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Gene structure and embryonic expression of mouse COP9 signalosome subunit 8 (*Csn8*)

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Abstract

Csn8 is the smallest subunit of the constitutively photomorphogenic 9 (COP9) signalosome (CSN), which consists of eight distinct components. CSN is homologous to the lid subcomplex of the 26S proteasome and is a regulator of the ubiquitin–proteasome pathway. Murine *Csn8* is an ortholog of Arabidopsis *COP9*, which was originally identified as a key regulator of photomorphogenic development. CSN is essential for the viability of plants and flies, but the role of this conserved protein complex in development of vertebrate animals remains obscure. We report the genomic structure of murine *Csn8* gene and the expression pattern of Csn8 during early embryo development. Mouse Csn8 protein contains 209 amino acid residues and is encoded by a single gene located on chromosome 1. Csn8 is expressed in embryonic stem (ES) cells and throughout early embryo development from zygote, preimplantation embryos, to post-implantation embryos. Immunostaining studies revealed that Csn8 is predominantly present in the inner cell mass (ICM) of E3.5 blastocyst and is widely expressed in E9.5-day embryos.
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Keywords: COP9 signalosome; Genomic structure; Embryogenesis; Early development

1. Introduction

The COP9 signalosome (CSN) is a highly conserved protein complex consisting of eight subunits named Csn1 to Csn8 (Deng et al., 2000). Each of the CSN subunit displays significant sequence homologies to the eight subunits of the lid subcomplex of the 26S proteasome (Glickman et al., 1998; Seeger et al., 1998; Wei et al., 1998). *Csn8*, also known as *COP9* in Arabidopsis, was the first gene cloned among all of the genes encoding CSN subunit (Wei et al., 1994) and was identified genetically as the *constitutively photomorphogenic 9* (COP9) locus of Arabidopsis (Wei and Deng, 1992). The CSN complex has been found in plant (Chamovitz et al., 1996), vertebrate animals (Seeger et al., 1998; Wei and Deng, 1998), fly (Freilich et al., 1999) and fission yeast *Schizosaccharomyces pombe* (Mundt et al., 1999). A CSN-like complex has also been reported in the budding yeast *Saccharomyces cerevisiae* (Wee et al., 2002; Maytal-Kivity et al., 2002). While the *csn* mutants of yeast are viable (Mundt et al., 1999), null mutants of *csn* in fly and in plant are lethal (Wei and Deng, 1992; Freilich et al., 1999; Suh et al., 2002). In plant, CSN is shown to be necessary for photomorphogenesis (Wei and Deng, 1992), auxin-mediated development (Schwechheimer et al., 2001), flower development (Wang et al., 2003) and defense response (Feng et al., 2003). In flies, it is involved in oogenesis, embryogenesis and axon guidance (Suh et al., 2002; Oron et al., 2002; Doronkin et al., 2002).

CSN has been shown to interact with the Skp1-Cul1-F-box protein (SCF) ubiquitin ligase complexes and acts as a de-neddylase that removes the ubiquitin-like protein, Nedd8 from the cullin component (Lyapina et al., 2001; Schwechheimer et al., 2001; Cope et al., 2002; Yang et al., 2002).

Abbreviations: COP9, constitutively photomorphogenic 9; CSN, COP9 signalosome; EST, expressed sequence tags; ICM, inner cell mass; PCR, polymerase chain reaction; RT, reverse transcriptase; SCF, Skp1-Cul1-F-box protein; UTR, untranslated region.

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CSN also exhibits ubiquitin depolymerization and deconjugation activities through association with a de-ubiquitin enzyme (Zhou et al., 2003; Groisman et al., 2003). Through these activities, CSN regulates SCF functions and the ubiquitin–proteasome pathway. In addition, CSN has been found to associate with protein kinases and participate in diverse signal transduction pathways (Seeger et al., 1998; Sun et al., 2002; Uhle et al., 2003).

Csn8 is an integral part of the CSN complex. The endogenous Csn8 has been detected exclusively as part of the CSN complex of approximately 500 kDa in mammalian or plant cell extracts (Wei et al., 1994; Wei and Deng, 1998). Furthermore, the Csn8 subunit is necessary for stable accumulation of the CSN complex in Arabidopsis, indicating it has a role in maintaining the structural stability of the complex (Staub et al., 1996; Kwok et al., 1998). Both Arabidopsis and human Csn8 proteins are nuclear enriched (Chamovitz et al., 1996; Wei and Deng, 1998). There was evidence that nuclear translocation of Csn8 requires assembly of the complex (Staub et al., 1996). However, the specific functions of Csn8 is unclear. The human *Csn8 (hCOP9)* cDNA sequence has been reported (Chamovitz and Deng, 1995; Wei and Deng, 1998). Here, we report characterization of the murine *Csn8* gene structure, protein sequence and the expression pattern during early mouse development.

