variance F test
p = 0.03


D
p = 0.378
volume (um3)
8×10^7
6×10^7
4×10^7
2×10^7
0
Ctrl
-VitC
Variance F test
p = 0.86


E
p = 0.044
implants per female
10
8
6
4
2
0
Ctrl
-VitC
n=17 n=14


F
p = 0.036
% plugs
100
50
0
Ctrl
-VitC
Unsuccessful matings
Successful matings


G
p = 0.0018
resorptions per female
5
4
3
2
1
0
Ctrl
-VitC
n=12 n=5

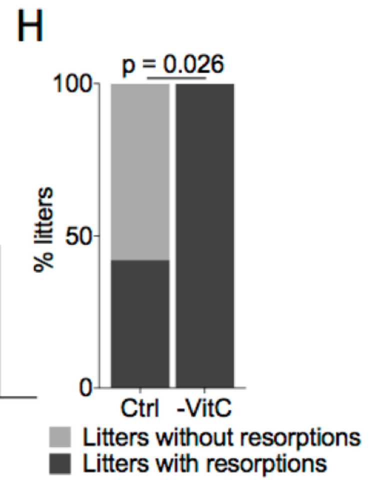

H
p = 0.026
% litters
100
50
0
Ctrl
-VitC
Litters without resorptions
Litters with resorptions

Figure 2-7: Gestational Vitamin C deficiency has a long-term impact on female fecundity.

a, Diagram of experiments to address female fecundity. Maternal females (F0) were taken off Vitamin C supplementation during the first two weeks of F1 embryo development (E0-E13.5). From E13.5 onwards, Vitamin C was resupplied in the drinking water. After birth, F1 females were assessed for ovarian reserve at day 7 or fecundity at 20 weeks. b, Representative whole-mount images of postnatal day 7 ovaries dissected from females developed under Vitamin C-deficient or control conditions. Red Nobox staining is marked with white spheres by imaging software to identify developing oocytes. c, Quantification of oocytes in day 7 ovaries by whole-mount imaging of Nobox staining. Geometric shapes represent individual female embryos; Identical shapes indicate ovary pairs. Control females contain between 3000-4500 Nobox+ oocytes per ovary (n=9). Females deprived of Vitamin C in utero contain between 1000-4500 Nobox+ oocytes per ovary (n=9). Vitamin C deficiency induces a significantly greater variance in the number of oocytes per ovary, with several females having very low numbers of developing oocytes relative to controls. Statistical significance assessed by the Student's t-test and variance determined by F ratio. d, Normal numbers and variance in P7 ovary volume from females that had developed in the absence of Vitamin C. Statistical significance assessed by the Student's t-test and variance determined by F ratio. e, F1 females that had developed in control or Vitamin C deficient conditions were mated to wild-type males, and the number of implantation sites per female displaying a vaginal plug was counted between E9.5-E18.5. Ctrl F1 females average 6.5 implants per pregnancy while -VitC F1 females average only 4 implants. Statistical significance assessed by the Student's t-test. f, There is a significant reduction in the number of successful matings in F1 females that had developed under Vitamin C deficient conditions. A successful mating is defined as the observation of at least 1 implanted embryo per female that had displayed a vaginal plug after mating. Statistical significance assessed by the Chi-square test. g, Within successful matings only, there is a higher number of resorptions in -VitC F1 females, relative to controls. The quantity of resorptions in -VitC females is increased compared to Ctrl F1 females. Statistical significance assessed by the Student's t-test. h, The number of pregnancies containing at least one resorbed embryo is significantly higher in -VitC F1 females, relative to controls. Statistical significance assessed by the Chi-square test.

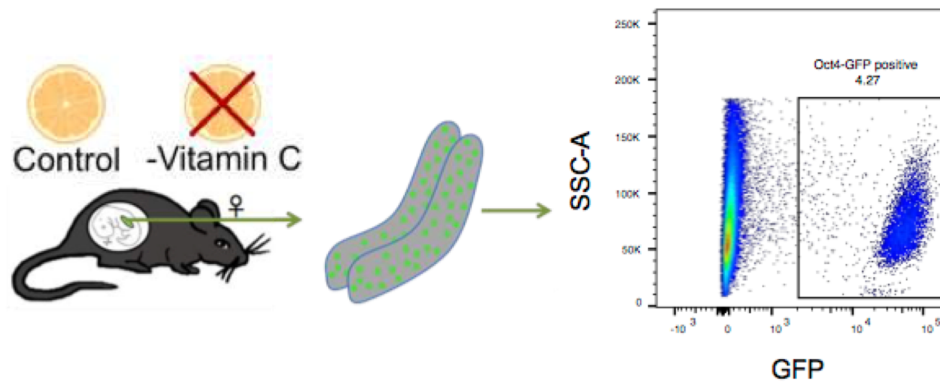


Figure 2-8: Diagram of experiments to determine the molecular impact of Vitamin C deficiency in the embryonic germline.
Pregnant mice were deprived of Vitamin C as before and Oct4/EGFP+ germ cells were isolated from single E13.5 embryos. A total of 6 Ctrl and 6 -VitC samples were collected across 4 litters each. Downstream transcriptional and DNA methylation analyses performed as described.

sample	Litter #	# of PGCs
1C F1	Ctrl 1	1482
1C F2	Ctrl 1	804
2C F2	Ctrl 2	651
3C F2	Ctrl 3	531
4C F1	Ctrl 4	1850
4C F2	Ctrl 4	839
1V F1	-VitC 1	729
1V F2	-VitC 1	674
2V F1	-VitC 2	480
3V F1	-VitC 3	852
4V F1	-VitC 4	142
4V F2	-VitC 4	488

Table 1: RNA-seq samples
Table outlines litter number and quantity of germ cells sorted per sample. Each sample represents a single E13.5 female.

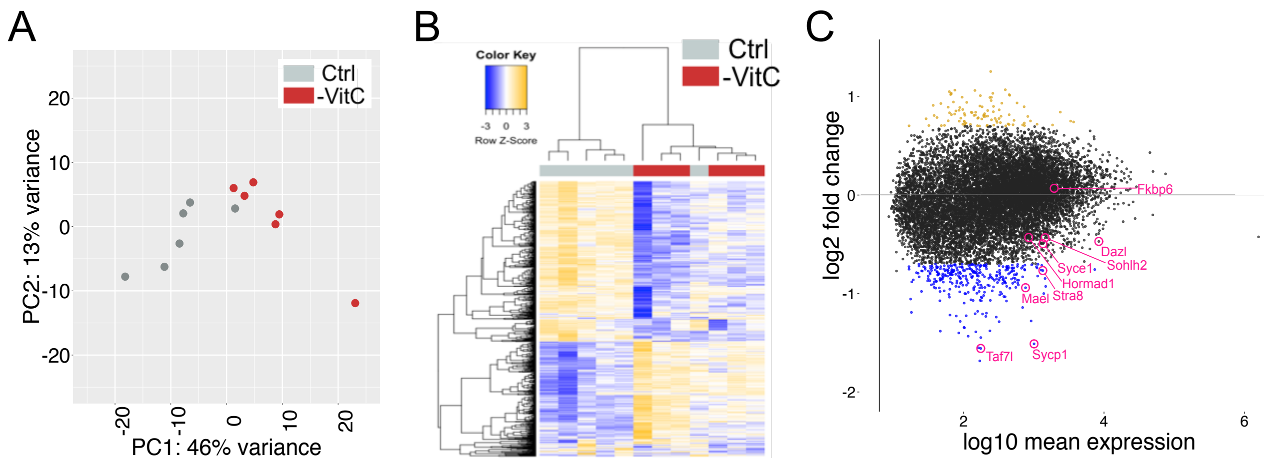


Figure 2-9: Detailed analyses of RNA-seq data.

a, Principle component analysis (PCA) of the transcriptional profile of E13.5 germ cells identifies a clear separation of samples according to Vitamin C availability along PC1. The Ctrl sample closest to the -VitC samples in the PCA plot clusters with them in hierarchical clustering. This sample is transcriptionally intermediate between Ctrl and -VitC samples, for reasons unknown. b, Unsupervised hierarchical clustering documenting the overall separation between Ctrl and -VitC samples (columns) and relative gene expression (rows). c, MA plot of the differential expression between Ctrl and -VitC samples. The 121 gold dots represent genes significantly up-regulated in -VitC germ cells. Conversely, the 390 blue dots represent genes down-regulated in -VitC germ cells. Select germline genes previously identified as Tet1-dependent in female germ cells (Yamaguchi et al., 2012), induced by Vitamin C in ES culture (Blaschke et al., 2013) are highlighted in pink.

