Cyclin D1 gene expression is essential for cell cycle progression from the maternal-to-zygotic transition during blastoderm development in Japanese quail

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Abstract

Embryogenesis proceeds by a highly regulated series of events. In animals, maternal factors that accumulate in the egg cytoplasm control cell cycle progression at the initial stage of cleavage. However, cell cycle regulation is switched to a system governed by the activated nuclear genome at a specific stage of development, referred to as maternal-to-zygotic transition (MZT). Detailed molecular analyses have been performed on maternal factors and activated zygotic genes in MZT in mammals, fishes and chicken; however, the underlying mechanisms remain unclear in quail. In the present study, we demonstrated that MZT occurred at blastoderm stage V in the Japanese quail using novel gene targeting technology in which the CRISPR/Cas9 and intracytoplasmic sperm injection (ICSI) systems were combined. At blastoderm stage V, we found that maternal retinoblastoma 1 (RB1) protein expression was down-regulated, whereas the gene expression of *cyclin D1* (*CCND1*) was initiated. When a microinjection of sgRNA containing *CCND1*-targeted sequencing and *Cas9* mRNA was administered at the pronuclear stage, blastoderm development stopped at stage V and the down-regulation of RB1 did not occur. This result indicates the most notable difference from mammals in which *CCND*-knockout embryos are capable of developing beyond MZT. We also showed that CCND1 induced the phosphorylation of the serine/threonine residues of the RB1 protein, which resulted in the degradation of this protein. These results suggest that CCND1 is one of the key factors for RB1 protein degradation at MZT, and the elimination of RB1 may contribute to cell cycle progression after MZT during blastoderm development in the Japanese quail. Our novel technology, which combined the CRISPR/Cas9 system and ICSI, has the potential to become a powerful tool for avian-targeted mutagenesis.

1. Introduction

The transcription of DNA replication factors in the G1-phase of the cell cycle is generally required for progression to the S-phase; however, transcription is typically inhibited to constrain cell proliferation. The retinoblastoma 1 (RB1) protein, a well-known tumor suppressor gene, plays a pivotal role in the arrest of transcription (Wang et al., 1994; Weinberg, 1995; Beijersbergen and Bernards, 1996; Sidle et al., 1996; Kaelin, 1997). RB1 exerts inhibitory effects on E2F, which is a key regulator that transactivates essential genes for entry into the S-phase (Grossel and Hinds, 2006; Tashiro et al., 2007). In somatic cells, cell cycle progression is regulated by external mitogens, such as growth factors and nutrients (Pardee, 1989; Sherr, 1996; Boonstra and Post, 2004). These external factors initiate the cascade of intracellular signal transduction. Previous studies revealed that D-type cyclins (*CCND*) and cyclin-dependent kinase 4/6 (*CDK4/6*) participated in multiple cell cycle regulatory pathways and played key roles in the phosphorylation of RB1 proteins in the G1/S phases (Buchkovich et al., 1989; Chen et al., 1989; Weinberg, 1995; Harbour et al., 1999). Phosphorylated RB1 proteins then lose this repressive activity for the E2F transcription factor.

Cell cycle progression in the embryo is regulated by internal factors because embryos undergo cleavage *in vitro* without the addition of any growth factors or serum (Whitten, 1971). The features of internal cell cycle regulators have mainly been reported in mammals and zebrafish vertebrate embryo studies, and two main regulatory systems have been identified for the G1/S transition. The first system suggests that maternal factors in the cytoplasm provided during oogenesis initially control cleavage, while the zygotic nuclear genome is quiescent. Nine maternal factors, *Mater*, *Hdf1*, *Dnmtlo*, *Pms2*, *Zar1*, *Npm2*, *stella*, *Zfp36I2*, and *Oogenesin*, have so far been identified in the mouse, and knockout mice of these genes showed developmental arrest and abnormal nuclear structures at the one- to two-cell stages (Newport and Kirschner, 1982; Christians et al., 2000; Tong et al., 2000; Howell et al., 2001; Gurtu et al., 2002; Burns et al., 2003; Minami et al., 2003; Payer et al., 2003; Wu et al., 2003; Ramos et al., 2004).

The second system suggests that maternal reliance ends, and developmental control is switched to the systems governed by an activated nuclear genome, during a period called the maternal-to-zygotic transition (MZT; Schultz, 1993; Lee et al., 2014). New nuclear reprograming networks are installed through gene expression, which is called zygotic genome activation (ZGA). The MZT in vertebrates was originally defined using α-amanitin, an inhibitor of RNA polymerase II and III. The application on fertilized eggs or later stages blocked zygotic transcription and the induced developmental arrest marks the MZT (Baroux et al., 2008). The MZT, under this definition, is distinct from the stage of ZGA, which corresponds to the onset of *de novo* transcription from the zygotic genome. In

particular, ZGA occurs in two waves (minor and major) in several vertebrates, and minor and major ZGA differ not only in the amount of transcription, but also in the number of genes transcribed (Aoki et al., 1997; Mathavan et al., 2005; Giraldez et al., 2006; Aanes et al., 2011; Xue et al., 2013; Yan et al., 2013). Minor ZGA commences at the pronuclear stage in many species (Park et al., 2013; Abe et al., 2015; Svoboda, 2018), whereas major ZGA occurs at species-specific stages (e.g. the 2-cell stage in mice, 4-cell stage in pigs, and mid-blastula transition in zebrafish; Jarrell et al., 1991; Schultz, 1993; Tadros and Lipshitz, 2009). The major ZGA and MZT coincide in mouse, but this is not always the case in all vertebrates (Baroux et al., 2008).

In mice, major egg MZT at the 2-cell stage is characterized by the down-regulation of *RB1* mRNA and maternally expressed proteins, and the absence of the RB1 protein lasts to the morula stage, indicating that the lack of the RB1 protein induces the constitutive activation of E2F in order to start DNA synthesis during these stages (Moore et al., 1996; Iwamori et al., 2002; Egashira et al., 2011). Recent studies on humans, mice, and zebrafish indicated that maternally expressed factors, such as *Nanog*, *Pou5f1*, *SoxB1*, and the *Dux* family, are indispensable for triggering ZGA; however, the potential relevance of these factors and the transcripts up-regulated by these factors for the degradation of RB1 remain unclear (Lee et al., 2013; De laco et al., 2017). Although it remains possible that other unknown factors are also involved in causing RB1 degradation at MZT, at least CCND does not contribute to the phenomenon, because embryo development of *CCND*-knockout mice proceeded past MZT stage (Kozar et al., 2000; Malumbres et al., 2004).

In avian species, RNA sequencing analyses using chicken embryo were recently performed, and the findings obtained revealed 2 waves of ZGA (Hwang et al., 2018a-c; Rengaraj et al., 2020). The first minor wave began at the one-cell stage and only the maternal genome was activated in the zygote. A second major wave then mainly occurred at the blastoderm stage between III and IV (Eyal-Giladi and Kochav, 1976). Previous studies reported the onset of gene activation during early blastoderm stage in quail (Olszańska et al., 1984, Nagai et al., 2015); however, its timing and the molecular mechanisms regulating MZT have not yet been elucidated in this species. To obtain a comprehensive understanding of the developmental control system within avian species, further studies to examine features gained using gene-manipulated birds in addition to *de novo* transcriptional analyses are needed.

In mammals, gene knockout animals are a powerful tool for elucidating the roles of numerous genes in development (Guan et al., 2010). In addition, recent programmable genome editing tools, including transcription activator-like effector nuclease (TALEN) and clustered regulatory interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) technologies

have been successfully adopted into fertilizing egg to establish genome-edited mice in one generation (Mashiko et al., 2013; Mashimo et al., 2013). In birds, TALEN as well as CRISPR/Cas9 systems have also been successfully adopted to edit the genome of cultured chicken primordial germ cells (cPGCs), which is a precursor cell of the gamete, and genome-edited chicken lines have been established by the transplantation of mutant cPGCs into recipient embryos (Park et al., 2014; Oishi et al., 2016; Taylor et al., 2017). Although this method demonstrates the high efficiency of germline chimerism, it is limited by the difficulties associated with the *in vitro* propagation of female PGCs in culture is difficult for unknown reasons (sex chromosome: ZW; van de Lavoir et al., 2006; Naito et al., 2015). The production of gene-edited mice or other animals by the injection of TALEN or CRISPR/Cas9 components into the egg cytoplasm or pronucleus as early as the one-cell stage combined with the intracytoplasmic injection of sperm (ICSI) or haploid cells is more efficient than cell culture–based strategies (Hwang *et al*. 2013; Li *et al*. 2013; Wang *et al*. 2013; Hai *et al*. 2014; Niu *et al*. 2014; Suzuki *et al*. 2014; Bai *et al*. 2016); however, this has not yet been applied to birds.

We previously established a ICSI method in quail by demonstrating that quail eggs injected with a single sperm and mixture of novel avian egg-activating factors (phospholipase Czeta1: *PLCZ1*, citrate synthase: *CS*, and aconitate hydratase: *AH*) were fertilized and completed full-term development to hatching (Mizushima et al., 2014). This technique may be applied to produce genome-edited quail embryos through the introduction of the CRISPR/Cas9 system into fertilizing eggs. The greatest advantage of the ICSI technique with a single sperm is that only one pair of male and female pronuclei is formed, thereby avoiding the pronuclear formation of supernumerary sperm, as observed in avian physiological polyspermic fertilization (Mizushima, 2017). Thus, ICSI with a single sperm may reduce the risk of these supernumerary sperm interfering with the translocation of the complex of sgRNA-Cas9 with the nuclear localization signal into the female pronucleus and principal male pronucleus, which fuses with the female pronucleus or zygotic nucleus. In the present study, we demonstrated that MZT occurred at blastoderm stage V with the degradation of the maternal RB1 protein in the Japanese quail. In addition, a microinjection of sgRNA-targeted *CCND1* and *Cas9* mRNA into ICSI-generated fertilized eggs prevented development beyond stage V and RB1 degradation, suggesting that CCND1 is one of the key regulators involved in ZGA via the down-regulation of the RB1 protein.

2. Materials and Methods

2.1. Animals and egg collection

Male and female Japanese quail, *Coturnix japonica*, 8-20 weeks of age (Motoki Corporation), were maintained individually under a photoperiod of 14L:10D (lights went on at 05:00) with *ad libitum* access to water and a commercial diet (Muroran Uzuraen). All experimental procedures for the care and use of animals were approved by the Animal Care and Use Committee of Hokkaido University (Approval number 14-0135).

