The role of Crumbs 2 in neural development

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Abstract

The formation of a functionally integrated nervous system is dependent on a highly organized sequence of events that includes timely division and differentiation of progenitors. Three evolutionarily conserved polarity protein complexes are crucial for defining the apical and basolateral boundaries of cells. In my thesis, I demonstrate that one of the vertebrate homologs of Crumbs- Crumbs2 (Crb2) plays context dependent roles in the developing nervous system using two model systems: the chick embryonic hindbrain and the mouse embryonic telencephalon.

In the developing telencephalon, conditional ablation of Crb2 leads to defects in recruitment of apical polarity proteins, cell junction proteins, positioning of mitotic cells and cortical neurogenesis.

In the chick embryonic hindbrain, misexpression of Crb2 affects morphology of the neural tube and also affects the apical localization of cell polarity proteins, mitotic cell divisions and neural differentiation. In addition to this, I demonstrate that a novel secreted splice variant of Crb2 plays an important role in regulating neural crest cell migration.

Taken together my analyses show that both loss and misexpression of Crb2 have similar effects on the apical domain and in confining mitotic cell divisions to the apical domain. This implies that the level of Crb2 is crucial for its various biological roles in the developing nervous system.

CHAPTER 1

Introduction to neural development and apical-basal cell polarity

1.0 Introduction

In my thesis, I have taken advantage of two model systems: a conditional knockout mouse model and the chick embryonic hindbrain to study the role of Crumbs 2 (Crb2) during neural development. The introduction aims to provide a general overview of 1. Neural induction and Neurulation 2. Cortical development 3. Segmentation of the hindbrain 4. Apical-basal cell polarity focussing mainly on the apical complex protein Crb2. Finally, I will outline the specific aims of my study.

1.1 Neural induction and neurulation

The central nervous system (CNS) is the most complex organ system in the vertebrate body. The complexity of the CNS belies its modest origin from a single sheet of polarized epithelium called the neuroectoderm (Gilbert, 2003). In the late 19th century, Ramon y Cajal proposed that the CNS is composed of discrete metabolic units. Cajal's exhaustive studies revealed the organizational complexity and precise connectivity between cells of the nervous system (Cajal, 1890, 1937; Guillery, 2005). More than a century after Cajal's studies, we are still trying to understand the mechanisms underlying the emergence of the complex CNS.

Neurons are individual functional units of the nervous system and are generated precisely in a spatio-temporal manner to mediate simple and higher order reflexes of vertebrates. Tight regulation of the specification, proliferation, migration and subsequent control of axonal path of neurons results in the intricate neural network observed in the adult CNS and the foundation for this neural circuitry is laid early during embryonic development (Gilbert, 2003).

Neural induction, the process by which naïve ectodermal cells adopt a neural fate over a non-neural fate is initiated during gastrulation (Wolpert et al., 1998; Zaraisky, 2007). Classical experiments in Xenopus

have shown that the cells underlying the prospective neural plate are a crucial source of neural inducing signals (Spemann and Mangold 1924, 2001; Harland, 1994). These signals induce the expression of neural-specific genes in competent ectodermal cells. The pathways implicated in neural induction are the BMP, FGF and Wnt signalling pathways (Wolpert et al., 1998). The induction of a 'neural-state' is not a single step process but involves sequential exposure to these signaling molecules (Wolpert et al., 1998; Stern, 1994).

As development progresses, the induced neural plate undergoes extensive morphogenesis. Initially, the neural plate elongates and bends around a medial groove. Subsequently, the neural folds elevate along the medio-lateral axis and the dorso-lateral apical surfaces of the neural folds meet, fusion occurs at the dorsal midline to form a neural tube with a lumen in the centre and this sequence of events occurring during late gastrulation is termed 'neurulation' (Wolpert et al., 1998) (Fig 1.1). The neural tube is formed beneath the overlying ectoderm and the dorsal most portion of the neural tube contributes to the neural crest cells (described in section 1.3.1).

The primitive neural tube can be grossly subdivided into a rostral part that develops into the brain and a caudal part that is the presumptive spinal cord. The rostral end of the neural tube enlarges and forms three linked primary vesicles – the prosencephalon, mesencephalon and rhombencephalon. The prosencephalon gives rise to the telencephalon and diencephalon. The rhombencephalon divides into the metencephalon and myelencephalon and connects to the presumptive spinal cord. Unlike the prosencephalon and rhombencephalon the mesencephalon does not expand significantly during subsequent brain development and remains as a single vesicle (Gilbert, 2003; Chizhikov & Millen, 2005) (Fig 1.2 B).

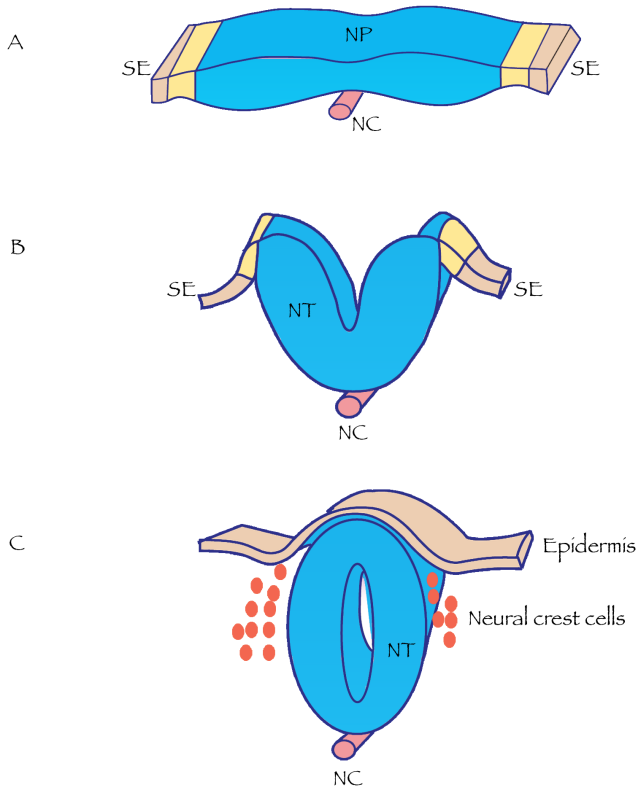


Fig 1.1 **Schematic illustration of neurulation.** The formation of the neural tube or neurulation involves three steps A- Induction of the neural plate B- Formation of neural folds and C-Formation of the neural tube. A. The neural plate is flanked on either side by the surface ectoderm and the notochord cells underlie the neural plate. B. The edges of the neural plate fold and elevate C. The neural folds meet in the midline to form the neural tube with a lumen/ventricle in the centre. The cells at the ends of the neural folds come to lie between the neural tube and the overlying epidermis, these cells delaminate and migrate out and are the neural crest cells. NC-notochord, NP-neural plate, NT-neural tube, SE- surface ectoderm.

Adapted from Wolpert, 1998.

Pioneering studies in chick embryos have indicated that the distinct demarcation of forebrain, midbrain, hindbrain and spinal cord territories may be defined in response to anteriorising and posteriorising signals (Fig 1.2 A) (Beddington & Robertson, 1999; Ericson et al., 1995; Patten & Placzek, 2002; Simon et al., 1995). These signals that pattern the neural tube along the anterior-posterior axis emanate from tissues such as anterior visceral endoderm, somites and notochord that lie in close proximity with the developing neural tube (Episkopou et al., 2001; Gavalas & Krumlauf, 2000; Muhr et al., 1997).

1.2 Organization of the forebrain

Forebrain organization involves patterning along the dorsoventral (DV) and anterioposterior (AP) axes (Rash & Grove, 2006; Rhinn et al., 2006). DV patterning specifies dorsal forebrain from the ventral forebrain. AP patterning delineates the telencephalon from the diencephalon and also specifies subdivisions of the telencephalon. The dorsal telencephalon gives rise to the cerebral cortex and the ventral telencephalon to the striatum, pallidum and septum (Kaufman & Bard, 1999).

A prosomeric model of forebrain development was put forward on the basis of morphological and gene expression boundaries (Puelles & Rubenstein, 1993; Rubenstein et al., 1994; Shimamura et al., 1995). At its core, the prosomeric model proposes sub-divisions of the forebrain into a grid-like pattern of neuromeric domains by AP and DV boundaries. These neuromeric domains called prosomeres are in turn grouped into diencephalon (prosomeres 1-3) and the secondary prosencephalon - the hypothalamus and telencephalon (prosomeres 4-6) (Rubenstein et al., 1994) (Puelles & Rubenstein, 2003). Apart from these transverse domains, the forebrain also shows organization along its longitudinal axis

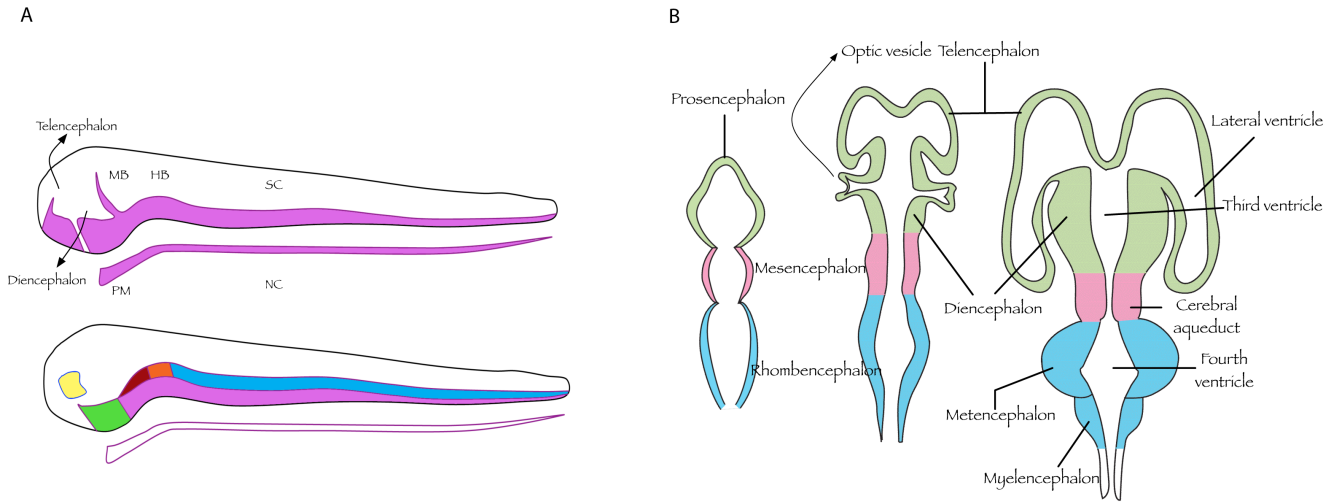


Fig 1.2 **Schematic illustration showing anterior-posterior patterning of the neural tube and formation of the three primary brain vesicles.** A. Signals along the anterior-posterior axis delineate the neural tube into four regions forebrain, midbrain, hindbrain and spinal cord. Signals from the underlying prechordal mesoderm and notochord confer dorso-ventral polarity to the neural tube. Specific cell types are generated with precise regional variation. Regions expressing Sonic hedgehog are indicated in pink. Different colours indicate the regional differences in cell types - Different colours indicate regional differences in the cell types . Yellow, ganglionic eminence; green, hypothalamic cells ; maroon, dopaminergic neurons; orange, serotonergic neurons; blue, motor and interneurons. PM-Prechordal mesoderm, MB-Midbrain, HB-Hindbrain, SC-Spinal cord, NC-Notochord. (Adapted from Patten and Placzek 2000) B. The rostral part of the primitive forebrain forms the telencephalon and the caudal part forms the diencephalon.The mesencephalon develops into the midbrain. The rostral and caudal parts of the primitive hindbrain form the metencephalon and myelencephalon respectively (Adapted from Kaufman and Bard 1999).

and these correspond to the roof, alar, basal and floor plates of the spinal cord (Shimamura et al., 1995).

Previous work has shown that gene expression profiles in the developing telencephalon correlates with morphological distinction of prosomeric boundaries and furthermore these genes play significant roles in defining position-specific identity of cells (Fishell, 1997; Shimamura & Rubenstein, 1997). In the telencephalon, specific transcription factors that include members of the distalless (Dlx), empty spiracles (Emx), forkhead (Fox), orthodenticle (Otx), paired-box (Pax) and sine oculis (Six) families are involved in the regional specification of the AP sub-divisions (Fishell, 1997; Shimamura & Rubenstein, 1997; Simeone et al., 1992). The expression profiles of some of the key transcription factors involved in patterning of the telencephalon are summarized in Fig 1.3 and Table 1.1.

1.2.1 Cortical histogenesis and proliferative zones in the cortex

Retroviral lineage tracing and birth-dating experiments have demonstrated that the cells lining the inner edge of the cortical wall contribute to the repertoire of cell types observed in the mature cerebral cortex (Price & Thurlow, 1988; Reid et al., 1995). The mammalian cerebral cortex is organized into six layers and each layer contains neurons with similar morphology. The cortical layers are laid down in an inside-out fashion (Fig 1.4). The early born neurons reside closer to their birth place whereas later born neurons migrate further to populate the superficial layers of the cortex (Angevine & Sidman, 1961; Berry & Rogers, 1965; Gotz & Bolz, 1992; Rakic, 1988).

Birthdating experiments using [^{3}H] thymidine were used to predict accurately the commitment of a neuronal cell to a particular laminar fate (Angevine & Sidman, 1961). In addition to these experiments, transplantation studies have shown that early cortical progenitors are multipotent and late progenitors have a more restricted fate potential. The association between the birth-date of a cell and its laminar fate made it

possible to study the timing of a cell's commitment to layer-specific neurons during development (McConnell & Kaznowski, 1991, Desai & McConnell, 2000).

It is generally accepted that during development the ventricular zone consists of a heterogenous cell population that includes: multipotent progenitors (Luskin et al., 1988), lineage-restricted progenitors (Williams & Price, 1995) and specified daughter cells (Reid et al., 1995). Initially, neuroepithelial cells span the width of the cortical wall and they undergo cell divisions to generate more of the proliferative cell population. The proliferating progenitors predominantly reside in a domain close to the ventricle and are called ventricular zone progenitors. Nuclei of the ventricular zone progenitors display interkinetic nuclear migration, where the position of the nuclei in relation to the ventricular surface is dependent on the phase of cell cycle. The nuclei move away from the ventricular surface during G1 and occupy outer ventricular zone during S-phase and undergo mitosis near the apical surface (Sauer, 1935). This dynamic nuclear migration gives a pseudo-stratified appearance to the neuroepithelium (Gotz & Huttner, 2005).

Table 1.1 List of some of the key genes involved in patterning of the telencephalon.

Gene	Expression pattern in the brain	Forebrain phenotype observed	Reference
Emx1	Dorsal telencephalon	Minor defects in forebrain development, reduced cortical plate thickness, disorganized fasciculation of anterior commissure and corpus callosum	Simeone et al., 1992 Qiu et al.,1996 Yoshida et al., 1997 Gorski et al., 2002
Emx2	Dorsal telencephalon and diencephalon	Decreased cortex size and absence of dentate gyrus	Simeone et al.,1992 Pellegrini et al., 1996 Yoshida et al., 1997
Pax6	Telencephalon and diencephalon	Loss of discrete prosomeric boundaries, aberrant cortical stratification and defects in telencephalic and diencephalic patterning	Stoykova et al., 1996, Warren and Price 1997, Gotz et al., 1998
Nkx 2.1	Ventral telencephalon	Defects in development of the septum and basal ganglia in the ventral telencephalon	Shimamura et al., 1995
Dlx1/2	Domains of diencephalon and ventral telencephalon	In Dlx1/2 double mutants, altered proliferation and differentiation in the basal telencephalic regions. No apparent patterning defects in the single mutants	Bulfone et al., 1993
Gli3	Telencephalon, dorsal mid and hindbrain	Reduced size of cortex, no defined boundary between telencephalon and diencephalon	Grove et al., 1998

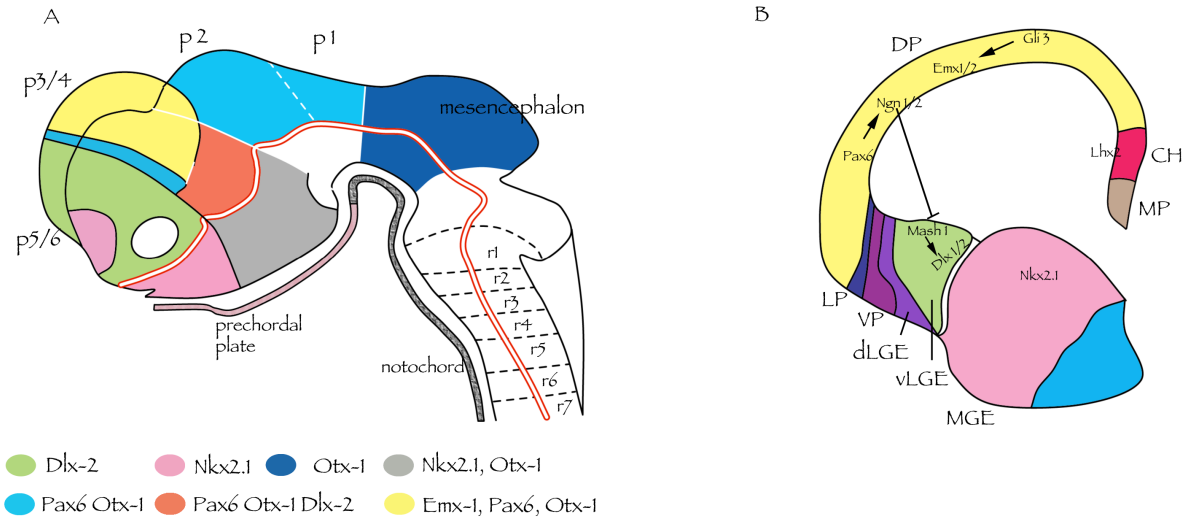


Fig 1.3 **Schematic representation of gene expression patterns and organization of the mouse forebrain.** A. The expression of Dlx-2, Emx-1, Pax6, Otx-1, Nkx2.1 is shown in the neural tube in E10.5 mouse embryonic brain. B. Schematic of a coronal secton through the telencephalon showing the dorsal and ventral domains defined by the gene expression patterns. In the dorsal telencephalon homeodomain proteins Emx1/2, Lhx2 and Pax6 are expressed. Nkx2.1 is expressed in the medial ganglionic eminence. In the ventral telencephalon Mash1 and Dlx1/2 are expressed. Cross-regulatory interactions between these genes maintains regional identity. r-rhombomeres, p- prosomeres, MP- medial pallium, CH-cortical hem, DP-dorsal pallium, LP-lateral pallium, VP- ventral pallium, dLGE - dorsal lateral ganglionic eminence, vLGE-ventral ganglionic eminence, MGE-medial ganglionic eminence. Adapted from Shimamura et al., 1995

For this section, I define neurogenesis as the generation of postmitotic cortical neurons and this take place over a period of seven days in the mouse – Embryonic ages E10.5 – E17.5 (Caviness, 1982) (Takahashi et al., 1995b). With the onset of neurogenesis, proliferating progenitor cells withdraw from the cell cycle and form the first cortical neurons. The cortical neurons migrate a short distance and form a distinct layer called the preplate. The preplate is subsequently divided by the cortical plate neurons into a superficial marginal zone that contains the Cajal-retzius cells and a deeper subplate (Allendoerfer & Shatz, 1994; Marin-Padilla, 1998).

With the onset of neurogenesis, neuroepithelial cells are transformed into radial glial cells (RGCs). RGCs are characterized by an intrinsic apical-basal polarity and long processes that contact both the apical and pial surfaces of the cortex. They act as scaffolds for the migration of newborn neurons to the cortical plate.

A second proliferative progenitor pool is present in the sub-ventricular zone -SVZ (Takahashi et al., 1995a) (Bayer & Altmann, 1991). The SVZ is associated with the emergence of upper layers (II-IV) of the neocortex. Basal or intermediate progenitor cells (IPCs) are neurogenic transient amplifying cells that populate the SVZ in the developing cerebral cortex. IPCs arise after the onset of neurogenesis and are prominent during mid and late neurogenesis. IPCs have been linked with determination of cortical surface area, laminar thickness and cortical neurogenesis during embryonic development and into adulthood. They undergo mitosis in the SVZ of the cortex unlike the neuroepithelial/radial glial cells that divide close to the ventricular surface (Haubensak et al., 2004) (Noctor et al, 2007). Additionally, IPCs lack the apical-basal polarity of neuroepithelial cells (Noctor et al., 2004; Attardo et al., 2008) and the majority undergoes symmetric terminal cell divisions. However, a relatively small population is still capable of undergoing symmetric

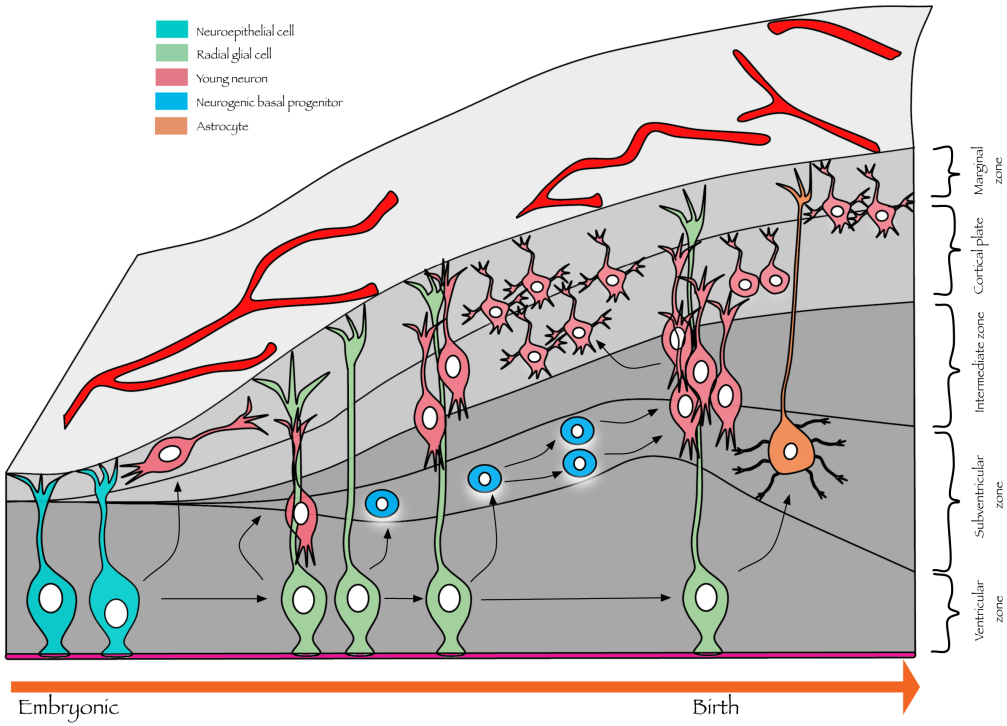


Fig. 1.4 **Schematic representation of neurogenesis in the developing mouse cortex.**
Initially, the developing cortex cosists of a single layer of neuroepithelial cells. These cells divide to generate the early neurons that migrate a short distance and settle in the marginal zone. As development proceeds, neuroepithelial cells are transformed into radial glial cells. These cells self-renew and also give rise to the next wave of neurons. Radial glia act as migratory guides for newborn neurons towards the superficial layers of the cortex. The radial glia also generate the intermediate progenitor cell pool that populate the subventricular zone. The intermediate progenitors divide to self-renew or generate neurons. The distinct layers of the cortex are laid-down in an inside out manner wherein later born neurons migrate past earlier born neurons. Adapted from: Kriegstein A. and Alvarez-Buylla, 2009.

proliferative divisions to expand the IPCs (Kowalczyk et al., 2009; Noctor et al., 2004).

Neuroepithelial cells, RGCs and IPCs are characterized by expression of different genes and these differentially impact on neurogenesis. The defining features of these cell types are summarized in Table 1.2.

Table 1.2 Summary of the similarities/differences of neural stem/progenitor cells.

Cell type	Neuroepithelial cell	Radial glial cell	Intermediate progenitor
Division potential	Multipotent	Multi/Bipotent	Bi/Unipotent
Apical-basal cell polarity	Present	Present	Downregulated
Molecular markers	Emx1/2, Hes5, Pax6, Par3, aPKC, CD133	Emx1/2, Hes5, Pax6, Par3, aPKC, CD133, GFAP, BLBP	Tbr2, Cux1/2, Neurog1/2
Interkinetic nuclear migration	√	√	X
Cortical layer	Ventricular zone	Ventricular zone	Sub-ventricular zone
	Mode of cell division		
Symmetric, proliferative	√	√	Small proportion
Symmetric, differentiative	√	√	√
Asymmetric	√	√	X

1.2.2 Specification of cortical progenitors:

The interplay between both intrinsic and extrinsic factors is thought to regulate progenitor cell proliferation, lineage restriction and cell fate specification. (Johansson et al., 2010; Temple & Qian, 1996). Strict control of cell cycle parameters is also critical in the specification of cortical progenitors (Dehay & Kennedy, 2007).

In this section, I will focus mainly on the intrinsic factors involved in the switch of a multipotent progenitor to a committed cell fate but also briefly discuss the roles of Notch and Reelin pathways in neural development. The candidate genes discussed below are classified into the following categories: Transcriptional regulators, Signalling pathways and apical/cell junction components.

Transcriptional regulators:

A combination of transcription factors work together to regulate cell fate decisions in the developing cortex (Hevner, 2006). The SoxB1 gene family that includes Sox1, 2 and 3 is crucial for maintaining the neural progenitor pool (Bylund et al., 2003; Graham et al., 2003). The basic helix loop helix (bHLH) proteins encoded by proneural genes such as Neurogenin 1/2, Mouse achaete-scute complex homolog 1 (Mash1) and mouse atonal homologs 5 (Math 5) have also been implicated in regulating cortical neurogenesis (Bertrand et al., 2002; Nieto et al., 2001; Britz et al., 2006). To maintain neural progenitors in an undifferentiated state, SoxB1 proteins inhibit the activity of the bHLH proneural proteins (Bylund et al., 2003; Holmberg et al., 2008). However, this is not the only mechanism involved in the switch of cell fate from a neural progenitor to a neuron.

The transcription factor Pax6, a conserved member of the paired-box family has been associated with establishing dorso-ventral patterning in the telencephalon, cell cycle progression of apical progenitors, specification of intermediate progenitors and also cortical neuronal

migration (Georgala et al., 2011). It functions both via regulation of proneural gene expression (Scardigli et al., 2003) and by mechanisms independent of proneural genes (Heins et al., 2002; Estivill-Torrus et al., 2002).

Tbr2 and Tbr1 are T-domain transcription factors expressed sequentially in the cells of the cortex. Tbr2 is expressed highly in the IPC population and has been used extensively as a marker for these cells (Cappello et al., 2006; Yoon et al., 2008). Conditional ablation of Tbr2 in the developing cortex results in a significant depletion of IPCs. This suggests that Tbr2 is critical for the specification of IPCs in the developing cortex (Sessa et al., 2008). Tbr1 expression is detected in early born cortical neurons of the preplate and layer 6 (Bulfone et al., 1995) and in glutamatergic neurons (Hevner et al., 2001). Loss of Tbr1 expression leads to impaired subplate division, molecular and functional defects in these neurons (Hevner et al., 2001).

In a simplified scheme of events, neurogenesis can be broadly classified into direct and indirect neurogenesis (Haubensak et al., 2004; Hevner, 2006). The direct transformation of RGCs to newborn neurons is regulated by proneural genes like Neurogenin 1/2 and Notch pathway target genes Hes1/5 and this process corresponds with a downregulation of progenitor fate determinants Pax6, Sox2 and a concomitant upregulation of postmitotic neuronal markers Tbr1, Math2 and NeuroD2 (Englund et al., 2005; Schuurmans et al., 2004). In the case of indirect neurogenesis, RGCs undergo transition to IPCs and subsequently into neurons. This involves an upregulation of Tbr2 and a downregulation of Pax6. Subsequently, the transition of IPCs to neurons then correlates with the downregulation of Tbr2 and an upregulation of Tbr1 and Math2 (Englund et al., 2005; Hevner, 2006).

Notch Signalling pathway

Notch signalling is an evolutionarily conserved intercellular signalling pathway that regulates cellular fate choices. It allows juxtacrine communication between neighbouring cells and mediates a variety of cellular responses (Louvi & Artavanis-Tsakonas, 2006). During development, Notch signalling mediates the segregation of specific cell lineages from a field of developmentally equivalent cells by linking the fate of adjacent cells (Cau & Blader, 2009; Louvi & Artavanis-Tsakonas, 2006). At the core of the Notch signalling pathway, is the receptor present on the surface of one cell and the ligands present on the surface of an adjacent cell. The signal is transduced from a 'sending cell' that displays the ligands and these ligands bind the receptors of the 'receiving cell'. This signalling transduction leads to a series of proteolytic events to release the Notch intracellular domain from the cell surface. NICD translocates to the nucleus and assembles an activated complex, which contains RBPjK (recombining binding protein suppressor of hairless) (Artavanis-Tsakonas et al., 1999; Louvi & Artavanis-Tsakonas, 2006) and triggers the transcription of its target genes. In the vertebrate CNS, the main Notch target genes are Hes1 and Hes5 (Kageyama & Ohtsuka, 1999; Ohtsuka et al., 1999). The canonical Notch signalling pathway described in this section is shown in Fig 1.5

Notch signalling maintains the balance between amplification of the neural progenitor pool and neural differentiation by a mechanism called lateral inhibition (Greenwald & Rubin, 1992; Raible & Eisen, 1995). The Notch pathway ligands are also targets of proneural genes and newly specificed neurons can transduce Notch signalling in adjacent neural progenitor cells (Bertrand et al., 2002; Casarosa et al., 1999; Cau et al., 2002). Active Notch signalling in adjacent cells inhibits acquisition of neuronal cell fate by antagonism of proneural genes. Thus the Notch pathway can contribute to waves of neuronal production as opposed to differentiation of all progenitor cells at a given time point.

In the vertebrate CNS, Notch signalling has been extensively studied for its diverse regulatory roles (Chambers et al., 2001; Louvi & Artavanis-Tsakonas, 2006; Mizutani et al., 2007). Notch signalling is crucial for maintaining the neural progenitor pool during neurogenesis and activation of the Notch signalling pathway maintains neural progenitors in an undifferentiated state by repressing the expression of proneural genes (Louvi & Artavanis-Tsakonas, 2006) (Holmberg et al., 2008). Interestingly, disruption of components of the Notch pathway such as its transcriptional target Hes5 mimic the phenotype observed in the telencephalon of Notch conditional knockout embryos: a reduction of the neural progenitors and premature neurogenesis (Chenn & McConnell, 1995; Mizutani et al., 2007; Ohtsuka et al., 1999; Yoon & Gaiano, 2005). This suggests that these transcriptional targets are the predominant downstream effectors of Notch signalling in the CNS (Ohtsuka et al., 1999; Yoon & Gaiano, 2005).

Both Hes1 and 5 are classical DNA-binding repressors that inhibit expression of proneural genes (Ohtsuka et al., 1999). Inhibition of proneural genes occurs when Hes proteins bind to the N-box sequences in their promoter region. Additionally, Hes proteins are also capable of directly interacting with proneural bHLH proteins to form non-functional dimers and inhibit neurogenesis (Cau & Blader, 2009) (Fischer & Gessler, 2007).

Reelin Signalling

Reelin, a secreted protein synthesized by Cajal-retzius cells acts through the extracellular milieu to regulate positioning of signal-responsive target cells (D'Arcangelo et al., 1995; Frotscher et al., 2009). The receptors for Reelin are VLDR (very low-density lipoprotein receptor) and apoER2 (apolipoprotein E receptor 2) receptors and Reelin signal is transduced by tyrosine phosphorylation of Dab1 (Disabled1), an intracellular adaptor protein (Fig 1.6). Dab1 and the Reelin receptors are expressed in the ventricular zone and this is consistent with the proposed

role of Reelin in influencing migration of neural cells (Tissir & Goffinet, 2003; Frotscher et al., 2009; Nomura, Hattori, & Osumi, 2009). In the *reeler* mouse model that has a spontaneous mutation in Reelin, severe abnormalities such as mislocalization of laminar specific neurons and disorganization of cortex are apparent. Interestingly, the inside-out pattern of cortical development (described in section 1.2.1) is lost in reeler mice and the postion of neurons in the cortex is inverted with layer specific neurons observed in an outside-in pattern (Rakic & Caviness 1995, Tissir and Goffinet 2003, D'Arcangelo et al., 1995).

Relationship between apical cell membrane constituents and neural cell fate determination

During *Drosophil*a neuroblast division, the orientation of the cleavage plane is influenced by apical-basal polarity cues (Knoblich, 2008; Zhong & Chia, 2008). It has been proposed that similar conserved mechanisms are in play during vertebrate neurogenesis (Wodarz & Huttner, 2003).

Neuroepithelial cells have an intrinsic apical-basal polarity and it was proposed that some of the self-renewing factors localize at the apical cell surface and the inheritance of these factors determines cell fate (Chenn, Zhang, Chang, & McConnell, 1998; Gotz & Huttner, 2005). Accumulating evidence from 'mouse knockout studies' supports the role for apical cell membrane constituents in neural cell fate determination. These studies are summarized in Table 1.3

Table 1.3 Summary of the phenotypes observed in mouse knockout models of polarity and cell-junction proteins.

Protein analysed	Knockout system/ Conditional knockout model	Phenotype observed	Reference
Crumbs complex **Pals1**	Emx1-Cre	Disruption of apical complex proteins Premature withdrawal from cell cycle Precocious neural differentiation Rapid cell death of neurons	Kim et al., 2010
Lin7 (MALS)	Null	Disruption of apical complex proteins, intact adherens junctions Altered neural progenitor cell proliferation only during early neurogenesis	Olsen et al., 2005 Srinivasan et al., 2008
Par complex **Par3**	shRNA	Premature cell cycle exit Increased symmetric divisions and in turn affects neural cell fate specification	Costa et al., 2008 Bultje et al., 2009
aPKC	Nestin-Cre	Loss of adherens junctions Impaired interkinetic nuclear migration No effect on neurogenesis	Imai et al., 2006

Cdc42	Emx1-Cre	Apical Par and adherens junctions disrupted Increase in mitosis at sub-ventricular zone Increased intermediate progenitor cell domain	Cappello et al., 2006
Scribble comple **Lgl**	Null	Disorganized localization of apical junctional complexes. Failure to exit cell cycle Hyperproliferation and increased apoptosis	Klezovitch et al., 2004
Cell junctions **N-Cadherin**	D6-Cre	Disruption of adherens junctions Disrupted laminar organization of cortex	Kadowaki et al., 2006
β-Catenin	Nestin-Cre D6-Cre	Disrupted adherens junctions Impaired interkinetic nuclear migration Precocious differentiation into astrocytes	Machon et al., 2003 ; Mutch et al., 2010.

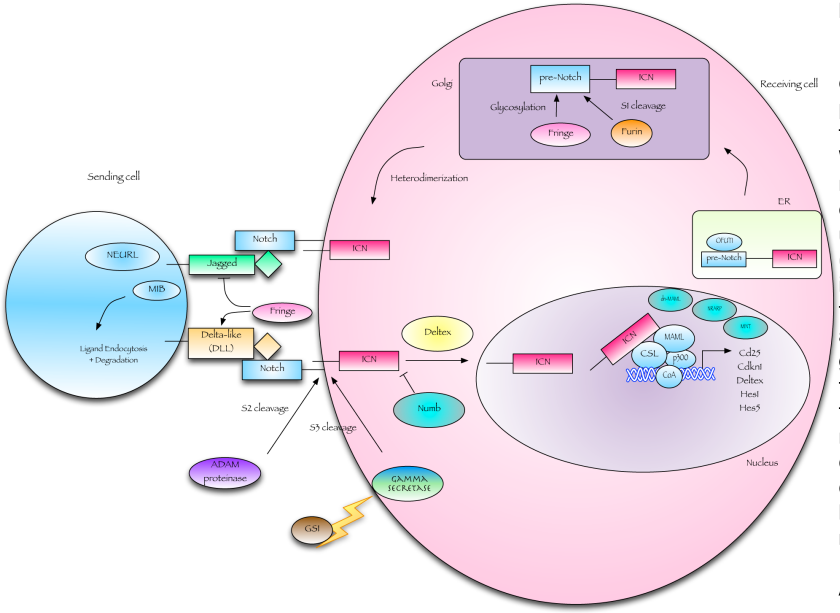


Fig. 1.5 **Schematic illustration of the Notch signalling pathway.**

In the endoplasmic reticulum (ER), intra and extracellular domains of Notch receptor are synthesized as pre-Notch and is transported to the Golgi. O-fucosyltransferase1 (OFUT1) functions as a chaperone. Within the Golgi apparatus, glycosylation is carried out by members of the Fringe family. Furin cleaves pre-Notch into extra and intracellular domains. In the signal sending cell, E3 ubiquitin ligases, Mindbomb (MIB) and Neuralized-like (NEURL) promote ligand turnover and ligand-receptor interactions. The binding of ligands Delta-like and Jagged to the receptor initiates successive proteolytic cleavages S2 and S3. S2 cleavage is mediated by ADAM proteinase. The gamma-secretase complex mediates the S3 cleavage and this releases the intracellular domain (ICN). The ICN is translocated to the nucleus and interactions with Master-mind-like proteins (MAML) and transforms the nuclear complex from a transcriptional repressed to an activator complex. This increases transcription of Notch target genes like Hes1/5, Cdkn1, Cd25. Numb and Deltex are known regulators of Notch signalling.

Adapted from: Grabher et al., 2006

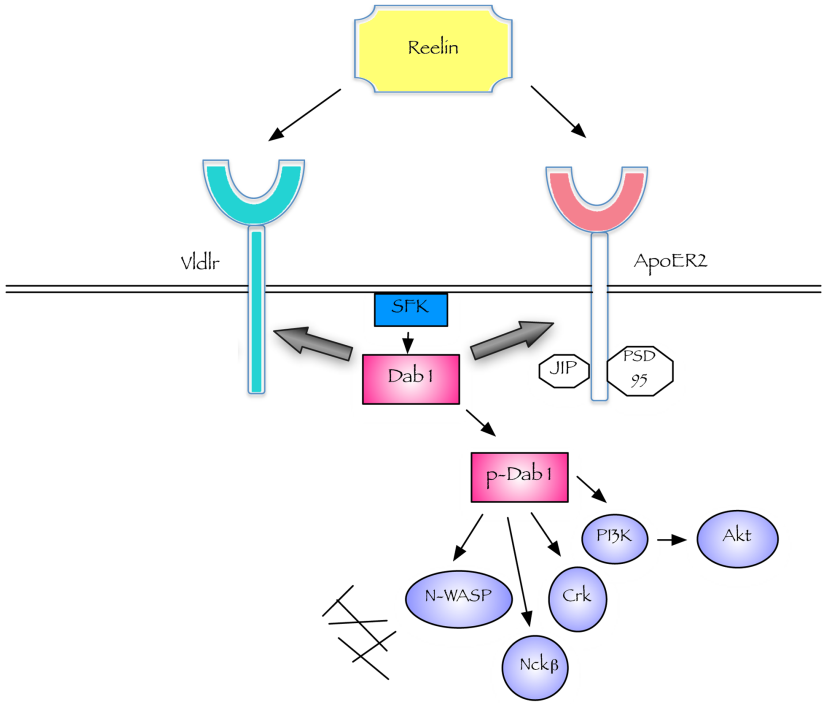


Fig 1.6 **Schematic illustration of Reelin signalling pathway**
Reelin binds to VLDLR and ApoER2 and activates src-family kinase (SFK) and phosphorylation of Dab-1. Dab1 is an adaptor protein that binds to VLDLR and ApoER2 receptors. ApoER2 interacts with synaptic and trafficking proteins(JNK-interacting protein) JIP and PSD-95. DAB-1 interacts with other signalling molecules such as Phosphatidyl-inositol3-kinase (PI3K), Nck β, Crk or N-WASP (neuronal Wiskott-Aldrich Syndrome protein).

Adapted from Zhang et al., 2007

Regulation of cell fate decisions by symmetric versus asymmetric divisions.

In vertebrates, the apical cell surface represents only a minor proportion of the total plasma membrane and so the mechanisms regulating cleavage plane orientation must be precisely orchestrated. Time-lapse studies have demonstrated that a vertical cleavage results in two identical daughter cells that remain in the ventricular zone (Attardo et al., 2008; Kosodo et al., 2004). If the daughter cells have the same potential as the mother, it is considered a symmetric/proliferative division and if the daughters are more committed in their lineage, it is a symmetric/differentiative division (Farkas & Huttner, 2008; Gotz & Huttner, 2005).

During early embryonic stages, prior to the onset of neurogenesis, neuroepithelial cells undergo symmetric, proliferative divisions to amplify the progenitor cell population. With the onset of neurogenesis, there is a gradual increase in the frequency of asymmetric divisions (Fig 1.7) (Gotz & Huttner, 2005; Huttner & Brand, 1997; Kriegstein et al., 2006; Zhong & Chia, 2008).

Several lines of evidence suggest that one of the mechanisms involved in generation of the diverse neural cell types is asymmetric cell division whereby the polarized distribution of cellular constituents and their differential inheritance by daughter cells determines cell fate (Horvitz & Herskowitz, 1992; Huttner & Brand, 1997; Kosodo et al., 2004; Wodarz & Huttner, 2003). The asymmetric inheritance of components may also regulate other factors crucial for cell fate determination, for instance, asymmetric distribution of Numb in turn regulates Notch protein expression and so influences the cellular response to extrinsic signals (Betschinger & Knoblich, 2004).

