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# Expression of Cdc25B mRNA in Duodenal Mucosa of Chicken*

**Junhui Qin[a], Hui Zhang[a], Huijun Bao, Qiang Zhou, Yi Liu, Chunsheng Xu, Xiaohong Chu and Qiusheng Chen****

College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, 210095, China

**ABSTRACT :** Cdc25B is a mitotic regulator that might act as a starter phosphatase to initiate the positive feedback loop at the entry into mitotic (M) phase. In the present study, distribution of Cdc25B mRNA in duodenal mucosa of the chicken was demonstrated by means of *in situ* hybridization histochemistry (ISHH) using sense and antisense digoxigenin (DIG)-labeled RNA probes. The results showed that there were many labeled cells distributing in the duodenal mucosa of the adult chicken. Of these labeled cells, 81.60±9.63% of Cdc25B mRNA positive cells was distributed in the basilar part and mid-portion of the intestinal gland and 36.21±8.81% in the middle and basilar portion of villi of the small intestine of the chicken, respectively. Most of these labeled cells were positive in the regions of the stem cell and proliferation. The signals of ISHH decreased from basilar to upper part in the crypt of Lieberkuhn and weakened in the inferior villi of the duodenum. Moreover, the positive signals were both in the cytoplasm and cell nucleus. However, the labeled cells were negative in both the lamina muscularis mucosae and muscular layer. The results of ISHH suggested the existence of Cdc25B mRNA and vigorous proliferation activities in the duodenal mucosa of adult chicken, replenishing the cells which had sloughed off from the superior part of the villus. Our results provide some molecular evidence for a regular pattern of avian intestinal epitheliosis and functional partition and provide an approach to further study of the locations of Cdc25B in the chicken. (**Key Words** : Duodenal Mucosa of Chicken, Cdc25B mRNA, Probe Synthesis, *In situ* Hybridization Histochemistry)

## INTRODUCTION

Cell division cycle 25(Cdc25) phosphatases family are key regulators of the eukaryotic cell cycle that are highly conserved in the process of organic evolution (Nisson et al., 2000). Cdc25 phosphatases are involved in the control of the earlier embryonic development and when the phosphatases were activated abnormally which is frequently associated with genesis of a series of human tumors. It is widely considered that overexpression of Cdc25B is correlated with the genesis, development and prognosis of various cancers (Hortwell, 1994). Detection of the expression level of Cdc25B in cells is promising to represent a molecular index judging the mitosis index and reproductive activity of tumour cell (Zai et al., 2006). Chicken is a most important model system in developmental biology. There is no duodenal gland in the muscularis mucosae of chicken which is different from that of mammals. And the studies about conservative genetic structure and function of chicken not only can explain its role in developmental biology, but also apply to the prophylaxis and control of diseases in mammal and human (Claudio, 2005). An extended ananlysis of Cdc25B expression in adult mice had been carried out by Dineli Wickramainghe et al. (1995), and the result revealed that transcripts were observed in the kidney, liver, heart and muscle. But the expression pattern of Cdc25B in adult chicken hasn't been reported yet. The intestinal crypt and epithelium mucosae of mammals was divided into the five regions, including the region of the stem cell and Paneth's cell in basilar part, the region of proliferation cell in the middle part of intestinal gland, the region of maturation in upper half of intestinal gland, function region and shedding part of upper half of intestinal villus. However, the functional partition of chicken intestinal gland has not been reported yet. Our studies are meant to investigate the distribution of Cdc25B in the duodenal mucosa of chicken, and compare it with the expression pattern of Cdc25B in adult tissues of mammals, providing some molecular evidences at the gene level that could elucidate the regular

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** Corresponding Author: Qiusheng Chen. Tel. +86-25-84395817, Fax: +86-25-84398699, E-mail: chenqsh305@yahoo.com.cn

[a] These authors have contributed equally to this work.

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pattern of avian intestinal tract epitheliosis and functional partition. Sense and antisense digoxigenin (DIG)-labeled RNA probes were designed to detect the distribution of Cdc25B mRNA in the duodenal mucosa of chicken, by means of *in situ* hybridization histochemistry (ISHH).

## MATERIALS AND METHODS

All protocols were approved by the Chinese Committee for Animal Use for Research and Education.