Importantly, the set of genes downregulated in -VitC germ cells is enriched for functions in meiosis and sexual reproduction. These results are in agreement with the physiological defects in meiosis and reproduction induced by gestational Vitamin C deficiency described above.

The results up to this point document a remarkable similarity in the phenotypes induced by gestational Vitamin C deficiency and whole-animal Tet1 null mutation. Similar to Vitamin C-deficient embryos, Tet1KO embryos are fully viable but display a reduced number of germ cells, reduced expression of meiotic regulators, defective meiosis and reduced fecundity (Yamaguchi et al., 2012; 2013). We explored the molecular parallels between Vitamin C deficiency and Tet1 mutation in the embryonic germline in more detail, using Gene Set Enrichment Analysis (GSEA) and a published dataset of RNA-seq in Tet1KO E13.5 germ cells (Yamaguchi et al., 2012). This analysis revealed that Vitamin C deficiency recapitulates the transcriptional defects of Tet1KO germ cells, with regards to both up-regulated and down-regulated genes (Figure 2-10). Importantly, the expression of Tet and Dnmt enzymes in PGCs are not affected by Vitamin C deficiency. These data are in agreement with our previous findings that Vitamin C directly enhances the enzymatic activity of Tet1 and induces the expression of germline regulators in ES cells in vitro in a Tet-dependent manner (Blaschke et al., 2013). This, Vitamin C is required for the execution of a transcriptional program that is orchestrated by Tet1 in the female embryonic germline and is essential for normal fecundity.

Tet enzymes contribute to two distinct windows of global DNA demethylation during embryogenesis, one after fertilization and a second window specifically in the midgestation germline (Wu & Zhang, 2014). In order to identify the window of

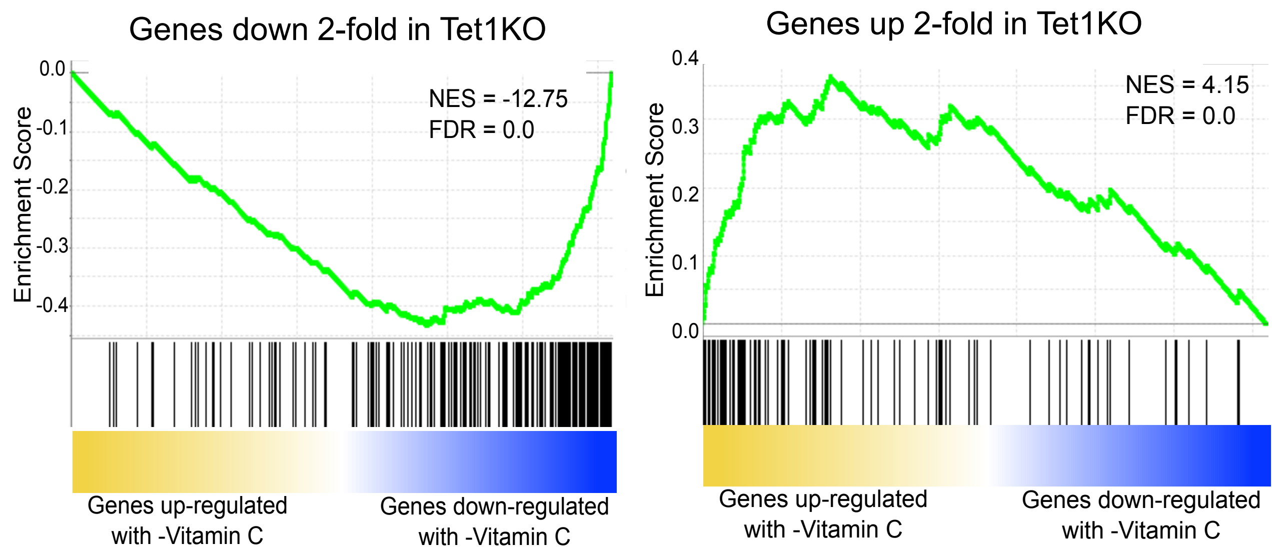


Figure 2-10: GSEA between Vitamin C deficiency and Tet1 KO in female PGCs. Gene Set Enrichment Analysis (GSEA) highlights the similarities between -VitC (this study) and Tet1KO (Yamaguchi et al., 2012) female E13.5 germ cells. Both up-regulated or down-regulated genes in Tet1KO germ cells are highly biased to be up-regulated or down-regulated, respectively, upon Vitamin C deficiency in E13.5 germ cells.

susceptibility to Vitamin C deficiency, we tested the re-addition of Vitamin C to the maternal diet at E3.5, after the first window of reprogramming, and quantified the expression of Vitamin C-dependent, Tet1-dependent germline regulators at E13.5. We found that re-supplementation of Vitamin C at implantation rescues the transcriptional defects and the reduction in germ cell counts induced by Vitamin C deficiency (Figure 2-11). These results suggest that Vitamin C is required for proper germline gene expression and germ cell development during a time frame after implantation but before E13.5, thus overlapping with the window of DNA demethylation in the embryonic germline.

Maternal Vitamin C regulates DNA methylation reprogramming in the embryonic germline

We next sought to determine the impact of maternal Vitamin C deficiency on the DNA methylation landscape of the embryonic germline. We used Reduced Representation Bisulfite Sequencing (RRBS) to compare the DNA methylome of -VitC and Ctrl E13.5 germ cells on a genome-wide scale and at base-pair resolution (H. Guo et al., 2013). As previously reported (Kobayashi et al., 2012; Seisenberger et al., 2012), E13.5 female murine germ cells are globally demethylated, and the presence or absence of Vitamin C does not affect the overall abundance of modified cytosines, genome-wide or at specific genomic features (average 3.5% mCpG genome-wide, Figure 2-12). These results are in agreement with the notion that global DNA demethylation in the germline occurs primarily via passive dilution, with Tet1 playing a secondary role (Ohno et al., 2013; Seisenberger et al., 2012; Yamaguchi et al., 2012). Using BiSeq (Hebestreit, Dugas, & Klein, 2013), we identified 460 Differentially Methylated Regions (DMRs) across the genome (p-value < 0.05, 5% minimum change). Two-thirds of the DMRs gain methylation

upon Vitamin C deficiency (285 hypermethylated DMRs vs 175 hypomethylated DMRs, Figure 2-13), indicating that Vitamin C is primarily required for DNA demethylation.

Interestingly, analyses using Genomic Regions Enrichment of Annotations Tool (GREAT) revealed that hypermethylated DMRs induced by Vitamin C deficiency in the germline are enriched for sequences associated with abnormal ovary development and female infertility (Figure 2-14A). Most of the DMRs are located distal from transcription start sites (TSS, Figure 2-14B), a trend very similar to that observed in Tet1KO germ cells (Yamaguchi et al., 2012). The 55 genes with hypermethylation within 5kb of their TSS upon Vitamin C deficiency are enriched for germline regulators. Examples include genes expressed in E13.5 germ cells and dependent on Vitamin C for proper expression, such as *Dazl* or *Sycp1*, as well as regulators of meiosis not expressed until later in development, such as *Spo11* or *Sohlh2* (Figure 2-14C). Of note, these Vitamin C-dependent germline regulators are classified as loci targeted for active demethylation in Seisenberger et al.

