Cdk4/6 is activated by DNA hypomethylation to cause DNA rereplication and organ outgrowth defects

Bhavani P. Madakashira, Elena Magnani, Shashi Ranjan, and Kirsten C. Sadler*

Program in Biology NYU Abu Dhabi, Abu Dhabi, UAE

*Corresponding author; Kirsten.edepli@nyu.edu

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Abstract

Coordinating epigenomic inheritance and cell cycle progression is essential for organogenesis. UHRF1 connects these functions, as it is required for DNA methylation, transposon suppression and cell cycle progression. *uhrf1* mutation in zebrafish embryos increases cells in S-phase, causes abnormal nuclei, heterochromatin loss, transposon activation and widespread cell death, preventing organ outgrowth and causing embryonic lethality. We determined that Uhrf1 deficient zebrafish hepatocytes undergo DNA rereplication but do not divide, despite activation of the entire set of E2F target genes. Blocking Cdk4/6 in *uhrf1* mutants with Palbociclib prevented DNA rereplication and cell death, restored nuclear morphology, normalized the expression of E2F target genes, rescued liver size and extended survival but did not rescue transposon expression or immune activation. The same genes were activated in a Cdk4/6 dependent fashion in the liver of *dnmt1* mutants. However, *dnmt1* mutant hepatocytes cannot undergo DNA replication and blocking *dnmt1* rescued the DNA rereplication defect in *uhrf1* mutant livers. This demonstrates that the developmental defects caused by DNA hypomethylation are attributed to downstream signaling through Cdk4/6.

Introduction

Cell cycle progression during organogenesis requires precise coordination of the machinery that replicates the genome and those that pattern the epigenome. Crosstalk between key cell cycle regulators and the factors that write and erase the epigenome assure that the timing and patterning of DNA replication and epigenomic fidelity are maintained from parental to daughter cells (Almouzni & Cedar, 2016). There is a reciprocal relationship, as the chromatin landscape dictates which regions of the genome get replicated early or late in S-phase and can influence when and where the origins of DNA replication fire (Petryk *et al*, 2016). Moreover, when there are widespread changes to the epigenetic landscape, such as during development when cell identity is established, during aging or in cancer, DNA damage can ensue, activating checkpoints that block DNA replication and prevent cell cycle progression (McIntosh & Blow, 2012; Recolin *et al*, 2014). How the pathways that control cell cycle progression are functionally related to those that edit or maintain the epigenome during development is not well understood.

Several pieces of data suggest that crosstalk between the proteins that regulate epigenomic patterning and those that regulate DNA replication influence the activity of each process. One example is the interaction between the proteins at the DNA replication fork with the maintenance DNA methylation machinery which copies the pattern of 5-methyl Cytosine (5-mC) from parental to daughter strand. In vertebrates, this requires Ubiquitin-like, containing PHD and RING finger domains, 1 (UHRF1) which recruits DNA methyltransferase 1 (DNMT1) to newly synthesized DNA (Bostick *et al*, 2007; Mancini *et al*, 2021; Sharif *et al*, 2007). These proteins are anchored to the DNA replication fork (Lopez-Contreras *et al*, 2013; Sirbu *et al*, 2013) through interactions with PCNA (Mancini *et al.*, 2021), LIG (Ferry *et al*, 2017) and modified histones (Kim *et al*, 2009; Mancini *et al.*, 2021; Nishiyama *et al*, 2013) and the DNA methylation pattern is copied as soon as the new strand is synthesized. Therefore, both UHRF1 and DNMT1 are highly expressed during S-phase. Interestingly, in both cancer cells and during development, inactivation of either UHRF1 or DNMT1 blocks cell proliferation (Anderson *et al*, 2009; Chen *et al*, 2007; Ge *et al*, 2016; Jacob *et al*, 2015; Jenkins *et al*, 2005; Qin *et al*, 2014; Sadler *et al*, 2007; Tittle *et al*, 2011; Zhang *et al*, 2020), but the mechanism of this is not known.

One possibility explaining this cellular phenotype is that UHRF1 and/or DNMT1 could regulate the cyclins and cyclin-dependent kinases (CDKs) that regulate progression through the different stages of the cell cycle (Malumbres & Barbacid, 2009). The transition from G1 to S-phase of the cell cycle is triggered by cyclin D binding to the cyclin dependent kinases (CDK) 4/6 which phosphorylates the retinoblastoma (RB) protein, causing it to dissociate from the E2F transcription factors, allowing them to activate target genes (Goel *et al*, 2018). These genes are required for the G1/S transition, licensing DNA replication origins and the polymerases and other factors that assemble and license DNA replication forks and regulate the processivity of the DNA replication fork. Both UHRF1 and DNMT1 are targets of E2F1 (Bonapace *et al*, 2002; Kimura *et al*, 2003; Magri *et al*, 2014; McCabe *et al*, 2005; Unoki *et al*, 2004) and this ensures that they are expressed during S-phase when they are needed. Another connection between the cell cycle regulators and UHRF1 is provided by our finding that UHRF1 is phosphorylated by the CDK2/Cyclin A2 complex and that this is required for embryonic development (Chu *et al*, 2012; Kent *et al*, 2016). Thus, UHRF1

is regulated by the cell cycle pathways during G1/S and that it is required for cell cycle progression.

Just as cell cycle regulatory pathways can coordinate DNA methylation, the chromatin landscape can influence the cell cycle. For instance, the chromatin environment restricts E2F interaction with the target genes needed for S-phase entry and progression, and removal of these repressive marks is essential for E2F function (Blais & Dynlacht, 2007). Another study suggested that epigenetic marks can suppress the gene encoding $p16^{INK4a}$ which is an effective suppressor of CDK4/6, and removing these repressive marks can activate $p16^{INK4a}$, causing inappropriate cell cycle entry (Jung *et al*, 2007). These examples indicate the complexity of the interplay between the ubiquitous cell cycle machinery and epigenetic modifiers to ensure fidelity of the epigenetic patterns as cells proliferate. Understanding of how these processes are regulated during vertebrate development is limited.

The developing liver provides an optimal model to study this relationship. The liver develops according to a series of stereotypical stages starting with specification of multipotent endodermal progenitors which become further restricted to the hepatic lineage and then committed to differentiate to the two main cells of the liver, hepatocytes and biliary cells. This is followed by the outgrowth and morphogenesis of the liver which occurs as the hepatocytes acquire cell specific functions. Hepatic outgrowth is a classic example of developmentally regulated cell cycle initiation and progression, with strong mitogenic signals that promote hepatocyte proliferation provided by the many growth factors produced during development of endodermally derived organs. This stage of hepatogenesis is the least well understood and the mechanisms of cell cycle regulation during hepatic outgrowth suggest that they are the same cell cycle signaling pathways that control proliferation in most other cell types. Studies in mice show that E2F regulates hepatocyte proliferation during liver development and regeneration (Delgado *et al*, 2011; Sladky *et al*, 2020) and loss of RB in mouse hepatocytes is sufficient to promote cell cycle entry, increased ploidy and large nuclei in post-natal and adult mouse hepatocytes (Mayhew *et al*, 2007; Wu *et al*, 2022).

In zebrafish, hepatocytes are specified and determined during the organogenesis stage of development (between 24-48 hours post fertilization, hpf) (Goessling & Stainier, 2016; Wang *et al*, 2017) and the differentiated hepatocytes proliferate while the liver undergoes morphogenesis during hepatic outgrowth. These stages are largely complete by 120 hpf (Chu & Sadler, 2009). *uhrf1* mutant zebrafish have an unusual developmental and cellular phenotype which restrict hepatic outgrowth. The developmental defects include a small liver, underdeveloped gut, malformed jaw and small eyes (Marjoram *et al*, 2015; Sadler *et al.*, 2007; Tittle *et al.*, 2011) which are first observed between 80-96 hpf and are exaggerated at 120 hpf, resulting in lethality by 10 days post fertilization (dpf) (Jacob *et al.*, 2015). The cellular phenotype of hepatocytes is characterized by large, misshapen nuclei, near complete loss of nuclear structures (Madakashira *et al*, 2021), and widespread cell death (Jacob *et al.*, 2015; Sadler *et al.*, 2007) which is dependent on Tnfa (Magnani *et al*, 2021). Most strikingly, at 120 hpf, nearly half of all hepatocytes incorporate nucleotides, and we concluded that this reflected unscheduled DNA replication (Jacob *et al.*, 2015). As in all other systems studied (Bostick *et al.*, 2007; Feng *et al*, 2010; Sharif *et al.*, 2007; Tittle *et al.*, 2011; Wang *et al*, 2019), *uhrf1* mutant zebrafish hepatocytes have global

loss of DNA methylation which leads to activation of transposons and an interferon response to these viral mimetics (Chernyavskaya *et al*, 2017; Magnani *et al.*, 2021). Here, we identify Cdk4/6 activity as the mechanism giving rise to most of the developmental and cellular defects in *uhrf1* zebrafish mutants, except for DNA hypomethylation and the subsequent derepression of transposons and activation of the interferon response. Cdk4/6 signaling is also activated in the liver of *dnmt1* mutants, suggesting DNA hypomethylation as the cause of this phenotype. We also show that DNA rereplication phenotype in *uhrf1* mutants requires *dnmt1*, consistent with a requirement of Dnmt1 at the replication fork. These data provide new mechanistic insight into the interaction between key cell cycle regulatory pathways and DNA methylation during development, indicate the importance of the Cdk4/6 pathway in cellular consequences that occur when Uhrf1 is modulated, and suggest that the important chemotherapeutic, Palbociclib, may function to modulate the cellular response to epigenetic defects.

Results

DNA re-replication is a primary cellular response to *uhrf1* loss

We previously reported that *uhrf1* mutant livers have an increased number of cells that incorporate nucleotides during hepatic outgrowth, despite the livers being small for size (Jacob *et al.*, 2015). This could reflect an increase in DNA damage repair or an increase in DNA replication. To differentiate between these possibilities, we carried out a series of studies on the pattern, timing and outcome of this cellular phenotype. If nucleotide incorporation was due to DNA replication, then the nuclei should have more DNA. FACS sorted hepatocytes from *uhrf1* and WT larvae at 120 hpf showed that 7% of *uhrf1* mutants had 4N DNA content compared to 2% of controls (Figure 1A). This is consistent with our previous finding of enlarged hepatocyte nuclei in *uhrf1* mutants (Madakashira *et al.*, 2021), a phenotype that reflects increased ploidy (Knouse *et al*, 2014). To determine the developmental timing of the EdU incorporation phenotype, we pulsed embryos at 80, 96 and 120 hpf for 30 minutes with EdU, sorted *uhrf1* mutants based on morphological phenotypes at 96 hpf and 120 hpf, and since the phenotype is not apparent at 80 hpf, all embryos for this time point were individually genotyped. This showed that nearly all hepatocytes in *uhrf1* mutants and their phenotypically normal sibling control larvae (heretofore referred to as controls) were EdU positive at 80 hpf. This was reduced to 25% and 10% of control hepatocytes at 96 hours and 120 hpf, respectively, but in *uhrf1* mutants 55% and 43% of hepatocytes were EdU positive at these same time points (Figure 1B-C). This time course mirrors the hepatocyte nuclear morphological defects in *uhrf1* mutants, which are very modest at 80 hpf and become more striking at 96 and 120 hpf (Madakashira *et al.*, 2021).

There is a stereotypical pattern of DNA replication, with euchromatic DNA replicated in early S-phase visualized as homogeneous EdU labeling in the nucleoplasm, followed by replication DNA in the nuclear lamina replicated during mid-S-phase and heterochromatin replicated last (Zhang *et al*, 2021). We reasoned that if the increase in EdU incorporation in *uhrf1* mutant hepatocytes was due to DNA rereplication, these patterns of EdU labeling would be apparent. We scored the S-phase stage of hepatocytes during hepatic outgrowth based on the patterns of EdU incorporation and found that at 80 hpf, most hepatocytes in controls and *uhrf1* mutants were in early S-phase, while at 96 hpf and 120 hpf there was a significant increase in the number of *uhrf1*$^{-/-}$ hepatocytes in mid and late S-phase, respectively (Figure 1D). These data rule

out the possibility that EdU incorporation in *uhrf1* mutant hepatocytes reflects nucleotide incorporation during DNA damage repair, as repair mediated nucleotide incorporation does not mirror the stereotypical pattern that we observe reflecting stages of DNA replication (Lee *et al*, 2011; Rieck *et al*, 2012). Interestingly, we observed that *uhrf1*$^{-/-}$ hepatocytes incorporate EdU in a pattern that reflects a pattern of heterochromatin, even though these hepatocytes are completely devoid of the heterochromatin markers H3K9me3, H3K9me2, Hp1α (Figure 1E) and the nuclear lamina marker lamin A (Madakashira *et al.*, 2021). Together, these data indicate that the increased number of EdU positive hepatocytes in *uhrf1* mutants reflects DNA rereplication, not DNA damage repair and suggest a defect that causes unregulated DNA replication origin firing, inappropriate firing of dormant origins or a failure of the DNA replication checkpoint that ensures that genome replication occurs only once during a cell cycle.

We hypothesized that the EdU incorporation phenotype in *uhrf1* mutant hepatocytes reflected either an acceleration of the fork progression rate or an increase in the number of active replication origins. To test this, we optimized the duration of the EdU pulse so to be just below the limit of detection in control hepatocytes at 120 hpf (Figure S1). Following a 12 minute pulse with EdU, no hepatocytes were labeled at either 80 or 120 hpf in control larvae or in *uhrf1*$^{-/-}$ larvae at 80 hpf but nearly 50% of *uhrf1* mutant hepatocytes were labeled at 120 hpf (Figures 1F and Figure S1), and nearly all of these EdU positive *uhrf1*$^{-/-}$ nuclei were in early S-phase (Figure 1G). This suggests that Uhrf1 deficiency either induces firing of more DNA replication origins or increases replication fork speed.

CDK4/6 is activated in *uhrf1* mutant livers

To identify the basis of this DNA replication defect, we analyzed bulk RNAseq data from pools of control and *uhrf1* mutant livers collected at 120 hpf (Magnani *et al.*, 2021), when the hepatic developmental and cellular phenotypes are fully penetrant. Gene ontology (GO) analysis using REVIGO was used to identify the pathways that were most significantly deregulated in the thousands of differentially expressed genes (DEGs). We found that downregulated genes were mainly involved in hepatic functions such as protein modification and lysis, signaling and metabolic process and that the upregulated genes were enriched in pathways involved in cell cycle regulation, DNA replication, anti-viral and immune response and cell death (Figure 2A, Table S1). UPSET analysis of the genes in the cell cycle pathways identified by REVIGO demonstrated that many of these genes were shared between the different genesets, however a cluster of genes involved in DNA replication and cell division were unique (Figure 2B; Table S1). These data confirm and extend our previous findings (Jacob *et al.*, 2015; Magnani *et al.*, 2021; Sadler *et al.*, 2007) that *uhrf1* mutant livers have aberrant cell cycle regulation.

To identify potential key regulators that could account for the deregulation of the cell cycle genes, we carried out Ingenuity Pathway Analysis (IPA) of the unified set of all DEGs in the GO term genesets related to the cell cycle. This uncovered a pattern whereby the G1/S and G2/M checkpoint pathways were downregulated and cell cycle regulation, proliferation and transition through the cell cycle were upregulated (Figure 2C, Table S2). Importantly, genes involved in the G1/S transition, including the key upstream regulator complexes of Cdk4/6-Cyclin D, Cdk2-Cyclin E and the DNA replication initiation factor, *Cdc25a*, were upregulated in *uhrf1* mutant livers.

Additionally, the cell cycle inhibitor *cdkn1b* and the cell cycle regulated gene *rbl2* which prevents access of E2F1 and is a UHRF1 interacting protein (Macaluso *et al*, 2007) were downregulated (Figure 2D). Together, these data suggest that loss of *uhrf1* results in repression of G1/S and G2/M cell cycle checkpoints and activation of Cdk4/6 downstream targets that promote G1/S transition and DNA replication.

The developmental timing of *uhrf1* mutant hepatic phenotypes follows a sequence whereby DNA hypomethylation (Magnani *et al.*, 2021), and nuclear size defects are first detected at 80 hpf (Madakashira *et al.*, 2021), followed by DNA rereplication (Figure 1B-D,F) and cell death at 96 and 120 hpf. No cell cycle DEGs were detected at 80 hpf (Jacob *et al.*, 2015) and qPCR analysis on RNA from pooled livers dissected at 96 hpf showed that key cell cycle regulatory genes (*ccne1, ccna2)*, replication licensing factors (*mdm2, mcm3, orc3, cdc6)* and processivity factors (*pola1, pold1* and *pcna)* were upregulated at this time point (Figure 2E). To determine if this effect is conserved, we assessed their expression in the liver of neonatal mice at P4 that are deficient for *Uhrf1* in hepatocytes ((*Alb:Cre*$^{Tg/+}$*;Uhrf1*$^{fl/f}$ *i.e. Uhrf1*^{HepKO}) (Wang *et al.*, 2019). There was robust expression of Cre (Figure 2F, inset) and downregulation of *Uhrf1*, indicating effective Cre-mediated Uhrf1 depletion in these samples. All cycle genes analyzed showed increased expression in *Uhrf1*^{HepKO} livers (Figure 2F), suggesting that Uhrf1 plays a conserved role in regulating the cell cycle during hepatic outgrowth by promoting S-phase entry and DNA rereplication.

Cdk4/6 activation is required for the *uhrf1* mutant phenotype

We hypothesized that Cdk4/6 and E2F activation combined with loss of G1/S checkpoints could drive Uhrf1 deficient cells into S-phase. To test this, we used the Cdk4/6 inhibitor Palbociclib (PD in figures), which is an approved chemotherapeutic for breast cancer (Laderian & Fojo, 2017) that effectively suppresses DNA replication and cell proliferation in liver cancer cells (Bollard *et al*, 2017). We optimized the dose and timing of Palbociclib exposure to obtain the maximal tolerable concentration and administration timing to achieve no effects on control larvae based on survival, overall embryo phenotype, morphological criteria and liver size (Table S3). The final treatment scheme outlined in Figure 3A involved adding 20 μM Palbociclib in 0.5% DMSO or 0.5% DMSO as a control to 48 hpf embryos generated from an incross of *uhrf1*$^{+/-}$ adults which expressed nuclear localized signal targeted cherry transgene in hepatocytes (*Tg(fabp10a: nls-mcherry)*) to mark the livers. The resulting larvae were collected at 120 hpf, imaged to assess morphological features characteristic of *uhrf1* mutants and then all larvae were individually genotyped. In some experiments, larvae at 120 hpf were pulsed for 30 minutes with EdU to assess DNA replication.

At 120 hpf, *uhrf1* mutants are distinguished by smaller head, lower jaw deformity, microphthalmia, underdeveloped gut and small liver (Figure S2A; (Jacob *et al.*, 2015; Sadler *et al.*, 2007)). These phenotypes are fully penetrant at 120 hpf, so that on average, 25% of embryos resulting from an incross of *uhrf1*$^{+/-}$ adults display all of these phenotypes (Figure S2B). We were able to reliably identify all hallmarks of the *uhrf1* mutant phenotype in only 7% of embryos that were produced from an incross of *uhrf1*$^{+/-}$ adults treated with 20 μM Palbociclib (Figure 3B). In treated clutches, 18% of embryos showed partial restoration of the lower jaw defect, change in liver size, or both (i.e. “partial” mutant phenotype; Figure S2B-C). When these embryos were genotyped, we uncovered the expected genotype: phenotype relationship in 100% of embryos in DMSO treated clutches, so that all embryos displaying the mutant phenotypes were

uhrf1$^{-/-}$ and all embryos that lacked these phenotypes were either *uhrf1*$^{+/+}$ or $^{+/-}$. However, in Palbociclib treated clutches the embryos with partial phenotype and a few scored as normal were *uhrf1*$^{-/-}$ (Figure S2C-D). Palbociclib had no significant effect on liver size in WT embryos, but significantly increased the size of the liver (Figure 3B-D) and eye (Figure S2E) in *uhrf1* mutants: the liver size of nearly all *uhrf1* mutants treated with Palbociclib exceeded the average liver size in DMSO treated mutants, and 20% of treated mutants had a liver size that was within the range of WT embryos (Figure 3C). Strikingly, Palbociclib also increased survival of *uhrf1* mutants to 9 dpf (Figure S2F).