Fig. 1.7 **Schematic illustration of progenitor cell divisions during neural development.** Simplified relationship between neuroepithelial cells, radial glial cells, intermediate progenitor cells and neurons. During early development, symmetric proliferative divisions dominate and after the onset of neurogenesis there is increased occurrence of symmetric differentiative cell divisions.

Adapted from Farkas and Huttner 2005; Gotz and Huttner 2005

Overall, it is clearly evident that the emergence of the diverse cell types of the vertebrate CNS cannot be attributed to a single factor and that it involves the concerted action of different transcription factors, cell intrinsic components and signalling molecules.

1.3 Hindbrain segmentation and cell lineage restriction

The vertebrate hindbrain (rhombencephalon) is the most posterior vesicle of the embryonic brain; it is characteristically diamond-shaped and extends caudally from the cerebral aqueduct to the central canal of the spinal cord. It is a complex structure that controls many autonomic and voluntary functions such as regulation of sleep patterns, the state of consciousness, breathing and blood circulation. The hindbrain is subdivided into metencephalon that develops in to the cerebellum and the myelencephalon; the latter subsequently gives rise to the pons and medulla oblongata in the adult brain (Kaufman & Bard, 1999).

The first clearly defined boundaries in the embryonic hindbrain are the transient segments observed along the AP axis called rhombomeres. In the chick embryo (Vaage, 1969), eight rhombomeres were defined with the last rhombomere being contiguous with the presumptive spinal cord. The roots and ganglia of cranial nerves – trigeminal (V), abducens (VI) facial (VII), vestibulocochlear (VIII), glossopharyngeal (IX) vagus (X) accessory (XI) and hypoglossal (XII) derive from the rhombomeres and are linked to the pons and medulla. These cranial nerves control and also receive sensory information from muscles in the eye, jaw and face. Axons from motor nuclei in rhombomeres 1, 2 and 3 gather at the trigeminal nerve and exit the hindbrain from rhombomere 2 to innervate the first branchial arch. The second and third branchial arches are innervated by the facioacoustic (VII/VIII) and glossopharyngeal nerves (IX). Axons from motor nuclei in rhombomeres 4/5 and 6/7 contribute to these cranial nerves (VII-IX) (Lumsden and Keynes, 1989; Kaufmann and Bard 1999).

Clonal analysis and ablation of inter-rhombomeric boundaries demonstrated that there was cell-lineage restriction within individual rhombomeres of the chick embryo (Fraser et al., 1990; Guthrie & Lumsden, 1991). These experiments indicated that cell-lineage restriction was established even before the delineation of the individual rhombomeres. However, it should be noted that this lineage restriction is

not absolute and a few cells are capable of crossing the inter-rhombomeric boundaries (Birgbauer & Fraser, 1994).

It has been reported that the transient segmentation of the hindbrain is crucial for appropriate neuronal specification and timely migration of cells from the hindbrain (Guthrie & Lumsden, 1991; Narita & Rijli, 2009; Trainor & Krumlauf, 2001). It has also been suggested that the expression of several rhombomere-specific genes is progressively refined during the setting up of morphological boundaries in the hindbrain (Cooke & Moens, 2002). In particular, the role of HOX genes that encode helix-turn helix transcription factors has been studied extensively in the embryonic hindbrain (Tumpel, et al., 2009). Their expression pattern correlates with the rhombomere boundaries and they play crucial roles in controlling both establishment and maintenance of regional identity along the AP axis of the hindbrain (Fig 1.8) (Alexander et al., 2009).

Two mechanisms have been proposed for the setting up of rhombomere limits: plasticity of cell fates and cell sorting (Cooke & Moens, 2002). To define inter-rhombomeric boundaries, cell sorting and cell plasticity could work in concert with each other or they could be redundant mechanisms that ensure precise formation of rhombomeric boundaries if one mechanism fails. Experiments in zebrafish and mouse embryonic hindbrain have shown that the identity of cells is plastic at early stages and that a cell is capable of altering the segment-specific genes it expresses (Schilling et al., 2001) (Trainor & Krumlauf, 2001). These data suggest that dynamic regulation of gene expression boundaries may play a role in establishing the morphological boundaries (Trainor & Krumlauf, 2001).

Initial evidence for cell sorting in rhombomeres was obtained from *in vitro* experiments. When odd-numbered and even-numbered rhombomeres were dissociated and cultured, the cells from the odd rhombomeres separated away from the even-rhombomeric cells (Wizenmann & Lumsden, 1997). Alternating rhombomeres demonstrate

similar cell-adhesion properties and the cell surface properties vary according to the rhombomeric units in the hindbrain (Guthrie & Lumsden, 1991; Schilling et al., 2001).

The Eph family of receptor tyrosine kinases and their membrane bound ligands, the ephrins, are candidates in mediating the differential affinity between rhombomeres. The expression of receptors and ligands is complementary: EphA4, EphB2 and EphB3 receptors are highly expressed in the odd rhombomeres r3 and r5 and the ephrin ligands – ephrin B1-B3 are expressed in the even rhombomeres r2, r4 and r6 (Xu et al., 2000). It has been reported that activation of the ephrins is sufficient to induce cell sorting in the rhombomeres (Mellitzer et al., 1999; Xu et al., 1999).

1.3.1 Neural Crest cell migration

Neural crest cells are a multipotent cell population capable of differentiating into a diverse array of cell types that include neurons and glia of peripheral nervous system, pigment cells, cartilage and bones (Wolpert et al., 1998). They originate from the dorsal neural tube in a rostrocaudal fashion and are spatially distributed along migratory paths to target regions. The neural crest population can be broadly classified on the basis of its positional origin in the neuraxis into cranial, vagal, trunk and sacral neural crest. Neural crest cells arising from the cranial level traverse through the cranial mesenchyme and make facial bones, cartilage and sensory glia (Ayer-Le Lievre & Le Douarin, 1982). Trunk neural crest cells generate melanocytes, sensory and sympathetic glia and chromaffin cells. Although both cranial and trunk neural crest cells produce sensory neurons, glia and melanocytes only cranial neural crest cells produce facial bone and cartilage suggesting that these cells have some properties in common but have intrinsic differences in their developmental potential (Nakamura & Ayer-Le Lievre, 1982).

The relationship between site of emergence of cranial neural crest from the hindbrain and the segmentation of the neural epithelium has been clearly defined. Neural crest cells migrating from rhombomeres 1 and 2 populate the first branchial arch and trigeminal ganglion. Rhombomere 4 neural crest cells contribute to vestibulo-acoustic ganglia, facial ganglia neurons and second branchial arch. Rhombomere 6 contributes to the third branchial arch and superior ganglion of the IX nerve (Guthrie & Lumsden, 1991; Lumsden, Sprawson, & Graham, 1991). Rhombomeres 3 and 5 do not contribute significantly to the neural crest cell population and the even-numbered rhombomeres flanking these segments repress neural crest production by inducing apoptosis (Graham, Heyman, & Lumsden, 1993) (Lumsden et al., 1991).

The specification of neural crest cells is dependent on extrinsic signals such as Notch, BMP, Wnt and intrinsic factors such as Pax3, Pax7, Snail, Slug and Sox9 (Saint-Jeannet, 2006). Improper migration of neural crest cells migration results in severe morphological defects in facial and cardiovascular development (Hutson & Kirby, 2003; Tobin, 2008).

Slug, a zinc finger transcription was identified as the earliest intrinsic marker of neural crest cells in Xenopus and chick embryos. Slug is highly expressed in neural crest cells prior to the onset of crest cell migration (Nieto et al., 1994) and triggers epithelial-mesenchymal transition during neural crest delamination (Duband et al., 1995; Cano et al., 2000). In addition to these roles, Slug is necessary for the formation of neural crest cell precursors and their migration (LaBonne & Bronner-Fraser, 2000). However, species-specific differences exist in the role of Slug in regulating neural crest specification and migration. For instance, mouse embryos lacking Slug showed severe developmental defects but loss of Slug had no impact on neural crest generation itself (Jiang et al., 1998).

Members of the Cadherin gene family have also been implicated in regulating neural crest migration cells (Nakagawa & Takeichi, 1998) (Stepniak et al., 2009). N-Cadherin and Cadherin6B are downregulated and Cadherin 7 expression is upregulated in migrating neural crest cells (Nakagawa & Takeichi, 1998). This distinct expression pattern of the Cadherins suggests that their roles in neural crest migration may not be restricted to intercellular adhesion but also influence epithelial to mesenchymal transition (Coles et al., 2007; Taneyhill, 2008). Ephs (section 1.3) also play divergent roles during avian neural crest cell migration (Mellott & Burke, 2008).

Different factors have been used extensively as markers to distinguish between premigratory and migratory neural crest cells. The glycoprotein/glycolipid epitope HNK-1 (Tucker et al., 1984) (Le Douarin & Dupin, 1993) and Slug (Nieto et al., 1994) are the most commonly used crest cell markers. Injection of HNK-1 antibody into the mesencephalic neural tube at the onset of crest cell migration resulted in aberrant migration of the cranial neural crest cells suggesting that HNK-1 epitope is important for neural crest migration (Bronner-Fraser, 1987). Whilst Slug is expressed in both premigratory and migratory cells, HNK-1 is only detected in the migratory cells (Del Barrio & Nieto, 2004).

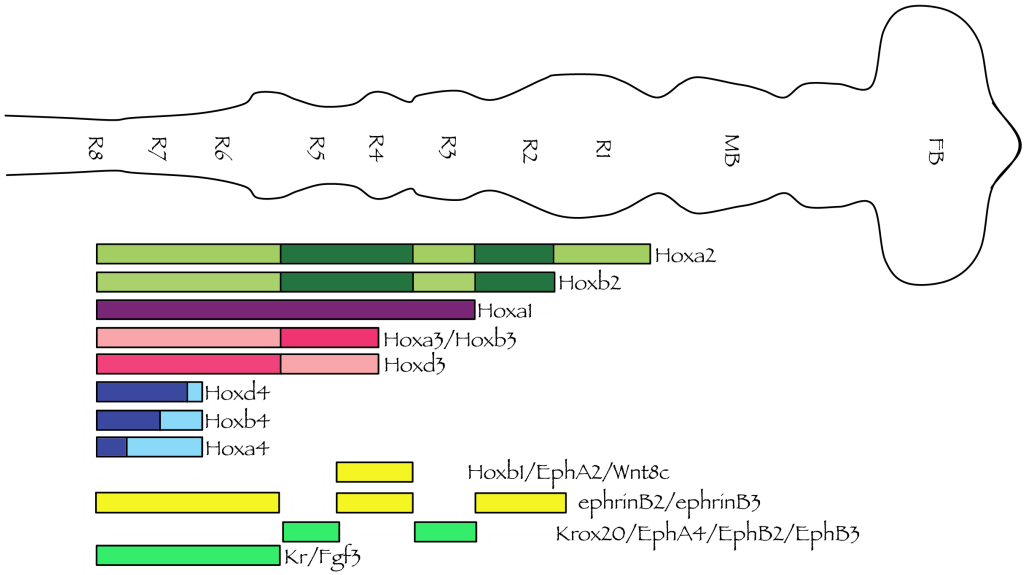


Fig.1.8 **Schematic illustration showing gene expression patterns in the embryonic hindbrain**. The hindbrain is segmented into cell-lineage restricted rhombomeres. There are distinct gene expression boundaries between rhombomeres. The gene expression patterns are highly dynamic and for sake of simplicity only principal domains of expression are represented here. The combined gene expression patterns ultimately determine the identity and fate of each rhombomeric unit.
Adapted from Melton et al., 2004

1.4 Apical-Basal Cell Polarity

In this section, I will introduce the apical-basal cell polarity protein complexes and briefly describe the functional roles of Crumbs. The role of apical polarity proteins during mammalian neurogenesis has been described in section 1.2.2 (Table 1.3).

Apical-basal cell polarity is crucial for a variety of biological processes such as proliferation, differentiation, vectorial transport of molecules, cell signalling and asymmetric cell division (Assemat et al., 2008) (Knoblich, 1997; Knoblich, 2008). It reflects intricate mechanisms that not only establish, but also maintain functionally specific plasma membrane and cytoplasmic domains by employing an elaborate network of polarity protein complexes. The detailed molecular mechanisms underlying the generation of cell polarity are just beginning to be understood. Three evolutionarily conserved protein complexes have been shown to be crucial in the setting up and maintenance of apical-basal polarity namely the **Scribble, PAR and Crumbs** complexes (Fig.1.9 A-B). The Crumbs and Par complexes define the apical domain whilst the Scribble complex is basally localized. The polarity complex proteins were initially identified in *C.elegans* and *Drosophila*. Mammalian homologs of all polarity proteins have been identified (Table 1.4).

1.4.1 Sub-cellular compartments- Cilia and cell junctions

The apical domain of epithelial cells can be broadly subdivided into three compartments: a) the ventricular/luminal surface b) the subapical domain that lies between the luminal surface and the lateral cell junctions and c) specialized membrane protuberances such as microvilli and cilia (Farkas & Huttner, 2008)

Cilia are microtubule-based organelles observed in almost all polarized cells and they perform crucial roles in signal reception and transduction of signals to the cell body. The role of cilia in signalling has

been associated with cellular events like cell proliferation, differentiation, migration and mechanotransduction (Eggenschwiler & Anderson, 2007; Gerdes, Davis, & Katsanis, 2009). Several key components of signalling pathways such as fibroblast growth factor, platelet-derived growth factor, sonic hedgehog and cell polarity proteins have been identified in primary cilia (Goetz & Anderson, 2010) (Fan et al., 2004). Primary cilia are crucial regulators of Hedgehog (Hh) signalling pathway and ligand binding to Patched (Ptc) receptor in the cilium removes Ptc from the cilium and results in enrichment of Smoothened in the cilium and the subsequent activation of Hh signalling pathway. In addition to this role, mutations in intraflagellar transport proteins also affect Hh signalling and lead to severe developmental defects in mammals. The primary cilium has also been linked with regulation of canonical and non-canonical Wnt signalling pathways (Gerdes et al., 2009; Goetz & Anderson, 2010; Goetz, Ocbina, & Anderson, 2009).

An elementary requirement for setting up a functionally integrated epithelium is the formation of cellular junctions. An adhesive belt of junctional complexes called adherens junctions is established at the apical-basal boundary of a cell. The adherens junctions are usually located basal to the tight junctions.

In vertebrates, cadherins are key regulators of cell-cell adhesion (Miyaguchi, 2000). They are single pass transmembrane proteins with homophilic interactions that are calcium dependent. Cadherins are linked to the cytoskeleton via α and β-catenins (Alberts et al., 2002). Apart from cadherins and catenins, nectin and nectin-like molecules are also associated with the adherens junctions and they interact with each other in a calcium independent manner (Miyaguchi, 2000; Mizoguchi et al., 2002). Adherens junctions not only mediate cell-cell adhesion but also cell signalling events by receiving and transmitting signalling cues (Erez et al, 2005; McCrea et al., 2009).

Table 1.4 Summary of gene names for the apical-basal polarity proteins in *C.elegans*, *Drosophila* and in Mammals.

Polarity complex	***C. elegans***	***Drosophila***	**Mammals**
Par complex (Par3/Par6/aPKC)	Par3 Par6 PKC-3	Bazooka DmPar6 DmaPKC	Pard3 (a,b) Pard6 (α, β, γ) Prkc (ζ, λ)
Scribble complex (Scrib/Dlg/Lgl)	LET-413 Dlg1 Tom-1	Scrib Dlg Dlgl	Scrib Dlg (1-5) Llgl (1-2)
Crumbs complex Crb/Pals/Patj	Crb1, Eat -20 TAG-117 MPZ-1	Crumbs Sdt Dpatj dLin7	Crb/CRB (1-3) MPP(1-7)/Pals INADL/PATJ MPDZ/MALS

Tight junctions are specialized vertebrate occluding membrane domains and are localized apically to the adherens junctions. They play a crucial role in regulating flow of molecules and ions through an epithelium. Formation of tight junctions involves interactions of transmembrane proteins from the following protein families: claudins, occludins, junctional adhesion molecules and zonula occludens (Gonzalez-Mariscal et al., 2000; Shin & Margolis, 2006).

Overall, a combination of transmembrane proteins, enzymes and adaptor proteins are involved in the organization of the dynamic cell-cell junctions. These components work together to regulate diverse functions and maintain structural integrity of complex tissues during development.

1.4.2 Scribble complex

The basally localized Scribble complex consists of Scribble (Scrib), Lethal giant larvae (Lgl) and Discs large (Dlg) proteins. Both Dlg and Lgl were primarily identified as tumor suppressors (Gateff, 1978; Stark &

Bridges, 1926). These tumor suppressors were linked to Scrib because they gave the same embryonic phenotype as observed in Scrib mutants (Bilder, Li, & Perrimon, 2000). It has been proposed that members of the Scrib complex function as scaffolds to regulate protein interactions (Bilder, 2004). The protein Scrib consists of 16 leucine rich repeats and 4 PDZ (PSD-95 (a 95 kDa protein involved in signaling in the post-synaptic density), Dlg (the *Drosophila* discs large protein), and ZO1 (the zonula occludens 1 protein involved in maintaining epithelial cell polarity) domains (Bilder et al., 2000). Scrib has been implicated in defining the basolateral boundary by exclusion of apical membrane determinants such as the Crumbs complex (Assemat et al., 2008; Bilder & Perrimon, 2000). Dlg has an L27 domain, a GUK domain and a SH3 domain and 3- PDZ domains (Woods & Bryant, 1991). In *Drosophila*, mutation in Dlg gene leads to neoplastic overgrowth in the eye imaginal disc (Woods & Bryant, 1991). Lgl protein has several tryptophan-aspartic acid repeats similar to proteins playing a role in cell adhesion (Lutzelschwab et al., 1987). During *Drosophila* larval development, mutations in Lgl result in growth and adhesion abnormalities.

In vertebrates, the Scrib complex consists of Scrib, 2 Lgl homologs –Lgl1 and Lgl2 and 5 Dlg homologs. Unlike the interactions between apical protein complexes little is known about the direct interactions between members of the Scrib complex (Assemat et al., 2008).

1.4.3 Par complex

The PAR (partitioning defective) genes, an integral part of the **PAR complex**, were the earliest cell polarity genes to be identified in a genetic screen carried out in the nematode *C. elegans* (Kemphues et al., 1988). In vertebrates, the PAR complex includes scaffold proteins Par3 and Par6 along with the atypical protein kinase C (aPKC λ/ζ) and Cdc42 (Macara, 2004). Previous studies have indicated that the PAR complex is

interdependent on its constituent proteins for its localization (Doe & Bowerman, 2001; Ohno, 2001).

Par3 colocalizes with aPKC in mammalian epithelial cells and is phosphorylated by aPKC *in vitro* (Izumi et al., 1998). Par3 is not always associated with the PAR complex and competes with Lgl for binding (Yamanaka et al., 2003) with Par6. Par6 binds aPKC to inhibit its kinase activity and the binding of Cdc42 to Par6 via its CRIB domain induces a conformational change to relieve aPKC inhibition. This in turn induces phosphorylation of downstream targets by aPKC. It has been proposed that competitive binding between Lgl and Par3 may mediate establishment and maintenance of apical-basal polarity (Margolis & Borg, 2005; Yamanaka et al., 2003)

1.4.4 Crumbs complex:

Crumbs, Protein associated with Lin 7 1 (PALS1), Lin 7 and PALS1 associated tight junction protein (PATJ) (Bachmann et al., 2008; Bachmann et al., 2001; Bhat et al., 1999; Bulgakova & Knust, 2009; Hong et al., 2003) form the core members of the mammalian **Crumbs complex**.

PALS1 and PATJ have multiple protein binding sites and function as scaffolds of the complex. Crumbs binds to PALS1 through its C-terminal tail to a PDZ domain present in PALS1 and PATJ interacts with Pals 1 through one of its L-27 multiple protein-protein interaction domains. Increasing evidence now seems to suggest existence of direct interactions between the Crumbs complex and Par complex (Hurd et al., 2003; Lemmers et al., 2004; Sotillos et al, 2004).

It is now accepted that the apical and basal complexes mutually antagonize each other to define the apical and basal limits of a cell (Margolis & Borg, 2005). Loss of gene function on either side results in

the expansion of the other and a subsequent alteration in normal growth and defects in epithelia and cell junction formation.

1.4.4.1 Homologs of Crumbs.

Drosophila Crumbs is a large transmembrane protein having 30 epidermal growth factor (EGF)-like repeats and 4 LamininA G-domain-like repeats in its extracellular domain, a membrane spanning domain and a short (37amino acid) highly conserved, intracellular domain (Tepass et al., 1990). Crumbs gene was first discovered in a Drosophila screen aimed at identifying genes affecting the larval cuticle (Jurgens, Wieschaus, Nusslein-Volhard, & Kluding, 1984). The cuticle in Crumbs mutant embryos was not contiguous, its appearance was reminiscent of breadcrumbs and Crumbs gene was named after this phenotype.

As previously mentioned, Crumbs genes are evolutionarily conserved from invertebrates to mammals. Human and mice Crumbs orthologs are represented as (Human/Mice): CRB1/Crb1 (den Hollander et al., 2002); CRB2/Crb2 (van den Hurk et al., 2005); CRB3/Crb3 (Lemmers et al., 2002). CRB1 has 19 EGF-like domains and 3 Laminin G like domains; CRB2 has 15 EGF like and 2 Laminin G like domins. CRB3 has a very short extracellular domain in contrast to CRB1 and CRB2, nevertheless, the intracellular domain is highly conserved between CRB1, 2 and 3 (Fig 1.9 C).

Drosophila Crumbs is expressed in all epithelia derived from the ectoderm (Tepass et al., 1990). Human CRB1 expression was predominantly confined to the brain and retina (den Hollander et al., 2002). There are reports (Roh et al., 2003; Watanabe et al., 2004) describing expression of mouse Crb1 in brain, retina, stomach, lung, testis and kidney. Both CRB2 and CRB3 are expressed in a broad range of tissues with CRB2 being expressed in retina, brain, kidney and at comparatively low levels in lung, heart and placenta (van den Hurk et al., 2005). Human CRB3 was expressed in the retina, colon, lungs, kidney,

heart and mammary glands (Makarova et al., 2003). The functions of Crumbs proteins in *Drosophila*, zebrafish and in mammals are summarized in Table 1.5

1.4.4.2 Alternative splice variants and secreted Crumbs.

It has been predicted that CRB1 and CRB2 both encode for transmembrane and truncated isoforms, with the truncated isoforms being putatively secreted (Katoh & Katoh, 2004; Watanabe et al., 2004). Differential splicing gives rise to these truncated isoforms that do not possess the transmembrane and intracellular domains typical of Crumbs protein.

A mouse Crb1 splice variant that encoded for a C-terminal truncated secretory protein (Crb1s) was previously identified (Watanabe et al., 2004). This study showed expression of Crb1s in the skin, lung and kidneys of adult mice and based on *in vitro* data suggested a role for Crb1s for stratified epithelial organization.

Based on bioinformatics studies, RT-PCR analyses and Northern blots different isoforms of mouse Crb2 were identified in our lab (unpublished, Walker and Rashbass) and are shown in Fig 1.10.

The first isoform encodes a full length form (**Crb2F)** and consists of a signal peptide, 10 epidermal growth factor (EGF) like repeats, 3 laminin G-like domains, 4 EGF repeats, a transmembrane domain and an evolutionarily conserved cytoplasmic tail. Isoform 2 has exon 9A spliced in and this introduces a premature stop before the transmembrane domain, thereby encoding a putatively secreted Crb2 protein (**Crb2S**) that contains 10 EGF repeats and 2 laminin-G like domains. Isoform 3 has an alternative start in exon 6A and encodes a shortened transmembrane protein that lacks the first 8 EGF-like repeats.

Table 1.5: Brief summary of known functions of Crumbs proteins in *Drosophila*, Zebrafish and Mammals.

Model organism	Functional role /Phenotypes observed	Reference
Drosophila Crumbs	Define apical domain Loss of cell polarity in embryonic, follicle epithelia, pupal and adult photoreceptors Disintegration of epithelia and extensive cell death Organ size control during head development Regulates growth via Hippo pathway	Bachmann et al., 2001; Hong et al., 2001; Klebes and Knust, 2000; Li et al., 2008; Tanentzapf et al., 2000
Zebrafish Crumbs	Apically localized Crb maintains apical basal gradient of Notch activity in zebrafish hindbrain	Ohata et al., 2011
crb1	No obvious phenotype observed	Omori and Malicki, 2006
crb2a (oko meduzy)	Morpholino induced knockdown - • Displacement of cell junctions in neuroepithelial cells • Neuronal patterning defects in the retina. Determinant of apical surface size in photoreceptors.	Malicki and Driever, 1999; Omori and Malicki, 2006
crb2b	Required for normal elongation of cilia and positioning of cilia in the pronephros.	Malicki and Driever, 1999; Omori and Malicki, 2006

crb3a, crb3b	Crucial determinant of auditory kinocilia length.	Omori and Malicki, 2006
Mammals Crb1	Progressive loss of photoreceptors Crb1 mutations in humans leads to retinal dystrophies	den Hollander et al., 1999; van de Pavert et al., 2004 van de Pavert et al., 2007
Crb2	Defective epithelial-mesenchymal transition during gastrulation in KO mouse embryos. Regulator of mouse embryonic stem cell derived neural progenitors	Xiao et al., 2011 Boroviak and Rashbass 2011
Crb3	Morphogenesis of tight junctions in mammalian epithelial cell lines Maintains apical basal polarity and cell junction formation and subsequently contact inhibits growth, suppress invasion and metastasis in tumour derived cell lines. Trafficking of Crb3 to Rab11 positive endosomes is crucial for lumen formation in MDCK cyst formation assays	Roh et al., 2003 Karp et al., 2008 Whiteman et al., 2008 Schluter et al., 2009

1.4.4.3 Functional role of the intracellular domain of Crumbs

A hallmark of polarized epithelial cells is the establishment and maintenance of junctions that demarcate the apical and basal boundaries of a cell. The specific functions carried out by epithelial cells depend heavily on the formation of these well-defined junctions. Studies in *Drosophila* have shown that the Crumbs protein influences the formation and maintenance of cellular junctions in epithelia (Tepass et al., 2001).

Drosophila Crumbs mutant embryos do not succeed in setting up the adherens junctions and in addition, demonstrate mislocalization of adherens junction components (Grawe et al., 1996; Tepass et al., 2001). Also, overexpression of Crumbs disrupts the integrity of epithelial junctions and consequently leads to formation of a multilayered epidermis, indicating that Crumbs plays a significant role in proper positioning and assembly of adherens junctions (Grawe et al., 1996). An interesting observation was the overexpression of either the full-length or transmembrane plus cytoplasmic domains of Crumbs was sufficient to partially rescue this mutant *crumbs* phenotype (Wodarz et al., 1995). This highlights the importance of interactions between Crumbs and the cytoplasmic protein machinery. In addition, the same study showed that an overexpression of only the extracellular Crumbs domain had no influence in determining the apical characteristics of the plasma membrane. This suggests that both the extra and intracellular domains of Crumbs have distinct functions, which may in turn be influenced by transient binding of proteins and the developmental stage and/or cell type.

The Crumbs complex also interacts with the apical spectrin cytoskeleton, via its juxtamembrane domain. The Crumbs complex is associated with the actin cytoskeleton through members of the Par complex and FERM protein family. Medina et al., 2002 have shown that Crumbs interacts with DMoesin and β-heavy spectrin, thereby, arbitrating interactions between the cytoskeleton and the Crumbs complex (Medina et al., 2002). It was also proposed that Moesin might exert its function by repressing the activity of the GTPase - Rho (Speck et al., 2003) or by associations between Crumbs and β-heavy spectrin. Extensive studies (Bulgakova & Knust, 2009) have now established the significance of Crumbs protein complex in defining apical-basal boundaries and stabilizing the adherens junctions in epithelia.

Overall, it can be said that setting up the apical/basal domains of epithelial cells involves an elaborate organization of protein scaffolds and interplay of proteins; with the Crumbs complex playing a crucial role.

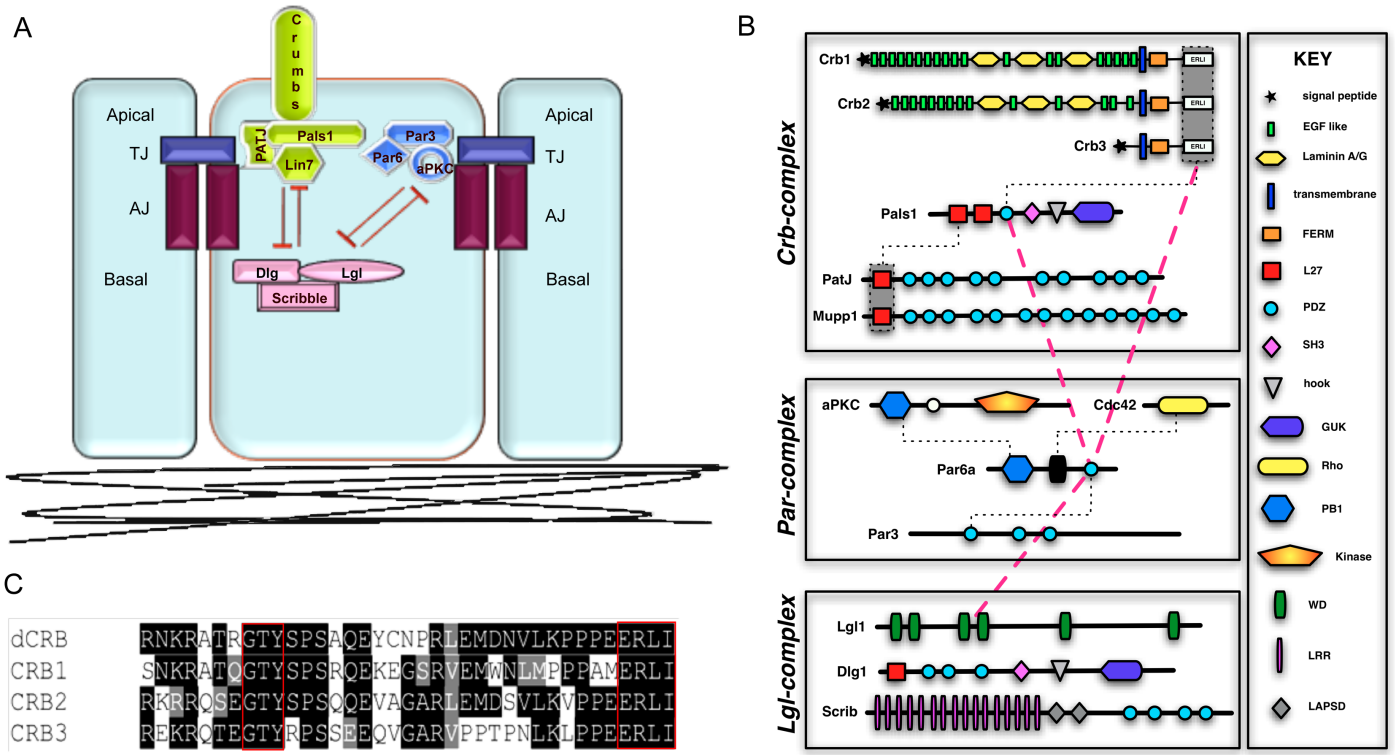
1.4.4.4 Functional Role of the extracellular domain of Crumbs

Apart from its role in determining apico-basal cell polarity in epithelial cells and assembling zonula adherens, Crumbs has also been implicated in photoreceptor morphogenesis (Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002). An important outcome of these studies (Johnson et al., 2002; Pellikka et al., 2002) was the identification of a role for the extracellular domain of Crumbs protein.

Both the extracellular and intracellular domains were shown to have distinct functions in photoreceptor cells of *Drosophila*. The intracellular domain was crucial and sufficient for the integrity of adherens junctions and rhabdomere elongation in contrast to the extracellular domain, which was important for modulating the length of stalk membrane in photoreceptors (Johnson et al., 2002; Pellikka et al., 2002).

Expression of the cytoplasmic membrane bound Crumbs domain was insufficient to rescue photoreceptor degeneration. In fact, deletion of the C-terminal domain had absolutely no effect on light-induced photoreceptor degeneration (Johnson et al., 2002).

This again seems to imply that the intracellular and extracellular domains of Crumbs have distinct functions; with the intracellular domain playing a crucial role in the formation of zonula adherens and morphogenesis; and the extracellular domain being a vital suppressor of light induced retinal degeneration (Johnson et al., 2002).


A
Crumbs
Apical
TJ
AJ
Basal
PATJ
Pals1
Lin7
Par3
Par6
aPKC
Dlg
Lgl
Scribble
Apical
TJ
AJ
Basal
B
Crb1
Crb2
Crb3
ERLI
Pals1
PatJ
Mupp1
Crb-complex
aPKC
Cdc42
Par6a
Par3
Par-complex
Lgl1
Dlg1
Scrib
Lgl-complex
KEY
signal peptide
EGF like
Laminin A/G
transmembrane
FERM
L27
PDZ
SH3
hook
GUK
Rho
PB1
Kinase
WD
LRR
LAPSD
C
dCRB RNKRATRGTYSPSAQEYCNPRLEMDNVLKPPPEERLI
CRB1 SNKRATQGTYSPSRQEKEGSRVEMWNLMPPPAMERLI
CRB2 RKRRQSEGTYSPSQQEVAGARLEMDSVLKVPPEERLI
CRB3 REKRQTEGTYRPSSEEQVGARVPPTPNLKLPPEERLI

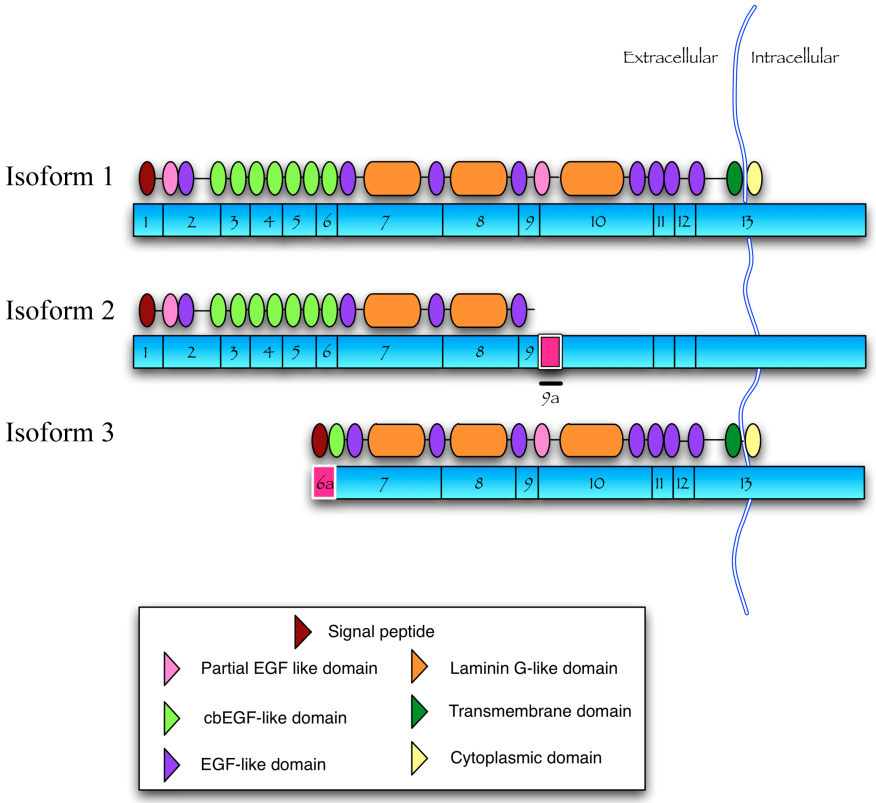


Fig 1.10 **Isoforms of Crb2**. Schematic illustration of the major isoforms of mouse homologue of Crb2. The open reading frame (ORF) is in blue and the protein domains encoded by the exons are depicted above the ORF. Isoform 1 encodes a full length protein that has an extracellular domain, a transmembrane domain and an intracellular domain. Isoform 2 has exon 9a spliced in and this introduces a premature stop codon. Isoform 2 encodes a truncated protein lacking the transmembrane and intracellular domains. Isoform 3 has an alternative start in exon 6a.

In human patients, abnormal Crumbs function has been implicated in causing severe retinal dystrophies such as Retinitis Pigmentosa (RP), Leber Congential Amaurosis and Pigmented Paravenous Chorioretinal Atrophy (den Hollander et al., 2001; McKay et al., 2005). It has been shown that majority of the mutations (more than 85%) in patients with these retinal dystrophies, map to the extracellular domain of human CRB1. However, it was predicted that all nonsense and frameshift mutations result in truncated isoforms lacking transmembrane and intracellular domains (den Hollander et al., 2004). The identification of missense mutations in the extracellular domain would be an indication of its association with retinal disorders.

Nonetheless, it has also been shown that the severity of the retinal dystrophy maybe dependent on environmental cues and/or genetic modifiers (den Hollander et al., 2004). For instance, when *crb* mutant flies have minimal exposure to light they show a mild phenotype unlike flies kept in constant light that demonstrate progressive and substantial retinal degeneration (Johnson et al., 2002). This suggests that reduced light intensity may assist in lowering the severity of RP in patients carrying CRB1 mutations.

1.4.4.5 Crumbs and its association with signalling pathways

The **Hippo** pathway is an evolutionarily conserved signal transduction pathway crucial for regulating tissue size in Drosophila and vertebrates (Reddy & Irvine, 2008). It has been reported that Crumbs regulates growth in Drosophila wing (Chen et al., 2010) and eye imaginal discs (Ling et al., 2010) by interacting with members of the Hippo signalling pathway. Interestingly, the Crumbs complex has also been implicated in coupling the Hippo and **TGF-ß** signalling pathways in regulating cell-density sensing mechanisms (Varelas et al., 2010).

The Crumbs complex has also been associated with the **mTORC** pathway (mammalian Target of Rapamycin) by directly interacting with

TSC1/2 (Tuberous sclerosis complex proten 1 or 2) an inhibitor of the mTORC pathway (Massey-Harroche et al., 2007).

Due to its high sequence homology with the Notch genes, it has been speculated that Crumbs may be a potential neurogenic gene. Additionally, cloned fragments of Crumbs were found to cross-hybridize with Notch under low stringency conditions in Drosophila (Tepass et al., 1990). A potential role for Crumbs in refining **Notch** signalling in Drosophila via the inhibition of γ-secretase was reported (Herranz et al., 2006). This work also demonstrated that the intracellular domain of Crumbs was dispensable for the inhibition of Notch signaling and implicated the extracellular domain in regulating Notch signaling. Consistent with this study, it was demonstrated in an *in vitro* system that human Crb2 inhibits γ-secretase cleavage of amyloid precursor protein (Mitsuishi et al., 2010). Crumbs has also been shown to biochemically interact with the extracellular domain of Notch in zebrafish (Ohata et al., 2011). This increasing evidence suggests a potential role for Crumbs in modulating Notch signalling and this may have important implications for neural development.

1.5 Thesis Aims

As outlined in the introduction, the development of a functionally integrated nervous system is a highly coordinated process involving stringent control of self-renewal, proliferation and differentiation. An important step towards understanding these processes is elucidating the underlying molecular mechanisms involved. As discussed previously, tremendous progress has been made in this direction and several cell polarity proteins have been identified as key cell fate determinants.

Recent work from our lab has shown that Crumbs homolog 2 (Crb2) is a novel regulator of mouse embryonic stem cell (mES) derived neural progenitors *in vitro* (Boroviak & Rashbass, 2011). In this *in vitro* system, Crb2 protein is upregulated at the onset of neural specification

and Crb2 knockdown mES cell lines failed (a) to stabilize apical polarity proteins and (b) to undergo neural differentiation. This suggested that Crb2 is critical for the recruitment of apical polarity complex proteins and it contributes to proliferation, survival of neural progenitors *in vitro*.

For the remainder of this section, I will discuss my hypotheses for how Crb2 could play a potential role in neural development *in vivo*, and how I have addressed some of them in my experiments.

1. Establishment and maintenance of cell junction components

There is evidence that the apical polarity proteins are essential for setting up and maintaining cell-cell junction components by recruiting proteins to the appropriate cellular compartments. Manipulating Crumbs protein levels in *Drosophila* and zebrafish severely impairs the formation of cell junctions and also results in mislocalization of other polarity proteins (Tepass et al., 1990; Malicki and Driever 1999, Omori and Malicki 2006, Ohata et al., 2011). This suggests that the vertebrate homolog of Crumbs- Crb2 might play a similar role in formation and maintenance of cell junctions. To test this, I have analysed Crb2 conditional knockout mouse mutants and chick embryos where Crb2 is misexpressed for altered expression of polarity and cell junction proteins

2. Role in cell fate specification

Initially, I analysed the expression pattern of Crb2 in the two model systems (chapter 3). Crb2 is predominantly expressed at the apical surface of the neural progenitors and this suggested that similar to other apical polarity proteins, Crb2 might also play a role in neural progenitor fate determination. To test this hypothesis, I carried out a candidate gene expression analysis (chapter 4) based on phenotypes observed in conditional knockout mouse models of apical polarity proteins (table 1.3) in the developing cortex of a Crb2 conditional-knockout mouse model at different stages of neurogenesis.

