

# Calpain 2 expression pattern and sub-cellular localization during mouse embryogenesis

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**ABSTRACT** Regulation of migration and proliferation by calpain has been shown in various cell types; however, no data are available concerning calpain 2 (CAPN2) localization in embryonic tissues. Here, we report the expression pattern of CAPN2 during mouse embryonic development. Expression of the *capn2* gene is observed throughout embryonic development. From ES cells and the 8-cell stage to late neurulation stages, CAPN2 is expressed in the cytoplasm and nuclear compartments, with a clear co-localisation with chromatin. Whole-mount *in situ* hybridization analysis from E8.5 to 14.5 stages indicates high levels of *capn2* expression in the nervous system, heart and mesodermal tissues. Up-regulation is maintained during later developmental stages in proliferating cells and in precursor cells involved in muscle (myoblasts) or bone formation (chondrocytes). At later developmental stages, elevated mRNA levels coincided with CAPN2 nuclear localization in these cell types, while differentiated cells maintained cytoplasmic expression. This detailed analysis reveals dynamic expression: nuclear localization was associated either with active cell mitosis in embryonic stem cells and early developmental stages or with precursor cells later during organogenesis. Thus, these data indicate that CAPN2 may represent a key factor in development from the first cell division.

**KEY WORDS:** *calpain2, mouse embryogenesis, subcellular localisation*

## Introduction

The calpain family of proteases has been associated with multiple functions such as migration, adhesion, and apoptosis. The key function of calpain 2 (CAPN2) in cell motility was demonstrated by the potential of talin proteolysis and consequently the regulation of dynamic adhesion (Franco *et al.*, 2004). However, recent data suggest a role in cell cycle regulation, specifically during mitosis (Honda *et al.*, 2004). Several studies have shown that calpains play a role in the proteolysis of nuclear transcription factors associated with proliferation and progression through the cell cycle such as c-Myc, p53, Jun and Fos (Carillo *et al.*, 1996, Huttenlocher *et al.*, 1997, Watt and Molloy, 1993, Welm *et al.*, 2002). Moreover calpains are involved in proteolysis of cyclin proteins such as cyclin D1 (Qin *et al.*, 2003).

Targeted inactivation of *capn2* leads to the death of homozygous mutants after the first zygote divisions (Dutt *et al.*, 2006). These recent data indicate an essential role of *capn2* concerning cell survival during development. We have shown previously that

CAPN2 is localized in the nucleus of proliferating myoblasts (Raynaud *et al.*, 2004) and proliferating cells in the adult brain (Konig *et al.*, 2003). In quiescent myoblasts (G0 phase), cytoplasmic, but no nuclear localization was detected. The close association between CAPN2 and chromosomes suggests a possible role in the regulation of mitosis (Raynaud *et al.*, 2004).

In order to assess the presence of CAPN2 during development, for which no data were available yet, we analyzed the expression pattern and the sub-cellular localization in mouse embryos.

## Results and Discussion

We examine the localization of calpain 2 at the protein and mRNA levels between the first cell divisions and E14.5 days of mouse development. *In situ* hybridizations with murine and hu-

*Abbreviations used in this paper:* CAPN, calpain; ES, embryonic stem; ISH, *in situ* hybridization.