Distal DMRs are enriched for Transposable Elements (TEs), specifically of the LINE1 and ERVK/IAP families, all of which display methylation gains (Figure 2-15). An unbiased analysis of the DNA methylation status of all TEs across the genome, regardless of whether they are called differentially methylated or not, revealed significant trends towards gains of DNA methylation (Figure 2-16). These results document that Vitamin C deficiency during development leads to retention of DNA methylation at meiosis regulators and TEs in the embryonic germline, in both cases mimicking the defects observed in Tet1KO germ cells (Yamaguchi et al., 2012).

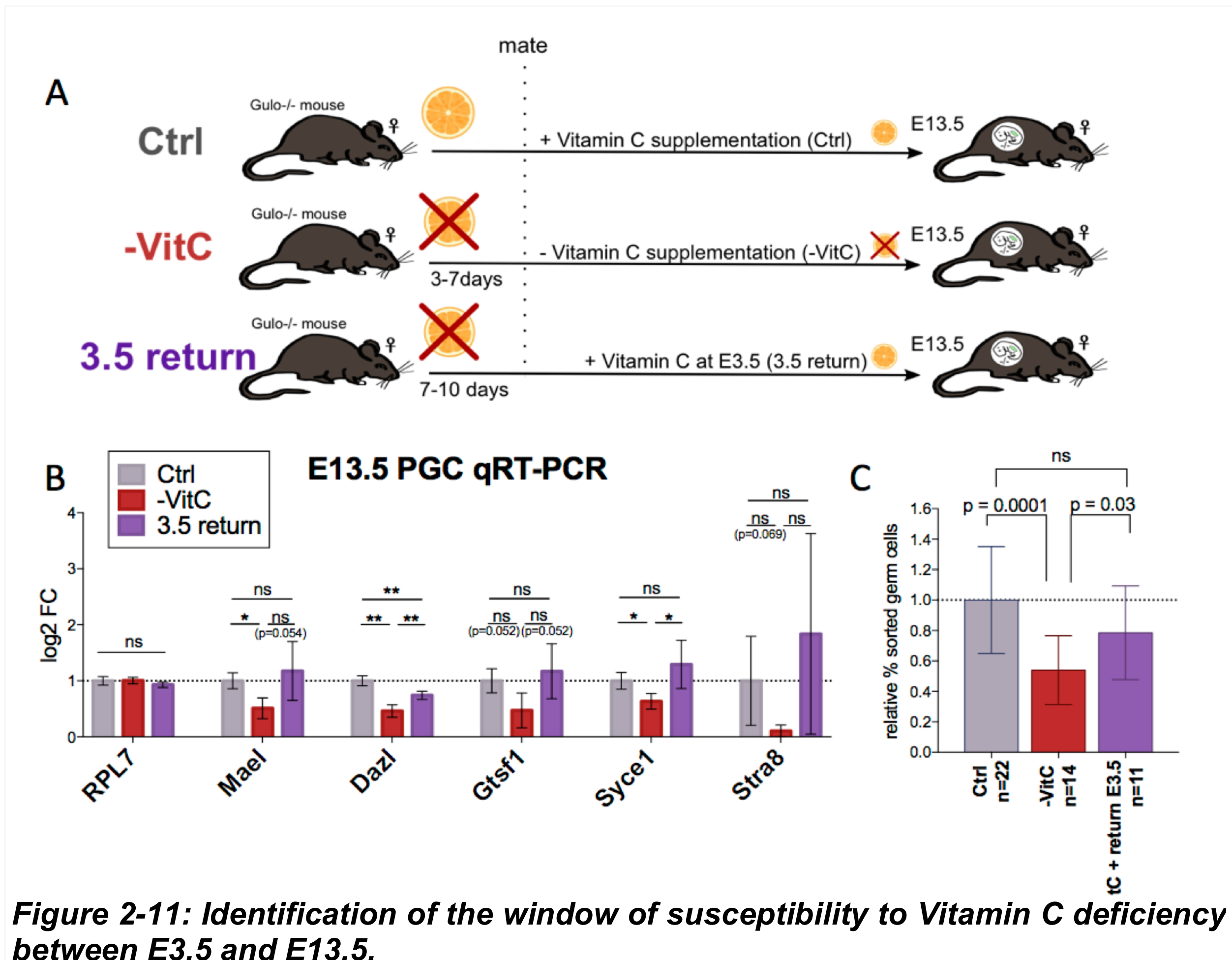


Figure 2-11: Identification of the window of susceptibility to Vitamin C deficiency between E3.5 and E13.5.

a, Pregnant females mated in Vitamin C-deficient conditions were either maintained without Vitamin C (-VitC) or returned to Vitamin C-containing water at E3.5 (3.5 return). b, Adding back Vitamin C from E3.5 to E13.5 rescues the defects in the expression of key germline regulators induced with full Vitamin C deficiency. Gene expression was measured by qRT-PCR in E13.5 female germ cells. Error bars depict mean ± SD of 4 biological replicates. Statistical significance assessed by Student's t-tests. * p<0.05; ** p<0.01. c, The numbers of E13.5 Oct4/EGFP+ germ cells are mostly recovered with return of Vitamin C at E3.5. Normalized to germ cell count of the Ctrl embryos. Statistical significance assessed by Student's t-test.

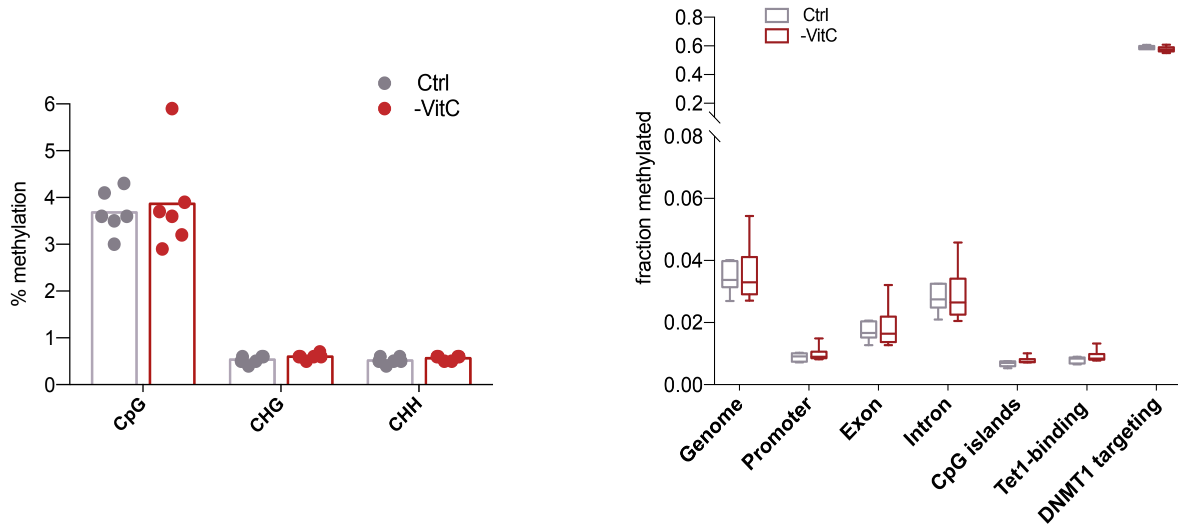


Figure 2-12: Average methylation of cytosine according to sequence context. Genome-wide CpG methylation is 3-6% regardless of Vitamin C supplementation. Methylation of cytosine in a CHG or CHH context is below 1%. Average methylation according to genomic context in Ctrl and -VitC samples does not significantly vary between samples with and without Vitamin C supplementation.