Additionally, previous work from our lab has shown that multiple splice variants of Crb2 exist. To begin to understand the role of one of these splice variants (a truncated isoform that is predicted to be secreted), I characterized this isoform – referred to as Crb2S (secreted Crb2). To further understand the role of Crb2 in neural development, the full length Crb2 (Crb2F) and Crb2S were misexpressed in the developing chick neural tube and I carried out marker expression analysis (chapter 5 and 6). I also analysed the effect of manipulating levels of Pals1, an intracellular binding partner of the Crumbs complex, in neural development (chapter 7).

Finally in the appendices, I present some preliminary data that suggests a role for Crb2 in the patterning of neural progenitors in the chick embryonic spinal cord (appendix 1). In addition to this, I have also included data from preliminary biochemical analyses that suggests a potential interaction between Crb2F and Crb2S (appendix 2)

CHAPTER 2

Materials and Methods

2.1 Cell Culture

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle medium (DMEM) + 10% Foetal Calf serum (Gibco)+ 1% L-Glutamine (Gibco)+ 1% Penicillin/ Streptomycin (Gibco) at 37 °C in 5% CO_2. Cells were routinely passaged using Trypsin-EDTA (0.25% Invitrogen) and seeded at appropriate dilutions for experiments.

2.1.1 Transfection

HEK 293 cells were plated to approximately 95% confluency at least 48 hours before transfection in 100 mm tissue culture dishes (Greiner Bio one). The cells were rinsed 2X in Opti-MEM I Reduced serum medium (Invitrogen) and the transfection mix was added to each dish. The transfection mix consisted of 10 μg DNA and 12 μl Lipofectamine 2000 (Invitrogen) in 600 μl Opti-MEM. This mixture was incubated at room temperature for an hour before adding it onto washed cells. Cells were incubated at 37 °C in 5% CO_2 for 5-6 hours. The transfection medium was then replaced with Opti-MEM+1% L-Glutamine and 1% Penicillin/Streptomycin. Cells were incubated at 37 °C in 5% CO_2 and maintained under serum-free conditions.

Table 2.1 List of constructs used for transfection/electroporation

Vector	Specific construct
pcDNA3.1 V5 His tag (Invitrogen)	Crb2 Full Length
pCDNA3.1 V5 His tag (Invitrogen)	Crb2 Secreted
pCDNA3.1 V5 His tag (Invitrogen)	Control Signal Peptide

2.1.2 Obtaining Crb2S protein containing cell culture supernatant

Transfected HEK 293 cells were cultured for 3-4 days post-transfection, allowing secretion of proteins into the serum-free medium. The cell culture supernatant was centrifuged at 1000g for 10minutes and the resulting supernatant was concentrated 25X using an Amicon Ultra centrifugal filter device 10kDa (Fisher) or Microcon filter unit YM-30 (Millipore)

2.1.3 Generation of stable cell lines

For generating stable cell lines, HEK 293 cells cultured in 6-well dishes (Greiner Bio one) were transfected as described above. After 24 hours, cells from each 6-well were expanded into two 100mm tissue culture dishes with HEK 293 cell culture medium. The following day the medium was changed to HEK 293 cell culture medium+ G418 (800 μg/ml-Sigma Aldrich) and replaced with fresh medium every 3 days. After 14 days, single colonies were picked using a sterile 20μl pipette tip and transferred into a 48 well plate with HEK293 medium+ G418. After the colonies had attached, they were dissociated using Trypsin-EDTA and allowed to reach confluency. Each well was then split into 2 wells of a 12-well plate (Greiner Bio one). Cells from one well were frozen down and cells from the other well were used for screening. For identification of positive clones, cell lysates or cell culture supernatants were collected as described in section 2.1.2. The expression level of protein of interest in the clones was determined by western blotting. Three positive clones were expanded stepwise into T-75 flasks and frozen down and transferred to liquid nitrogen. All the transgenic cell lines were routinely maintained in HEK293 cell culture medium+G418medium.

2.2 Crb2S protein purification and sequencing

A HEK 293 stable cell line overexpressing Crb2S was used for obtaining purified protein. The transgenic Crb2S cell line was passed onto

BioServ UK for scale-up of cells and immobilized metal ion affinity chromatography (IMAC). The cells were maintained in G418 selection antibiotic (800 μg/ml) throughout the culture period. The purified Crb2S protein (100 μg/ml) was sequenced as described below, aliquoted and stored at -80°C.

For protein sequencing, SDS gel electrophoresis was carried out as described below; care was taken to minimize external keratin contamination from the environment. All processing was carried out in a clean biosafety cabinet. The gel was fixed and stained with Coomassie Brilliant Blue (Sigma-Aldrich) as per manufacturer's instructions and the bands of interest were excised using a clean blade and stored at 4°C in a sterile tube. LC-ESI-Mass spectrometry was carried out by a commercial company (Eurogentec) using an LC (*nano*-Ultimate 3000- Dionex)-ESI-ion trap (AMAZONE-Bruker) in positive mode.

2.3 SDS Protein Gel/Western Blot

Cells were washed 2X in Phosphate Buffer Saline (PBS) at 4°C and harvested using RIPA (Radio-immunoprecipitation assay) lysis buffer supplemented with 1 Complete Mini EDTA free protease inhibitor tablet (Roche). Cells were scraped off the surface of the culture dish and passed through a syringe fitted with a 21-gauge needle. The lysate was transferred to a microcentrifuge tube and incubated on ice for 30min. The lysed samples were then microfuged at 2800g for 20min. The protein concentration was determined by Bradford assay using dye reagent concentrate (Biorad) according to manufacturer's instructions. For long-term storage, the lysates were stored at -20°C.

NuPAGE 4-12% Bis Tris gradient precast gels (Invitrogen) were loaded with 20*μ*g total protein of the cell lysates or 30*μ*l of concentrated cell culture supernatant under denaturing conditions. SDS PAGE gel electrophoresis was carried out using the X-Cell Novex MiniCell system (Invitrogen). The gels were run at 180V for 90 min and wet transferred

using the same X-Cell system to a Hybond-C extra nitrocellulose membrane (GE Healthcare). After transfer for 2-3 hours at 20V, the membrane was blocked in blocking solution for 1hour at room temperature. Blocking solution was made with PBS, 5% w/v dried skimmed milk powder (Marvel) and 0.1% Tween (Sigma-Aldrich).

The membrane was then incubated with the appropriate primary antibody (refer table 2.2) in blocking solution at 4°C on a rotating shaker overnight. The following day, 3X PBS+0.1% Tween washes: first wash 15min and subsequent 5min washes were carried out at room temperature. The appropriate HRP-conjugated secondary antibody (Jackson Immunolabs) diluted in blocking solution (1:1000) was added. Membrane was incubated for 1 hour at room temperature on a rotating shaker. After 4X PBS+0.1% Tween washes, the membrane was developed using ECL Plus chemiluminescent detection kit (GE Healthcare). X-Ray films (Amersham Hyperfilm ECL-GE Healthcare) were developed using an X-Ray developer.

2.4 Harvesting embryos

Mice

C57black/6J mice were used to obtain wild type mouse embryos. Timed mating was used to obtain embryos at the appropriate stages; the day of vaginal plug discovery following mating was designated as E0.5. Pregnant mice were killed by cervical dislocation. The embryos were dissected out from the uterine pouch into ice-cold L-15 medium (Gibco).

The embryos were fixed in 4% Paraformaldehyde (PFA) for 2hours at 4°C, transferred to 30% sucrose in PBS and shipped with blue ice. After receiving the embryos, they were transferred to fresh 30% sucrose solution, incubated for 2 hours at 4°C and embedded in optimal cutting temperature (OCT) compound. The frozen tissue blocks were stored at -80°C before being processed for immunostaining.

Transgenic mouse models

The Emx-1 Cre; Crb2 conditional knockout mice and the Nestin Cre; Pals1 conditional knockdown mice were generated by our collaborators at the Netherlands Institute for Neuroscience (Henrique Alves and Bokyung Park working in the laboratory of Jan Wijnholds). A schematic of the Cre-lox technology used for conditional gene knockout is shown in Fig 2.1. A conditional gene-targeting construct for Crb2 was generated using bacterial artificial chromosomes (BACs) and Cre/loxP technology. A 3' *loxP* site was inserted in exon 13 behind the stop codon in the 3' untranslated region of *Crb2*. A neomycin cassette flanked by *frt* recombination sites and a 5' *loxP* site was inserted in intron 9 behind exon 9A. The targeting vector was released from the BAC into a plasmid using homologous recombination. The *loxP* and *frt* recombination sites were tested by expression of the floxed *Crb2* targeting vector in bacterial cells expressing CRE or FLP recombinases. The targeting vector was used to generate $Crb2^{F/+}$ mouse 129 E14 ES cells by homologous recombination. The $Crb2^{F/+}$ conditional knockout mice were generated by blastocyst injections of $Crb2^{F/+}$ ES cells. Chimeric mice gave germ line transmission, thereafter the neomycin cassette was successfully removed by crossing the $Crb2^{F/+}$ mice with a transgenic mouse that expressed FLP recombinase in the germ line (129S4/SvJaeSor-t(ROSA)26Sortm1(*FLP1*)Dym/J mice; Jackson lab). Two $Crb2^{F/+}$ mouse lines were generated from two independent ES cells clones these lines were designated P1E9 and P11D6. The two lines gave identical phenotypes. The conditional knockout mice were crossed with Emx1-*Cre* mice *(B6.129S2-Emx1tm1(cre)Krj/J*; Jackson lab) expressing Cre recombinase in the developing neuroepithelium of the cerebral cortex. *shPals1* mice previously described in Park et al., 2011 were crossed with Nestin-*Cre* transgenic mice to obtain shPals1 conditional knockdown mice.

For the analysis of the mutant mouse models, embryos were genotyped and sent from Amsterdam in 30% sucrose solution. I embedded and processed the embryos as described in sections 2.7 and 2.8. A minimum of three control and three conditional knockout embryos were used for marker analysis.

Chick

Fertile brown chicken eggs (Henry Stewart & Co. Ltd) were incubated at 39°C and the embryos were staged according to Hamburger and Hamilton staging system (Hamburger and Hamilton, 1951). Embryos were dissected by cutting into the vitelline membrane and around the embryo with a pair of dissection scissors. Embryos were then transferred to ice-cold Leibovitz's L-15 medium (L-15) or Hank's balanced salt solution-HBSS (Gibco)

2.5 Explant culture

Hamburger and Hamilton (H&H) Stage 10-11 chick embryos were dissected into ice-cold L-15 medium. Embryos were treated with Dispase (Roche) at room temperature for 5-15min. The treatment was stopped by addition of L-15 medium + 2% foetal calf serum and embryos transferred to ice. After 30 min, neural tubes were dissected away from the surrounding embryonic tissue. The notochords were left intact at this point to distinguish dorsal from ventral. The neural tubes were transferred to 2x changes of OptiMEM medium. The neural tube was sub-dissected into rhombomeres. Each rhombomere was carefully transferred using a

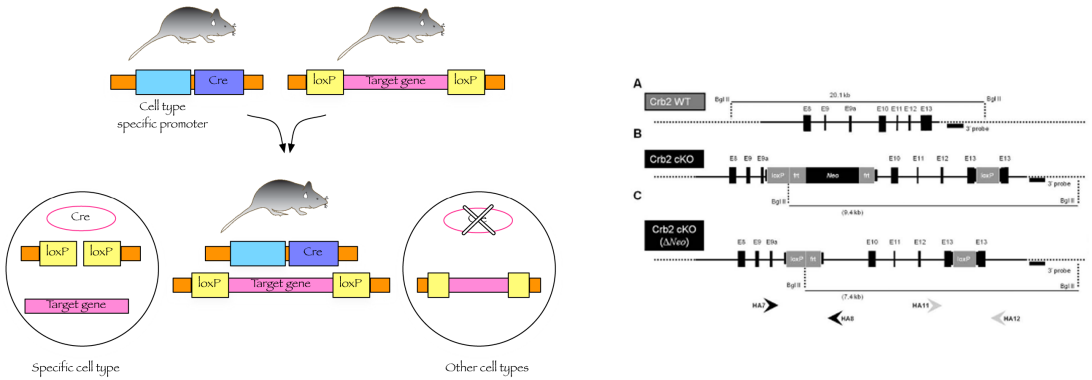


Fig 2.1 **Schematic illustration of the Cre-lox mouse breeding strategy.** Transgenic mice expressing Cre protein in specific cell lineages are crossed with transgenic mice that contain a target gene flanked by loxP sites. Target gene expression is only disrupted in the specific cell type expressing Cre Adapted from Sauer 1998. **Schematic representation of the Crb2 targeting construct and genotyping strategy** (A) Crb2 wild type gene (WT) composed of 13 exons (B) Crb2 targeting construct, the loxP recombination sites are flanking the last 4 exons (10 and 13), the targeting construct also contains a neomycin cassette flanked by frt recombination sites. (C) Crb2 targeting construct after frt recombination and deletion of the neomycin cassette. The localization of the 3' end arm probe used to characterize the targeting construct, and of the primer pairs, HA7/8 and HA11/12, located around the loxP recombination sites and used for genotyping. Two Bgl II restriction sites located outside the targeted DNA, one extra Bgl II restriction site is present in the targeting construct near the 5' end loxP site.

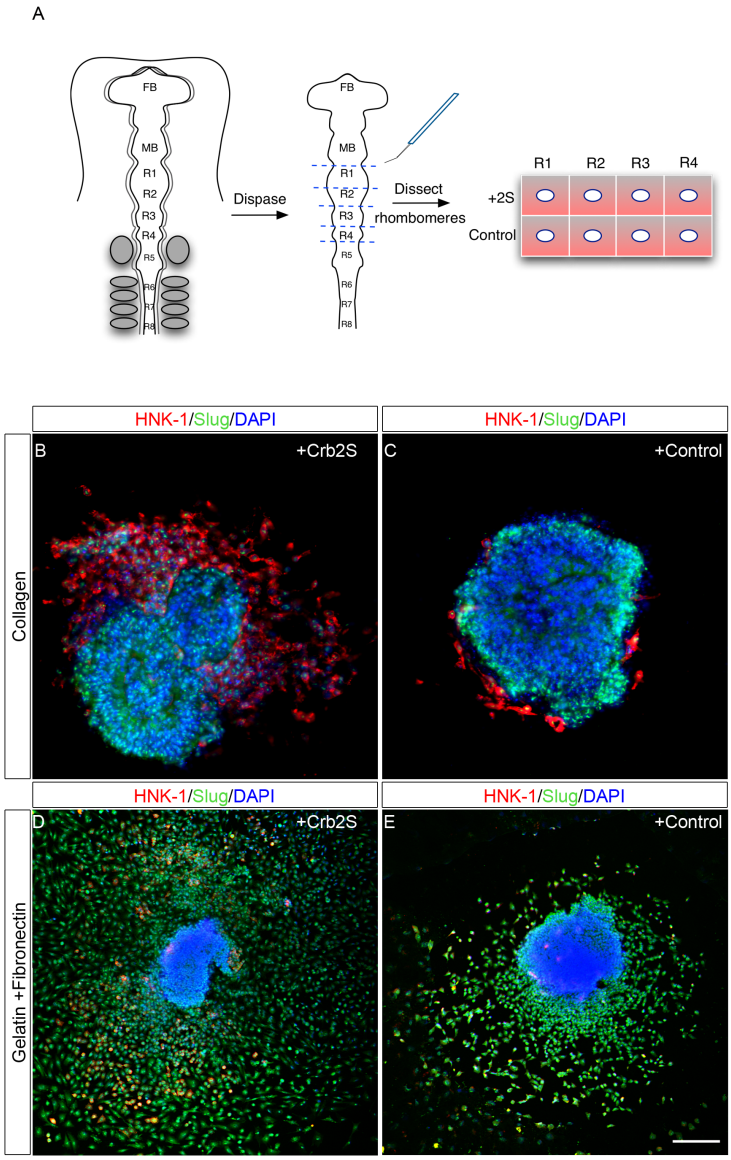


Fig 2.2 ***In vitro*** **explant culture setup** A. Schematic illustration of the in vitro explant culture system used to assay neural crest migration. The neural tube was dissected from an H&H stage11 chick embryo after dispase treatment. The hindbrain was sub-dissected into rhombomeres, plated onto a gelatin and fibronectin coated dish and cultured in serum-free medium alone or with purified Crb2S protein. B. Gelatin+Fibronectin was a permissive substrate for neural crest migration in vitro under serum-free conditions. Hindbrain explants from H&H stage 11 chick embryos cultured for 24hours on collagen (B-C) and Gelatin+Fibronectin (D-E) immunostained as for HNK-1 (red) and Slug (green). B,D represent control explants E,F represent explants cultured with Crb2S. Note the increased migration in Crb2S treated explants cultured in collagen (C) and gelatin/fibronectin compared to the control explants (E). Nuclei are counterstained with DAPI and shown in blue. Scale bar= 20 µm

200 μl pipette tip to 0.1% Gelatin (Sigma) and 50μg/ml Fibronectin (Invitrogen) coated dishes (Ibidi) with Opti-MEM I reduced serum medium, 1% Penicillin-Streptomycin and 1% L-Glutamine. The explants were cultured for 24 hours at 37°C and 5% CO2 prior to fixing in 4% PFA and further processing.

Preliminary analyses suggested that a combination of fibronectin and gelatin is a suitable substrate for adhesion of explants and neural crest migration under serum-free conditions compared to collagen (Fig 2.2).

2.6 *In ovo* electroporation

H & H St10 embryos were electroporated as described previously by (Itasaki et al., 1999). Briefly, eggs were windowed and the extra-embryonic membrane partially removed. A few drops of sterile HBSS medium were added to the embryo and DNA solution was injected into the lumen of neural tube. Excess DNA was washed away with HBSS and electroporation was carried out using a BTX ECM830 square wave electroporator with 4X 26V square wave pulses of 10millisec duration and 1 second interval between each pulse. The eggs were then sealed using Parafilm and incubated at 39 °C (Sanyo) for 24h-48h.

2.7 Cryostat embedding and sectioning

Embryos were fixed in 4% PFA at 4°C for 2 hours in a rotating shaker, washed 3X 5min at room temperature. Embryos were then transferred to 30% sucrose in PBS at 4°C and left overnight. Tissue was embedded in OCT and rapidly frozen on dry ice. Frozen blocks were stored at -80°C in a sealed container.

2.8 Immunostaining of cryosections

Solutions used for immunostaining:

1. Permeabilization solution – PBS + 0.5% Triton-X 100
2. Blocking solution – PBS+5% Heat inactivated donkey serum (HIDS)
3. Antibody solutions- Primary antibody (refer table 2.2) diluted in blocking solution, secondary antibody diluted in PBS.

15μm thick sections were cut using a cryostat (Bright). Before cutting the frozen blocks were mounted on a chuck and allowed to reach the cryostat chamber temperature for 30 min. Sections were collected on Superfrost slides and air-dried for 2hours. After 1X wash for 5min in PBS, sections were permeabilized in 0.5% Triton-X 100/PBS for 10min. 5% HIDS and 0.1% Triton-X 100 in PBS was used for blocking. After 1hour of blocking, 250μl primary antibody was added and the slides were incubated in a humidified chamber at 4°C overnight. Slides were then washed 3X - 5min in PBS and corresponding secondary antibody with DAPI (4,6-diamidino-2phenylindole, Molecular Probes) was added. They were incubated for 1hour at room temperature in a dark humidified chamber, washed 3X in PBS and then mounted in Vectashield (Vector laboratories) with a glass coverslip sealed with clear nail varnish (Boots No.7). Slides were stored in the dark at 4°C before imaging.

Note: For chick embryo sections, there was no separate permeabilization step before blocking. Also, sections were blocked only for 30min.

2.9 Immunostaining explant culture/cells on glass coverslips

Cell culture dishes were washed 2X in PBS. Cells/explants were fixed in 4% PFA for 10min at room temperature. 2X PBS washes, followed by blocking in PBS+ 0.1%Triton-X 100 + 1% heat inactivated serum for 30 min. Primary antibody diluted in blocking solution was added

and culture dishes were stored at 4°C overnight. After 3X changes of PBS, appropriate secondary antibodies (+DAPI) were added and incubated for an hour at room temperature. After 3X PBS washes, the coverslips were mounted on glass slides and sealed with clear nail varnish. Cell culture dishes were left in PBS solution at 4°C in a sealed box before imaging.

Table 2.2 List of antibodies used for immunofluorescence (IF) and western blotting (WB)

Antibody	Source	Dilution
Crumbs2	Custom made Eurogentec- (EMDSVLKVPPEERLI) and (AWEGPRCEIRAD)	1:500 (IF) 1:1000 (WB)
Pals1	Abcam, rabbit polyclonal	1:200 (IF)
Par3	Millipore, rabbit polyclonal	1:200 (IF)
PKC-zeta	Santa Cruz, rabbit polyclonal	1:200 (IF)
PKD/PKC*μ*	Cell Signalling, rabbit polyclonal	1:100 (IF)
ZO-1	Zymed, mouse monoclonal	1:200 (IF)
ß-Catenin	BD Biosciences, mouse monoclonal	1:500 (IF)
N-Cadherin	BD Biosciences, mouse monoclonal	1:100 (IF)
NCAM	Chemicon, rabbit polyclonal	1:200 (IF)
pH3	Upstate, rabbit polyclonal	1:500 (IF)
Ki67	Novocastra, rabbit polyclonal	1:500 (IF)
Pax6	DSHB, mouse monoclonal	1:50 (IF)
Sox2	Millipore, rabbit polyclonal	1:500 (IF)
Nestin	Abcam, mouse monoclonal	1:300 (IF)
CD133	Abcam, rabbit polyclonal	1:250 (IF)
Tuj1	Covance, mouse monoclonal	1:500 (IF)
BLBP	Chemicon, rabbit polyclonal	1:250 (IF)
GFAP	Abcam, rabbit polyclonal	1:250 (IF)

HNK-1	Sigma, mouse monoclonal	1:100 (IF)
Slug	Abcam, rabbit polyclonal	1:100 (IF)
V5 Tag	Abcam, chicken polyclonal	1:200 (IF) 1:2000 (WB)
His Tag	Cell signalling, rabbit polyclonal	1:1000 (WB)
Calnexin	Cell signalling, rabbit polyclonal	1:100 (IF)

FITC, RRX and Cy5 conjugated secondary antibodies raised in donkey were from Jackson Immunolabs. They were used at 1:200.

2.10 Microscopy and Image processing

Images were captured using ZEISS LSM 510 META confocal microscope or ZEISS Apotome microscope with Axioimager. Imaging of explant cultures was done using a Leica fluorescent dissecting microscope using Leica Firecam software. Images were processed using ImageJ (NIH, http://rsb.info.nih.gov/ij), Photoshop CS4 and Bridge CS4 (Adobe). Statistical analysis was performed using GraphPad Prism.

2.11 General molecular biology

Bacterial cell culture

LB Agar was prepared by dissolving 35g of LB-Agar (Sigma-Aldrich) in 1l of deionized water. After autoclaving, the agar was allowed to cool down and appropriate antibiotic was added before pouring the agar into bacterial dishes.

LB Broth was prepared by dissolving 20g of LB-Broth (Sigma-Aldrich) in 1l of deionized water and autoclaving.

Bacterial cells were grown at 37 °C in LB medium+ antibiotic on a shaker at 225rpm or on LB agar plates. To make glycerol stocks for long term storage, two part volumes of bacterial culture in the exponential

growth phase was mixed with 1 part of 80% glycerol in LB-Broth and stored at -80°C.

Plasmid extraction and purification

Plasmids were extracted and purified using commercially available kits (Qiagen), according to manufacturer's instructions.

RNA extraction, cDNA synthesis

RNA was extracted using RNAeasy kit (Stratagene) following manufacturer's instructions. For RNA extraction from mouse liver and skeletal muscle, phenol chloroform was used instead of the kit. The purified RNA was eluted in dH_20 and the concentration was determined using NanoDrop ND1000 (Labtech). 3μg of RNA per 20μl reaction was used for reverse transcription using SuperScript III reverse transcriptase (Invitrogen), according to manufacturer's instructions with random primers from Promega.

RT-PCR

DNA amplification was done using a PTC-200 Thermocycler (MJ Research). For a reaction volume of 25μl, double distilled water, 1X PCR buffer (Promega) 1mM $MgCl_2$, 0.2mM dNTPs, 1μl Taq polymerase, 0.25μM forward primer and 0.25μM reverse primer.

The PCR program used was as follows:

Initial denaturation	95°C	2 min	
Denaturation	95°C	20 sec	30-35 cycles
Annealing	60°C	40 sec	
Extension	72°C	1 min	
Final elongation	72°C	5min	

Table 2.3 Primer sequences used for RT-PCR/ cloning

Gene	Forward Primer	Reverse Primer
Mouse Crb2S	CTTGGTGATGCTCAGCTTTG	AGCTTCGGTTGGTAGACTGC
Mouse GAPDH	AACGGGAAGCCCATCACC	CAGCCTTGGCAGCACCAG
Mouse Crb2F	AAGTCTAAGgcggccgctCAGGCAGAG CCGGCTGCCAT Not1 restriction site	GTACGTCCGgtcgacGGCACCAGCAG CCAGGCAAAC Sal1 restriction site
Mouse Crb2S	AAGTCTAAG gcggccgctATGGCGCTG GTGGGGCCTA Not1 restriction site	GTACGTCCG gtcgac CTAAGAAGGC ACAGTCGAGGCTGA Sal1 restriction site

Primers were supplied by Sigma Aldrich UK and DNA sequencing was carried out by the Core Genetic Facility, University of Sheffield.

Transformation of bacterial cells

One shot TOP10 (Invitrogen) chemically competent cells were routinely used for transformation of plasmid DNA according to manufacturer's instructions.

DNA electrophoresis

Agarose gels were made using 1% ultra-pure Agarose (Invitrogen) in 1X TAE (50mM TrisHCl (Sigma Aldrich), pH 8.0; 1mM EDTA (VWR International), 0.02M acetic acid (Fisher Scientific), heated in a microwave until completely dissolved. After the solution had cooled, Ethidium Bromide (Bio-Rad) was added to a final concentration of 0.7 ug/ml. 10X loading buffer [4% v/v saturated bromophenol blue (Sigma Aldrich) solution, 20% 50X TAE, 40% glycerol (Fisher Scientific) in

deionized water] was added to the sample at a final concentration of 1X. Gel was run in 1X TAE at 80 V. Bio-Rad DNA Mini Sub Cell GT electrophoresis kit was used for electrophoresis. The bands were visualized using a UV transilluminator. 1kb DNA ladder (New England Biolabs or Promega) was always run on the gel for size reference.

Sub-cloning, Restriction digestion and ligation

To amplify DNA for making constructs, Pfu high fidelity polymerase (Rovalab) was used. All restriction enzymes used were from New England Biolabs and used according to manufacturer's instructions. PCR product and digested vector backbone were purified using QIAquick PCR purification kit (Qiagen).

The cut vector backbone and insert were analysed by gel electrophoresis and subsequently mixed at a ratio of 1:3 for ligation. T4 DNA ligase was used according to manufacturer's instructions. The ligation reaction was then used for transformation into TOP10 cells. Colonies were screened and diagnostic restriction digests carried out to identify positive clones. DNA from positive clones was sequenced and glycerol stocks were made.

2.12 *In situ* hybridisation

Solutions used for the protocol

Prehybridisation solution – 50% Formamide, 5X SSC (saline sodium citrate) buffer –pH 7, 2% Boehringer Blocking powder, 0.1% Triton X-100, 0.5% CHAPS, 100μg yeast RNA, 50μM EDTA and 50μg/ml Heparin.

Solution I- 50% Formamide, 5X SSC pH4.5, 1% SDS

SolutionII- 50% Formamide, 2X SSC pH 4.5 0.1% Tween 20

NTMT- 0.1M NaCl, 0.1M Tris pH9.5, 0.05 $MgCl_2$, 0.1% Tween 20

Embryos were harvested as described in section 2.4 and fixed in 4% PFA overnight at 4°C. After 2X PBS+0.1% Tween-20 (Sigma Aldrich)

washes, the embryos were dehydrated by taking them through a series of methanol washes – 25%, 50%, 75% and 100%. The embryos were then rehydrated using the reverse graded methanol series -100%, 75%, 50% and 25% and incubated in prehybridisation solution at 68°C for 3-4 hours. 0.2-1μg digoxigenin (DIG) riboprobe was diluted in prehybridisation buffer, denatured at 68° C and incubated with embryos overnight at 68°C.

The following day embryos were washed 2X with solution I and 2X with solution II for 30min each at 68°C and blocked in 10% heat inactivated goat serum in PBS-T for 90min at room temperature. After blocking the embryos were incubated with 1:2000 anti-DIG Alkaline phosphatase Fab fragments overnight at 4°C. After 8X post-antibody washes the embryos were developed in NTMT containing NBT and BCIP. When the colour had developed to the desired extent, the reaction was terminated by washing with PBS-T. Embryos were re-fixed in 4% PFA and stored at 4°C before imaging.

The following template DNA was used to generate a DIG-labelled antisense RNA probe: Plasmid pBS Hes5 (from Verdon Taylor) containing a cDNA fragment encoding mouse Hes5 was linearised with Hind III restriction enzyme and transcribed with T3 polymerase. Chicken EST (ChEST) clone Crb2-663n24 in pBluescript II vector was used to generate antisense Crb2 riboprobe. Not1 was used for linearization and T3 polymerase for *in vitro* transcription.

For *in situ* hybridization on sections, previously published protocols were followed (Manning et al., 2006; Strahle et al., 1993). Briefly, 20 μm cryostat sections were collected on Superfrost plus glass slides (VWR) and air-dried. The slides were kept dry and stored at -20°C. The following day sections were rehydrated with 3X washes of PBS and acetylated using 11.6μl/ml Triethanolamine and 2.5μl/ml acetic anhydride in water. Acetylation was followed with equilibration in 5X SSC buffer (pH 6) for 7min and incubation in prehybridisation solution for a minimum of 2 hours at 65 °C. The antisense probe was diluted in 100μl of prehybridisation

buffer and incubated overnight at 65 °C. The following day slides were processed similar to the whole mount *in situ* protocol. After stopping the developing reaction, slides were washed 3X in PBS and mounted using Aquamount (BDH)

2.13 *In ovo* manipulation

Dorsal and dorso-lateral telencephalic tissue from E17.5 WT brains was dissected using a fine tungsten needle from 200μm vibratome sections of the brain. The tissue was incubated in 500nM SYTO green dye at 37°C for 10min and transplanted into H&H St10 chick embryos in the caudal neuropore region, after making a small incision. Eggs were sealed and incubated at 39°C for 24hours prior to further processing.

Affigel beads (Biorad) were soaked for 24 hours in approximately 100nM of purified Crb2S protein. H&H Stage 10 chick embryos were accessed *in ovo* by making a small window in the eggshell. Beads were implanted in the caudal neuropore or inside the hindbrain region, resealed and incubated at 39°C for 24 hours prior to fixation and analysis by immunostaining.

CHAPTER 3

Expression profile of Crb2 in the developing central nervous system of chick and mouse embryos

3.1 Introduction

The earliest reported expression of *Drosophila* Crumbs protein is during gastrulation (Tepass et al., 1990). After gastrulation, Crumbs expression is detected in all the ectodermally derived epithelia analysed (Bulgakova & Knust, 2009).

The vertebrate homologs of Crumbs exhibit dynamic expression patterns during development and in adult tissues that are suggestive of tissue-specific functions. The expression of the different Crumbs homologs has previously been discussed in Chapter 1 (1.4.4.1).

Recently, the expression pattern of Crb2 mRNA in the early stages of mouse embryonic development was described (Xiao et al., 2011). However, the expression profile of Crb2 protein in the developing central nervous system of chick and mouse embryos has not yet been reported.

The aim of this chapter was to analyze the temporal and spatial expression pattern of Crb2 in the developing chick and mouse embryonic nervous system. The expression studies were restricted to two specific regions in each of these model systems: the hindbrain in the chick embryo and the dorsal telencephalon in the mouse embryo. In the chick embryonic system, *in ovo* electroporation and manipulations described in chapter 6 were carried out at Hamburger and Hamilton (H&H) stage10 and embryos were allowed to develop to H&H stage17 to study the role of Crb2 during neural development. The expression profile of Crb2 during the different developmental stages from H&H stages 10-17 is described in this chapter. In chapter 4, I have analysed a dorsal telencephalon specific Crb2 conditional knockout and this chapter shows the expression profile of Crb2 in the telencephalon of wild type mouse embryos during early (E12.5), mid (E14.5) and late (E17.5) stages of cortical neurogenesis.

3.2 Results

CHICK

3.2.1 *Crb2* mRNA is expressed in the neural tube of a developing chick embryo

To detect the expression of chick *Crb2* transcripts *(cCrb2)* in Hamburger and Hamilton (H&H) stage 10-stage 17 chick embryos, I carried out whole mount *in situ* hybridization. At stage 10, *cCrb2* is expressed in the developing forebrain, midbrain, caudal hindbrain and in the spinal cord (Fig 3.1A). At stage 11, *cCrb2* is expressed in the forebrain, midbrain and at the midbrain-hindbrain boundary (Fig 3.1B). By stage 11, the rhombomeres of the hindbrain are distinctly visible and *cCrb2* is strongly expressed only in the caudal rhombomeres (arrow heads in Fig 3.1B) but not in the rostral rhombomeres (arrows in Fig 3.1B).

In contrast to its expression at stage 10 and 11 wherein *cCrb2* is expressed in a rhombomere-specific manner, by stage 12 cCrb2 is expressed uniformly in the developing neural tube (Fig 3.1C). *cCrb2* expression is also detected in the optic vesicle (arrow in Fig. 3.1C).

Analysis of stage 13, stage 15 and stage 17 embryos showed that *cCrb2* is expressed in the developing eye and along the anterior-posterior axis of the neural tube (Fig. 3.2). Additionally, at stage 17 *cCrb2* expression is also detected in the branchial arches (arrow in Fig 3.2E).

15μm transverse sections were cut through the whole mount embryos and the expression of *cCrb2* at different levels of the neural tube was examined. At stages 10 and 11, *cCrb2* is expressed in the neuroepithelial cells (Fig 3.3, 3.4). At both these stages, particularly at stage 10 there is a dorsal ^{low}-ventral ^{high} gradient expression of *cCrb2* in the hindbrain region (Fig 3.3 arrow in E'-G').

As development proceeds, the dorsal ^{low}-ventral ^{high} gradient expression of *cCrb2* is no longer evident and the expression of cCrb2 transcripts within the neural tube is noticeably more concentrated at the apical surface of the neuroepithelial cells (Fig 3.5 and Fig 3.6). In the stage 15 embryonic eyes, *cCrb2* is expressed in the lens cup and is apically enriched in the retinal pigment epithelium (Fig. 3.6 B").

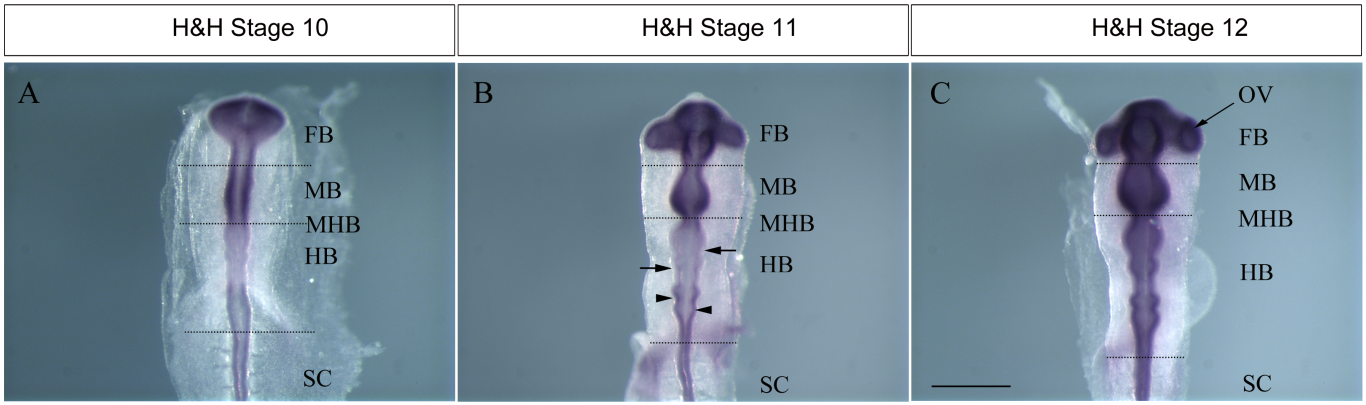


Fig 3.1 **Whole mount *in situ* hybridization analysis of Crb2 expression in chick embryos.** A-C Dorsal views of H&H stage 10-stage 12 chick embryos show expression of Crb2 mRNA in the neural tube. A. In H&H stage 10 chick embryo, Crb2 is expressed in the developing forebrain, midbrain, caudal hindbrain and spinal cord B.In H&H stage 11 chick embryo, Crb2 is expressed in the developing forebrain, midbrain, midbrain-hindbrain boundary, caudal hindbrain and spinal cord. C. H&H Stage12 chick embryo shows Crb2 expression in the optic vesicle, developing forebrain, midbrain and hindbrain. Dotted lines delineate the different regions of the brain. Arrows in B denote rostral rhombomeres with less Crb2 transcripts and arrow heads denote caudal rhombomeres where Crb2 is highly expressed. H&H - Hamburger and Hamilton FB-Fore-brain, MB-Midbrain, HB-Hindbrain, MHB-Midbrain-Hindbrain boundary, SC-Spinal cord, OV-Optic vesicle. Scale bar = 1 mm

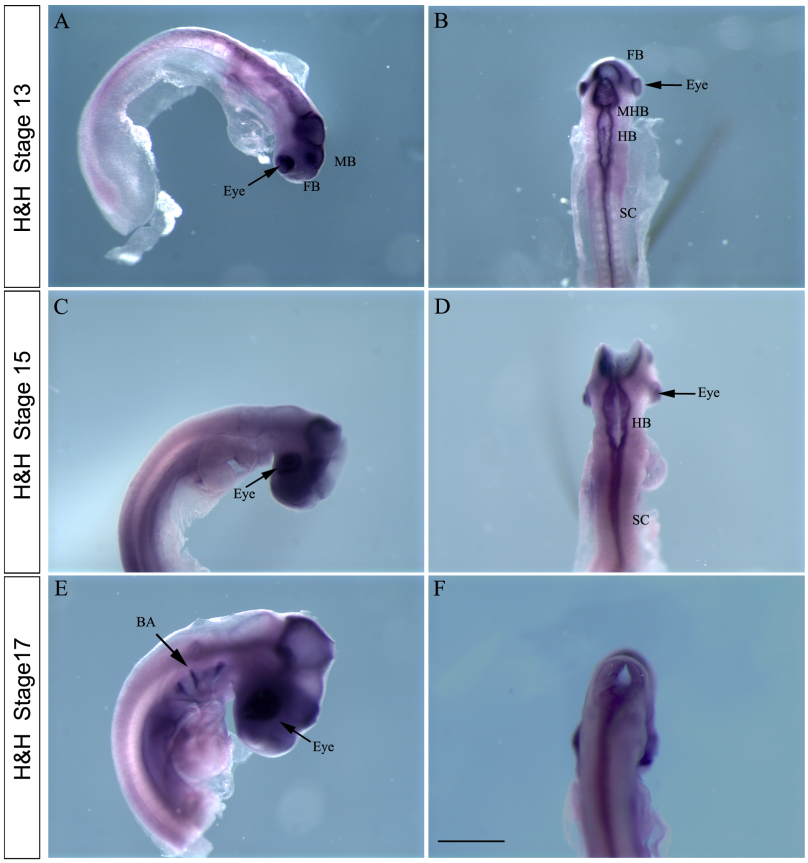


Fig 3.2 **Expression pattern of Crb2 mRNA in H&H Stage 13-Stage 17 chick embryos.** Lateral and dorsal views of H& H stage 13, 15 and 17 whole mount *in situ* hybridised chick embryos show Crb2 mRNA expression in the developing neural tissue. A-B. In H&H stage 13 embryo, Crb2 is expressed in the developing eyes (arrows in A and B), FB, MB, HB and SC. C-D. In H&H stage 15 embryo, Crb2 is detected in the developing neural tube and eyes (arrows in C and D) E-F In H&H stage 17 embryo, Crb2 is expressed in the developing neural tube, eyes and in the branchial arches (arrows in E). FB-Forebrain, MB-Midbrain HB-Hindbrain SC-Spinal Cord MHB Midbrain-Hindbrain boundary BA-Branchial arch H&H-Hamburger and Hamilton. Scale bar = 1 mm

