Developmental Biology 357 (2011) 259–268

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Phosphorylated β-catenin localizes to centrosomes of neuronal progenitors and is required for cell polarity and neurogenesis in developing midbrain

Dmitri Chilov [a,*], Natalia Sinjushina [a], Hannu Rita [d], Makoto M. Taketo [c], Tomi P. Mäkelä [a], Juha Partanen [a,b,*]

[a] Institute of Biotechnology, University of Helsinki, Finland
[b] Department of Biosciences, University of Helsinki, Finland
[c] Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[d] Faculty of Agriculture and Forestry, Department of Forest Resource Management, University of Helsinki, Finland

ARTICLE INFO

Article history:
Received for publication 16 November 2010
Revised 16 May 2011
Accepted 21 June 2011
Available online 26 June 2011

Keywords:
Wnt signaling
Neurogenesis
Cell polarity
Microtubule
Centrosome
Mitotic spindle
Asymmetric cell division
Midbrain

ABSTRACT

β-catenin has well-established functions in cell growth and differentiation as part of the Wnt signaling pathway and in regulation of cellular adhesion with E-cadherin. Here we studied its significance in midbrain development by temporally controlled deletion of *β-catenin* allowing simultaneous analysis of complete (β-cat-null) and partial (β-cat-low) loss-of-function phenotypes in progenitor cells. β-cat-null cells did not contain centrosomes or a microtubule network and were unpolarized forming delaminated bulges. β-cat-low cells displayed defects in the orientation of the mitotic spindle, increased asymmetric cell divisions and premature differentiation in absence of alterations in polarity or adhesion. The spindle defect was associated with decreased centrosomal S33/S34/T41 phosphorylated β-catenin (p-β-cat) and centrosomal and microtubule defects. Interestingly, neural progenitor cells in mice expressing only unphosphorylatable β-catenin share several phenotypes with β-catenin loss-of-function mice with defects in microtubules and polarity. The results demonstrate a novel function for p-β-cat in maintaining neuroepithelial integrity and suggest that centrosomal p-ß-cat is required to maintain symmetric cleavages and polarity in neural progenitors.

Introduction

The Wnt/β-catenin (β-cat) signaling pathway is important for embryogenesis via the regulation of patterning, cell fate decisions, and cell polarity. A well-characterized part of the pathway involves regulation of the amount of β-cat available to act as a co-activator in transcription through β-cat phosphorylation and regulation of its stability. In the absence of Wnt ligands, cytoplasmic β-cat is phosphorylated on Ser-33, Ser-37, and Thr-41 by glycogen synthase kinase 3β (GSK3β) in a destruction complex with APC, axin1, and axin2/conductin leading to ubiquitination and degradation of the cytoplasmic phosphorylated β-cat (p-β-cat) in the proteasome. Binding of Wnts to cell surface receptors releases β-cat from the destruction complex and results in accumulation and nuclear translocation of unphosphorylated β-cat, which acts as a co-activator for TCF/LEF to regulate transcription and promote proliferation during organogenesis (reviewed in (Grigoryan et al., 2008; Huang and He, 2008)). In neuronal development transcriptional regulation by Wnt/β-cat is important in the spinal cord and forebrain (Chenn and Walsh, 2002; Machon et al., 2003, 2007; Megason and McMahon, 2002; Woodhead et al., 2006; Zechner et al., 2003, 2007) and for patterning, cell proliferation and neuron differentiation also in the developing midbrain (Andersson et al., 2008; Castelo-Branco et al., 2004, 2005; Chilov et al., 2010; Panhuysen et al., 2004; Prakash et al., 2006; Tang et al., 2009).

β-catenin also plays a pivotal role in regulating cell adhesion as a component of adherens junctions (AJ) suggested to be important in neuronal development. Conditional deletion of β-cat early in cortical development causes disruption of the neuroepithelium, loss of cell polarity, and apoptosis (Brault et al., 2001; Junghans et al., 2005; Machon et al., 2003). These effects were primarily attributed to the breakdown of cadherin-based cell adhesion (Junghans et al., 2005; Tang et al., 2009) although a direct effect of β-cat on neuronal nuclear translocation through influencing cytoskeleton dynamics was also considered (Machon et al., 2003).