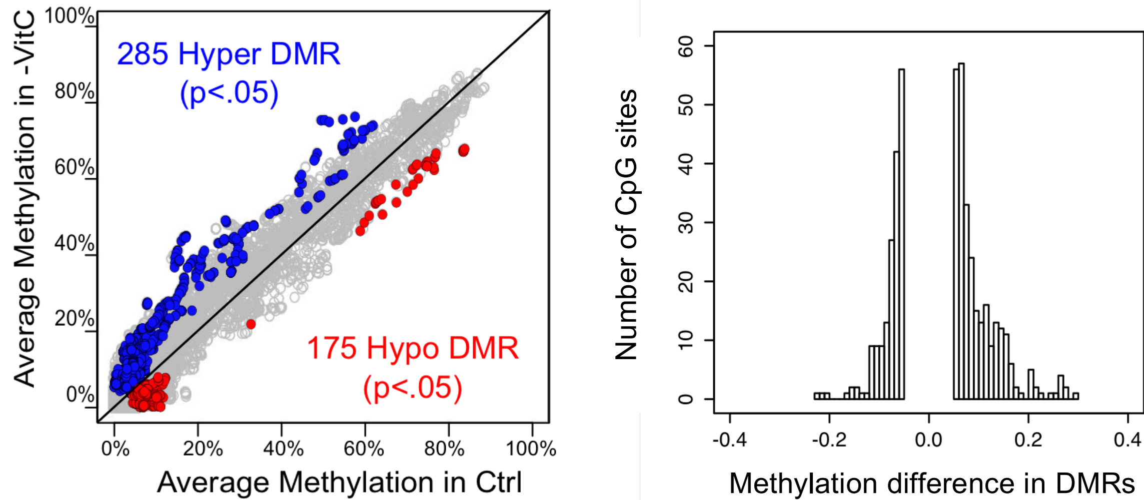
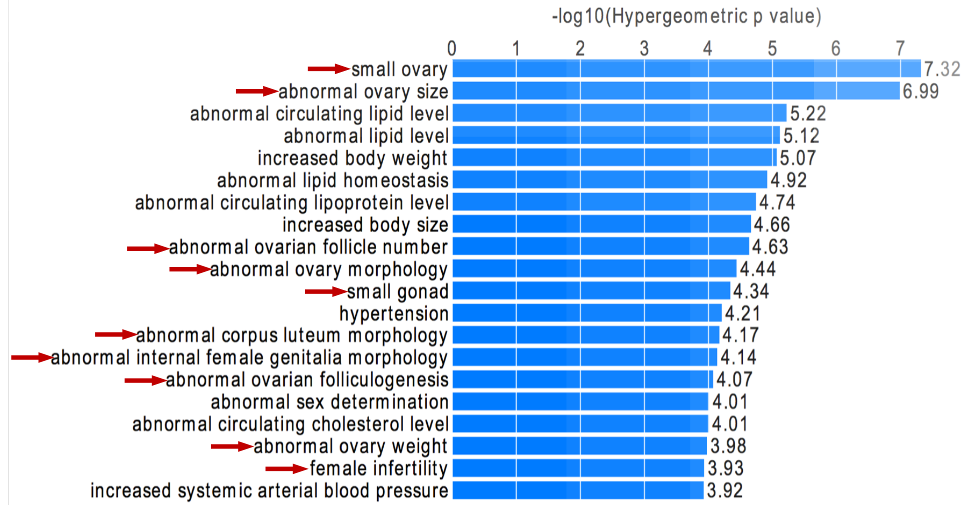
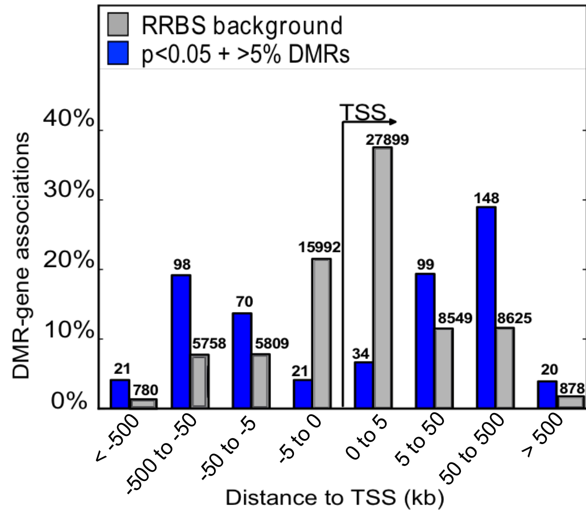


Figure 2-13: Differential DNA methylation with Vitamin C deficiency.
Left, Average methylation per CpG across 6 replicates each of Ctrl versus -VitC female E13.5 germ cells. Colored dots represent CpGs belonging to significant Differentially Methylated Regions (DMRs). Blue and red dots represent 285 hypermethylated DMRs and 175 hypomethylated DMRs, respectively. Right, Density plot of DMRs with >5% methylation reveals an overall increase in the number and magnitude of methylation gains over losses upon Vitamin C deficiency.

A



B



C

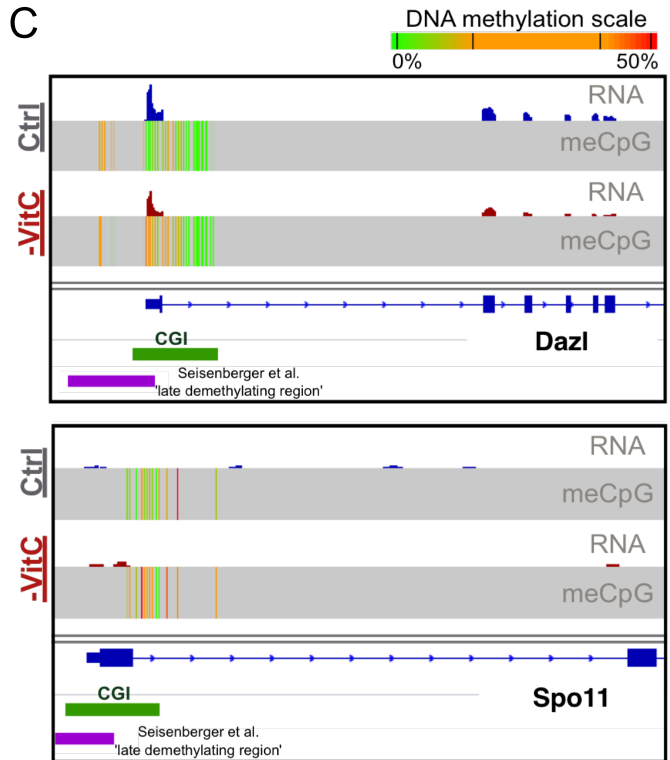


Figure 2-14: Vitamin C deficiency leads to incomplete loss of DNA methylation at meiosis regulators.

a, GREAT analysis of hypermethylated DMRs induced by Vitamin C deficiency identifies a high enrichment for annotations associated with abnormal ovary development and female infertility (red arrows). b, Distance of hypermethylated DMRs to gene Transcription Start Sites (TSS) compared to the universe of genomic regions covered by RRBS. DMRs are found primarily at a distance from TSSs. This trend is consistent with hypomethylated DMRs. c, *Dazl* and *Spo11* are both examples of hyper DMRs at the promoter of germline regulators. The reduced expression of *Dazl* at E13.5 is correlated with a gain in promoter methylation. Interestingly, the promoter of *Spo11* is found hypermethylated in -VitC E13.5 germ cells, several days before its expression is induced.