To differentiate those aspects of the *uhrf1* mutant phenotype that were a direct consequence of Uhrf1 loss compared to those that were mediated by CDK4/6 activation, we assessed the effects of Palbociclib on the cellular aspects of the hepatic phenotype: EdU incorporation, nuclear size and shape and cell death. These parameters were unchanged by Palbociclib treatment of control embryos, but were significantly rescued in *uhrf1* mutants. The number of hepatocytes with EdU incorporation at 120 hpf was reduced from 55% to 30% in untreated compared to Palbociclib treated *uhrf1* mutants (Figure 3E). This was accompanied by a dramatic change in the nuclear morphology: DMSO and Palbociclib treated control hepatocyte nuclei displayed clear nuclear rim and distinct heterochromatin foci, with prominent H3K9me3 staining, whereas *uhrf1* mutant nuclei were large and oblong, with significant nuclear debris (Figures 3F). The nuclear size, shape, DNA patterning and H3K9me3 staining in *uhrf1* mutant hepatocytes were nearly completely restored to the WT phenotype by Palbociclib treatment (Figure 3F-G). Finally, the amount of cell death in *uhrf1* mutant livers was significantly reduced by Palbociclib (Figure 3H). Therefore, Cdk4/6 activity is required for both the developmental and cellular phenotypes caused by Uhrf1 loss.

Since Uhrf1 is a required component of the maintenance methylation machinery (Bostick *et al.*, 2007; Sharif *et al.*, 2007), we expected DNA hypomethylation to be a direct consequence of Uhrf1 loss. Assessment of bulk DNA methylation levels confirmed this, as both DMSO and Palbociclib treated *uhrf1* mutants had significant DNA hypomethylation (Figure 3I). Since Palbociclib treatment rescued most of the developmental and cellular defects in *uhrf1* mutants despite persistent DNA hypomethylation, we conclude that the developmental and cellular phenotypes caused by Uhrf1 loss is due to persistent activation of Cdk4/6 and is not a direct consequence of DNA hypomethylation.

Cdk4/6 inhibition blocks cell cycle and Tnfa target genes activation in *uhrf1* mutant livers but does not suppress TE activation or the interferon response

We hypothesized that the cellular and developmental phenotypic rescue of *uhrf1* mutants by Palbociclib was attributed to downregulation of the G1/S transition and S-phase promoting genes. In contrast, we expected that activation of transposable elements (TEs) and the ensuing interferon response (Chernyavskaya *et al.*, 2017; Magnani *et al.*, 2021) would not be affected by Palbociclib, since these are directly mediated by DNA methylation loss in *uhrf1* mutants.

We assessed this using bulk RNAseq analysis of the livers of 120 hpf *uhrf1* mutants and their phenotypically normal siblings (control) treated with DMSO or Palbociclib. Principal component analysis showed that controls cluster together regardless of treatment, while mutants treated with Palbociclib were closer to control samples

(Figure 4A). Palbociclib treatment of control larvae resulted in modest downregulation of genes that play roles in hepatocyte function and metabolism, but had no effect on genes that regulate the cell cycle (Figure S3A-B; Table S4, S5). In contrast, Palbociclib dramatically changed the hepatic transcriptome in *uhrf1* mutants, reducing the number of DEGs from 2,272 in DMSO treated mutants to 650 in the liver of *uhrf1* mutants treated with Palbociclib, 560 DEGs common to both (Figure 4B, Table S6). While the common DEGs showed a very high correlation in their expression (r=0.974), the genes that were unique to each dataset genes were less correlated (r= 0.666; Figure S3C). In contrast, the upregulation of TEs in *uhrf1* mutant livers was not suppressed by Palbociclib (Figure S3D).

We predicted that Cdk4/6 inhibition in *uhrf1* mutants would reduce the induction of E2F targets, but would have no effect on the interferon response as this is caused by TE activation. Unsupervised clustering of the unified set of DEGs from both DMSO and Palbociclib treated mutants showed that most of the upregulated genes in DMSO treated *uhrf1* mutant livers were still upregulated, but to a lesser extent in Palbociclib treated mutants (Figure 4C, cluster 5), however, a subset of genes remain elevated in both samples (cluster 4 and 5) (Figure 4C, Table S7). GO analysis of these two clusters shows that DNA replication related genesets were predominant in cluster 5, and that immune pathways were predominant in cluster 4 (Figure 4D-E, Table S8). Specific analysis of key genes involved in the pre-replication phase of cell cycle, such as *ccna2*, the *orcs*, *mcms* and *cdc45* which is important for origin firing at the replication forks in association with *mcms* (Pacek et al, 2006; Tanaka et al, 2007) showed that they were highly induced in DMSO treated mutants, but expressed at significantly lower levels in the liver of Palbociclib treated *uhrf1* mutants (Figure 4F, Figure S3E and Table S9).

Interestingly, the immune genes which were all upregulated in *uhrf1* mutants showed a nuanced response to Palbociclib treatment: interferon response genes remained highly expressed while genes downstream of Tnfa were downregulated in the liver of *uhrf1* mutants exposed to Palbociclib (Figure 4F, Table S10). Since CDK6 positively regulates TNFa (Schmitz & Kracht, 2016), we conclude that Tnfa induction in *uhrf1* mutants is attributed to Cdk4/6 activation, in contrast to interferon related genes which are due to TE activation.

We further investigated the effect of Palbociclib on the expression of cell cycle regulatory genes in *uhrf1* mutants using IPA. We first analyzed the genes activated in *uhrf1* mutants compared to controls treated with DMSO and found significant activation of genes regulating G1/S *ccnd1*, *ccne* and *cdk4/6,* which lead to upregulation of transcription factors *e2fs, hdac, ftdp1*, as well as activation of the DNA damage sensors *atm, atr* and *tp53* (Figure S4A). qPCR analysis of several of these genes confirmed that Palbociclib downregulated their expression the liver of *uhrf1* mutants (Figure S4B). We next compared the gene expression patterns in *uhrf1* mutants treated with Palbociclib to mutants treated with DMSO, and identified 280 DEGs (Figure S4C; Table S11). IPA analysis of these DEGs showed that genes involved in cell cycle checkpoints were upregulated and genes involved in cell cycle control and S-phase were downregulated in *uhrf1* mutant livers treated with Palbociclib (Figure 4G) but that Palbociclib had no effect on *atm/atr* and *tp53* induction in *uhrf1* mutants (Figure 4G, Figure S4D, Table S12), suggesting that DNA damage persists even when DNA replication is suppressed.

Together, these data indicate that Cdk4/6 activation does not affect DNA methylation, TE induction, interferon response and DNA damage caused by Uhrf1 loss, but is required for the downregulation of G1/S checkpoints, E2F targets, DNA rereplication and cell death. Based on our previous finding that blocking Tnfa reduced cell death in *uhrf1* mutant livers but has no effect on the mutant phenotype or liver size at 120 hpf (Magnani *et al.*, 2021), combined with the current finding that Tnfa activation was reduced by Palbociclib, we conclude that the rescue of cell death by Palbociclib is due to inactivation of Tnfa.

Cdk4/6 is activated by *dnmt1* mutation

To understand if the cellular phenotypes that cause hepatic outgrowth failure in *uhrf1* mutants are attributed to DNA hypomethylation, we assessed larvae with a loss of function mutation in *dnmt1* (Anderson *et al.*, 2009). These have a strikingly similar phenotype to *uhrf1* mutants, including DNA hypomethylation, large and disorganized hepatocyte nuclei and a small liver (Ganz *et al*, 2019; Jacob *et al.*, 2015; Madakashira *et al.*, 2021; Magnani *et al.*, 2021; Tittle *et al.*, 2011). We asked whether the cell cycle related gene set that we found to be differentially expressed in *uhrf1* mutant livers (the unified set of all cell cycle genes in Figure 2B) were also differentially expressed in bulk RNAseq data from the liver of 120 hpf *dnmt1* mutants (Magnani *et al.*, 2021).This showed that, similar to *uhrf1* mutants, many of the genes required for DNA replication (*pcna, orcs, mcms*, *cdc45* and *pold2*) were upregulated in *dnmt1* mutant livers (Figure 5A). Surprisingly, however there were a lower number of hepatocytes that incorporate EdU in *dnmt1* mutants at both 96 hpf and 120 hpf (Figure 5B). This indicates that Dnmt1 loss prevents DNA replication, consistent with a requirement for Dnmt1 at the replication fork (Lopez-Contreras *et al.*, 2013; Sirbu *et al.*, 2013). Previous analysis showed that *uhrf1* and *dnmt1* mutant livers have a similar pattern whereby genes required for DNA replication were upregulated (Jacob *et al.*, 2015; Magnani *et al.*, 2021). IPA provided a more detailed analysis showing that while the Atm and Tp53 mediated DNA damage responses were upregulated in both samples, some genes that regulate S-phase (*chek1, chek2, tlk1/2, cdc25),* M phase progression (*cdk1, cyclin B*) and Atr mediated signaling were upregulated only in *uhrf1* mutant livers (Figure S5). Thus, while both mutants have a global upregulation of cell cycle promoting genes and the Atm mediated response to double stranded breaks, *uhrf1* loss specifically causes the response to single stranded breaks.

If Cdk4/6 is upstream of cell cycle activation in *uhrf1* and *dnmt1* mutants, treatment of *dnmt1* mutants with Palbociclib should downregulate these genes. While Palbociclib had no effect on the developmental phenotype or liver size in *dnmt1* mutants (Figure 5C-D), qPCR analysis showed that the expression of cell cycle genes, but not interferon response genes, were decreased in the liver of Palbociclib treated *dnmt1* mutants (Figure 5E). Together, these data show that *dnmt1* and *uhrf1* loss activate Cdk4/6 and E2F target genes and also derepress transposons and induce an interferon response, but that these responses are independent, so that induction of cell cycle genes is dependent on Cdk4/6 signaling while TE activation and the interferon response are not. Moreover, the finding that *dnmt1* mutants are not rescued by Palbociclib reflects the fact that Dnmt1 deficient cells cannot undergo DNA replication, likely due to the requirement of Dnmt1 at the replication fork (Milutinovic *et al*, 2003; Sirbu *et al.*, 2013; Unterberger *et al*, 2006).

DNA re-replication in *uhrf1* mutant hepatocytes requires *dnmt1*
We hypothesized that Uhrf1 deficient hepatocytes are able to undergo DNA replication because of the presence of Dnmt1. Cdk4/6 activity has been shown to stabilize Dnmt1 (Acevedo *et al*, 2016) and thus one mechanism by which Palbociclib could reduce DNA rereplication in *uhrf1* mutants is by reducing Dnmt1 levels. To test this, we assessed Dnmt1 protein levels in 120 hpf hepatocytes using immunofluorescence, which showed no Dnmt1 in control hepatocytes, but showed prominent expression in 70% of *uhrf1* mutant hepatocytes, which was significantly decreased by Palbociclib treatment (Figure 6A). Therefore, Dnmt1 protein stabilization in *uhrf1* deficient hepatocytes requires Cdk4/6, either as a direct effect or as a consequence of rereplication defect driving expression of many S phase genes, *dnmt1* being one of them.

To determine if Dnmt1 was required for the EdU incorporation phenotype in *uhrf1* mutant hepatocytes, we used CRISPR-Cas9 to target *dnmt1*. Efficacy was verified using an *in vivo* assay for DNA methylation in which the UAS:GFP cassette is silenced by DNA methylation, and GFP expression occurs in response to Gal4 when methylation is lost on the UAS promoter (*Tg(c269off$^{+/-}$; 14X UAS:dsRed$^{+/-}$;fabp10a:Gal4;cmlc2:GFP))* (Goll *et al*, 2009; Magnani *et al.*, 2021). All *Tg(c269off$^{+/-}$; 14X UAS:dsRed$^{+/-}$;fabp10a:Gal4;cmlc2:GFP);dnmt1^{cr}* larvae had GFP expression in the liver at 120 hpf (Figure S6A), but had a mild phenotype compared to *dnmt1* mutants (Figure 5C). Crispr deletion of *dnmt1* did not change liver size of *uhrf1* mutants (Figure 6B-C) or the volume or shape of the *uhrf1* mutant hepatocyte nuclei (Figure S6B-D), but did significantly reduce EdU incorporation and modest cell death rescue in hepatocytes compared to uninjected *uhrf1$^{-/-}$* (Figure 6D-E). We conclude that Dnmt1 is required for DNA rereplication and cell death in *uhrf1* mutant hepatocytes.

Discussion

We report that Cdk4/6 activation is the mechanism accounting for the unusual cell cycle defect caused by *uhrf1* mutation in zebrafish and present a model that illustrates the relationship between DNA methylation, cell cycle regulation, DNA replication and development (Figure 7). There was striking rescue of the cellular and developmental defects in Uhrf1 deficient zebrafish embryos by Cdk4/6 inhibition without rescuing DNA hypomethylation, TE activation or the antiviral response. This differentiates between the direct effects of Uhrf1 depletion – i.e. DNA hypomethylation – and those that are secondary consequences. The finding of similar profile of Cdk4/6 dependent gene expression in *dnmt1* mutants suggests DNA hypomethylation as the mechanism for Cdk/4/6 activation, although the direct relationship by which this occurs is not clear. We previously published that *uhrf1* mutant hepatocytes have both an abnormal DNA replication pattern and widespread cell death (Jacob *et al.*, 2015; Sadler *et al.*, 2007). The data presented here showing that Cdk4/6 inhibition rescues liver size and, surprisingly, nearly all developmental defects in *uhrf1* mutants combined with our finding that reducing cell death does not rescue liver size (Magnani *et al.*, 2021) indicate that the cell cycle defect is the main mechanism of hepatic outgrowth failure. Moreover, this rescue was achieved despite persistent DNA hypomethylation,

indicating that the cellular and developmental phenotypes in Uhrf1 and Dnmt1 deficient cells is due to the downstream consequences of DNA hypomethylation, and not to the loss of DNA methylation *per se*. Importantly, the role of Uhrf1 in regulating the hepatic cell cycle is conserved in the developing mouse liver. We demonstrate that both *uhrf1* and *dnmt1* loss in the developing liver result in activation of cell cycle genes in a Cdk4/6 dependent manner, but while *uhrf1* mutant hepatocytes undergo DNA rereplication, *dnmt1* mutant hepatocytes do not, and that DNA rereplication in *uhrf1* mutant hepatocytes was rescued by *dnmt1* depletion. This indicates that either the *uhrf1* mutant phenotype could be partly attributed to elevated Dnmt1 levels or, more likely, it is due to the requirement for Dnmt1 for a functional replication fork (Milutinovic *et al.*, 2003; Unterberger *et al.*, 2006). Hence, Dnmt1 is both upstream and downstream of Cdk4/6. Taken together, these results provide mechanistic insight into cell cycle regulation during development and point to a functional relationship between epigenetic regulation and cell cycle control.

The cell cycle phenotype in *uhrf1* mutants is unusual, as it appears to have conflicting pathways activated simultaneously. First, *uhrf1* mutant hepatocytes are actively undergoing DNA replication, and our data indicate that the genome is replicated more than once in each hepatocyte. Uhrf1 loss has been implicated in the mis-regulation of both G1 and G2/M phases of the cell cycle (Arima *et al*, 2004; Bonapace *et al.*, 2002; Jenkins *et al.*, 2005) and here we provide evidence for a functional role in regulating DNA replication. We speculate that the rereplication phenotype in *uhrf1* deficient hepatocytes is due to a defect in DNA replication origin licensing, in which origins are not deactivated once utilized or that dormant replication origins are fired inappropriately. Replication licensing factor Cdt1 degradation is required to prevent reuse of replication origins, and the levels of Cdt1 protein are regulated by the Cul4–Ddb1–Cdt2 E3 ubiquitin ligase complex (Truong & Wu, 2011). DNA rereplication resulting from Cdt1 stabilization has been shown in tissue culture cells (Aggarwal *et al*, 2007) and in mouse models and human cancer cells, increased nuclear Cyclin D1/CDK4 complexes stabilize Cdt1 (Aggarwal *et al*, 2010). Recent work showed that quiescent cells re-enter the cell cycle by increasing the expression of Cyclin D and inhibiting p21 (Stallaert *et al*, 2022). In our model, *uhrf1* loss triggered high levels of *ccnd1* and *cdk4*, and it is possible that it could lead downstream to stabilization of Cdt1, whose degradation is critical to prevent DNA re-replication (Kim & Kipreos, 2007).

Second, gene expression analysis indicates that a cell cycle checkpoint in response to DNA damage is active, as the Atm pathway which senses double strand breaks is activated in both *uhrf1* and *dnmt1* mutants and the Atr pathway is activated only in *uhrf1* mutants (Figure S5). Since the liver does not expand in either mutant, the mutant cells do not divide, potentially reflecting checkpoint activation. We speculate that the double stranded breaks that activate Atm could be due to transposon activation or the euchromatinization of the genome which is evident in both mutants. Single stranded breaks in *uhrf1* mutants could reflect DNA replication stress, or perhaps could reflect an increase in oxidative stress, as we recently found in the context of human UHRF1 overexpression in zebrafish livers (Ajouaou *et al*, 2023). Interestingly, in *uhrf1* mutants, since there is induction of genes controlling mitosis, we conclude that that these cells ultimately exit S-phase and become arrested in G2/M, fail to undergo cytokinesis, or both. Future studies deploying single cell analysis sequencing will allow distinguishing

the gene expression signatures that accompany cells in different stages of the cell cycle.

During normal DNA replication, fork speed has to be moderated for the fidelity of normal cell cycle, with an increased fork speed triggering replication stress and DNA damage response (Maya-Mendoza *et al*, 2018; Merchut-Maya *et al*, 2019). However during DNA rereplication, DNA synthesis was reported to progress slowly, and repeatedly utilize the same main origins (Fu *et al*, 2021). In contrast, unscheduled firing of dormant origins can lead to increased and uncoordinated DNA synthesis (Bohly *et al*, 2022; McIntosh & Blow, 2012). While the experiments required to assess fork speed is challenging in whole embryos, our data supports the hypothesis that the DNA rereplication in *uhrf1* deficient hepatocytes is caused by firing multiple dormant origins, not a repeated firing of the same origins. The question is why origins of replication can be fired at all if there is a checkpoint activated by ATM and ATR. One possibility is that though ATR is upregulated, its function might be abrogated possibly due to inhibition of some of its downstream effectors. Investigation of the role of the players in the ATR pathway will provide better insight into how replication stress fails to prevent DNA rerereplication in *uhrf1* deficient hepatocytes.

The most straightforward conclusion to explain how Palbociclib prevents DNA replication in *uhrf1* mutants is through reinstatement of Rb mediated suppression of E2f. Another possibility is that Cdk4/6 inhibition depletes Dnmt1, which we have shown is required for DNA rereplication. Uhrf1 targets Dnmt1 for degradation (Du *et al*, 2010; Felle *et al*, 2011; Mudbhary *et al*, 2014; Qin *et al*, 2011; Zhang *et al*, 2019) and CDK4 phosphorylates DNMT1, preventing its degradation and therefore Palbociclib treatment reduces DNMT1 protein levels (Bourdeau & Ferbeyre, 2016). Our data suggest that in *uhrf1* mutant embryos, Dnmt1 accumulation is attributed to increased Cdk4/6 activity, as inhibition of Cdk4/6 caused significant reduction in Dnmt1 levels. However, it is also possible that this effect was mediated through decreased E2f mediated *dnmt1* transcription. We conclude that Dnmt1 levels are permissive to the unscheduled replication phenotype in *uhrf1* mutant hepatocytes.