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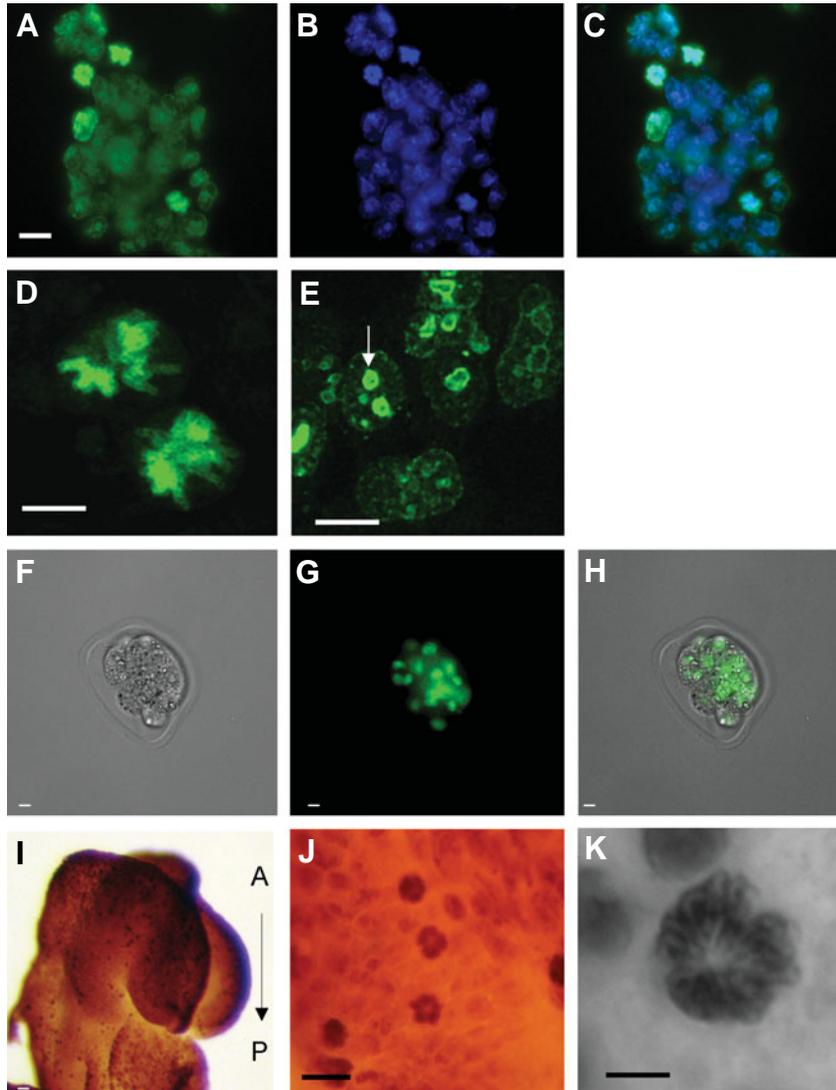
man probes were performed in parallel and gave identical results.

### Cytoplasmic and nuclear localisation of CAPN2 at early developmental stages

As no data concerning protein distribution during embryonic development were available, our study relies on a report showing that *capn2* is necessary for the survival of the 8-cell stage embryo (Dutt et al., 2006). As the question of the presence and localiza-

tion of the protein was essential to analyze at very early stages, CAPN2 expression was explored in ES cells. The 8-cell stage corresponds to the beginning of compaction process that will lead to fully compact morula embryo. This stage was important to explore because it represents the survival stage for mutant embryo.

In mouse ES cells (Fig. 1A-E) as well as 8-cell embryos (Fig. 1F-H), CAPN2 (green) is localized in the cytoplasm and in the nucleus. Close association with chromosome DNA (DAPI staining) was particularly prominent in mitotic figures and in particular when metaphase plate was observed (Fig. 1A,C,D). We also performed immunohistochemistry on whole embryo at E8.5: CAPN2 was expressed ubiquitously with intense labelling in mitotic cells (Fig. 1I-K) throughout the embryo.



**Fig. 1. Nuclear localization of CAPN2 during early developmental stages: from ES cells to E 8.5 embryo.** CAPN2 expression was observed in all ES cells (A-E) in the cytoplasm as well as in the nucleus; green in (B,C). Nuclei position was assessed with DAPI nuclear staining (B). The close association of CAPN2 with DNA and in particular with chromosomes during metaphasis highlights the nuclear localization (D). These high magnification images were taken at low gain to avoid saturation of the detector by the intense chromosomal signal; as a consequence, the weaker cytoplasmic signal is not visible here (D-E). (E) Nucleolus labelling (arrow). CAPN2 expression was observed in all cells of 8-cell stage embryo with a strong nuclear signal (F-H). (F) Image with transmission light; (G) same embryo with fluorescent staining; (H) merged image. Whole-mount immunohistochemistry at E 8.5 (I-K). CAPN2 labelling was observed within the whole embryo (I) with very dark spots corresponding to mitotic figures (metaphases plate) with condensed chromosomes [antero-posterior (A-P) orientation is indicated]; higher magnifications of chromosome labelling (J,K). Scale bars, 10  $\mu$ m.

