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RESEARCH ARTICLE

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Lrwd1 impacts cell proliferation and the silencing of repetitive DNA elements

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Summary

LRWD1, also known as ORCA, is a nuclear protein functioning in multiple biological processes. Using its WD40 domain LRWD1 interacts with repressive histone marks and maintains the silencing of heterochromatin regions in mammalian cells. ORCA also associates with the origin recognition complex (ORC) and facilitates prereplication complex formation at late-replicating origins. However, whether LRWD1 plays a role during development and the functional significance of LRWD1 in vivo remains largely unknown. Using gene-trap approach we generated *Lrwd1* knockout mice and examined the expression of *Lrwd1* during embryonic development. We found that *Lrwd1* is ubiquitously expressed in the majority of the developing mouse embryo. Depletion of LRWD1 did not affect embryonic development but the postnatal growth of the homozygous mutants is retarded. In vitro cultured mouse embryonic fibroblasts (MEFs) depleted of LRWD1 displayed a reduced proliferation compared to wild type cells. We also showed that the knockout of *Lrwd1* in MEFs increased the expression of the epigenetically silenced repetitive elements but with minimal effect on the expression of protein coding genes. Together, these results suggest that LRWD1 plays an important, but not essential, role in postnatal development by regulating cell proliferation likely through modulating DNA replication.

KEYWORDS
epigenetics, transcription process, process

1 | INTRODUCTION

The replication of genomic DNA in eukaryotic cells is tightly regulated to ensure the genome is replicated only once during each cell division. DNA replication initiates at genomic regions termed replication origins and occur in three main stages through the sequential loading of replication complex proteins (Masai, Matsumoto, You, Yoshizawa-Sugata, & Oda, 2010). Firstly, the origin recognition complex (ORC), consisting of six subunits ORC1-6, recognizes and assembles on replication origins. This is followed by origin "licensing" during which CDC6 and CDT1 proteins are recruited to ORC-associated sites where they promote the assembly of MCM2-7 proteins to form the pre-replication complex (pre-RC) in G1 phase. Finally, DDK and CDK kinases activate pre-RC by phosphorylating several replication factors to establish the functional replisome in S phase. Replication origins fire at different times within the S phase. Origins in open chromatin

regions normally fire early whereas origins in transcriptionally inactive, heterochromatin regions fire later in the S phase (Aladjem & Redon, 2017).

In *Saccharomyces cerevisiae* (*S. cerevisiae*), yeast ORC binds to specific genomic regions containing autonomously replicating sequences. In contrast, metazoan replication origins are not sequence specific. Although some genetic and epigenetic features have been identified among metazoan origins, origin specification in metazoans remains poorly understood (Aladjem & Redon, 2017). Several proteins that directly interact with replication origins or facilitate ORC loading to the chromatin have also been identified in mammalian cells (Aladjem & Redon, 2017), which adds another layer of complexity to origin specification by ORC.

The leucine-rich repeats and WD40 repeat domain-containing protein 1 (LRWD1), also known as ORC-associated (ORCA), assembles into a complex with ORC and plays an important role in stabilizing ORC on the chromatin (Bartke et al., 2010; Shen et al., 2010, 2012; Vermeulen et al., 2010). The LRWD1 protein is highly conserved among higher eukaryotes and is not present in *S. cerevisiae* (Shen et al., 2010). Aside from its role in pre-RC assembly, LRWD1 is also involved in heterochromatin organization and maintenance. LRWD1 interacts with repressive histone modifications that mark the heterochromatin, maintains the silencing of major satellite repeats in mouse pericentric heterochromatin and mediates the establishment and maintenance of H3K9me3 enrichment in specific genomic regions in human cells (Bartke et al., 2010; Chan & Zhang, 2012; Giri et al., 2015; Vermeulen et al., 2010; Wang et al., 2017). The C-terminus WD40 repeat domain of LRWD1 mediates LRWD1's direct interaction with ORC subunits and the repressive histone marks H4K20me3, H3K9me3, and H3K27me3 (Bartke et al., 2010; Chan & Zhang, 2012; Shen et al., 2010). Through its association with both ORC and repressive histone marks, LRWD1 facilitates pre-RC formation on late-replicating origins by stabilizing ORC's association on the heterochromatin and maintains replication timing on these origins (Brustel et al., 2017; Wang et al., 2017). The expression of LRWD1 is cell-cycle dependent; LRWD1 level is the highest during G1 phase and decreases during S phase (Shen et al., 2010). Furthermore, LRWD1 depletion in human cells leads to cell cycle arrest in G1, suggesting LRWD1's essential role in pre-RC assembly (Shen et al., 2010, 2012).