2. Methods

2.1. cDNA cloning, alignment and phylogenic three of Csn8

The mouse cDNA sequence was identified from a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) of the expressed sequence tag (EST) database using human *Csn8 (hCOP9)* sequence. The EST clone (AA545290) was obtained from Genome Systems (St. Louis, MO), and was completely sequenced. The clone contained the full-length *Csn8* cDNA. Alignment and the phylogenic tree analyses of the Csn8 sequences from mouse and other organisms were done using the Clustal W method (DNA-Star).

2.2. Genomic library screening and Southern blot

A Lambda phage library (Zhong et al., 2000) was screened using the mouse *Csn8* cDNA clone by random-priming (Roche Molecular Biochemicals). Lambda DNA was isolated, restriction digested and subcloned into the pPCRscript vector (Stratagene). The plasmid clones were used for most of the sequencing. For Southern bolt, genomic DNA from a 129/SVJ mouse strain was obtained from The Jackson Laboratory (Bar Harbor, ME). Five micrograms of DNA was digested with the restriction enzymes as indicated in Fig. 2A. The DNA fragments were separated in a 0.6% agarose gel. After electrophoresis, the DNA was transferred to a Hybond-N membrane (Amersham). A $[^{32}P]$-labeled DNA probe was prepared by random priming according to the manufacturers instructions (Boehringer Mannheim) using a full length *Csn8* cDNA as a template.

2.3. Western blot and whole mount immunohistochemistry

ES and HeLa cells were lysed in the SDS loading buffer for western blotting. After SDS-PAGE, the proteins were transferred to a PVDF membrane (Amersham Pharmacia Biotech). The membrane was blocked in 2% skim milk in PBST (PBS containing 0.1% Tween 20) for 1 h and incubated with anti-CSN8 antibody at 4 °C overnight (Wei and Deng, 1998). The membrane was washed three times in PBST and incubated with alkaline phosphatase conjugated anti-rabbit secondary antibody for 45 min at room temperature. Finally, the immunoreaction was detected by soaking the membrane in 100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$, 0.5% (v/v) 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP), 0.5% (v/v) Nitroblue tetrazolium chloride (NBT).

Whole amount immunohistochemistry was performed as described (Hogan et al., 1994). Mouse embryos were collected in cold PBS from dated pregnant female mice at E9.5 and were fixed in 4% paraformaldehyde, permeabilized in methanol/DMSO (4:1). The endogenous peroxidase activity was blocked by methanol/DMSO/H_2O_2 (4:1:1) as described. The embryos were first incubated in PBSMT (PBS containing 2% skim milk, 0.5% triton X-100) overnight at 4 °C followed by overnight incubation with anti-CSN8 antibody (1:100 dilution, Affiniti Research) in PBSMT at 4 °C. The embryos were washed five times for 1 h in PBSMT prior to an overnight incubation with a peroxidase-conjugated secondary antibody (Sigma). Embryos were washed as described above and the peroxidase color reaction was developed by incubating in PBS containing 50 mM 3-3′-diaminobenzidine tetrahydrochloride (DAB), 4.6 mM $NiCl_2$ and 0.03% H_2O_2. The embryos were then cleared by incubating in increasing concentrations of methanol to 100% (Hogan et al., 1994), followed by 10 min incubation in benzylalcohol/benzyl benzoate, 1:2 (BABB).

The E3.5 blastcysts were collected by flushing the uterine with M2 media (Specialty Media). The embryos were cultured in vitro in embryo medium containing ES grade DMEM, 20% FBS, supplemented with L-glutamine, antibiotics, non-essential amino acids, nucleotides and β-mercaptoethanol. Immunofluorescence staining was performed as described (Wei and Deng, 1998).