We were intrigued by the finding that Palbociclib blocked cell death in *uhrf1* mutant hepatocytes. In other studies, Palbociclib has been shown to activate NF-κB and TNF-induced gene expression in mammalian cells (Buss *et al*, 2012). Given our previous finding that Tnfa depletion in *uhrf1* mutants prevented hepatocyte cell death (Magnani *et al.*, 2021), we attribute the rescue of cell death in Palbociclib treated *uhrf1* mutants to the downregulation of Tnfa. Since rescue of cell death by inhibiting *tnfa* in *uhrf1* mutants did not rescue liver size (Magnani *et al.*, 2021) we conclude that cell death is not caused by cell cycle defects and also indicates that cell death is not the cause of the small liver phenotype in this context. Instead, we conclude that the small liver phenotype is rather due to failure of hepatocyte proliferation.

Our data point to DNA hypomethylation as an upstream signal that leads to Cdk4/6 activation in hepatocytes. How this occurs is not known, but other studies suggest that the $p16^{INK4a}$ inhibitor of CDK4/6 could be suppressed through DNA methylation (D'Arcangelo *et al*, 2017; Jung *et al.*, 2007), and inhibiting $p16^{INK4a}$ lead to increased cell cycle progression and increased DNMT1 expression (Jung *et al.*, 2007). While it would be surprising if *uhrf1* and *dnmt1* mutation caused hypermethylation of the $p16^{INK4a}$ or any other Cdk4/6 inhibitor, it is possible that loss of methylation causes

redistribution of another repressive epigenetic mark to the $p16^{INK4a}$ regulatory regions. Alternatively, loss of DNA methylation could derepress Cyclin D, leading to perpetual activation of Cdk4/6.

In summary, this work provides the first insight into two distinct functions of Uhrf1 during liver development and is also relevant to cancer. Here we show that Uhrf1 loss induces Cdk4/6, E2f and all the genes that are required for S-phase. This is paradoxical, as UHRF1 is overexpressed in nearly all types of solid tumors (Ashraf *et al*, 2017), including HCC (Liang *et al*, 2015; Mudbhary *et al.*, 2014), Palbociclib effectively suppressed cell proliferation in tumor growth in preclinical models of HCC by promoting a reversible cell cycle arrest (Bollard *et al.*, 2017). Therefore, it is possible that in HCC, UHRF1 overexpression can act as a dominant negative to activate CDK4/6 and promote cell proliferation, and future studies may investigate whether Palbociclib functions as a therapeutic by counteracting the oncogenic effects of UHRF1.

Materials and Methods

Zebrafish Husbandry and Genotyping

Adult zebrafish were maintained in a circulating aquaculture system on a 14:10 h light: dark cycle at 28 °C. The *uhrf1*^{hi272} allele (Amsterdam *et al*, 2004) was maintained by genotyping adults to identify heterozygous carriers as described (Amsterdam *et al.*, 2004). Homozygous mutant embryos were generated by crossing heterozygous adults and were identified based on distinctive phenotypes as described (Jacob *et al.*, 2015; Sadler *et al.*, 2007; Tittle *et al.*, 2011) or by genotyping individual embryos as described (Sadler *et al.*, 2007). *uhrf1*$^{hi272+/-}$ adults were genotyped by PCR as described (Sadler *et al.*, 2007). *Tg(fabp10a: nls-mcherry)* in *uhrf1*$^{hi272+/-}$ and *Tg(fabp10a: CAAX-EGFP)* in *uhrf1*$^{hi272+/-}$ background was used for all experiments for ease of liver detection (Mudbhary *et al.*, 2014). Homozygous *uhrf1*$^{hi272-/-}$ mutant larvae are hereafter called *uhrf1*$^{-/-}$. Homozygous *dnmt1*$^{s904-/-}$ mutants (*dnmt1*$^{-/-}$) were generated by crossing *dnmt1*$^{s904+/-}$ adults in *dnmt1*$^{s904+/-}$*; Tg(c269*^{off}*; 10XUAS:dsRed); (fabp10a: Gal4; cmlc2: GFP)* background to monitor for DNA methylation in the liver as described (Magnani *et al.*, 2021). All protocols were approved by the NYU Abu Dhabi Institutional Animal Care and Use Committee (IACUC).

Mice husbandry and usage

Mice maintenance and experiments were approved by the New York University Abu Dhabi Animal Care and Use Committee. Temperature, humidity, and light:dark cycles were controlled and mice were fed food and water ad libitum. Mice with hepatocyte-specific deletion of the Uhrf1 gene (*Uhrf1*$^{fl/fl}$*;Alb-Cre*; referred to as *Uhrf1*^{HepKO} mice) were generated by crossing mice homozygous for floxed *Uhrf1* (*Uhrf1*$^{fl/fl}$) with mice expressing the Cre recombinase under the liver-specific albumin promoter back-crossed onto the *Uhrf1*$^{fl/fl}$ background (*Alb-cre*$^{Tg/+}$*; Uhrf1*$^{fl/+}$) described previously (Wang *et al.*, 2019). P4 mouse embryos were sacrificed and their livers were collected, flash frozen in liquid nitrogen, and stored at -80 °C for subsequent analysis.

FACS analysis of the cell cycle

For FACS, 20 *uhrf1*$^{-/-}$*; Tg(fabp10a: nls-mcherry)* and 20 *Tg(fabp10a: nls-mcherry)* sibling larvae were collected and used for single cells suspension. Single cell suspension was prepared by incubating larvae at 37 °C for 20 minutes in Trypsin-EDTA 0.05% (Thermo Fisher). After 20 minutes 10% FBS was added to stop the activity of the trypsin and cells were centrifuged at 2000 g for 7 minutes at 4 °C followed by 2 washes in PBS. Cells were resuspended in PBS and fixed with ice cold ethanol overnight. Cells were staining in PBS containing 10 μg/ml Hoechst (Thermo Fisher) for 10 minutes in dark and washed twice with PBS. Cells were finally resuspended in PBS containing 1% BSA (Sigma Aldrich) and 10 μg/ml RNaseA (Invitrogen) and acquired at FACS Aria II (BD Bioscience). Hepatocytes were selected by gating for mCherry^{+} population, and Hoechst levels recorded for analysis. Analysis was performed with FlowJo 10 and plotted in GraphPad Prism 9.

CRISPR/Cas9 Generation and T7 Endonuclease Assay

4 sgRNAs for *dnmt1 was* designed by using ChopChop (https://chopchop.cbu.uib.no/). sgRNA for *slc45a2* (gene involved in pigmentation) were previously designed and validated (Irion *et al*, 2014; Marjoram *et al.*, 2015). Genotyping primers were designed by Primer3 (https://bioinfo.ut.ee/primer3- 0.4.0/) and validated in USCS Genome Browser (https://genome.ucsc.edu/cgi-bin/hgPcr). sgRNAs were produced by sgRNA IVT kit (Takara Bio) by following the manufacturer's instructions and RNA was isolated by Trizol (Invitrogen). sgRNAs were quantified by Qubit RNA BR kit and diluted at 50 ng/µl and stored as single use aliquots. The efficiency of the sgRNAs were assessed by injecting all 4 *dnmt1* sgRNAs into WT embryos with equal volume of previously diluted nls-Cas9 protein (IDT; 0.5 µl of nls-Cas9 added with 9.5 µl of 20 mM HEPES; 150 mM KCl, pH 7.5) and sgRNAs, incubated at 37 °C for 5 minutes and then 1 nl was injected in 1–2 cells stage embryos. At 24–72 hpf, 12–16 sgRNA injected embryos were individually collected and genomic DNA was extracted by heat shock denaturation in 50 mM NaOH (95 °C for 20 minutes). For each embryo, PCR was performed on genomic DNA by using Q5 High-Fidelity Taq Polymerase (New England Biolabs) followed by T7 endonuclease I assay (New England Biolabs) to detect indel mutations. For T7 endonuclease I assay, 10 µl of PCR product was incubated with 0.5 µl of T7e1 enzyme (New England Biolabs) for 30 minutes at 37°C. Digested and undigested fragments were run in parallel in 2% agarose gel to assess the presence of indels. Efficiency was calculated as the number of embryos that show a positive result based on T7e1 assay divided by the total number of embryos assayed for the sgRNAs. Once established for efficiency, the pool of 4 *dnmt1* sgRNAs were injected into the 1 cell embryos generated by an incross of *uhrf1*$^{+/-}$ adults as previously described. Not injected embryos or *slc45a2* crispants were used as control. The resulting F0 larvae were considered crispants. For each clutch, *uhrf1*$^{-/-}$ mutants were divided from phenotypically WT siblings at 5 dpf based on morphological differences and T7e1 assay. sgRNA sequence information and genotyping primers are provided in Table S13.

Inhibitor Treatment

The CDK4/6 inhibitor Palbociclib (PD-0332991, Sigma Aldrich) (Bollard *et al.*, 2017) was dissolved in DMSO (Sigma) to yield a 10 mM stock solution and stored at -80 °C. For the *in vivo experiments*, Palbociclib was dissolved in 0.5% DMSO embryo water at a concentration of 20 µM. 48 hpf dechorionated embryos in 6 well plates were treated either with Palbociclib in 0.5% DMSO or 0.5% DMSO alone and collected at 120 hpf for further analysis**.**

RNA and DNA Extraction

For each clutch, 10 to 20 livers were microdissected from *dnmt1*$^{-/-}$ and their controls or *uhrf1*$^{-/-}$ and their controls in Tg(fabp10a: CAAX-EGFP) background larvae to facilitate liver identification according to published protocols (Evason *et al*, 2015). RNA was extracted using Trizol (Invitrogen) following the manufacturer's instructions with modifications. Briefly, during precipitation in isopropanol, 10 µg of Glycoblue (Thermo Fisher Scientific) was added, and precipitation performed overnight at −20 °C followed by 1 h centrifugation at 12,000× g at 4 °C. The resultant RNA pellet was resuspended in water and quantified by Qubit RNA High Sensitivity kit (Thermo Fisher). For genomic DNA extraction, each embryo or 10 livers were subjected to DNA extraction buffer (10 mM Tris-HCl pH9, 10 mM EDTA, 200 mM NaCl, 0.5% SDS, 200 µg/mL proteinase K) followed by DNA precipitation with isopropanol as previously described

(Chernyavskaya *et al.*, 2017). DNA was resuspended in water and quantified by Qubit dsDNA High Sensitivity kit (Thermo Fisher).

Slot Blot Analysis of 5-MeC

Slot blot was performed as previously described (Jacob *et al.*, 2015). Briefly, 2 ng of genomic DNA was denatured in 400 mM NaOH/10 mM EDTA and blotted onto nitrocellulose membrane in duplicate for dsDNA and 5-MeC using a slot blot apparatus. Membranes were incubated 1 h at 80 °C, blocked with 5% skim milk in TBST (37 mM NaCl, 20 mM Tris pH 7.5, 0.1% Tween 20), and incubated overnight at 4 °C in either anti-dsDNA (Abcam, 1:8000 in 2% BSA in TBST) or anti-5-methyl-cytosine (m5C– Aviva Biosystem clone 33D3, 1:2000 in 2% BSA in TBST). Membranes were washed in TBST and probed with anti-mouse HRP secondary antibody (Promega; 1:5000 in 2% BSA in TBST) for 1 h at room temperature followed by development in ECL (Thermo Fisher Scientific). ChemiDoc (BioRad) was used to detect and quantify the chemiluminescent signal. Gel Analyzer was used to measure the signals and ratio between 5-MeC and dsDNA was plotted for controls and mutants in each clutch using GraphPad Prism 9.

cDNA Production and qPCR

RNA extracted from microdissected livers was retrotranscribed to cDNA using Qscript cDNA synthesis kit (Quanta Bio) following the manufacturer's instructions. cDNA was diluted to 1 ng/µL, and 5 µL was used per reaction for qPCR using Maxima R SYBR green/ROX master mix (Thermo Fisher Scientific). *rplp0* was used to normalize expression levels by using the calculations for delta-Ct, and WT siblings were used to calculate delta-delta-Ct (DDCt) as previously described (Schmittgen & Livak, 2008). To determine changes in expression between control and experimental samples, the fold change was calculated and expressed as the log 2 value (L2FC) for display. All experiments were performed on samples from at least two independent clutches, with the number of biological replicates indicated for each experiment. Primer information is provided in Table S13.

Left lobe liver size measurements

Liver size measurements were carried out based on the 2-dimensional area of the left liver lobe in 120 hpf larvae. Embryos from incross of *Tg(fabp10a: nls-mcherry)* in *uhrf1*$^{hi272+/-}$ and *Tg(fabp10a: CAAX-EGFP)* in *uhrf1*$^{hi272+/-}$ background was used for all experiments for ease of liver detection. The live embryos were mounted in 3% methylcellulose on the right side and the left liver lobe was imaged using Nikon Elements on a Nikon SMZ25 Stereoscope. Liver size measurements were calculated in ImageJ using free hand selection of the EFGP/ cherry labeled area corresponding to the left liver lobe, and analyzed using the shape descriptor sub-function to determine the area of the left liver lobe, expressed in µM^2. For Palbociclib treatment, *uhrf1*$^{-/-}$ liver size of 20,000 µM^2 and below was considered small liver phenotype and *uhrf1*$^{-/-}$ liver size above 20,000 µM^2 considered as rescued.

Immunofluorescence

120 hpf control siblings and *uhrf1*$^{-/-}$ zebrafish embryos containing the *Tg(fabp10a: nls-mcherry)* transgene to identify hepatocyte nuclei were fixed in 4% paraformaldehyde for 4 h at room temperature, washed in PBS, and treated with 150 mM Tris-HCl at pH 9.0 for 5 minutes, followed by heating at 70 °C for 15 minutes according to an established protocol (Inoue & Wittbrodt, 2011) and following incubation at room

temperature to cool the embryos and washed in PBS. Livers were dissected as described, permeabilized with 10 µg/mL Proteinase K (Macherey-Nagel) in PBS containing 0.1% tween (PBST) for 10 minutes, washed 3 times with PBS, and incubated in a blocking solution containing 5% Fetal Bovine Serum (GIBCO) in PBS for 60 minutes at room temperature. The blocking solution was removed, and the livers were then incubated in 100 µL of H3k9me3 (39161, Activ Motif), H3k9me2 (39753, Activ Motif), Heterochromatin protein (Hp1) (MAB3866, Millipore), or Dnmt1 (SantaCruz) antibody in blocking solution (1:200 dilution) overnight at 4 °C. After 3 washes in PBST, the livers were incubated in secondary antibody (Molecular Probes) in blocking solution (1:400 dilution) in the dark for 2 h on a shaker. After 5 serial washes with PBST, the nuclei were counterstained with Hoechst (Thermo Fisher Scientific) diluted 1:1000 in PBS, washed twice with PBS to remove excess Hoechst, and mounted on a microscope slide with Vectashield (Vector Laboratories) and covered with a 0.1 mM coverslip for imaging using Leica SP8 confocal microscope.

Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling Assay (TUNEL)
Larvae collected at 5 dpf were fixed in 4% Paraformaldehyde for 4 h at room temperature, and gradually dehydrated through a graded series of methanol and stored in 100% methanol at 4 ◦C overnight. Gradual rehydration to PBS through a graded series of methanol/PBS dilutions was carried out at room temperature. Larvae were permeabilized with 10 µg/ml Proteinase K (Macherey-Nagel) in PBS containing 0.1% tween (PBST) and fixed in 4% Paraformaldehyde for 10 minutes at room temperature. Livers were then dissected out of the larvae and subjected to TUNEL assay according to manufacturer's instructions (*In Situ* Cell Death Detection kit, Fluorescein; Roche). Nuclei were counterstained with Hoechst (Thermo Fisher Scientific) diluted 1:1000 in PBS, mounted on a microscope slide with Vectashield (Vector Laboratories) and covered with a 0.1 mM coverslip for imaging using Leica SP8 confocal microscope.

EdU incorporation for S-phase analysis
10 live larvae at 80 hpf, 96 hpf or 120 hpf were transferred into 50 ml falcon tube with 250 uL solution 1 mM EdU (ThermoFisher) in 10% DMSO in embryo water, and incubated on ice for 20 minutes. The tubes were moved to room temperature, filled with embryo water to 50 ml, and incubated for either 12 minutes or 30 minutes at 28.5 °C. After removing the embryo water, the embryos were fixed in 4% Paraformaldehyde (Electron Microscopy Sciences) overnight at 4 °C, then dehydrated through a graded series of methanol and incubated in 100% methanol overnight at 4°C. The embryos were then gradually rehydrated back to 100% PBS by serial dilutions of methanol. The embryos were then permeabilized with PBS containing 1% Triton-X for 15 minutes prior to the Click-iT reaction to attach the Alexa azide (Invitrogen). The larvae were transferred to a solution of 172 µl PBS, 8 µl CuSO4 (100 mM), and 0.2 µl Alexa azide in a 0.6 µl microcentrifuge tube, and rocked in the dark for 10 minutes. 20 µl of ascorbic acid (0.5 M) was added after, and was rocked in the dark for additional 20 minutes. Then the mixture was removed and a fresh Alexa azide solution was added, and the same steps were repeated once more. 80 hpf embryos were individually genotyped by collecting tail samples. The larvae were washed 4 times in PBST prior to dissection and mounted in Vectashield mounting medium with DAPI before they were imaged with the Leica SP8 confocal microscope.

Confocal Imaging, Image Processing, and Analysis

Confocal imaging was performed using Leica TCS SP8 microscope with a 40x oil immersion at a scan speed of 100 Hz. For antibody immunofluorescence stains and TUNEL experiments, images were acquired at different focal distances from each liver and LAS X software (Leica software) was used for quantification from 3 separate optical sections per liver, which were then averaged from 3 livers per clutch per condition, and a minimum of 2 clutches were analyzed. For EdU and nuclear morphology measurements, Z-stacks were acquired using the galvo stage, with 2 µm intervals. Bit depth was 12, and to enhance image quality, field of view and laser intensity were adjusted separately for each sample. The acquired images were visualized using LASX software. 3D analysis of Z-stacks of Hoechst-stained nuclei was performed using the interactive 3D measurement tool in LASX for volume, surface area, sphericity, elliptical mean, and nuclei counting with Hoechst and EdU stains. Z-stacks were compiled into a 3D image, were adjusted for threshold and noise to define the nuclei clearly, set for minimum size to be measured as 1000 voxels, and measurements of the previously mentioned parameters obtained and exported in excel spreadsheets. For each time point and parameter measured, around 150 nuclei were sampled from at least 3 livers per condition. Results were plotted in GraphPad Prism 9.

RNA-Seq

50 livers were dissected at 120 hpf from Palbociclib or DMSO treated control sibling or *uhrf1*$^{-/-}$; *Tg(fabp10a: CAAX-EGFP)* larvae as previously described. The livers were individually collected and stored at -80 °C while the carcasses were genotyped for *uhrf1* mutation (Sadler *et al.*, 2007). Once genotyped, the *uhrf1*$^{-/-}$ livers and control livers from each individual clutch were pooled and RNA extracted according to established protocols (Magnani *et al.*, 2021). 100 ng of RNA was used for library preparation according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Libraries were sequenced on NovaSeq (Illumina) to obtain 150 bp paired-end reads. Raw Fastq files quality was assessed by using FASTQC and the reads were quality trimmed using Trimmomatic (Bolger *et al*, 2014) to remove low quality reads and adapters. Qualified reads were mapped to the reference genome Danio rerio GRCz10. To estimate and compare gene expression in different data sets, raw reads were uploaded to http://tsar.abudhabi.nyu.edu/ for pre-processing and DeSeq2 for Differential gene expression analysis. Datasets of RNAseq performed on 120 hpf *uhrf1*$^{-/-}$ and *dnmt1*$^{-/-}$ and their controls is previously published (Magnani *et al.*, 2021) and available at GEO (GSE160728). Dataset of RNAseq performed on 120 hpf *uhrf1*$^{-/-}$ and sibling controls treated with Palbociclib or DMSO will be available on GEO.