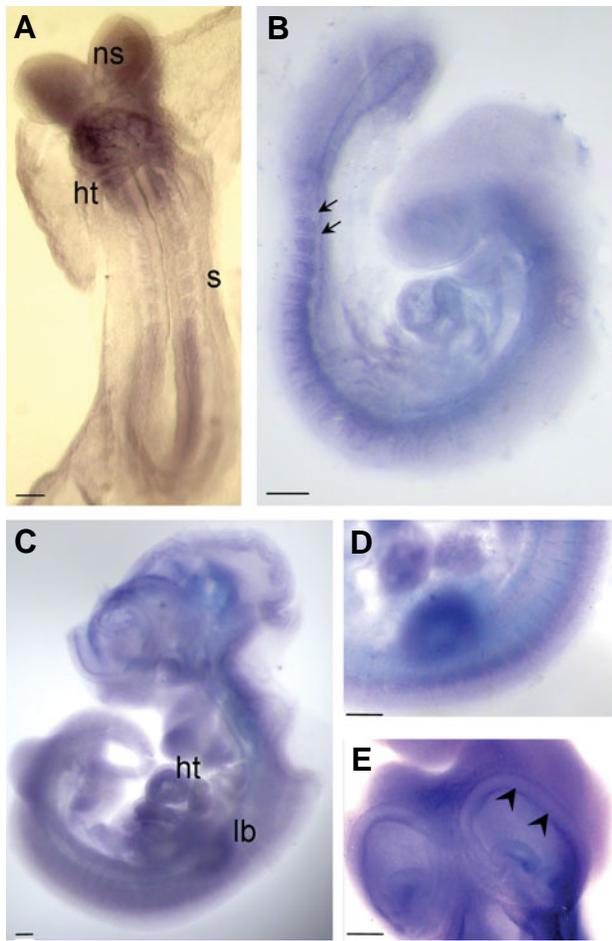
### Expression of the *capn2* gene between E8.5 and E11.5

Expression pattern of *capn2* was studied from embryonic day 8.5 to 11.5 (E8.5-E11.5) by *In situ* hybridization (ISH) in whole-mount embryos.

At E8.5 and E9.5, *capn2* expression is observed in most tissues with high mRNA levels in the nervous system, heart and somites (Fig. 2A,B). In order to obtain a more detailed view of the somite expression at E9.5, vibratome sections were performed and counterstained with DAPI nuclear marker to outline the tissue after the ISH processing (Fig. 3A-E). Sagittal sections through the whole-mount preparation of E9.5 embryos indicated restricted expression in the ventral half of the somites, while in the dorsal part of the somites no signal could be detected (Fig. 3C-E). The ventral region will give rise to the sclerotome progenitors and is specified as early as E9.5 by transcription factor expression (Goldman et al., 2000; Tam et al., 2000). At E10.5 (Fig. 2 C,D) and E11.5 (Fig. 2E), *capn2* mRNA labelling was also found at high levels within the whole embryo with particularly strong labelling of the limb buds, the branchial arches and somites (Fig. 2 C,D) and in the telencephalic ventricular zone (Fig. 2 D). At these early developmental stages, *capn2* expression was relatively broad and present in cells with elevated proliferation, most tissues, or intense cell migration or organogenesis (limb buds, heart, somites).