### Tissue preparation

Ten adult male or female tender chickens with age of 65 days and weight of 1.5-2.0 kg were purchased from Nanjing Farm product market (Nanjing, Eastern China). The birds were housed for an adaptation period of 3-4 days and were then anaesthetized with an intraperitoneal injection of ethylurethane at 1 g/kg and were fastened onto a stereotaxic frame. A horizontal incision, about 5 cm long, was made in the infrapubic region of the abdominal wall, 2 cm ventrally to the shaft of the pubis.

The musculus obliquus externus abdominus, musculus rectus abdominus, musculus transversus abdominus, and the very thin peritoneum were then cut open. The small intestine was exposed through this incision. The small intestine from the pylorus of stomach to duodenojejunal flexure was tentatively regarded as the duodenum. The chickens were deeply anaesthetized and were perfused downward through the left common carotid artery with physiological saline, followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer, (4°C, pH 7.4). The duodenum portion was dissected out separately from the small intestine. Tissues were immersed in the same fresh fixative for 4 h and then stored overnight in a 20% sucrose solution at 4°C. Frozen serial sections (10 μm) were prepared by cutting in the longitudinal plane of the duodenum, and were placed on polylysine-coated glass slides.

### Embryos

Fertile hens' eggs were incubated at 38°C in a humidified incubator to yield embryos of appropriate stages (HH 10) (Hamburger and Hamilton, 1951) according to Hamburger and Hamilton, and isolated embryos from yolk (Mozdziak et al., 2008).

### Probe preparations

For the preparation of sense and antisense Digoxigenin (DIG)-labeled RNA probes, total RNA was isolated from chicken embryos using TRIzol reagent (Invitrogen, Shanghai, China). Fragment of Cdc25B mRNA genes was obtained by reverse transcription (RT)-PCR from the total RNA of chicken embryos. The following primers which according to Benazeraf et al. (2006) were used to amplify a 575 bp cDNA of Cdc25B mRNA by PCR: 5′-GGAGCCCTGCGTGCCGAG-3′(AJ720997:800-818) and 5′-CAATTAACCCTCACTAAAGGGTCCTGGTGTTTG CC-3′(AJ720997:1359-1375). The PCR products were analyzed by electrophoresis on 2% agarose gel.

The amplified cDNA fragment by PCR was subcloned into pGM-T easy vector (TIANGEN, Peking, China) using the TA cloning system. And then the plasmid was transformed into *Escherichia coli* strain DH5α and chosen by "white-blue plaque selection". Subsequently, the positive recombinant Cdc25B/ pGM-T easy vector plasmids were identified by sequencing. The recombinants were linearized with the restriction enzymes *Nco*I and *Spe*I (TaKaRa, Dalian, China). The linearized recombinant plasmid was used as templates for *in vitro* transcription. Sense and antisense Digoxigenin-labeled riboprobes of the Cdc25B RNA were produced *in vitro* transcription by using SP6 and T7 RNA polymerase (Roche, Mannheim, Germany), respectively, according to the manufacturer's instructions of "DIG RNA Labeling Kit (SP6/T7)" (Roche). Certificated by electrophoresis on 2% agarose gel and determined by spectrophotometer, both the sense and antisense RNA probes were prepared.

### *In situ* hybridization

For the *in situ* hybridization experiments, the custom service from Young et al. (1986) and Su (1998) was performed essentially with the following modifications for the detection of Cdc25B mRNA transcripts. Briefly, tissue sections were rinsed 2 times in 0.1 M phosphate buffer (PBS) at pH 7.4 for 5 min, in glycine-PBS at 0.1 mol/L for 5 min, in 0.3% TritonX-100-PBS for 5 min and were digested with proteinase-K-PBS for 30 min at 37°C, followed by fixing in 4% paraformaldehyde in PBS, treating with triethanolamine-acetic anhydride, and washing. Then sections were prehybridized for 2 h and hybridized at 42°C for 18 h in hybridization solution containing DIG-labeled RNA antisense probe at 2 μg/ml.

After hybridization the tissue was subjected to stringent washing in 50% 2×standard saline citrate (SSC), 1×SSC, and 0.1×SSC, at 37°C, for 10 min each. Then the sections were rinsed again in PBS, blocked for 30 min with 1.5% normal goat serum (TIANGEN BIOTECH CO., LTD. Peking, China) in PBS, and rinsed in PBS. The sections were then incubated with goat anti-DIG antibody (Roche, Nutley, NJ) at 1:100 to 1:1,000 for 2 h at 4°C. After a rinse in PBS for 10 min, the sections were incubated in 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT; Roche USA) complex for 1 h-16 h at room temperature and examined under a standard light