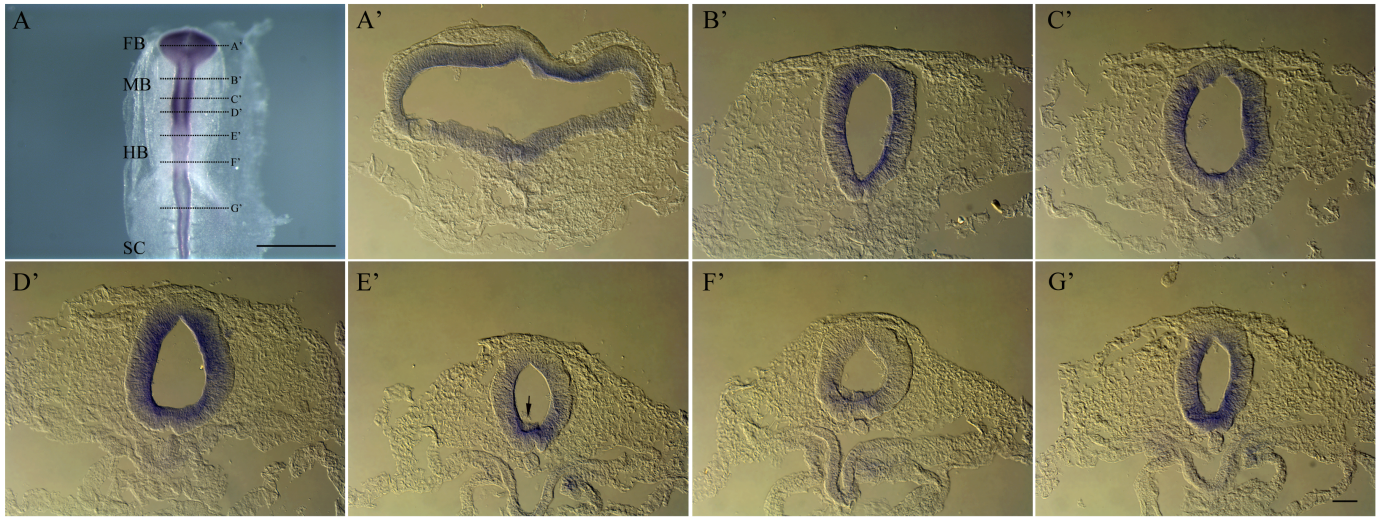


Fig 3.3 **Expression of Crb2 in the neural tube of H&H Stage10 chick embryo.** A. Dorsal view of H&H stage 10 chick embryo hybridised with Crb2. Dotted lines indicate the approximate position of transverse sections through the embryo. A' In the forebrain region, Crb2 mRNA is expressed in the neuroepithelium B'-C' In the midbrain region, Crb2 expression is confined to the neural tube. Crb2 mRNA expression is stronger close to the lumen. D' Crb2 is highly expressed in the midbrain-hindbrain region. E'-F' In the rostral hindbrain, Crb2 is expressed in a dorsal low -ventral high manner. Arrow in E shows high Crb2 expression in the ventral neural tube. G' In the presumptive spinal cord region, Crb2 mRNA is apically enriched. H&H- Hamburger and Hamilton FB-Forebrain, MB-Midbrain, HB-Hindbrain, SC-Spinal cord. Scale bars = A 1mm, A'-G' 100 μm

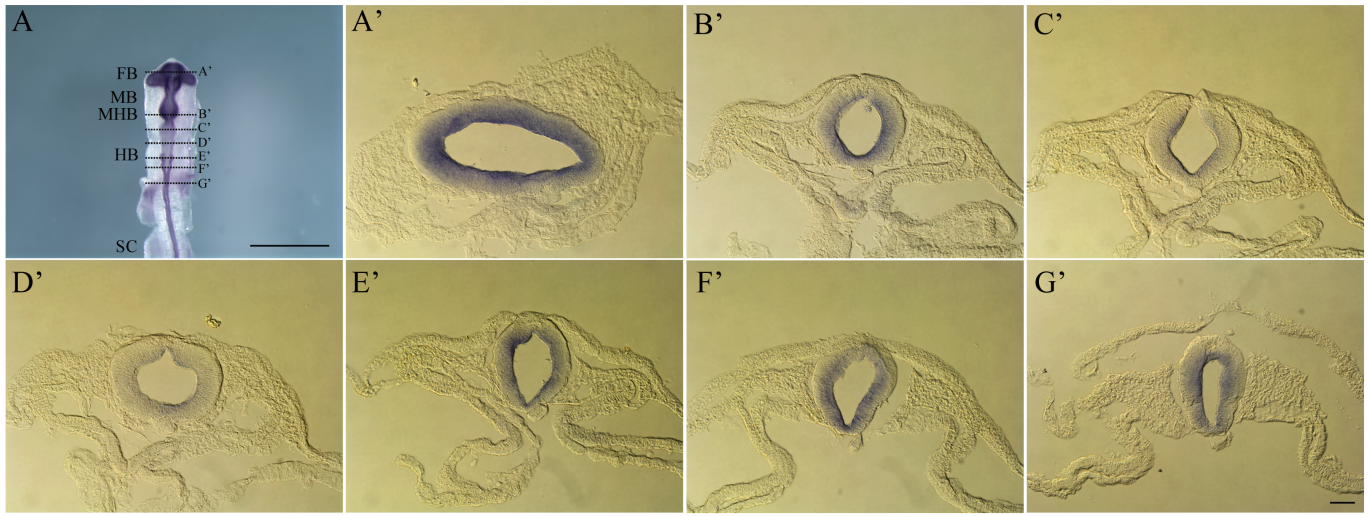


Fig 3.4. **Expression of Crb2 in the hindbrain of H&H stage 11 chick embryo is rhombomere-specific.** A. Dorsal view of H&H stage 11 chick embryo hybridised with Crb2. Dotted lines indicate approximate level of the transverse sections shown in A'-G'. A' In the forebrain region, Crb2 is expressed in the neuroepithelial cells. B' In the midbrain region, Crb2 transcripts are confined to the neural tube C'-D' In the rostral hindbrain region, Crb2 is expressed at low levels in the neural tube. E'-F' In the caudal hindbrain region, Crb2 is enriched closer to the lumen G' Apical Crb2 expression is detected in the developing spinal cord region. Scale bars = A 1 mm, A'-G' 100 μm

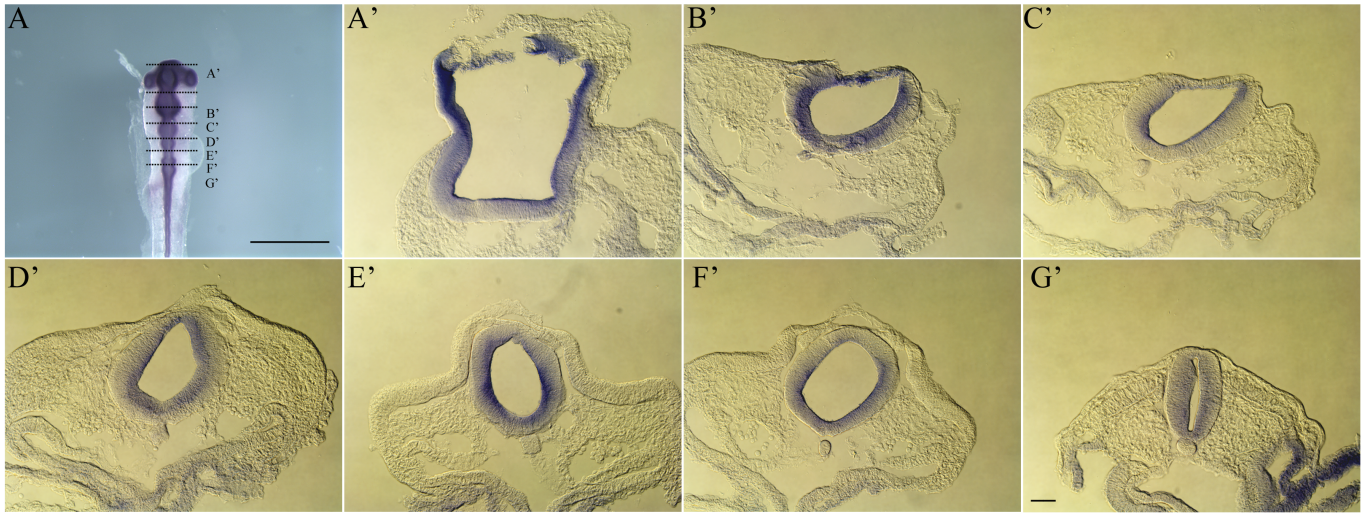


Fig 3.5. **Expression of Crb2 in the hindbrain of H&H stage 12 chick embryo is rhombomere-specific.** A. Dorsal view of H&H stage 12 chick embryo hybridised with Crb2. Dotted lines indicate approximate level of the transverse sections shown in A'-G'. A' In the forebrain region, Crb2 is expressed in the neuroepithelial cells. B' In the midbrain region, Crb2 transcripts are confined to the neural tube C'-F' In the rostral hindbrain region, Crb2 is enriched at the lumenal surface of the neural tube. G' Crb2 expression is detected in the developing spinal cord region. Scale bars = A 1 mm, A'-G' 100 μm

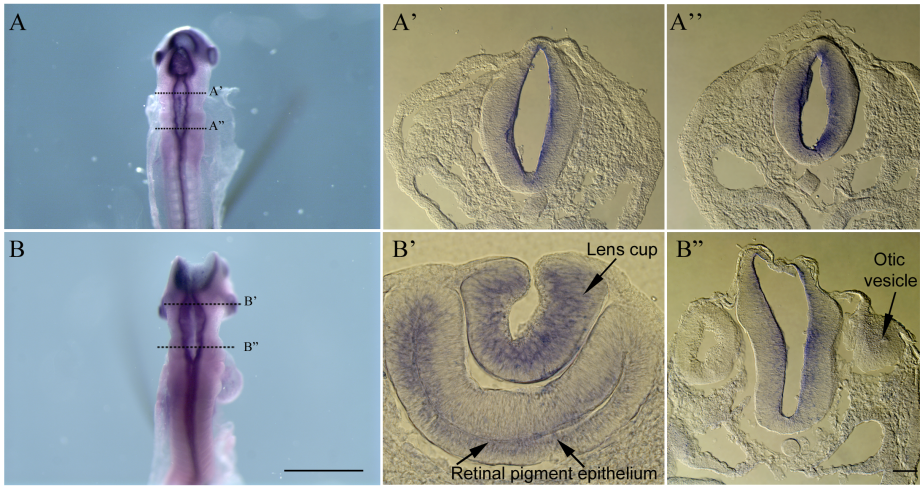


Fig 3.6 **Expression of Crb2 in H&H Stage 13 and Stage 15 chick embryos.** A-B Dorsal views of stage 13 and stage 15 embryos hybridised with Crb2. Dotted lines indicate the approximate position of transverse sections through the embryos. A'-A" In the hindbrain region of H&H stage13 embryo, apical accumulation of Crb2 transcripts is detected within the neural tube. B' In the developing eye of H&H stage 15 embryo, Crb2 is expressed in the lens cup and retinal pigment epithelium. B" In the hindbrain region, Crb2 is apically enriched in the neuroepithelial cells. Scale bars = A-B 1 mm, A'-B" 100 μm.

1.2.2 Crb2 protein is apically localized in the developing chick neural tube

To determine the sub-cellular localization of cCrb2 protein I carried out immunostaining on coronal and transverse sections through the chick embryo for Crb2.

Coronal sections through an H&H stage 11 chick embryo were immunostained for Crb2. cCrb2 is expressed in the forebrain, midbrain, midbrain-hindbrain boundary and spinal cord (Fig 3.7). cCrb2 protein is apically localized in the neural epithelium. Consistent with the expression of cCrb2 mRNA, cCrb2 protein expression is also rhombomere-specific in the hindbrain. Crb2 protein is weakly detected in rostral rhombomeres (asterisk in Fig 3.7 B). Conversely, in caudal rhombomeres Crb2 protein is highly expressed at the apical surface of the neural tube (arrow head in Fig 3.7 B). Intriguingly, Crb2 staining is also detected at the basal surface (arrows in Fig 3.7 B).

Immunostaining of transverse representative sections through the developing brain of an H&H stage11 chick embryo show that Crb2 protein is also variably expressed in the hindbrain region, where apically localized Crb2 staining is observed only in sections through the caudal hindbrain (Fig 3.7 H) but not in sections through the rostral hindbrain (Fig 3.7 E-G).

Whole mount immunostaining and immunostaining of transverse sections through an H&H stage 13 embryo show Crb2 expression in the neural tube along the anterior-posterior axis. In the transverse sections, Crb2 is detected at the luminal surface of the neuroepithelial cells (Fig 3.8 B-C). Similar to the basal staining detected at stage 11, at stage 13, Crb2 staining is also detected at the basal surface (asterisk, Fig 3.8 A)

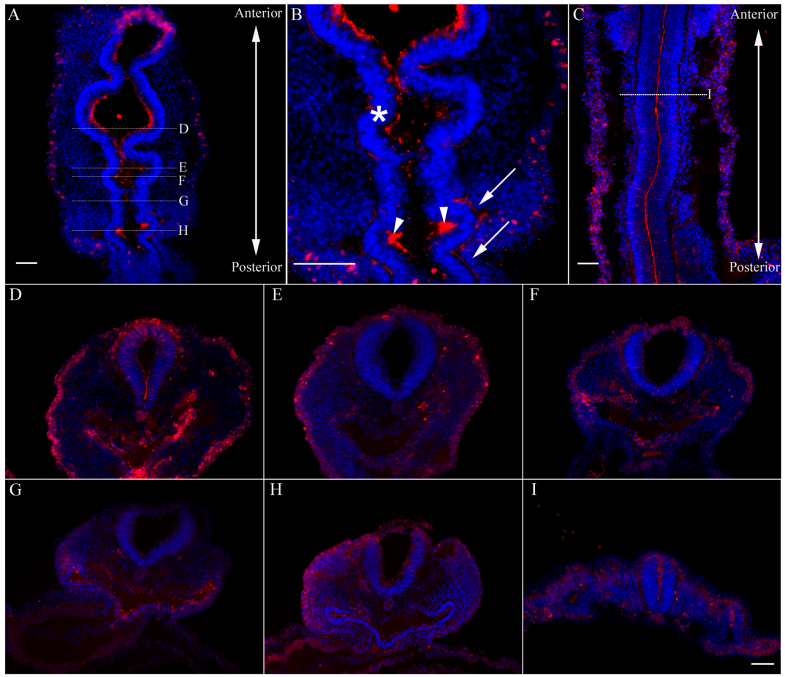


Fig. 3.7 **Expression of Crb2 protein in H&H stage 11 chick embryo.**
A. Coronal section through H&H stage 11 chick embryo immunostained for Crb2 shows Crb2 expression in the forebrain, midbrain and in the caudal hindbrain. Dotted lines indicate approximate level of the transverse sections shown in D-H. Higher magnification of A is shown in B. Arrows in B show basal staining of Crb2 in the neural tube. * - weak Crb2 expression in the rostral hindbrain, arrow heads - Crb2 expression in the caudal hindbrain region C. Coronal section through H&H stage 11 chick embryo immunostained for Crb2 shows apical Crb2 expression in the spinal cord. Dotted line in C indicates approximate position of the transverse section shown in I. D- In transverse sections through the midbrain region, Crb2 staining is apically enriched in the neural tube. Crb2 is also expressed in the dorsal roof plate, overlying ectoderm and the surface ectoderm. E-G In the rostral hindbrain weak or no expression of Crb2 protein is detected at the lumenal surface of the neural tube. H-In the caudal hindbrain, Crb2 is apical enriched in the neural tube. Crb2 is also expressed in the gut. I- In the spinal cord region, Crb2 is apically localized in the neural tube. Crb2 is shown in red. Nuclei counterstained with DAPI is shown in blue. Scale bars = A-C 100μm, D-I 50μm

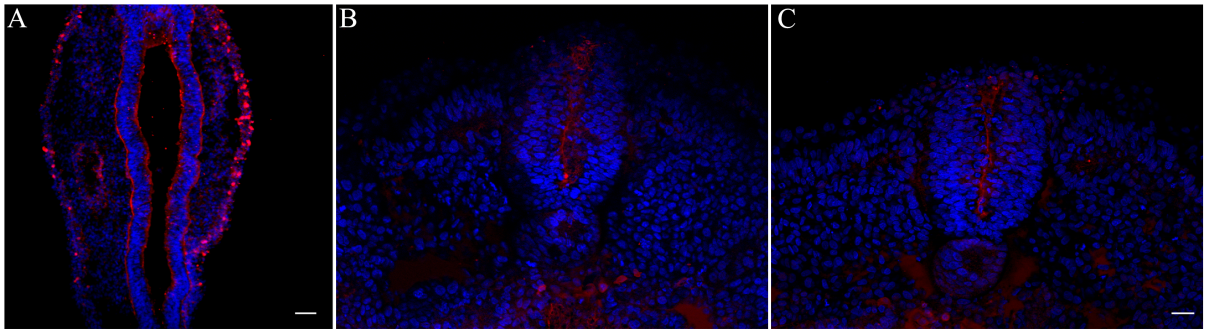


Fig. 3.8 **Expression of Crb2 in H&H Stage 13 chick embryo.** A. Coronal section through the hindbrain region of H&H stage 13 embryo immunostained for Crb2. Crb2 staining is detected both apically and basally. B-C Transverse sections through the hindbrain and spinal cord regions of H&H stage 13 chick embryo immunostained for Crb2. Crb2 protein is apically localized in the neural tube. Crb2 is shown in red and nuclei counterstained with DAPI shown in blue. H&H - Hamburger and Hamilton Scale bars = A-100 μm B-C 50 μm.

Overall, in the early chick embryo the expression of Crb2 mRNA and protein is specifically restricted to the developing neural tube. Notably in the hindbrain, Crb2 mRNA and protein is highly expressed only in the caudal rhombomeres at early stages, but as development proceeds Crb2 is uniformly expressed in all the rhombomeres.

MOUSE

Using an antibody predicted to cross-react with all three vertebrate Crumbs homologs, it was reported that the mouse Crumbs proteins are restricted to the apical surface of neuroepithelial cells in E8.5 mouse embryos (Lee et al., 2007). Additionally, during cortical development in the rat, Crumbs proteins are apically localized along with other polarity proteins- Pals1, MALS3 and PATJ (Srinivasan et al., 2008).

Previous unpublished work from our lab has shown that Crb2 mRNA is expressed in the developing neural tube of a mouse embryo as early as E8.5. However, little is known about the expression pattern of Crb2 protein in the developing mouse cortex.

1.2.2 Crb2 protein is apically localized in the telencephalon of a developing mouse embryo.

Initially, I examined the expression of Crb2 in the developing cortex at three ages – E12.5, E14.5 and E17.5. These stages correspond to early, mid and late neurogenesis (Caviness et al., 2003; Caviness et al., 1995).

In E12.5 wild-type mouse telencephalon, Crb2 protein is apically localized in the cortical neuroepithelial cells (Fig 3.9). The staining observed at the pial surface of the cortex is non-specific staining (asterisk in Fig 3.9) as it is also observed in telencephalic tissue immunostained with only the secondary antibody (data not shown). By E14.5, the cortex has a well-defined laminar organization and Crb2 expression continues to be restricted to the apical cell surface (Fig 3.10). At E17.5, there is an

appreciable increase in the thickness of the cortex and a decrease in the size of the lateral ventricle. Crb2 protein is expressed apically in the cells lining the ventricle. Interestingly at E17.5, the expression of Crb2 is not confined only to the apical domain but extends to a distinct region in the sub-ventricular zone in the dorsal telencephalon (Fig 3.11). Double immunostaining with Nestin, a neural stem cell marker, revealed that there is a close association between Crb2 and Nestin expression in the dorsal telencephalon at E17.5 (Fig 3.12).

Overall, in both chick and mouse embryos Crb2 is highly expressed in the neural tube and its expression is more pronounced at the apical surface of neural progenitor cells.

3.3 Discussion

Both in terms of morphology and localization of cellular constituents, neuroepithelial cells demonstrate apical-basal polarization (Chenn et al., 1998). The evolutionarily conserved apical polarity proteins Pals1, Par3, aPKC have previously been associated with the apical surface of developing neuroepithelia (Afonso & Henrique, 2006; Costa et al, 2008; Kim et al., 2010; Srinivasan et al., 2008; Stohr et al., 2005). Recently these apical polarity proteins have been implicated in cell fate determination during neurogenesis (Kim et al., 2010; Bultje et al., 2009; Imai, 2006). Despite this, comparatively little is known about the expression and role of the vertebrate homologs of Crumbs in neural development.

In this chapter, I have shown that one of the vertebrate homologs of Crumbs, Crb2, is apically enriched in the neuroepithelium of a developing chick neural tube and in the developing murine telencephalon.

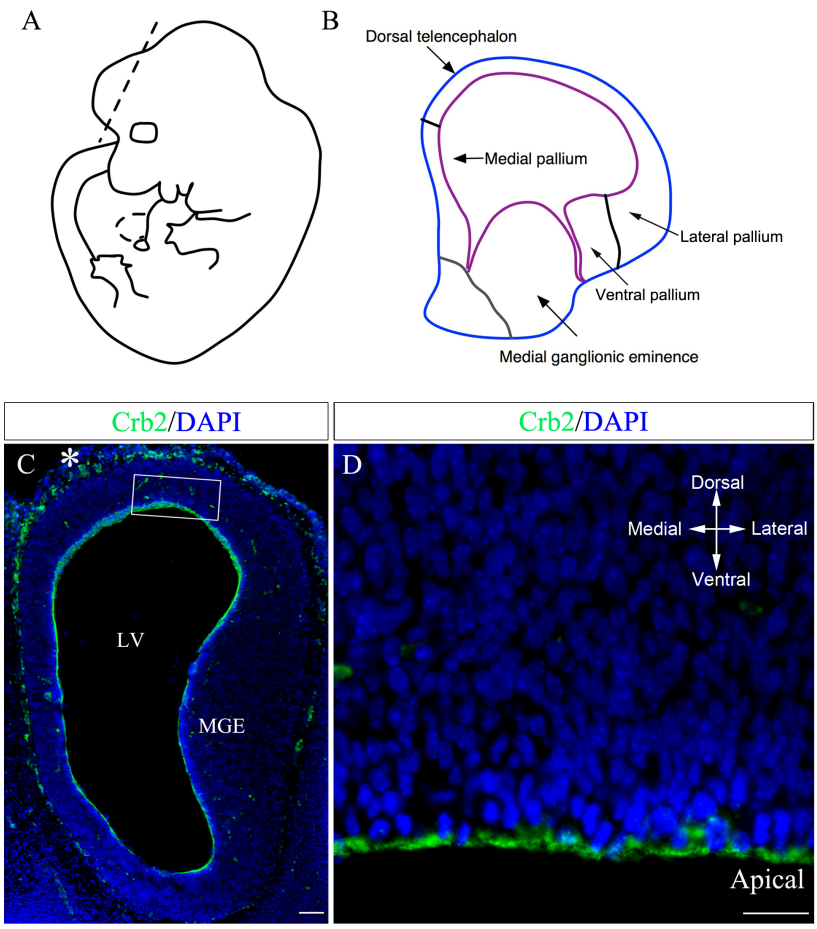


Fig 3.9 **Expression of Crb2 in the telencephalon of E12.5 mouse embryo.** A. Schematic representation of an E12.5 mouse embryo. Dotted line in A indicates position of coronal section through the telencephalon shown in B, C, D. B. Schematic of a coronal section through an E12.5 telencephalic hemisphere shows the dorsal, medial, lateral, ventral pallial domains and the medial ganglionic eminence. C. Crb2 protein is apically enriched in the telencephalon of an E12.5 mouse embryo.* - indicates non-specific staining. D. Higher magnification image of the boxed area in C. Crb2 staining shown in green and nuclei counterstained with DAPI shown in blue. LV- Lateral ventricle MGE- Medial ganglionic eminence Scale bar = C-100 μm, D -20 μm

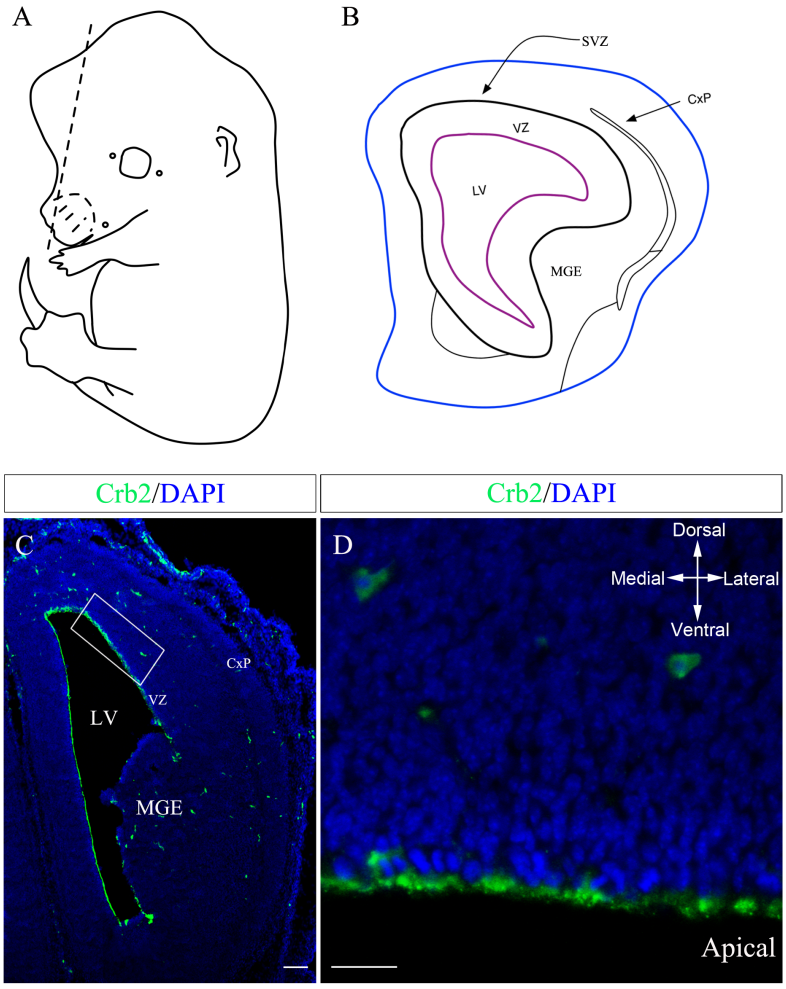


Fig. 3.10 **Expression of Crb2 in the telencephalon of an E14.5 mouse embryo.** A. Schematic representation of an E14.5 mouse embryo. Dotted line in A indicates position of coronal section through the telencephalon shown in B-D. B. Schematic illustration of a coronal section through an E14.5 telencephalic hemisphere shows the ventricular zone (VZ), sub-ventricular zone (SVZ), cortical plate (CxP), medial ganglionic eminence (MGE) and the lateral ventricle (LV). C. Crb2 protein is apically enriched in the telencephalon of an E14.5 mouse embryo D. Higher magnification image of the boxed area in C. Crb2 staining shown in green and nuclei counterstained with DAPI shown in blue.

Scale bar= C-100 μm, D -20 μm

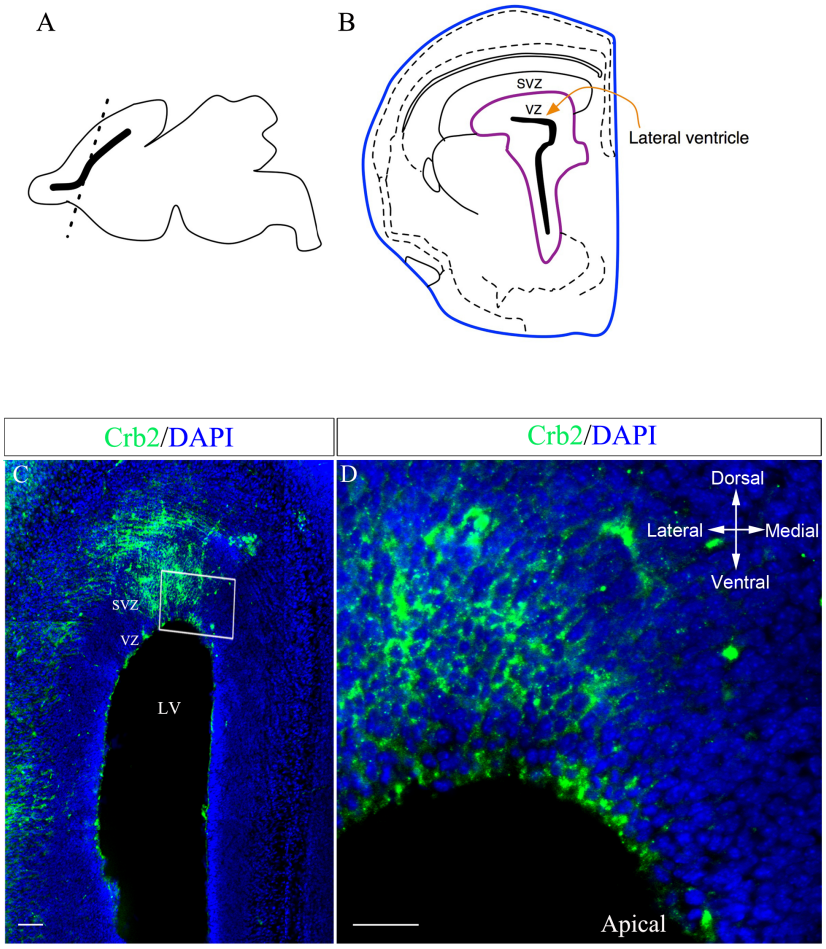


Fig. 3.11 **Expression of Crb2 in the dorsal telencephalon of an E17.5 mouse brain**. A. Schematic representation of an E17.5 mouse brain. Dotted line in A indicates position of coronal section through the telencephalon shown in B-D. B.Schematic illustration of a coronal section through the left telencephalic hemisphere shows the ventricular zone (VZ), sub-ventricular zone (SVZ) and the lateral ventricle (LV). C. Crb2 protein is apically enriched in the telencephalon. Note the expansion of Crb2 expression dorsally. D. Higher magnification image of the boxed area in C shows apical Crb2 staining and an expansion of the Crb2 expression domain, specifically in the dorsal SVZ. Crb2 staining shown in green and nuclei counterstained with DAPI shown in blue.

Scale bar= C-100 μm, D -20 μm

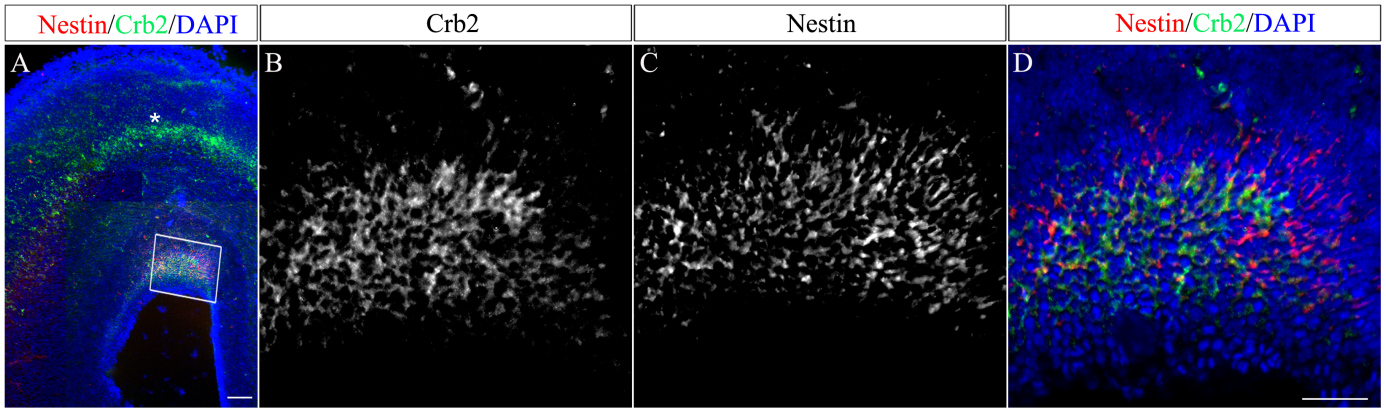


Fig. 3.12 **Expression of Crb2/Nestin in the telencephalon of an E17.5 mouse embryo.** A. Coronal section through the telencephalon of an E17.5 mouse brain shows a close association between Crb2 and Nestin staining in the the dorsal sub-ventricular zone. B-D Higher magnification of the boxed area in A. B-C Grey scale images and D- Overlay shows Crb2 staining in green, Nestin in red and nuclei counterstained with DAPI in blue. * shows non-specific Crb2 staining in the upper cortical layers Scale bar= A-100 μm, B-D -20 μm.

The expression of Drosophila Crumbs (dCrb) has been associated with all ectodermally derived epithelia, the only exception being the expression of dCrb in the peripheral nervous system (Tepass et al., 1990). It has been shown previously that dCrb plays a crucial role in the dynamic reorganization of epithelia during morphogenesis (Campbell et al., 2009). Given this role for Crumbs in tissue remodeling, it is not surprising that Crb2 is highly expressed in the developing neural tube, a tissue that undergoes extensive morphogenesis.

cCrb2 mRNA is apically enriched in the chick embryonic neural tube. Asymmetric mRNA localization of *cCrb2* transcripts could be beneficial for the production of multiple protein copies that are enriched at the apical neural epithelium. It is also possible that specific asymmetric localization of mRNA is a regulatory mechanism to prevent Crb2 protein from acting ectopically during translocation.

Interestingly, *cCrb2* mRNA is not detected within rhombomeres 1-4 at H&H stages 10 and 11. This pattern of *cCrb2* expression may be crucial for defining rostral rhombomere identity from that of caudal rhombomeres during early stages of chick embryonic development. This c*Crb2* free zone can be used to ectopically express Crb2 and to analyse its role during chick embryonic hindbrain development.

It is now well established that the apical neural progenitors of the neural tube reside close to the ventricles (the apical surface) and as they become post-mitotic they migrate to the basal layers in an orderly fashion (Gotz & Huttner, 2005). Crb2 protein expression in both the chick neural tube and mouse neocortex is predominantly restricted to the apical surface of the neuroepithelial cells. However, some Crb2 expression is also detected at the basal surface of the neural tube during H&H stages 10-11. It is possible that this is non-specific antibody staining or that Crb2 during early stages of development is also basally localized and is enriched at the apical surfaces as development proceeds. Overall, the preferential localization of Crb2 at the apical surface of the proliferating

progenitors is suggestive of a role for Crb2 in regulating the proliferation and/or cell fate of neural progenitors.

In addition to its apical expression in the dorsal telencephalon of E17.5 mouse embryos, Crb2 shows an intriguing expression pattern in and is expressed in a particular subset of cells in the dorsal sub-ventricular zone. These Crb2 positive cells are also closely associated with the neural stem cell marker, Nestin. It is possible that this cell population within the dorsal sub-ventricular zone is a specialized sub-type of progenitor cells that migrate to and settle-down in a different environment such as the olfactory bulb or that they give rise to specific neuronal sub-types in the cortex. It is tempting to speculate that the non-apically expressed Crb2 may play roles independent of apical-basal polarity during murine cortical neurogenesis.

CHAPTER 4

Analysis of Crb2 conditional knockout mouse embryos

4.1 Introduction

Recent work from our lab has shown that Crb2 is a novel regulator of neural differentiation *in vitro* (Boroviak & Rashbass, 2011). The main aim of this chapter was to elucidate if Crb2 plays a role in neural development *in vivo*. To study the potential effect of conditional removal of Crb2, transgenic *Crb2* floxed mice were generated by our Dutch collaborators (Henrique Alves working in the lab of Jan Wijnholds). However, the targeting construct is directed only against full-length Crb2 and not the secreted isoform described in chapter 6.

The homeodomain protein encoded my Emx1 is predominantly restricted to cortical subdivisions of the telencephalon. Emx1 is expressed in progenitor cells and neurons of dorsal, medial and lateral pallia (Simeone, et al., 1992; Puelles & Rubenstein, 1993). The neuronal expression of Emx-1 is mainly restricted to projection neurons (Chan et al., 2001) (Gorski et al., 2002). The defects observed in brains of Emx-1 homozygous mutants were subtle and restricted to the forebrain. Emx-1 mutant mice were born in normal Mendelian ratio and survived into adulthood (Yoshida et al., 1997).

To restrict Cre mediated recombination to the developing dorsal telencephalon, homozygously floxed *Crb2* mice were crossed with Emx1-Cre transgenic mice. In this system, Cre mediated recombination is restricted to the dorsal telencephalon (Gorski et al., 2002; Guo et al., 2000). The Crb2 cKO mice are viable and survive into adulthood they also do not display any overt morphological or behavioural defects (H. Alves, personal communication).

As summarized in table 1.3 many polarity proteins play crucial roles during murine cortical neurogenesis by regulating apical-basal polarity. These studies suggested that apically enriched proteins such as Par3, Par6, Pals1, MALS and Cdc42 influence the fate of daughter cells

and disruption of any of these proteins affects apical-basal polarity of neuroepithelial cells and subsequently affects normal cortical development (Bultje et al., 2009; Cappello et al., 2006; Kim et al., 2010; Manabe et al., 2002; Srinivasan et al., 2008). Based on the observations made in these studies, we hypothesized that conditional removal of Crb2 from the cortex may affect a) cell junction components b) recruitment of other apical polarity proteins c) apical restriction of mitoses d) cell fate decisions of neural progenitors.

4.2 Results:

The experiments described in this chapter were carried out at three stages of embryonic development – E12.5, E14.5 and E17.5. For analysis of the Crb2 conditional knockout embryos, a candidate marker approach was taken and I have focused on the dorsal telencephalic region of the mouse embryonic brain. A minimum of three control and three conditional knockout embryos were analysed for each marker at each embryonic stage.

Immunohistochemical analysis was carried out for markers classified into the following categories:

1. Apical polarity proteins
2. Cell junction proteins,
3. Cell proliferation markers
4. Neural progenitor markers
5. Neuronal markers

Analysis of Crb2; Emx1-Cre conditional knockout mouse embryos

E12.5

4.2.1 Conditional deletion of Crb2 results in the loss of apical Crb2 protein expression

Initially, I analysed the expression of Crb2 protein in the dorsal telencephalon of Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$ and/or Crb2 $^{F/F}$ (control) and

Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ (cKO) embryos. Crb2 protein is enriched at the apical surface of the cells lining the ventricle in the dorsal telencephalon of a control embryo (Fig 4.1 A-C). Conversely, in the cKO cortex where Cre expression is detected there is a complete loss of Crb2 expression (Fig 4.1 D-F).

4.2.2. Loss of Crb2 affects the expression of polarity proteins and cell junction-associated proteins

To determine if loss of Crb2 has an effect on localization of apical polarity proteins and junction-associated proteins, I analysed the control and cKO littermate embryos for alterations in marker expression. ZO-1, a cell junctional protein (Aaku-Saraste, Hellwig, & Huttner, 1996) is localized at the luminal surface of the dorsal telencephalon in the control embryos (Fig 4.2 A, B). In the cKO cortex, the expression of ZO-1 is unaltered (Fig 4.2 E, F). N-Cadherin is one of the major cadherins associated with neuroepithelial cells (Kadowaki et al., 2007). At E12.5 in control embryos, N-Cadherin expression is enriched in the apical domain of the VZ- ventricular zone (Fig 4.2 C, D). Interestingly in the cKO cortex, the apically enriched expression of N-Cadherin is perturbed and N-Cadherin is expressed in a diffuse manner in the ventricular zone. Weak N-Cadherin expression was also detected in the SVZ-sub-ventricular zone (Fig 4.2 G, H).

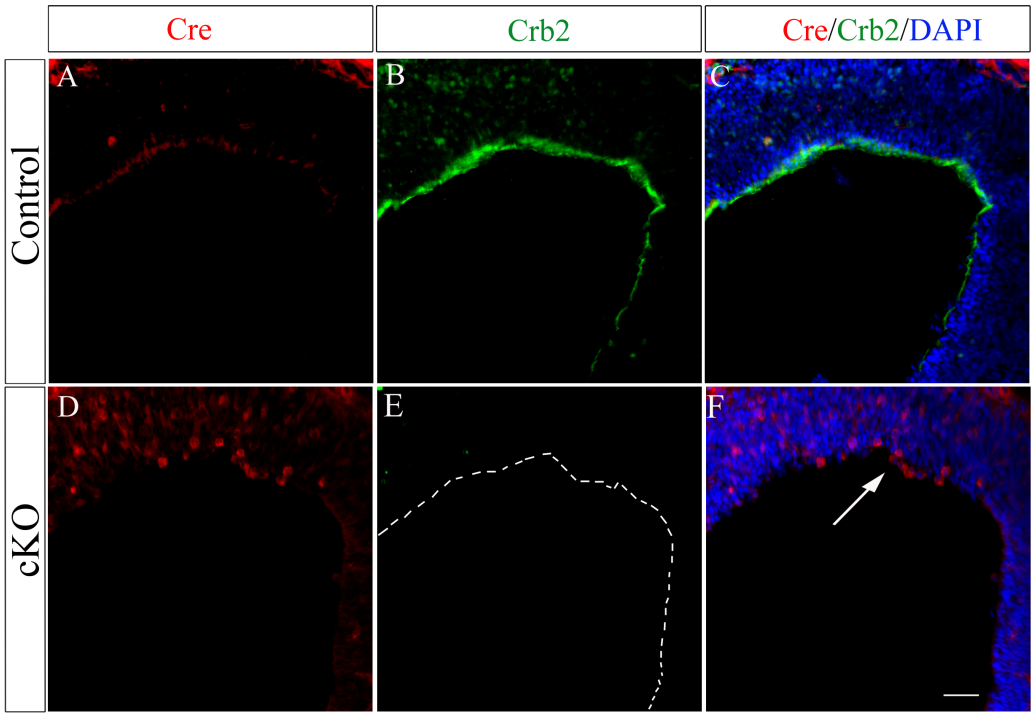
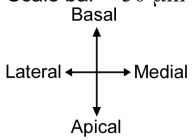


Fig 4.1 **Expression of Crb2 and its deletion by Emx-1 Cre in the dorsal telencephalon.** Coronal sections through the telencephalon of E12.5 control and cKO littermate embryos immunostained as indicated.