Defects in the replication machinery have detrimental effects in organism growth. Mutations in the replication genes are linked to several microcephalic primordial disorders (MPDs) that are characterized by pre- and postnatal growth retardation (Bellelli & Boulton, 2021). The classic MPD, Meier–Gorlin syndrome (MGS), is a rare autosomal recessive disease that is characterized by primordial dwarfism, microtia, and patellar aplasia/hypoplasia. In 2011, homozygous or heterozygous compound mutations in the core prereplication genes *ORC1*, *ORC4*, *ORC6*, *CDT1*, and *CDC6* were identified in individuals with MGS (Bicknell et al., 2011; Guernsey et al., 2011). Cell lines derived from two MGS individuals with biallelic *ORC1* mutations showed reduced chromatin bound ORC1 and reduced pre-RC assembly, confirming that the *ORC1* mutations caused origin licensing defects (Bicknell, Walker, et al., 2011). An *orc1* zebrafish morphant model recapitulated MGS-like features; almost all zebrafish morphants exhibited reduced overall body size and 80% of the morphants developed normally besides some minor craniofacial features (Bicknell, Walker, et al., 2011). Taken together, these studies suggest that primordial dwarfism is caused by replication origin licensing defects.

Here, we generated *Lrwd1* mutant mice to examine LRWD1's biological functions during mouse development. A gene trap insertion in the *Lrwd1* locus disrupts *Lrwd1* expression and prevents the expression of the WD40 domain that associates directly with ORC and repressive histone marks. Homozygous *Lrwd1* mutant mice are viable and display growth retardation without major developmental defects. In addition, transcriptome analysis by RNA-seq revealed that the knockout of *Lrwd1* has minimal effect on the expression of protein coding genes in mouse embryonic fibroblast, suggesting that the phenotypes observed in the *Lrwd1* mutant mice are primarily caused by replication defects. Overall, the growth restriction and cell proliferation defect phenotypes observed in *Lrwd1* mutant mice are reminiscent of the dwarfism phenotypes observed in human disorders containing defects in the pre-RC machinery.

2 | RESULTS

2.1 | Homozygous *Lrwd1* mutant mice exhibit dwarfism phenotype

To examine LRWD1's biological function in vivo, we generated *Lrwd1* gene trap mutant mice by targeting the *Lrwd1* locus via homologous recombination. An ES cell clone with a *Frt*-flanked β-gal-neo (neomycin) cassette inserted within intron 5 and *LoxP* sites flanking exons 6–7 was injected into blastocysts to generate $Lrwd1^{+/neo}$ heterozygous mice (Figure 1a). Heterozygous $Lrwd1^{+/neo}$ mice were intercrossed to produce *Lrwd1* homozygous mutant ($Lrwd1^{neo/neo}$) mice. The correct integration was confirmed by PCR (Figure 1b). The mouse and human LRWD1 protein contain a leucine-rich repeat domain, a linker region and a WD40 repeat domain that is responsible for LRWD1's interaction with both ORC and repressive histone marks. The gene trap disrupted gene expression downstream of the linker region and abrogated the expression of the WD40 region. RT-qPCR analysis of primary embryonic fibroblasts (pMEFs) isolated from 13.5 dpc $Lrwd1^{neo/neo}$ embryos confirmed the complete disruption of *Lrwd1* transcription from exon 6 (Figure 1c). X-gal staining of 10.5 and 13.5 dpc $Lrwd1^{+/neo}$ embryos showed that *Lrwd1* is expressed ubiquitously during mid-late embryonic development (Figure 1d). Both heterozygous and homozygous *Lrwd1* mutant mice developed normally and did not display apparent defects. Intriguingly however, the homozygous mutant mice exhibited overall reduced body sizes after birth. Both male and female homozygous Lrwd1 mutant mice ($Lrwd1^{neo/neo}$) were smaller and weighed less than the WT and $Lrwd1^{+/neo}$ mice during the follow up from 4 weeks until 4 months (Figure 2a). Moreover, pMEFs isolated from 13.5 dpc $Lrwd1^{neo/neo}$ embryos proliferated very slowly compared to WT pMEFs (Figure 2b). These data indicate that LRWD1 is important for growth and development.