2.4. RNA isolation, Northern blot and reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using Trizol reagent (GibcoBRL). Fifteen micrograms of total RNA extracted from embryos of various developmental stages was separated on a formaldehyde/[3-(*N*-morpholino)]propanesulfonic acid (MOPS) agarose gel, transferred to a Hybond-N+ Membrane (Amersham Pharmacia Biotech) by capillary blotting and probed with an

antisense RNA riboprobe (Ambion) against the *Csn8* cDNA. The [^{32}P]-labeled probe was added to the hybridization solution to 1×10^6 dpm/ml. Hybridization was done at 50 °C overnight in 50% formamide, 5 × SSPE (20 × SSPE is 3.6 M NaCl, 0.2 M $NaPO_4$, 20 mM EDTA (pH 8.0), 2% SDS), 1 × Denhardt's solution (5 × Denhardt's solution is 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone). The filter was washed twice for 30 min with 2 × SSPE, 0.1% SDS at room temperature, 30 min with 0.1 × SSPE, 0.1% SDS at 65 °C and 30 min with 0.1 × SSPE, 0.1% SDS at 65 °C. The same filter was stripped by adding boiling 0.1% SDS and left at room temperature for 30 min and was re-hybridized with a [^{32}P]-labeled riboprobe against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (accession number AW541288) to confirm equal loading of RNA samples in each lane.

RT-PCR was performed according to the manufacturers instruction (Roche Molecular Biochemicals). Briefly, denatured total RNA (10 μg per reaction) was incubated with 50 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Stratagene) in the buffer containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 800 μM of each dNTP, 1 μg of oligo (dT18) at 42 °C for 1 h, followed by RNAse treatment (10 μg/ml) at 37 °C for 1 h. The sample was purified on a G50 Sephadex spin column (Pharmacia Biotech) and extracted in phenol/chloroform. The precipitated cDNA pellet was resuspended in 50 μl TE buffer and 1 μl was used in a PCR reaction using 5 U of cloned Pfu DNA polymerase (Stratagene) using suppliers buffer (10 × is 200 mM Tris–HCl (pH 8.8), 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton X-100, 1 mg/ml nuclease-free BSA). For the PCR step, the following oligonucleotides was used: 88D (5′-ggaattgccacaccgcca) and 502U (5′-ctggcttcctgggaagaa), which gives rise to a product of 414 base pairs (bp). The PCR condition was 95 °C for 30 s, 50 °C for 1 min, 72 °C for 5 min for 25 cycles.

3. Results and discussion

3.1. Identification of Csn8

We identified mouse *Csn8* cDNA clones by database homology searches using the human *Csn8* (*hCOP9*) sequence. One EST clone (AA545290) was obtained and completely sequenced. This clone contains 2058 bp including a complete open reading frame encoding 209 amino acids, a 586-bp 5′ untranslated region and 847-bp 3′ untranslated region (Fig. 1A, Genbank accession AF482000). The murine Csn8 protein sequence is 94% identical to its human ortholog. The Csn8 protein contains a PCI domain in the central region. The PCI domain is a signature protein motif found in components of CSN, the proteasome lid and the eukaryotic translation initiation factor 3 (eIF3) complexes (Hofman and Bucher, 1998). It has been shown to function as a structural scaffold for subunit–subunit interactions within the complex (Freilich et al., 1999; Tsuge et al., 2001). Csn8 has orthologs from various multicellular organisms including *Distyostelium discoideum* (Fig. 1B and C), revealing its evolutionary conservation. The *Distyostelium* Csn8 appears to be more distant from its counterparts in animals and plants (Fig. 1C). Despite the presence of a functionally conserved CSN complex, the Csn8 subunit has not been found in the fission yeast *S. pombe*. It is possible that Csn8 is simply absent from the yeast CSN complex, or that *Csn8* has diverged to the extent that it is difficult to be identified by homology search of databases. Notably, *Csn8* is the smallest and the least conserved subunit of the CSN complex.

Southern hybridization revealed that mouse *Csn8* has a single locus in the genome (Fig. 2A). To obtain the genomic clone of *Csn8*, we screened a lambda phage genomic library (Zhong et al., 2000) using the cDNA as a probe. Two clones were isolated which together covered the entire genomic locus of *Csn8*. The genomic sequence was determined from the plasmid subclones derived from the lambda phage clones. The murine *Csn8* gene consists of eight exons that distribute along approximately 12 kb of DNA (Fig. 2B, Genbank accession AF502144). The sequences of the exon–intron boundaries and the length of exon/intron are summarized in Table 1.