Bioinformatic analysis

For Gene Ontology and Ingenuity Pathways Analysis (IPA) zebrafish gene names were converted into human gene names using Biomart. Differentially expressed genes were determined by p-value adjusted 0.05 and log 2 fold change 0. GO enrichment analysis of differential expressed genes was conducted using REVIGO (Supek *et al*, 2011) to find enriched terms for different DEG data sets. An adjusted p-value < 0.05 was considered significant for all analyses. UPSET plots were generated to show intersections of enriched terms from REVIGO, their size, and unique terms (Conway *et al*, 2017). Heatmaps were generated with Pheatmap on z-score normalized counts. For plotting and statistical analysis, R package 'ggplot' and GraphPad Prism 9

software were used. The R codes used are available at https://github.com/SadlerEdepli-NYUAD.

Statistical Analysis

All experiments were carried out on multiple larvae from at least 2 clutches, as indicated in each figure and figure legend. Statistical significance included a two tailed Students t-test with adjustment for multiple comparisons as required. All plots along with statistical analysis were generated in GraphPad Prism. Bioinformatic analysis and visualization of genomic data were performed and plotted in RStudio.

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References

Acevedo M, Vernier M, Mignacca L, Lessard F, Huot G, Moiseeva O, Bourdeau V, Ferbeyre G (2016) A CDK4/6-Dependent Epigenetic Mechanism Protects Cancer Cells from PML-induced Senescence. *Cancer Res* 76: 3252-3264

Aggarwal P, Lessie MD, Lin DI, Pontano L, Gladden AB, Nuskey B, Goradia A, Wasik MA, Klein-Szanto AJ, Rustgi AK *et al* (2007) Nuclear accumulation of cyclin D1 during S phase inhibits Cul4-dependent Cdt1 proteolysis and triggers p53-dependent DNA rereplication. *Genes Dev* 21: 2908-2922

Aggarwal P, Vaites LP, Kim JK, Mellert H, Gurung B, Nakagawa H, Herlyn M, Hua X, Rustgi AK, McMahon SB *et al* (2010) Nuclear cyclin D1/CDK4 kinase regulates CUL4 expression and triggers neoplastic growth via activation of the PRMT5 methyltransferase. *Cancer Cell* 18: 329-340

Ajouaou Y, Magnani E, Madakashira B, Jenkins E, Sadler KC (2023) atm Mutation and Oxidative Stress Enhance the Pre-Cancerous Effects of UHRF1 Overexpression in Zebrafish Livers. *Cancers (Basel)* 15

Almouzni G, Cedar H (2016) Maintenance of Epigenetic Information. *Cold Spring Harb Perspect Biol* 8

Amsterdam A, Nissen RM, Sun Z, Swindell EC, Farrington S, Hopkins N (2004) Identification of 315 genes essential for early zebrafish development. *Proc Natl Acad Sci U S A* 101: 12792-12797

Anderson RM, Bosch JA, Goll MG, Hesselson D, Dong PD, Shin D, Chi NC, Shin CH, Schlegel A, Halpern M *et al* (2009) Loss of Dnmt1 catalytic activity reveals multiple roles for DNA methylation during pancreas development and regeneration. *Dev Biol* 334: 213-223

Arima Y, Hirota T, Bronner C, Mousli M, Fujiwara T, Niwa S, Ishikawa H, Saya H (2004) Down-regulation of nuclear protein ICBP90 by p53/p21Cip1/WAF1-dependent DNA-damage checkpoint signals contributes to cell cycle arrest at G1/S transition. *Genes Cells* 9: 131-142

Ashraf W, Ibrahim A, Alhosin M, Zaayter L, Ouararhni K, Papin C, Ahmad T, Hamiche A, Mely Y, Bronner C *et al* (2017) The epigenetic integrator UHRF1: on the road to become a universal biomarker for cancer. *Oncotarget* 8: 51946-51962

Blais A, Dynlacht BD (2007) E2F-associated chromatin modifiers and cell cycle control. *Curr Opin Cell Biol* 19: 658-662

Bohly N, Schmidt AK, Zhang X, Slusarenko BO, Hennecke M, Kschischo M, Bastians H (2022) Increased replication origin firing links replication stress to whole chromosomal instability in human cancer. *Cell Rep* 41: 111836

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114-2120

Bollard J, Miguela V, Ruiz de Galarreta M, Venkatesh A, Bian CB, Roberto MP, Tovar V, Sia D, Molina-Sanchez P, Nguyen CB *et al* (2017) Palbociclib (PD-0332991), a selective CDK4/6 inhibitor, restricts tumour growth in preclinical models of hepatocellular carcinoma. *Gut* 66: 1286-1296

Bonapace IM, Latella L, Papait R, Nicassio F, Sacco A, Muto M, Crescenzi M, Di Fiore PP (2002) Np95 is regulated by E1A during mitotic reactivation of terminally differentiated cells and is essential for S phase entry. *J Cell Biol* 157: 909-914

Bostick M, Kim JK, Esteve PO, Clark A, Pradhan S, Jacobsen SE (2007) UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science* 317: 1760-1764

Bourdeau V, Ferbeyre G (2016) CDK4-CDK6 inhibitors induce autophagy-mediated degradation of DNMT1 and facilitate the senescence antitumor response. *Autophagy* 12: 1965-1966

Buss H, Handschick K, Jurrmann N, Pekkonen P, Beuerlein K, Muller H, Wait R, Saklatvala J, Ojala PM, Schmitz ML *et al* (2012) Cyclin-dependent kinase 6 phosphorylates NF-kappaB

P65 at serine 536 and contributes to the regulation of inflammatory gene expression. *PLoS One* 7: e51847
Chen T, Hevi S, Gay F, Tsujimoto N, He T, Zhang B, Ueda Y, Li E (2007) Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. *Nat Genet* 39: 391-396
Chernyavskaya Y, Mudbhary R, Zhang C, Tokarz D, Jacob V, Gopinath S, Sun X, Wang S, Magnani E, Madakashira BP *et al* (2017) Loss of DNA methylation in zebrafish embryos activates retrotransposons to trigger antiviral signaling. *Development* 144: 2925-2939
Chu J, Loughlin EA, Gaur NA, SenBanerjee S, Jacob V, Monson C, Kent B, Oranu A, Ding Y, Ukomadu C *et al* (2012) UHRF1 phosphorylation by cyclin A2/cyclin-dependent kinase 2 is required for zebrafish embryogenesis. *Mol Biol Cell* 23: 59-70
Chu J, Sadler KC (2009) New school in liver development: lessons from zebrafish. *Hepatology* 50: 1656-1663
Conway JR, Lex A, Gehlenborg N (2017) UpSetR: an R package for the visualization of intersecting sets and their properties. *Bioinformatics* 33: 2938-2940
D'Arcangelo D, Tinaburri L, Dellambra E (2017) The Role of p16(INK4a) Pathway in Human Epidermal Stem Cell Self-Renewal, Aging and Cancer. *Int J Mol Sci* 18
Delgado I, Fresnedo O, Iglesias A, Rueda Y, Syn WK, Zubiaga AM, Ochoa B (2011) A role for transcription factor E2F2 in hepatocyte proliferation and timely liver regeneration. *Am J Physiol Gastrointest Liver Physiol* 301: G20-31
Du Z, Song J, Wang Y, Zhao Y, Guda K, Yang S, Kao HY, Xu Y, Willis J, Markowitz SD *et al* (2010) DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitination. *Sci Signal* 3: ra80
Evason KJ, Francisco MT, Juric V, Balakrishnan S, Lopez Pazmino Mdel P, Gordan JD, Kakar S, Spitsbergen J, Goga A, Stainier DY (2015) Identification of Chemical Inhibitors of beta-Catenin-Driven Liver Tumorigenesis in Zebrafish. *PLoS Genet* 11: e1005305
Felle M, Joppien S, Nemeth A, Diermeier S, Thalhammer V, Dobner T, Kremmer E, Kappler R, Langst G (2011) The USP7/Dnmt1 complex stimulates the DNA methylation activity of Dnmt1 and regulates the stability of UHRF1. *Nucleic Acids Res* 39: 8355-8365
Feng S, Cokus SJ, Zhang X, Chen PY, Bostick M, Goll MG, Hetzel J, Jain J, Strauss SH, Halpern ME *et al* (2010) Conservation and divergence of methylation patterning in plants and animals. *Proc Natl Acad Sci U S A* 107: 8689-8694
Ferry L, Fournier A, Tsusaka T, Adelmant G, Shimazu T, Matano S, Kirsh O, Amouroux R, Dohmae N, Suzuki T *et al* (2017) Methylation of DNA Ligase 1 by G9a/GLP Recruits UHRF1 to Replicating DNA and Regulates DNA Methylation. *Mol Cell* 67: 550-565 e555
Fu H, Redon CE, Thakur BL, Utani K, Sebastian R, Jang S-M, Gross JM, Mosavarpour S, Marks AB, Zhuang SZ *et al* (2021) Dynamics of replication origin over-activation. *Nature Communications* 12: 3448
Ganz J, Melancon E, Wilson C, Amores A, Batzel P, Strader M, Braasch I, Diba P, Kuhlman JA, Postlethwait JH *et al* (2019) Epigenetic factors Dnmt1 and Uhrf1 coordinate intestinal development. *Dev Biol* 455: 473-484
Ge TT, Yang M, Chen Z, Lou G, Gu T (2016) UHRF1 gene silencing inhibits cell proliferation and promotes cell apoptosis in human cervical squamous cell carcinoma CaSki cells. *J Ovarian Res* 9: 42
Goel S, DeCristo MJ, McAllister SS, Zhao JJ (2018) CDK4/6 Inhibition in Cancer: Beyond Cell Cycle Arrest. *Trends Cell Biol* 28: 911-925
Goessling W, Stainier DY (2016) Endoderm specification and liver development. *Methods Cell Biol* 134: 463-483
Goll MG, Anderson R, Stainier DY, Spradling AC, Halpern ME (2009) Transcriptional silencing and reactivation in transgenic zebrafish. *Genetics* 182: 747-755

Inoue D, Wittbrodt J (2011) One for all--a highly efficient and versatile method for fluorescent immunostaining in fish embryos. *PLoS One* 6: e19713
Irion U, Krauss J, Nusslein-Volhard C (2014) Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development* 141: 4827-4830
Jacob V, Chernyavskaya Y, Chen X, Tan PS, Kent B, Hoshida Y, Sadler KC (2015) DNA hypomethylation induces a DNA replication-associated cell cycle arrest to block hepatic outgrowth in uhrf1 mutant zebrafish embryos. *Development* 142: 510-521
Jenkins Y, Markovtsov V, Lang W, Sharma P, Pearsall D, Warner J, Franci C, Huang B, Huang J, Yam GC *et al* (2005) Critical role of the ubiquitin ligase activity of UHRF1, a nuclear RING finger protein, in tumor cell growth. *Mol Biol Cell* 16: 5621-5629
Jung JK, Arora P, Pagano JS, Jang KL (2007) Expression of DNA methyltransferase 1 is activated by hepatitis B virus X protein via a regulatory circuit involving the p16INK4a-cyclin D1-CDK 4/6-pRb-E2F1 pathway. *Cancer Res* 67: 5771-5778
Kent B, Magnani E, Walsh MJ, Sadler KC (2016) UHRF1 regulation of Dnmt1 is required for pre-gastrula zebrafish development. *Dev Biol* 412: 99-113
Kim JK, Esteve PO, Jacobsen SE, Pradhan S (2009) UHRF1 binds G9a and participates in p21 transcriptional regulation in mammalian cells. *Nucleic Acids Res* 37: 493-505
Kim Y, Kipreos ET (2007) Cdt1 degradation to prevent DNA re-replication: conserved and non-conserved pathways. *Cell Div* 2: 18
Kimura H, Nakamura T, Ogawa T, Tanaka S, Shiota K (2003) Transcription of mouse DNA methyltransferase 1 (Dnmt1) is regulated by both E2F-Rb-HDAC-dependent and -independent pathways. *Nucleic Acids Res* 31: 3101-3113
Knouse KA, Wu J, Whittaker CA, Amon A (2014) Single cell sequencing reveals low levels of aneuploidy across mammalian tissues. *Proc Natl Acad Sci U S A* 111: 13409-13414
Laderian B, Fojo T (2017) CDK4/6 Inhibition as a therapeutic strategy in breast cancer: palbociclib, ribociclib, and abemaciclib. *Semin Oncol* 44: 395-403
Lee SH, Hao E, Levine F, Itkin-Ansari P (2011) Id3 upregulates BrdU incorporation associated with a DNA damage response, not replication, in human pancreatic beta-cells. *Islets* 3: 358-366
Liang D, Xue H, Yu Y, Lv F, You W, Zhang B (2015) Elevated expression of UHRF1 predicts unfavorable prognosis for patients with hepatocellular carcinoma. *Int J Clin Exp Pathol* 8: 9416-9421
Lopez-Contreras Andres J, Ruppen I, Nieto-Soler M, Murga M, Rodriguez-Acebes S, Remeseiro S, Rodrigo-Perez S, Rojas Ana M, Mendez J, Muñoz J *et al* (2013) A Proteomic Characterization of Factors Enriched at Nascent DNA Molecules. *Cell Reports* 3: 1105-1116
Macaluso M, Montanari M, Noto PB, Gregorio V, Bronner C, Giordano A (2007) Epigenetic modulation of estrogen receptor-alpha by pRb family proteins: a novel mechanism in breast cancer. *Cancer Res* 67: 7731-7737
Madakashira BP, Zhang C, Macchi F, Magnani E, Sadler KC (2021) Nuclear Organization during Hepatogenesis in Zebrafish Requires Uhrf1. *Genes (Basel)* 12
Magnani E, Macchi F, Madakashira BP, Zhang C, Alaydaroos F, Sadler KC (2021) uhrf1 and dnmt1 Loss Induces an Immune Response in Zebrafish Livers Due to Viral Mimicry by Transposable Elements. *Front Immunol* 12: 627926
Magri L, Swiss VA, Jablonska B, Lei L, Pedre X, Walsh M, Zhang W, Gallo V, Canoll P, Casaccia P (2014) E2F1 coregulates cell cycle genes and chromatin components during the transition of oligodendrocyte progenitors from proliferation to differentiation. *J Neurosci* 34: 1481-1493
Malumbres M, Barbacid M (2009) Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* 9: 153-166

Mancini M, Magnani E, Macchi F, Bonapace IM (2021) The multi-functionality of UHRF1: epigenome maintenance and preservation of genome integrity. *Nucleic Acids Res* 49: 6053-6068
Marjoram L, Alvers A, Deerhake ME, Bagwell J, Mankiewicz J, Cocchiaro JL, Beerman RW, Willer J, Sumigray KD, Katsanis N *et al* (2015) Epigenetic control of intestinal barrier function and inflammation in zebrafish. *Proc Natl Acad Sci U S A* 112: 2770-2775
Maya-Mendoza A, Moudry P, Merchut-Maya JM, Lee M, Strauss R, Bartek J (2018) High speed of fork progression induces DNA replication stress and genomic instability. *Nature* 559: 279-284
Mayhew CN, Carter SL, Fox SR, Sexton CR, Reed CA, Srinivasan SV, Liu X, Wikenheiser-Brokamp K, Boivin GP, Lee JS *et al* (2007) RB loss abrogates cell cycle control and genome integrity to promote liver tumorigenesis. *Gastroenterology* 133: 976-984
McCabe MT, Davis JN, Day ML (2005) Regulation of DNA methyltransferase 1 by the pRb/E2F1 pathway. *Cancer Res* 65: 3624-3632
McIntosh D, Blow JJ (2012) Dormant origins, the licensing checkpoint, and the response to replicative stresses. *Cold Spring Harb Perspect Biol* 4
Merchut-Maya JM, Bartek J, Maya-Mendoza A (2019) Regulation of replication fork speed: Mechanisms and impact on genomic stability. *DNA Repair (Amst)* 81: 102654
Milutinovic S, Zhuang QL, Niveleau A, Szyf M (2003) Epigenomic stress response - Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes. *Journal of Biological Chemistry* 278: 14985-14995
Mudbhary R, Hoshida Y, Chernyavskaya Y, Jacob V, Villanueva A, Fiel MI, Chen X, Kojima K, Thung S, Bronson RT *et al* (2014) UHRF1 overexpression drives DNA hypomethylation and hepatocellular carcinoma. *Cancer Cell* 25: 196-209
Nishiyama A, Yamaguchi L, Sharif J, Johmura Y, Kawamura T, Nakanishi K, Shimamura S, Arita K, Kodama T, Ishikawa F *et al* (2013) Uhrf1-dependent H3K23 ubiquitylation couples maintenance DNA methylation and replication. *Nature* 502: 249-253
Pacek M, Tutter AV, Kubota Y, Takisawa H, Walter JC (2006) Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. *Mol Cell* 21: 581-587
Petryk N, Kahli M, d'Aubenton-Carafa Y, Jaszczyszyn Y, Shen Y, Silvain M, Thermes C, Chen CL, Hyrien O (2016) Replication landscape of the human genome. *Nat Commun* 7: 10208
Qin W, Leonhardt H, Spada F (2011) Usp7 and Uhrf1 control ubiquitination and stability of the maintenance DNA methyltransferase Dnmt1. *J Cell Biochem* 112: 439-444
Qin Y, Wang J, Gong W, Zhang M, Tang Z, Zhang J, Quan Z (2014) UHRF1 depletion suppresses growth of gallbladder cancer cells through induction of apoptosis and cell cycle arrest. *Oncol Rep* 31: 2635-2643
Recolin B, van der Laan S, Tsanov N, Maiorano D (2014) Molecular mechanisms of DNA replication checkpoint activation. *Genes (Basel)* 5: 147-175
Rieck S, Zhang J, Li Z, Liu C, Naji A, Takane KK, Fiaschi-Taesch NM, Stewart AF, Kushner JA, Kaestner KH (2012) Overexpression of hepatocyte nuclear factor-4alpha initiates cell cycle entry, but is not sufficient to promote beta-cell expansion in human islets. *Mol Endocrinol* 26: 1590-1602
Sadler KC, Krahn KN, Gaur NA, Ukomadu C (2007) Liver growth in the embryo and during liver regeneration in zebrafish requires the cell cycle regulator, uhrf1. *Proc Natl Acad Sci U S A* 104: 1570-1575
Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3: 1101-1108
Schmitz ML, Kracht M (2016) Cyclin-Dependent Kinases as Coregulators of Inflammatory Gene Expression. *Trends in Pharmacological Sciences* 37: 101-113