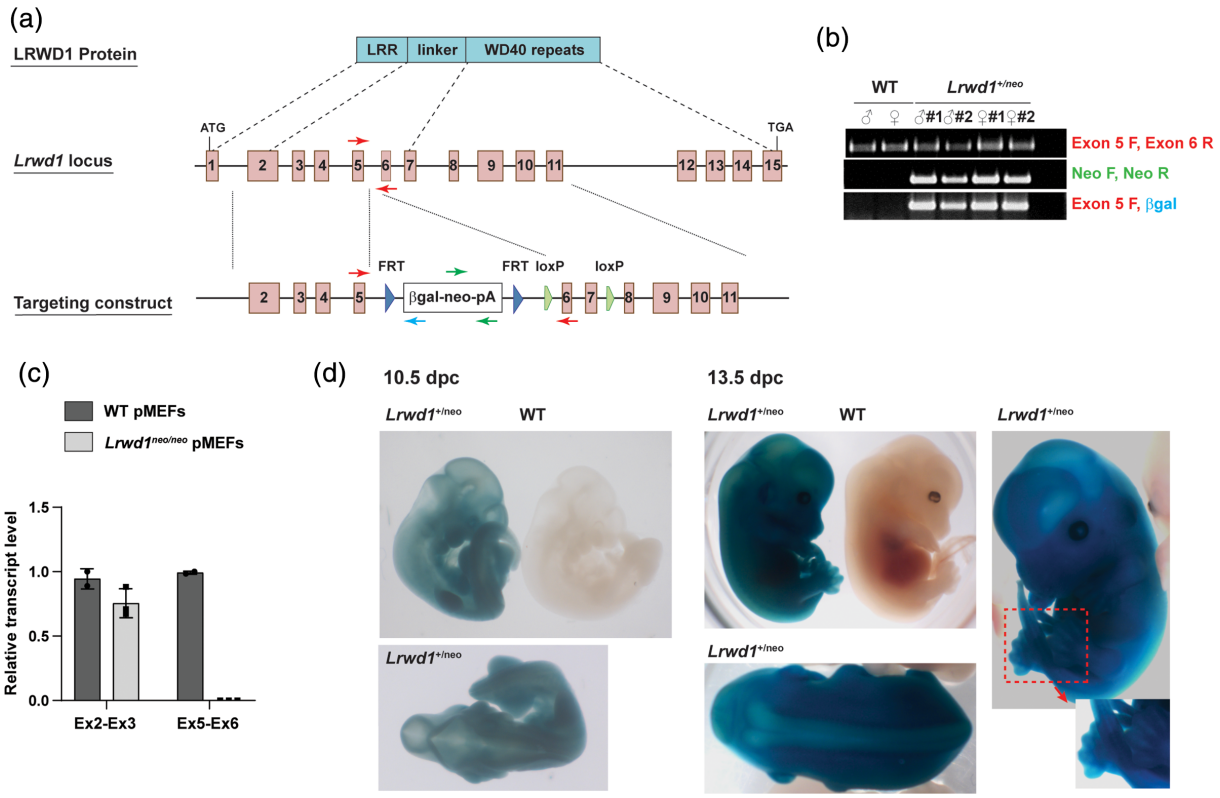
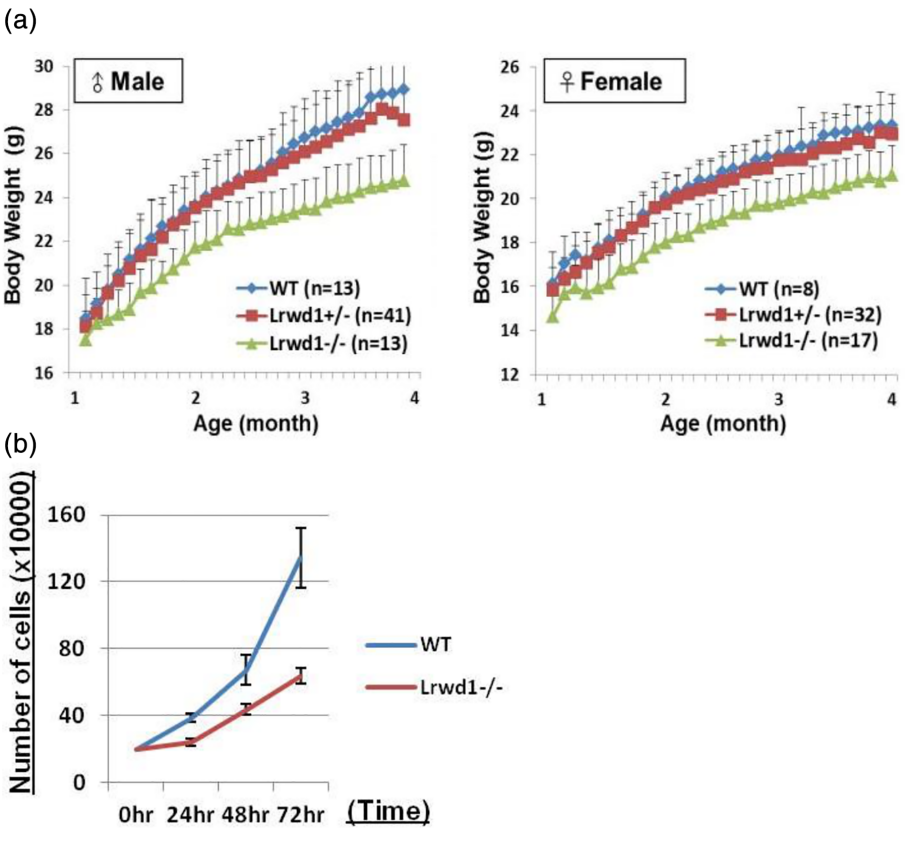