### Restricted intense expression at later developmental stages

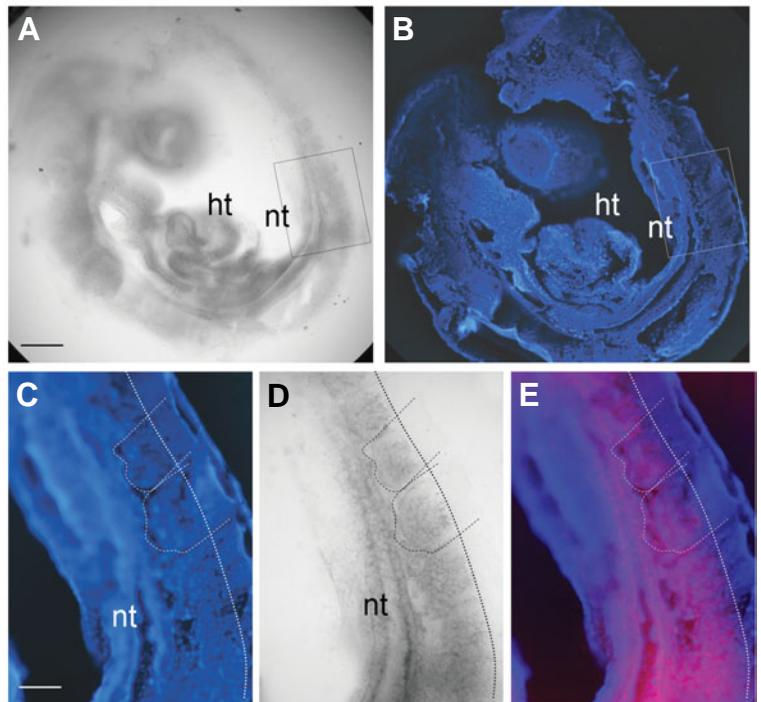
At later developmental stages (E14.5), frozen sections were performed. Elevated *capn2* mRNA signal was detected in restricted cell types (Fig. 4A-I). We observed strong expression in myoblasts engaged in muscle fiber formation. Cell identity was assessed using a *myoD* probe on serial sections of the same embryos (Fig. 4B). Myoblasts expressing *myoD* were also positive



**Fig. 2. Expression of calpain2 in whole-mount E8.5-11.5 embryos.** Whole-mount in situ hybridization at E8.5 (A), E9.5 (B), E10.5 (C,D) and E11.5 (E). (A) mRNA is detected within the nervous system (ns), heart (ht) and somites (s). At E9.5 capn2 expression is observed within the whole embryo and at a higher level in the mesoderm: the somite boundaries are highlighted; see arrows in (B). At E10.5, capn2 mRNA was maintained at high levels in the heart (ht) and the limb buds (lb) (C). Higher magnification of the somites (D). At E11.5 (E), capn2 expression underlined the ventricular surface within the forebrain (arrowheads). Scale bars, 70  $\mu$ m (A-D), 140  $\mu$ m (E).

for the *capn2* probe (Fig. 4A-B, arrowheads). The expression of both genes, *myoD* and *capn2*, is coherent with previous studies on the regulation of human *capn2* transcription by *myoD*/*myogenin* (Dedieu *et al.*, 2003). This regulation is probably conserved during mouse development. Identical patterns were found for the thoracic muscle cell precursors, the shoulder muscles (D, arrow), the tongue and limb muscles (data not shown).

*Capn2* was also expressed in a specific cell type, the chondrocytes within the cartilage primordia of the ribs (Fig. 4A-C arrows). On longitudinal sections of the developing forelimb, strong expression was noticed at the chondrocyte level. (Fig. 4E-F). Within the growing bone, a specific proliferative area, the growing plate of the epiphysis, is characterized by proliferation of chondrocytes and their hypertrophy. This typical feature was clearly recognizable and *capn2* expression was maintained in hypertrophic chondrocytes (Fig. 4F). Nuclear localisation of CAPN2 in chondrocytes was also clearly observed on bone sections. It has been shown that calpains are necessary for the cartilage mineralization mediated by chondrocytes. This mineralization process occurs through proteoglycan proteolysis achieved by calpain activity (Yasuda *et al.*, 1995). *Capn2* is involved in the major remodelling occurring



**Fig. 3. Expression of calpain2 in the somites of an E9.5 embryo.** Sagittal vibratome sections (30  $\mu$ m) through a hybridized E9.5 embryo (A,D) counterstained with DAPI (B,C). Expression was confined to the ventral part of the somites and the adjacent neural tube (A-B). Higher magnification of the somites (C-D) and a combined image (E). The dotted line indicates the capn2 expression limit. Expression is mostly detected in ventral mesodermal cells. Columnar epithelial cells of the dorsal half are devoid of signal (C-E). nt, neural tube; ht, heart. Scale bars, 70  $\mu$ m (A,B), 12  $\mu$ m (C-E).

during cartilage growth. *In vitro* studies using mouse pre-osteoblasts indicated that CAPN2 activity increases during differentiation of osteoblastic cells and is regulated by Bone Morphogenic Proteins, which are well-known regulatory factors throughout embryogenesis (Murray *et al.*, 1997).