**Table 1.** Percentages of cell devision cycle 25 mRNA-positive cells in the basilar part and midportion of crypt of lieberkuhn and middle and basilar portion of villi of small intestine in adult chickens

| n | Basilar part and midportion of crypt of lieberkuhn | | | Middle and basilar portion of villi of small intestine | | |
|---|---|---|---|---|---|---|
| | Result of ISHH[1] | Haematoxylin and eosin staining | Percentage (%) | Result of ISHH | Haematoxylin and eosin staining | Percentage (%) |
| 1 | 297 | 324 | 91.67 | 34 | 145 | 23.45 |
| 2 | 54 | 73 | 73.97 | 15 | 39 | 38.46 |
| 3 | 394 | 459 | 85.84 | 67 | 169 | 39.64 |
| 4 | 553 | 712 | 77.67 | 87 | 317 | 27.44 |
| 5 | 306 | 324 | 94.44 | 55 | 176 | 31.25 |
| 6 | 578 | 644 | 89.75 | 91 | 209 | 43.54 |
| 7 | 287 | 375 | 76.53 | 24 | 56 | 42.86 |
| 8 | 418 | 477 | 87.63 | 68 | 257 | 26.46 |
| 9 | 467 | 632 | 73.90 | 78 | 206 | 37.86 |
| 10 | 115 | 178 | 64.61 | 23 | 45 | 51.11 |
| | Mean | | 81.60 | | | 36.21 |
| | S.D. | | 9.63 | | | 8.81 |

[1] ISHH = *In situ* hybridization histochemistry.

microscope. Every 3 serial sections of duodenum portion of intestine were designated as a group, and within each group, the first section was stained with Haematoxylin and eosin (HE) to display epithelia cells; the second one was stained with antisense probe of Cdc25B mRNA, and the last one was used as control of ISHH, in which the antisense probe was replaced with sense probe of CDC25B mRNA.

**Statistical data**

The reacted sections were examined with a BH-2 microscope (Olympus, Tokyo, Japan). The positive cells were distributed in corresponding campus visualize in the 2 serial sections that had been hybridized with Cdc25B antisense probe, and stained with Haematoxylin and eosin, respectively, were counted. The numbers of Cdc25B-positive cells and Haematoxylin and eosin-stained cells observed at each location were expressed as the mean±SE (n = 10 chickens) in Table 1.

## RESULTS

**Results of probe preparation**

Figure 1 showed the gelectrophoresis of the fragments from RT-PCR. After PCR, 3 μl of product was run on agarose gel of 2% to check the DNA band. There were two bands, one was between 500 bp and 750 bp and the other one was with the size of 250 bp. Then the bands were cut out, followed by purification using the gel kit of DNA purification kit (TIANGEN, Peking, China). Both of the fragments were subcloned into pGM-T easy vectors (TIANGEN, Peking, China) using the TA cloning system respectively. And then the plasmids were transformed into *Escherichia coli* strain DH5α and chosen by "white-blue plaque selection". Subsequently, the positive recombinant Cdc25B/pGM-T easy vector plasmids were identified by sequencing. The sequence results confirmed that the 575 bp does the target Cdc25B gene. And the product with the size of 250 bp was a non-specific PCR reaction. Figure 2 showed the result of Cdc25B/pGM-Teasy digested with EcoRI.

**Results of ISHH**

The mucous membrane chorioepithelium of avian digestive tract is composed of absorptive cells, goblet cells and endocrine cells. In addition to above three kinds of cells, intestinal crypt of chicken contains the undifferentiated cells and Paneth's cells. The undifferentiated cells are

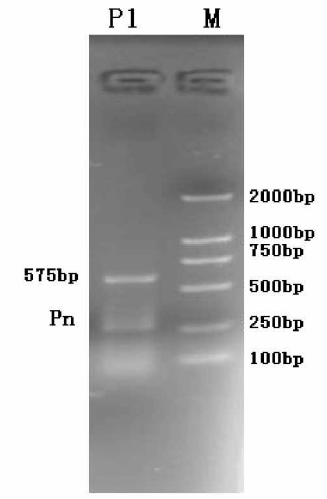


**Figure 1.** Gelectrophoresis of the fragments from RT-PCR. 575 bp: the target Cdc25B gene. P1: RT-PCR production; M:DL2000 marker $P_n$: a non-specific PCR product.