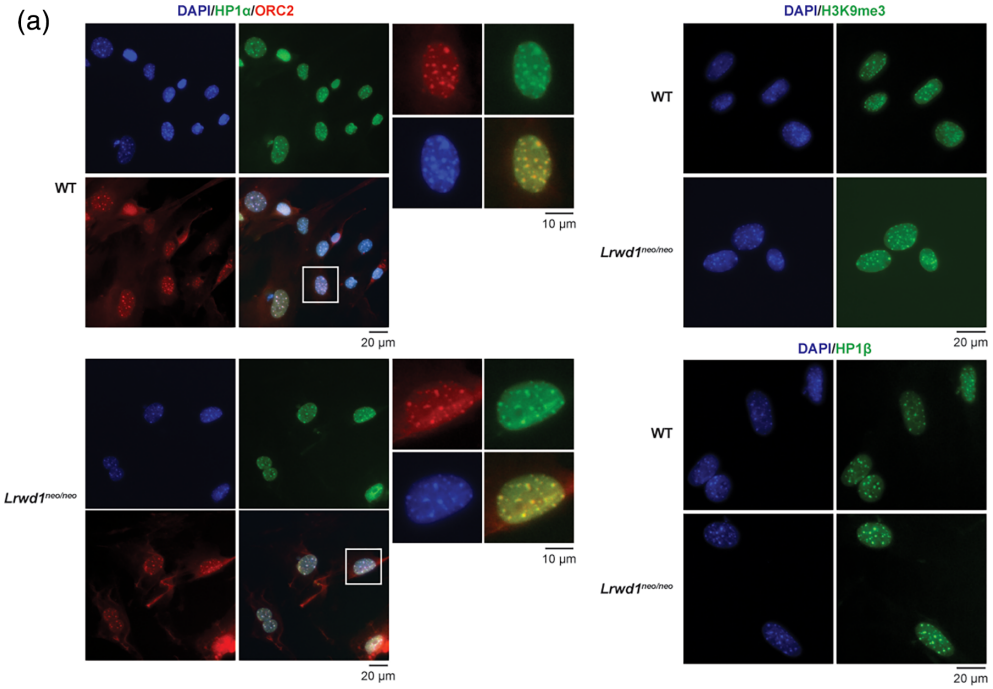
FIGURE 1 *Lrwd1* is expressed in the majority of the developing mouse embryo. (a). Schematic showing the strategy of knocking out *Lrwd1* using gene trap approach. A "beta-galactocidase -neomycin -poly A" cassette was inserted between exon 5 and exon 6 to disrupt the expression of the*Lrwd1* gene in mouse. (b). RT-PCR genotyping of the *Lrwd1* heterozygous mice. Primers targeting certain regions of the *Lrwd1* locus were used for the genotyping PCR reactions. (c). RT-qPCR data showing the depletion of exon 6 in *Lrwd1* homozygous mutant primary MEFs. The Exon 2-Exon 3 RT-qPCR reaction serves as a control. (d). X-gal staining of the wildtype and *Lrwd1* heterozygous mice from 10.5 and 13.5dpc reveals the expression of *Lrwd1* during mouse embryonic development

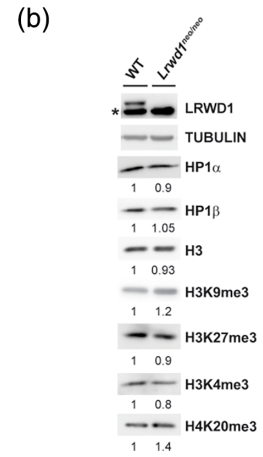
FIGURE 2 The knockout of *Lrwd1* disrupt the postnatal growth of mouse. (a). The growth of both the male and female wildtype (WT), heterozygous ($Lrwd1^{+/-}$) and homozygous mutant ($Lrwd1^{-/-}$) mice were monitored from week 4 to 4 months. The mice were weighted every 3 days during the selected period. (b). Proliferation of wildtype and $Lrwd1^{-/-}$ primary MEF was measured in a 72-hour time course. Cell number was counted every 24 hours in the selected period. Graph showing the average of 3 independent experiments



2.2 | *Lrwd1* knockout does not affect ORC localization to the pericentromeric heterochromatin in primary MEFs