Restricted *capn2* expression was observed in the nervous system. At this stage, the brain has undergone extensive morphogenic changes generating complex neural structures where cellular differentiation is progressing. The proliferation at the hind-brain-spinal cord level has decreased by E13.5-E14.5 (Haubensak *et al.*, 2004) and is limited to small regions. *Capn2* positive cells were then confined to few positive cells in the ventricular zone of the neural tube (Fig. 4G,H). Moreover sub-cellular localisation indicates that cytoplasmic CAPN2 was observed in post-mitotic neurons in the mantle region, while nuclear staining was observed in ventricular precursors (Fig. 5A, arrows). Neither dorsal root ganglia nor cranial ganglia were labelled except the trigeminal ganglion, where all cells showed *capn2* mRNA (Fig. 4I).

Taken together, our results suggest that *capn2* is highly expressed both in proliferating precursor cells and in differentiating cells in a transition process (chondrocytes, migrating myoblasts).

We have previously demonstrated that the sub-cellular localization of CAPN2 in the myoblast cell line C2.7 is correlated with the quiescent versus proliferating status of the cells: nuclear localization was associated with proliferation while cytoplasmic localization was linked to quiescent and differentiating conditions (Raynaud *et al.*, 2004).

In order to further assess the physiological meaning of these previous results, we analyzed the sub-cellular localization of CAPN2 in primary embryonic explants.

#### Sub-cellular localisation and differentiation status

To further analyze the relationship between proliferation and nuclear localization, we used primary culture of embryonic cells from various tissues of E10.5 embryo.

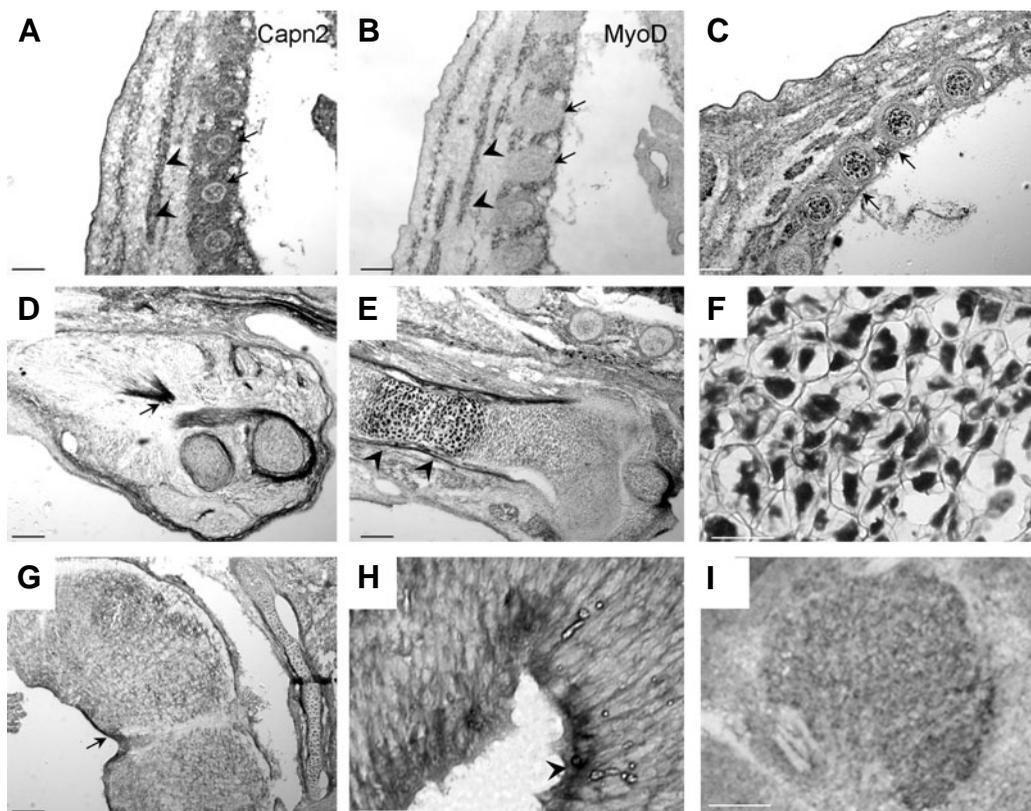
Neural tube explants from the head region generated neurosphere-like structures. After one day in culture, cells started to differentiate into neurons extending neuritic processes and expressing beta3tubulin (Fig. 5D-F). We performed double labelling with CAPN2 and anti-beta3tubulin antibodies. Nuclear CAPN2 (red, arrowheads) was never observed in post-mitotic beta3tubulin (green, arrows) positive neurons (Fig. 5D-F). In a previous study, we reported nuclear CAPN2 expression in adult nervous tissue. Interestingly, expression was mainly observed in areas where adult neurogenesis is happening, namely the rostral migratory stream toward the olfactory bulb and the subventricular zone of the striatum (König *et al.*, 2003). Nuclear localization of CAPN2 may therefore be considered as a marker of neural proliferating cells in embryonic as well as adult tissues. CAPN2 nuclear localization and activity have been demonstrated in cerebellar granule cells in culture. In granule cells, nuclear activity is associated with selective CaMKIV proteolysis (Tremper-Wells and