3.2. Widespread expression of Csn8 mRNA during embryo development

It has been shown that the Csn8 protein can be found in most of the adult organs (Wei and Deng, 1998). Here, we examined the expression of *Csn8* during early mouse embryo development by a combination of Northern blot and RT-PCR analyses. Total RNA isolated from E3.5 to E10.5 revealed a single transcript of approximately 1.6 kb, which is close to the size range of *Csn8* cDNA (Fig. 3A). The 1.6-kb transcript was detected in all the embryonic stages examined, and the expression level seems similar throughout implantation, gastrulation and early organogenesis (from E3.5 to E10.5). The presence of the *Csn8* mRNA in the embryos including the preimplantation stages was further confirmed by RT-PCR using sequence specific oligonucleotides (Fig. 3B). Even though this methods was not optimized for a quantitative measurement, our result revealed that *Csn8* is expressed throughout early embryonic development starting from zygotes.

In agreement with accumulation of the mRNA, the immunoblot analysis showed that the Csn8 protein is present in early embryos (data not shown) as well as in embryonic stem (ES) cells (Fig. 3C). Note that Csn8 appeared as a typical doublet bands in the SDS-PAGE gel as previously reported for human and porcine Csn8 (Wei and Deng, 1998). There was evidence from Mass Spectrometry analysis of purified porcine CSN that the two Csn8

Fig. 1. cDNA sequence and alignment of Csn8 orthologs. (A) Nucleotide sequence of the *Csn8* cDNA along with the encoded amino acid sequence. The PCI domain is shaded in gray. The poly-A signal is shown in bold. (B) Alignment of the mouse Csn8 protein sequence with its orthologs from various organisms: humans (NP_006701), zebrafish (AAH46071.1), *Arabidopsis thaliana* (A54842), *Nicotiana benthamiana* (AAO85511.1), *Dictyostelium discoideum* (AAO53102.1). The identical residues to those of the mouse Csn8 are shown in white on black shading. The sequence alignment was obtained using Clustal W (DNA Star). Base on this alignment, the relationship in terms of their sequence homologies is plotted in the form of a phylogenetic tree (C).

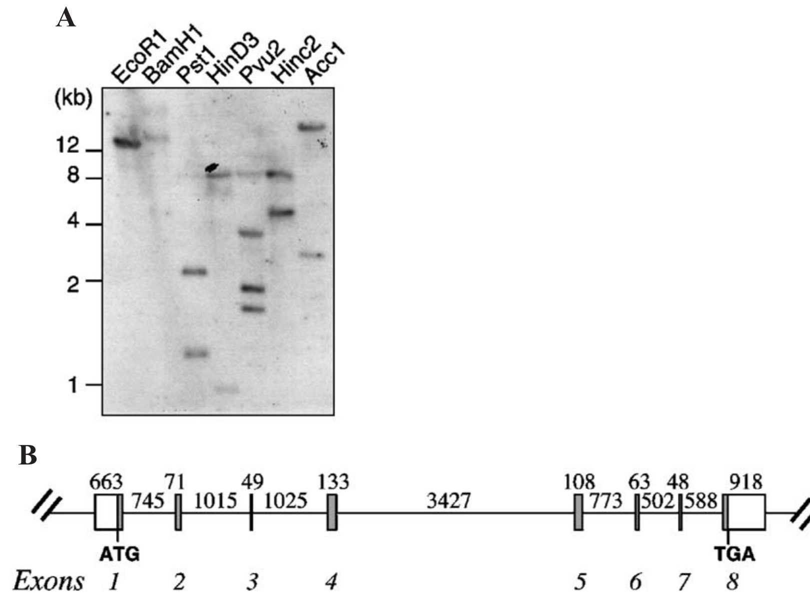

Fig. 2. The genomic structure of the *Csn8* locus and its genomic structure. (A) Southern hybridization. The genomic DNA from mouse strain 129/SVJ was digested with the restriction enzymes as indicated. The *Csn8* cDNA was used as a hybridization probe. (B) Diagram of *Csn8* genomic structure. The length of each exons (boxes) and introns (lines) are labeled as basepairs. Shaded boxes indicate the coding region between the "ATG" start codon and the "TGA" stop codon. The blank boxes indicate the 5′ and 3′ untranslated regions. The exon numbers are labeled at the bottom.

proteins differ in the amino terminal fragments of the Achromobacter protease I digestion (Wei et al., 1998). Accordingly, the doublet Csn8 proteins could arise from possible post-translational modifications at its amino-terminal region, or from alternative translation initiation sites at both Met^1 and Met^6 (Fig. 1).