LRWD1 stabilizes ORC on the chromatin and maintains heterochromatin organization (Giri et al., 2015; Shen et al., 2010). Previous studies showed that LRWD1 depletion was accompanied by defects in heterochromatin organization which include dysregulation of H3K9me3 organization and defects in Hp1α and H3K9me3 localization on heterochromatin foci in human cells (Wang et al., 2017). In mouse cells, tandem arrays of 234 bp AT-rich major satellite repeats mark the pericentromeric heterochromatin (PCH), forming clusters that appear as DAPI-dense foci (chromocenters) in interphase nuclei (Kuznetsova, Podgornaya, & Ferguson-Smith, 2006). Aside from its role in DNA replication, ORC2 also has a functional role in heterochromatin silencing. ORC2 localizes to mouse chromocenters and is required to maintain HP1α and HP1β localization on the heterochromatin in human cells (Prasanth, Prasanth, Siddiqui, Spector, & Stillman, 2004). To address the effect of *Lrwd1* knockout on ORC localization and heterochromatin organization during mouse development, we examined ORC2 and other heterochromatin factors in WT and *Lrwd1*$^{neo/neo}$ pMEFs isolated from 13.5 dpc embryos by immunofluorescence. The distribution of heterochromatin foci marked by the pericentromeric markers HP1α, HP1β, and H3K9me3 was comparable between WT and *Lrwd1*$^{neo/neo}$ MEFs. ORC2 localization on the heterochromatin foci also appeared unaffected by *Lrwd1* abrogation (Figure 3a). To further examine LRWD1's role in heterochromatin silencing, we examined levels of the repressive histone modifications by immunoblotting. Western blotting analysis with a LRWD1 antibody recognizing the linker region confirms LRWD1's depletion to undetectable levels in *Lrwd1*$^{neo/neo}$ pMEFs (Figure 3b), further confirming the knockout of the Lrwd1 by our gene trap approach. Consistent with a previous study, LRWD1 depletion did not affect total H3K9me3 levels (Giri et al., 2015). Interestingly, the levels of other histone modifications, such as H3K27me3 and H3K4me3 are slightly reduced while the H4K20me3 is significantly increased in *Lrwd1*$^{-/-}$ MEFs. These data indicate that LRWD1 is not essential for ORC recruitment to the PCH and for PCH organization.





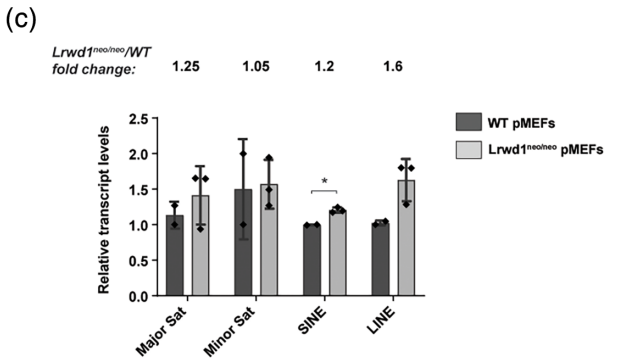


FIGURE 3 LRWD1 silences certain repetitive elements in mouse. (a). Primary WT and *Lrwd1* mutant MEFs were immunostained for HP1α, Orc2, H3K9me3 and HP1β. (b). Immunoblot analysis of WT and *Lrwd1* mutant MEFs. Whole cell lysates were analyzed by immunoblotting using the indicated antibodies. Asterisk (*) marks an unspecific band. Protein levels of HP1α and HP1β are quantified relative to tubulin. Protein levels of histone modifications are quantified relative to histone H3. (c). mRNA expression of major and minor satellite repeat, SINE and LINE transcripts. Relative expression levels from two WT and three *Lrwd1* mutant samples are shown (mean ± *SD*, $^*p \le .05$). *p*-values were calculated using two-tailed Welch *t*-test between WT and *Lrwd1* mutant samples

2.3 | *Lrwd1* knockout had a minor effect on heterochromatin silencing

Previously, we showed that LRWD1 is required for the maintenance of heterochromatin silencing on major satellite repeats in MEF cells. A large proportion (44%) of the mouse genome consists of repetitive elements that include major and minor satellite repeats in the constitutive heterochromatin and interspersed repetitive elements that are spread throughout the genome. Major and minor satellite repeats account for approximately 3.5% and interspersed repetitive elements including LINE and SINE account for approximately 18.78% and approximately 2.66% of the mouse genome, respectively (Martens et al., 2005). Transcriptional silencing of these repetitive elements is maintained epigenetically and mediated by repressive histone modifications. To examine LRWD1's role in heterochromatin silencing, levels of major and minor satellite repeats, LINE and SINE transcripts in WT and *Lrwd1*$^{-/-}$ pMEFs were examined by RT-qPCR. A minimal increase of major satellite and LINE transcript levels (1.25- and 1.6-fold increase, respectively) and a significant increase of SINE were found in *Lrwd1*$^{-/-}$ pMEFs (Figure 3c), indicating that LRWD1 plays a role in silencing certain repetitive elements in mouse.