Sharif J, Muto M, Takebayashi S, Suetake I, Iwamatsu A, Endo TA, Shinga J, Mizutani-Koseki Y, Toyoda T, Okamura K *et al* (2007) The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. *Nature* 450: 908-912
Sirbu BM, McDonald WH, Dungrawala H, Badu-Nkansah A, Kavanaugh GM, Chen Y, Tabb DL, Cortez D (2013) Identification of Proteins at Active, Stalled, and Collapsed Replication Forks Using Isolation of Proteins on Nascent DNA (iPOND) Coupled with Mass Spectrometry*. *Journal of Biological Chemistry* 288: 31458-31467
Sladky VC, Knapp K, Soratroi C, Heppke J, Eichin F, Rocamora-Reverte L, Szabo TG, Bongiovanni L, Westendorp B, Moreno E *et al* (2020) E2F-Family Members Engage the PIDDosome to Limit Hepatocyte Ploidy in Liver Development and Regeneration. *Dev Cell* 52: 335-349 e337
Stallaert W, Kedziora KM, Taylor CD, Zikry TM, Ranek JS, Sobon HK, Taylor SR, Young CL, Cook JG, Purvis JE (2022) The structure of the human cell cycle. *Cell Syst* 13: 103
Supek F, Bosnjak M, Skunca N, Smuc T (2011) REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One* 6: e21800
Tanaka S, Tak YS, Araki H (2007) The role of CDK in the initiation step of DNA replication in eukaryotes. *Cell Div* 2: 16
Tittle RK, Sze R, Ng A, Nuckels RJ, Swartz ME, Anderson RM, Bosch J, Stainier DY, Eberhart JK, Gross JM (2011) Uhrf1 and Dnmt1 are required for development and maintenance of the zebrafish lens. *Dev Biol* 350: 50-63
Truong LN, Wu X (2011) Prevention of DNA re-replication in eukaryotic cells. *J Mol Cell Biol* 3: 13-22
Unoki M, Nishidate T, Nakamura Y (2004) ICBP90, an E2F-1 target, recruits HDAC1 and binds to methyl-CpG through its SRA domain. *Oncogene* 23: 7601-7610
Unterberger A, Andrews SD, Weaver ICG, Szyf M (2006) DNA methyltransferase 1 knockdown activates a replication stress checkpoint. *Molecular and Cellular Biology* 26: 7575-7586
Wang S, Miller SR, Ober EA, Sadler KC (2017) Making It New Again: Insight Into Liver Development, Regeneration, and Disease From Zebrafish Research. *Curr Top Dev Biol* 124: 161-195
Wang S, Zhang C, Hasson D, Desai A, SenBanerjee S, Magnani E, Ukomadu C, Lujambio A, Bernstein E, Sadler KC (2019) Epigenetic Compensation Promotes Liver Regeneration. *Dev Cell* 50: 43-56 e46
Wu SC, Kim A, Gu YJ, Martinez DI, Zocchi L, Chen CC, Lopez J, Salcido K, Singh S, Wu J *et al* (2022) UHRF1 overexpression promotes osteosarcoma metastasis through altered exosome production and AMPK/SEMA3E suppression. *Oncogenesis* 11
Zhang H, Gao Q, Tan S, You J, Lyu C, Zhang Y, Han M, Chen Z, Li J, Wang H *et al* (2019) SET8 prevents excessive DNA methylation by methylation-mediated degradation of UHRF1 and DNMT1. *Nucleic Acids Res* 47: 9053-9068
Zhang J, Bellani MA, Huang J, James RC, Pokharel D, Gichimu J, Gali H, Stewart G, Seidman MM (2021) Replication of the Mammalian Genome by Replisomes Specific for Euchromatin and Heterochromatin. *Front Cell Dev Biol* 9: 729265
Zhang J, Yang C, Wu C, Cui W, Wang L (2020) DNA Methyltransferases in Cancer: Biology, Paradox, Aberrations, and Targeted Therapy. *Cancers (Basel)* 12

Figure legends

Figure 1. DNA re-replication leads to increased DNA content and heterochromatin loss in *uhrf1* mutant hepatocytes

(A) FACS sorting of 5 dpf *Tg(fabp10a:nls:mcherry)* hepatocytes with control or *uhrf1*$^{-/-}$ at 120 hpf (B) Whole control and *uhrf1*$^{-/-}$ embryos were pulsed with EdU for 30 minutes at 80 hpf, 96 hpf and 120 hpf and processed for EdU fluorescence by Click-it (C) Quantification of EdU incorporation in B (D) 80 hpf, 96 hpf and 120 hpf hepatocytes were sorted into early, mid and late S phase based on EdU pattern and quantified (E) Representative immunofluorescent images of 120 hpf *uhrf1*$^{-/-}$ and control livers with heterochromatin markers H3K9me3, H3K9me2 and Heterochromatin and their quantification (F) Quantification of EdU incorporation from a 12 minute EdU pulse at 80 hpf and 120 hpf control and *uhrf1*$^{-/-}$ hepatocytes and (G) sorted based on their S phase stage. C indicates control siblings and M indicates *uhrf1*$^{-/-}$. Scale: 50 µm, the number of samples and clutches indicated for each condition. p-value *< 0.05, **< 0.005, *** < 0.0005 by two tailed Students t-test with adjustment for multiple comparisons.

Figure 2. CDK4/6 is activated in *uhrf1* mutant livers

(A) Gene ontology (REVIGO) of the significant upregulated and downregulated genes in 120 hpf *uhrf1*$^{-/-}$ liver RNA-Seq (p_{adj} < 0.05) for each category. The cell cycle GO terms are highlighted in bold (B) UPSET plot of all the cell cycle GO category genes from A (C) Ingenuity Pathway Analysis (IPA) of all the cell cycle genes differentially expressed in 120 hpf *uhrf1*$^{-/-}$ liver RNA-Seq, pathways involved in cell cycle checkpoint regulation in bold, z- score in IPA indicates a predicted activation or inhibition of a pathway (D) Genes from the G1/S checkpoint pathway generated by IPA plotted with log2fold change, important cell cycle genes highlighted in red (E) qPCR on key cell cycle genes of 96 hpf *uhrf1*$^{-/-}$ and control livers (F) qPCR on key cell cycle genes in the livers of P4 neonatal mice deficient for *Uhrf1* in hepatocytes (*Uhrf1*^{HEPKO}) and controls, inset shows band for Cre transgene by RT-PCR. The number of clutches is indicated for each condition. p-value *< 0.05, **< 0.005, *** < 0.0005 by two tailed Students t-test with adjustment for multiple comparisons.

Figure 3. Cdk4/6 inhibition by Palbociclib (PD) rescues the *uhrf1* mutant liver phenotype

(A) Treatment scheme for inhibitor Palbociclib (PD) (B) Representative images of 120 hpf *uhrf1*$^{-/-}$ and control embryos after DMSO or PD treatment, liver indicated in red (C) Quantification of the left liver lobe area of 120 hpf PD treated and untreated livers (D) Liver phenotype rescue in 120 hpf *uhrf1*$^{-/-}$ treated with PD (E) quantification of 30 minute EdU incorporation at 120 hpf in DMSO and PD treated controls and *uhrf1*$^{-/-}$ (F) Representative confocal image of 120 hpf Hoechst stained *uhrf1* control and *uhrf1*$^{-/-}$ hepatocytes from DMSO or PD treatment and quantification of the nuclear surface area (G) Representative immunofluorescent images of 120 hpf livers with heterochromatin marker H3K9me3 and quantification of H3K9me3 positive hepatocytes (H) TUNEL assay was performed to detect cell death in 120 hpf in DMSO and PD treated controls and *uhrf1*$^{-/-}$ and quantified (I) Representative image of 5mC levels in *uhrf1*$^{-/-}$ embryos and controls as measured by slot blot and quantification of the methylation levels from three clutches. Scale: 5000 µm in B, 50 µm in F,G, the number of samples and clutches indicated for each condition. p-value *< 0.05, **< 0.005, *** < 0.0005 by two tailed Students t-test with adjustment for multiple comparisons.

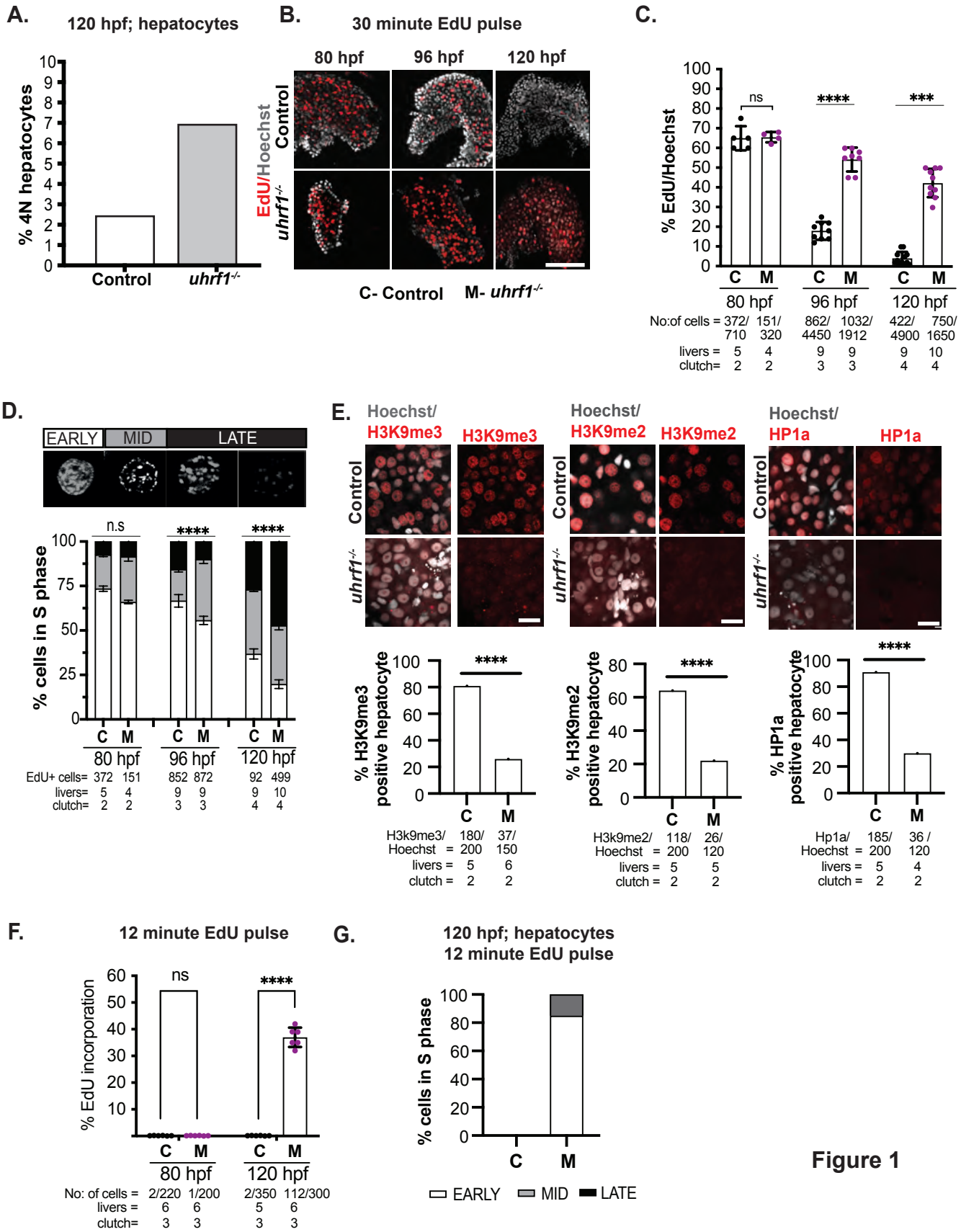
Figure 4. Cdk4/6 inhibition by Palbociclib (PD) rescues abnormal cell cycle gene expression in *uhrf1* mutants

(A) PCA analysis of RNA Seq from 120 hpf Palbociclib (PD) treated and DMSO treated *uhrf1*$^{-/-}$ and control livers (B) Venn diagram of differential expressed genes (DEGs) (cutoff p_{adj} < 0.05) between *uhrf1*$^{-/-}$ and their control siblings treated with DMSO and PD (C) Unsupervised clustering heatmap of the expression profile of 120 hpf DMSO and PD treated *uhrf1*$^{-/-}$ liver RNA-Seq with z-scores based on rows. Rows are divided into 10 clusters calculated on the hierarchical clustering of dendrogram (Euclidean distance) (D) REVIGO gene ontology of clusters 4 and (E) and cluster 5 (F) Key cell cycle genes, immune and *tnfa* response gene expression between PD and DMSO treated *uhrf1*$^{-/-}$ plotted as a heatmap with log2fold change (G) IPA of the cell cycle genes differentially expressed between 120 hpf *uhrf1*$^{-/-}$ PD and DMSO livers, cell cycle checkpoint regulation pathways indicated in bold.
p-value *< 0.05, **< 0.005, *** < 0.0005 by two tailed Students t-test with adjustment for multiple comparisons.

Figure 5. Loss of *uhrf1* leads to Cdk4/6 mediated Dnmt1 protein stabilization and smaller liver size
(A) Unsupervised clustering heatmap of all the cell cycle genes of 120 hpf *dnmt1*$^{-/-}$ liver RNA-Seq with z-scores based on rows, important S-phase genes are indicated (B) EdU quantification (pulse 30 minute) of 96 hpf and 120 hpf control and *dnmt1*$^{-/-}$ hepatocytes (C) Representative images of 120 hpf *dnmt1*$^{-/-}$ and controls with *c269off+/-; 14X UAS:dsRed+/-; Tg(fabp10a:Gal4;cmlc2:GFP)+/-* treated with DMSO or Palbociclib (PD), showing the liver (dsRed), the heart (GFP) as a marker of transgenesis and in green, the liver with hypomethylation in mutants only (D) Quantification of the left liver lobe area of *dnmt1*$^{-/-}$ and controls, treated with PD or DMSO. Scale: 50 µm, the number of samples and clutches indicated for each condition (E) QPCR of key cell cycle genes and immune genes in *dnmt1*$^{-/-}$ livers. p-value *< 0.05, **< 0.005, *** < 0.0005 by two tailed Students t-test with adjustment for multiple comparisons.

Figure 6. *dnmt1* mutation rescues DNA re-replication defect in *uhrf1* mutant hepatocytes
(A) Representative immunofluorescent images with Dnmt1 staining of 120 hpf control and *uhrf1*$^{-/-}$ livers treated with DMSO or PD and Dnmt1 positive cell quantification (B) Representative images of 120 hpf *uhrf1*$^{-/-}$ and control embryos uninjected or injected with *dnmt1*^{cr} (C) Quantification of the left liver lobe area of 120 hpf *uhrf1*$^{-/-}$ and controls uninjected or injected with *dnmt1*^{cr} (D) Representative images of 30 minute EdU incorporation at 120 hpf in uninjected and *dnmt1*^{cr} injected controls and *uhrf1*$^{-/-}$ livers (E) Quantification of the TUNEL assay to detect cell death in 120 hpf *uhrf1*$^{-/-}$ and controls uninjected or injected with *dnmt1*^{cr}. p-value *< 0.05, **< 0.005, *** < 0.0005 by two tailed Students t-test with adjustment for multiple comparisons.

Figure 7. Model of the relationship between *uhrf1* mutation, DNA methylation, cell cycle regulation, DNA replication and small liver
Uhrf1 and Dnmt1 loss cause DNA hypomethylation, upregulation of Transposable Elements (TEs) and inflammatory response. Cdk4/6 activation upon DNA hypomethylation mediate Tnfa/b mediated cell death in *uhrf1* deficient livers. Cdk4/6 dependent activation of DNA replication genes leads to DNA rereplication and replication stress. Both TE activation and replication stress trigger DNA damage response to inhibit cell division restricting organ outgrowth and cause lethality in *uhrf1* mutants.


A.
120 hpf; hepatocytes
% 4N hepatocytes
Control
uhrf1-/-
B.
30 minute EdU pulse
80 hpf
96 hpf
120 hpf
EdU/Hoechst
Control
uhrf1-/-
C- Control
M- uhrf1-/-
C.
% EdU/Hoechst
ns
****
***
C
M
80 hpf
96 hpf
120 hpf
No:of cells = 372/710 151/320 862/4450 1032/1912 422/4900 750/1650
livers = 5 4 9 9 9 10
clutch= 2 2 3 3 4 4
D.
EARLY
MID
LATE
% cells in S phase
n.s
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C
M
80 hpf
96 hpf
120 hpf
EdU+ cells= 372 151 852 872 92 499
livers= 5 4 9 9 9 10
clutch= 2 2 3 3 4 4
E.
Hoechst/ H3K9me3
H3K9me3
Hoechst/ H3K9me2
H3K9me2
Hoechst/ HP1a
HP1a
Control
uhrf1-/-
% H3K9me3 positive hepatocyte
% H3K9me2 positive hepatocyte
% HP1a positive hepatocyte
****
C
M
H3k9me3/ Hoechst = 180/200 37/150
livers = 5 6
clutch = 2 2
H3k9me2/ Hoechst = 118/200 26/120
livers = 5 5
clutch = 2 2
Hp1a/ Hoechst = 185/200 36/120
livers = 5 4
clutch = 2 2
F.
12 minute EdU pulse
% EdU incorporation
ns
****
C
M
80 hpf
120 hpf
No: of cells = 2/220 1/200 2/350 112/300
livers = 6 6 5 6
clutch= 3 3 3 3
G.
120 hpf; hepatocytes
12 minute EdU pulse
% cells in S phase
C
M
EARLY
MID
LATE

Figure 1

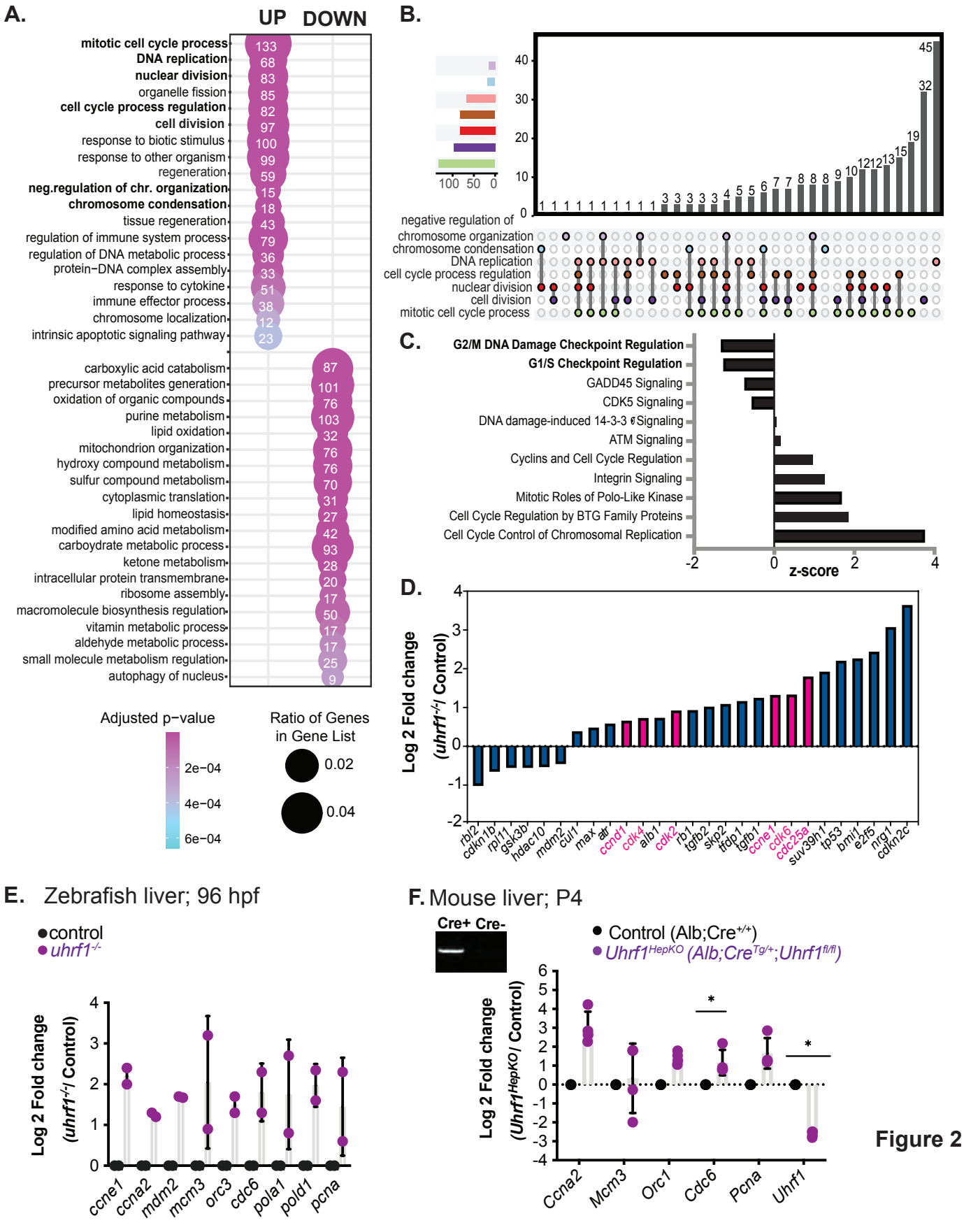

A.
UP DOWN
mitotic cell cycle process 133
DNA replication 68
nuclear division 83
organelle fission 85
cell cycle process regulation 82
cell division 97
response to biotic stimulus 100
response to other organism 99
regeneration 59
neg.regulation of chr. organization 15
chromosome condensation 18
tissue regeneration 43
regulation of immune system process 79
regulation of DNA metabolic process 36
protein−DNA complex assembly 33
response to cytokine 51
immune effector process 38
chromosome localization 12
intrinsic apoptotic signaling pathway 23
carboxylic acid catabolism 87
precursor metabolites generation 101
oxidation of organic compounds 76
purine metabolism 103
lipid oxidation 32
mitochondrion organization 76
hydroxy compound metabolism 76
sulfur compound metabolism 70
cytoplasmic translation 31
lipid homeostasis 27
modified amino acid metabolism 42
carboydrate metabolic process 93
ketone metabolism 28
intracellular protein transmembrane 20
ribosome assembly 17
macromolecule biosynthesis regulation 50
vitamin metabolic process 17
aldehyde metabolic process 17
small molecule metabolism regulation 25
autophagy of nucleus 9
Adjusted p−value
2e−04
4e−04
6e−04
Ratio of Genes in Gene List
0.02
0.04
B.
negative regulation of chromosome organization
chromosome condensation
DNA replication
cell cycle process regulation
nuclear division
cell division
mitotic cell cycle process
C.
G2/M DNA Damage Checkpoint Regulation
G1/S Checkpoint Regulation
GADD45 Signaling
CDK5 Signaling
DNA damage-induced 14-3-3 σ Signaling
ATM Signaling
Cyclins and Cell Cycle Regulation
Integrin Signaling
Mitotic Roles of Polo-Like Kinase
Cell Cycle Regulation by BTG Family Proteins
Cell Cycle Control of Chromosomal Replication
z-score
D.
Log 2 Fold change (uhrf1-/- / Control)
rbl2 cdkn1b rpl11 gsk3b hdac10 mdm2 cul1 max atr ccnd1 cdk4 alb1 cdk2 rb1 tgfb2 skp2 tfdp1 tgfb1 ccne1 cdk6 cdc25a suv39h1 tp53 bmi1 e2f5 nrg1 cdkn2c
E. Zebrafish liver; 96 hpf
control
uhrf1-/-
Log 2 Fold change (uhrf1-/- / Control)
ccne1 ccna2 mdm2 mcm3 orc3 cdc6 pola1 pold1 pcna
F. Mouse liver; P4
Cre+ Cre-
Control (Alb;Cre+/+)
Uhrf1HepKO (Alb;CreTg/+;Uhrf1fl/fl)
Log 2 Fold change (Uhrf1HepKO / Control)
Ccna2 Mcm3 Orc1 Cdc6 Pcna Uhrf1