3.3. Enriched expression of Csn8 in the inner cell mass (ICM) of preimplantation embryos

We next examined Csn8 accumulation in preimplantation embryos or blastocysts and during in vitro outgrowth of blastocysts by immunofluorescence staining using anti-Csn8 antibody. In E3.5–E4.0-day blastocysts, Csn8 protein accumulated highly in the ICM region and weakly in the trophectoderm cell layer (TC) (Fig. 4a–c). The blastocysts were cultured in vitro to allow outgrowth for 3 days (Fig. 4d). Anti-Csn8 immunoreaction in the outgrowth showed strong signals in the ICM region, whereas the giant trophoblast cells displayed weak nuclear staining (Fig. 4f). In comparison, double staining using the anti-alpha tubulin antibody revealed an intense cytoplasmic staining in both

Table 1
Exon–intron junctions in the mouse CSN8 locus

Exon	3′ splice acceptor	5′ splice donor	Exon size (bp)	Intron size (bp)
1		CTCGAG**gt**agcg	>663	745
2	ctct**ag**GCTCCT	CGACAT**gt**aagt	71	1015
3	atc**ag**GAATAA	AAGTCT**gt**aaag	49	1025
4	tttt**ag**GCAAAT	TTAGAG**gt**agtg	133	3427
5	caat**ag**ATGCAA	TGAAAG**gt**actt	108	773
6	Tttc**ag**GCGTGT	AGCCAG**gt**aggt	63	502
7	Gttt**ag**CCTCAG	TATCAG**gt**catg	48	588
8	Atgc**ag**AGCCTG		>918	

Introns and exons are shown in lower and uppercase letters, respectively. Splicing acceptor and donor are shown in bold.

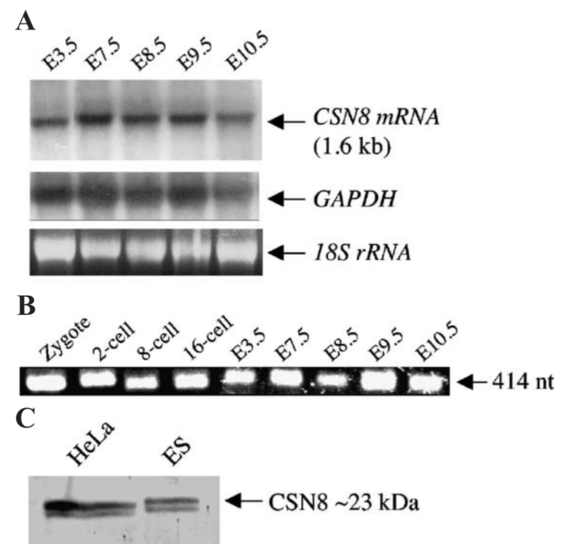

Fig. 3. Analysis of Csn8 expression. (A) Northern blot analysis of *Csn8* expression in embryos of various stages as indicated. Total RNA of 15 µg was loaded in each lane. The blots were hybridized with a *Csn8* probe or a GAPDH probe. The ethidium bromide staining of the gel showing the ribosomal RNA was shown (18S rRNA). Note a single 1.6-kb transcript of *Csn8* mRNA was detected in the embryos. (B) *Csn8*-specific RT-PCR on total RNA isolated from embryos as indicated showed an expected PCR product of 414 nt. (C) Western blot analysis using anti-Csn8 antibody showed expression of the Csn8 protein in ES cells as compared to HeLa cells.