To obtain developing blastoderms of different stages, quail were killed at different times after ovipositioning, namely, 20 min and 3, 5, 8, 12, 14, 20, and 23 hr, and *in vivo* fertilized eggs were retrieved surgically form the magnum or uterine part. The developmental stages of collected egg were determined according to Eyal-Giladi and Kochav (1976) under a stereomicroscope (M165 FC, Leica Corp.), and these samples were analyzed by RT-PCR and Western blotting as described later. Unfertilized eggs were recovered from the anterior of the magnum within 2 hr of ovipositioning (Mizushima et al., 2014), and subjected to ICSI and subsequent CRISPR/Cas9 or CCND1 overexpression treatments.

2.2. *Ex vivo* culture of one-cell stage eggs and α-amanitin treatment

All *ex vivo* culture procedures of one-cell stage eggs were performed as described by Mizushima et al. (2014). After the collection of eggs from the hen oviducts, one-cell stage eggs were cultured in Dulbecco's modified Eagle's medium (DMEM) in plastic cups at 41.5°C for 24 hr in an atmosphere containing 5% CO_2 (Ono et al., 1994). To investigate the effects of a specific inhibitor of RNA polymerase on blastoderm development, one-cell stage eggs were incubated in medium containing 24 μg/ml α-amanitin during the *ex vivo* culture (Moore et al., 1996).

2.3. cDNA cloning of *RB1* and *CCND1* and RT-PCR analysis of *RB1*, *CCND1*, *CDK4*, and *E2F1* mRNAs

The quail germinal disc was dissected from the blastoderm stage X-egg and total RNA was extracted using TRIzol^{TM} reagent (Invitrogen, CA, USA). Total RNA (0.1 μg) was reverse transcribed (RT) with oligo $(dT)_{12\text{-}18}$ primers using the Reva-Tra Ace kit (Toyobo Bio.) according to the manufacturer's protocol. Based on the sequence of chicken *RB1* (Accession No. NM_204419.1) and chicken *CCND1* (Accession No. NM_205381), specific primer sets for *RB1* and *CCND1* were synthesized and subjected to first PCR (Suppl. Table S1). The full-length sequences encoding quail *RB1* and *CCND1* were obtained using the 5′ and 3′ RACE kits (Invitrogen) according to the manufacturer's protocol. PCR products were cloned into the pGEM-T Easy vector (Promega) and

the nucleotide sequences of *RB1* and *CCND1* cDNA were elucidated using an ABI Prism 3100 genetic analyzer (Applied Biosystems Japan ltd.).

To investigate the mRNA expression of cyclin-related genes at different stages of the blastoderm, total RNA from the germinal discs of each stage was transcribed using a ReverTra Ace kit after DNA was digested by DNase I (Takara Biomedicals). PCR amplification was performed using specific primer sets (Suppl. Table S1).

2.4. Western blotting

The quail germinal discs were dissected from oviductal blastoderm stage-eggs or *ex vivo* cultured eggs as described above. Each germinal disc was homogenized, sonicated in intracellular-like medium (ICM: 120 mM KCl, 0.1 mM EGTA, 10 mM Na-β-glycerophosphate, 0.2 mM PMSF, 1 mM DTT, and 20 mM HEPES-NaOH, pH 7.5), vortexed for 30 min, and the supernatant was collected by centrifugation at 10,000 × *g* for 10 min. Protein concentrations were measured by the BCA protein assay kit (Wako). Each extract (20 μg protein per lane) was resolved by SDS-PAGE (Laemmli, 1970) on a 10% polyacrylamide gel and transferred on the PVDF membrane (Millipore). The membrane was incubated with a detector block (SeraCare Life Sciences) for 30 min, reacted with an anti-human RB1 polyclonal antibody (Santa Cruz Biotechnology), anti-human CCND1 polyclonal antibody (BD Pharmingen), anti-mouse CDK4 polyclonal antibody (Santa Cruz Biotechnology), anti-human E2F1 polyclonal antibody (GeneTex), or anti-chicken γ-tubulin monoclonal antibody (Sigma-Aldrich), and then visualized using a chemiluminescence agent (Millipore) with HRP-conjugated anti-mouse or rabbit IgG (Santa Cruz Biotechnology). In order to evaluate the phosphorylation status of the RB1 protein, immunoprecipitation using an anti-RB1 antibody and Protein A Sepharose (GE Healthcare) was performed prior to Western blotting. After SDS-PAGE and subsequent transfer onto the PVDF membrane, the membrane was incubated with an anti-phosphoserine/threonine antibody (Sigma-Aldrich).

2.5. Construction of sgRNA of the CRISPR/Cas9 system

To construct the sgRNA and *Cas9* mRNA expression plasmid, sgRNA or *Cas9* sequences in pX330 were placed downstream of the T7 promotor sequences in the pUC19 plasmid (Takara) or pTNT plasmid (Promega), respectively. Two pairs of the targeting site close to the initiation codon and in 3' untranslated region (3'-UTR) of *CCND1* gene, respectively, were designed to create sgRNA using CRISPOR program (http://crispor.tefor.net/crispor.py) (Suppl. Fig. S1). Complementary oligonucleotides corresponding to the target sequence of *CCND1* were inserted into

the BbsI site of the pUC19 plasmid containing sgRNA sequences, and plasmids were subjected to RNA synthesis using a Ribomax RNA synthesis system (Promega) (*CCND1* sgRNA). The sequences of the resulting constructs were verified by DNA sequencing with the ABI 3100 Genetic Analyzer. The PCR products of partial exon 1 and 5 were used to evaluate target cleavage efficiency of sgRNA using the Guide-it sgRNA screening system (Takara Biomedicals). Primers for PCR amplification are shown in Suppl. Table S1.

2.6. Production of CCND1 cRNA

Quail *CCND1* products amplified with T7 promoter sequences from the pGEM-T easy vector using the primer set (sense: 5′ - GTC TCG AGT AAT ACG ACT CAC TAT AGG G - 3′; antisense: 5′ - GTG GAT CCC GAA TGT TCA CAT CTC GCA - 3′) were subcloned into the pTagGFP2-N vector (EVROGEN)(pTagGFP2-N-CCND1). Quail *CCND1*-EGFP cRNA was then synthesized as described above after the linearization of pTarGFP2-N-CCND1 by digesting with the *Afl*2 restriction enzyme.

2.7. ICSI and cRNA injection

In order to reenact avian-specific egg-activating signaling by polyspermy, *PLCZ1* (60 ng/µl), *CS* (100 ng/µl), and *AH* (100 ng/µl) cRNA were microinjected with a single sperm into the unfertilized egg collected from the oviduct, as described by Mizushima et al. (2014). Briefly, 1 nl of the cRNA mixture was drawn into an injection micropipette, followed by a single ejaculated spermatozoon in the same micropipette, and they were then injected into the center of a germinal disc. Each egg was cultured in DMEM in a plastic cup at 41.5 °C in an atmosphere containing 5% CO_2. After a 2-hr incubation, 1 nl of the RNA mixture of sgRNA (50 ng/µl) and *Cas9* mRNA (50 ng/µl) or 1 nl of *CCND1*-EGFP cRNA (50 ng/µl) was microinjected and the egg was subsequently cultured for 22-24 hr. The stages of blastoderm development were classified according to Eyal-Giladi and Kochav (1976) under a stereomicroscope.

2.8. Genomic DNA sequence analysis

Genomic DNA was extracted from the blastoderm and genomic DNA fragments containing the sgRNA target sequence were amplified by PCR using specific primers for exon 1 (sense: 5′ - TAG CAG CAG CAG CAT CCA AG - 3′; antisense: 5′ - TCC AGC ATC CAA GTG GCG AC - 3′) and for exon 5 (sense: 5′ - GGA TTG CCT GTG CAT TTG TA - 3′; antisense: 5′ - ATA TCT AAT GGA CTG AAA GT - 3′), and the PCR products were sequenced with the ABI 3100 Genetic Analyzer.

2.9. Immunocytochemistry

To observe the localization of RB1 and CCND1 in the blastoderm, isolated blastoderms were fixed in 4% paraformaldehyde fixative, embedded in Paraplast (Oxford Labware, St. Louis, MO, USA), and sectioned at a thickness of 10 μm. Sections were deparaffinized, rehydrated in graded ethanols, and then autoclaved at 105°C for 5 min in 10 mM citric acid buffer. After blocking with Phosphate Buffer Saline (PBS) containing 10% normal goat serum, 1% BSA, and 0.1% (w/v) Triton X-100 for 30 min, the sections were incubated with a mixture of primary antibodies (anti-RB1 antibody (1:250) and the anti-CCND1 antibody (1:250) or normal rabbit and mouse IgG (MBL) at 4°C overnight and were washed three times in PBS. The specimen was visualized after the incubation with the mixture of secondary antibodies containing goat anti-rabbit IgG labeled with the fluorophore Alexa 488 (Abcam) and goat anti-mouse IgG labeled with the fluorophore Alexa 546 (Abcam). After wash three times in PBS and subsequent staining with Hoechst 33342 (Dojindo), the immunolabeled sections were finally mounted in glycerol and examined under a fluorescence microscope (E800; Nikon).

3. Results

3.1. Effects of α-amanitin on blastoderm development

To investigate the timing of ZGA in the developing embryo, one-cell stage eggs were incubated with α-amanitin, an inhibitor for transcription, in the *ex vivo* culture. Although all zygotes in the absence of the inhibitor developed over blastoderm stage IX, the α-amanitin treatment completely blocked development at blastoderm stage V (Table 1).

3.2. G1-related gene expression during blastoderm development

The messenger RNA and protein expression of *RB1*, *CCND1*, *CDK4*, and *E2F1* were examined during blastoderm development (Fig. 1). *RB1* mRNA was detected at all stages examined; however, RB1 proteins disappeared between stages VI and VIII. In contrast, *CCND1* mRNA and protein expression initially appeared at blastoderm stage V and continued to be detected in subsequent stages. *CDK4* and *E2F1* gene expression persisted during the fertilizing stage to blastoderm development.

The immunohistochemical analysis of sections of blastoderm stages IV, V, and VI showed that immunoreactive RB1 proteins mainly localized to the cytoplsm of blastomeres in stage IV and to nuclei in stage V; however, these signals were not detected in stage VI (Fig. 2M, O, Q, Y, a). In accordance with the results of Western blotting, CCND1 proteins were not detected at stage IV, but were observed at stages V and VI (Fig. 2S, U, W). As an additional finding, the CCND1 protein, which accumulated in the nuclei of blastomeres at stage V and colocalized with RB1protein, translocated mainly to the cytoplasm near plasma membrane at stage VI (Fig.2a, c).