A-C Crb2 (green) protein is apically localized in the cortex of control embryos. D-F Crb2 expression is completely reduced in the cKO cortex compared to the control. Dotted line in E marks apical surface of the cortex. Arrow in F indicates loss of Crb2 immunoreactivity in the Cre (red) positive region. Nuclei counterstained with DAPI (blue)
Control= Crb2 $^{F/F}$
cKO= Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$
Scale bar = 50 μm



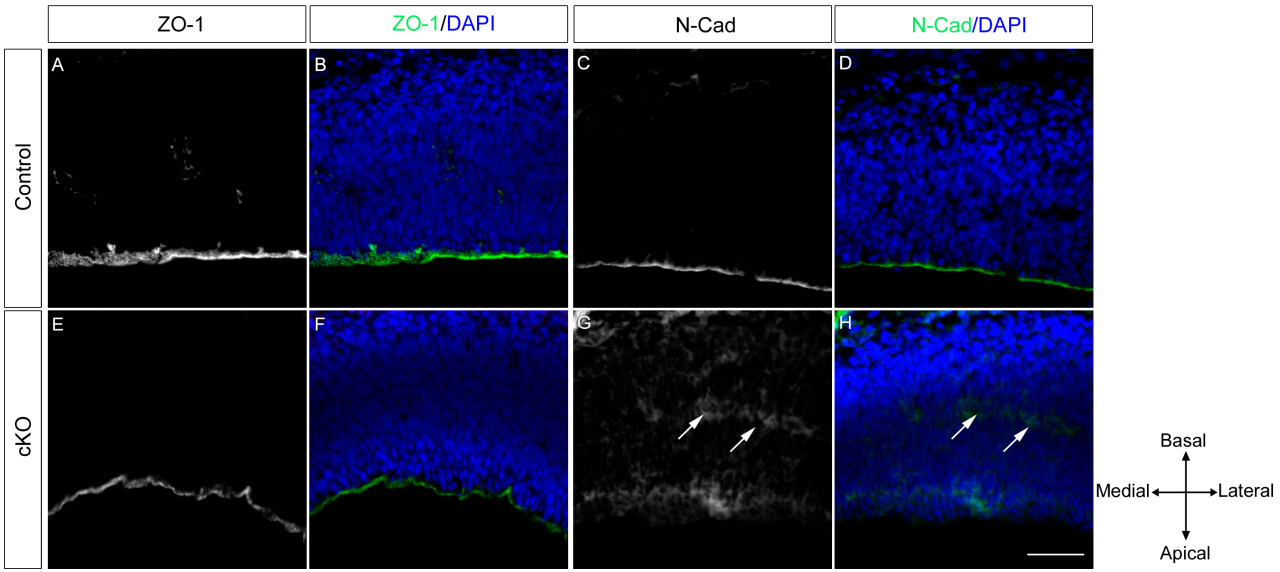


Fig.4.2 **Expression of adherens junction markers in the Crb2 cKO cortex**. Coronal sections through the telencephalon of E12.5 control and cKO littermate embryos immunostained as indicated. ZO-1 is apically localized in the control (A-B) and cKO telencephalon (E-F). N-Cadherin is apically enriched at the lumenal surface of ventricular zone cells in the control (C-D). In the cKO cortex, there is a significant reduction in N-Cadherin expression (G,H). Arrows in G,H indicate weak N-Cadherin staining observed in the sub-ventricular zone. A,C,E,G show antibody staining alone.Overlays (B,D,F,H) show antibody staining in green and nuclei counterstained with DAPI in blue. Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$ cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ Scale bar = 50 μm

To investigate what effect Crb2 deletion has on the expression of apical polarity components, I analysed the expression of two candidate genes - Pals1, a member of the Crumbs complex and Par3, a member of the Par complex. In the control littermate embryos, Pals1 is expressed in the apical domain of the ventricular zone cells. In contrast, in the Crb2 cKO embryos, the expression of Pals1 is barely detectable (Fig 4.3 A-B, E-F).

Consistent with previously published data (Manabe et al., 2002) (Bultje et al., 2009), Par3 expression is enriched at the apical surface of the ventricular zone progenitors in the control embryos (Fig 4.3 C-D). Similar to Pals1 expression, the expression of Par3 is also disrupted in the Crb2 cKO cortex. However, some Par3 staining is detected in the lateral cortex (Fig 4.3 G-H). It is possible that Cre mediated recombination has not yet occurred in the lateral cortex at this stage.

Overall, the data suggests that at E12.5, loss of Crb2 expression in the dorsal telencephalon leads to a disruption of the apical polarity protein complexes and the adherens junction protein N-Cadherin but does not affect the localization of ZO-1 that is usually associated with tight junctions.

4.2.3 Loss of Crb2 affects the neural progenitor cell pool

To elucidate the role of Crb2 in neurogenesis, I analysed the control and cKO littermate embryos for alterations in markers of neural progenitors (Sox2, Nestin), intermediate progenitors (Tbr2) and early-born neurons (TuJ1).

In the control telencephalon, Sox2 positive progenitors are observed in the ventricular zone (Fig 4.4 A-B). Compared to the controls, very few cells in the cKO cortex show distinct nuclear localization of Sox2 instead Sox2 staining is diffuse throughout the cell body (Fig 4.4 E-F).

At E12.5, immunostaining for Nestin revealed an intense staining pattern throughout the control cortex labeling cells with typical radial glial morphology (Fig 4.4 C-D) but in the cKO cortex Nestin expression was markedly reduced (Fig 4.4 G-H)

To identify any alterations in the progression of apical neural progenitors to basal intermediate progenitors, I analysed the control and cKO littermate embryos for expression of Tbr2, a specific marker for intermediate progenitors (Sessa et al., 2008). At E12.5, Tbr2 is expressed in the sub-ventricular zone of the control cortex (Fig 4.4 I-J). Interestingly, in the cKO cortex Tbr2 positive cells are detected not only in the sub-ventricular zone but also in the ventricular zone (Fig 4.4 M-N).

I also examined the control and cKO cortices for alterations in the expression of early neuronal marker TuJ1. It has been previously reported that intermediate progenitors divide to generate more neurons than the apical progenitors (Noctor et al., 2007; Haubensak et al., 2004). Despite the apparent increase in Tbr2 positive intermediate progenitors after conditional deletion of Crb2, I did not observe a significant alteration in TuJ1 positive neurons in the cKO cortex compared to the control cortex, at E12.5.

Taken together, these results indicate that conditional deletion of Crb2 from the cortex leads to a depletion of the apical neural progenitor pool and a concomitant increase in intermediate progenitors suggestive of a population shift from apical to intermediate progenitors. However, terminal neural differentiation remains unaffected at this stage.

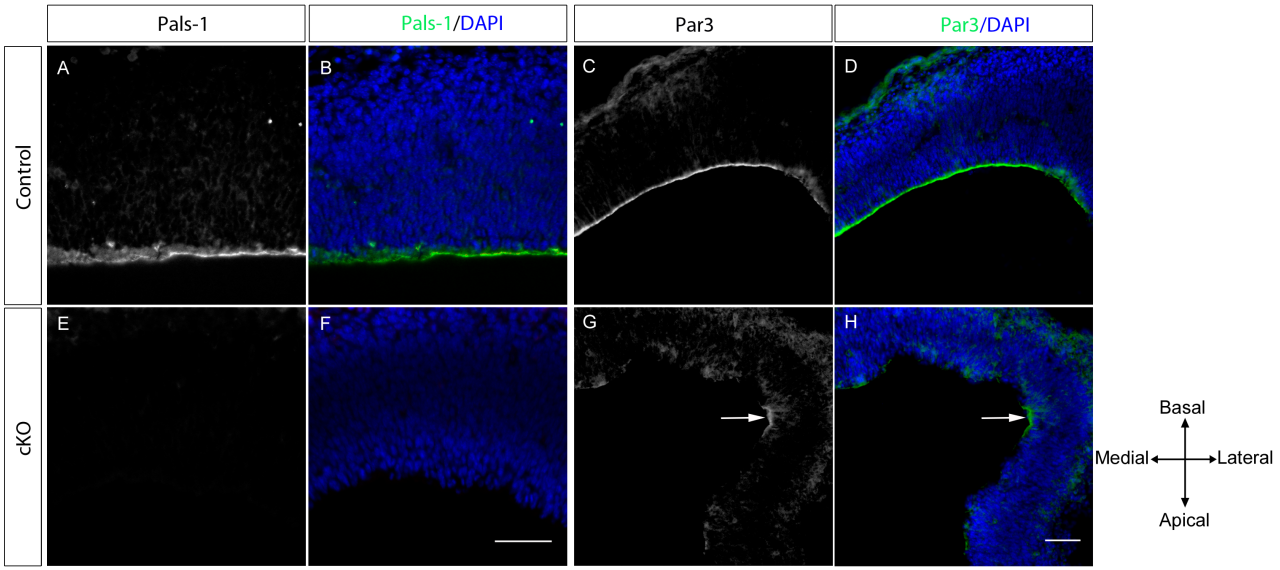


Fig 4.3 **Crb2 deletion affects expression of apical polarity proteins.** Coronal sections through the telencephalon of E12.5 control and cKO littermate embryos immunostained as indicated. A-B In the control cortex, Pals1 expression is apically enriched in the ventricular zone progenitor cells. E-F In the cKO cortex, Pals1 expression is barely detected in the dorsal telencephalon. C-D Par3 is apically localized in the control cortex. G-H In the cKO cortex, Par3 expression is disrupted. Arrows in G,H indicate some Par3 expression in the lateral cortex. Overlays show antibody staining in green and nuclei counterstained with DAPI in blue. Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$ cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ Scale bar (A-B,E-F) = 50 μm Scale bar(C-D,G-H)= 50 μm

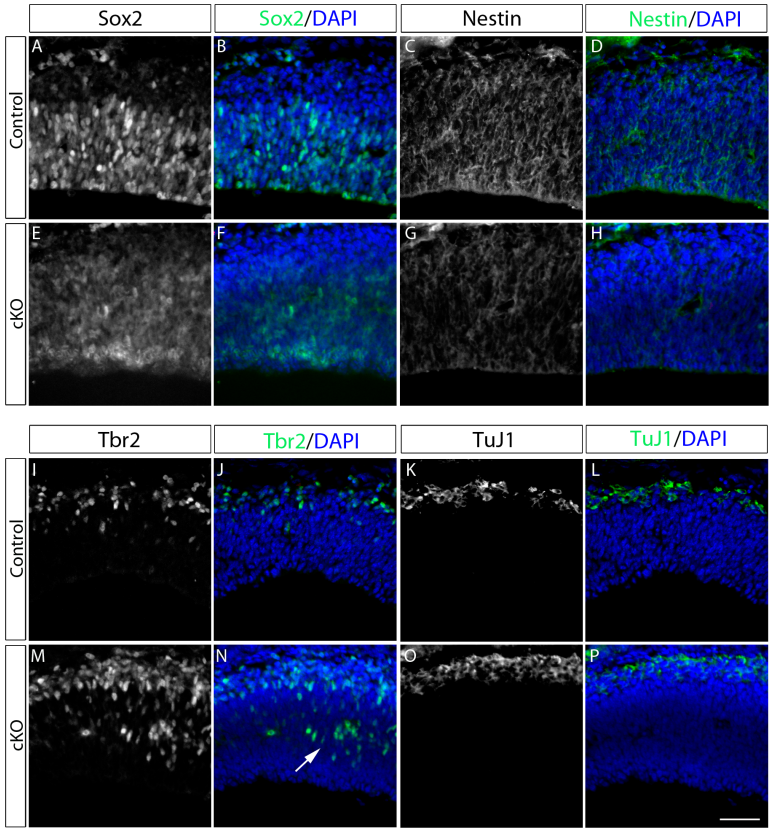
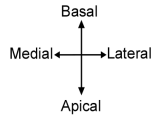


Fig 4.4 **Depletion of Crb2 affecs the neural progenitor population in the developing telencephalon.** Coronal sections through the telencephalon of E12.5 control and cKO littermate embryos immunostained as indicated. Sox2 is predominantly expressed in the VZ and at low levels in the SVZ in the control embryos (A-B).In the Crb2 cKO embryos, Sox 2 staining is diffuse in the VZ and SVZ (E-F).
In the control, Nestin is detected spanning the cortex (C,D) In the cKO cortex, Nestin expression is significantly reduced (G-H).
In the control, Tbr2+ve IPCs are detected in the SVZ and preplate (I-J). In the Crb2 cKO cortex, Tbr2 +ve cells are detected in the SVZ and preplate layers. Mislocalized Tbr2 +ve cells are detected in the VZ and basal VZ, arrow in N.
β-Tubulin III positive neurons are observed in the upper SVZ largely exclusive of Sox2 expression in both the control (K-L) and Crb2 cKO embryos (O-P). Overlays show antibody staining in green and nuclei counterstained with DAPI in blue.
Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$
cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$
VZ- ventricular zone SVZ-subventricular zone
IPC-intermediate progenitor cells.
Scale bar= 50 μm



4.2.4 Hes5 expression is downregulated in the Crb2 cKO cortex

The Notch signaling pathway has been implicated in the maintenance of progenitors during vertebrate neural development (Chenn & McConnell, 1995; Mizutani et al., 2007). To investigate whether loss of Crb2 has an effect on the Notch pathway, I analysed control and cKO cortices for the expression of Hes5, a well-established downstream effector of the Notch pathway (Ohtsuka et al., 1999).

In the control telencephalon, Hes5 mRNA expression is observed in the neural progenitors of the ventricular zone (Fig 4.5 A, C). Interestingly, in the cKO cortex there is a significant reduction in Hes5 mRNA transcripts. However, Hes5 is not completed downregulated in the cortex and the decrease in Hes5 mRNA expression occurs in a medial to lateral manner (Fig 4.5 B, D). In the Crb2 cKO cortex, there appears to be a distinct boundary between regions where Hes5 expression is unaffected and where Hes5 expression is markedly reduced (dotted lines in Fig 4.5 B, D).

In light of the above observation, the depletion of the apical progenitor population in the Crb2 cKO cortex and the subsequent switch to an intermediate Tbr2 positive cell fate may be a consequence of Hes5 downregulation. Alternatively, the loss of Hes5 could be a secondary effect to the loss of apical progenitors and this could be regulated by the Cdc42-mTOR pathway (Endo et al., 2009).

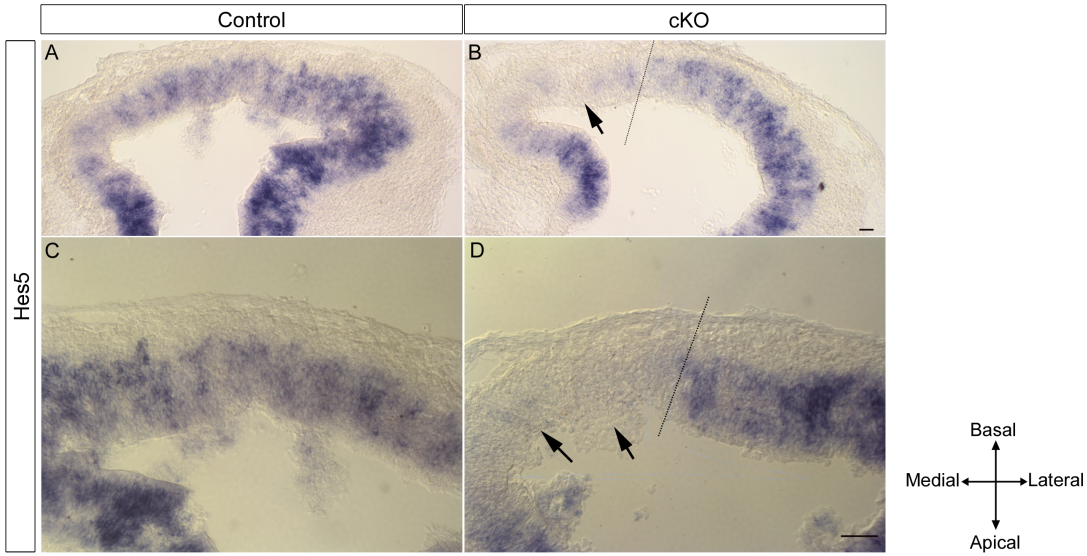


Fig 4.5 **Crb2 deletion affects Hes5 mRNA expression.** Coronal sections through the telencephalon of E12.5 control and cKO littermate embryos. *In situ* hybridisation for Hes5 shows Hes5 expression in the control telencephalon (A,C). In the cKO cortex, there is a significant reduction in Hes5 expression on the medial side, arrows in B, D.
Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$
cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ Scale bars = 50 μm

E14.5

4.2.5 Loss of Crb2 affects the expression of polarity proteins and cell junction-associated proteins

To follow the progressive effect of Crb2 deletion on the expression of polarity proteins, I analysed control and cKO littermate embryos at E14.5. Similar to the expression at E12.5 in the control cortex, Pals1 and Par3 (Fig 4.6 A-B, E-F) are localized in the apical cell compartment. In the cKO situation, apical Pals1 expression is lost (Fig 4.6 C-D). Although Par3 was disrupted at E12.5, rather surprisingly its expression is restored in the cKO cortex by E14.5 (Fig 4.6 G-H). I next examined N-Cadherin localization. In the control cortex N-Cadherin staining is detected in the SVZ and in the apical surface of the cells lining the ventricle (Fig 4.6 I-J). In the cKO cortex, N-Cad expression is significantly reduced (Fig 4.6 K-L).

Overall, the data suggests that at E14.5, loss of Crb2 expression in the dorsal telencephalon continues to affect recruitment of Pals1 and N-Cadherin to the apical compartment. However, it no longer influences Par3 localization.

4.2.6 Loss of Crb2 affects expression of neural progenitor/neuronal markers

At E14.5, Sox2 expression in the control and cKO cortex is not significantly different (Fig 4.7 A-D). In contrast to the diffuse cytoplasmic Sox2 staining observed at E12.5 in the Crb2 cKO cortex, nuclear Sox2 expression is restored by E14.5. Interestingly, the expression of Prominin (CD-133) an apical neuroepithelial stem cell marker (Marzesco et al., 2005), is completely lost in the cKO cortex (Fig 4.7 E-H) when compared to the control cortex. Nestin positive cells extend from the apical to pial surface in the control cortex (Fig 4.7 I-J).

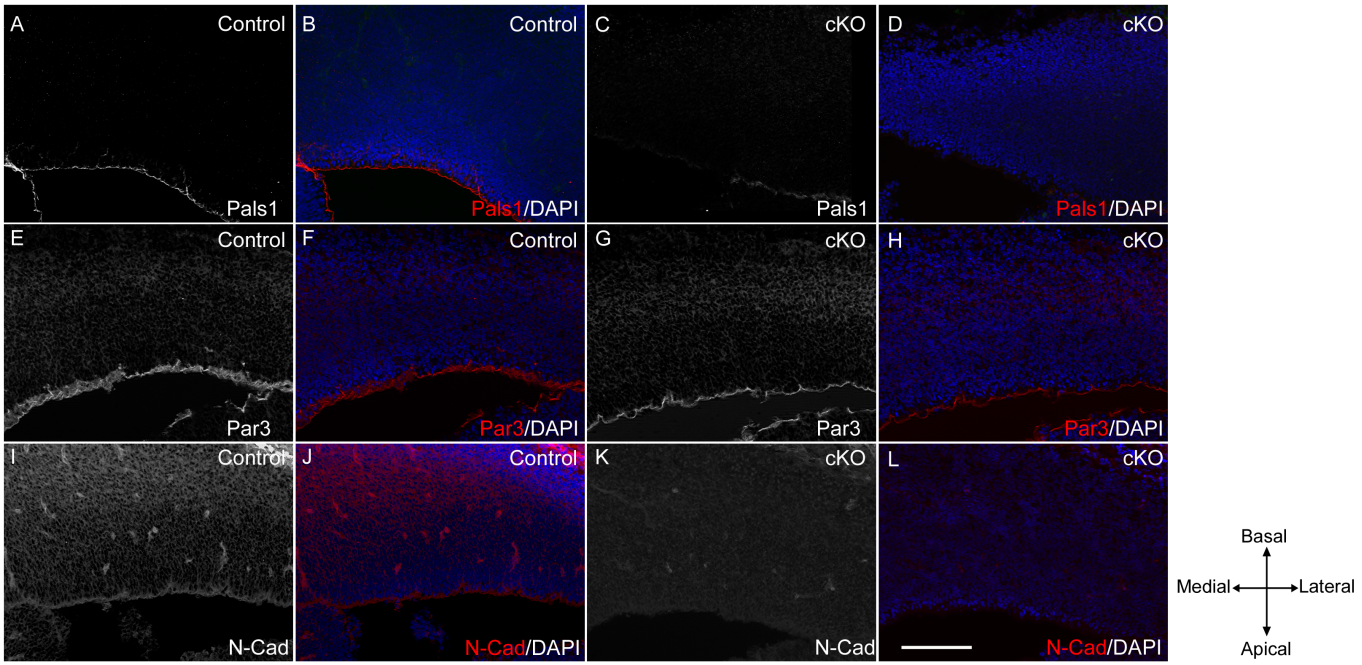
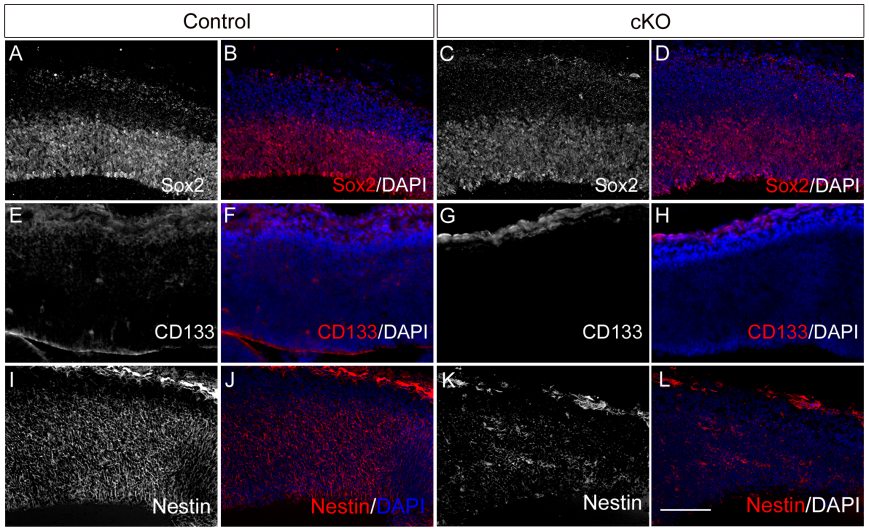


Fig 4.6 **Loss of Crb2 affects the expression of apical cell junction proteins.** Coronal sections through the telencephalon of E14.5 control and cKO littermate mouse embryos immunostained as indicated. Pals1 is apically localized in the control cortex (A-B) and its expression is disrupted in the cKO cortex (C,D). Par3 expression is apically enriched in the control (E-F) and cKO cortex (G-H). N-Cadherin expression is detected apically and in the sub-ventricular zone in the control (I,J) In the cKO telencephalon, N-Cad expression is undetectable (K-L). Overlays (B,D,F,H,J,L) show antibody staining in red and nuclei counterstained with DAPI in blue Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$ cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ Scale bar = 100 μm



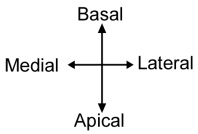


Fig 4.7 **Crb2 deletion affects the VZ progenitors.**
Coronal sections through the telencephalon of E14.5 control and cKO littermate embryos immunostained as indicated. Sox2 is expressed in the VZ and SVZ progenitors in the control (A-B) and cKO cortex (C-D). In the control cortex, CD133 is detected in the apical domain of the neuroepithelial cells (E-F). CD133 is undetectable in the cKO cortex (G-H). In the control, Nestin expression spans the cortex (I-J). There is a significant decrease in Nestin expression in the cKO cortex (K-L).Overlays show antibody staining in red and nuclei counterstained with DAPI in blue.
VZ-ventricular zone, SVZ- sub-ventricular zone. Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$
cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ Scale bar = 100 μm

In the cKO cortex, Nestin positive fibers are disorganized and significantly reduced (Fig 4.7 K-L).

Tbr2 positive cells are detected in both the SVZ and basal VZ layers of the control (Fig 4.8 A-B) and cKO (Fig 4.8 C-D) cortex. However, there appears to be a subtle increase in Tbr2 positive cells in the cKO cortex when compared to the controls.

To ascertain whether the depletion of apical neural progenitors in the Crb2 cKO cortex at E12.5 has a pronounced effect on neurogenesis at E14.5, I analysed the control and cKO embryos for alterations in neural marker expression. Immunostaining the control and cKO cortices for early neuronal marker TuJ1 revealed a significant expansion of TuJ1 positive neuronal domain in the cKO cortex compared to the control (Fig 4.8 E-H). In addition to this expanded domain, I also observed mislocalized TuJ1 positive neurons in the ventricular zone. The expression of Tbr1, a marker for post-mitotic neurons (Bulfone et al., 1995) is unaltered in the control (Fig 4.8 I-J) and cKO (Fig 4.8 K-L) cortex at this stage.

Taken together, the data implies that at E14.5 loss of Crb2 continues to affect the expression of apical polarity components and neural progenitor markers and leads to precocious neural differentiation.

E17.5

4.2.7 Loss of Crb2 affects neural differentiation and expression of cortical - layer specific markers.

To examine the effect of Crb2 deletion on neurogenesis at later stages, I analysed control and cKO brains at E17.5. Intriguingly, in the dorsal telencephalon of a control brain, Nestin is specifically restricted to a population of cells close to the ventricle (Fig 4.9 A-B). In the cKO brain, Nestin expression is completely downregulated (Fig 4.9 C-D). Compared to Sox2 expression at earlier stages, by E17.5 there are fewer Sox2 positive cells lining the ventricles. In the control cortex, Sox2 labelled cells are dispersed in the SVZ (Fig 4.9 E-F). In the cKO cortex, although Sox2

positive cells are present in the lateral cortex, Sox2 positive cells are not detected in the dorsal telencephalon (Fig 4.9 G-H).

By E17.5, there is a remarkable increase in TuJ1 positive neurons in all layers of the cortex (Menezes & Luskin, 1994). A few TuJ1 positive neurons are also detected in the SVZ (Fig 4.9 I-J). The intensity of TuJ1 staining is more in cKO cortex compared to the controls. Also, there appear to be more TuJ1 labelled neurons in the SVZ of a cKO cortex (Fig 4.9 K-L) compared to a control cortex.

To investigate the spatial pattern of neural differentiation in the cKO brains, I analysed the expression of two cortical neuronal markers - Tbr1 and Reelin. Tbr1 is expressed in the upper layers of the cortex and in the subplate of the control cortex (Fig 4.10 A-B). In the cKO cortex, Tbr1 expressing cells are detected in the same regions as in the controls. However, a few Tbr1 positive cells are detected outside their normal expression domain (Fig 4.10 C-D). In the control, Reelin marks Cajal-retzius cells in the superficial marginal zone of the cortical plate (Fig 4.10 E-F). In contrast to the compact laminar staining observed in the controls, in the cKO brain Reelin labeled cells are detected in a disorganized fashion at the marginal zone (Fig 4.10 G-H).

4.2.8 Loss of Crb2 results in increased SVZ mitoses

Neuroepithelial cells undergo interkinetic nuclear migration and the nucleus translocates to the apical surface during mitosis (Chenn & McConnell, 1995). Intermediate progenitor cells that populate the SVZ undergo mitosis away from the ventricles. To determine if loss of apical Crb2 expression influences the positioning of mitotic cells, I analysed the control and cKO cortices for alterations in pH3 (phosphorylated histone H3) a late G2/mitotic phase marker. Immunostaining for pH3 helps distinguish between apical VZ and basal SVZ mitoses based on the spatial location of pH3 labelled cells in the cortex.

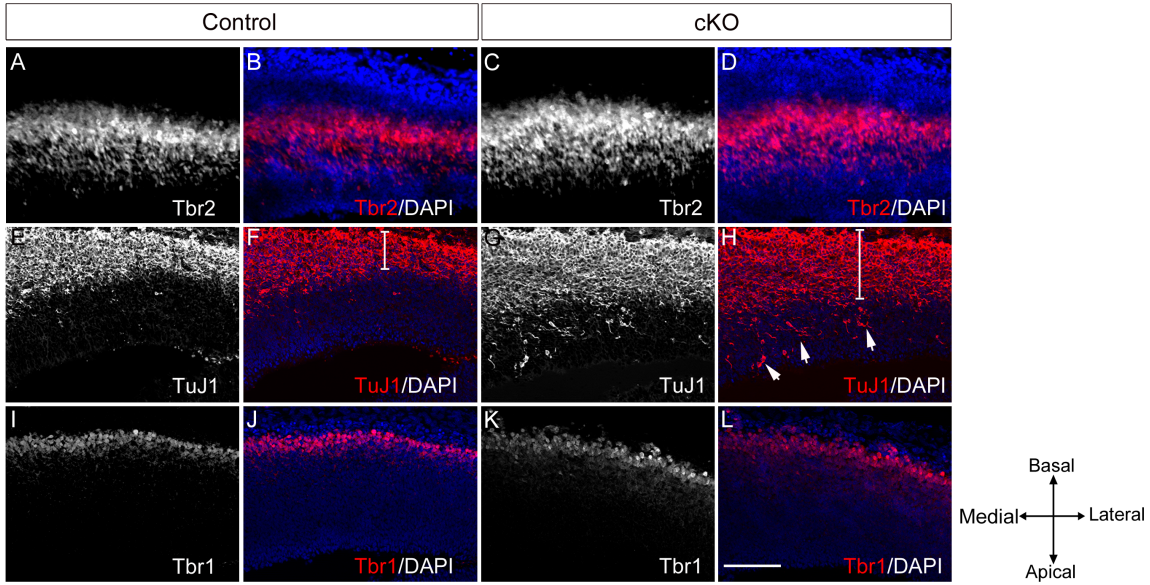


Fig 4.8 **Crb2 deletion affects neurogenesis** Coronal sections through the telencephalon of E14.5 control and cKO littermate embryos immunostained as indicated. In the control, Tbr2 is predominantly expressed in the SVZ (A-B). There is a subtle increase in Tbr2 positive intermediate progenitors in the cKO cortex (C-D). TuJ1 positive neurons are predominantly detected in the basal layers of the control cortex (E-F)). In the cKO cortex, there is a marked increase in TuJ1 positive neurons (G-H) present in the VZ and SVZ , arrows in G, line in F and H. Tbr1 positive post-mitotic neurons are present in the cortical plate of the control (I-J) and cKO cortices (K-L). Overlays show antibody staining in red and nuclei counterstained with DAPI in blue. VZ-ventricular zone, SVZ- subventricular zone. Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$ cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ Scale bars = 100 μm

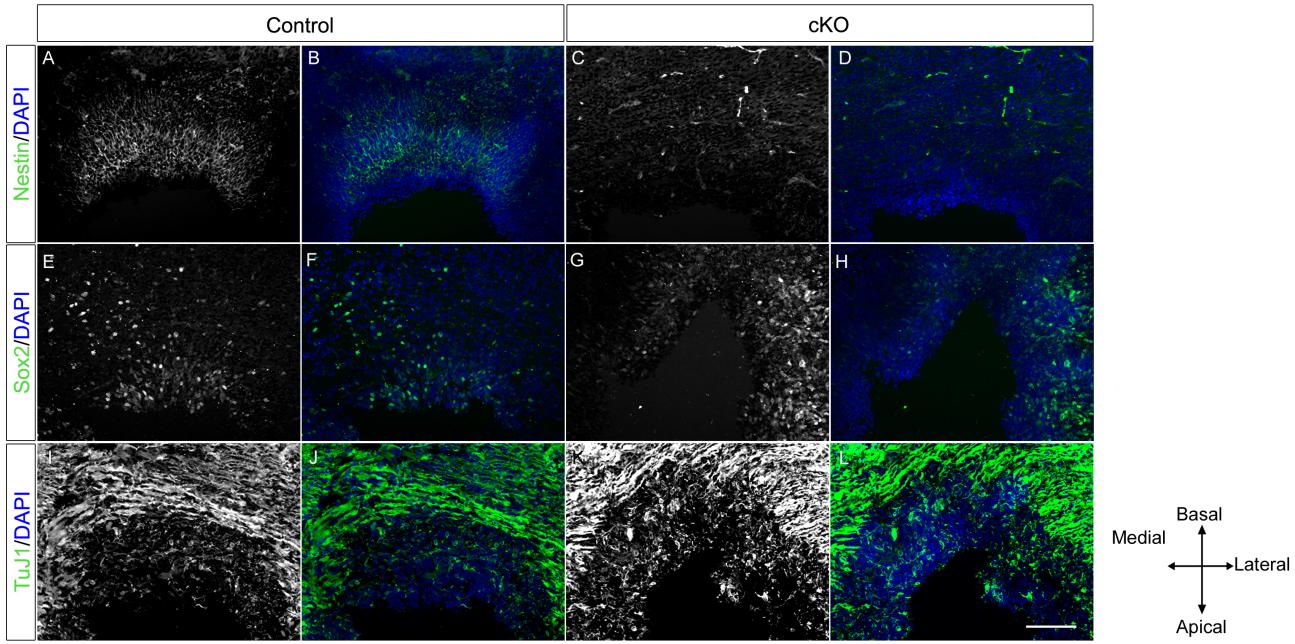


Fig.4.9 **Loss of Crb2 affects neural differentiation**. Coronal sections through the telencephalon of E17.5 control and cKO littermate embryos immunostained as indicated. Nestin expression is specifically restricted to a population of cells in the dorsal telencephalon of a control brain (A-B). In the cKO, Nestin expression is barely detected in the dorsal telencephalon (C-D). In the control, Sox2 positive progenitors are localized in the VZ and SVZ (E-F). There is a significant downregulation of Sox2 in the dorsal telencephalon of a cKO brain (G-H). There is a subtle increase in β-tubulin III positive neurons in the cKO (K-L) brain compared to the control (I-J). Overlays show antibody staining in green and nuclei counterstained with DAPI in blue. VZ- ventricular zone, SVZ- sub-ventricular zone.
Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$ cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ Scale bar = 100 μm

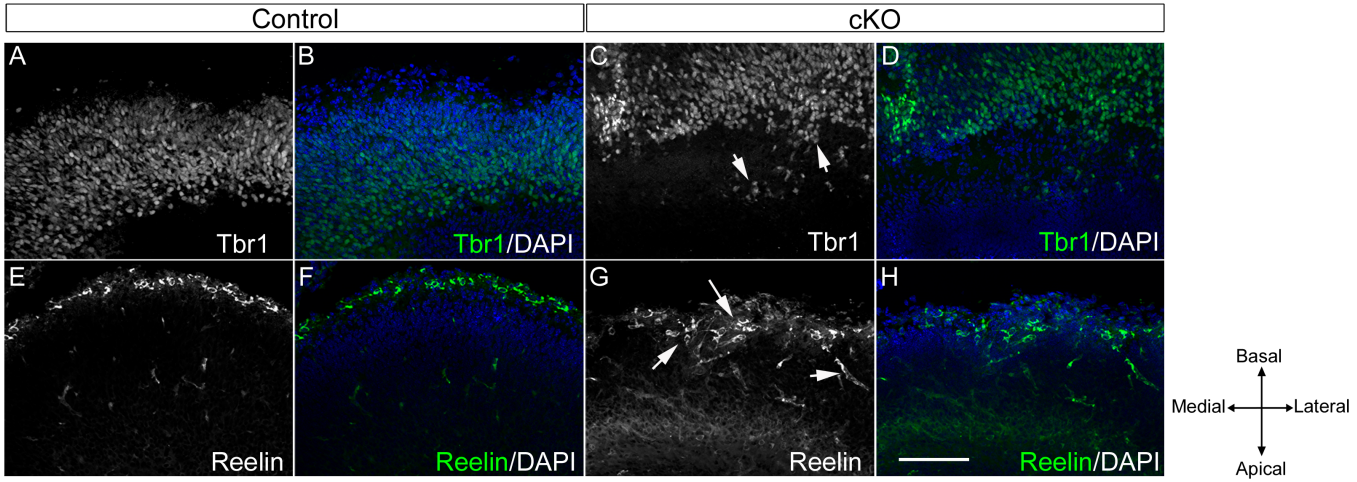


Fig.4.10 **Mislocalization of Tbr1 and Reelin in the Crb2 cKO cortex.** Coronal sections through the telencephalon of E17.5 control and cKO littermate brains immunostained as indicated. A-B Tbr1 positive post-mitotic neurons are detected in the cortical plate and marginal zone in the control brain. C-D In the cKO brain, Tbr1 positive cells are mislocalized and are indicated by arrows in C. E-F In the control, Reelin positive Cajal-retzius cells are localized in the marginal zone G-H In the cKO brain, the defined layer of Reelin positive cells is disrupted and Reelin positive cells are aberrantly localized (arrows in G). Overlays show antibody staining in green and nuclei counterstained with DAPI in blue.
Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$ cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ Scale bar = 100 μm

At E12.5 in the control cortex, pH3 positive are detected at the apical cell surface (Fig 4.11 A-B). There is a dramatic reduction in pH3 positive mitotic cells only in the dorsal telencephalon of the cKO cortex (Fig 4.11 C-E).

At E14.5, although there is no apparent alteration in the number of pH3 positive cells detected at the apical surface in the cKO compared to the control (Fig 4.11 F-G) there is an increased occurrence of mitotic cells in the SVZ of the cKO cortex (Fig 4.11 J-K).

At E17.5, I could not detect any pH3 positive cells at the apical cell surface in the cKO cortex (Fig 4.11 L-M) but observed a significant proportion of pH3 positive cells localized away from the ventricular surface when compared to the control cortex (Fig 4.11 H-I).

The data suggests that apical Crb2 expression is essential for the appropriate localization of mitotic cells in the developing telencephalon. The increased mitosis at the SVZ correlates with the increase in Tbr2 positive intermediate progenitors.

4.2.9 Loss of Crb2 influences PKD expression in the cortex

The protein kinase D (PKD) gene family has been implicated in neural differentiation and neuronal protein trafficking *in vitro* (Bisbal et al., 2008; Yin et al., 2008; Maier et al., 2007).

To determine if in the Crb2 cKO, PKD is affected I analysed control and cKO cortices for the expression of PKD1/PKCμ. In the control situation, PKD1 is weakly expressed in the VZ of the cortex at E12.5 (Fig 4.12 A-B). Surprisingly, in the cKO cortex PKD1 is highly expressed throughout the dorsal telencephalon (Fig 4.12 C-D).

At E14.5, PKD1 expression is confined to the basal VZ and SVZ in the control cortex (Fig 4.12 E-F). In the cKO cortex, there is a remarkable upregulation of PKD1 expression. PKD1 is expression is detected in all

layers of the dorsal telencephalon (Fig 4.12 G-H). This increased PKD1 expression was observed only in the dorsal telencephalon and not in the lateral and ventral telencephalon (data not shown).

By E17.5, PKD1 expression is specifically confined to a population of cells in the dorsal telencephalon of a control cortex (Fig 4.12 I-J). However, in the cKO cortex PKD1 expression was undetectable (Fig 4.12 K-L).

The data suggests that there is a progressive upregulation of PKD1 expression in the control cortex from E12.5 to E17.5. However, in the cKO cortex PKD1 is prematurely expressed at earlier stages and its expression is downregulated by E17.5. Thus, loss of Crb2 affects the normal spatial and temporal expression of PKD1 in the dorsal telencephalon.