More recently, β-cat has also been identified at centrosomes and implicated in both anchoring microtubules (MTs) to the centrosome (Huang et al., 2007) and in regulation of centrosome cohesion in cultured cell lines (Bahmanyar et al., 2008; Hadjihannas et al., 2010).

* Corresponding authors.
E-mail addresses: Dmitri.chilov@helsinki.fi (D. Chilov), Juha.M.Partanen@Helsinki.Fi (J. Partanen).

0012-1606/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2011.06.029

Centrosomal β-cat is also phosphorylated by GSK3β and this phosphorylation is inhibited following Wnt signals. However, the centrosomal p-β-cat appears to be less susceptible to degradation as it is readily detectable in the absence of Wnt signals (Hadjihannas et al., 2010) suggesting a specific role for p-β-cat in centrosomes.

For neuronal development, it is crucial to establish apico-basal polarity in the ventricular zone progenitor cells and precisely regulate the plane of their division. Symmetrical vertical divisions are thought to be important for maintenance of progenitor cell identity, whereas oblique asymmetric divisions result in neurogenic cell cycle exit. Both apico-basal polarity and regulation of cell cleavage plane depend on the MT network and centrosome function. Several centrosomal proteins regulating mitotic spindle orientation in neuronal progenitors have been identified (reviewed in Fietz and Huttner, 2010). Interestingly, some of these have been recently shown to interact with β-cat (Godin et al., 2010a, 2010b).

Here we have dissected the roles of the various β-catenin functions in neurogenesis in the embryonic midbrain using both temporally controlled inactivation of β-cat as well as replacement of β-cat with a non-phosphorylatable stabilized form. We find phosphorylated β-cat in centrosomes of neuroepithelial progenitors, and suggest involvement of p-β-cat in maintenance of MTs, orientation of the mitotic spindle and regulation of the neurogenic cell-cycle exit.

Methods

Generation and genotyping of mice and embryos

Generation and genotyping of an *Engrailed 1 (En1)* allele carrying Cre-recombinase knock-in (*En1*^{cre}; Kimmel et al., 2000), Rosa26 locus carrying tamoxifen inducible *R26cre-ert* allele (Vooijs et al., 2001), conditional *β-catenin* inactivating allele (*β-cat*^{flox}) (Brault et al., 2001), and the conditional stabilizing *β-catenin* allele (*β-catenin*$^{flox(ex3)}$) (Harada et al., 1999) were described elsewhere. Herein *R26*$^{cre\text{-}ert/+}$; *β-catenin*$^{flox/flox}$ embryos are called β-cat^{lof} (for loss of function), *En1*$^{cre/+}$; *β-catenin*$^{flox(ex3)/flox}$ as β-cat$^{non\text{-}phospho}$ and *En1*$^{cre/+}$; *β-catenin*$^{flox(ex3)/+}$ as β-cat^{stab} in accordance with (Chilov et al., 2010). For staging, the day of vaginal plug was counted as embryonic day 0.5 (E0.5). For BrdU incorporation analysis, pregnant females were given intraperitoneal injection of BrdU (3 mg/100 g body weight) 1 h before dissecting the embryos. To induce Cre-recombinase in *R26cre-ert* mice, pregnant females were given intraperitoneal injection of tamoxifen (Sigma) (8 mg/40 g body weight) 48 h prior to embryo harvesting. All animal work has been conducted according to relevant national and international guidelines. Approval has been obtained from the Finnish Committee of Experimental Animal Research.

Immunofluorescence

Immunofluorescent staining on paraffin sections was performed as described previously (Jukkola et al., 2006). Primary antibodies used were mouse monoclonals against BrdU (GE Healthcare); HuC/D (Invitrogen, Eugene, OR); pancadherins, γ-tubulin (Abcam, Cambridge, UK); aPKC, β-catenin, p27, EB1 (BD South San Francisco, CA); ZO1 (Zymed, South San Francisco, CA); and α-tubulin (Developmental Studies Hybridoma bank, Iowa City, IA). Rabbit polyclonal antibodies used were against SOX2, PAR3 (Millipore); β-catenin, γ-tubulin (Sigma, Saint Louis, USA); and phospho-β-catenin (Ser33/37/Thr41; Cell Signaling, #9561), α-tubulin (Cell Signaling), phosphohistone H3 (Upstate, Lake Placid, NY). All images were acquired using consecutive channel laser scanning.