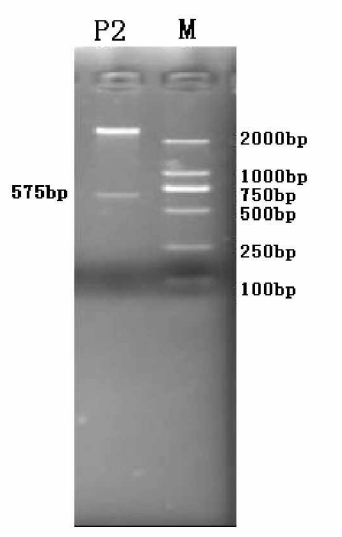


**Figure 2.** Cdc25B/pGM-Teasy digested with *Eco*RI. P2: Cdc25B/pGM-Teasy digested with *Eco*RI; M.DL2000 marker.

mainly distributed in lower half of intestinal gland and have columnar shape in interphase of mitosis. The cytoplasm of these cells appears basophilia and stains with eosin and their nucleus are elongated near to basal surface (Shown as Figure 3A and B). The labeled cells stained with BCIP/NBT and appear amethyst. The positive signals of Cdc25B mRNA hybridizated with antisense were observed mainly distributing in the undifferentiated cells of intestinal gland (Shown as Figure 3C-E).

Under lower power lens, there were many labeled cells distributing in the duodenal mucosa of adult chicken. The signals of ISHH were decreased from basilar part to upper in the crypt of lieberkuhn and disappeared in the inferior of villi of small intestine. Figure 3C showed that the labeled cells were both negative in the lamina muscularis mucosae and muscular layer. And turned to the high power lens, most of these labeled cells observed were positive in the regions of the stem cell and proliferation localized districts of undifferentiated cells. The positive signals were both in the cytoplasm and cell nucleus (Shown as Figure 3E). Control experiment was performed as above method of ISHH by sense probe of DIG-labelled Cdc25B- mRNA instead of anti-sense probe and the signal of hybridization was negative (Shown as Figure 3F).

**Results of statistics**

Many Cdc25B mRNA-positive cells that were labeled by Cdc25B mRNA antisense probes were distributed in the basilar and upper part of the crypt of lieberkuhn, and the positive signals were both in the cytoplasm and cell nucleus. The signals of ISHH were decreased from basilar part to upper in the crypt of lieberkuhn and weakened in the inferior of villi of small intestine observed from Table 1. The percentages of Cdc25B mRNA-positive neurons in the basilar part and midportion of crypt of lieberkuhn were 81.60% and 36.21% in the upper part of the crypt of lieberkuhn and inferior of the intestinal villis of adult chicken, which were shown in Table 1.

## DISCUSSION

The previous studies suggested that Cdc25 homologues control the entry into mitosis through dephosphorylating and activating specific substrate cyclin-dependent kinases (CDK) to regulate the progression of cell division cycle and transition (Gould and Nurse, 1989; Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991; Strausfeld et al., 1991; Lee et al., 1992). Cdc25 phosphatase was first identified in *Schizosaccharomyces pombe* (*S. pombe*; Russell and Nurse, 1986). Then it was found that Cdc25 existed in all eukaryotic organisms except plants (Boudolf, 2006). Three isoforms have been identified in mammalian: Cdc25A, Cdc25B and Cdc25C (Sadhu, 1990; Galaktionov, 1991; Nagata, 1991). Only Cdc25A and Cdc25C have been found in xenopus laevis and two isoforms (*Gallus gallus*; Cdc25A and Cdc25B) were found in chicken (Lzumi et al., 1993; Okazaki et al., 1996; Benaeraf et al., 2006). To date, the existence of Cdc25C in chicken has not been reported yet (Rose Boutros, 2007).

As an inducer, Cdc25B phosphatase plays a crucial role at the onset of mitosis. Several studies support the concept that the G2/M checkpoint response in cells to DNA damage is closely correlated with tumorigenesis and Cdc25B functions as a key regulator of the cell cycle progression which represents an effective target for cancer therapy (Ma et al., 2006). In the study of Lammer (1998), injection of anti-body against Cdc25B caused cells to arrest after they reach G2 phase. Dineli et al. used the method of ISHH to detect the existence of Cdc25B in most organs of adult mouse, and the results indicated that lower level of Cdc25B were observed in the adrenal gland, kidney, liver and muscle. The expression level of Cdc25B was higher in the brain and small intestine, and was the highest in lung and spleen (Dineli et al., 1995). The expression pattern of Cdc25B suggested that the expression level of Cdc25B was related with the development of mouse. With regard to the expression pattern of Cdc25B in chicken, only few have been reported by Bertrand Benazeraf (2006) in the early embryos experiment. There is not any paper about the distribution of Cdc25B in duodenal mucosa of chicken till now. In order to investigate the association between the expression level of Cdc25B and proliferative cells, we designed the sense and antisense probes of digoxigenin (DIG)-labeled RNA probes to analysis the expression condition of Cdc25B mRNA in chicken and found that Cdc25B mRNA was distributed widely in duodenal mucosa