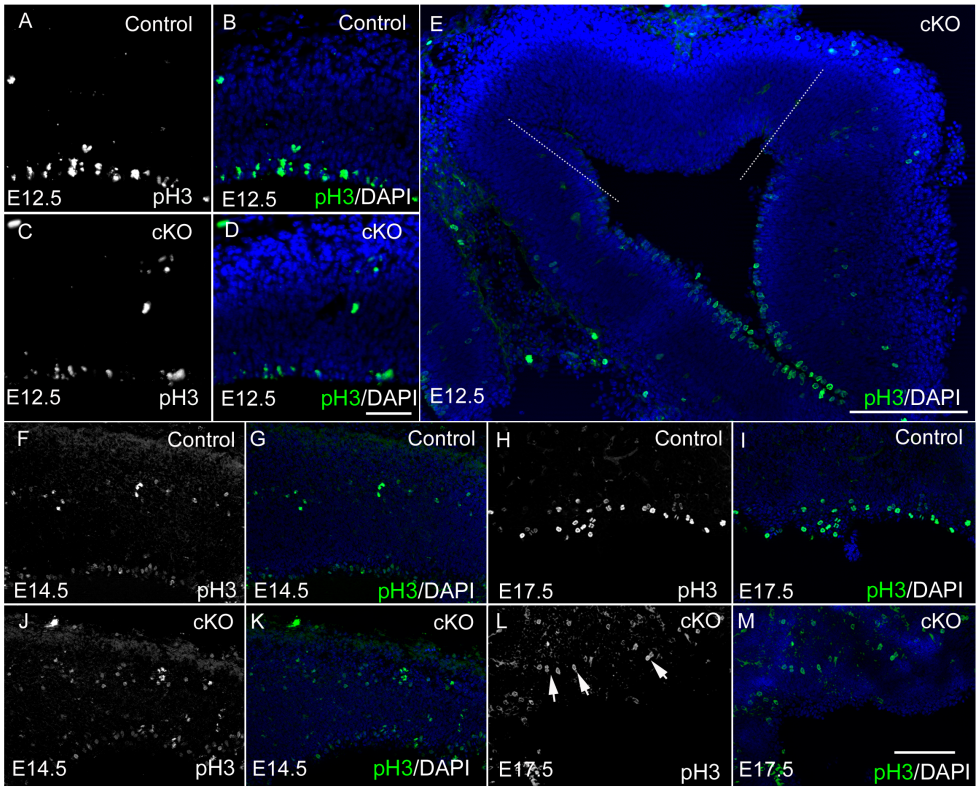
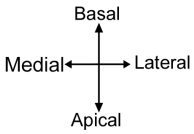
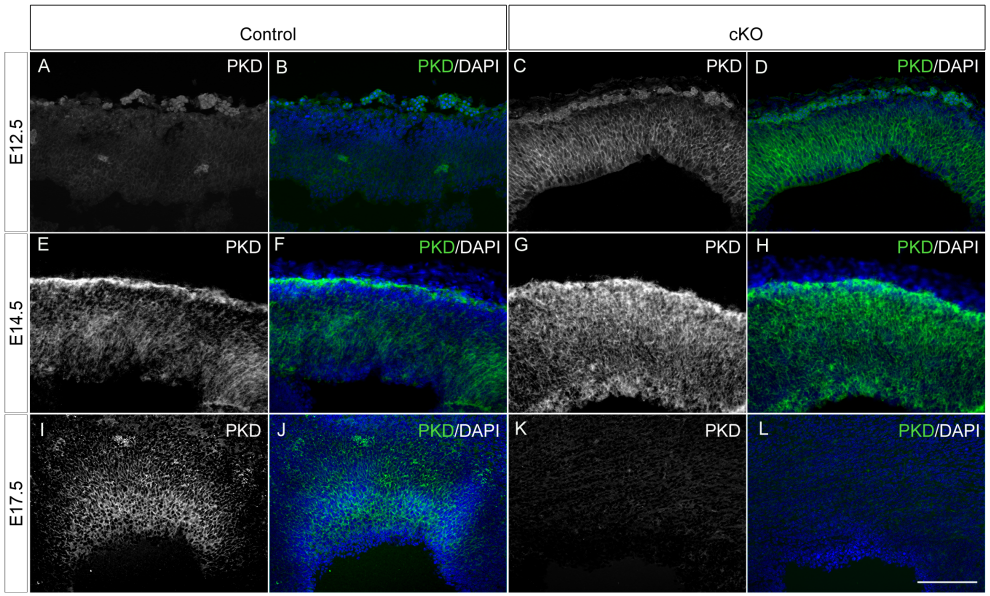


Fig. 4.11 **Loss of Crb2 results in a progressive increase in basal mitoses.** Coronal sections through the telencephalon of E12.5, E14.5 and E17.5 control and cKO littermate embryos were immunostained using phospho-histone 3 (pH3) antibody. pH3 positive mitotic cells are observed near the ventricle in the control cortex (A-B). In the cKO, there is a clear reduction in the pH3 positive cells (C-E) in the dorsal telencephalon region, indicated by dotted lines in E. At E14.5, pH3 positive cells are detected at the ventricular surface and in the sub-ventricular zone of a control cortex (F-G). In the cKO cortex there is an increase in basally located pH3 positive cells (J-K). At E17.5 a majority of pH3 positive cells are localized away from the ventricles in the cKO cortex, arrows in L (L-M) compared to the control cortex (H-I). Overlays show antibody staining in green and nuclei counterstained with DAPI in blue.
Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$
cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$
Scale bars = A-D 50 μm, E-M 100 μm,





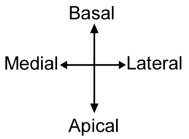


Fig 4.12 **Expression of PKD-1 in the dorsal telencephalon**. Coronal sections through the telencephalon of E12.5, E14.5 and E17.5 mouse embryos were immunostained for PKD-1. At E12.5, PKD-1 is weakly expressed in the control cortex (A-B) and is strongly expressed in the cKO cortex (C-D). At E14.5, PKD-1 is expressed in the SVZ and IZ of the control cortex (E-F) but is highly expressed in all layers of the cKO cortex (G-H). At E17.5, PKD-1 expression is restricted to a specific-population of cells in the dorsal telencephalon of a control brain (I-J) but PKD-1 expression is absent in the cKO brain (K-L). SVZ=sub-ventricular zone, IZ= intermediate zone. Overlays show antibody staining in green and nuclei counterstained with DAPI in blue.
Control = Crb2 $^{F/+}$; Emx1-Cre $^{Tg/+}$ cKO = Crb2 $^{F/F}$; Emx1-Cre $^{Tg/+}$ Scale bar = 100 μm

Table 4.1 Summary of data from the analysis of Crb2; Emx-1Cre cKO embryos.

Markers	**Control**			**cKO**		
	E12.5	**E14.5**	**E17.5**	**E12.5**	**E14.5**	**E17.5**
Crb2						
Pals1						
Par3						
ZO-1						
N-Cad						
Sox2						
Nestin						
Prominin1						
Tbr2						
TuJ1						
Tbr1						
Reelin						
pH3						
Hes5						
PKD-1	+	++	++++	++++	++++	

Normal Decrease Mislocalized

Complete loss Increase + Level of expression

4.3 Discussion

In this chapter, I have shown that Crb2, one of the vertebrate homologs of Drosophila Crumbs plays important roles during cortical neurogenesis. In the developing telencephalon, where Crb2 is conditionally removed, defects are apparent in the localization of apical polarity proteins, cell junction proteins and mitotic cells. In addition to these effects, loss of Crb2 leads to a dramatic decrease in apical neural progenitors and a concomitant increase in intermediate progenitors and neurons. These findings suggest a crucial role for Crb2 in regulating murine cortical neurogenesis.

Crb2 depletion from the cortex led to decreased apical localization of polarity and adherens junction proteins such as Pals1, Par3 and N-Cadherin. It has been shown previously that Pals1 is an intracellular binding partner of Crb2 (Kim et al., 2010); therefore the loss of Pals1 expression in the Crb2 cKO cortex was not unexpected.

The loss of Par3 expression at E12.5 is in agreement with previous studies in mammalian epithelial cell lines that have reported a direct interaction between the Crumbs and Par complexes (Hurd et al., 2003). Surprisingly, apical expression of Par3 expression was restored in the cKO cortex by E14.5. It is plausible that two separate mechanisms regulate Par3 localization at different stages of neural development: the earlier mechanism is Crb2 dependent whilst at later stages a Crb2 independent mechanism is in place.

Overall, the data presented in the chapter suggests that conditional removal of Crb2 in the developing telencephalon affects apical recruitment and stabilization of Crumbs complex components and interacting apical proteins. These defects in recruitment of polarity proteins are consistent with previously reported phenotypes in both *Drosophila* and zebrafish Crumbs mutants (Hsu et al., 2006; Omori & Malicki, 2006b; Pellikka et al., 2002).

At early stages of cortical development, there is a decrease in mitotic cells at the apical surface of the Crb2 cKO cortex. This correlates with a decrease in apical progenitor markers Nestin, Sox2, Prominin-1 and Hes5. Furthermore in the Crb2 cKO cortex, there is an expansion of Tbr2 positive intermediate progenitor cells and a subsequent increase in post-mitotic neuronal markers TuJ1, Tbr1. This suggests that Crb2 is required for maintenance of neural progenitors in the developing telencephalon.

As discussed in Chapter 1, Crumbs has been associated with the Notch signalling pathway. Although it is tempting to speculate that a similar association between Crb2 and Notch exists in the developing cortex, further experiments need to be carried out to addresses this. It remains unclear if the depletion of Hes5 a Notch target gene, in the Crb2 cKO cortex is the underlying cause for the depletion of the apical progenitor cell population or if it represents a mere loss of apical progenitors.

It is also possible that Crb2 affects cortical neurogenesis via removal of apical Pals1, Par3 or N-Cadherin. Previous studies have implicated all three proteins in cell fate determination in the developing cortex (Bultje et al., 2009; Kadowaki et al., 2007; Kim et al., 2010).

Conditional removal of Pals1 leads to premature withdrawal of neural progenitors from the cell cycle and precocious neural differentiation. These prematurely born neurons rapidly apoptose and the entire cortical structure of Pals1 mutants is compromised (Kim et al., 2010). Similar to the phenotype observed in Crb2 cKO embryos in my study, in Pals1 cKO embryos adherens junctions, apical complex proteins, cell proliferation and neural progenitor fate is affected. Intriguingly, the massive cell death phenotype observed in Emx-1 Cre; Pals1 cKO embryos (Kim et al., 2010) is not observed in Emx-1 Cre; Crb2 cKO cortex, despite the absence of apically enriched Pals1. It is plausible that the timing of Pals1 ablation, together with its effect on interacting proteins influences cell survival and

that this temporal sequence of events is different between the Pals1 cKO and Crb2 cKO embryos.

In addition to the loss of cell junction components, a member of the PKD gene family, Protein kinase D1 is prematurely upregulated in the Crb2 cKO cortex. Protein kinase D1 is implicated in trans-Golgi network-derived sorting of dendritic proteins (Bisbal et al., 2008; Yin et al., 2008). Its early upregulation in the Crb2 cKO cortex suggests that Crb2 may also influence trafficking of neuronal proteins to the correct cellular domains during murine cortical development. Alternatively, PKD-1 could be a marker that is indicative of premature neural differentiation and/or altered lamination in the cortex.

Mislocalization of Reelin and Tbr1 positive cells in the absence of Crb2 suggests that secondary to defects in cell polarity, lamination of the developing cortex is also affected. Reelin is crucial for the inside-out layering of the cortex (Caviness et al., 1982) and mislocalization of Reelin positive cells in the Crb2 cKO cortex could in turn affect the precise localization of layer-specific neurons in the cortex. It is plausible that absence of apical Crb2 expression renders cells unresponsive to extrinsic guidance cues and subsequently affects their spatial localization. It will be interesting to investigate the role of Crb2 in cortical projections of neurons and migration.

Overall, the above-proposed mechanisms are not mutually exclusive and Crb2 may act via the concerted action of several interacting proteins and interplay of signalling pathways.

Despite these defects in cortical development, Crb2 mutant mice survive and do not display any overt morphological or behavioural defects (H. Alves, personal communication). This could probably be due to functional redundancy between Crb1 and Crb2 as Crb1 is also expressed in the brain (den Hollander et al., 2002). Alternatively, it is also possible that the secreted Crb2 isoform (described in Chapter 5) compensates in

the absence of full length Crb2. It will be interesting to investigate the effect of Crb2 on neurogenesis in a system where both the full-length and secreted Crb2 isoforms are targeted.

CHAPTER 5

Characterization of an alternative splice variant of Crumbs 2

5.1 Introduction

As described in Chapter 1 (section 1.4.4), different splice variants of Crb2 have been previously identified. The main aims of this chapter were to address the following questions:

1. Does the alternative splice variant of Crb2 – Crb2S encode a secreted protein?
2. Does Crb2S exist physiologically?
3. Does Crb2S have an expression profile that is distinct from that of full length Crb2 (Crb2F)?

5.2 Results

5.2.1 Crb2S protein can be detected in the cell culture supernatant

The cDNA of Crb2S isoform was previously cloned (R. Walker, previous post-doc in the lab) into a mammalian expression vector. The expression vector has a CMV promoter for high-level constitutive expression and a C-terminal V5 epitope tag and a polyhistidine tag. The Crb2 signal peptide encoding sequence was cloned into the same expression vector and used as a control (Fig 5.1 A).

To determine if the Crb2S-V5 His tag fusion protein (Crb2S protein) was secreted into the cell culture medium, HEK293 cells were transfected with the expression vectors inserted with either Crb2S cDNA or Crb2 signal peptide cDNA. V5 tagged protein in the processed cell culture supernatant was detected by Western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene was used as a loading control for the lysates. GAPDH was not detected in the supernatants, indicating that the supernatants were not contaminated with cytoplasmic debris. Crb2S-V5 His tagged protein was detected in the supernatant and lysate from cells transfected with the Crb2S expression vector but not in the supernatant and lysate from cells transfected with the control expression vector (Fig 5.1 B). Unfortunately, I could not detect the low molecular weight signal peptide in the control

transfected cell culture supernatant (data not shown). However, this does not negate the use of this expression vector as a suitable experimental control.

Overall, data from this section shows that Crb2S isoform does indeed encode a protein that can be secreted in *in vitro* assays.

5.2.2 Generation of stable cell lines overexpressing Crb2S protein

Stable clonal cell lines constitutively expressing Crb2S were derived from HEK293 cells transiently transfected with the Crb2S expression vector. After selection in G418 antibiotic, individual clones were expanded and the processed cell culture supernatants from 26 different clones were analysed by western blotting to detect the V5 tagged fusion protein (Fig 5.2 A). The data from this analysis is summarized in table 5.1. Of the 26 clones, Clone 9 (C-9) and Clone 16 (C-16) had the highest level of V5 tagged fusion protein expression (Fig 5.2 B) and were therefore used for further experiments.

Table 5.1: Summary of western blot screening of the stable clonal cell line supernatants

Clone Number	Protein bands detected by V5 tag antibody
1- 5 and 18	No bands detected
6-12 and 24-26	Three bands of apparent molecular masses between 180kDa and 110kDa. Clone 9 has the highest level of expression
13-15, 20-22	Single band of apparent molecular mass between 180 kDa and 110 kDa
16,17, 19, 23	Four bands of apparent molecular masses between 180kDa and 110kDa. Clone 16 has the highest level of expression

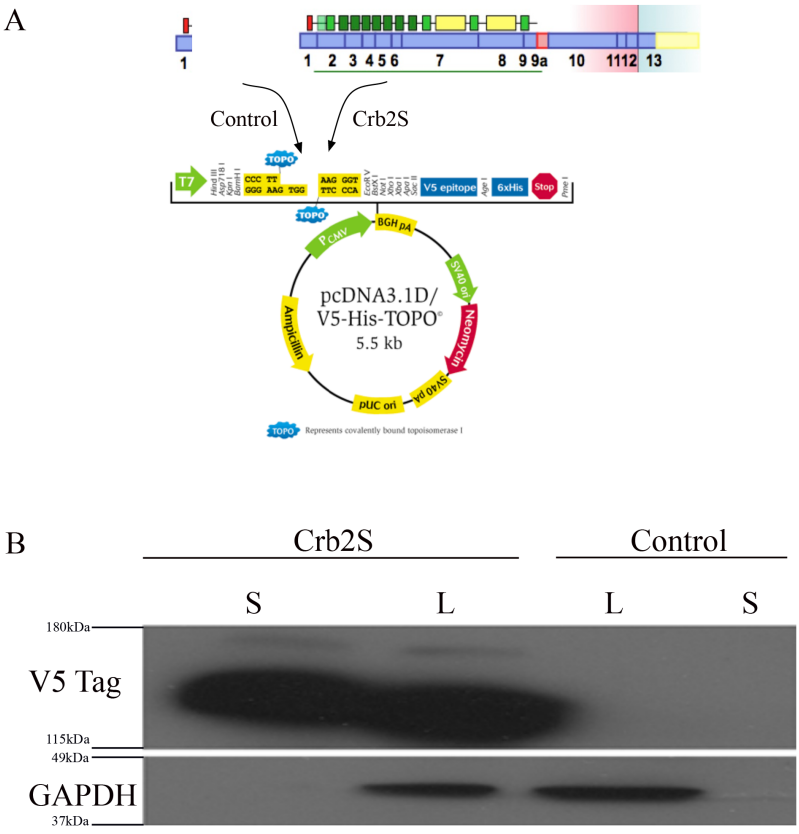


Fig 5.1 **Crb2S is detected in the cell culture supernatant after exogenous overexpression in HEK293 cells.** A. Crb2S cDNA and Crb2S signal peptide coding cDNA were cloned into pcDNA3.1 V5-His-TOPO expression vector. B. Western blotting to detect the V5 tagged recombinant protein shows that Crb2S can be detected in the supernatant (S) and lysate (L) from cells transfected with Crb2S expression vector but not in the cells transfected with the control expression vector. Cells were cultured in serum-reduced conditions for 72hours before harvesting.GAPDH was used as a loading control. Apparent molecular weights are indicated on the left in B.

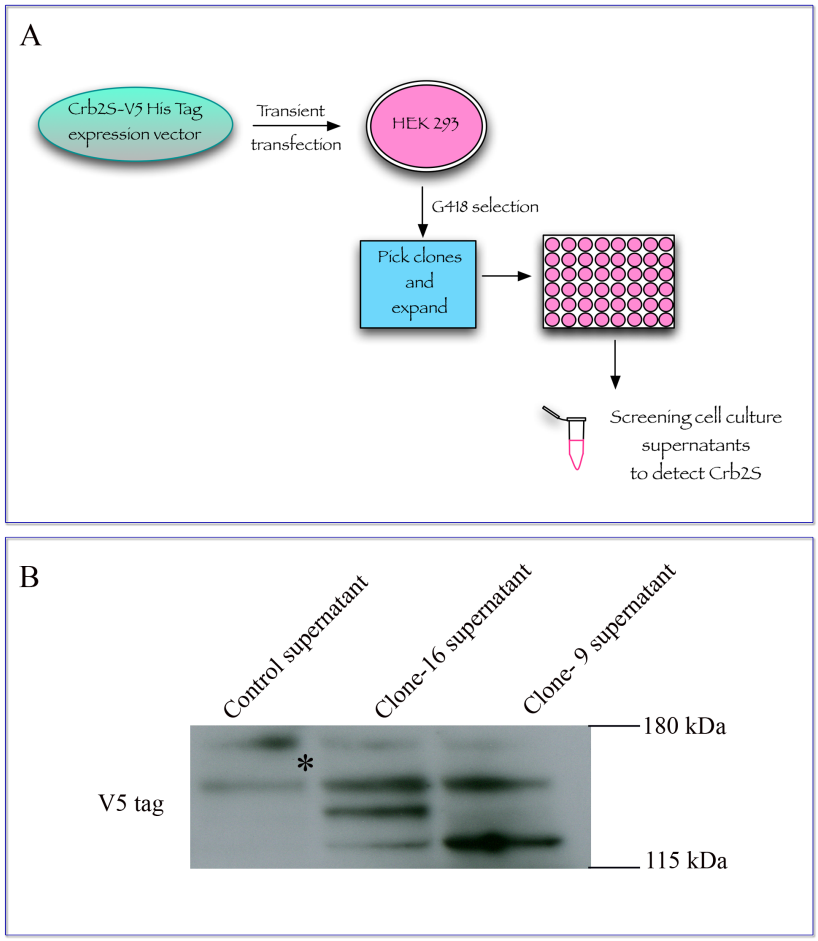


Fig 5.2 **Generation of stable cell lines overexpressing Crb2S protein** A.Schematic showing the generation and screening strategy to obtain a stable cell line producing Crb2S V5 His tag re-combinant protein. HEK293 cells were transiently transfected with a Crb2S expression vector and cultured in medium supplemented with G418.The resultant geneticin resistant colonies were picked into 48 well plates and expanded. Cell culture supernatant from each clonal cell line was concentrated and screened by western blotting to detect the V5 tag. B. Western blot shows detection of V5 tag in the cell culture supernatant of two representative cell lines Clone-16 and Clone-9.*- indicates non-specific bands detected in the control supernatant. Apparent molecular masses are indicated on the right in B.

It is well established that the endoplasmic reticulum (ER) plays a key role in protein and lipid biosynthesis. The ER is a site for nascent secretory protein translocation and any protein designed for secretion is localized to the ER (Alberts et al., 2002).

To determine if the exogenously overexpressed Crb2S protein localizes to the ER, I carried out immunocytochemistry for Calnexin, an integral chaperone protein in the ER (Kleizen & Braakman, 2004). In C-16 cells, Crb2S-V5 His tag fusion protein co-localizes with Calnexin (Fig 5.3). However, I could not detect any sub-cellular localization of V5 tagged protein in C-9 or HEK293 cells (Fig 5.3).

Overall, the data suggests that C-16 cell line is overexpressing Crb2S and constitutively secreting the Crb2S protein into the cell culture supernatant.

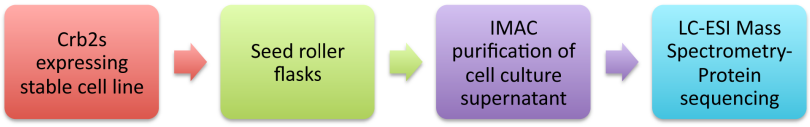
5.2.3 Purification and Protein sequencing of Crb2S

To obtain larger volumes of purified Crb2S V5 His tag fusion protein that can be used in biological assays, the C-16 cell line was passed to Bioserv UK Ltd. at the University of Sheffield. I analyzed samples from the loading, column washes and imidazole elution steps of the protein purification process by western blotting for V5 tag antibody (data not shown). Crb2S is detected in the final eluted sample using both V5 tag (Fig 5.4C) and His tag antibody (data not shown).

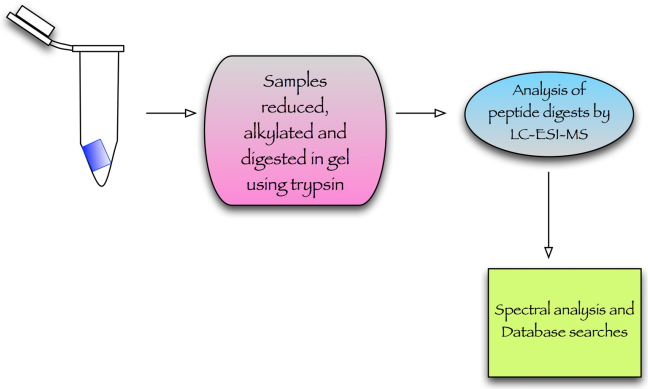
To confirm if the purified protein was Crb2S, the protein sample was reduced, denatured and fractionated by SDS-PAGE. The gel was stained using Coomassie Brilliant blue compatible with mass spectrometry analysis (Fig 5.4C). 4 candidate bands (Sample I-IV) were systematically excised from the gel and sent to Eurogentec for sequencing by LC-ESI (liquid chromatography-electrospray ionization) mass spectrometry (Fig 5.4 B).

Fig 5.3 **Crb2S V5 His tag protein localizes to the endoplasmic reticulum.** A-C Immunostaining of HEK293 cells and 2 stable cell lines of Crb2S Clone-9 (C-9) and Clone-16 (C-16) for V5 tag antibody. V5 tag is present in the cytoplasm in a punctate pattern around the nucleus in C-16 cells but not in HEK293 and C-9 cells.
C-H Immunostaining of HEK293 and C-16 cells for V5 tag antibody and Calnexin, an ER chaperone protein. In C-16 cells, V5 tag (red) co-localizes with Calnexin (green) as shown by the yellow staining in H.Nuclei counterstained with DAPI in blue. Scale bar A-C = 50μm; C-H = 50μm.

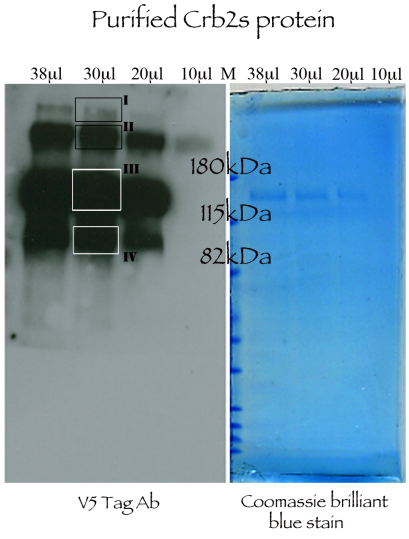
A


Crb2s expressing stable cell line
Seed roller flasks
IMAC purification of cell culture supernatant
LC-ESI Mass Spectrometry-Protein sequencing

B


Samples reduced, alkylated and digested in gel using trypsin
Analysis of peptide digests by LC-ESI-MS
Spectral analysis and Database searches

C


Purified Crb2s protein
38µl 30µl 20µl 10µl M 38µl 30µl 20µl 10µl
I
II
III
IV
180kDa
115kDa
82kDa
V5 Tag Ab
Coomassie brilliant blue stain

Sample I and III had two bands each, an intense and a weak band (Fig 5.4 C and Fig 5.5 A). Each band was treated separately and was reduced, alkylated and digested in gel by trypsin. Peptide digests from the individual samples were analyzed by LC-ESI mass spectrometry.

Database searches restricted to the mouse taxonomy provided significant identification scores. Sample I (Intense) and Sample III (Intense and Weak) were identified as Crb2. Sample II and IV correspond to spectrin alpha chain and heat shock protein respectively. The peptide sequences hits and MASCOT ID score are shown in the Appendix 3. The sequencing data is briefly summarized in Fig 5.5 B.

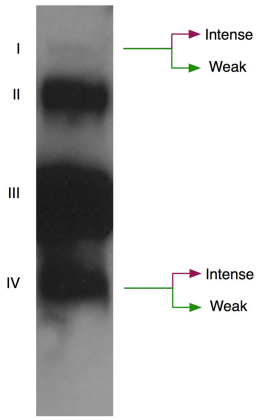
Overall, the results show that the Crb2S expression vector encodes a Crb2S-V5 His tag fusion protein that is localized in the ER and readily secreted into the cell culture medium. The purified Crb2S protein is a useful resource that can be used in biological assays to study the potential functional roles of Crb2S isoform.

5.2.4 Expression of Crb2S mRNA in mouse tissue

In the previous section, I have shown that the Crb2S isoform encodes for a secreted protein *in vitro*. However, an outstanding question remains – does Crb2S isoform exist physiologically?

To begin to address this question, I analysed mouse embryonic and adult tissues to detect the endogenous expression of Crb2S. Total RNA from mouse embryonic tissues E10.5, E12.5 head and bodies and adult mouse eye, forebrain, cerebellum, medulla, kidney, liver, lung, spleen and heart was analyzed by RT-PCR using specific primers for Crb2F and Crb2S.

A



B

Sample ID	Bands	Protein ID	Mascot ID Score
I	Intense	**1. Crumbs2**	**209**
		2. Keratin typeII	63
		3. Peroxidasin	43
		4. Splicing factor 3B subunit	39
	Weak	**1. Spectrin alpha chain**	**349**
		2. Keratin typeI	86
		3. Keratin typeI	76
		4. Keratin typeII	57
		5. Nidogen	51
		6. Crumbs2	49
		7. Keratin typeII	43
II		**1. Spectrin alpha chain**	**295**
		2. Nidogen	78
		3. Keratin	60
III	Intense	**1. Crumbs2**	**315**
		2. Nell2-PKC binding protein	103
	Weak	**1. Crumbs2**	**183**
		2. Nell2-PKC binding protein	68
		3. Splicing factor 3B subunit	66
		4. DNA damage binding protein	49
IV		**1. Heat shock protein**	**350**
		2. Heat shock cognate protein	316
		3. Crumbs2	277
		4. Heat shock HSP90-beta	121
		5. Splicing factor 3A	111
		6. Heat shock protein	106
		7. Transketolase	87
		8. Endoplasmin	84
		9. Albumin	48
		10. Plastin	41

Crb2F mRNA is ubiquitously expressed in the embryonic and adult tissues. In contrast, Crb2S has a more restricted expression compared to Crb2F. Crb2S is expressed in the embryo and also expressed in the adult eye, forebrain, cerebellum but not in the medulla, liver, kidney, spleen, heart and lung (Fig 5.6). This suggests that the splicing of exon 9A is regulated in a tissue-specific manner and that Crb2S expression is more prominent during early development and in neural tissue.

5.3. Discussion

In this chapter, I have shown that Crb2S, an alternative splice variant of Crb2 encodes a secreted protein. This is consistent with bioinformatic predictions that human homologs of CRB1 and CRB2 genes encode full-length proteins that have a transmembrane domain and also putatively secreted truncated proteins that lack the transmembrane domain (den Hollander et al., 2002; Katoh & Katoh, 2004). Additionally, a mouse Crb1 splice variant encoding a secreted protein has been identified (Crb1S) (Watanabe et al., 2004). Similar to the expression of Crb2S, the expression profile of Crb1S mRNA is distinct from that of full length Crb1 mRNA (Watanabe et al., 2004) suggesting that the splicing of Crumbs isoforms is regulated in a tissue -specific manner.

In cultured cell lines overexpressing Crb2S, I have shown that Crb2S is localized to the endoplasmic reticulum and is secreted into the cell culture supernatant. It will be interesting to investigate whether secretion of Crb2S is mediated by the classical ER-Golgi pathway by using inhibitors of protein transport from the ER-Golgi such as Brefeldin A or Exo1 (Feng et al., 2003) and monitoring the expression of Crb2S.

Unfortunately, our custom-made antibodies against Crb2S failed to work. The lack of tools to specifically detect the endogenous Crb2S protein has hampered in detailed analysis of this isoform. All studies relating to Crb2S isoform in this thesis were carried out using the Crb2S-V5 His tagged fusion protein.

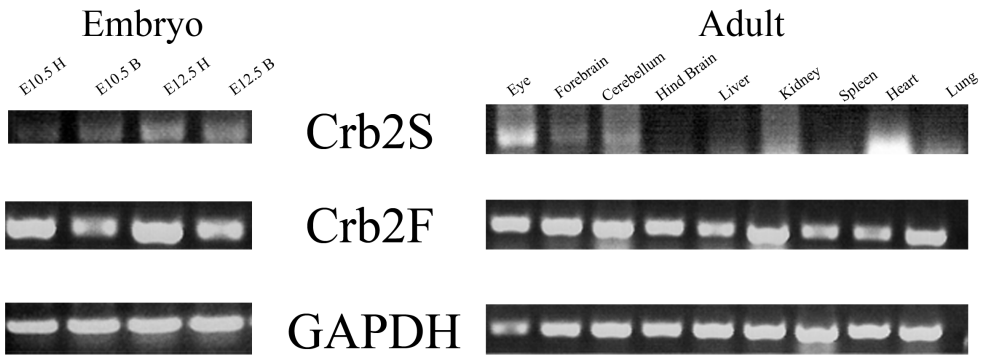


Fig 5.6 **Expression of Crb2F and Crb2S in embryonic and adult mouse tissue.** The expression pattern of Crb2F and Crb2S were analysed by RT-PCR on RNA samples from E10.5-E12.5 mouse embyronic tissue and from adult mouse tissue. Crb2S is expressed in the embryonic and specifically in the eye, forebrain and cerebellum of the adult mouse. Crb2F is expressed in the embryo and all adult mouse tissues analysed. GAPDH is used as a control.

Crumbs is the “only polarity protein” to have an extracellular domain. Whilst the interactions of the cytoplasmic tail of Crumbs with other polarity proteins have been widely studied (discussed in Chapter 1), not much is known about the proteins binding to the extracellular domain of Crumbs. The extracellular domain of Crumbs may aid in sequestering molecules and in turn contribute to functional diversity of the Crumbs protein complex dependent on the cell type/developmental stage. The purified Crb2S protein can be used as a biochemical tool to identify proteins interacting with Crb2S (Appendix 2). This may offer insight into not only the function of the extracellular domain of Crb2, but also enhance our understanding of the influence of transient and novel complex members on the activity of the entire protein complex.

CHAPTER 6

Misexpression of Crb2 isoforms in the chick embryonic hindbrain

6.1 Introduction

In the previous chapter, I have discussed the multiple isoforms of Crb2 and described the characterization of a novel secreted isoform Crb2S. The main aim of this chapter was to determine if the full length (Crb2F) and the secreted (Crb2S) isoforms have distinct functional roles in the development of the chick embryonic hindbrain.

I have taken advantage of two well-established and powerful approaches to manipulate the levels of Crb2 in the chick embryonic hindbrain: 1. *in ovo* electroporation (Itasaki et al., 1999) 2. *in vitro* explant culture (Placzek & Dale, 1999) . *In ovo* electroporation facilitates the analysis of gene function by overexpression or depletion of the protein of interest in the chick embryo. This technique has been successfully used to study neural development (Itasaki et al., 1999; Nakamura et al., 2004). The neural tube is easily accessible and after electroporation, the cDNA or short hairpin of interest is expressed only on one side of the neural tube whilst the contralateral side serves as an untransfected control (Fig 6.1A). The idea behind the chick electroporation studies was to misexpress Crb2 in a region where endogenous Crb2 is not expressed and analyse the effect on neural development by carrying out a candidate marker analysis similar to the studies performed on Crb2 conditional knockout mouse embryos. Briefly, looking at the effect of Crb2 misexpression on a) recruitment of apical polarity proteins and cell junction proteins b) neural progenitor cell fates c) apically restricted mitoses.

In contrast to *in ovo* electroporation, explant culture offers the advantage of isolated culture of the tissue of interest under defined *in vitro* culture conditions. As mentioned in the introduction, the neural differentiation program is initiated in even-numbered rhombomeres and the odd numbered rhombomeres follow on. However, rhombomeres 3 and 5 do not contribute significantly to the neural crest cell population and the even-numbered rhombomeres flanking these segments repress

neural crest production by inducing apoptosis (Graham, Heyman, & Lumsden, 1993) (Lumsden et al., 1991). In the explant culture system, I wanted to investigate the effect of secreted Crb2 isoform (described in Chapter 5) on neural crest migration in rhombomeres 1-4 of Hamburger and Hamilton stage 10 chick embryos, where endogenous Crb2 expression is not detected (data shown in Chapter 3).

6.2 Results

In ovo electroporation of only RFP or GFP control constructs per se had no apparent deleterious effect on neural development (Fig 6.1 B, C) and no alterations in marker expression profile were observed (data not shown) compared to the untransfected contralateral side. Therefore, the contralateral side that does not express the gene of interest/reporter was used as a control.

6.2.1 Manipulation of Crb2 levels in the chick embryonic hindbrain produces isoform-dependent phenotypes

In order to ascertain what function full length (Crb2F) and secreted Crb2 (Crb2S) isoforms may play during neural development, Crb2F and Crb2S expression vectors were co-electroporated with an RFP vector into an H&H stage 10 chick embryonic hindbrain. The embryos were analysed twenty-four hours after electroporation. All the electroporated embryos were analysed at the level of the rostral hindbrain.

Overexpression of a control RFP construct shows RFP expression in one side of the neural tube (Fig 6.1 B&C). The control RFP electroporated embryos showed no alteration in neural tube morphology and RFP expression was predominantly confined to the neural tube.

Interestingly, misexpression of Crb2F and Crb2S isoforms produced different phenotypes. Misexpression of Crb2F resulted in a remarkable change in neural tube morphology (Fig 6.1 D, E). Compared to the contralateral control side, there was an apparent increase in the

width of the neural tube on the Crb2F+RFP co-electroporated side. It also appears that misexpression of Crb2F + RFP in the neural tube induces hyperplasia within 24 hours.

In contrast to this, after misexpression of the Crb2S isoform, I observed a stream of RFP^{+ve} cells migrating away from the neural tube. Moreover misexpression of Crb2S in the hindbrain had no overt effect on neural tube morphology unlike the full-length isoform (Fig 6.1F,G)

6.2.2 Misexpression of Crb2F in the hindbrain alters marker expression profile

To determine if misexpression of Crb2F affects cell polarity and cell junction components, I carried out candidate marker expression analysis on the Crb2F electroporated embryos. As already described, twenty-four hours after electroporation of Crb2F there is an alteration in the neural tube morphology on the electroporated side compared to the unelectroporated control side. In addition, the adherens junction proteins N-Cadherin (Fig 6.2 A-B) and ß-Catenin (Fig 6.2 C-D) are apically enriched in the control side. Within the Crb2F+RFP expressing region, the expression of both N-Cadherin and ß-Catenin is not apically restricted. Whilst N-Cadherin staining is detected in a broader domain away from the apical surface (Fig 6.2 A-B), ß–Catenin staining is observed outlining a mesh-like arrangement of neural tube cells (Fig 6.2 C-D).

Similarly, expression of the polarity protein aPKC is no longer confined to the apical surface of the neural epithelium in the Cr2b2F+RFP expressing region (Fig 6.2 E-F). Intriguingly, in contrast to the broader expression domain of ß-Catenin, N-Cadherin and aPKC, the expression of Pals1, a member of the Crumbs complex is completely lost (Fig 6.2 G-H) upon misexpression of Crb2F.

Interestingly, the apical expression of Rab11, a GTPase shown to play key regulatory roles in endocytic trafficking and cytokinesis

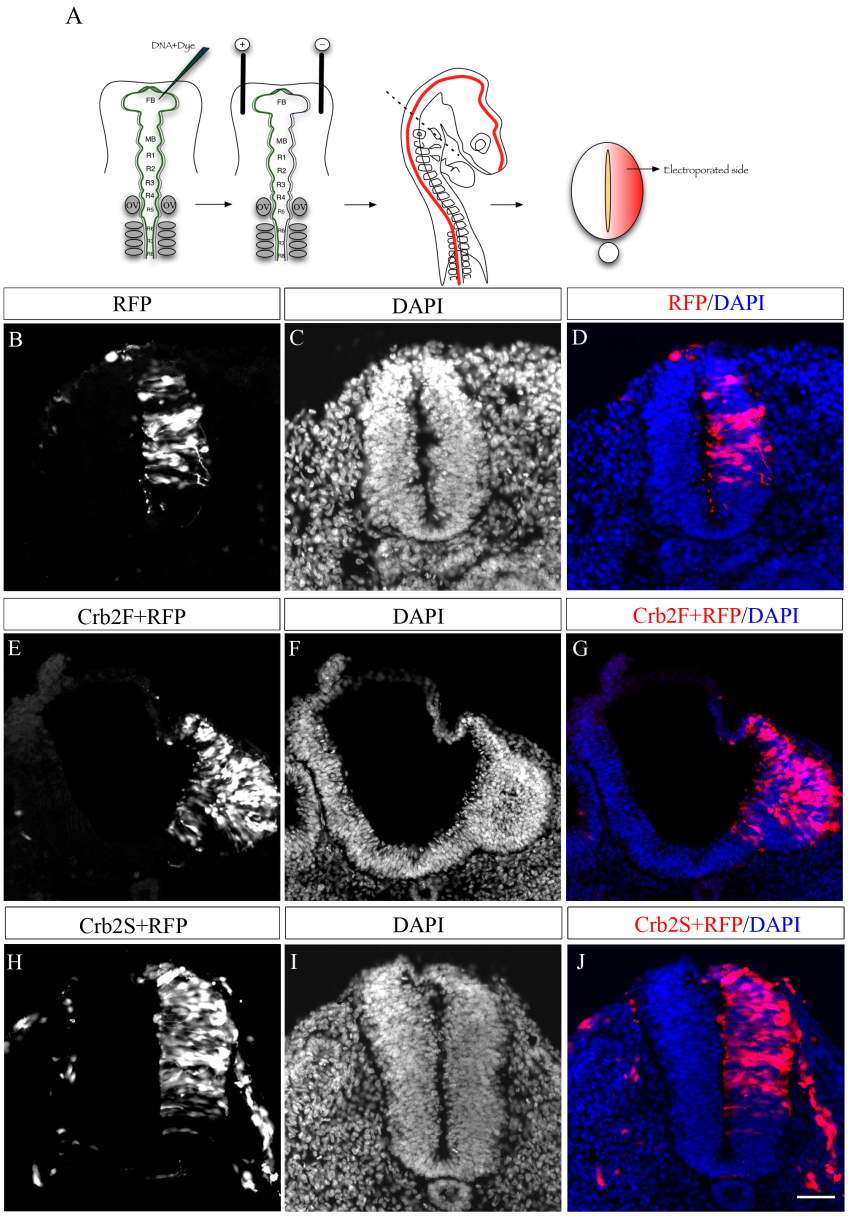
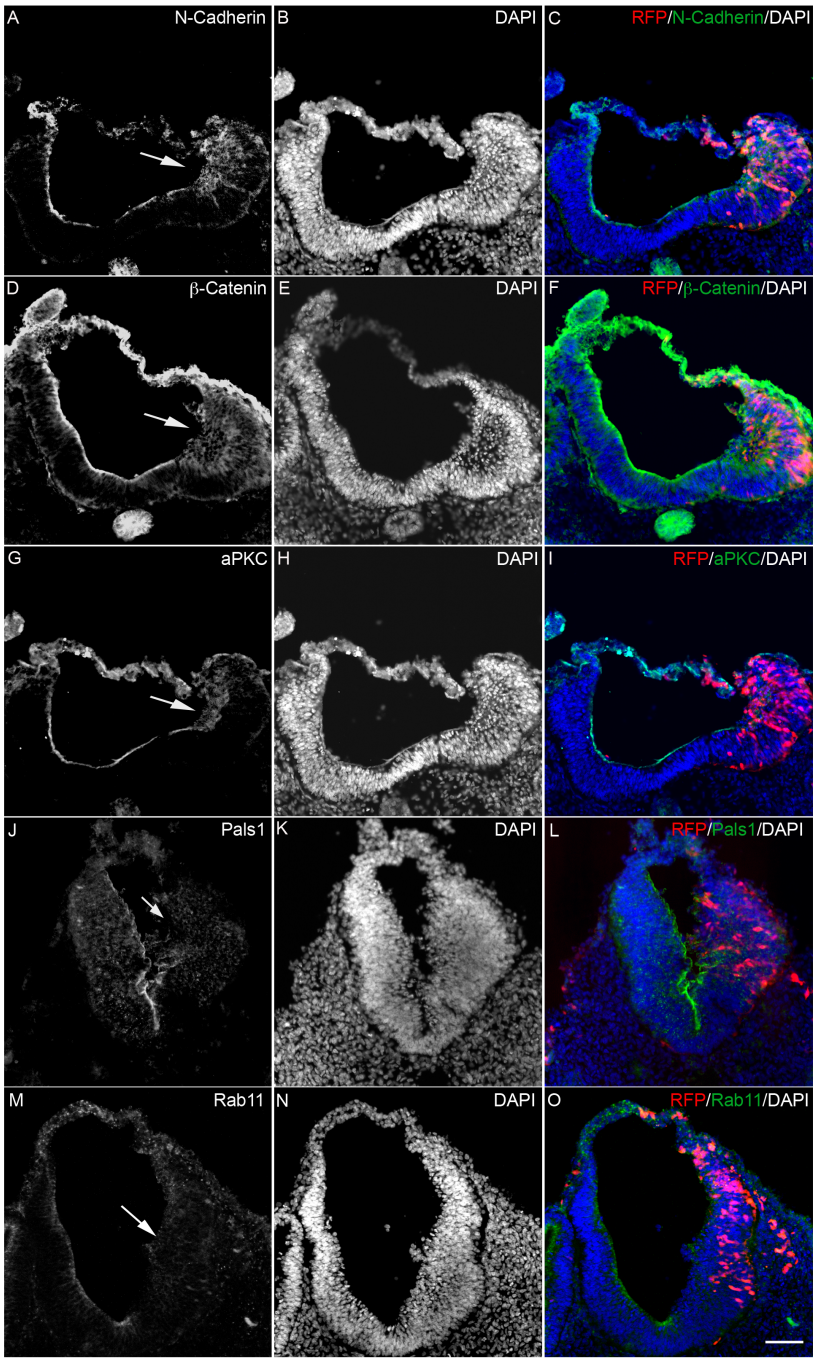


Fig 6.1 ***In ovo*** **electroporation of Crb2 isoforms in the developing chick neural tube.** A. Schematic illustration of the setup used for electroporation. For electroporation, DNA and fast green dye solution is injected into the lumen of the neural tube and electrodes are placed on either side of the embryo. Application of electric pulse results in unilateral transfer of DNA and the embryos are cultured *in ovo* for 24hours. Dotted line indicates the level of a transverse section through the embryo where fluorescent reporter expression is observed on only one side of the neural tube. Transverse sections through chick embryos electroporated with an RFP expression vector (B, C, D), Crb2F+RFP vector (E,F,G) and Crb2S+RFP (H,I,J). In the overlays transfected cells are shown in red and nuclei counterstained with DAPI in blue. OV- Otic vesicle Scale bar = 50µm


A
N-Cadherin
B
DAPI
C
RFP/N-Cadherin/DAPI
D
β-Catenin
E
DAPI
F
RFP/β-Catenin/DAPI
G
aPKC
H
DAPI
I
RFP/aPKC/DAPI
J
Pals1
K
DAPI
L
RFP/Pals1/DAPI
M
Rab11
N
DAPI
O
RFP/Rab11/DAPI

(Hoekstra et al., 2004) (Strickland & Burgess, 2004) was also dramatically reduced in the electroporated side (Fig 6.2 I-J). Taken together, Crb2F misexpression affects the apical localization of not only adherens junction markers, but also that of the apical localized proteins aPKC, Pals1 and Rab11.