3.3. Role of CCND1 in blastoderm development

To investigate whether CCND1 is essential for blastoderm development beyond stage V, *CCND1* sgRNA and *Cas9* mRNA were microinjected into the pronuclear stage of eggs. As shown in Suppl. Fig. S2, since designed sgRNA all exhibited cleavage activity in *in vitro* sgRNA/Cas9-mediated cleavage assays, CCND1-33 and CCND1-U1 sgRNAs were selected from protein-coding regions and 3'-UTR, respectively, and used for microinjection in the present study. The microinjection of Cas9 mRNA and sgRNA, which did not contain the *CCND1* target sequence, did not inhibit normal development, whereas the microinjection of *Cas9* mRNA along with *CCND1* sgRNA-33 prevented development beyond blastoderm stage V in the majority of blastoderms (Table 2). Further sequencing analyses of stage V-arrested blastoderms revealed the indels of *CCND1* sequences in all blastoderms, with homozygous 2-bp indels in *CCND1* alleles in 7 blastoderms and compound homomosaicity with a single indel and 2-bp indels in the remaining one blastoderm (Fig. 3A). In contrast, the three other blastoderms in 11 microinjected samples developed to stages VI-VII; however, all of them carried alleles of the wild type and 2-indel mutations.

On the other hand, the microinjection of Cas9 mRNA and sgRNA-U1, which was designed in 3'-UTR of the *CCND1* locus, did not inhibit normal development (Table 2). Sequencing analyses revealed indels in the targeted sequences of all developing blastoderms, with homozygous 4-bp indels in *CCND1* alleles in 4 blastoderms and two different homozygous single indel mutations in the remaining 3 blastoderms (Fig. 3A).

When these samples injected with *CCND1* sgRNA-33 were subjected to Western blotting, we failed to detect CCND1 proteins, whereas intact RB1 proteins were retained despite a 24-hr *in vitro* culture (Fig. 3B), which is in contrast to control blastoderm stage VI after a 14-hr culture. In addition, the retention of RB1 proteins in these blastoderms and no change in the cytoplasmic localization of the RB1 protein were confirmed by an immunohistochemical analysis (Fig. 4O). On the other hand, when these samples injected with CCND1 sgRNA-U1, changes in the expression and

the localization of the RB1 and CCND1 proteins in stage VI were similar to those in the control blastoderm (Fig. 3B and 4M, Q, S, W).

To evaluate the off-target effects of CRISPR in quail, in the present study, we searched for potential off-target sites that exactly match 10-13 bases followed by 5'-NGG-3' with web tools, such as PrimerBLAST and NCBI's BLAST. Three candidates (SAM and HD domains containing deoxynucleoside triphosphate triphosphohydrolase 1(SAMHD1), protein O-fucosyltransferase 2 (POFUT2), and potassium voltage-gated channel subfamily C member 4 (KCNC4)) were selected because they carried sequences that matched the sgRNA-33 sequences. In addition to these genes, 3'-UTR of the *CCND1* locus was analyzed because the off-target DNA edit were occurred in the 3' UTR region in human cells by the CRISPR/Cas9 system (Grünewald et al., 2019). We amplified and sequenced the 500-2,000-bp regions containing potential off-target sites, but did not detect any mutations in the blastoderms examined (data not shown).

3.4. Role of CCND1 in RB1 protein degradation

To elucidate the direct effects of CCND1 on RB1 degradation, *CCND1*-EGFP or EGFP cRNA was microinjected into pronuclear stage of zygotes that did not express *CCND1*. We confirmed the expression of CCND1 proteins after the injection of *CCND1*-EGFP cRNA (Fig. 5). The Western blot analysis revealed the fragmentation of RB1 proteins 30 min after the *CCND1*-EGFP cRNA injection and the complete degradation of these proteins was noted 1 hr after the injection (Fig. 5B). The anti-phosphoserine/threonine antibody detected a strong signal in RB1 proteins immunoprecipitated from the germinal disc 15 min after the *CCND1*-EGFP cRNA injection (Fig. 5C). These results indicate that CCND1 phosphorylated the RB1 protein, which resulted in its degradation. Furthermore, the microinjection of *CCND1*-EGFP cRNA completely prevented development, whereas EGFP cRNA did not disturb development during blastoderm stages (Fig. 6). This result suggests that initial blastoderm development is independent of the G1/S transition regulated by E2F.

4. Discussion

We herein report the results of the first detailed study on CCND1 during avian blastoderm development. In mice, embryo development proceeds no further than second mitosis without the down-regulation of the RB1 protein (Iwamori et al., 2002). Although mouse major ZGA occurs at the two-cell stage (Schultz, 1993; Wiekowski et al., 1993; Lee et al., 2014), the gene expression of

CCND in mammals has not yet been studied in detail during preimplantation embryo development. However, neither double-knockout mice of *CDK4* and *CDK6* nor triple-knockout mice of *CCND1*, *CCND2*, and *CCND3* had abnormal embryos during preimplantation, indicating that CCNDs and CDK4/6 are not responsible for the down-regulation of RB1 (Kozar et al., 2000; Malumbres et al., 2004). This assumption is also supported by the finding showing that the embryo developed to the blastocyst stage after the overexpression of $p16^{INK4a}$, a potent endogenous suppressor of the CDK4/6-CCND complex (Egashira et al., 2011).

In contrast to mammals, the present results showed that *CCND1*-genome edited quail blastoderms were completely arrested at stage V, indicating that *CCND1* genes are key regulators of embryonic development beyond stage V in quail. It is important to note that the quail RB1 protein was down-regulated after development past stage V; however, this down-regulation did not occur when the *CCND1* gene was disrupted by genome editing. Furthermore, the onset of *CCND1* gene expression was detected at stage V, which is consistent with the timing of RB1 protein down-regulation. In addition to these observations, the colocalization of CCND1 with RB1 at stage V suggests that CCND1 contributed to decreases in RB1 protein levels, which is markedly different from mammals. Developmental arrest and the lack of *CCND1* mRNA expression at stage V after the α-amanitin treatment supports the hypothesis that *CCND1* is one of the α-amanitin-sensitive ZGA genes in the quail (Suppl. Fig. S3). *CCND1* gene expression is generally triggered by external factors in somatic cells; however, quail fertilizing eggs complete blastoderm development without any external stimulus (Mizushima et al., 2014). Although no additional information is available on internal factors that elicit *CCND1* gene expression, maternal factors are a good candidate for the Japanese quail blastoderm.

We overexpressed CCND1 in one-cell stage eggs, which do not express endogenous *CCND1* mRNA or the protein, in order to clarify whether CCND1 triggers the degradation of RB1 proteins. The degradation of RB1 proteins was observed 30 min after the microinjection of *CCND1*-EGFP cRNA, and RB1 proteins were completely degraded 1 hr after the microinjection. More importantly, the serine and/or threonine residues of immunoprecipitated RB1 proteins 15 min after the microinjection were phosphorylated, indicating that the phosphorylation of RB1 proteins precedes their degradation. A previous study reported that serine/threonine residues in human RB1 phosphorylated by the CCND1-CDK4 complex were highly conserved in the quail (Suppl. Fig. S4; Zarkowska and Mittnacht, 1997). Taken together with the expression of CDK4 throughout the blastoderm stage, the CCND1-CDK4 complex may be involved in the phosphorylation of RB1 in the quail. On the other hand, many experiments on human epithelial cells and tumor cells suggest that

the ubiquitin-proteasome system plays a direct role in RB1 protein degradation (Boyer et al., 1996; Wang et al., 2001; Sengupta and Henry, 2015). Further studies on the possible involvement of the ubiquitin-proteasome system in quail RB1 degradation are warranted.

Despite the presence of *RB1* mRNA throughout blastoderm development, the loss of RB1 protein expression was observed between stages VI and VIII and subsequently reappeared by stage X. A similar phenomenon was observed in the mouse embryo in which the absence of RB1 proteins from the two-cell stage lasted to the morula stage (Iwamori et al., 2002; Egashira et al., 2011). In vertebrate embryos, the G1 phase is markedly shortened, and DNA synthesis is initiated soon after the M-phase for the production of molecules required for the G1/S transition (Smith and Johnson, 1986; Moore et al., 1996). In somatic cells, the expression of the molecules required for the G1/S transition is regulated by E2F, which is generally inactivated by RB1 binding (Nevins, 1992; Dyson, 1998). Collectively, the present results and previous findings indicate that the degradation of RB1 in stages VI and VIII induces the constitutive activation of E2F1 and, thus, may be required for subsequent embryo development past stage V. Although it currently remains unknown whether the *de novo* transcription of *E2F1* and *RB1* mRNA occurs after stage V due to their continuous expression throughout the blastoderm stage, maternal E2F1 proteins dissociated from RB1 proteins by the effects of CCND1 may initiate E2F1-dependent transcription at stage V.

The reappearance of the RB1 protein in the normal stage X-egg in the present study suggests blastoderm-specific cell cycle changes into the somatic cell cycle at this developmental stage. Chicken and quail blastoderm formation is completed in the oviduct of the mother hen at approximately 41°C; however, after being expelled from the oviduct, this temperature changes to approximately 37°C for the resumption of subsequent embryo development in their own shell. To guarantee the fidelity of cell division, checkpoint systems that monitor the replication process were shown to be activated in mammalian cells (Elledge, 1996), and RB is suggested to be involved in cellular checkpoint signaling (Hartwell and Welnert, 1989; Flatt et al., 2000), indicating that similar checkpoint events occur in the quail egg at the timing of the blastodermal-to-embryonal developmental transition. More detailed studies are needed to establish whether these observations involve checkpoint activation and cell cycle progression.

On the other hand, the microinjection of *CCND1*-EGFP cRNA into one-cell stage eggs inhibited initial development, which suggests that zygotic gene expression induced by activated E2F1 disordered the original signal cascade toward initial mitosis. In other words, this developmental arrest means that the functions of E2F1 proteins were repressed by RB1 protein binding until blastoderm stage V. In addition, α-amanitin-treated eggs survived and developed to blastoderm stage

V, indicating that early embryos contain intrinsic molecules that promote early mitosis. Large amounts of maternal mRNAs and proteins accumulate in vertebrate ovulated eggs. These molecules themselves are considered to regulate the G1/S transition of embryonic development before ZGA as well as ZGA itself (Schultz, 1993; Li et al., 2010) and many maternal factors that control cell-cycle progression in vertebrate embryos have been identified (Newport and Kirschner, 1982; Tong et al., 2000; Howell et al., 2001; Gurtu et al., 2002; Minami et al., 2003; Burns et al., 2003; Payer et al., 2003; Wu et al., 2003; Ramos et al., 2004; Lee et al., 2013 and 2014). Further studies are needed to identify the maternal key regulators for early blastoderm development in birds.

To the best of our knowledge, this is the first study to demonstrate effective gene disruption in the quail zygote by a co-injection of sgRNA and *Cas9* mRNA. Eight out of 11 microinjected blastoderms carried the biallelic mutation at the protein coding region of the *CCND1* locus, while seven homozygous mutants carried the same mutations in alleles, suggesting that quail carrying homozygous mutations may be generated in one generation. Furthermore, these homozygous mutations at 3'-UTR of the *CCND1* locus were identified in all blastoderms. Since the conventional PGC gene targeting strategy requires at least 1 year to obtain male heterozygote maturation in chickens (6 months for chimera maturation and 6 months for germ line transmission), the current approach to one-cell stage eggs significantly shortens the period needed for the production of mutants in birds.