Vallano, 2005).

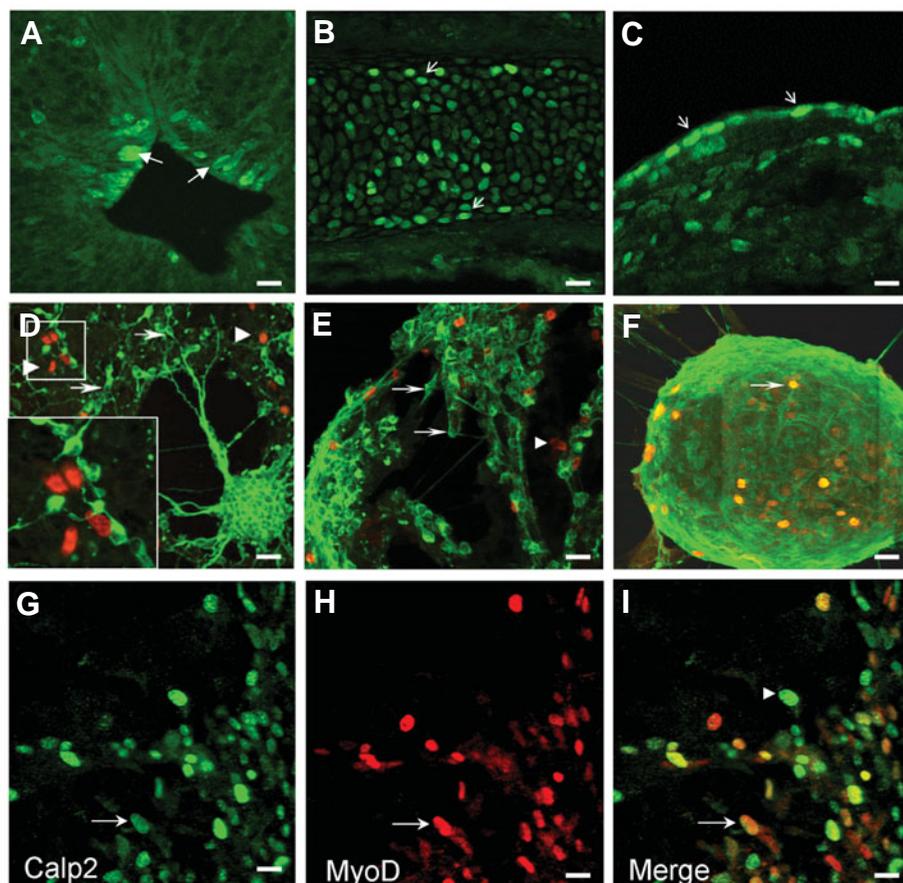
Explants from the trunk and caudal region were used to generate primary myoblast cultures. Myoblast labelling was comparable to that of neuronal cells: undifferentiated cells showed nuclear CAPN2 expression (Fig. 5G-I, green). Co-labelling with myoD transcription factor assessed myoblast identity (red). The merge figure shows that both markers co-localized (Fig. 5I, arrow), whereas some fibroblasts were labelled by CAPN2 only (Fig. 5I, arrowhead). Our results are in agreement with the previous observation of CAPN2 sub-cellular localization during the C2.7 cell differentiation. Cytoplasmic localization was correlated with the differentiation status of the myotubes (Raynaud *et al.*, 2004).