To determine if Crb2F misexpression affected cell proliferation, I carried out immunostaining for phospho-histone3 (pH3), a mitotic cell marker (Hendzel et al., 1997; Van Hooser et al., 1998). In the control unelectroporated side, $pH3^{+ve}$ cells are present on the ventricular surface of the neuroepithelium. In the Crb2F electroporated side, many $pH3^{+ve}$ mitotic cells are detected away from the ventricular surface, at ectopic locations within the neural tube (arrowheads in Fig 6.3 A-C).

To elucidate if Crb2F misexpression and the subsequent alterations in the expression of apical cell components and localization of mitotic cells had an effect on neural differentiation, I analysed electroporated embryos for alterations in the expression of neural progenitor markers Pax6, Sox2 and early neuronal marker TuJ1. Twenty-four hours after electroporation, there was no apparent alteration in the neural progenitor marker expression. Sox2 and Pax6 co-localized with Crb2F+RFP in the electroporated side of the neural tube (Fig 6.4 A-B, E-F). However, I observed aberrantly localized TuJ1 (ß-Tubulin III) positive neurons in the electroporated side (Fig 6.3 D-F). Interestingly, Crb2F+RFP expression did not overlap with the $TuJ1^{+ve}$ cells. This suggests that there may be some non-cell autonomous effect perhaps due to incorrect epithelial integrity and adhesion so that cells already destined to differentiate prior to electroporation can no longer migrate to the correct basal location.

To investigate if misexpression of Crb2F had an effect on neural crest cell migration, I analysed electroporated embryos for the expression of Slug, an early neural crest marker (Nieto et al., 1994) and HNK-1, a marker for migratory neural crest cells (Del Barrio & Nieto, 2004).

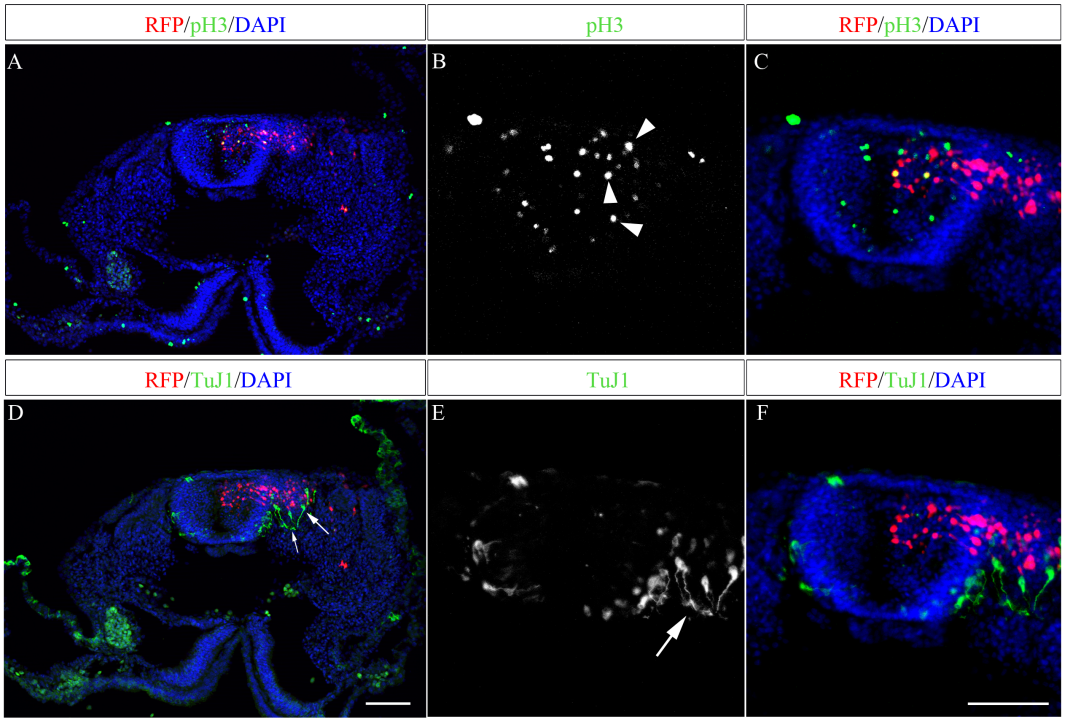


Fig 6.3 **Crb2F misexpression affects apical mitoses and neural differentiation** 24hours after electroporation.
Transverse sections through the rostral hindbrain of embryos co-electroporated with Crb2F and RFP were immunostained for mitotic marker pH3 and an early neuronal marker TuJ1.
A- Ectopically localized pH3+ve mitotic cells are observed on the electroporated side indicated by arrow heads in B.
D- TuJ1+ve cells are aberrantly localized on the electroporated side indicated by arrows in D and E.
B-C, E-F are higher magnification images of A and D.
Overlays show antibody staining in green, RFP in red and nuclei counterstained with DAPI in blue.
Scale bars = A,D and B-C, E-F=100 µm

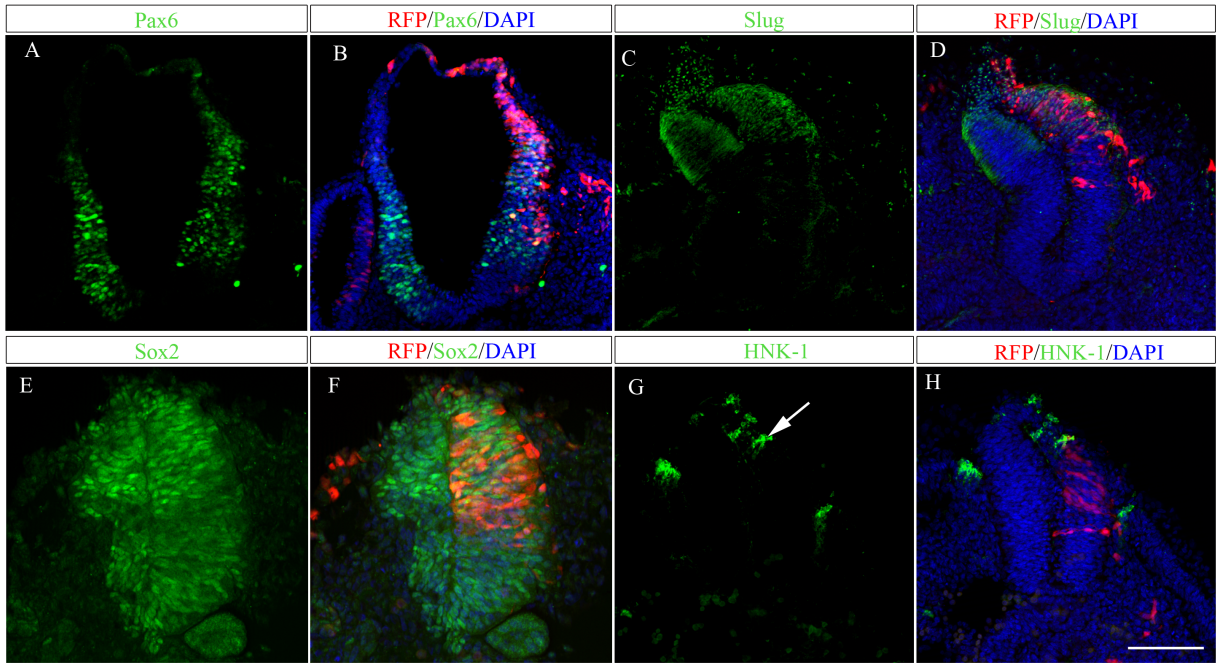


Fig 6.4 **Crb2F misexpression does not affect the expression of neural progenitors but ectopically induces neural crest cell marker expression**, 24 hours after electroporation. Transverse sections through the rostral hindbrain of embryos co-electroporated with Crb2F and RFP were immunostained for Pax6 (A-B), Slug (C-D), Sox2 (E-F) and HNK-1 (G-H). Arrow in G indicates ectopically localized HNK-1 +ve cells. A,C,E,G show antibody staining in green. B,D,F,H are overlays where antibody staining is in green, RFP in red and nuclei counterstained with DAPI in blue. Scale bar = 100 μm

Misexpression of Crb2F had no obvious effect on Slug expression (Fig 6.4 C-D). However, HNK-1 $^{+ve}$ neural crest cells were ectopically located in the dorsal neural tube on the electroporated side (Fig 6.4 G-H).

Overall, the results suggest that misexpression of Crb2F leads to altered neural tube morphology and also interferes with the apical distribution of adherens junction proteins; polarity proteins, localization of mitotic cells and neural crest cells.

6.2.3 Misexpression of Crb2S in the hindbrain induces migration of neural crest cells.

To determine if the Crb2S isoform (discussed in Chapter5) has a distinct functional role in neural development, the effect of misexpressing Crb2S in the chick embryonic hindbrain was analysed twenty-four hours post electroporation. Pax6 is expressed predominantly in the dorsal and intermediate progenitors of the neural tube (Ericson et al., 1997). Within the neural tube there was no apparent difference in Pax6 expression in the Crb2S+RFP side compared to the control side. However, a few RFP^{+ve} cells co-localized with Pax6 were detected outside the neural tube (Fig 6.5 A-B). Pax7 is expressed in the dorsal neural tube and in the cranial neural crest cells (Kawakami et al., 1997). No Pax7^{+ve} cells were detected in the contralateral control side however Pax7 expressing cells co-localized with RFP were also observed outside the neural tube (Fig 6.5 C-D). Misexpression of Crb2S had no apparent effect on the expression of Sox2 (Fig 6.5 E-F), pH3 (Fig 6.5 G-H), Pals1 (Fig 6.5 I-J), N-Cadherin (Fig 6.5 K-L) and ß-Catenin (data not shown)

Interestingly, the stream of RFP $^{+ve}$ cells observed in Crb2S+RFP electroporated embryos coexpressed neuronal class III β-Tubulin detected by TuJ1 antibody (Fig 6.6 A-C). In the control side, HNK-1 expression is detected only in neural crest cells that have migrated away from the neural tube. In the Crb2S+RFP electroporated side, HNK-1 staining was detected in the dorsal neural tube and the stream of

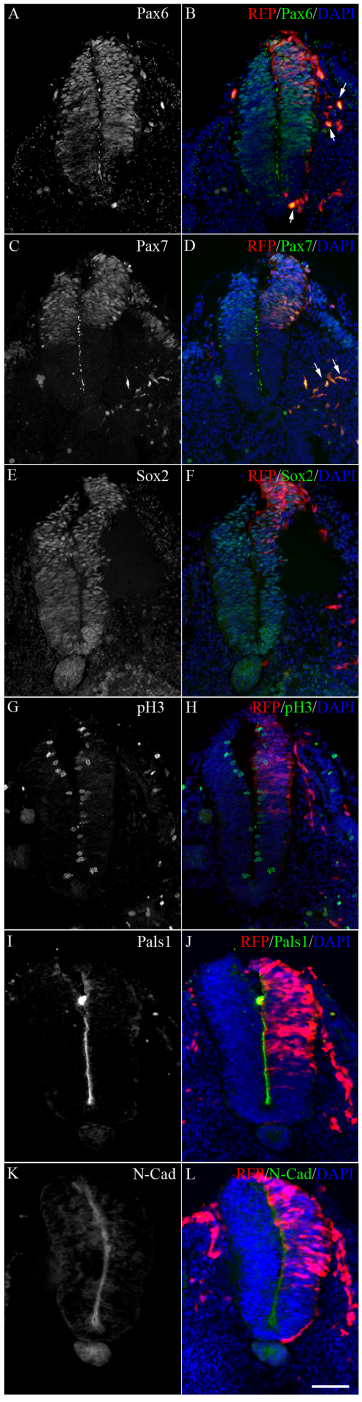


Fig 6.5 **Effect of Crb2S misexpression in the hindbrain of chick embryos.** Transverse sections through the rostral hindbrain of embryos co-electroporated with Crb2S and control RFP expression vector were immunostained for Pax6 (A-B), Pax7 (C-D), Sox2 (E-F), pH3 (G-H), Pals1 (I-J) and N-Cad (K-L). Arrows in B and D indicate Pax6 and Pax7 +ve cells present outside the neural tube. Overlays show RFP in red, antibody staining in green and nuclei counterstained with DAPI in blue.
Scale bar = 50 μm

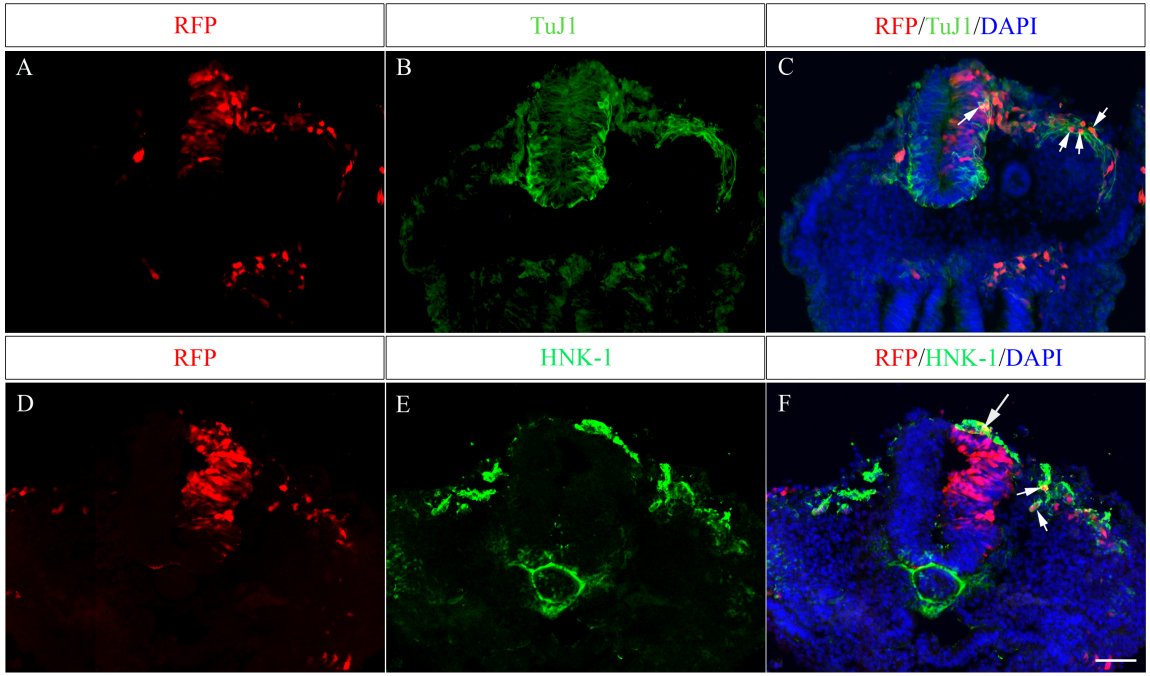


Fig 6.6 **Misexpression of Crb2S in the hindbrain affects neuronal and neural crest cell marker expression.** Transverse sections through the rostral hindbrain of embryos co-electroporated with Crb2S and RFP were immunostained for TuJ1 (A-C) and HNK-1 (D-F) Arrows in C and F indicate co-localization of TuJ+ve and HNK-1+ve cells with RFP expressing cells. Overlays show antibody staining in green, RFP in red and nuclei counterstained with DAPI in blue. Scale bar = 100 µm

Crb2S+RFP^{+ve} cells outside the neural tube also co-localized with HNK-1(Fig 6.6 D-F).

Overall, the data suggests that misexpression of Crb2S leads to a different phenotype from that of Crb2F misexpression. Crb2S misexpression has no obvious effect on polarity protein expression but affects the expression of neural crest markers and the migratory behaviour of cells.

6.2.4 Misexpression of Crb2S in the hindbrain induces migration of neural crest cells *in vitro*

To further examine the role of Crb2S in migration of neural crest cells, I setup an *in vitro* explant culture system where hindbrain segments were explanted and cultured.

The explant system was used to:

1. Study the effect of Crb2S on the number of migrating neural crest cells from hindbrain explants.

2. Test differences between migration patterns of neural crest cells from individual rhombomeres of H&H stage11 chick embryos cultured in the presence or absence of Crb2S.

Rhombomere explants were cultured in OptiMEM media alone (Control) or with purified Crb2S protein (Experimental). After 24 hours in culture, these explants were analysed. I observed that under the minimal serum-free culture conditions, the number of cells migrating from the experimental samples were more than the cells migrating from the control samples (Fig 6.7 A-B).

To identify the population of migrating cells, I carried out immunostaining for neural crest cell markers Slug and HNK-1. A majority of the migrating cells expressed Slug, cells co-expressing Slug and HNK-

1 were also detected (Fig 6.7 C-D). In agreement with the *in ovo* electroporation data, exogenous addition of Crb2S to the hindbrain explants resulted in increased neural crest cell migration.

To quantify the increase in neural crest cell migration following exogenous overexpression of Crb2S, the number of cells that had migrated from each explant were counted and recorded for at least 10 explants from 3 independent experiments. There was a statistically significant increase (unpaired t-test, $p < 0.05$) in neural crest migration in the Crb2S treated explant compared to the control (Fig 6.7E).

To examine if the increased migratory phenotype observed upon Crb2S overexpression is restricted to specific rhombomeres of the chick embryonic hindbrain, individual rhombomeres (Rhombomere1-4) were sub-dissected from H&H stage11 chick embryos and cultured alone or with Crb2S. Twenty-four hours after culture the explants were assayed for neural crest cell migration. The number of migrating cells from each rhombomere explant was counted and recorded. There was a statistically significant increase in the migration of neural crest cells from rhombomeres 2 and 4 (unpaired t-test, $p < 0.05$, $p< 0.005$) when cultured with Crb2S (Fig 6.8 B-B'', D-D''). Although there was an increase in neural crest migration from rhombomeres 1 and 3 in the Crb2S treated explants (Fig 6.8 A-A'', C-C''), the effect was not significant when compared to the control explants (unpaired t-test).

Intriguingly, the increased neural crest cell migration phenotype observed *in vitro* using the purified Crb2S protein could not be replicated by *in ovo* transplantation of Crb2S protein-soaked beads into the hindbrain (Fig 6.9). However, the Crb2S protein-soaked beads had an effect on the expression of dorsal neural progenitor markers in caudal neuropore transplantation experiments (Appendix 1) Taken together, the data suggests that misexpression of Crb2S induces migration of cranial neural crest cells in the chick embryonic hindbrain.

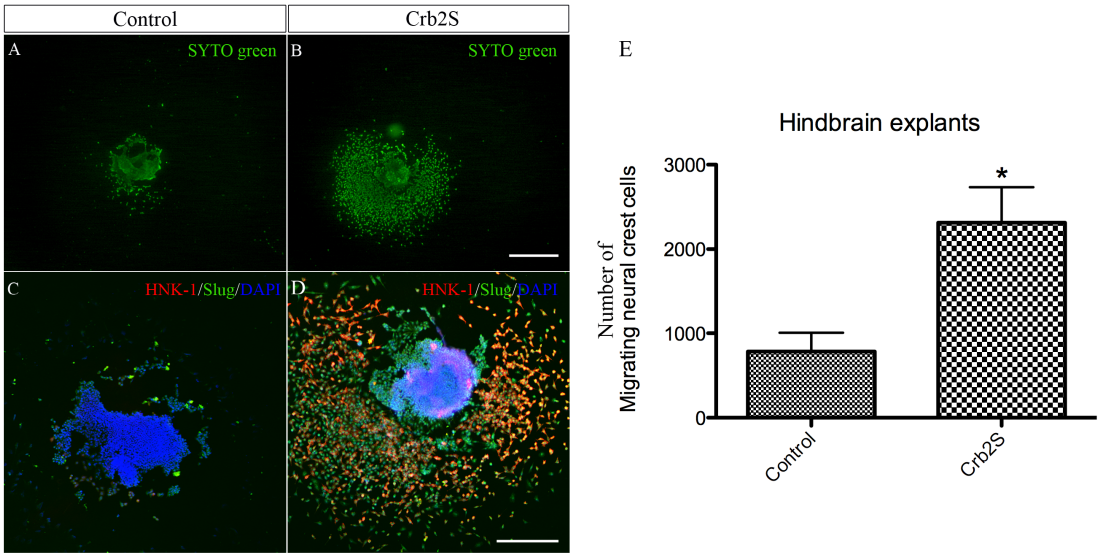
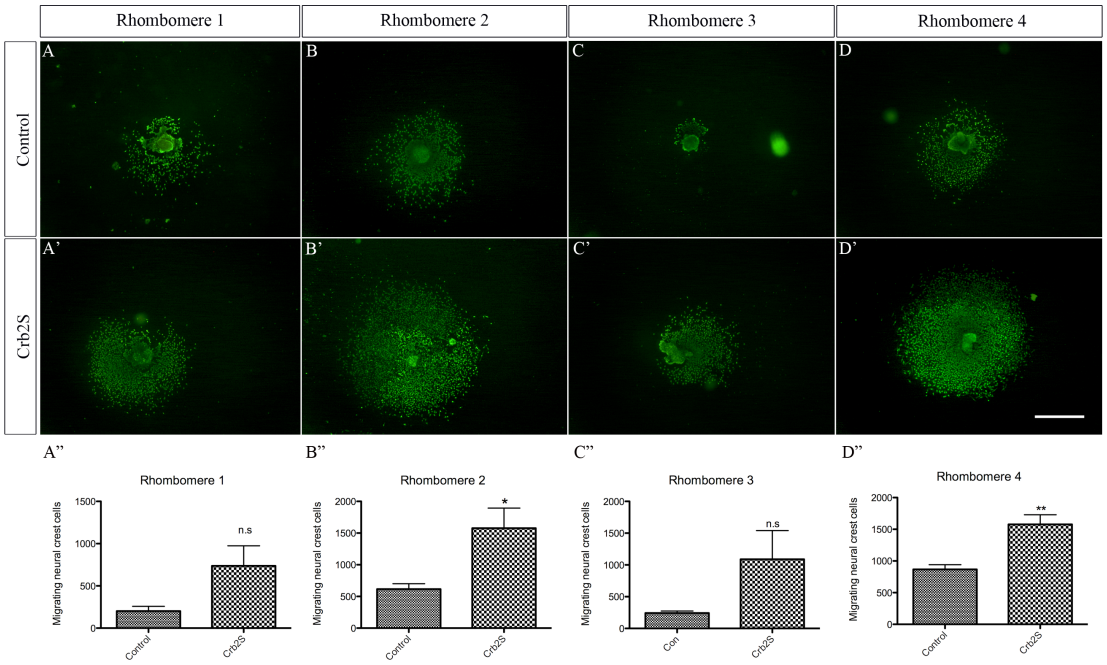


Fig 6.7 **Exogenous addition of purified Crb2S protein induces neural crest cell migration.** Hindbrain explants from H&H stage 11 chick embryos cultured for 24hours on Gelatin+Fibronectin - alone (A, C) or in the presence of Crb2S in serum-free media (B, D). Live cells were imaged using SYTO green fluorescent dye. Explants were fixed and immunostained for HNK-1 (red) and Slug (green). Nuclei counterstained with DAPI shown in blue. E. Numerical graph representing explant data. Cell counting and statistical analysis shows a significant increase in the number of cells migrating from Crb2S treated hindbrain explants when compared to the control explants (*= $p<0.05$, unpaired t-test). Scale bars = 100 μm


Rhombomere 1
Rhombomere 2
Rhombomere 3
Rhombomere 4
Control
Crb2S
A
B
C
D
A'
B'
C'
D'
A''
B''
C''
D''
Rhombomere 1
Rhombomere 2
Rhombomere 3
Rhombomere 4
Migrating neural crest cells
n.s
*
n.s
**
Control
Crb2S

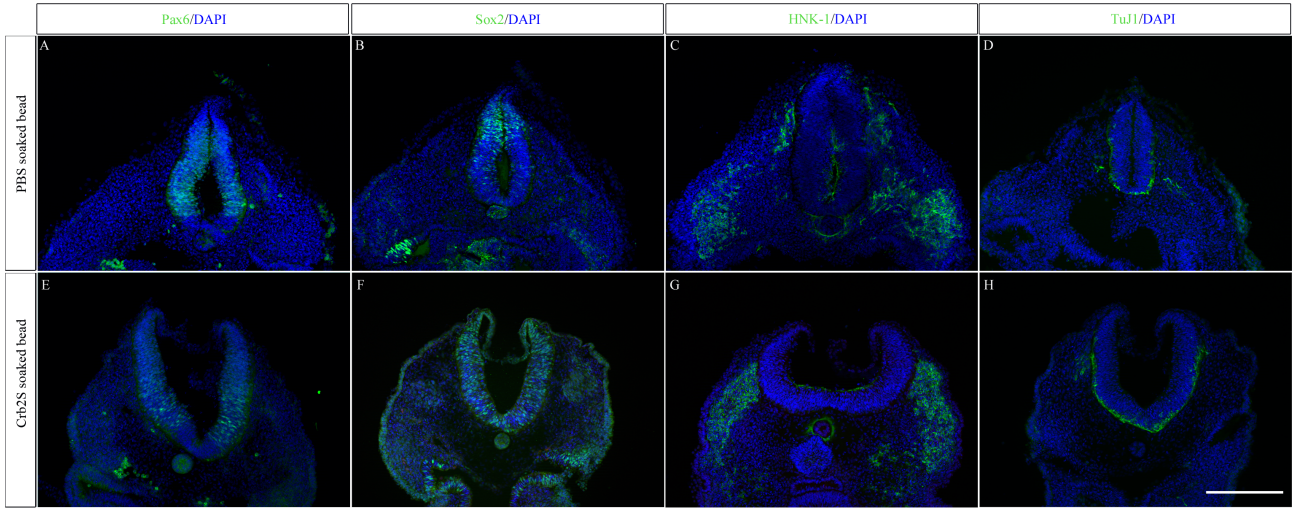


Fig 6.9 **Transplantation of Crb2S protein-soaked beads into the hindbrain** of H&H St11 chick embryos has no overt effect on neural cell types. Transverse sections through the rostral hindbrain of embryos implanted with PBS or Crb2S protein soaked beads immunostained after 24 hours culture in ovo, for Pax6 (A, E), Sox2 (B, F), HNK-1 (C, G) and TuJ1 (D, H). Overlays show antibody staining in green and nuclei counterstained with DAPI in blue. Scale bar = 100 μm

The effects of manipulating Crb2 isoforms in the chick embryonic hindbrain are summarized in Table 6.1. Together the data implies that altering the expression of Crb2 isoforms in the chick embryonic hindbrain as early as H&H stage10 has a significant effect on localization of mitotic cells, neural differentiation and neural crest migration.

Table 6.1 Summary of the phenotypes observed after Crb2 misexpression in the chick embryonic hindbrain.

24 hours post- electroporation	**Misexpression of Crb2 isoforms**	
	Crb2F	**Crb2S**
Altered neural tube morphology	√	X
Changes in apical expression of adherens junction proteins and polarity proteins	√	X
Mislocalized mitotic cells	√	X
Mislocalized TuJ1 $^{+ve}$ neuronal cells	√	√
Increased migration of neural crest cells	X	√

6.3 Discussion

In this chapter, I have shown that misexpressing two different isoforms of Crb2, Crb2F (full length) and Crb2S (secreted) in the chick embryonic hindbrain results in distinct phenotypes.

Crb2F misexpression results in an alteration in neural tube morphology. On the basis of morphology, it appears that misexpression of Crb2F induces hyperplasia within the neural tube. However, this needs to be confirmed with BrdU cell proliferation assays which I would have

performed had time allowed. Alternatively, it is possible that misexpression of Crb2F affects cell-cell adhesion and the cells are reorganized and reshaped within the neural tube. Consistent with this latter possibility, the expression of cell junction components like N-Cadherin and β-Catenin are altered in the Crb2F+RFP expressing regions and TuJ1 $^{+ve}$ cells are mislocalized. In addition to this, apical cell polarity components are also affected upon Crb2F misexpression.

Several lines of evidence suggest that Crb2 may regulate both cell cycle dynamics and cell-cell adhesion (Ohata et al., 2011; Omori & Malicki, 2006a). In zebrafish, Crumbs genes have been implicated in defining the apical domain of neural tube epithelia and in restricting mitosis to the apical surface (Jensen et al., 2001; Malicki & Driever, 1999; Ohata et al., 2011; Zou et al., 2008). Recently, it has been shown that zebrafish Crumbs proteins directly bind to the extracellular domain of Notch. This binding inhibits Notch activity and the Crumbs-Notch pathway is important for the maintenance of apical- basal polarity and also for restricting mitosis to the apical cell surface (Ohata et al., 2011). In line with the role of Crumbs genes in restricting mitosis to the apical surface, misexpression of Crb2 leads to ectopically localized pH3 positive mitotic cells away from the apical surface.

As discussed in previous chapters, polarity proteins and cell junction components play a role in cell fate determination. A profound disorganization of neuronal architecture was previously reported in zebrafish Crumbs loss of function mutants (Omori & Malicki, 2006a). The data from this chapter shows that misexpression of Crb2 in the chick embryonic hindbrain resulted in abnormal localization of TuJ1 positive neurons. Although Crb2F misexpressing cells in the neural tube co-localize with neural progenitor markers like Sox2 and Pax6 there was no co-localization with TuJ1. It is possible that the defects observed in polarity of neuroepithelial cells affect the migration of neurons in a cell or non-cell autonomous manner. These studies were carried out by co-

electroporating the Crb2 vectors with a control RFP expression vector. It was therefore not possible to determine from these studies whether the observed effects were cell-autonomous or not. Unfortunately, bidirectional expression constructs expressing Crb2 isoforms and the fluorescent reporter protein worked efficiently in mammalian cell lines but despite several attempts, I could not observe fluorescent protein expression in the chick system (data not shown).

As described in Chapter 1 (section 1.2.2), asymmetric versus symmetric cell divisions also determine cell fate. The expansion of apical components upon Crb2 misexpression and their differential inheritance may contribute to mislocalized mitosis and increased proliferation of neural progenitors by influencing the balance of symmetric versus asymmetric divisions. Crb2 may regulate this through control of the Notch signaling pathway. Further experiments are required to investigate the role of Crb2 and Notch in the developing chick hindbrain. In addition to this, it will be interesting to investigate if there is an expansion of the basolateral components in the Crb2F+RFP expression region. It has been reported that loss of Lgl – a basolateral protein causes hyperproliferation in the embryonic mouse brain (Klezovitch et al., 2002). The currently accepted model is that the apical and basal complexes mutually antagonize each other to define the respective cell boundaries (Bilder, 2004; Margolis & Borg, 2005). Consistent with this model, the phenotype observed after loss of a basolateral protein is similar to the phenotype observed after overexpression of an apical complex member.

In contrast to the phenotype observed after misexpression of Crb2F, Crb2S misexpression has no apparent effect on localization of cell junction or cell polarity markers. Surprisingly, however, Crb2S affects the migration of neural crest cells. In the explant system, Crb2S had a significant effect only on neural crest migration from the even numbered rhombomeres but not on the odd numbered rhombomeres. This observation is consistent with the endogenous specification of neural

crest cells where even-numbered rhombomeres generate more neural crest than the odd-numbered rhombomeres. It will be interesting to investigate if the endogenous expression of Crb2S in the hindbrain is rhombomere-specific and if this in turn is responsible for the rhombomere-specific effects of Crb2S. Additionally, it is crucial to validate the dissection of the individual rhombomeres used in the *in vitro* explant culture experiments. This could be addressed by analyzing the dissected rhombomeres for the expression of rhombomere-specific markers like Ephrins or Krox20 (Chapter 1 section 1.3, Fig 1.8).

The observed increase in neural crest migration after exogenous addition of Crb2S could be due to an alteration in 1. cell behaviour (premature or increased number of migrating cells) 2. proliferation of neural crest cells 3. cell fate (favour cells to become neural crest at the expense of other cell populations). These possibilities are not necessarily mutually exclusive to each other.

In Xenopus, a secreted protein **X**enopus **E**GF-like **r**epeat with **l**aminin-G protein- Xerl was identified (Kuriyama et al., 2000). This novel CNS secretory protein demonstrated an expression profile that was similar to Crumbs, with predominantly high expression in the eye and brain of Xenopus embryo. Xerl and Crb2 have a high sequence homology (Kuriyama, Miyatani, & Kinoshita, 2000). According to Xenbase (http://ftp.xenbase.org) and Ensembl (http://www.ensembl.org) Xerl is the *Xenopus* homolog of Crb2. It was shown that this novel secretory protein is crucial for establishing the boundary between the neural plate and neural crest in *Xenopus* embryo and it excludes neural crest differentiation from the neural plate region (Kuriyama, Ueda, & Kinoshita, 2003). This suggests that Crb2S isoform may be the vertebrate homolog of Xerl and that it might also play a role in defining the neural plate and neural crest boundary during neural development in vertebrates.

In both *in ovo* electroporation and *in vitro* explant culture systems, misexpression or exogenous addition of Crb2S resulted in increased

HNK-1 positive migratory neural crest cells. Surprisingly, no obvious increase in the pre-migratory neural crest cell marker, Slug was observed in the Crb2S misexpressing embryos. The migration of neural crest cells is a dynamic process and in the Crb2S misexpressing embryos the expansion of the pre-migratory neural crest domain could have occurred at a time point earlier than 24 hours post-electroporation. *In ovo* transplantation of Crb2S protein soaked beads into the hindbrain did not have any apparent effect on neural crest migration. The Crb2S bead soaked assays were successfully used in chick embryonic spinal cord transplantation experiments (Appendix 1). It is possible that the concentration of Crb2S required for eliciting a neural crest migration response needs to be optimized for the hindbrain transplantation experiments.

Overall, both Crb2F and Crb2S play context dependent roles in the chick embryonic hindbrain. Deregulation of the balance between these isoforms may have major implications for development of the nervous system.

CHAPTER 7

Analysis of Pals1 conditional knockdown mouse embryos

7.1 Introduction

sh (short-hairpin) RNA mediated knockdown is an alternative to the conventional gene knockout approaches. The Cre/loxP system is used to activate RNA in a temporal and tissue-specific manner. The knockdown system is ideal to study intermediate phenotypes where there is a 70-80% reduction in expression of a particular gene (Kunath et al., 2003). So, using shRNA knockdown over conventional knockout mice is more beneficial to study intermediate phenotypes caused by a particular gene. Additionally, a knockdown model could be a closer representation of a disease caused by point mutations in a specific gene (Kleinhammer et al., 2011).

Recent work has shown that Pals1, an intracellular binding partner of Crumbs 2 is essential for cell survival and loss of Pals1 leads to premature cell cycle exit and precocious neural differentiation (Kim et al., 2010). In this study, Pals1 was deleted from the dorsal telencephalon using Emx-1 Cre and Pals1 protein expression was undetectable by E11.5. Interestingly, the heterozygous Pals1 mutants showed an intermediate phenotype compared to the homozygous mutants suggestive of a dosage-sensitive effect of Pals1 during neural development.

Despite the complete loss of Pals1 in the Crb2 cKO model (chapter 4) the massive cell death phenotype reported in the Pals1 cKO was not observed. To investigate the underlying reasons for this disparity, I analysed *shPals1* conditional knockdown mice (cKD - details in next section). *shPals1* transgenic offspring were crossed to Nestin-Cre transgenic mice to activate the shRNA vector and obtain *shPals1* cKD. An important distinction between Nestin-Cre and Emx-1 Cre, apart from their expression domains is the timing of recombination produced by Cre. Using reporter lines it has been shown that efficient recombination is evident at E10.5 in Emx-1 Cre and at E11.5 in Nestin-Cre transgenic mice (Chou et al., 2009).

7.2 Results

To study the potential effect of depleting Pals1 levels, transgenic *shPals1* mice were generated by our Dutch collaborators (Bokyung Park working in the laboratory of Jan Wijnholds) using previously published *shPals1* sequences (Kim et al., 2010; van Rossum et al., 2006). Recently, these *shPals1* mice were crossed to different Cre lines expressing Cre in retinal progenitor cells. It was reported that reduced Pals1 levels led to retinal disorganization and degeneration (Park et al., 2011). To restrict Cre mediated recombination mainly to the developing nervous system, *shPals1* mice were crossed with Nestin-Cre transgenic mice (Dubois, Hofmann, Kaloulis, Bishop, & Trumpp, 2006). I performed a preliminary analysis of the *shPals1*; Nestin-Cre conditional knockdown mouse embryos. The experiments described in this chapter were carried out at two embryonic stages E12.5 and E14.5.

7.2.1 Apical enrichment of Pals1 is reduced in the cortex of *shPals1*; Nestin-Cre embryos

Pals1 protein is apically enriched in the dorsal telencephalon of wild type and Nestin- Cre control embryos at E12.5 and E14.5 (Fig 7.1 A, B, D, E). In contrast, the *shPals1*; Nestin-Cre embryos show a reduction in the apical enrichment of Pals1 protein at both these stages (Fig 7.1 C, F). This indicates that the hairpin construct has successfully depleted endogenous Pals1 protein levels.

7.2.2 Apical localization of cell-junction associated proteins is unaffected in the *shPals1*; Nestin-Cre cortex.

To determine if depletion of apical Pals1 protein influences recruitment of cell-junction associated proteins I analysed the Nestin-Cre control and *shPals1*; Nestin-Cre embryos for alterations in marker expression.

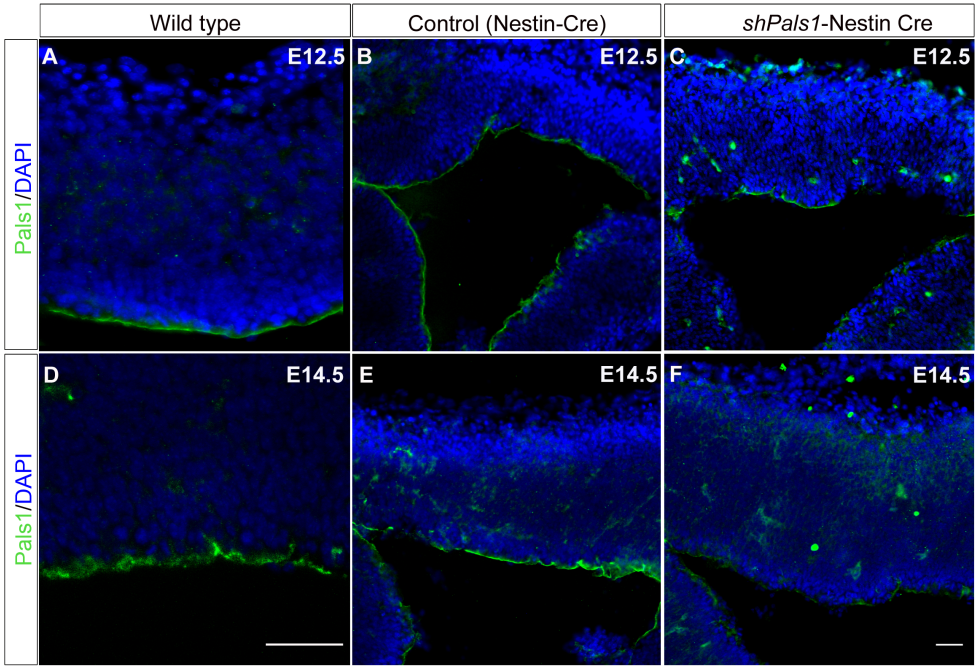


Fig 7.1 **Expression of Pals1 in the dorsal telencephalon of wild type, control and *shPals*1-Nestin Cre mouse embryos**

Coronal sections through the telencephalon of E12.5 (A-C) and E14.5 mouse embryos (D-F) immunostained for Pals1. When compared to the wild type (A, D) and control embryos (B, E) conditional knockdown of Pals1 results in reduced apical Pals1 expression (C, F). Antibody staining shown in green and nuclei counterstained with DAPI shown in blue. Scale bars - A,D= 50 μm B,C,E,F= 50 μm

At E12.5, the apical enrichment of Par3, ß-Catenin and ZO-1 is unaltered in the *shPals1*; Nestin-Cre cortex compared to the Nestin-Cre control embryos (Fig 7.2). ß-Catenin staining observed in the upper layers of the cortex (Fig 7.2 K) is non-specific as it is also observed in sections stained with secondary antibody alone (data not shown). This suggests that the reduced level of Pals1 is insufficient to disrupt cell polarity and cell junctions in the telencephalon at these stages.