In summary, the present results suggest that *CCND1* is an important ZGA gene during MZT occurring at blastoderm stage V in the Japanese quail. However, *de novo* transcriptional analysis has not been studied in this experiment. Based on detailed information from chickens and other animals, minor ZGA may also occur in the early development in quail. Further characterization is required in future studies to elucidate the molecular mechanisms of minor and major ZGA. The present study also provides important indications that not only the down-regulation of RB1 is required to activate E2F for subsequent development after stage V, but also that CCND1 initiates the phosphorylation of RB1 at stage V for its degradation. Moreover, the CRISPR/Cas9 system in the pronuclear stage egg generated by ICSI with a single sperm resulted in high mutagenesis, suggesting the potential of the combination of a genome editing technology and ICSI as a powerful tool for avian-targeted mutagenesis.

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References

Aanes, H., Winata, C.L., Lin, C.H., Chen, J.P., Srinivasan, K.G., Lee, S.G., Lim, A.Y., Hajan, H.S., Collas, P., Bourque, G., Gong, Z., Korzh, V., Alestrom, P., and Mathavan, S. (2011). Zebrafish mRNA sequencing deciphers novelties in transcriptome dynamics during maternal to zygotic transition. Genome Res. 21, 1328-1338.

Abe, K., Yamamoto, R., Franke, V., Cao, M., Suzuki, Y., Vlahovicek, K., Svoboda, P., Schultz, R.M. and Aoki, F. (2015). The first murine zygotic transcription is promiscuous and uncoupled from splicing and 4' processing. EMBO J. 34, 1523-1537.

Aoki, F., Worrad, D.M., Schultz, R.M. (1997). Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. Dev Biol. 181, 296-307.

Bai, M., Wu, Y., and Li, J. (2016). Generation and application of mammalian haploid embryonic stem cells. J Intern Med. 280, 236–245.

Barroux, C., Autran, D., Gillmor, C.S., Grimanelli, D., and Grossniklaus, U. (2008). The maternal to zygotic transition in animals and plants. Cold Spring Harb Symp Quant Biol. 73, 89-100.

Beijersbergen, R.L., and Bernards, R. (1996). Cell cycle regulation by the retinoblastoma family of growth inhibitor proteins. Biochim. Biophys. Acta. 1287, 103-120.

Boonstra, J., and Post J.A. (2004). Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. Gene 337, 1-13.

Boyer, S.N., Wazer, D.E. and Band, V. (1996). E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res. 56, 4620-4624.

Buchkovich, K., Duffy, L.A. and Harlow, E. (1989). The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 58, 1097-1105.

Burns, K.H., Viveiros, M.M., Ren, Y., Wang, P., DeMayo, F.J., Frail, D.E., Eppig, J.J., and Matzuk, M.M. (2003). Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos, Science 300, 633-636.

Chen, P.L., Scully, P., Shew, J.Y., Wang, J.Y. and Lee, W.H. (1989). Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 58, 1193-1198.

Christians, E., Davis, A.A., Thomas, S.D., and Benjamin, I.J. (2000). Maternal effect of Hsf1 on reproductive success, Nature 407, 693-694.

De laco, A., Planet, E., Coluccio, A., Verp, S., Duc, J., and Trono, D. (2017). DUX-family of double-

homeodomain transcription factors regulates zygotic genome activation in placental mammals. Nat. Genet. 49, 941-945.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. Genes Dev. 12, 2245–2262.

Egashira, A., Kano, K., and Naito, K. (2011). Pre-implantation-embryo-specific cell cycle regulation is attributable to a low expression of retinoblastoma protein rather than its phosphorylation. J. Reprod. Dev. 57, 492-499.

Elledge, S.J. (1996). Cell cycle checkpoints: Preventing an identity crisis. Science 274:1664-1672.

Eyal-Giladi, H., and Kochav, S. (1976). From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. Dev. Biol. 49, 321-337.

Flatt, P.M., Tang, L.J., Scatena, C.D., Szak, S.T., and Pietenpol, J.A. (2000). P53 regulation of G2 checkpoint is retinoblastoma protein dependent. Mol. Cell. Biol. 20, 4210-4223.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J., and Schier, A.F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science 312, 75-79.

Grossel, M.J. and Hinds, P.W. (2006). From cell cycle to differentiation: an expanding role for cdk6. Cell Cycle 5, 266-270.

Grünewald, J., Zhou, R., Garcia, S.P., Lyer, S., Lareau, C.A., Aryee, M.J. and Joung, K.K. (2019). Transcriptome-wide off-target RNA editing induced by CRISPR-guided DNA base editors. Nature 569, 433-437.

Guan, C., Ye, C., Yang, X., and Gao, J. (2010). A review of current large-scale mouse knockout efforts. Genesis 48, 73-85.

Gurtu, V.E., Verma, S., Grossmann, A.H., Liskay, R.M., Skarnes, W.C., and Baker, S.M. (2002). Maternal effect for DNA mismatch repair in the mouse, Genetics 160, 271-277.

Hai, T., Teng, F., Guo, R.F., Li, W., and Zhou, Q. (2014). One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Res. 24, 372–375.

Harbour, J.W., Luo, R.X., DeiSanti, A., Postigo, A.A. and Dean, D.C. (1999). Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 98, 859-869.

Hartwell, L.H., and Welnert, T.A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science 246, 629-634.

Howell, C.Y., Bestor, T.H., Ding, F., Latham, K.E., Mertineit, C., Trasler, J.M., and Chaillet, J.R. (2001). Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene, Cell 104,

829-838.

Hwang, Y.S., Seo, M., Bang, S., Kim, H., and Han, J.Y. (2018a). Transcriptional and translational dynamics during maternal-to-zygotic transition in early chicken development. FASEB J. 32, 2004-2011.

Hwang, Y.S., Seo, M., Kim, S.K., Bang, S., Kim, H., and Han, J.Y. (2018b). Zygotic gene activation in the chicken occurs in two waves, the first involving only maternally derived genes. Elife 7, e39381.

Hwang, Y.S., Seo, M., Lee, B.R., Lee, H.J., Park, Y.H., Kim, S.K., Lee, H.C., Choi, H.J., Yoon, J., Kim, H., and Han, J.Y. (2018c). The transcriptome of early chicken embryos reveals signaling pathways governing rapid asymmetric cellularization and lineage segregation. Development 145,157453.

Hwang, W.Y., Fu, Y.F., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.R., and Joung, J.K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol. 31, 227–229.

Iwamori, N., Naito, K., Sugiura, K., and Tojo, H. (2002). Preimplantation-embryo-specific cell cycle regulation is attributed to the low expression level of retinoblastoma protein. FEBS Lett. 526, 119-123.

Jarrell, V.L., Day, B.N., and Prather, R.S. (1991). The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig, *Sus scrofa*: quantitative and qualitative aspects of protein synthesis. Biol. Reprod. 44, 62-68.

Kaelin, W.G. Jr. (1997). Recent insights into the functions of the retinoblastoma susceptibility gene product. Cancer Invest. 15, 243-254.

Kozar, K., Ciemerych, M.A., Rebel, V.I., Shigematsu, H., Zagozdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R.T., Akashi, K., and Sicinski, P. (2000). Mouse development and cell proliferation in the absence of D-cyclins. Cell 118, 477-491.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lee, M.T., Bonneau, A.R., and Giraldez, A.J. (2014). Zygotic genome activation during the maternal-to-zygotic transition. Annu. Rev. Cell Dev. Biol. 30, 581-613.

Lee, M.T., Bonneau, A.R., Takacs, C.M., Bazzini, A.A., DiVito, K.R., Fleming, R.S. and Giraldez, A.J. (2013). Nanog, Pou5f1 and SoxB1 activate zygotic gene expression during the maternal-to-zygotic transition. Nature 213, 360-364.

Li, L., Zheng, P., and Dean, J. (2010). Maternal control of early mouse development. Development

137, 859-870.

Li, W., Teng, F., Li, T.D., and Zhou, Q. (2013). Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. Nat Biotechnol. 31, 684–686.

Malumbres, M., Sotillo, R., Santamaría, D., Galán, J., Cerezo, A., Ortega, S., Dubus, P., and Barvacid, M. (2004). Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. Cell 118, 493-504.

Mashiko, D., Fujihara, Y., Satouh, Y., Miyata, H., Isotani, A., and Ikawa, M. (2013). Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. Sci. Rep. 3, 3355.

Mashimo, T., Kaneko, T., Sakuma, T., Kobayashi, J., Kunihioro, Y., Voigt, B., Yamamoto, T., and Serikawa, T. (2013). Efficient gene targeting by TAL effector nucleases coinjected with exonucleases in zygotes. Sci. Rep. 3, 1253.

Mathavan, S., Lee, S.G., Mak, A., Miller, L.D., Murthy, K.R., Govindarajan, K.R., Tong, Y., Wu, Y.L., Lam, S.H., Yang, H., Ruan, Y., Korzh, V., Gong, Z., Liu, E.T., and Lufkin, T. (2005). Transcriptome analysis of zebrafish embryogenesis using microarrays. PLoS Genet. 1, 260-276.

Minami, N., Aizawa, A., Ihara, R., Miyamoto, M., Ohashi, A., and Imai, H. (2003). Oogenesin is a nobel mouse protein expressed in oocytes and early cleavage-stage embryo. Biol. Reprod. 69, 1736-1742.

Mizushima, S. (2017). Fertilization 2: polyspermic fertilization. (Ed T. Sasanami). Springer, New York, In Avian reproduction from behavior to molecules. pp.105-123.

Mizushima, S., Hiyama, G., Shiba, K., Inaba, K., Dohra, H., Ono, T., Shimada, K., and Sasanami, T. (2014). The birth of quail chicks after intracytoplasmic sperm injection. Development 141, 3799-3806.

Moore, G.D., Ayabe, T., Kopf, G.S., and Schultz, R.M. (1996). Temporal patterns of gene expression of G1-S cyclins and cdks during the first and second mitotic cell cycles in mouse embryos. Mol. Reprod. Dev. 45, 264-275.

Nagai, H., Sezaki, M., Kakiguchi, K., Nakaya, Y., Lee, H. C., Ladher, R., Sasanami, T., Han, J. Y., Yonemura, S., and Sheng, G. (2015). Cellular analysis of cleavage-stage chick embryos reveals hidden conservation in vertebrate early development. Development 142, 1279-1286.

Naito, M., Harumi, T., and Kuwana, T. (2015). Long-term culture of chicken primordial germ cells isolated from embryonic blood and production of germline chimaeric chickens. Anim. Reprod. Sci. 153, 50-61.