In summary, we show that *capn2* is expressed during mouse embryonic development as soon as the first cell divisions, and maintained during later stages in specific cell types at the nuclear level. Previous expression analysis of the other calpains indicated distinct spatial and temporal patterns. Calpain 3 expression starts in myotome cells at E11.5 in mouse (Fougerousse *et al.*, 1998, 2000). Calpain 4 (the regulatory subunit of calpains) is expressed at the first zygote stages and later on (Arthur *et al.*, 2000; Zimmerman *et al.*, 2000) in an ubiquitous pattern, which is also the case for calpain 1 (Azam *et al.*, 2001). Here we show that CAPN2 is expressed in a ubiquitous pattern at the earliest stages of development and becomes tightly regulated during organogenesis and differentiation steps of development.

Nuclear localization is probably linked to chromosomal activity since transient inactivation in cell culture indicated that CAPN2 is necessary for proper chromosome alignment at the metaphase plate (Honda *et al.*, 2004). Taken together, the results of the targeted *capn2* gene inactivation (embryonic lethality after the 8-



**Fig. 4. Calpain2 expression at E14.5.** In situ hybridization on 14  $\mu$ m frozen sections showed *capn2* expression in myoblasts (A,B), chondrocytes (C-F) and neural cells (G-I). Sagittal sections through the thorax showing *capn2* mRNA in myoblasts (A), identified with myoD probe on adjacent sections (B). Identical precursor groups were labelled with both markers (arrowheads). Strong signal was also observed in the rib primordia which were devoid of myoD expression; see arrows in (B). Section through the shoulder also showed myoblast labelling; arrow in (D). Labelling of chondrocytes was observed in sections of forelimb bones (D,E) and higher magnification confirmed their hypertrophic status (F). Strong *capn2* expression was detected within neural tissue in cells of the ventricular zone at the hindbrain level (Garrow, H, arrowhead). *Capn2* was expressed in all cells of the trigeminal ganglion (I) but not in other cranial or dorsal root ganglia. Scale bars, 250  $\mu$ m (A-E, G), 22  $\mu$ m (F, H, I).



**Fig. 5. Nuclear localization of CAPN2 during organogenesis and in explant tissues.** On frozen sections, strong expression at the protein level was observed in: neural cells (A), chondrocytes (B) and fibroblasts (C). (A) Prominent nuclear labelling was detected in proliferating neural cells (arrows), and cytoplasmic staining was observed in post-mitotic neurons in the neural tissue. Within the growing epiphysis of the bones, chondrocytes were labelled at the nuclear and cytoplasmic levels (B, arrows). Fibroblasts showed mainly a nuclear CAPN2 localization in the conjunctive tissue (C, arrows). Tissue explants of E10.5 embryos from head and trunk regions generated neurosphere-like structures (D-F) and primary myoblasts (G-I). Immuno-localization of CAPN2 (red, arrowheads) indicates that neuronal cells loose nuclear expression during differentiation, which was assessed by co-labeling with the post-mitotic neuronal marker  $\beta$ 3tubulin (green, arrows) (D-F). Neurosphere-like structures with ongoing proliferation maintain nuclear expression (arrow) (F). Myoblasts were identified based upon *myoD* expression (red, H) and co-labelling indicated that most of the myoblasts retained nuclear expression of CAPN2 (arrow, G, I). Fibroblasts were only labelled by CAPN2 antibody (green, arrowhead, I). Scale bar, 10  $\mu$ m.