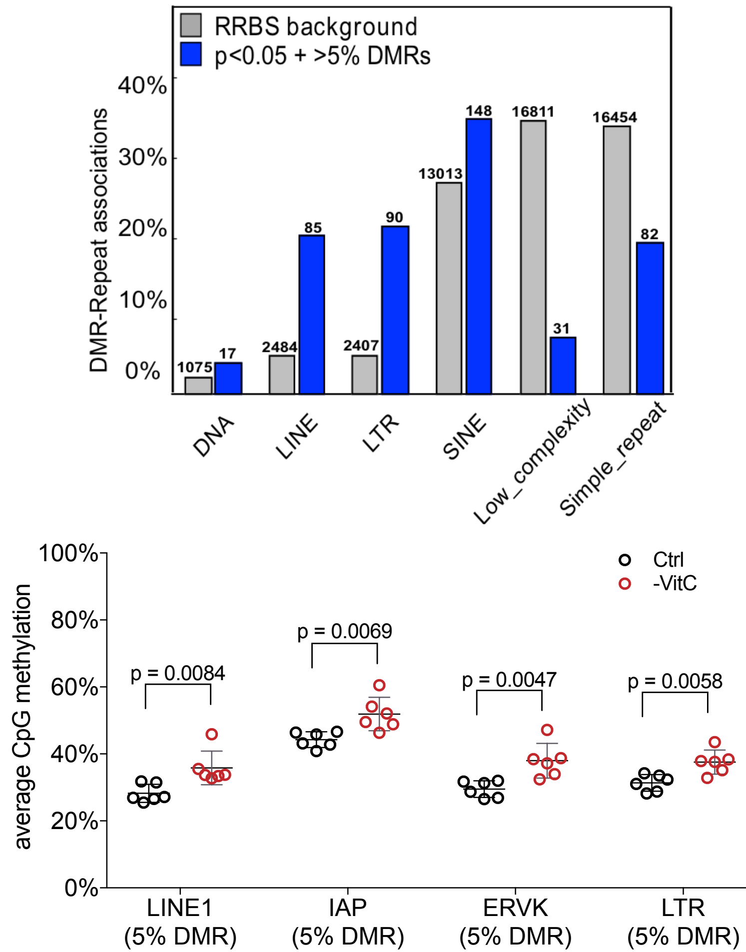


Figure 2-15: DMRs associated with Transposable Elements (TEs)
DMR-TE analyses reveals that DMRs (hyper and hypo) are enriched at LINE, LTR and SINE elements relative to the RRBS background. TEs of the LINE1 and LTR/ERVK/IAP families that are associated with DMRs show a consistent pattern of hypermethylation upon Vitamin C deficiency.

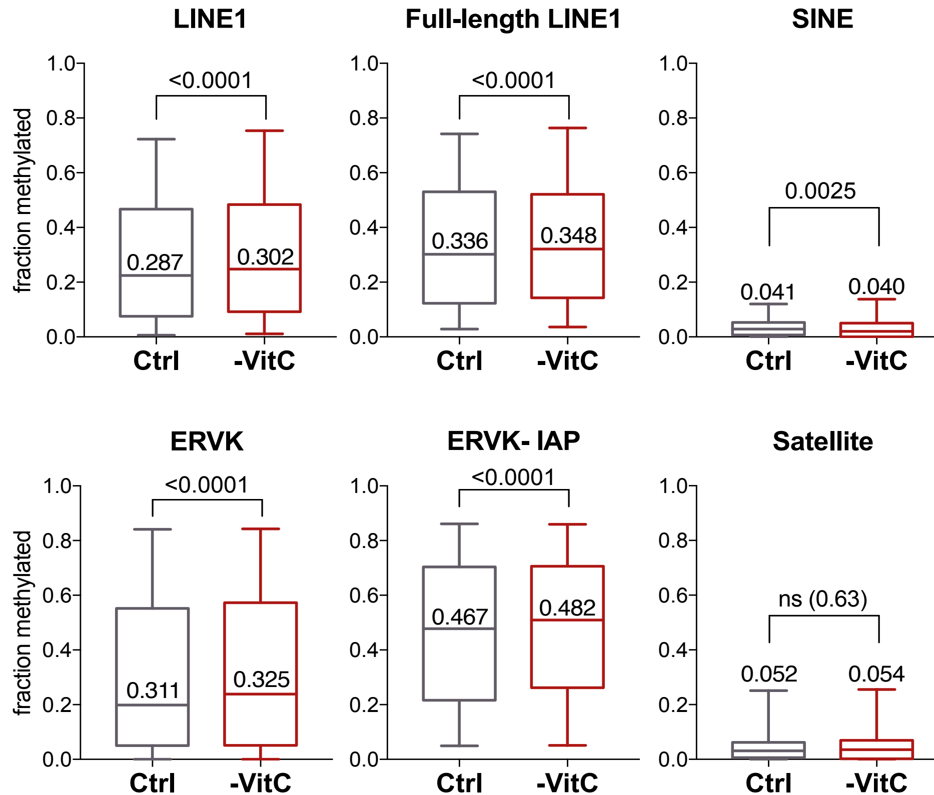


Figure 2-16: Average methylation at TE families across genome.
Average methylation in Ctrl or -VitC samples in different TE families across the genome. Data are from all uniquely mapped TEs annotated by RepeatMasker and captured by RRBS, regardless of the DMR calls, based on 558 elements and 3180 CpGs (LINE1), 185 elements and 1065 CpGs (Full-length LINE1; >6Kb), 384 elements and 1724 CpGs (SINE), 568 elements and 2691 CpGs (ERVK), 226 elements and 1057 CpGs (ERVK-IAP), 10 elements and 73 CpGs (Satellite). Error bars depict 5 and 95 percentiles. Statistical significance assessed by Wilcoxon matched-pairs signed rank test.

Chapter 3: Conclusions and future directions

We report here that Vitamin C deficiency during mouse embryogenesis induces defects in germline DNA methylation and transcriptional profile, meiosis and fecundity, recapitulating to a remarkable extent the defects caused by mutation of the Tet1 gene (Figure 3-1). Although we cannot exclude that Vitamin C deficiency has an impact on somatic tissues, the fact that morphologically normal adult animals develop indicates that the embryonic germline is particularly sensitive to the levels of Vitamin C in the maternal diet. Our findings uncover a novel role for a key micronutrient in the epigenetic regulation of mammalian reproduction and raise the possibility that fecundity may be mechanistically tuned to environmental quality.

There is a growing appreciation that environmental insults during mammalian development can induce long-term defects in adult physiology (Barker, 2004; Boekelheide et al., 2012). The two waves of genome-wide DNA demethylation and re-establishment, first during germline development and later during pre-implantation of the next generation, are thought to erase the transmission of epigenetic perturbations induced by environmental insults (Heard & Martienssen, 2014). However, these reprogramming events are not complete, with some genomic regions being partially resistant to DNA demethylation and therefore potentially being carriers of environmentally-induced epialleles. In particular, certain TEs such as IAP elements in mouse are partially resistant to DNA demethylation during germline reprogramming (Hackett et al., 2013; Seisenberger et al., 2012). Thus, environmental insults that impact DNA methylation reprogramming of TEs in the germline, such as the case of Vitamin C

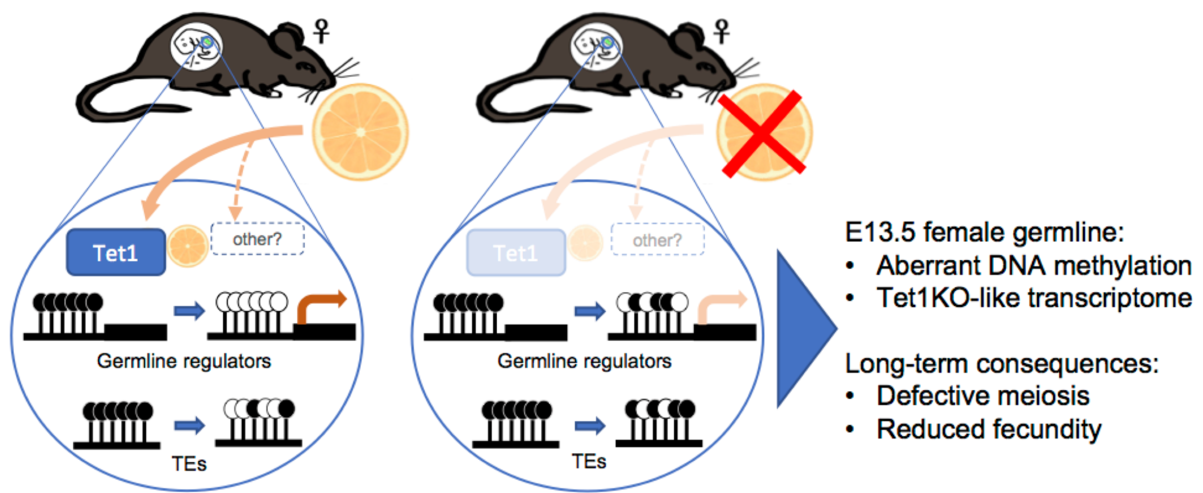


Figure 3-1: Model of Maternal Vitamin C deficiency and consequences on female germline development.