Nevins, J.R. (1992). E2F: a link between the Rb tumor suppressor protein and viral oncoproteins.

Science 258, 424-429.

Newport, J., and Kirschner, M. (1982). A major developmental transition in early Xenopus embryos: II. Control of the onset of transcription. Cell 30, 687-696.

Niu, Y., Shen, B., Cui, Y., Chen, Y., Wang, J., Wang, L., Kang, Y., Zhao, X., Si, W., Li, W., Xiang, A.P., Zhou, J., Guo, X., Bi, Y., Si, C., Hu, B., Dong, G., Wang, H., Zhou, Z., Li, T., Tan, T., Pu, X., Wang, F., Ji, S., Zhou, Q., Huang, X., Ji, W., and Sha, J. (2014). Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156, 836–843.

Oishi, I., Yoshii, K., Miyahara, D., Kagami, H., and Tagami, T. (2016). Targeted Mutagenesis in Chicken Using CRISPR/Cas9 System. Sci. Rep. 6, 1-10.

Olszańska, B., Kludkiewicz, B., and Lassota, Z. (1984). Transcription and polyadenylation processes during early development of quail embryo. J Embryol Exp Morph. 79, 11-24.

Ono, T., Murakami, T., Mochii, M., Agata, K., Kino, K., Otsuka, K., Ohta, M., Mizutani, M., Yoshida, M., and Eguchi, G. (1994). A complete system for avian transgenesis, supporting quail embryos from the single-cell stage to hatching. Dev. Biol. 161, 126-130.

Pardee, A.B. (1989). G1 events and regulation of cell proliferation. Science 246, 603–608.

Park, S.J., Komata, M., Inoue, F., Yamada, K., Nakai, K., Ohsugi, M., Shirahige, K. (2013). Inferring the choreography of parental genomes during fertilization from ultralarge-scale whole-transcriptome analysis. Genes Dev. 27, 2736-2748.

Park, T.S., Lee, H.J., Kim, K.H., Kim, J.S., and Han, J.Y. (2014). Targeted gene knockout in chickens mediated by TALENs. Proc. Natl. Acad. Sci. U. S. A. 111, 12716-12721.

Payer, B., Saitou, M., Barton, S.C., Thresher, R., Dixon, J.P., Zahn, D., Colledge, W.H., Carlton, M.B., Nakano, T., and Surani, M.A. (2003). Stella is a maternal effect gene required for normal early development in mice, Curr. Biol. 13, 2110-2117.

Ramos, S.B., Stumpo, D.J., Kennington, E.A., Phillips, R.S., Bock, C.B., Ribeiro-Neto, F., and Blackshear, P.J. (2004). The CCCH tandem zincfinger protein Zfp36l2 is crucial for female fertility and early embryonic development. Development 131, 4883-4893.

Rengaraj, D., Hwang, Y.S., Lee, H.C., and Han, J.Y. (2020). Zygotic genome activation in the chicken: a comparative review. Cell Mol Life Sci. 77, 1879-1891.

Schultz, R.M. (1993). Regulation of zygotic gene activation in the mouse. Bioessays 15, 531-538.

Sengupta, S., and Henry, R.W. (2015). Regulation of the retinoblastoma-E2F pathway by the ubiquitin-proteasome system. Biochim. Biophys. Acta 1849, 1289-1297.

Sherr, C.J. (1996). Cancer cell cycles. Science 274, 1672–1677.

Sidle, A., Palaty, C., Dirks, P., Wiggan, O., Kiess, M., Gill R.M., Wong, A.K., and Hamel P.A. (1996). Activity of the retinoblastoma family proteins, pRB, p107, and p130, during cellular proliferation and differentiation. Crit. Rev. Biochem. Mol. Biol. 31, 237-271.

Smith, R.K., and Johnson, M.H. (1986). Analysis of the third and fourth cell cycles of mouse early development. J. Reprod. Fertil. 76, 393-399.

Suzuki, T., Asami, M., and Perry, A.C. (2014). Asymmetric parental genome engineering by Cas9 during mouse meiotic exit. Sci Rep. 4, 7621.

Svoboda, P. (2018). Mammalian zygotic genome activation. Semin Cell Dev Biol. 84, 118-126.

Tadros, W., and Lipshitz, H.D. (2009). The maternal-to-zygotic transition: a play in two acts. Development 136, 3033-3042.

Tashiro, E., Tsuchiya, A. and Imoto, M. (2007). Functions of cyclin D1 as an oncogene and regulation of cyclin D1 expression. Cancer Sci. 98, 629-635.

Taylor, L., Carlson, D.F., Nandi, S., Sherman, A., Fahrenkrug, S.C., and McGrew, M.J. (2017). Efficient TALEN-mediated gene targeting of chicken primordial germ cells. Development 144, 928-934.

Tong, Z.B., Gold, L., Pfeifer, K.E., Dorward, H., Lee, E., Bondy, C.A., Dean, J., and Nelson, L.M. (2000). Mater, a maternal effect gene required for early embryonic development in mice, Nat. Genet. 26, 267-268.

van de Lavoir, M.C., Diamond, J.H., Leighton, P.A., Mather-Love, C., Heyer B.S., Bradshaw, R., Kerchner, A., Hooi, L.T., Gessaro, T.M., Swanberg, S.E., Delany, M.E., and Etches, R.J. (2006). Germline transmission of genetically modified primordial germ cells. Nature 441, 766-769.

Xue, Z., Huang, K., Cai, C., Cai, L., Jiang, C.Y., Feng, Y., Liu, Z., Zeng, Q., Cheng, L., Sun, Y.E., Liu, J.Y., Horvath, S., and Fan, G. (2013). Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature 500, 593-597.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153, 910–918.

Wang, J.Y.J., Knudsen, E.S., and Welch. P.J. (1994). The retinoblastoma tumor suppressor protein. Adv. Cancer Res. 64, 25-85.

Wang, J., Sampath, A., Paychaudhuri, P., and Bagchi, S. (2001). Both Rb and E7 are regulated by the ubiquitin proteasome pathway in HPV-containing cervical tumor cells. Oncogoene 20, 4740-4749.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell 81, 323-330.

Whitten, W.K. (1971). Intrinsic and extrinsic factors in early mammalian development. In Advanced in the bioscience 6 (Edl G. Raspa). Pegamon Press, New Yolk, pp.139-163.

Wiekowski, M., Miranda, M., and DePamphilis, M.L. (1993). Requirements for promoter activity in mouse oocyte and embryos distinguish paternal protenuclei from maternal and zygotic nuclei. Dev. Biol. 159, 366-378.

Wu, X., Viveiros, M.M., Eppig, J.J., Bai, Y., Fitzpatrick, S.L., and Matzuk, M.M. (2003). Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. Nat. Genet. 33, 187-191.

Yan, L., Yang, M., Guo, H., Yang, L., Wu, J., Li, R., Liu, P., Lian, Y., Zheng, X., Yan, J., Huang, J., Li, M., Wu, X., Wen, L., Lao, K., Li, R., Qiao, J., and Tang, F. (2013). Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nat Struct Mol Biol. 20, 1131-1139.

Zarkowska, T., and Mittnacht, S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J. Biol. Chem. 272, 12738-12746.

Figure legends

Figure 1 Expression of G1-phase cell cycle-related genes in developing blastoderms. Quail *RB1*, *CCND1*, CDK4, *E2F1*, and *γ-tubulin* mRNA expression by semiquantitative RT-PCR (A) and protein expression by Western blotting (B) at the indicated stages. A representative gel of three independent experiments was shown.

Figure 2 Immunohistochemical observations using anti-RB1 and anti-CCND1 antibodies in blastoderm stages IV, V, and VI. Blastoderms cultured for 8 hr (A, B, G, H, M, N, S, T, Y, Z), 12 hr (C, D, I, J, O, P, U, V, a, b), and 14 hr (E, F, K, L, Q, R, W, X, c, d) were incubated with anti-RB1 (M, O, Q) and anti-CCND1 antibodies (S, U, W) or normal rabbit IgG (N, P, R) and normal mouse IgG (T, V, X). Antibodies were visualized with anti-rabbit or mouse-IgG antibodies conjugated with Alexa Fluor-488 or 546, respectively. Nuclei were counterstained with Hoechst 33342 (G-L), and blue, green and red fluorescence images were merged (Y-d). Bright field microscopic observations were shown in A-F. Bar=30 μm.

Figure 3 CRISPR/Cas9-mediated *CCND1* mutations in the quail. (A) Sequence analysis of *CCND1* sgRNA-induced mutations in a blastoderm. The sgRNA-targeted locus and PAM sequences are represented in red and blue bold letters, respectively. (B) Detection of CCND1 and RB1 proteins in the blastoderm of an egg microinjected with *CCND1* sgRNA or control blastoderm stage V-VI after a culture for 14 hr.

Figure 4 Immunohistochemical observations in *CCND1* mutant blastoderms.
Control blastoderm stage VI (A, B, G, H, M, N, S, T), *CCND1* sgRNA-33-induced stage V (C, D, I, J, O, P, U, V) or *CCND1* sgRNA-U1-induced stage VI (E, F, K, L, Q, R, W, X) were incubated with anti-RB1 (M, O, Q) and anti-CCND1 antibodies (S, U, W) or normal rabbit IgG (N, P, R) and normal mouse IgG (T, V, X). Nuclei were counterstained with Hoechst 33342 (G-L), and bright field microscopic observations were shown in A-F. Bar=30 μm.

Figure 5 CCND1 mediates the phosphorylation of the RB1 protein and its subsequent degradation. (A) GFP or GFP-fused CCND1 expression in germinal discs 10 min after the microinjection of EGFP or *CCND1*-EGFP cRNA. (B) Representative example of RB1 degradation in the one-cell stage egg 0.5 and 1 hr after the microinjection of *CCND1*-EGFP cRNA. (C)

Immunoblotting analysis using an anti-phosphoserine/threonine antibody. Proteins were extracted from the germinal disc of one-cell stage eggs 10 min after the microinjection of EGFP or *CCND1*-EGFP cRNA and were subsequently immunoprecipitated using an anti-RB1 rabbit antibody.