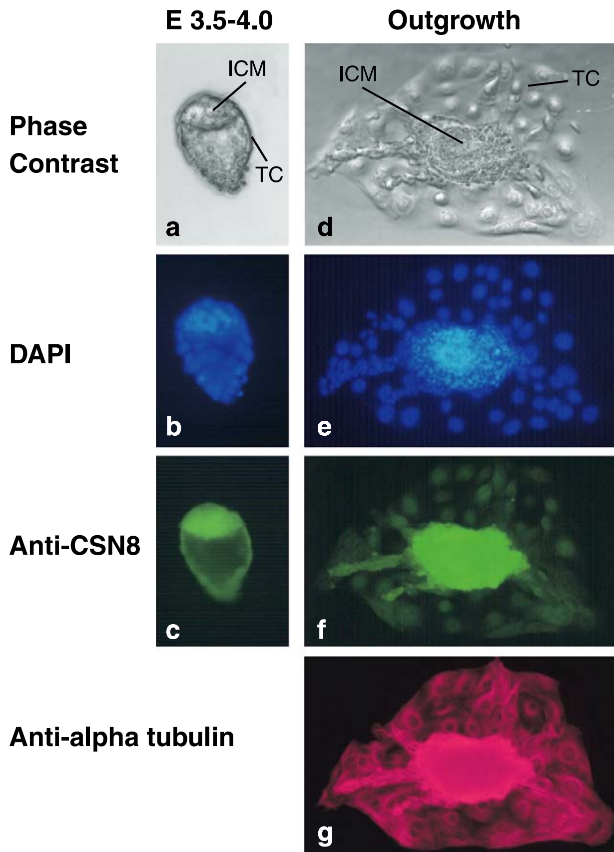

Fig. 4. Localization pattern of the Csn8 protein in mouse preimplantation embryos and blastocyst outgrowth. Newly isolated E4.0 blastocysts (a) or the blastocysts that have been allowed to outgrow for 3 days in culture (d) were immunoflourescently stained with anti-Csn8 (c and f), anti-tubulin (g) and a nuclei marker DAPI (b and e). Arrows point to ICM and TC. Csn8 is highly expressed in ICM cells and also in the nuclei of the trophectoderm cells.

ICM and trophoblast cells (Fig. 4g). The cell nuclei were stained with DAPI (Fig. 4a and c).

3.4. Postimplantation expression of Csn8 protein

To examine the expression pattern of Csn8 in postimplantation mouse embryos, we conducted whole amount immunostaining on E9.5 embryos. As shown in Fig. 5, the Csn8 protein was widely expressed in most parts of the embryo, and it appeared to be enriched in somite, bronchial arch, cardio-system, limb buds and parts of the brain. The overall pattern of Csn8 expression is similar to the recently reported anti-Csn2 (Alien) whole-mount immunostaining (Tenbaum et al., 2003), although the anti-Csn2 antibody appears to stain particularly strong in the neural crest cells compared to anti-Csn8.

We have identified the murine *Csn8* cDNA, which contains an open reading frame (ORF) giving rise to a protein of 209 amino acids. Murine Csn8 has a PCI domain, which has postulated to act as subunit interaction domain within the complex. The murine *Csn8* gene contains eight exons, encompassing approximately 12-kb region on chromosome one.

Little is known about the role of Csn8 in vertebrate development. Our data showed that *Csn8* expresses very early in embryo development and is expressed in embryonic stem cells. This expression pattern would be consistent with a role of the CSN complex in early embryogenesis and in organogenesis which involve complex cell proliferation and differentiation events. Indeed, CSN has been implicated in a wide range of cellular activities and developmental processes including a role in cell cycle control in mammalian cells (Malingham et al., 1998; Tomoda et al., 1999; Yang et al.,

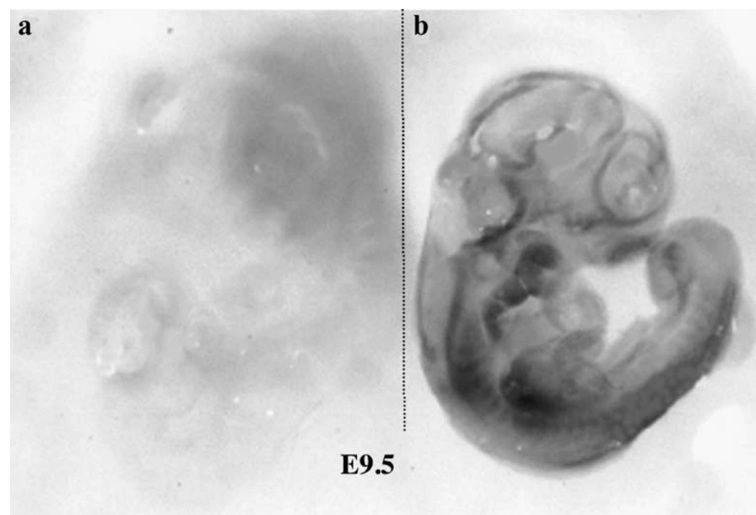

Fig. 5. Whole mount immunostaining of E9.5 embryo. Embryos were isolated from a pregnant female mouse of timed mating at E9.5 day. The embryos were stained with (a) pre-immune serum or (b) anti-Csn8 antibody as described in Section 2. Csn8 appears to express in most of the organs and tissues and is enriched in certain organs, a pattern that is consistent with the hypothesis that Csn8 is involved in multiple developmental processes during vertebrate embryogenesis.

2002). The widespread expression of Csn8 is consistent with a hypothesis that Csn8, or the CSN complex, is involved in multiple developmental processes. Further genetic and functional investigation will be necessary to reveal the specific functions of CSN in vertebrate development.

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