Figure 2

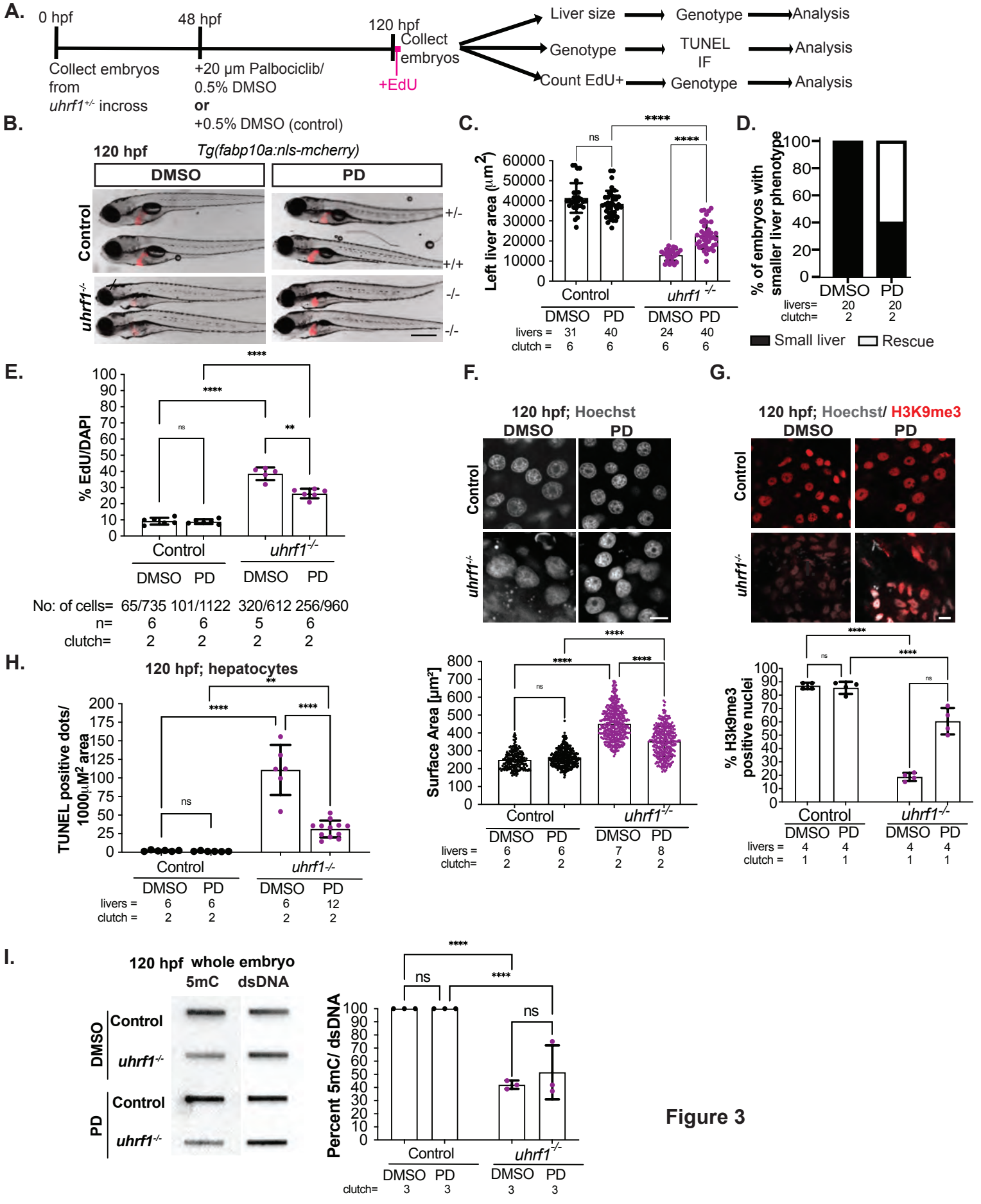

A.
0 hpf
48 hpf
120 hpf
Collect embryos from uhrf1+/- incross
+20 µm Palbociclib/ 0.5% DMSO or +0.5% DMSO (control)
Collect embryos
+EdU
Liver size
Genotype
Count EdU+
TUNEL IF
Analysis
B.
120 hpf
Tg(fabp10a:nls-mcherry)
DMSO
PD
Control
uhrf1-/-
+/-
+/+
-/-
C.
Left liver area (µm2)
Control
uhrf1 -/-
livers = 31 40 24 40
clutch = 6 6 6 6
D.
% of embryos with smaller liver phenotype
livers= 20 20
clutch= 2 2
Small liver
Rescue
E.
% EdU/DAPI
No: of cells= 65/735 101/1122 320/612 256/960
n= 6 6 5 6
clutch= 2 2 2 2
F.
120 hpf; Hoechst
Surface Area [µm2]
livers = 6 6 7 8
clutch= 2 2 2 2
G.
120 hpf; Hoechst/ H3K9me3
% H3k9me3 positive nuclei
livers = 4 4 4 4
clutch = 1 1 1 1
H.
120 hpf; hepatocytes
TUNEL positive dots/ 1000µM2 area
livers = 6 6 6 12
clutch = 2 2 2 2
I.
120 hpf whole embryo
5mC
dsDNA
Percent 5mC/ dsDNA
clutch= 3 3 3 3

Figure 3

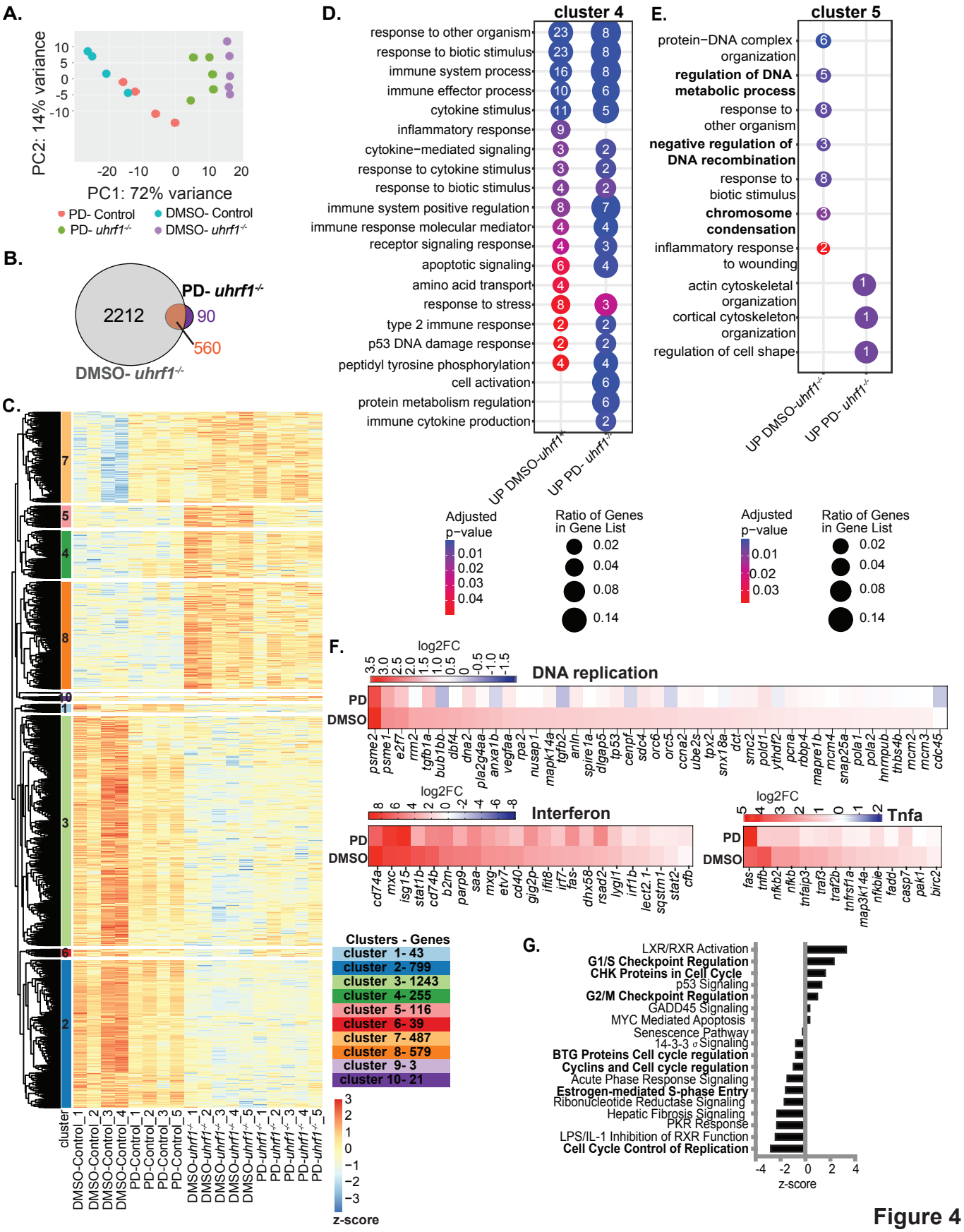

A.
PC2: 14% variance
PC1: 72% variance
PD- Control
PD- uhrf1-/-
DMSO- Control
DMSO- uhrf1-/-
B.
2212
90
560
PD- uhrf1-/-
DMSO- uhrf1-/-
C.
cluster
DMSO-Control_1
DMSO-Control_2
DMSO-Control_3
DMSO-Control_4
PD-Control_1
PD-Control_2
PD-Control_3
PD-Control_5
DMSO-uhrf1-/-_1
DMSO-uhrf1-/-_2
DMSO-uhrf1-/-_3
DMSO-uhrf1-/-_4
DMSO-uhrf1-/-_5
PD-uhrf1-/-_1
PD-uhrf1-/-_2
PD-uhrf1-/-_3
PD-uhrf1-/-_4
PD-uhrf1-/-_5
z-score
Clusters - Genes
cluster 1- 43
cluster 2- 799
cluster 3- 1243
cluster 4- 255
cluster 5- 116
cluster 6- 39
cluster 7- 487
cluster 8- 579
cluster 9- 3
cluster 10- 21
D.
cluster 4
response to other organism
response to biotic stimulus
immune system process
immune effector process
cytokine stimulus
inflammatory response
cytokine−mediated signaling
response to cytokine stimulus
response to biotic stimulus
immune system positive regulation
immune response molecular mediator
receptor signaling response
apoptotic signaling
amino acid transport
response to stress
type 2 immune response
p53 DNA damage response
peptidyl tyrosine phosphorylation
cell activation
protein metabolism regulation
immune cytokine production
UP DMSO-uhrf1-/-
UP PD- uhrf1-/-
Adjusted p−value
Ratio of Genes in Gene List
E.
cluster 5
protein−DNA complex organization
regulation of DNA metabolic process
response to other organism
negative regulation of DNA recombination
response to biotic stimulus
chromosome condensation
inflammatory response to wounding
actin cytoskeletal organization
cortical cytoskeleton organization
regulation of cell shape
UP DMSO-uhrf1-/-
UP PD- uhrf1-/-
Adjusted p−value
Ratio of Genes in Gene List
F.
log2FC
DNA replication
PD
DMSO
psme2 psme1 e2f7 rrm2 tgfb1a bub1bb dbf4 dna2 pla2g4aa anxa1b vegfaa rpa2 nusap1 mapk14a tgfb2 anln spire1a dlgap5 tp53 cenpf sdc4 orc6 orc5 ccna2 ube2s tpx2 snx18a dct smc2 pold1 ythdf2 pcna rbbp4 mapre1b mcm4 snap25a pola1 pola2 hnrnpub thbs4b mcm2 mcm3 cdc45
Interferon
cd74a mxc isg15 stat1b cd74b b2m parp9 saa mxg etv7 cd40 gig2p ifit8 irf7 fas dhx58 rsad2 lyg1 irf1b lect2.1 sqstm1 stat2 cfb
Tnfa
fas tnfb nfkb2 nfkb tnfaip3 traf3 traf2b tnfrsf1a map3k14a nfkbie fadd casp7 pak1 birc2
G.
LXR/RXR Activation
G1/S Checkpoint Regulation
CHK Proteins in Cell Cycle
p53 Signaling
G2/M Checkpoint Regulation
GADD45 Signaling
MYC Mediated Apoptosis Signaling
Senescence Pathway
14-3-3 σ Signaling
BTG Proteins Cell cycle regulation
Cyclins and Cell cycle regulation
Acute Phase Response Signaling
Estrogen-mediated S-phase Entry
Ribonucleotide Reductase Signaling
Hepatic Fibrosis Signaling
PKR Response
LPS/IL-1 Inhibition of RXR Function
Cell Cycle Control of Replication
z-score

Figure 4

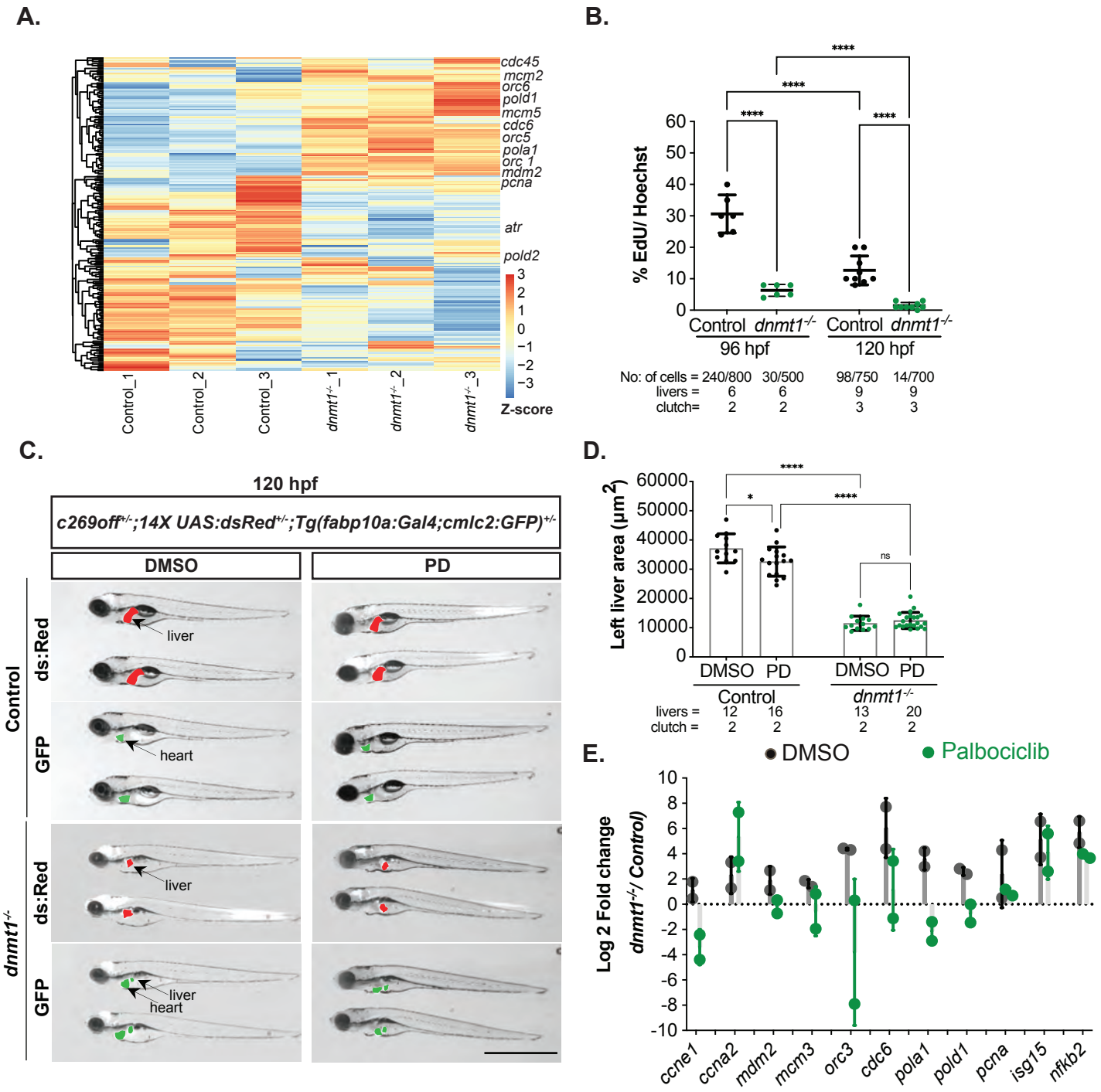

A.
cdc45
mcm2
orc6
pold1
mcm5
cdc6
orc5
pola1
orc 1
mdm2
pcna
atr
pold2
Z-score
Control_1
Control_2
Control_3
dnmt1-/- 1
dnmt1-/- 2
dnmt1-/- 3
B.
% EdU/ Hoechst
Control
dnmt1-/-
96 hpf
120 hpf
No: of cells = 240/800 30/500 98/750 14/700
livers = 6 6 9 9
clutch= 2 2 3 3
C.
120 hpf
c269off+/-;14X UAS:dsRed+/-;Tg(fabp10a:Gal4;cmlc2:GFP)+/-
DMSO
PD
Control
dnmt1-/-
ds:Red
GFP
liver
heart
D.
Left liver area (µm2)
DMSO
PD
Control
dnmt1-/-
livers = 12 16 13 20
clutch = 2 2 2 2
ns
E.
DMSO
Palbociclib
Log 2 Fold change
dnmt1-/-/ Control)
ccne1
ccna2
mdm2
mcm3
orc3
cdc6
pola1
pold1
pcna
isg15
nfkb2