2.4 | LRWD1 does not regulate gene expression in primary MEFs

Since LRWD1 has been shown to be involved in heterochromatin maintenance and silencing in previous studies, we next examined if *Lrwd1* knockout affected gene expression using our *Lrwd1* knockout model. We analyzed the gene expression profiles of 13.5 dpc WT and *Lrwd1*$^{neo/neo}$ pMEFs by RNA-sequencing (RNA-seq) and found that gene expression was largely unaffected by *Lrwd1* knockout (Figure 4). Aside from *Lrwd1* ($\log_2$ fold change of −2.21), only one other gene, *Gm7120* was found to be differentially expressed in *Lrwd1*$^{neo/neo}$ pMEFs. These data suggest that LRWD1 does not play a major role in regulating the expression of protein coding genes in cultured primary mouse embryonic fibroblast.

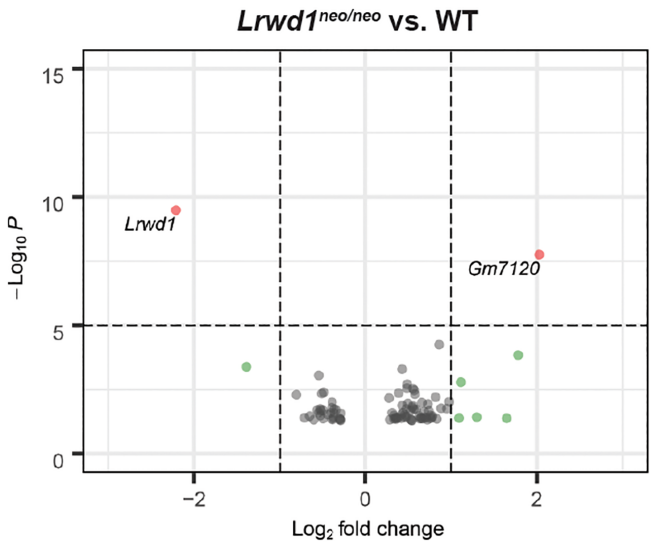


FIGURE 4 LRWD1 has minimal effect on the expression of protein coding genes. Gene expression profiles of WT and *Lrwd1* homozygous primary MEF were examined by RNA-seq. The expression of *Lrwd1* (red dot) is significantly downregulated in the knockout cells. *Gm7120* (red dot on the right) is the only significantly upregulated gene in the *Lrwd1*$^{-/-}$ cells. Green dots annotate the genes with log2 fold change >1 but not statistically significant based on their *p*-values

3 | DISCUSSION

Mutations of the pre-RC and other replisome components are linked to disorders that share growth restriction as a clinical feature. These include MGS which feature mutations in the ORC genes: *ORC1*, *ORC4* and *ORC6*. Furthermore, *mcm5* and *orc1* knockdown in zebrafish similarly reduced overall body sizes, providing strong evidence that impaired origin licensing causes dwarfism. The dwarfism phenotype is also linked to histone methylation-mediated recruitment of ORC, as depletion of H4K20me2/3 levels resulted in reduced body sizes similar to the phenotype shown by *orc1* morphants (Kuo et al., 2012). Growth retardation in the *orc1* zebrafish morphants could be partly rescued by WT *orc1* but not the H4K20me2-binding *orc1* mutants, suggesting that ORC1 recognition of H4K20me2-associated origins is important for origin licensing. In fact, *ORC1* mutations in MGS individuals mostly occur in the BAH domain responsible for H4K20me2 recognition (Bicknell, Walker, et al., 2011). LRWD1 is a stable interactor of ORC and is required for ORC association to the chromatin (Bartke et al., 2010; Shen et al., 2010; Vermeulen et al., 2010). During origin licensing, LRWD1 mediates pre-RC assembly on replication origins bound by repressive histone marks and is required for normal cell-cycle progression at G1 phase (Brustel et al., 2017; Shen et al., 2010). Altogether, these data suggest that LRWD1 is an important factor in DNA replication initiation.