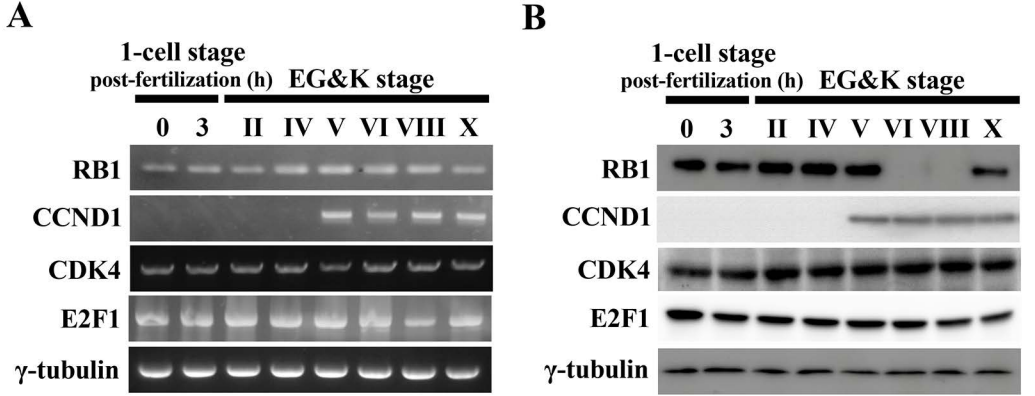
Figure 6　Effects of CCND1 cRNA in one-cell stage eggs on blastoderm development. Blastoderm stage X (A-C) and unfertilized eggs (D-F) 24 hr after the microinjection of EGFP or *CCND1*-EGFP cRNA. GFP expression was examined under fluorescent stereomicroscopy (B, E) and shown with a bright field (A, D). After stereoscopic observations, the germinal disc was cut out and stained with DAPI (C, F). Bar=1 mm (A, D) and 50 μm (C, F).

Supplementary Figure S1　Schematic representation of sgRNA targeting the *CCND1* locus. Exon-intron organization and the DNA sequences corresponding region is indicated by the red arrow. The sgRNA targeting site is represented by a black bar below the nucleotide sequence. Adjoining protospacer adjacent motif (PAM) sequences are highlighted in the red bar.

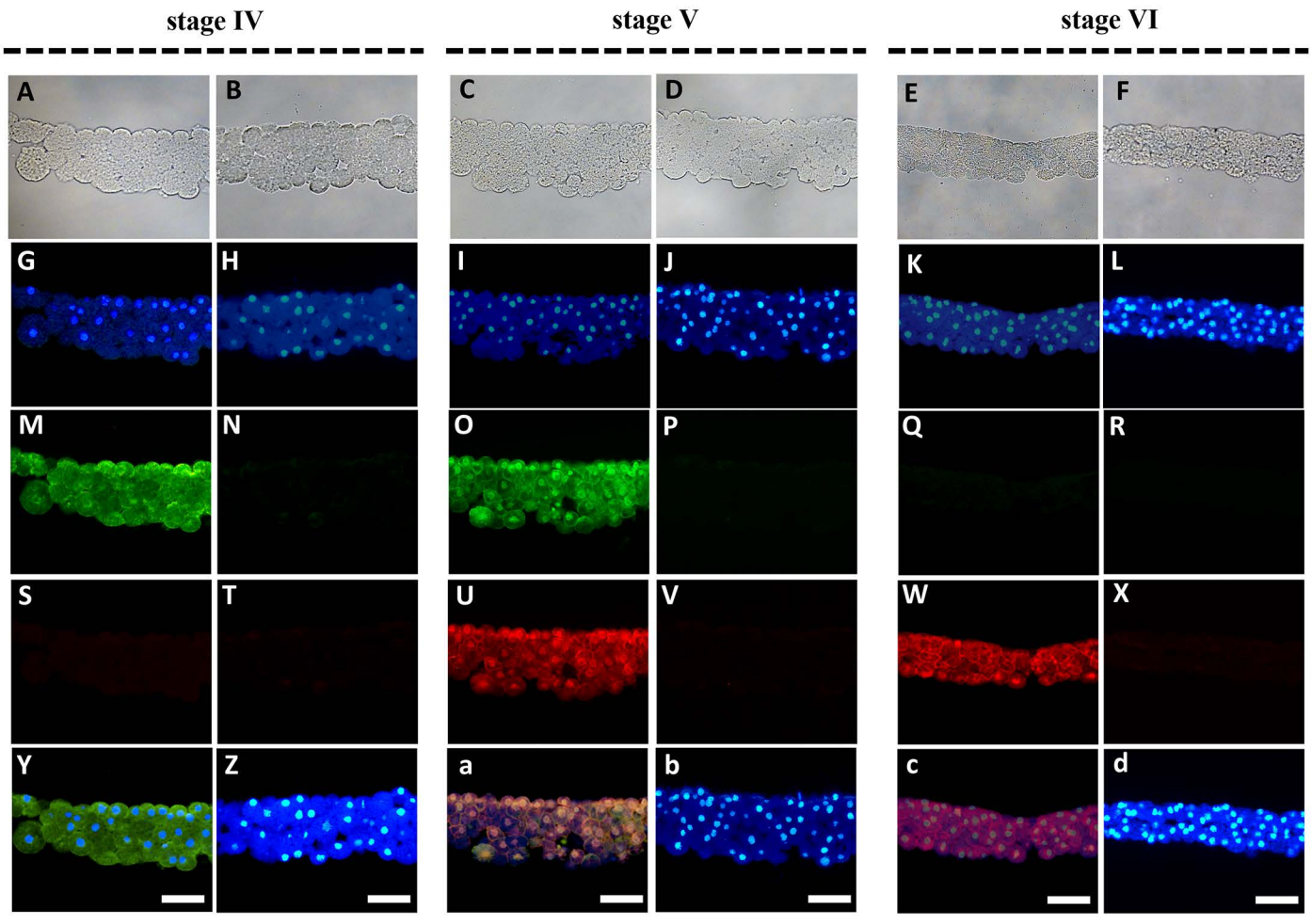
Supplementary Figure S2　sgRNA/Cas9-mediated cleavage assay of four target sites. CCND1-33 or 81, and CCND1-U1 or U2 sgRNA were combined with Cas9 enzyme and added to PCR product that were amplified partial exon 1 region (around 800 bp) or partial exon 5 region (around 900 bp), respectively. Uncleaved PCR product is shown as the largest band. The two smaller bands indicate the cleaved fragments of PCR product for the sgRNA/Cas9 complex.

Supplementary Figure S3　Expression of *CCND1* and *γ-tubulin* mRNA expression in the blastoderm following the α-amanitin treatment. Semiquantitative RT-PCR products were amplified from the total RNA extract from the blastoderm at stage V.

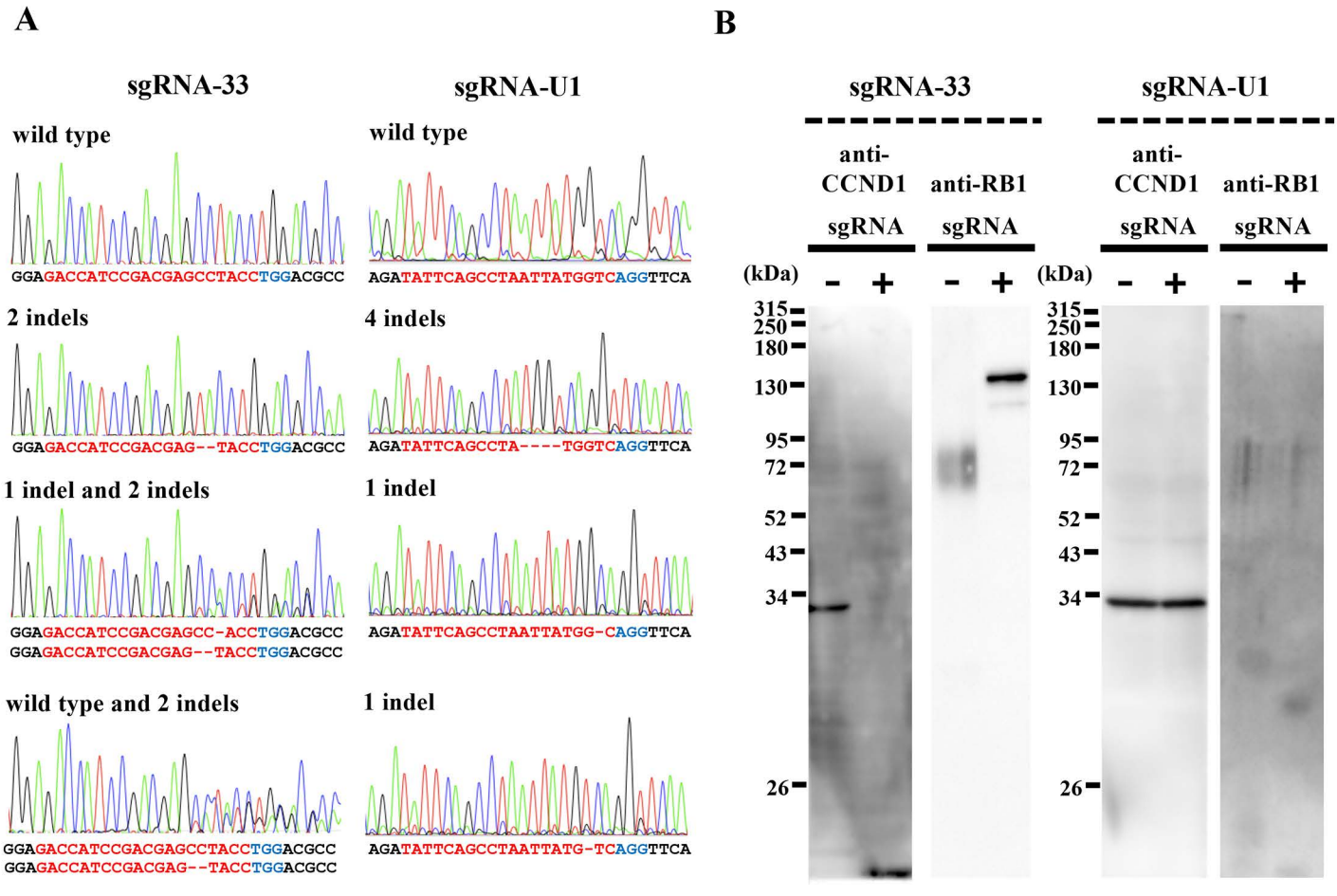
Supplementary Figure S4　Comparison of amino acid sequences of RB1 in a quail, chicken, mouse, and human. Underlines and dotted lines indicate CCND- and E2F-binding domains, respectively. Squares indicate the serine and threonine residues of human RB1 phosphorylated by the CDK4-CCND1 complex.