Figure 5

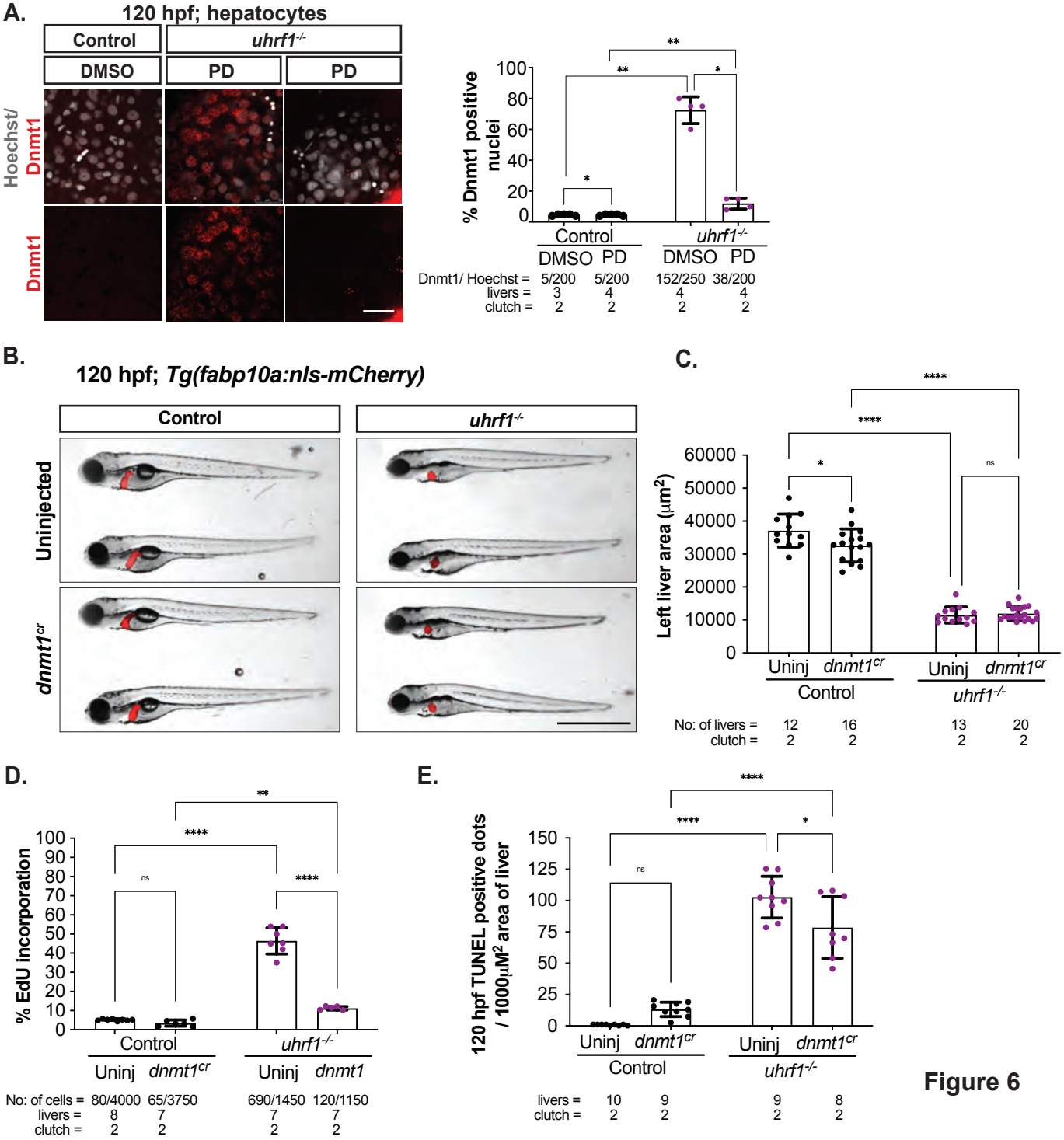

A.
120 hpf; hepatocytes
Control
uhrf1-/-
DMSO
PD
PD
Hoechst/ Dnmt1
Dnmt1
% Dnmt1 positive nuclei
Control
uhrf1-/-
DMSO PD
DMSO PD
Dnmt1/ Hoechst = 5/200 5/200 152/250 38/200
livers = 3 4 4 4
clutch = 2 2 2 2
B.
120 hpf; Tg(fabp10a:nls-mCherry)
Control
uhrf1-/-
Uninjected
dnmt1cr
C.
Left liver area (µm2)
Uninj dnmt1cr
Control
Uninj dnmt1cr
uhrf1-/-
No: of livers = 12 16 13 20
clutch = 2 2 2 2
D.
% EdU incorporation
Control
uhrf1-/-
Uninj dnmt1cr
Uninj dnmt1
No: of cells = 80/4000 65/3750 690/1450 120/1150
livers = 8 7 7 7
clutch = 2 2 2 2
E.
120 hpf TUNEL positive dots / 1000µM2 area of liver
Uninj dnmt1cr
Control
Uninj dnmt1cr
uhrf1-/-
livers = 10 9 9 8
clutch = 2 2 2 2

Figure 6

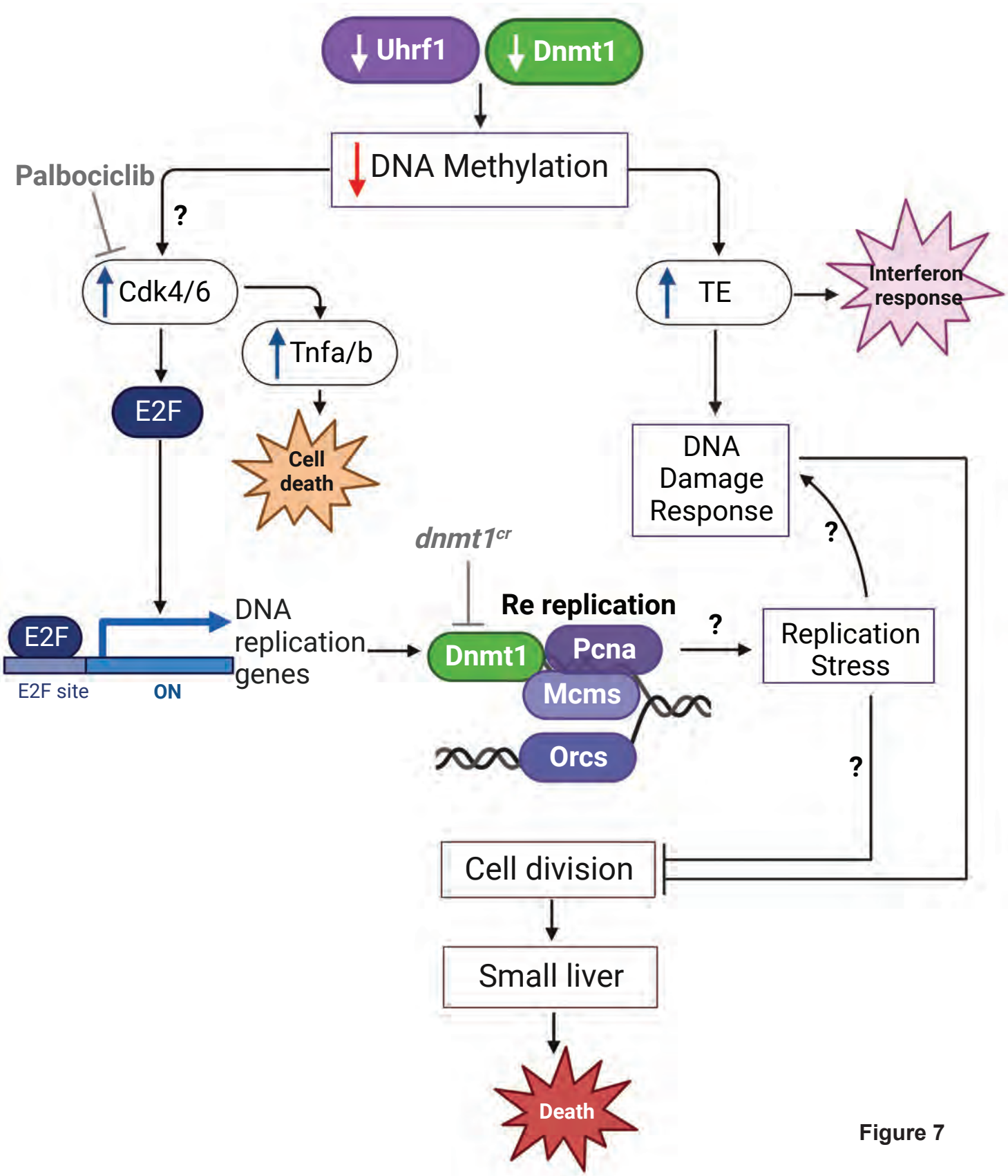

Uhrf1
Dnmt1
DNA Methylation
Palbociclib
?
Cdk4/6
Tnfa/b
E2F
Cell death
TE
Interferon response
DNA Damage Response
?
dnmt1cr
Re replication
?
Replication Stress
E2F
E2F site
ON
DNA replication genes
Dnmt1
Pcna
Mcms
Orcs
?
Cell division
Small liver
Death

Figure 7

Supplemental Materials

Supplement Figure Legends

Figure S1. Representative images of 5 minute, 12 minute and 30 minute EdU incorporation in 120 hpf *uhrf1* control and *uhrf1*$^{-/-}$ livers

Figure S2. Phenotype of Palbociclib (PD) treated *uhrf1* control and mutants
(A) 120 hpf embryos from incross of *uhrf1*$^{+/-}$ *;Tg(fabp10a: nls-mcherry)* adults treated with DMSO and imaged, livers are in red (B) Phenotypic assessment of the embryos at 120 hpf (C) 120 hpf embryos from incross of *uhrf1*$^{+/-}$ *;Tg(fabp10a: nls-mcherry)* adults treated with PD and imaged, livers in red, embryos numbered and genotype on the right side of the image (D) PCR based genotyping to ascertain the *uhrf1*$^{-/-}$ with PD treatment (E) Eye size measurement of 120 hpf *uhrf1*$^{-/-}$ and control embryos with DMSO and PD treatment (F) Survival of DMSO and PD treated embryos at 9 dpf. Scale: 50µm, the number of samples and clutches indicated for each condition. p-value *< 0.05, **< 0.005, *** < 0.0005 by two tailed Students t-test with adjustment for multiple comparisons.

Figure S3. Palbociclib (PD) treatment differentially regulates cell cycle gene expression but does not rescue transposon expression in *uhrf1* mutants
(A) Volcano plot of Differently Expressed Genes (DEGs) from RNA-Seq between 120 hpf *uhrf1* control PD treated and control DMSO treated livers (B) IPA showing the pathways differentially expressed from RNA-Seq between 120 hpf *uhrf1* control PD treated and control DMSO treated livers (C) Four quadrant cross-plot of the DEGs with log2fold change from RNA-Seq between 120 hpf *uhrf1*$^{-/-}$ PD treated and DMSO treated livers (D) Correlation plot of repetitive elements in 120 hpf *uhrf1*$^{-/-}$ mutant livers treated with DMSO and PD. Upregulated TEs have log 2 fold change > 0; downregulated TEs have log 2 fold change < 0. Log 2 fold change is calculated between mutants and their own control siblings. (E) Gene ontology (REVIGO) of the upregulated and downregulated genes in 120 hpf DMSO and PD treated *uhrf1*$^{-/-}$ liver RNA-Seq using significant genes (p_{adj} < 0.05) for each category.

Figure S4. Palbociclib (PD) treatment on *uhrf1* mutants rescues key cell cycle gene overexpression
(A) IPA analysis of the G1-S pathway in DMSO *uhrf1*$^{-/-}$ compared to DMSO treated controls. The color of the circles represents the observed results (red for activated/ upregulated and blue for repressed/ downregulated) (B) qPCR of key cell cycle genes of 120 hpf *uhrf1*$^{-/-}$ and control livers treated with DMSO or PD, data from 2 clutches shown (C) Volcano plot of Differently Expressed Genes (DEGs) from RNA-Seq between 120 hpf *uhrf1*$^{-/-}$ PD treated and *uhrf1*$^{-/-}$ DMSO treated livers (D) IPA analysis of the G1-S pathway between PD and DMSO treated *uhrf1*$^{-/-}$. The color of the circles represents the observed result (red for activated/ upregulated and blue for repressed/ downregulated).

Figure S5. IPA analysis of ATM pathway
IPA analysis of the ATM pathway in 120 *uhrf1*$^{-/-}$ liver and 120 *dnmt1*$^{-/-}$ liver transcriptomes. The color of the circles represents the observed results (orange for

activated/ upregulated only in *uhrf1*$^{-/-}$, red for activated in both, light blue for repressed/ downregulated only in *uhrf1*$^{-/-}$ and dark blue for repressed in both.

Figure S6. Testing the efficiency of *dnmt1*^{cr} *in c269off+/-; 14X UAS:dsRed+/-; Tg(fabp10a:Gal4;cmlc2:GFP)+/-* and nuclear morphology of *dnmt1*^{cr} injected *uhrf1* control and mutants
(A) Representative images of 120 hpf uninjected and *dnmt1*^{cr} embryos imaged for dsRed and GFP in the head. GFP positivity indicates hypomethylation (B) 120 hpf embryos from incross of *uhrf1*$^{+/-}$;*Tg(fabp10a: nls-mcherry)* adults injected or uninjected with *dnmt1*^{cr} were genotyped, PFA fixed, livers dissected, Hoechst stained and imaged as a Z-stack which were then used to calculate (A) volume, (B) Surface area and (C) Ellipsoid mean of the hepatocytes. The number of samples and clutch indicated for each condition. p-value *< 0.05, **< 0.005, *** < 0.0005 by ANOVA.

Supplemental tables
Table S1. REVIGO analysis of RNA-Seq from 120 hpf *uhrf1* mutant livers
Table S2. Cell cycle pathways identified by IPA of RNA-Seq from 120 hpf *uhrf1* mutant livers
Table S3. Optimization of PD exposure protocol on WT embryos
Table S4. DEGs in the liver of PD and DMSO treatment of 120 hpf control larvae
Table S5. IPA of DEGs from the liver of PD and DMSO treated 120 hpf control larvae
Table S6. DEGs from the liver of DMSO treated control and *uhrf1* mutant larvae and from PD treated control and *uhrf1* mutants at 120 hpf
Table S7. ENSEMBL IDs of the heatmap clusters from DMSO and PD treated control and *uhrf1* mutant larvae
Table S8. REVIGO analysis of clusters 4 and 5 from DMSO and PD treated control and *uhrf1* mutant larvae
Table S9. REVIGO analysis of DEGs between *uhrf1* mutants treated with DMSO or PD (from Table S6)
Table S10. DNA replication DEGs, immune DEGs and TNFa DEGs between *uhrf1* mutants treated with DMSO or PD
Table S11. DEGs from the liver of DMSO vs. PD treated *uhrf1* mutants at 120 hpf
Table S12. IPA of DEGs from DMSO vs. PD treated *uhrf1* mutants at 120 hpf
Table S13. Primers for qPCR and CRISPR sgRNA

Supplementary materials and methods

Survival assay
Heterozygous *uhrf1*$^{hi272+/-}$; *Tg(fabp10a: nls-mcherry)* $^{+/-}$ adults were incrossed and split into 40 embryos per 90mm petriplate, with around around 25% *uhrf1*$^{-/-}$ per plate. The embryos were dechorionated and treated with either 0.5% DMSO or 20 μm Palbociclib at 48 hpf**.** At 120 hpf, the embryos were transferred to fresh embryo water and monitored till 9 dpf when the live embryos were individually collected and genotyped for the *uhrf1* mutation.

Eye size measurement
Eye size measurements were carried out based on the 2-dimensional area of the left eye in 120 hpf larvae. Live larvae were mounted in 3% methylcellulose on the right side and the left eye was imaged using Nikon Elements on a Nikon SMZ25

Stereoscope. Eye size measurements were calculated in ImageJ using free hand selection of the eye area and analyzed using the shape descriptor sub-function to determine the area expressed in μM^2.

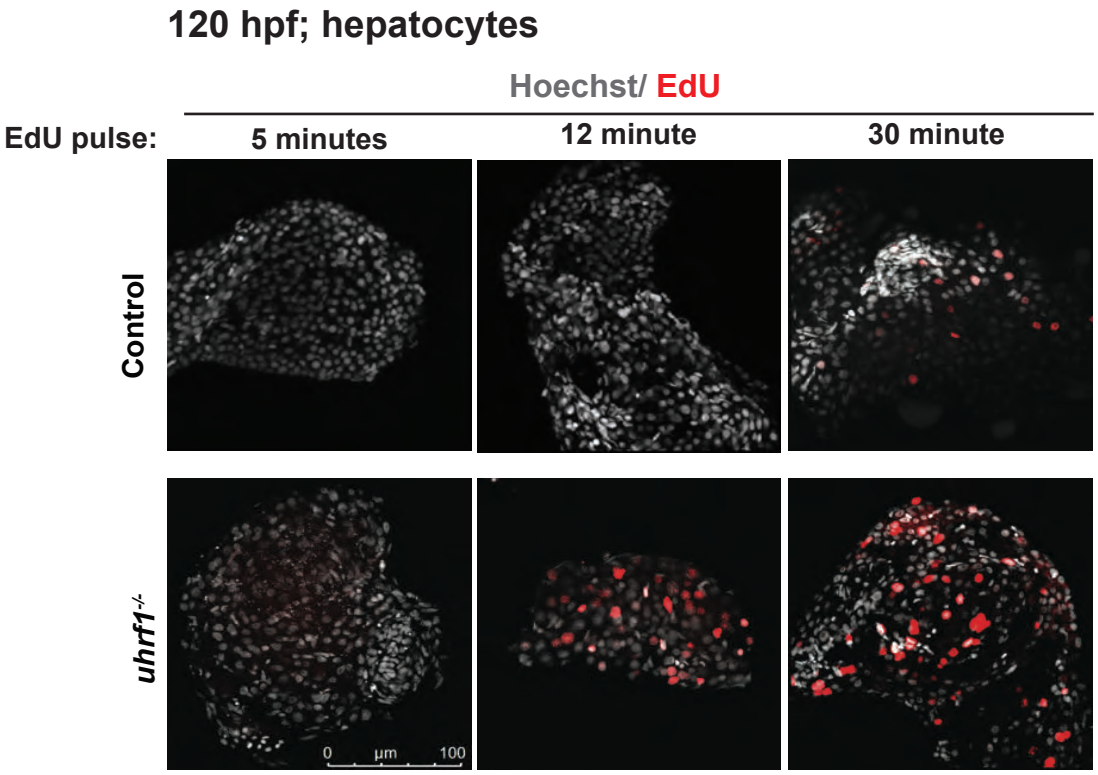

120 hpf; hepatocytes
Hoechst/ EdU
EdU pulse:
5 minutes
12 minute
30 minute
Control
uhrf1-/-
0 µm 100