Here, we generated a *Lrwd1* knockout mouse model that exhibited MGS-like features. *Lrwd1* mutant mice had overall reduced body sizes but were viable and structurally normal. Disease severity appears to correlate with a greater loss-of-function as MGS individuals with compound heterozygous missense and loss of function mutations in prereplication genes were associated with more severe phenotypes (Bicknell, Bongers, et al., 2011; Takehara, Makise, Takenaka, Asano, & Mizushima, 2008). Consistent with these observations, the homozygous *Lrwd1* mutant mice displayed growth retardation whereas heterozygous *Lrwd1*$^{+/neo}$ mutant mice were unaffected. Since LRWD1 facilitates ORC binding to a subset of replication origins, the effect of *LRWD1* mutations on pre-RC function may not be as severe as those caused by mutations in genes encoding the core pre-RC components. In a *Orc1* mutant mouse model, germline deletion of the ORC1 ATPase domain necessary for pre-RC assembly in one allele did not affect the mutant mice while deletion in both alleles caused embryonic lethality (Okano-Uchida et al., 2018; Shibata et al.,

2016). Consistent with the effect of *ORC1/2* knockout in HCT116 cells and *Orc1* knockout in MEFs (De Munnik et al., 2012; Okano-Uchida et al., 2018), *Lrwd1* mutant pMEFs experienced proliferation defects.

Previous work from Giri et al revealed that the LRWD1 interacts with histone methyltransferases including the EZH2, Suv39h1, and Suv420h1/h2. We observed that the level of H4K20me3 is elevated but the H3K9me3 is not significantly affect in the Lrwd1$^{-/-}$ MEFs, which is possibly an indirect effect of LRWD1 protein depletion in out *Lrwd1* mutant cells. Intriguingly, our RNA-seq data revealed a subtle elevated expression of genes involved in the positive regulation of cell division pathway, including *Igf2*, *Aurkb*, *Tgfb2*, and *Kif20b*, with absolute fold change <1.5-fold. It is possible that the proliferation defects in the *Lrwd1* mutant pMEFs is primarily caused by impaired replication initiation which indirectly affects the expression of cell cycle related genes. In line with the MGS pathogenesis, our findings suggest that the reduced body sizes of the *Lrwd1* mutant mice stem from impaired cellular proliferation caused by DNA replication defects (Bicknell, Bongers, et al., 2011).

Previous studies have revealed the expression of Lrwd1 in testicular tissues (Teng, Chuang, & Liu, 2013) and shown that LRWD1 is crucial for human spermatogenesis (Hung et al., 2021; Miyamoto et al., 2014). Here, using genetic approach we were able to demonstrate the expression of *Lrwd1* expression during mouse embryonic development by following the expression of the reporter beta galactosidase driven by the endogenous *Lrwd1* promoter. Our findings indicate that the *Lrwd1* is expressed in the majority of the developing mouse embryo. This data suggests LRWD1 might be an important factor regulating chromatin function as well as DNA replication in a variety of tissues and organs.

4 | MATERIAL AND METHODS

4.1 | Primary MEF isolation

Primary MEFs were collected from embryos at developmental stage 13.5dpc. Internal organs and head are removed and the remaining tissues are mince with razor blade followed by trypsin digestion for 15 min. Tissues were pipetted 20 times to break up tissues and were filtered using cell strainer. The cells were plated in culture disuse and were allowed to grow in DMEM in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and cultured at 37°C with 5% CO_2 up to four passages.

4.2 | Proliferation assay

Wildtype and *Lrwd1*$^{-/-}$ primary MEFs of passage number two were seeded on 10-cm plates with confluency 50%. Cells were allowed to grow for 72 hr and were counted using hemocytometer every 24 hours following standard subculture protocol.

TABLE 1 Primers used for genotyping

Primers	Sequence
Neo F	TTATCGATGAGCGTGGTGGTTATGC
Neo R	GCGCGTAGATCGGGCAAATAATATC
Exon 5 F	GCAGGTGCCTCCAAGTTCAGG
Exon 6 R	CGTTTTGAAGCCACCTCTCTGGC
Bgal	TTCGCAGCCAGGACGGAATG

4.3 | Mouse generation and PCR genotyping

The Lrwd1 gene trap mutant mice were generated from blastocyst infection of ES cells containing the gene trap between the exon 5 and exon 6 of the Lrwd locus (Lrwd1$^{Gt(IST14002D2)Tigm}$, available from Texas A&M Institute for Genomic Medicine). Primers for genotyping are listed in Table 1. Mice with *Lrwd1*$^{+/-}$ genotypes were crossed to generate the Lrwd1 homozygous mutant (*Lrwd1*$^{-/-}$) mice. The mouse line will be made available to the research community upon acceptance of this manuscript of publication.