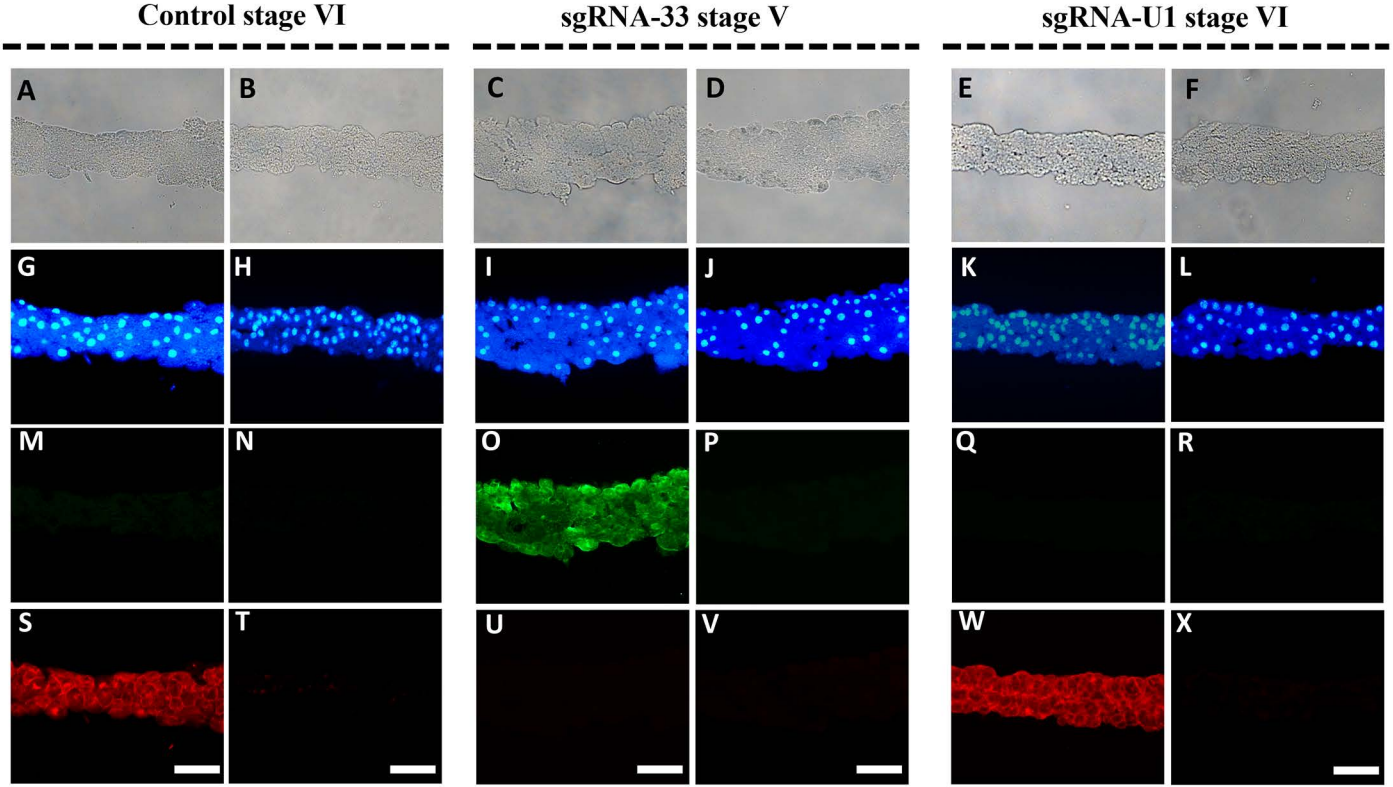
Mizsuhima et al. Figure 1



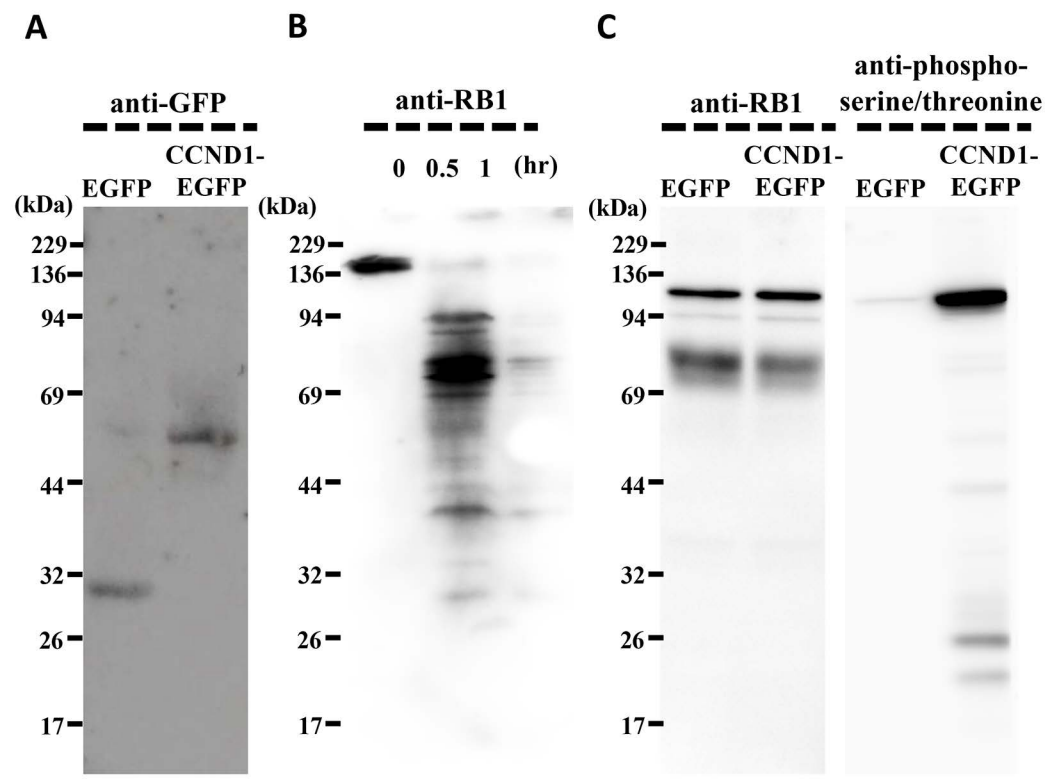
Mizushima et al. Figure 2



Mizsuhima et al. Figure 3

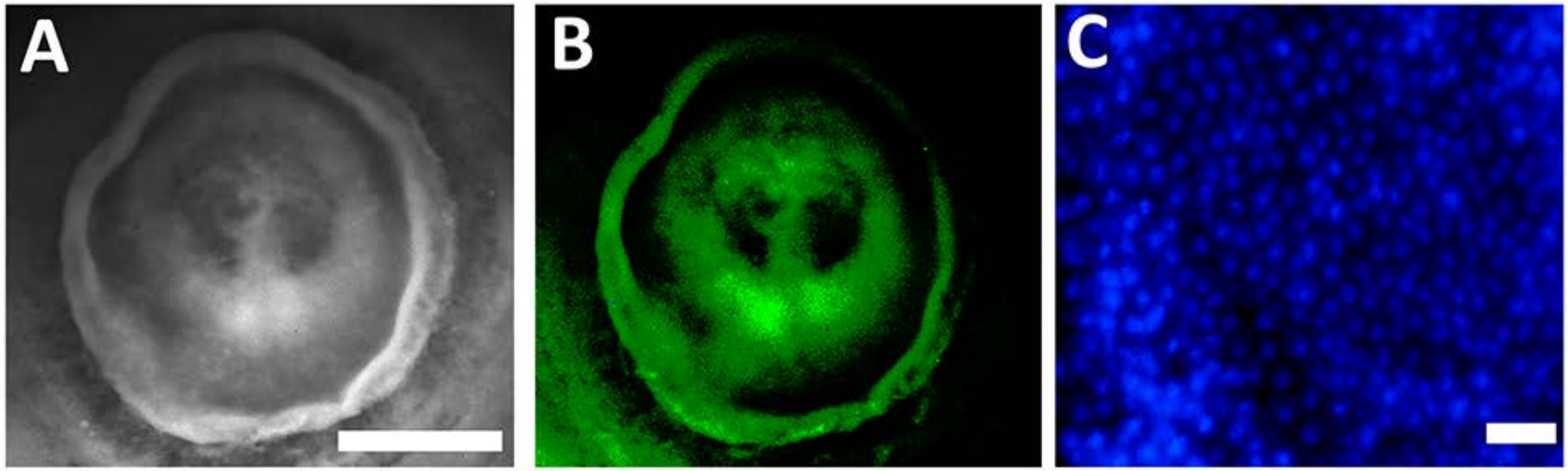


Mizushima et al. Figure 4

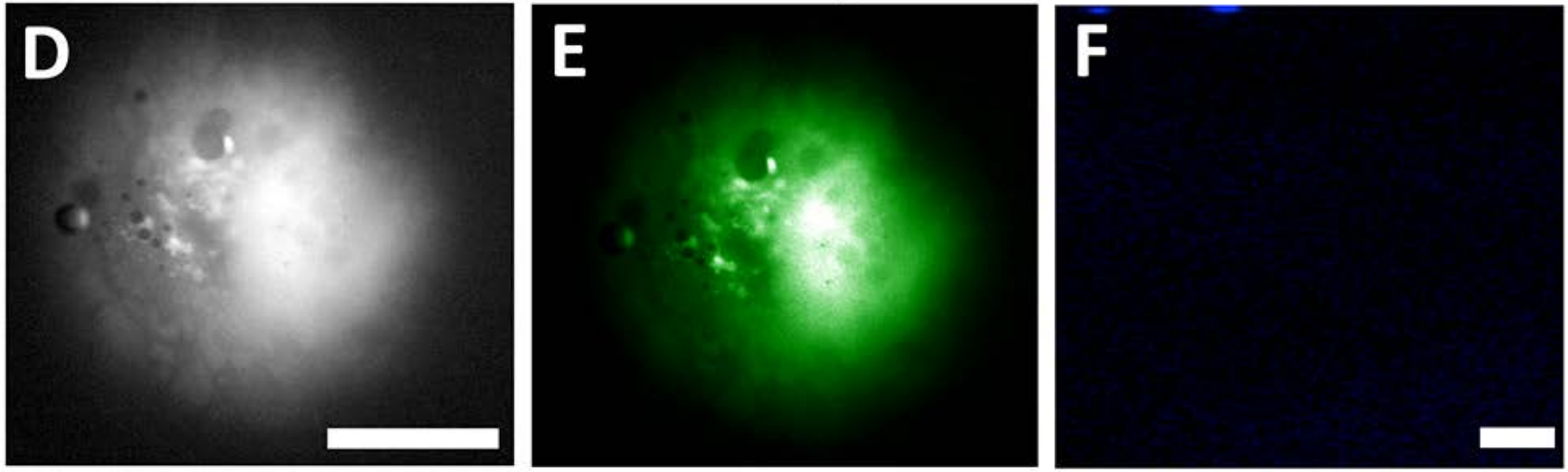


Mizsuhima et al. Figure 5

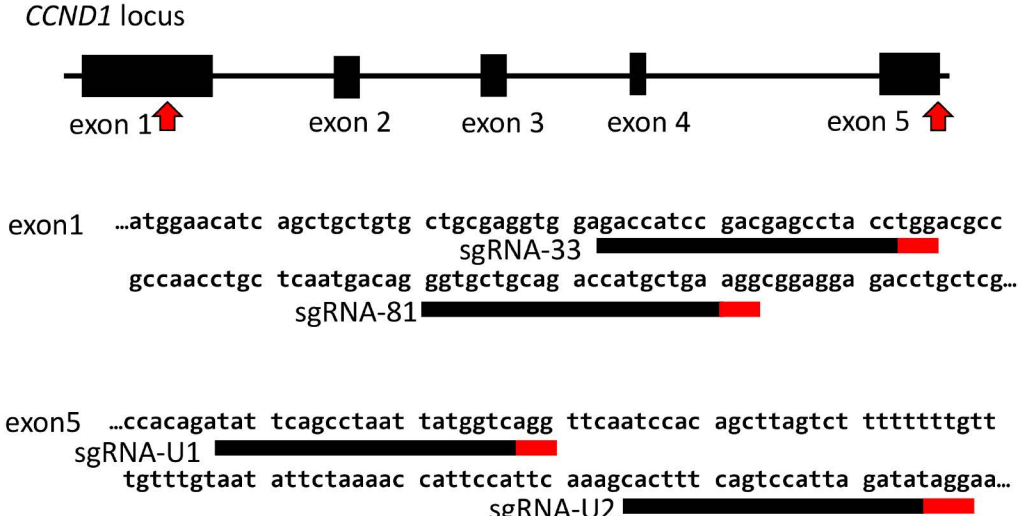
EGFP cRNA injection