cell developmental stage, Dutt *et al.*, 2006), and the nuclear expression in ES cells as well as the 8-cell embryos, indicate a decisive role during cell mitosis and/or cell cycle regulation. Moreover our present report and previous *in vitro* data support the notion that CAPN2 can be shuttled between nuclear and cytosolic compartments and has distinct functions in proliferating and differentiated cells (Shao *et al.*, 2006). These data constitute a strong basis to understand the role of CAPN2 during embryonic development.

## Materials and Methods

### Animals

All experiments were conducted in accordance with the European

Communities Council Directive and INSERM guidelines. Pregnant OF1 mice were purchased from Charles River Research Laboratories (L'Arbresle, France). The day after nocturnal mating was considered as embryonic day (E) 0.5. 8-cell stage embryos were collected between 68 and 72 hours after the record of the vaginal plug. Embryos were flushed from the oviducts with culture medium, staged and then fixed in PFA4% (Honda *et al.*, 2005). For *In situ* hybridization (ISH) and immunohistochemistry, embryos were obtained from pregnant females at E 8.5 to 14.5 stages. Embryos were collected in cold PBS, fixed by immersion in 4% paraformaldehyde, dehydrated in methanol, and stored at -20 °C until processing. After re-hydration, whole embryos or sagittal cryostat sections (14  $\mu$ M) were processed for ISH.

### In situ hybridization

We used mouse and human *capn2* cDNA as templates for riboprobe: the human full length cDNA fragment (GenBank Accession number: M23254) shows 88% identity with the mouse cDNA (GenBank Accession number Y10139) (cloning vector pCAF1-calp2). The sequence verified clone IRAVp968F10124D6 containing the mouse cDNA was obtained from the RZPD resource center. The mouse probe gave the same results as the human probe on whole-mount embryos as well as on cryostat sections. The *myoD* probe corresponded to the mouse *myoD* cDNA (GenBank Accession number: M18779, (Davis *et al.*, 1987)).

We used digoxigenin-labeled probes as described previously (Manley and Capecchi, 1995, Poluch *et al.*, 2003). Hybridization signal was detected using alkaline phosphatase-conjugated antibodies against digoxigenin (Roche, Mannheim, Germany) and BM purple substrate (Roche). Whole-mount embryo ISH was performed as previously described (Carroll *et al.*, 2001, Goddard *et al.*, 1996). Hybridization with the sense probes was performed on control embryos and serial frozen sections under the same conditions; no signal was detected in examined tissues. Vibratome sections (30 $\mu$ m) were carried out after clearing the embryos through serial glycerol solutions.

### Immunocytochemistry

Immunofluorescent staining was performed on ES cells and 8-cell stage embryos as described previously (Raynaud *et al.*, 2004). Whole-mount immunostaining was performed on E8.5 embryos as described previously using a secondary antibody against mouse IgG conjugated to horseradish peroxidase diluted at 1/400 (Jackson) (Rossel and Capecchi, 1999).

Mouse monoclonal antibodies against  $\beta$ 3 tubulin (Sigma) and against MyoD (Pharmingen) were diluted to 1/200. Rabbit anti-calpain2 was used at 1/100 as described previously (Raynaud *et al.*, 2004). Secondary antibodies directed against mouse IgG or rabbit IgG were conjugated to rhodamine or FITC (Santa Cruz); both combinations were used.

### Explant tissue culture and embryonic stem cells

E10.5 embryos were dissected and tissues were placed in skeletal

muscle growth medium (Pharmingen), maintained for 5 days in standard culture conditions and processed for immunocytochemistry. ES cell line BS1 was cultured as described previously (Papadimou *et al.*, 2005) and processed for immunofluorescence.

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