4.4 | Immunoblotting

Whole cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked with 5% non-fat milk in TBST and incubated with primary antibodies overnight at 4°C followed by incubation with HRP-conjugated secondary antibodies (Jackson ImmunoResearch). Proteins were detected by ECL. Primary antibodies of LRWD1 (homemade, 1:1000), α-Tubulin (Immunoway, YM3115, 1:10000), HP1α (CST, 2616, 1:1000), HP1β (CST, 8676, 1:1000), H3 (Immunoway, YM3038, 1:10000), H3K9me3 (Active Motif, 61,013, 1:500), H3K27me3 (CST, 9733, 1:500), H3K4me3 (Active Motif, 61,379, 1:500), H4K20me3 (Active Motif, 39,671, 1:500) were used at indicated concentrations for immunoblotting.

4.5 | Antibody generation

Polyclonal antibody against LRWD1 was produced in rabbits by immunizing using peptide NNSPKDLETQLWACAFEPAR (human LRWD1 284-303aa) (ABClonal).

4.6 | Immunofluorescence

Primary MEFs were seeded in chamber slides, washed with PBS and fixed with 3% PFA/PBS for 12 min at room temperature. Cells were permeabilized with 0.5% Triton solution (20 mM HEPES, 50 mM NaCl, 3 mM MgCl2, 300 mM Sucrose, 0.5% Triton X-100) for 5 min, blocked with 5% normal goat serum for 1 hr and stained with primary

TABLE 2 Primers used for RT-qPCR

Primers	Sequence
Lrwd1 Ex2-Ex3	F- CAACTTGCTGGAGACGCTGCC
	R- GGAGGCCGTGTCCTTGCCATTC
Lrwd1 Ex5-Ex6	F- GTGCGGATGATCGCCGAGGAAC
	R- CTGCCCTCCATCAGCACCCATAG
Major satellite	F- GGCGAGAAAACTGAAAATCACG
	R- CTTGCCATATTCCACGTCCT
Minor satellite	F- TTGGAAACGGGATTTGTAGA
	R- CGGTTTCCAACATATGTGTTTT
LINE	F- TGGCTTGTGCTGTAAGATCG
	R- TCTGTTGGTGGTCTTTTTGTC
SINE	F- GAGCACACCCATGCACATAC
	R- AAAGGCATGCACCTCTACCACC

antibodies overnight at 4°C. Cells were washed with PBS and stained with FITC/TRITC secondary antibodies. Samples were then incubated with DAPI for 1 min, washed with PBS and mounted with ProLong™ Gold Antifade Mountant (Invitrogen). Cells were imaged with Nikon Eclipse Ni-E upright fluorescence microscope. Primary antibodies of HP1α (CST, 2616, 1:200), HP1β (CST, 8676, 1:800), H3 (Immunoway, YM3038, 1:10000), H3K9me3 (Abcam, Ab8898, 1:500), H3K27me3 (CST, 9733, 1:1000), H3K4me3 (Active Motif, 61379,1:500), H4K20me3 (Active Motif, 39,671, 1:500), Orc2 (gift from Bruce Stillman, 920-4, 1:200) were used at indicated concentrations for immunofluorescence.

4.7 | RNA extraction, RT-qPCR

Total RNA was extracted from pMEFs isolated from two WT and three *Lrwd1* mutant mice with the MiniBEST Universal RNA extraction kit (Takara). RNA was reverse transcribed to cDNA using PrimeScript RT Master Mix (Takara). Relative fold expression was calculated using the $2^{-\Delta\Delta Ct}$ method with *Actin* as the endogenous control. Primers are listed in Table 2.

4.8 | RNA-seq library preparation

Ribosomal RNA was depleted from total RNA using the NEBNext rRNA Depletion kit and libraries were prepared with NEBNext Ultra II Directional RNA Library Prep kit for Illumina (NEB). Library quality was assessed using Bioanalyzer 2100. Libraries were sequenced using 150 bp paired-end sequencing on a HiSeq platform.

4.9 | RNA-seq data analysis

Adapters were removed by "cutadapt." Reads were aligned to mouse reference genome mm10 from UCSC using "STAR" (v.2.7.6a) with default parameters. Differential gene expression analysis between *Lrwd1* mutant and WT samples was performed using the R package "DESeq2." Genes passing both thresholds, absolute $\log_2$FC > 1 and Benjamini-Hochberg adjusted $p < .05$ are considered differentially expressed.

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CONFLICT OF INTEREST

Authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Tze Zhen Evangeline Kang, Zhiguo Zhang and Kui Ming Chan conceived the project, designed the experiments and analyzed the data. Tze Zhen Evangeline Kang, and Kui Ming Chan performed the experiments. Tze Zhen Evangeline Kang, Yi Ching Esther Wan, and Kui Ming Chan interpreted the data and wrote the manuscript with comments from Zhiguo Zhang. All authors read and approved the final manuscript

DATA AVAILABILITY STATEMENT

The data is available upon request.

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