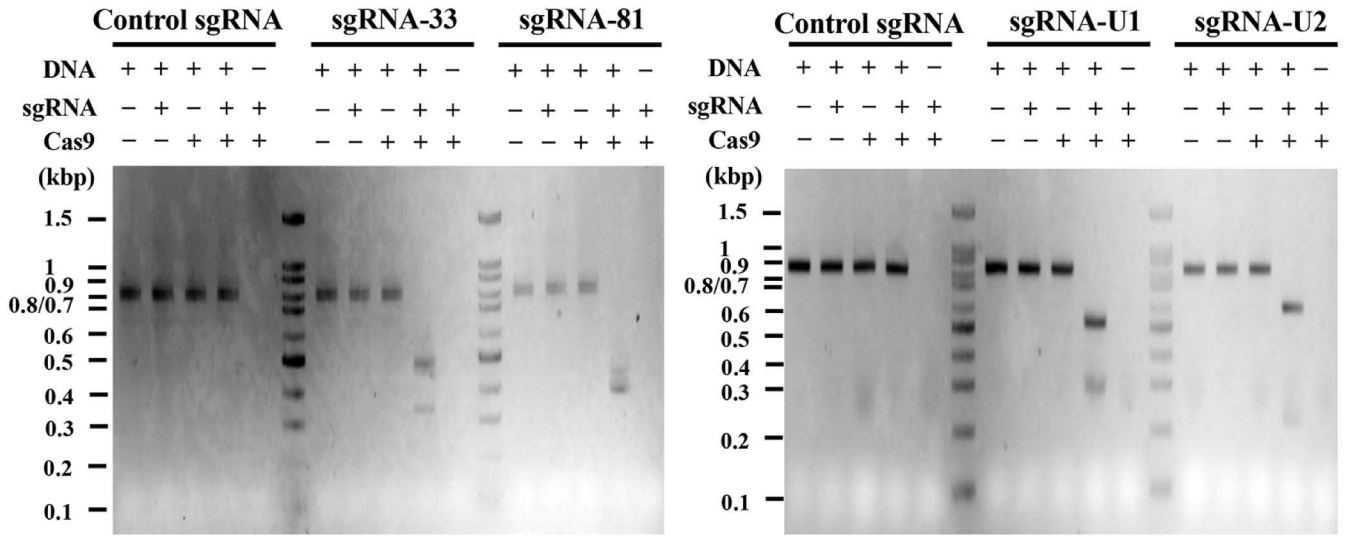
CCND1-EGFP cRNA injection



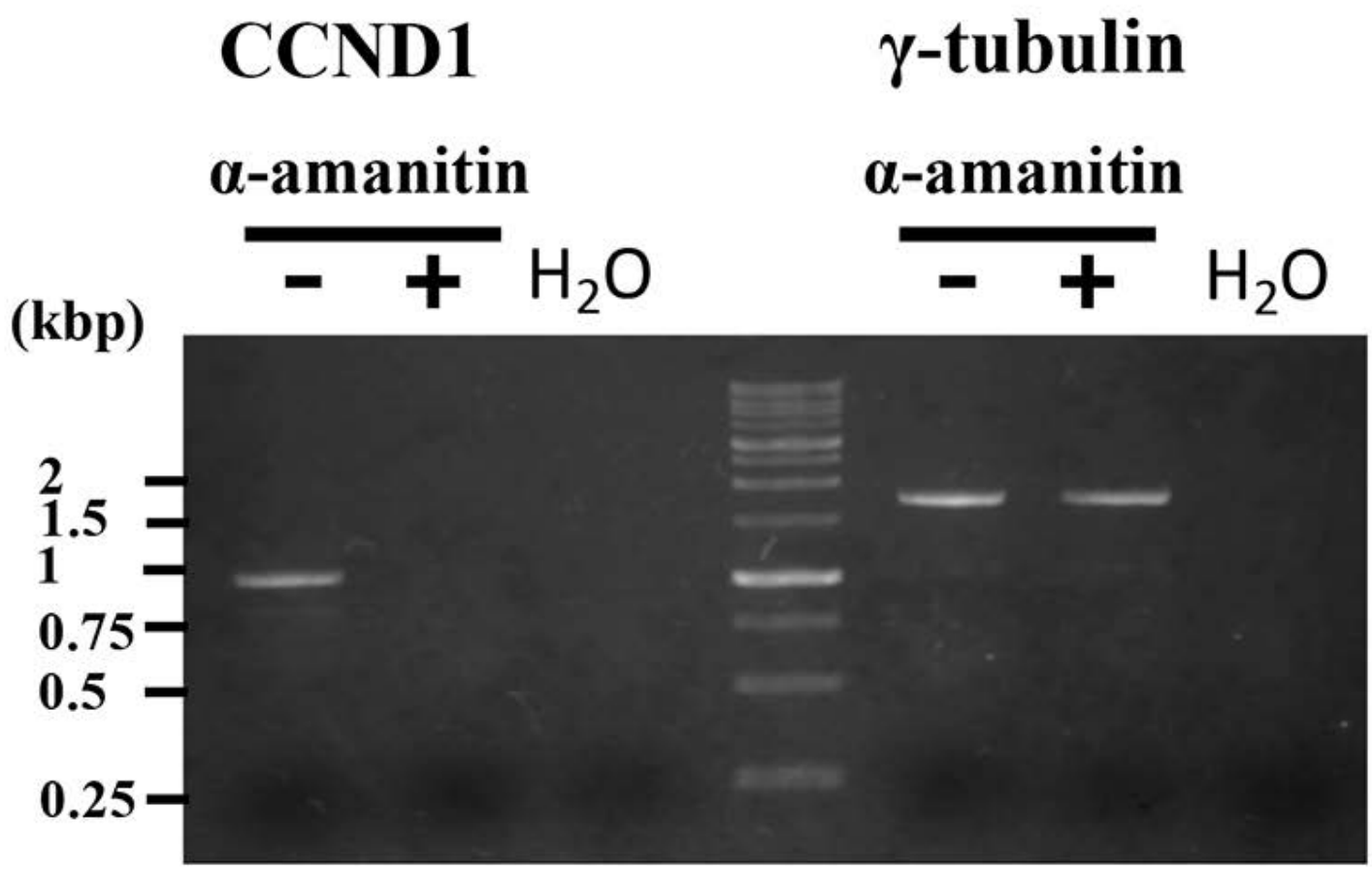
Mizsuhima et al. Figure 6



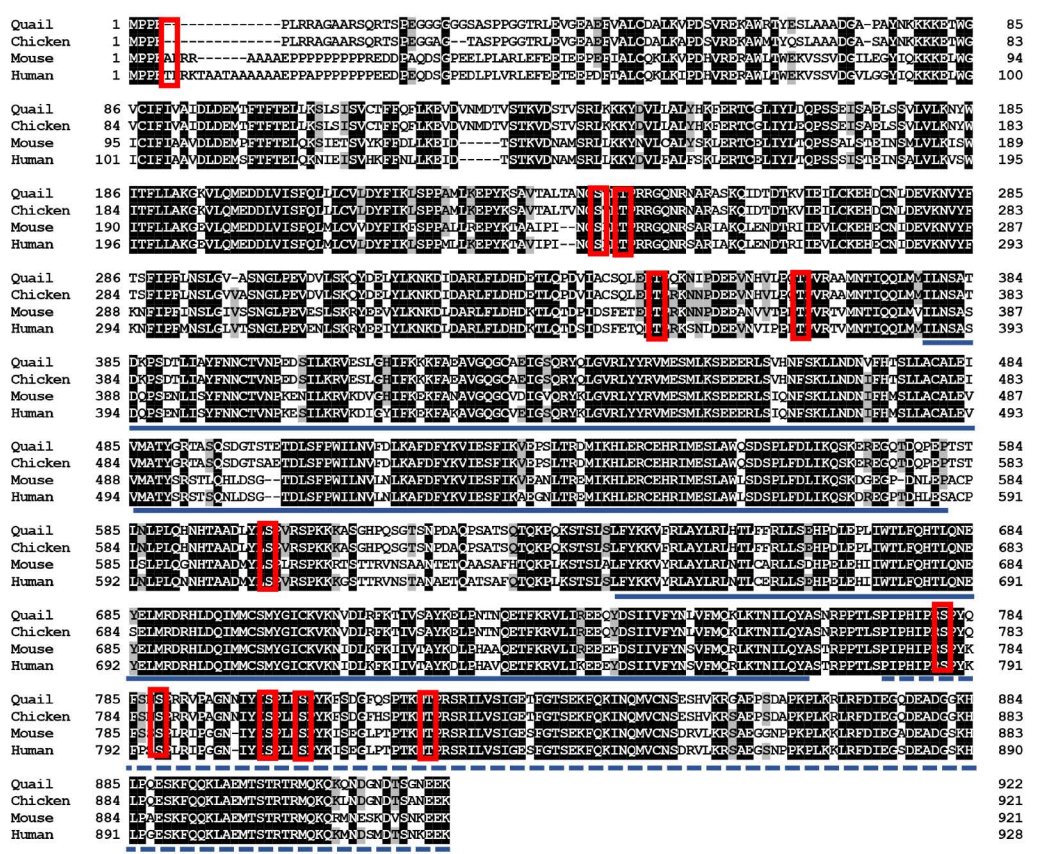
Mizushima et al. Supplementary Figure S1



Mizushima et al. Supplementary Figure 2



Mizushima et al. Supplementary Figure S3



Mizushima et al. Supplementary Figure S4