Embryonic germline cells require Vitamin C for proper DNA demethylation of key meiosis regulators and transposable elements. Gestational Vitamin C deficiency is compatible with development to term and adulthood, but induces a phenotype akin to a Tet1 hypomorph, with incomplete DNA demethylation and down-regulation of germline genes, reduced germ cell numbers, meiosis defects and decreased fecundity. Vitamin C deficiency may also impact other enzymatic reactions in the germline.

deficiency reported here, may induce epialleles that manifest intergenerationally, or allow for the manifestation of otherwise silent epialleles. In this regard, it will be of interest to combine gestational Vitamin C deficiency with other genetic or environmental perturbations to uncover potential epigenetic interactions between them.

In addition to the potential removal of epialleles, DNA demethylation is also required for the activation of a meiosis gene expression program. Tet enzymes, in particular Tet1, regulate DNA demethylation and activation of meiosis genes in the embryonic germline (Yamaguchi et al., 2012). The low numbers of PGCs and their very low levels of DNA methylation preclude a quantification of the impact of Vitamin C deficiency on hmC in the embryonic germline. However, the ability of Vitamin C to induce Tet-mediated generation of hmC has been extensively documented in many cell types, including ES cells, embryonic fibroblasts, T cells and hematopoietic progenitors (Agathocleous et al., 2017; Blaschke et al., 2013; Cimmino et al., 2017; Minor, Court, Young, & Wang, 2013; Sasidharan Nair, Song, & Oh, 2016; Yin et al., 2013). The present study documents the extensive parallels between gestational Vitamin C deficiency and the Tet1 mutation with regards to germline reprogramming, transcriptional profile, meiosis and fecundity. Our results highlight that a nutritional deficit can recapitulate a genetic mutation with a central role in mammalian reproduction.

The window of Vitamin C deficiency used in this study (17-20 days) allows mice to remain viable and fertile. Decreased body weight and mortality of Gulo-/- mice only manifests after 5 weeks of Vitamin C deficiency (Maeda et al., 2000). Nevertheless, Vitamin C is a powerful anti-oxidant and a co-factor for several 2-oxoglutarate-dependent dioxygenases, and therefore it is expected to have roles beyond enhancement of Tet

activity. We have recently shown that in ES cells Vitamin C induces the specific demethylation of H3K9me2, but no other histone mark tested, by enhancing the activity of the histone demethylases Kdm3a/b (Ebata et al., 2017). Although the function of H3K9me2 in the germline remains unknown, it is sharply lost specifically in PGCs within the same time frame as the loss of DNA methylation (Seki et al., 2007; 2005). It will therefore be of interest to compare the role of Vitamin C in the activity of Tet enzymes versus Kdm3a/b enzymes during germline development.

Meiosis is essential for gamete formation, and errors in meiosis are the leading cause of miscarriage in human reproduction. Given the central role of Tet1 in meiosis initiation in female mice (Yamaguchi et al., 2012), it is notable that Tet enzymes receive a diversity of inputs from the cell's metabolic state, be it in the form of oxygen, 2-oxoglutarate, iron, O-GlcNAc or Vitamin C (Blaschke et al., 2013; Carey et al., 2015; Wu & Zhang, 2011; Yin et al., 2013; Q. Zhang et al., 2014). Thus, the initiation of meiosis in mammals may be regulated at an epigenetic level by the metabolic state of the embryonic germline, which in turn may be modulated by the external environment. We propose that the interconnectivity of Tet1 with metabolism and maternal nutrition during germline development, rather than being a liability, provides a mechanism for adjusting the investment on reproduction across generations to the abundance of key nutrients in the environment. In support of this notion, several studies in invertebrate species have documented that poor nutrition during early life can lead to defects in reproduction in adults [e.g., (Chippindale, Leroi, Kim, & Rose, 1993; Moatt, Nakagawa, Lagisz, & Walling, 2016; Walker, Houthoofd, Vanfleteren, & Gems, 2005)]. While dietary factors may regulate fecundity in a variety of indirect ways including by modulating gonad

development or mating behavior, our findings suggest that the Tet1-Vitamin C axis may be one mechanism by which the germline is directly able to interpret environmental quality.

Most fungi, plants and animals can synthesize their own Vitamin C. Given the importance of Vitamin C in several processes, including Tet-regulated reproductive potential, it is interesting that certain vertebrate species have lost the ability to synthesize Vitamin C, such as teleost fishes, bats, guinea pigs and anthropoid primates, including humans (Drouin et al., 2011). In all cases this has occurred by accumulation of mutations in the gene coding for L-Gulonolactone oxidase (Gulo), the enzyme that carries out the last step in Vitamin C synthesis from glucose. These species thus evolved to become entirely dependent on their dietary intake of Vitamin C. Although the evolutionary reasons behind these events are the subject of debate (Drouin et al., 2011; Monfort & Wutz, 2013), our results suggest that, at least in some cases, this may have provided an evolutionary advantage by allowing the species to adjust for a higher investment in reproduction in nutritionally-rich environments. Of note in this regard, our insights arise from a mouse model of Vitamin C deficiency where the Gulo mutation was engineered by gene targeting (Maeda et al., 2000). It will therefore be of interest to test the role of Vitamin C in germline development in a non-synthesizing species. It may also prove insightful to test whether reengineering the synthesis of Vitamin C (by reestablishing a functional Gulo gene) in a naturally non-synthesizing species would affect its reproductive potential or other Tet-regulated processes.

Vitamin C may be a more general readout of environmental conditions beyond availability/quality of food. Oxidative stress induced by environmental contaminants can

lead to oxidation of Vitamin C (Cyr & Domann, 2011). A remarkably long list of environmental contaminants has been shown to alter the redox state of the cell and potentially impact Vitamin C levels, including heavy metals such as lead, cadmium, arsenic and copper, dioxins, alcohol and cigarette smoke [e.g., (Alsharif, Lawson, & Stohs, 1994; C. E. Cross, Traber, Eiserich, & van der Vliet, 1999; Cyr & Domann, 2011; Ercal, Gurer-Orhan, & Aykin-Burns, 2001; Matsumura, 2003; Salnikow & Zhitkovich, 2008)]. More broadly, several environmental contaminants are now known to have a detrimental effect on reproduction, although in most cases the mechanisms of action remain unclear (Manfo, Nantia, & Mathur, 2014; A. Wang, Padula, Sirota, & Woodruff, 2016). It will be important to investigate whether inactivation of Vitamin C contributes to subfertility induced by some environmental contaminants. In a planet increasingly affected by human-caused climate change and pollution, the sensitivity of the germline to environmental inputs has important implications for the reproductive potential of many species, including humans.

Chapter 4: Materials and Methods

Mouse model for gestational Vitamin C deficiency

Gulo$^{-/-}$ mice (Maeda et al., 2000) were obtained from the UC Davis Mutant Mouse Resource & Research Center (strain B6.129P2-*Gulo*^{tm1Mae}/Mmucd, RRID:MMRRC_000015-UCD) and bred with *Oct4/GFP* mice (Szabó et al., 2002) to establish a homozygous *Gulo*$^{-/-}$*;Oct4/GFP* colony on a C57BL/6 background. All mice were supplemented with 3.3g/L L-Ascorbic Acid (Sigma-Aldrich #A92902) in their drinking water, refreshed weekly. Experimental (-VitC and Ctrl) females were maintained on a custom mouse chow devoid of Vitamin C (Teklad Custom Rodent Diet number TD.130707). Vitamin C supplemented drinking water was replaced with non-supplemented water 3-7 days before mating for experimental -VitC females. Pregnant females remained on non-supplemented water through day 13.5 of gestation (E13.5) with morning of observed vaginal plug denoted as E0.5. All pregnant mice (Ctrl and -VitC) were supplied with fresh Vitamin C supplemented water at E13.5 and were visually indistinguishable. Experiments were performed in accordance with the guidelines of the UCSF Institutional Animal Care and Use Committee.