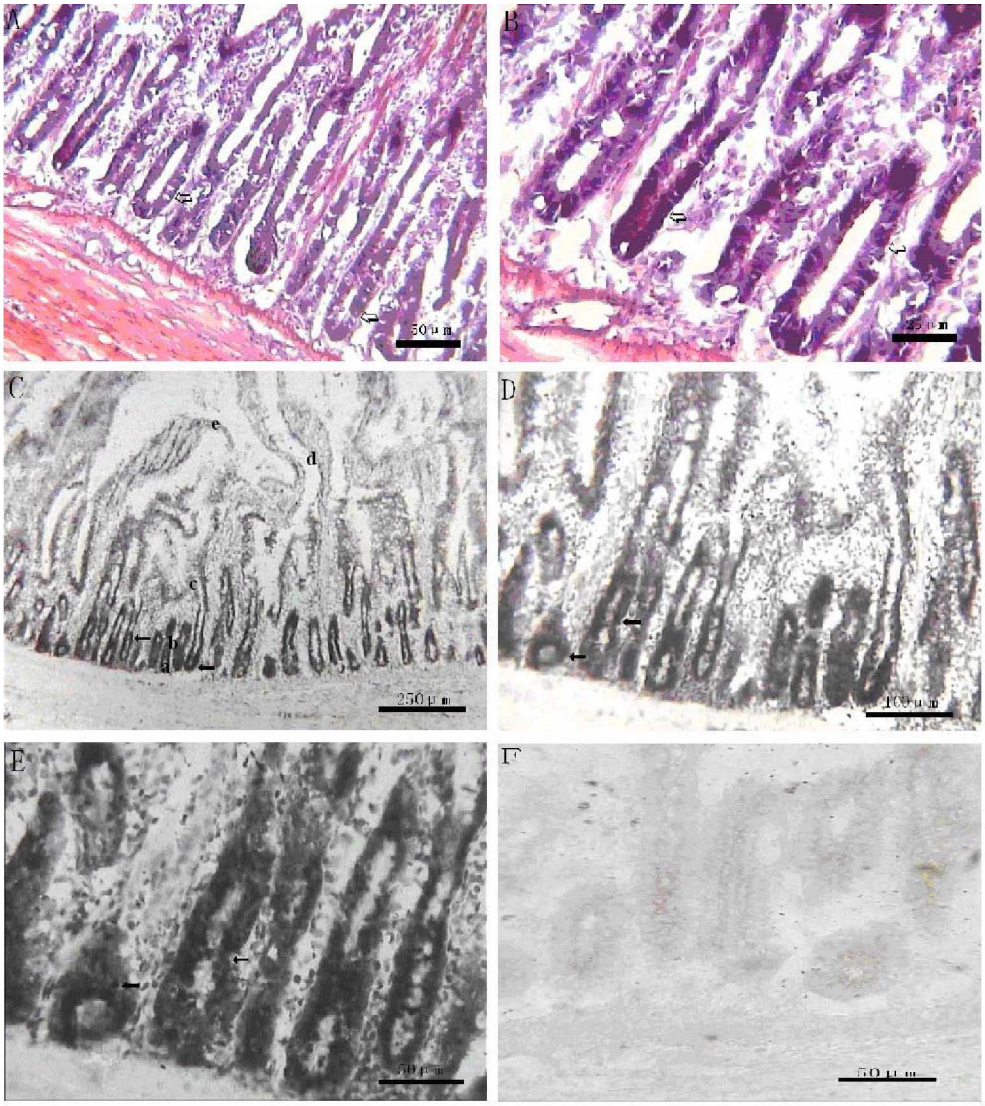


**Figure 3.** Distribution of cell division cycle 25(Cdc25) mRNA in the duodenal mucosa of the chicken. A. Haematoxylin and eosin (HE) staining on the duodenal mucosa of chicken ×100. B. Duodenal mucosa of chicken (HE staining) ×200. C. Reaction of *in situ* hybridization histochemistry (ISHH) on the duodenal mucosa, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT) staining ×40. a. the basilar part of intestinal gland. b. the midportion part of intestinal gland. c. the upper part of intestinal gland. d. the inferior part of the intestinal villi. e. the upper part of the intestinal villi. D. Reaction of ISHH on the duodenal mucosa of chicken, BCIP/NBT staining ×100. E. Reaction of ISHH on the duodenal mucosa of chicken, BCIP/NBT staining ×200. F. Negative control with sense probe ×200 (⇦: the intestinal gland of chicken. ➡: the Cdc25 mRNA-Positive cells).