Figure S1

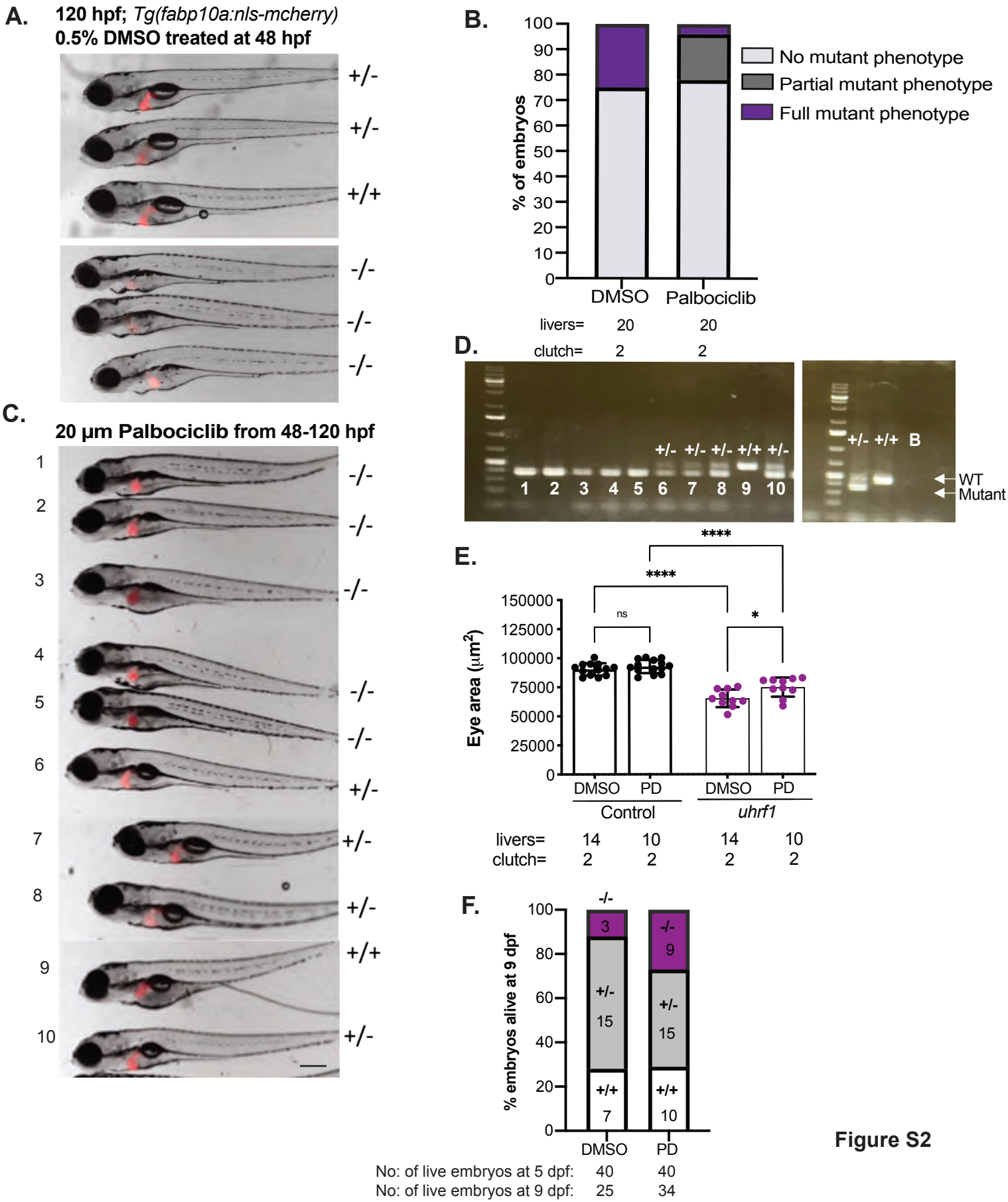

A.
120 hpf; Tg(fabp10a:nls-mcherry)
0.5% DMSO treated at 48 hpf
+/-
+/-
+/+
-/-
-/-
-/-
B.
% of embryos
No mutant phenotype
Partial mutant phenotype
Full mutant phenotype
DMSO
Palbociclib
livers= 20 20
clutch= 2 2
C.
20 µm Palbociclib from 48-120 hpf
1 -/-
2 -/-
3 -/-
4
5 -/-
-/-
6 +/-
7 +/-
8 +/-
9 +/+
10 +/-
D.
+/- +/- +/- +/+ +/-
1 2 3 4 5 6 7 8 9 10
+/- +/+ B
WT
Mutant
E.
Eye area (µm²)
****
****
ns
*
DMSO PD DMSO PD
Control
uhrf1
livers= 14 10 14 10
clutch= 2 2 2 2
F.
% embryos alive at 9 dpf
-/-
3
+/-
15
+/+
7
-/-
9
+/-
15
+/+
10
DMSO PD
No: of live embryos at 5 dpf: 40 40
No: of live embryos at 9 dpf: 25 34

Figure S2

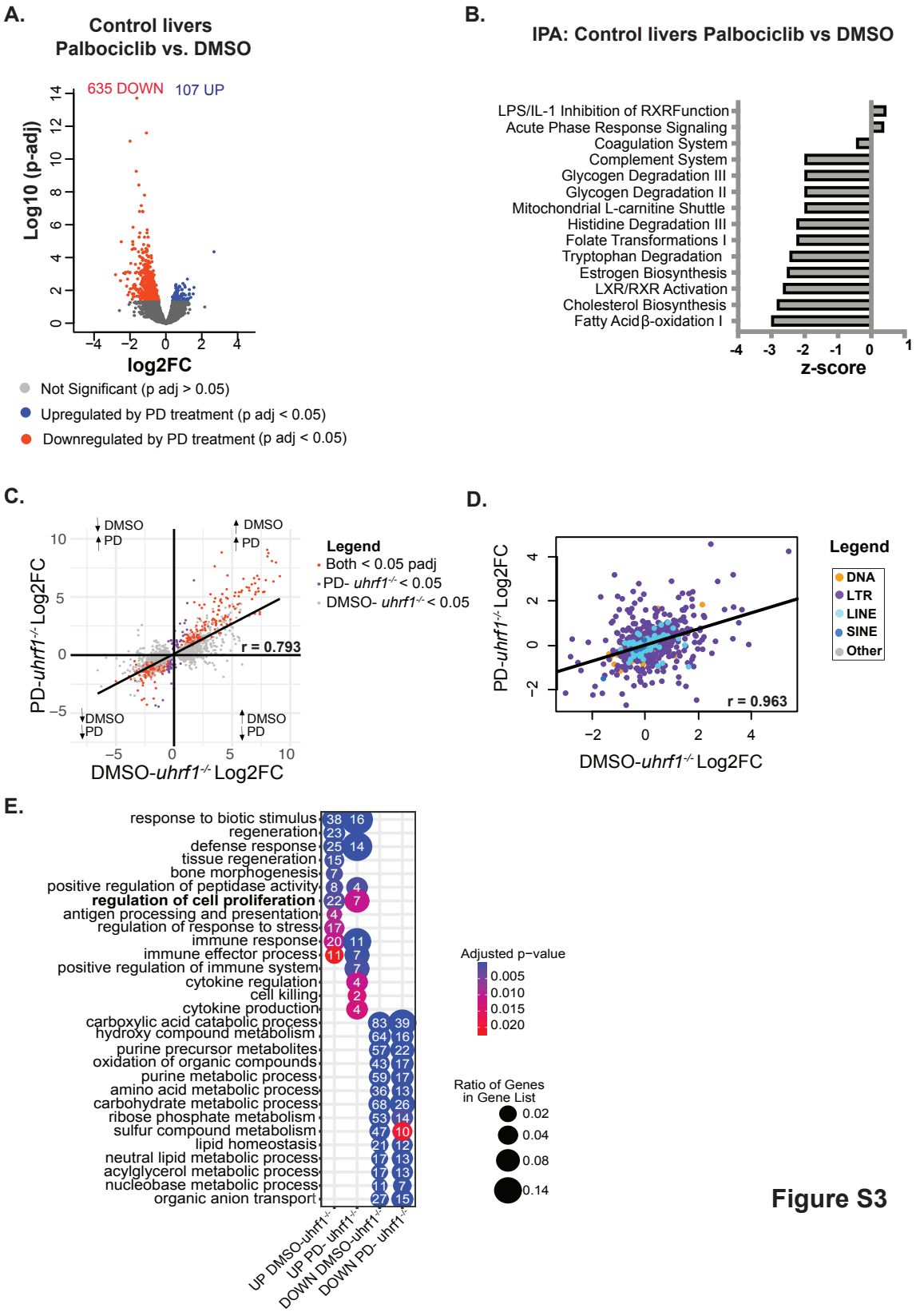
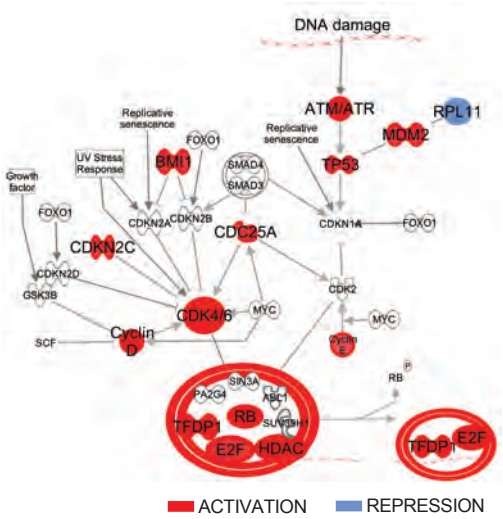

A.
Control livers
Palbociclib vs. DMSO
635 DOWN
107 UP
Log10 (p-adj)
log2FC
Not Significant (p adj > 0.05)
Upregulated by PD treatment (p adj < 0.05)
Downregulated by PD treatment (p adj < 0.05)
B.
IPA: Control livers Palbociclib vs DMSO
LPS/IL-1 Inhibition of RXRFunction
Acute Phase Response Signaling
Coagulation System
Complement System
Glycogen Degradation III
Glycogen Degradation II
Mitochondrial L-carnitine Shuttle
Histidine Degradation III
Folate Transformations I
Tryptophan Degradation
Estrogen Biosynthesis
LXR/RXR Activation
Cholesterol Biosynthesis
Fatty Acid β-oxidation I
z-score
C.
DMSO
PD
r = 0.793
PD-uhrf1-/- Log2FC
DMSO-uhrf1-/- Log2FC
Legend
Both < 0.05 padj
PD- uhrf1-/- < 0.05
DMSO- uhrf1-/- < 0.05
D.
r = 0.963
PD-uhrf1-/- Log2FC
DMSO-uhrf1-/- Log2FC
Legend
DNA
LTR
LINE
SINE
Other
E.
response to biotic stimulus
regeneration
defense response
tissue regeneration
bone morphogenesis
positive regulation of peptidase activity
regulation of cell proliferation
antigen processing and presentation
regulation of response to stress
immune response
immune effector process
positive regulation of immune system
cytokine regulation
cell killing
cytokine production
carboxylic acid catabolic process
hydroxy compound metabolism
purine precursor metabolites
oxidation of organic compounds
purine metabolic process
amino acid metabolic process
carbohydrate metabolic process
ribose phosphate metabolism
sulfur compound metabolism
lipid homeostasis
neutral lipid metabolic process
acylglycerol metabolic process
nucleobase metabolic process
organic anion transport
UP DMSO-uhrf1-/-
UP PD- uhrf1-/-
DOWN DMSO-uhrf1-/-
DOWN PD- uhrf1-/-
Adjusted p−value
0.005
0.010
0.015
0.020
Ratio of Genes in Gene List
0.02
0.04
0.08
0.14

Figure S3

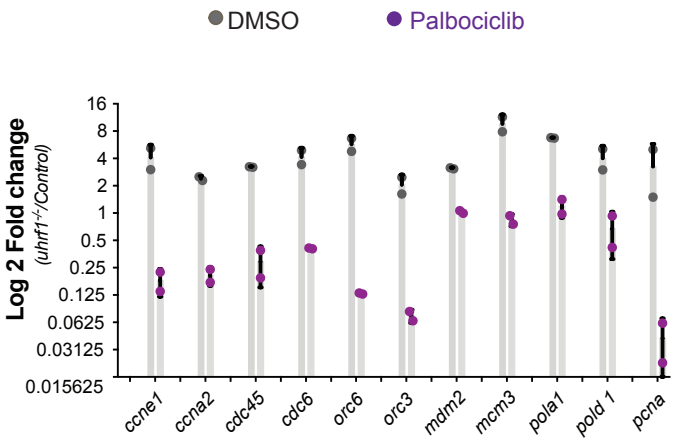
A.

IPA: G1-S pathway
DMSO *uhrf1*$^{-/-}$ vs Control livers



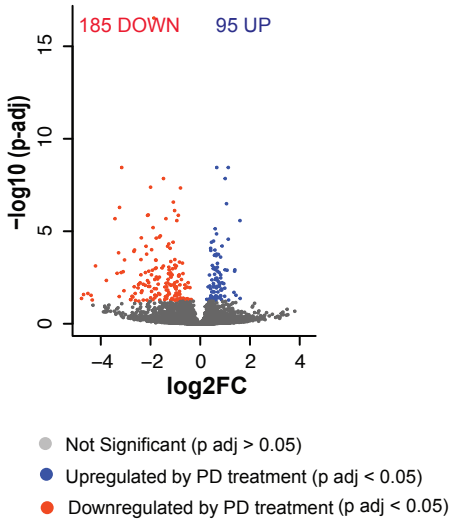
B.

120 hpf *uhrf1*$^{-/-}$ livers vs. Controls



C.

***uhrf1*$^{-/-}$ livers**
Palbociclib vs. DMSO



D.

IPA: G1-S pathway
Palbociclib *uhrf1*$^{-/-}$ vs DMSO *uhrf1*$^{-/-}$

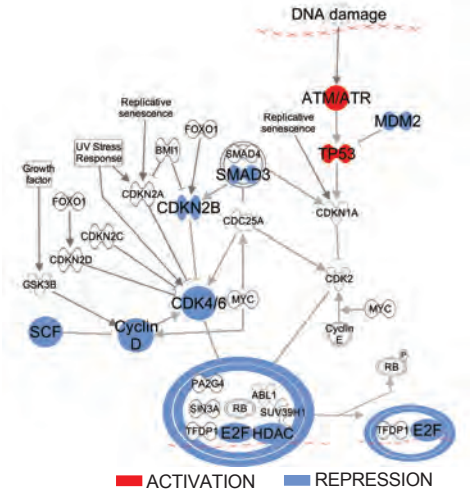
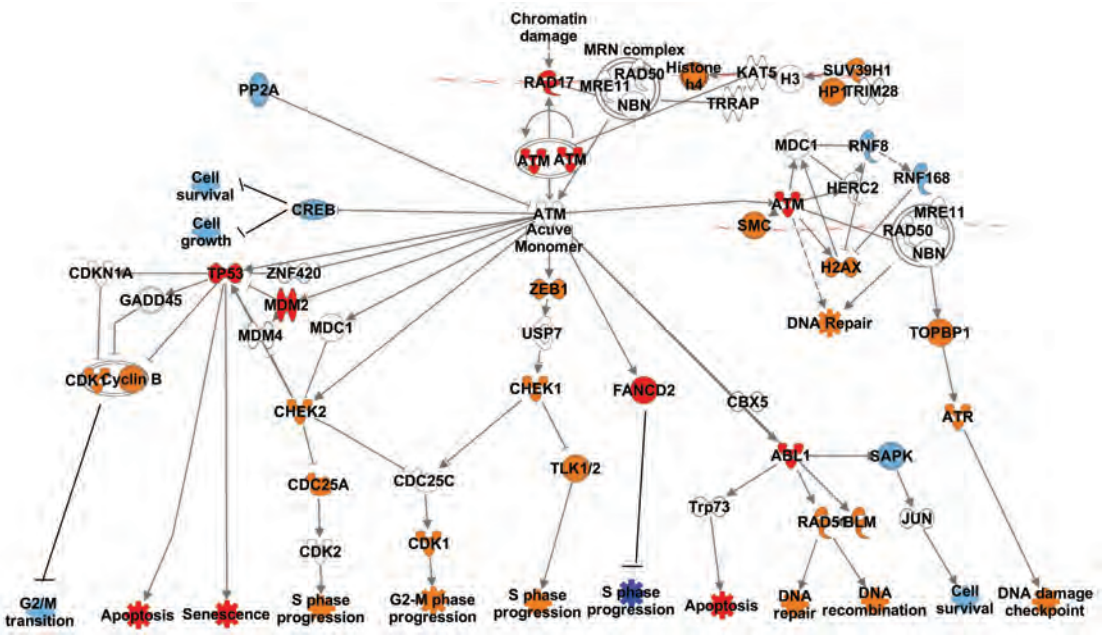


Figure S4



Legend

- UP *uhrf1*$^{-/-}$ only
- DOWN *uhrf1*$^{-/-}$ only
- UP *uhrf1*$^{-/-}$ *and dnmt1*$^{-/-}$
- DOWN *uhrf1*$^{-/-}$ *and dnmt1*$^{-/-}$

Figure S5

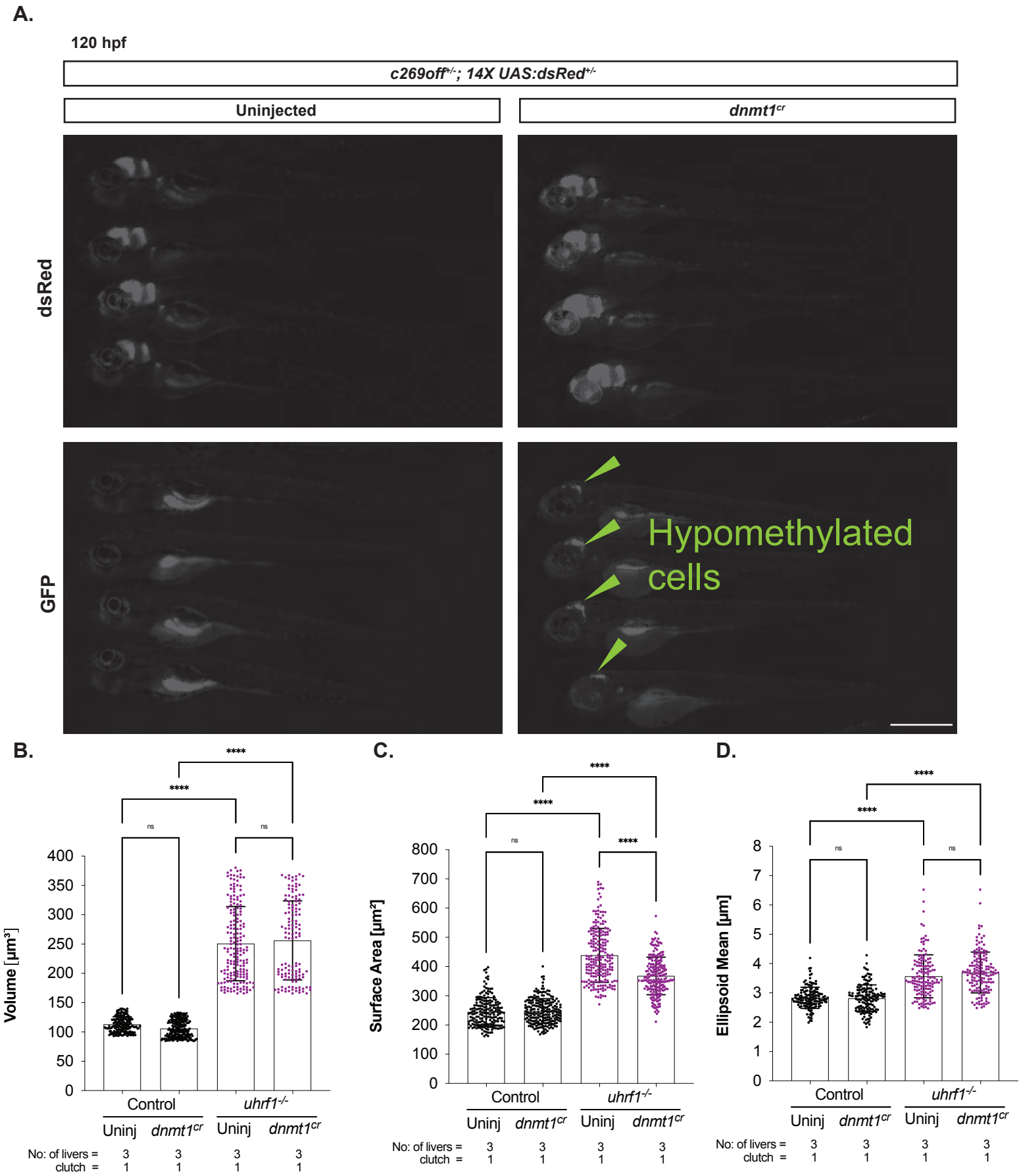

A.
120 hpf
c269off+/-; 14X UAS:dsRed+/-
Uninjected
dnmt1cr
dsRed
GFP
Hypomethylated cells
B.
Volume [µm³]
Control
uhrf1-/-
Uninj
dnmt1cr
No: of livers =
clutch =
C.
Surface Area [µm²]
D.
Ellipsoid Mean [µm]
****
ns

Figure S6