Measurement of circulating maternal Vitamin C

After deep isoflurane anesthesia, blood was collected from the vena cava of pregnant female mice using a syringe. ~1mL of blood was allowed to clot for 30min at room temperature in a 1.5 mL Eppendorf tube. Clotted blood was centrifuged at 10,000 rpm for 10min. Serum was collected and immediately stored at -80 or processed using a

colorimetric Ascorbic Acid Assay Kit (Sigma-Aldrich MAK074-1KT) according to manufacturer's protocol.

Isolation of germ cells

Dissections were performed on day E11.5, E13.5, E14.5 or E18.5, depending on the experiment, after euthanasia of pregnant females under deep isoflurane anesthesia. Embryonic gonads were dissected in sterile cold PBS and immediately fixed or dissociated for further analyses. Embryonic ovaries were enzymatically digested in 0.5% Trypsin and 0.8 mg/mL DNAse I (Worthington) at 37°C for 3-5 minutes, then manually dissociated by pipetting. PGCs and matched somatic cells were isolated by FACS on an AriaII instrument (BD Biosciences) based on GFP and SSEA1 expression. PGCs and soma were sorted directly into RLT lysis buffer or PBS, for downstream RNA or DNA extraction respectively, and immediately frozen on dry ice.

qRT-PCR

RNA was extracted using RNeasy micro columns (Qiagen) and cDNA generated using a High Capacity cDNA Reverse Transcription kit (ABI) according to manufacturer's protocol. qRT-PCR was performed on an ABI-Prism PCR machine with primers listed in (Table 2). The relative amount of each gene was normalized using two housekeeping genes (*Rpl7* and *Ubb*).

Histology and Immunohistochemistry

E14.5 ovaries were fixed with Bouin's fluid (for haematoxilin/eosin staining) or 4% formaldehyde (for immunohistochemistry) for 45 minutes and dehydrated through an ethanol series, embedded in paraffin and cut into 5μm-thick sections. Sections were mounted on glass, dewaxed, rehydrated and stained with haematoxilin and eosin. Meiotic staging was based on nuclear shape and chromatin compaction as previously described (Abby et al., 2017; Arora et al., 2016; Guerquin et al., 2010). The Histolab analysis software (Microvision Instruments, Evry, France) was used for counting.

For immunohistochemistry, tissue sections were mounted on glass slides, dewaxed, rehydrated and submitted to antigen retrieval by boiling for 20 min in citrate buffer (pH 6). Endogenous peroxidase activity was blocked by a 10-minute incubation with 3% hydrogen peroxide. Slides were then washed in PBS and blocked for 30 minutes in 2% horse serum in PBS. Slides were incubated overnight at 4°C with primary antibody diluted in PBS with 20% blocking buffer containing 2.5% of horse serum. Peroxidase-conjugated secondary antibodies (ImmPRESS reagent kit, Vector Laboratories) were incubated for 30 minutes, followed by 3,3′-diaminobenzidine (DAB substrate reagent kit, Vector Laboratories) or VIP (Vector VIP substrate reagent kit, Vector Laboratories) reactions. Three sections were randomly chosen from each gonad to quantify the percentage of DDX4/Vasa+ (Abcam Ab13840 Rabbit 1:200 or Ab27591 Mouse 1:500) germ cells co-stained for Stra8 (Abcam Ab49602 Rabbit 1:400), Sycp3 (Abcam Ab97672 Mouse 1:500), cleaved Caspase 3 (Cell signaling D175 9661S Rabbit 1:200) or Ki67 (BD Pharmingen 550609 Mouse 1:200). At least 200 Ddx4+ germ cells were scored per section.

Centromere staining in meiotic spreads

Glass slides were prepared by submerging in 70% ethanol while E14.5 ovaries were dissected and prepared. Freshly dissected E14.5 ovary pairs were dissociated in 200μl of enzymatic solution (0.025% Trypsin; 2.5mg/mL Collagenase; 0.1mg/ml DNase I) 37°C for 30 minutes, pipetting every 10 minutes. Trypsin was quenched with 50μl FBS and 250μl hypotonic buffer (30mM Tris pH 8.2; 50mM sucrose; 17mM Sodium-citrate; 5mM EDTA; 0.5mM DTT; 0.5mM PMSF) was added to each sample for 30 minutes at room temperature. The cell suspension was centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in 90μL 100mM Sucrose. At this point, slides were dried of ethanol and prepared with fixative solution (1% PFA; 0.15% Triton X100; 3mM DTT). Aliquots of cell suspension (20μL) were added to each drop of fixative solution per slide and allowed to dry at room temperature. Dried slides were quickly submerged in 0.4% Kodak Photo-Flo 200 and air dried. Meiotic spreads were stored at -80°C until staining.

Slides containing E18.5 female germ cell spreads were allowed to thaw for 15 minutes at room temperature before staining. Thawed slides were washed twice in PBS then permeabilized in PBS plus 0.2% triton for 20 minutes at room temperature. Next, spreads were blocked in a buffer containing PBS plus 5% BSA and 0.1% Tween (PBSST) for 45 minutes. The primary antibodies for Sycp1 (Abcam Ab15090) and CREST (Antibodies Incorporated #15-234-0001) were incubated at a 1:400 dilution overnight in a 4°C humidity chamber. On day two, slides were washed in PBSST 3 times for 10 minutes each and blocked in PBSST plus 10% donkey serum for 30 minutes. The slides were incubated in secondary antibodies (Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor

647 AffiniPure donkey anti-human IgG cat# 709-605-149) for 1 hour in the dark. Slides containing immunofluorescent stained meiotic spread were then washed for a final time (PBSST 3 x 10 minutes at room temperature in the dark) before mounting with Vectashield + DAPI and imaging on a Leica confocal.

Whole-mount imaging

Whole-mount immunostaining and confocal imaging of postnatal day 7 ovaries were previously described (Faire et al., 2015). The primary antibody used was Nobox (1:100, a gift from Aleksandar Rajkovic) and the stain Hoechst (1:100, Fisher H3569). The secondary antibody used was Alexa-555 Donkey anti-Goat from Fisher and used at 1:200. Whole-mount ovaries were imaged using the Leica DMi8 confocal microscope, using a 20x objective. Imaris software (Bitplane) was used to quantify Nobox objects. An Imaris surface was created around entire ovary to exclude oviduct and surrounding tissue and channels were masked to this surface for analysis. The ovary volume was generated from this Imaris surface contour. Images were filtered using Background Subtraction with a 30um filter and Gaussian Filter 1 voxel size. Nobox objects were quantified using the Imaris spot module with a diameter of 8 um and all spot objects were selected.

Statistical analyses

All statistical calculations were performed with GraphPad Prism 7.0 software. Details of individual tests are outlined within each figure legend.