Explant and cell culture

E9.5 embryos were dissected from NMRI females followed by isolation of rhombomere1/midbrain tissues. The neuronal tube was opened dorsally, placed on filters and positioned on organotypical culture grids. The explants and MDCK cells were cultured in DME medium with 10% fetal calf serum in presence or absence of 5 μM of BIO (Calbiochem) for 2 days. MDCK cells were obtained from ATCC (CCL-34).

Microscopy

Confocal images were acquired using the Leica TCS SP5 confocal system and LAS-AF software. Confocal stacks and images were processed and deconvoluted using Imaris 6.1 (Bitplane) and AutoQuantX (AutoQuant) software. All multi-color images were acquired using consecutive channel laser scanning. Co-localization of p-β-cat and γ-tubulin was analyzed using image-processing software Imaris. The program calculates a co-localization channel based on pixels that feature both green and red channel intensities above a certain threshold level. To increase stringency of calculation, pixels with intensities lower than 25% of average were cut off. EB1 particle numbers were calculated using ImageJ software.

Statistical analyses

The division plane angles were measured using ImageJ software and analyzed using two-sample *t*-test.

Results

To identify β-catenin functions in neurogenesis in the embryonic midbrain a strategy circumventing lethality associated with early deletion (Brault et al., 2001) was required. For this we generated mice with a widely expressed tamoxifen-inducible deletor allele (*R26cre-ert*) (Vooijs et al., 2001) together with a conditional *β-catenin* allele (Brault et al., 2001). Deletion of the floxed region of *β-catenin* in *R26*$^{cre\text{-}ert/+}$; *β-catenin*$^{flox/flox}$ embryos (abbreviated herein as β-cat^{lof} for loss of function) (Chilov et al., 2010) was induced by intraperitoneal injection of tamoxifen into pregnant dams at E9.5 (Brault et al., 2001; Vooijs et al., 2001) 2 days prior to harvesting of the embryos to analyze neurogenesis in the midbrain neuroepithelium.

Analysis of E11.5 midbrain sections of β-cat^{lof} embryos revealed occasional discontinuities in the neuroepithelial lining where cells were delaminated and bulged into the ventricle. β-cat staining revealed that cells in these bulges were β-cat protein null (Figs. 1e–h, arrows). In addition, there were areas of cells with altered β-cat staining within an apparently intact neuroepithelium (Figs. 1e–h). In these areas basolaterally localized β-cat staining was absent, whereas β-cat adjacent to apical cell edges was still present suggesting that these cells had undergone recombination recently and had partial loss of β-cat protein. These cells are referred to herein as "β-cat-low" to distinguish them from the genotypically identical β-cat-null cells.

β-catenin deficiency leads to loss of cell polarity

To investigate polarization of β-cat-low and β-cat-null cells, E11.5 midbrain sections were stained with the major apical polarity complex proteins PAR3 and atypical PKC (aPKC), which are critical for formation and the maintenance of mammalian neuroepithelial cell polarity, maintenance of cell asymmetry and thereby progenitor cell proliferation and differentiation (reviewed in Barnes and Polleux, 2009; Suzuki and Ohno, 2006). In β-cat-low cells with apically localized β-cat protein all polarity markers as well as cadherins are comparable to controls (Figs. 1e, f, g, and h). In contrast, in β-cat-null cells PAR3 is undetectable and aPKC is redistributed from the apical membrane (Figs. 1e and f, arrows). In addition the apical marker ZO1 is absent in β-cat-null cells (Fig. 1g, arrow). Thus, loss of β-cat leads to pronounced defects in polarity in midbrain neural progenitors. The delamination of the cells is indicative of defects also in cell adhesion,