of chicken, especially in the stem cells of intestinal gland which were in the mitosis phase. The widely distribution pattern of Cdc25B mRNA indicated that Cdc25B played a critical role in proliferation, migration and differentiation of small intestinal epithelium mucosa in chicken.

The avian small intestinal epithelium mucosae is composed of absorptive cells, goblet cells and endocrine cells. Like epithelium, intestinal crypt contains undifferentiated cells and Paneth's cells besides above three kinds of cells. The undifferentiated cells lie in the lower halves of the gland and represent the major stem cells which are often in the mitosis phase. The replenishment of the absorptive cells and goblet cells fallen off from the top of intestinal villus is provided by the continual proliferation of the undifferentiated cells. Both the enteroendocrine cell and Paneth's cell are deriving from the undifferentiated cell which is considered as stem cell of enteric epithelium. When the undifferentiated cell differentiates and migrates from basilar to upper part of intestinal villus, the microvillus become more and longer and the content of RNA becomes fewer. According to the renewal process of enterocytes, epithelium mucosae of small intestine of mammal is divided into five regions, containing the region of the stem cell and Paneth's cell in basilar part, the region

of proliferation cell in the middle of intestinal gland, the region of maturation in upper half of intestinal gland, function region and shedding part of upper half of intestinal villus. The stem cells of proliferation region migrate upward and displace millions of cells falling off from upper half of gastrointestinal tract. Only intestinal crypt has proliferative activities in small intestine (Cheng et al., 2003). Large numbers positive Cdc25B mRNA cells were observed in the present study. Of these labeled cells, 81.60±9.63% and 36.21±8.81% Cdc25B mRNA positive cells were distributed in the basilar and midportion part and middle and inferior portion of intestinal villi of adult chickens respectively. The distribution pattern suggested that the existence of Cdc25B mRNA and athletic proliferation activities in the duodenal mucosa of adult chicken. This result corresponded with the version that intestine crypt has proliferating and the proliferation tendency kept concordance with the divided regions of mammals. Consequently, we presumed that the intestinal crypt and epithelium mucosae of chicken were also divided into the same five regions as that of mammals.

Cdc25B is a short-half-life protein (Gabrielli et al., 1996). There are five Cdc25B splice variants and they represent different levels in human and mammalian cells. The physiology discrepancy of these variants is still unclear because most previous related studies did not distinguish the variants (Rose Boutros, 2006). However, it is clear that Cdc25B variants shuttle between the nucleus and the cytoplasm between S phase and mitosis, and this process is mediated by a combination of 14-3-3 binding and the existence of both Nuclear Export Signal(NES) and Nuclear Localization Signal (NLS) in Cdc25B sequence (Bulavind et al., 2001; Lindqvist, 2004; Uchida, 2004). The expression levels of Cdc25 mRNA, protein, activity of kinase appear to be constant during the cell cycle. And the expression of Cdc25B is detected throughout the whole cell cycle. Inactived Cdk1/cyclinB complex localizes in the cytoplasm and Cdc25B in the nucleus during the interphase. But in the G1 phase, Cdc25B localizes both in cytoplasm and nucleus, then it accumulates during the late S and early G2 phases of the cell cycle and its activity peaks at the G2/M transition. In the late G2 phase, Cdc25B is transported into the cytoplasm again and activated through dephosphorylating and activating Cdk1/cyclinB complex to enhance the entry into mitotic phase (Gabrielli, 1996; Lindqvist, 2005). However, Cdc25B is transported to nuclear zone again in the mitotic phase (Gasparotto, 1997). The positive signals of ISHH observed in the present study were both in the cytoplasm and cell nucleus, which is strongly supported the concept in molecule morphologically that Cdc25B is presented during each stage of cell cycle and accumulates during the late S and early G2 phases of the cell cycle and its activity peaks at the G2/M transition, shuttling between the nucleus and the cytoplasm.

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