RNAseq

Germ cell RNA isolated from single embryos was quantified using an Agilent Bioanalyzer RNA Pico Kit. Barcoded libraries were created from 1.5 ng DNAseI-treated total RNA using Clonetech SMARTR-seq RNA library prep kit according to the manufacturer's recommendations. Successful libraries were bioanalyzed for quality and size distribution and then quantified using the Qubit dsDNA HS Assay Kit. Diluted libraries were pooled at equal 5nM concentrations for sequencing. Final product was sequenced 50bp SE on 1 lane of an Illumina HiSeq4000 at the UCSF Center for Advanced Technology. Six embryos were sequenced per condition, totaling 12 libraries in all. 25-40 million reads were obtained and analyzed per sample. Single-end reads were quality-controlled and adaptor-trimmed using Trim Galore! (Babraham Bioinformatics), using standard settings. Filtered reads were mapped to the mm9 mouse genome utilizing Tophat2 (D. Kim et al., 2013). Reads mapping uniquely to known genes were counted using htseq-count (Anders, Pyl, & Huber, 2015). Count data were subsequently imported into R with Bioconductor (Huber et al., 2015) and filtered to remove non-expressed genes. For read-depth normalization, filtered gene count data were analyzed using the R package DESeq2 (Love, Huber, & Anders, 2014). Data were input into a DESeq object and normalized according to package recommendations. Gene Ontology term enrichment was analyzed using DAVID (Huang, Sherman, & Lempicki, 2009), and comparisons to the data on Tet1KO E13.5 germ cells (Yamaguchi et al., 2012) were carried out using GSEA (Subramanian et al., 2005).

Reduced Representation Bisulfite Sequencing (RRBS) on single embryo PGCs

We modified previously published protocols for RRBS and scRRBS (H. Guo et al., 2013; Meissner et al., 2008) to create a streamlined single-tube workflow for low-input. Each RRBS library was created from 2ng of gDNA collected from PGCs isolated by FACS from single E13.5 embryos. Initially, gDNA was extracted from Oct4/GFP+ PGCs using Purelink Genomic DNA kit (K1820-00 Invitrogen) according to the manufacturer's instructions. 2ng gDNA + 0.25% Lambda DNA were then digested with 10 units of MspI enzyme (Fermentas) in a total reaction volume of 18μL at 37°C for 3 hours, followed by heat inactivation at 80°C for 20 minutes. End repair and A-addition was then carried out to repair and tail the 3' end of each digested fragment. This reaction was done immediately following MspI digestion by adding 2uL of 5U Klenow exo- with additional dNTPs (0.04mM dATP, 0.004mM dGTP, 0.004mM dCTP) directly to the 18μL digest. This reaction was incubated at 37°C for 40 minutes followed by heat inactivation at 75°C for 15 minutes. After repair, NEBnext USER methylated adaptors were ligated onto the DNA by adding 25 units of T4 DNA Ligase, 1.5mM ATP, and 25nM NEB Adaptors directly to the 20μL reaction, giving a final reaction volume of 30μL. Adapter ligation was performed at 16°C for 30 minutes and then left at 4°C overnight. The following day, 1μL of NEB USER enzyme was added to each reaction, followed by an incubation at 37°C for 15 minutes and then inactivation at 65°C for 20 minutes. Per the manufacturer's protocol, NEB USER enzyme is required for NEBnext adapter cleavage.

Bisulfite conversion was performed on each 30μL sample containing DNA fragments with methylated sequencing adapters. We used the low-concentration protocol

of the EpiTect Fast Bisulfite Conversion Kit (Qiagen), eluting in 15μL. 13μL of converted DNA were used directly in a library amplification PCR, which was optimized for 2ng input, including 1μL NEBNext Primer F, 1μL NEBNext R, and 15μL of 2x HiFi Uracil+ Mix (KAPA). This reaction was incubated at 95°C for 2 minutes, followed by 15 cycles of 95°C for 20 seconds; 60°C for 30 seconds; 72°C for 1 minute, with a final step of 72°C for 5 minutes and a hold at 4°C. At this point all samples were quantified using the Qubit dsDNA HS Assay Kit.

Successfully amplified libraries were completed with size selection and cleanup. Size selection of 200-600bp fragments was performed using Axygen Fragment Select-I beads. First, 0.5 volume of beads (15μL) was added to the 30μL sample, pipetted to mix, and incubated at room temperature for 5 minutes. The bead-sample solution was then magnetically separated and 45uL of supernatant was placed in a clean strip-tube. An additional 10μL of beads were then added to each 45μL sample for collection. After a 5-minute incubation, beads were magnetically recovered and washed twice in 80% ethanol. Size selected libraries were eluted off the beads with 20μl ddH2O. Completed libraries were then quantified in an Agilent Bioanalyzer using the High Sensitivity DNA Assay, and diluted to 10nM. Libraries were pooled and sequenced at the UCSF Center for Advanced Technology on an Illumina HiSeq4000 sequencer with adequate PhiX spike-in DNA (~30%). 13-20 million reads were obtained and analyzed per sample.

RRBS Data Analysis

RRBS fastq files were first trimmed for quality and MspI-induced overhangs using Trim Galore! (Babraham Bioinformatics) with RRBS specific settings. Trimmed files were then aligned to the mouse mm9 genome and methylation extracted using Bismark, a program specific for alignment of bisulfite-treated DNA (Krueger & Andrews, 2011). Given the very low genome-wide methylation in E13.5 PGCs (<5% total methylation, Fig. S5A), the R program BiSeq (Hebestreit et al., 2013) was used to detect differential methylation. BiSeq is a DMR-detecting method that enables testing within target regions, like with RRBS, and allows calculation of FDR. Coverage and methylation calls per CpG calculated with Bismark were imported into RStudio, and these files were then used to build the BiSeq data-frame, accounting for replicates. Coverage data was filtered with requirements of >5x coverage in a minimum of 9/12 samples per CpG. Each sample contained 1.3 – 1.6 million CpGs passing this coverage threshold. CpGs were categorized into clusters, with a cluster defined as a string of >5 CpGs with a maximum of 20bp distance between adjacent CpGs. 52707 clusters were defined across the mouse genome ranging from 6 to 370bp per cluster (53bp average cluster size). Functional annotation of DMRs was done using GREAT (McLean et al., 2010). Tet1-binding sites in ES cells were taken from (Wu et al., 2011) and Dnmt1-targeting sites in ES cells were taken from (Li et al., 2015). TE genomic locations were downloaded from UCSC RepeatMasker for TE-methylation analysis (Karolchik et al., 2004).

Appendices

Table 2: qRT-PCR primers

Primer Name	Sequence
Rpl7-qRT-F	AGC GGA TTG CCT TGA CAG AT
Rpl7-qRT-R	AAC TTG AAG GGC CAC AGG AA
Ubb-qRT-F	GCG GTT TGT GCT TTC ATC AC
Ubb-qRT-R	GGC AAA GAT CAG CCT CTG CT
Mael-qRT-F	GAG CAG CCC AGG GAA AGG AT
Mael-qRT-R	TAC AAG CAT GCC TGG CCT CC
Dazl-qRT-F	CAA CTG TTA ACT ACC ACT GCA G
Dazl-qRT-R	CAA GAG ACC ACT GTC TGT ATG C
Gtsf1-qRT-F	GAC TCC CTG GAC CCT GAA AA
Gtsf1-qRT-R	GCC AAT TTG TTT GCG ACA TC
Syce1-qRT-F	GGC AGT ATG GGT CCA CAC AGA
Syce1-qRT-R	CAA TCC GGG GCT CTA GGC TT
Sycp1-qRT-F	TTC ATA AAG GAG CGC ACG CG
Sycp1-qRT-R	TTC TCC ATG CTG CCT CCT GG
Stra8-qRT-F	CCC AAC AGC TTA GAG GAG GTC A
Stra8-qRT-R	TCC AAC AGC CTC AGA GGG GA
Gng13-qRT-F	TGT CGT CCA AGA CCA TCC CC
Gng13-qRT-R	TGG TGC ACT TGG CCT TCT CT
Magi2-qRT-F	AGT CAA TGG AAA CGG CGT GG
Magi2-qRT-R	TAC ACG GGT GCA GGG TAA GG
Anpep-qRT-F	CCT GCT GGC CTA CAT CGT GA
Anpep-qRT-R	ACC CTG GCC CTC ATC AAT GG
Foxl2-qRT-F	GCT CAC TCT GTC CGG CAT CT
Foxl2-qRT-R	GCG GAT GCT ATT CTG CCA GC
Wnt4-qRT-F	ACA CTG GAC TCC CTC CCT GT
Wnt4-qRT-R	GTC ACT GCA AAG GCC ACA CC

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