7.2.3 Pals1 levels are critical for maintaining the neural progenitor pool in the developing dorsal telencephalon.

To determine if depletion of Pals1 affects cortical neurogenesis, I analysed control and *shPals1*-Nestin Cre littermates for alterations in markers of neural progenitors (Sox2, Nestin), intermediate progenitors (Tbr2) and early born neurons (TuJ1). At E14.5, there is a marked reduction of Sox2 positive neural progenitors in the *shPals1*; Nestin-Cre cortex (Fig 7.3 C-D) compared to the controls (Fig7.3 A-B). This is concomitant with an increase in the Tbr2 positive intermediate progenitors in the *shPals1*; Nestin-Cre cortex (Fig 7.3 K-L, arrows in L) compared to the littermate controls (Fig 7.3 I-J). In contrast, no obvious difference was observed in the expression profiles of Nestin positive radial glial cells that span the entire wall of the cortex (Fig 7.3 E-H) or TuJ1 positive neurons (Fig 7.3 M-P) at the same stage.

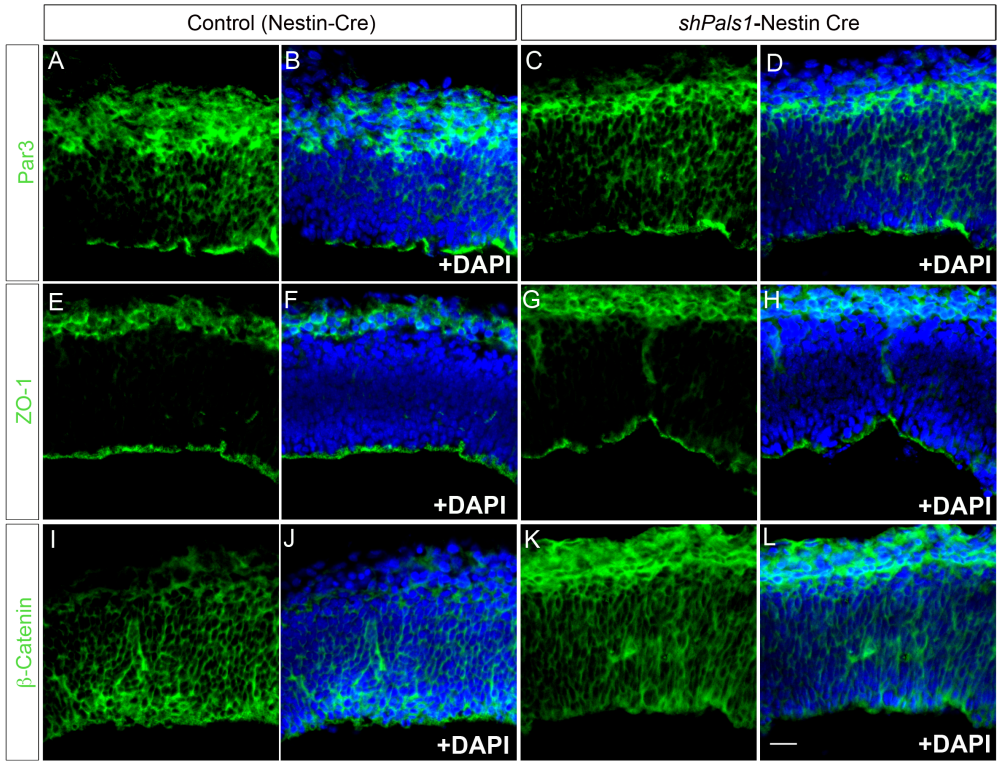


Fig 7.2 **Reduced apical Pals1 expression does not affect apical localization of cell junction-associated proteins**. Coronal sections through the telencephalon of E12.5 control and *shPals1*-Nestin Cre mouse embryos immunostained as indicated. Par3 expression is enriched at the apical surface and pial surface in both the control (A-B) and cKD (C-D) cortex. ZO-1 is apically enriched in the control. The expression of ß-Catenin is not significantly different between the control (I-J) and cKD (K-L) cortex. Overlays show antibody staining in green and nuclei counterstained with DAPI in blue. Scale bar = 50 μm

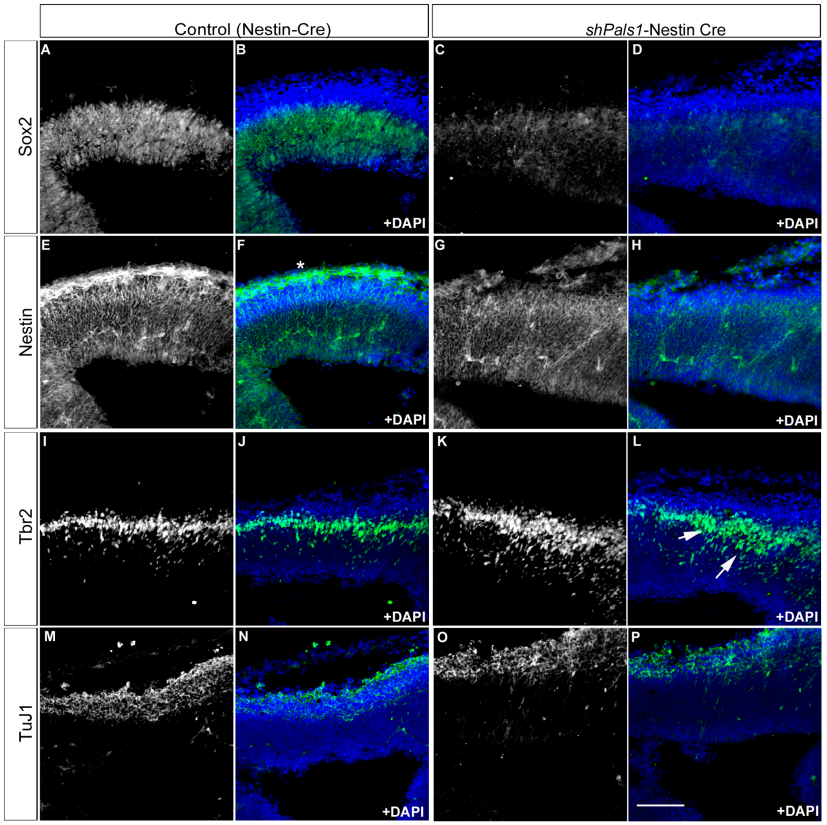


Fig 7.3 **Conditional knockdown of Pals1 affects the neural progenitor cells.** Coronal sections through the telencephalon of E14.5 control and *shPals1*-Nestin Cre mouse embryos immunostained as indicated. Sox2 is expressed in the VZ and SVZ in the control embryos (A-B). In the Pals1 cKD embryos, Sox2 is significantly reduced (C-D). Nestin is detected spanning the apical-basal surface of the cortex in the control (E-F) and cKD (G-H). In the control, Tbr2 +ve intermediate progenitors are detected predominantly in the SVZ. A few Tbr2 +ve progenitors are also present in the lower SVZ (I-J). In the cKD cortex, there is an increase in the Tbr2 +ve progenitor domain (K-L, arrows in L). β-Tubulin III positive neurons are mainly detected at the pial surface in the control and cKD cortex. Overlays show antibody staining in green and nuclei counterstained with DAPI in blue.
VZ- ventricular zone SVZ- sub-ventricular zone * -indicates non-specific staining
Scale bar = 50 μm.

7.2.4 Pals1 protein levels influence positioning of mitotic cells in the developing dorsal telencephalon

I analysed the control and *shPals1*-Nestin Cre embryos for the expression of mitotic cell marker- phosphorylated histone H3 (pH3). In the control littermates, $pH3^{+ve}$ mitotic cells are predominantly apical at E12.5 (Fig 7.4 A) and E14.5, with a few mitotic cells localized at the sub-ventricular zone at E14.5 (Fig 7.4 E). In contrast, in the E12.5 and E14.5 *shPals1*; Nestin-Cre telencephalon, there is a reduction of apical pH3 positive cells and an increase in ectopically localized pH3 positive cells (Fig 7.4 B, F, arrows in B and F). Ki67 marks all proliferating cells throughout the control cortex (Fig 7.4 C), however Ki67 is reduced in the apical cells of the *shPals1*; Nestin-Cre cortex (Fig 7.4 D) at E12.5.

Combined, the data suggests that Pals1 protein levels are critical for the regulation of cell proliferation in the cortex and that depletion of Pals1 causes a premature switch of cell fate from Sox2 positive to Tbr2 positive cells.

7.3 Discussion

The results show that apical enrichment of Pals1 is reduced in the cortex of *shPals1*-NestinCre embryos. Surprisingly, there is no alteration in expression of apical polarity proteins and junctional proteins in the *shPals1*; Nestin-Cre cortex. However, it has been reported that complete removal of Pals1 from the developing cortex affects localization of apical polarity and adherens junction proteins (Kim et al., 2010). This implies that the levels of Pals1 were not depleted sufficiently to disrupt recruitment of other apical components. Despite this, I do observe mislocalization of pH3 positive cells, depletion of apical Ki67 positive cells and alterations in neural differentiation markers in the *shPals1*-NestinCre telencephalon.

This data shows both similarities and some significant differences from a recent paper that used an Emx1-Cre conditional knockout strategy

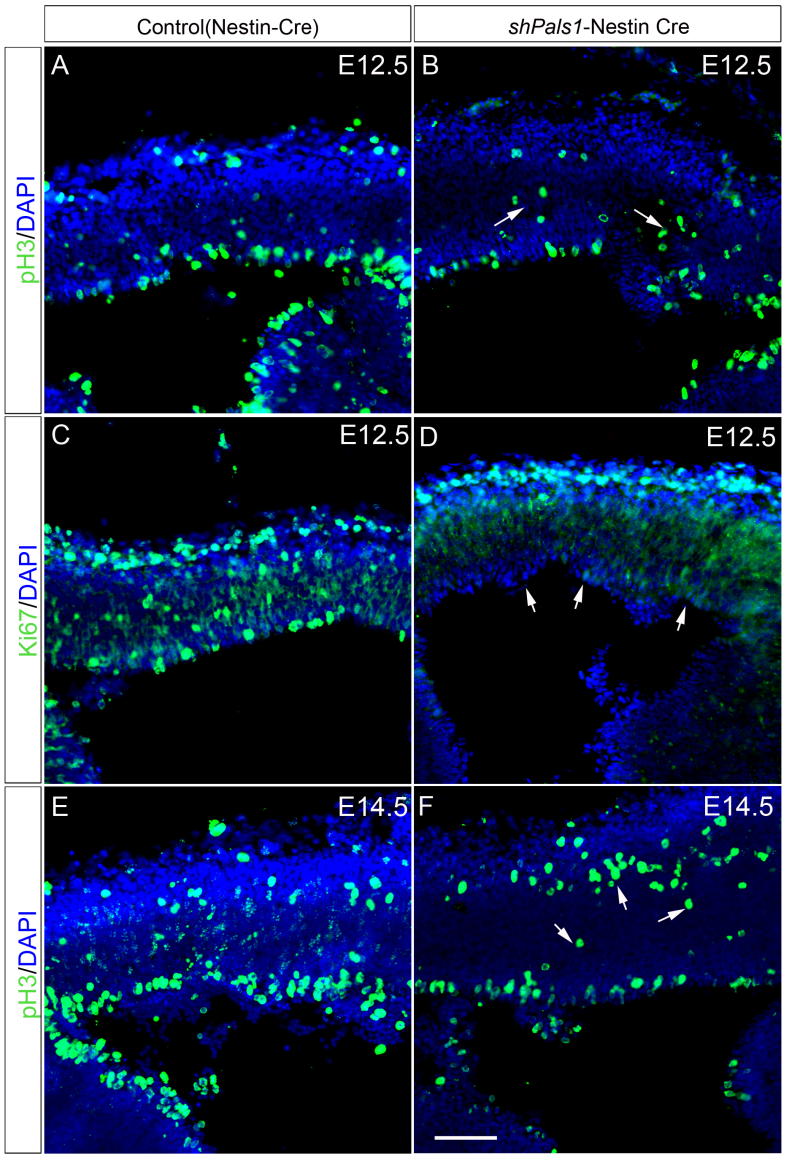


Fig 7.4 **Depletion of Pals1 protein affects the localization of mitotic cells.** Coronal sections through the telencephalon of control and *shPals1*-Nestin Cre mouse embryos immunostained for pH3, a marker for mitotic cells (A-B, E-F) and for Ki67 (C, D) a cell proliferation marker. Arrows in B and F show mislocalized pH3+ve cells in the *shPals1*-Nestin Cre embryos at E12.5 and E14.5. Note the decrease in Ki67+ve proliferating cells in the *shPals1*-Nestin Cre compared to the control (C, D, arrows in D). Overlays show antibody staining in green and nuclei counterstained with DAPI in blue. Scale bar= 50 μm

to remove Pals1 completely in the developing dorsal telencephalon (Kim et al., 2010). Both studies show decreased proliferation, however I also see an increase in mislocalized mitotic cells in the *shPals1*; Nestin-Cre cortex that was not observed in the Pals1-/- Emx1-Cre telencephalon. Moreover, in the *shPals1*; Nestin-Cre telencephalon there is a decrease in Sox2 positive cells and an increase in Tbr2 positive cells. In contrast, the Pals1-/- Emx1Cre study describes a decrease in Tbr2 positive cells (Kim et al., 2010). There are several reasons that could explain this apparent disparity. Firstly, it is possible that Pals1 plays diverse roles at different stages of neurogenesis and that the two different promoters driving Cre expression have revealed this role. Alternatively, there may be different dose-sensitive functions for Pals1 in cortical development: complete loss of Pals1 results in a decrease of Tbr2 positive cells whilst a reduced level of Pals1 in my study causes an increase in Tbr2 positive cells.

Cdc42 regulates neural progenitor cell fate in the developing mouse brain (Cappello et al., 2006; Chen et al., 2006) and Cdc42 deficiency causes a decrease in Pax6 positive cells and an increase in Tbr2 positive cells in the developing cortex (Cappello et al., 2006). It is plausible that Pals1 controls the switch from Sox2 positive to Tbr2 positive cells via Cdc42, as there is a biochemical and functional link between the Crb-Pals1-PATJ and Par3-Par6-Cdc42 complexes (Hurd et al., 2003). If time had allowed, I would have tested this hypothesis by immunostaining for Cdc42 in the Pals1 conditional knockdown cortex and further investigated if the Pals1 conditional knockdown phenotype mimics the Cdc42 mutant phenotype. In addition to this, it will be useful to determine if there is a direct biochemical interaction between Pals1 and Cdc42 in the telencephalon.

CHAPTER 8

General discussion

8. General discussion

In this chapter, I will briefly summarize the key findings of my work and try to put them in context with previously known roles for apical cell polarity proteins in neurogenesis. Additionally, I will suggest further experiments that have not been performed in this thesis but may help identify additional roles for Crb2 during neural development.

8.1 Overview of main results:

Most of our current knowledge about the role of the Crumbs family is from studies in *Drosophila* or zebrafish (Assemat et al., 2008; Ohata et al., 2011; Omori & Malicki, 2006b). The identification of significant roles for Crb1 in retinal development opened up a new field of investigation into the role of Crumbs genes (den Hollander et al., 1999; den Hollander et al., 2001; den Hollander et al., 2004; van de Pavert et al., 2004). More recently, Crumbs has been associated with growth control and Crumbs proteins are emerging as potential tumour suppressors (Laprise, 2011). However, Crb2 function has, until now, not been examined in mammalian neural development *in vivo*.

In my thesis, by analysing the effect of Crb2 deletion (mouse embryo) and Crb2 misexpression (chick embryo) during different developmental stages, I have shown that Crb2, a vertebrate homolog of *Drosophila* Crumbs is

- expressed at the apical surface of neural progenitors in the developing telencephalon of mouse embryos and in the chick embryonic hindbrain.
- Conditional removal of Crb2 from the dorsal telencephalon leads to defects in cortical neurogenesis.
- Misexpression of Crb2 affects neural tube morphology.
- A truncated isoform of Crb2 is secreted *in vitro.*
- This secreted isoform has potential implications for regulating neural crest cell migration.

In addition to this, I have also shown that the levels of Pals1, an intracellular binding partner of Crb2 is important in cell cycle control and cortical cell fate specification.

8.2 Crb2 and cortical neurogenesis

As shown in Chapter 3, Crb2 protein expression is specifically enriched at the apical surface of neural progenitors in the developing telencephalon. The asymmetric segregation of several cell-junction components and cell polarity proteins has been shown to affect proliferation of progenitors and/or neuronal specification (Bultje et al., 2009; Costa et al., 2008; Marthiens & ffrench-Constant, 2009; Neumuller & Knoblich, 2009).

Here, to determine if the asymmetric distribution of Crb2 in cortical progenitors affects cell fate specification, I analysed the telencephalon of Crb2 conditional knockout (cKO) embryos. A schematic illustration of cortical neurogenesis in the wild type versus Crb2 knockout situation is shown in Fig 8.1 A-B.

Depletion of Crb2 from apical progenitors affects the recruitment of proteins to the apical compartment. As development progresses, in the Crb2 cKO cortex there is a premature shift in cell fate from ventricular zone progenitors to sub-ventricular zone progenitors/neurons. At later stages, the well-defined stratification of the cortex is disrupted and post-mitotic neuronal cells are aberrantly positioned. In addition to this, mitotic cell divisions are not confined to the apical surface but are more randomly localized.

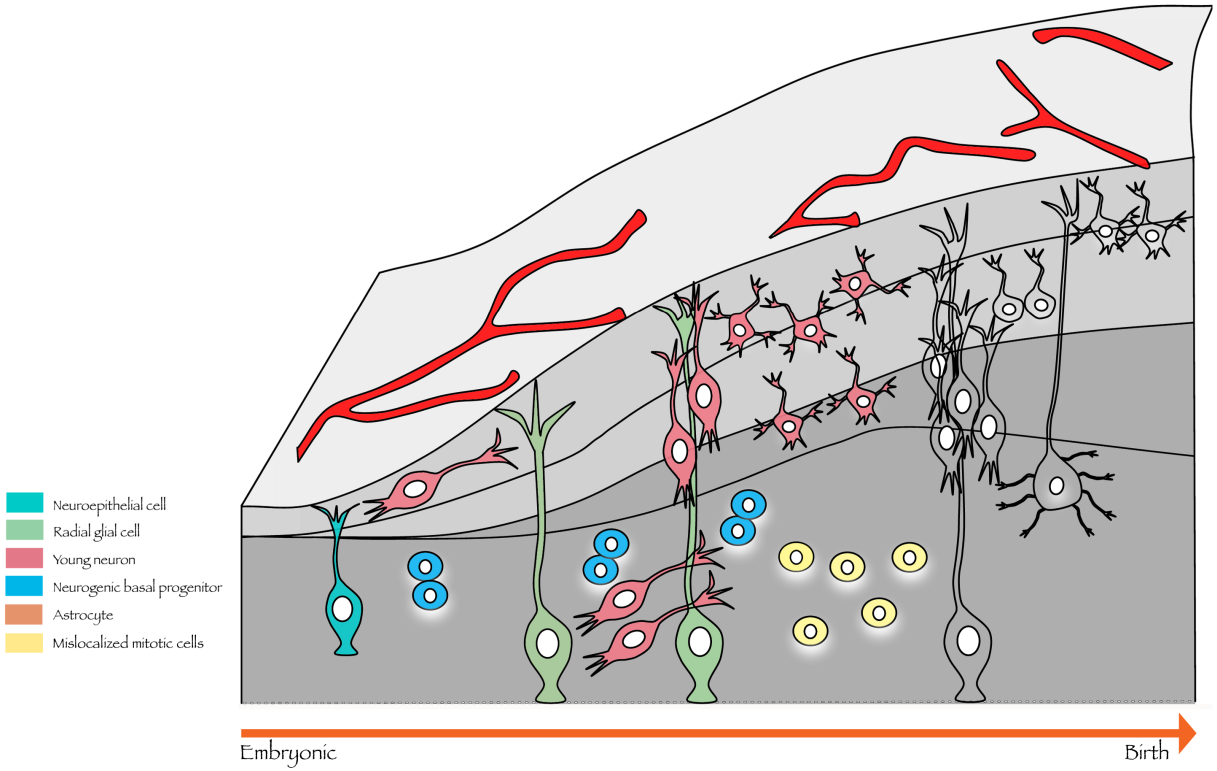


Fig 8.1 B Schematic illustration of the phenotype observed in Crb2 cKO cortex

Based on the effect of Crb2 depletion on apical cell polarity proteins, cell junction proteins, mitotic cell divisions, neural progenitors and post-mitotic neurons, it is reasonable to propose that Crb2 is a crucial regulator of neurogenesis in the developing telencephalon.

However, given that Crb2 protein may have pleiotropic functions, the roles of Crb2 during neural development in the conditional knockout system need to be interpreted carefully.

Previous studies have shown that apical and basal polarity proteins play crucial roles during neural development in vertebrates:

1. Members of the Par complex Par3 and Par6 localize apically and promote proliferative progenitor divisions (Bultje et al., 2009; Costa et al., 2008).

2. The Rho GTPase Cdc42, associated with the Par complex is important for neurogenesis. Neural progenitors deficient in Cdc42 undergo a shift in cell fate towards intermediate progenitor cell types (Cappello et al., 2006).

3. Apically localized aPKCζ in the chick embryo regulates neural stem cell proliferation and also plays a role in the overall stratification of cells within the embryonic neural tube (Ghosh et al., 2008).

4. MALS3 (Lin7c) a member of the Crumbs complex has been reported to maintain apical-basal polarity in neural progenitors. In the developing murine CNS depletion of MALS3 leads to slower cycling rates of progenitors followed by an increase in cell cycle exit and neuronal differentiation (Srinivasan et al., 2008).

5. Recently, Pals1 a member of the Crumbs complex has been implicated in control of cell fate and cell survival during cortical development (Kim et al., 2010).

6. Loss of Lgl1, member of the basal Scribble complex, during neural development leads to hyperproliferation and formation of rosette-like structures reminiscent of neuroectodermal tumours (Klezovitch et al., 2002).

Given these roles for polarity proteins during neural development, it is highly plausible that the switch in cell fate to generate neurons instead of maintaining a progenitor-state in the Crb2 cKO cortex may stem from the disruption of the apical domain. The absence of apical components may lead to inadequate tethering of the cells to the ventricular zone, thereby, exposing these cells to different extrinsic cues and subsequently affecting their fate.

It has been reported that intact cell junctions are a fundamental prerequisite for normal neural progenitor cell proliferation in *Drosophila* (Lu et al., 2001). However, the presence of intact apical junctions is not an absolute requirement in regulation of vertebrate neurogenesis. For instance, conditional knockout of aPKCλ disrupted adherens junctions yet failed to have an impact on neurogenesis (Imai, 2006). Conversely, in MALS triple knockout mutant embryos, adherens junctions were unaffected but significant defects in proliferation of neural progenitors were observed (Srinivasan et al., 2008). Taken together, this suggests that the Crb2 cKO phenotype cannot be solely attributed to loss of apical junctional components.

8.3 Role of Crb2 in chick embryonic hindbrain

Misexpressing two different isoforms of Crb2, Crb2F (full length) and Crb2S (secreted) in the chick embryonic hindbrain results in distinct phenotypes.

Crb2F misexpression results in a remarkable alteration in the morphology of neural tube. A schematic illustration of the morphology observed after Crb2F misexpression is shown in Fig 8.2. Within this region of altered morphology, the apical localization of cell junction and

polarity proteins is affected. Crb2F misexpressing neural tube cells appear to be reshaped and rearranged within the neural tube. If time had allowed, I would have performed three-dimensional reconstruction of sections and also have carried out cell proliferation/cell survival assays in the Crb2F misexpressing embryos.

In *Drosophila*, Crumbs has been associated with the Hippo pathway for regulation of tissue size. Both loss and overexpression of Crumbs resulted in overgrowth, hyperproliferation and induction of Hippo target genes (Chen et al., 2010). It will be interesting to investigate if Hippo signalling is altered after misexpression of Crb2F in the chick embryonic system.

Although no overt difference in neural progenitor marker expression was observed upon Crb2F misexpression, post-mitotic neurons were aberrantly positioned outside the neural tube. It is possible that the defects observed in polarity of neuroepithelial cells in turn affect the migration and positioning of neuronal cells. Overall, both loss of Crb2 and misexpression of Crb2 affect the localization of apical junctional components. As mentioned in the previous section, the apical cell junctional components are important determinants of cell fate. Crb2F may alter neural tube morphology due to defects in apical-basal polarity and/or regulation of cell fate decisions by interacting with signalling pathways.

In contrast to Crb2F, Crb2S does not affect localization of apical cell polarity components and results in a distinct phenotype from that of Crb2F. This also clearly demonstrates that the phenotype observed in Crb2F misexpressing embryos is not an artifact of electroporation.

Intriguingly, Crb2S misexpression affects neural crest cells. Neural crest cells contribute to the neural and non-neural cell types of the peripheral nervous system (Le Douarin & Dupin, 1993). Xerl, a novel secreted protein having high sequence homology to *Drosophila* Crumbs was identified in Xenopus (Kuriyama et al., 2000). It was proposed that

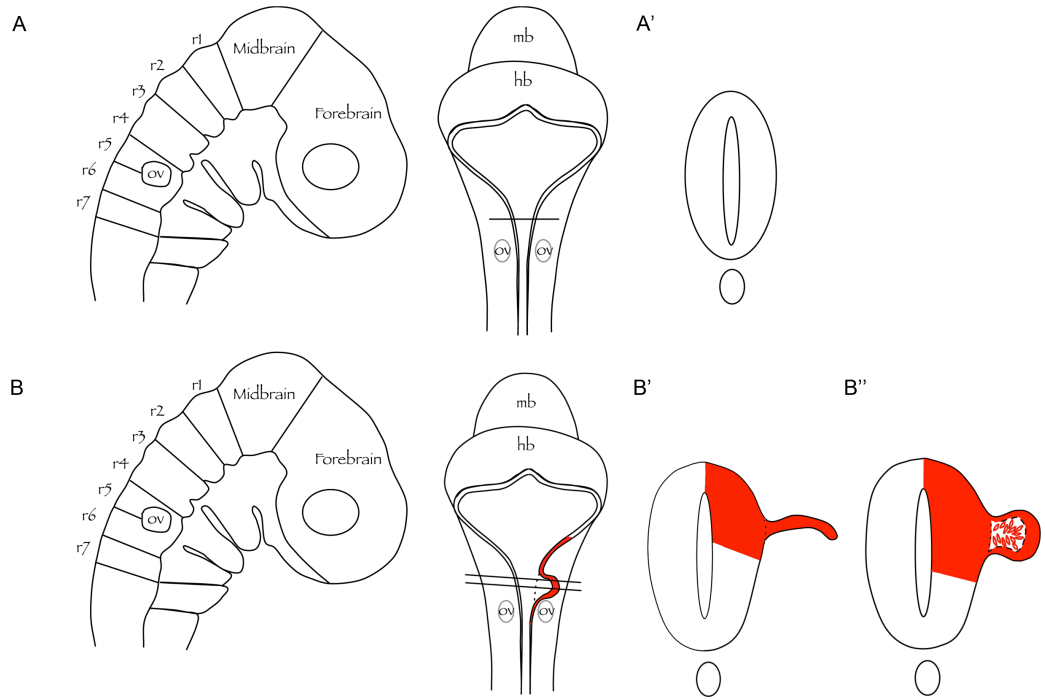


Fig 8.2 **Schematic illustration of the phenotype observed after Crb2F misexpression** in the chick embryonic hindbrain. A. Lateral and dorsal views of an unelectroporated chick embryo. A' Transverse section through the hindbrain region. B. Lateral and dorsal view of an embryo electroporated with Crb2F and a control RFP expression vector. B' and B" Transverse sections through the hindbrain region. RFP is shown in red.

Xerl establishes the boundary between neural plate and neural crest (Kuriyama & Kinoshita, 2001; Kuriyama et al., 2003). It is plausible that Crb2S may play a role similar to that of Xerl in the developing chick embryo to influence the neural crest cell population.

Crb2S misexpression both *in vitro* and *in vivo* leads to increased migration of neural crest cells. Even though an increased migration of neural crest cells after Crb2S misexpression (*in vitro)* is observed in all rhombomeres, the effect is significant only in even-numbered rhombomeres. It has been previously reported that the odd-rhombomeres generate fewer neural crest cells (Birgbauer et al., 1995; Farlie et al., 1999) and that the even-rhombomeres control the apoptosis of neural crest cells migrating from odd-numbered rhombomeres (Graham et al., 1993). It is plausible that Crb2S only influences migration in cells already committed to a neural crest cell fate.

The identification of a role for Crb2S in neural crest migration indicates that Crb2 affects not only development of central nervous system but also that of the peripheral nervous system. However, it is crucial to analyze expression of endogenous Crb2S in the chick embryonic hindbrain.

8.4 Crumbs and Notch signalling

One key question that needs to be addressed is how Crb2 links signalling events during neurogenesis to regulate neuronal output? Given that Crumbs is the only known apical polarity protein to have an extracellular domain it is tempting to speculate that it is an ideal candidate for the transduction of signals originating at the luminal surface to the neural progenitor cells.

The Notch signalling cascade is essential for maintenance of progenitor pools and in the control of neurogenesis in the developing and adult brain. Inactivation of Notch signalling results in depletion of the progenitor population and induces precocious neural differentiation. On

the other hand, activation of Notch signalling keeps the neural stem cells in a progenitor state and thereby maintains the progenitor pool (Bertrand et al., 2002; Ross et al., 2003; Kageyama et al., 2008; Kopan and Ilagan, 2009).

A potential interaction between Crumbs and Notch was initially reported in *Drosophila* (Herranz et al., 2006). In Crumbs mutant clones Notch signalling pathway is activated. The mutant wing phenotype mimics gain-of-function of Notch and can be rescued by overexpressing either full length Crumbs or a truncated form of Crumbs lacking the intracellular domain. The authors suggested that Crumbs refines Notch signalling by inhibition of γ-secretase at the wing margin in *Drosophila.*

Consistent with the *Drosophila* study, it was shown that human Crb2 binds to the presenilin complex and inhibits γ-secretase mediated cleavage of amyloid precursor protein. Crb2 mediated inhibition of γ-secretase leads to reduced proteolytic production of Notch intracellular domain (Mitsuishi et al., 2010).

During *Drosophila* head development, Crumbs plays an important role in the control of organ size and in Crumbs mutant clones there is an increase in ligand-dependent Notch signalling. It was also reported that ectopic Notch signalling observed in Crumbs mutant clones corresponds to an increase in Notch and Delta endocytosis and this function was independent of the role of Crumbs in apical-basal polarity (Richardson and Pichaud, 2010).

Recently it was reported that in zebrafish, the Crumbs-Notch pathway is important for restriction of mitosis to apical surface and also in the maintenance of neuroepithelial polarity. Crumbs proteins were shown to directly interact with the extracellular domain of Notch and inhibit its activity (Ohata et al., 2011).

These studies suggest that the interaction between Notch signalling cascade and Crumbs is evolutionarily conserved and that

Crumbs is part of a negative feedback loop during Notch signalling. Based on this it can be predicted that the conditional removal of Crb2 would lead to ectopic activation of Notch signalling. Surprisingly, in the dorsal telencephalon loss of Crb2 results in the opposite phenotype-downregulation of Hes5 mRNA expression. Hes5 is a bona fide downstream target of the canonical Notch signalling pathway (de la Pompa et al., 1997; Ohtsuka et al., 1999).

This discrepancy could be due to a) temporal differences in Crb2 and Notch interactions b) tissue-specific regulation of Crb2 and Notch signalling pathway. It is also important to take into account that the reports showing interactions between Crumbs and Notch were biochemical studies carried out in non-*in vivo* situations and possibly predict misleading functions for Crumbs and Notch. The physiological relevance of these interactions remains to be addressed. Furthermore, the phenotype observed in Notch, Hes1 and Hes5 loss of function mutant brains is remarkably similar to that of Crb2 cKO phenotype; the loss of progenitor pools and precocious neural differentiation (Chenn & McConnell, 1995; Mizutani et al., 2007; Ohtsuka et al., 1999; Yoon & Gaiano, 2005).

This suggests that Crumbs loss of function in the brain leads to reduced Notch activity and in turn affects cell fate decisions. However, it is unclear from my studies whether Crumbs-Notch interaction is positive or negative. It is plausible that in the brain, Crb2 and Notch positively regulate each other and Crb2 could directly bind to extracellular domain of Notch and sequester progenitor cells from neural differentiation signals.

Although it may be favourable to put forth a unifying model to define the roles of Crb2 during neural development this could be a naïve approach, given that Crumbs plays isoform and context -dependent roles in different systems. Further studies need to be carried out to confirm an

association between Crb2 and Notch signalling in the developing vertebrate nervous system. Crb2 may play an instructive, permissive or inhibitory role in the transduction of signals to neural progenitors.

8.5 Future work

The broad scope of investigation in this study using two different model systems leaves room for further experiments. In this section I will briefly summarize some possible areas of future work to investigate further the role of Crb2.

8.5.1 Role of Crb2 in control of cell cycle dynamics

In both the mouse and chick systems, loss and misexpression of Crb2 resulted in mitotic cells frequently localized at aberrant positions. However, due to time constraints I could not perform detailed analysis of the effect of Crb2 on the cycling of neural progenitors. The role of Crb2 in regulating cell cycle progression could be examined by using cell cycle specific markers such as cyclins and cyclin-dependent kinases (Nigg, 1995; Sherr, 1994) and time-lapse imaging and/or BrdU assays (Estivill-Torrus et al., 2002) to determine cell-cycle length.

8.5.2 Role of Crb2 in cell survival

Recently work from our lab has shown that Crb2 is a novel regulator of mouse embryonic stem cell derived neural progenitors. At the onset of neuroepithelial specification, Crb2 localized to the apical surface of ES-cell derived neural structures called neural rosettes. Crb2 knockdown embryonic stem cells fail to survive neural differentiation (Boroviak & Rashbass, 2011). Given this role for Crb2 in cell survival of neural progenitors *in vitro*, it will be interesting to investigate if a similar mechanism operates *in vivo* by using markers to detect apoptotic cells in Crb2 cKO embryos using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) cell death detection methods (Labat-Moleur et

al., 1998; Negoescu et al., 1996) and/or Cleaved caspase3 antibody (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995).

8.5.3 Cell autonomous versus non-cell autonomous roles of Crb2

In both the chick and mouse embryonic systems, the cell autonomous versus non-cell autonomous effects of Crb2 in neural development needs to be determined. This could be approached by *in utero* electroporation of hairpin constructs in the mouse embryonic system and/or using bidirectional overexpression vectors. Similar electroporation studies can be performed in the chick embryonic system. In addition to this, analysis of a knockout system where both Crb2F and Crb2S isoforms are targeted would reveal if these isoforms have overlapping functions in neural development.

Some preliminary data suggestive of potential non-cell autonomous effects of Crb2F and Crb2S is presented in Chapter 9 (Appendix1).

8.6 Concluding remarks

Although Crb2F and Crb2S modulate distinct aspects of neural organization, maintaining the balance between these isoforms may be critical for normal neural development. In conclusion, the roles of Crb2 in neural development are far from simple and Crb2 may perform context-dependent functions throughout development and possibly into adulthood. The identification of a role for Crb2 during embryonic neurogenesis and its potential association with Notch signalling pathway makes it an attractive candidate for playing similar roles in adult neurogenesis. Interestingly, Crb2 is expressed in the sub-ependymal region (Allen brain atlas), characterized as a stem cell niche (Riquelme et al., 2008) in the adult brain. Further investigations in this direction may contribute significantly to the fields of adult stem cell research and neuroscience.

CHAPTER 9

Appendices

9. Appendices

In Appendix 1, I have included data from preliminary experiments to determine if the 'non-typical' expression of Crb2 at E17.5 has a role in neural development. The data included here is suggestive of additional roles for Crb2 and Crb2S during neural development. In Appendix 2, I have included data from experiments aimed at identifying proteins interacting with the secreted Crb2 isoform. However, the data is fairly preliminary and does not fit in with the main crux of this thesis and hence is included as an appendix. Finally in Appendix 3, I have included the protein sequencing data from the mass spectrometry analysis.

Appendix 1

Analysis of Crb2 positive tissue/ Crb2S protein-soaked bead transplanted embryos

As described in Chapter 3, in the mouse embryonic brain at E17.5, Crb2 expression is not restricted to the apical surface but is expanded to a specific-population of cells in the dorsal telencephalon. To identify this cell population, I carried out marker expression analysis. Interestingly, the expression of Nestin, a well-established stem cell marker is closely associated with Crb2 expression in this region (Fig 9.1 A-D). At E17.5, a similar association of Crb2 and Nestin expression is also observed in the olfactory bulb (Fig 9.1 E-G) and in the developing spinal cord (Fig 9.1 H-J) (K.Chinnaiya).

To understand if this expression pattern has any significance, we carried out *in ovo* transplantation experiments (Fig 9.2) in collaboration with K. Chinnaiya and Prof. M.Placzek. We transplanted dorsal telencephalic (DT) tissue (Crb2 and Nestin positive region) into the caudal neuropore of H&H stage 10 chick embryos and analysed the embryos after 24 hours. Dorso-lateral telencephalic tissue (Control) that did not

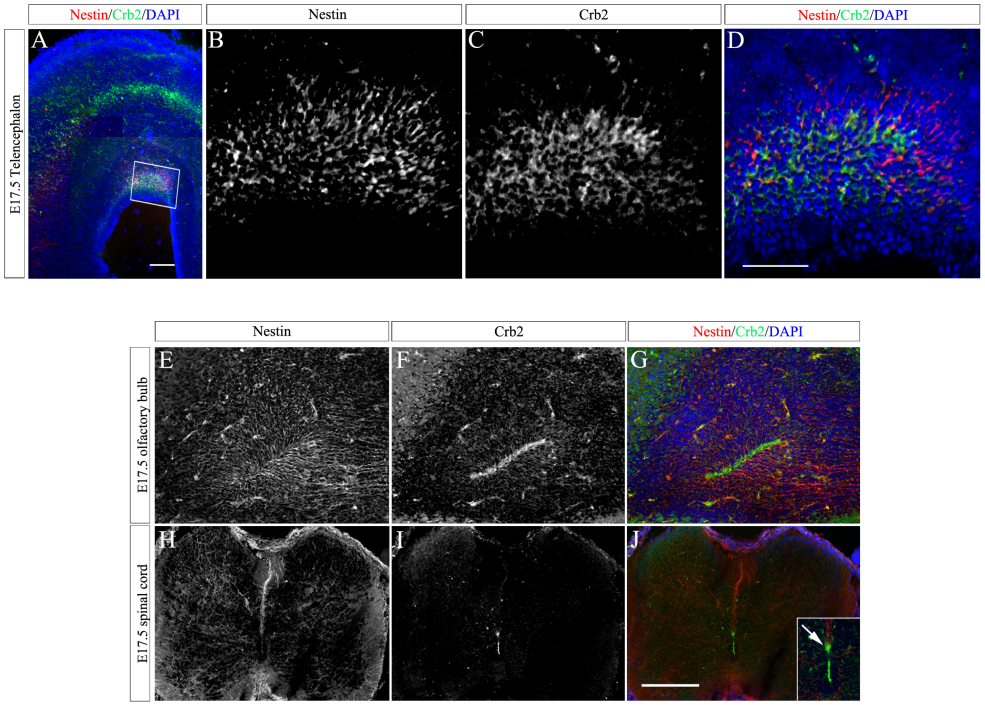


Fig 9.1 **Expression pattern of Crb2 and Nestin at E17.5.** A- Coronal section through the telencephalon of an E17.5 mouse brain shows close association of Nestin (red) and Crb2 (green) expression in a specific population of cells in the dorsal telencephalon. B-D Higher magnification images of the boxed region in A. E-F Coronal section through the olfactory bulb immunostained for Nestin (red) and Crb2 (green). I-J Transverse section through the spinal cord of E17.5 immunostained for Nestin (red) and Crb2 (green). Inset in J shows a higher magnification of the central canal region. Images in panel H-J were kindly provided by K.Chinnaiya. Nuclei counterstained with DAPI shown in blue.
Scale bars = A, E-J 100 μm; B-D 20 μm

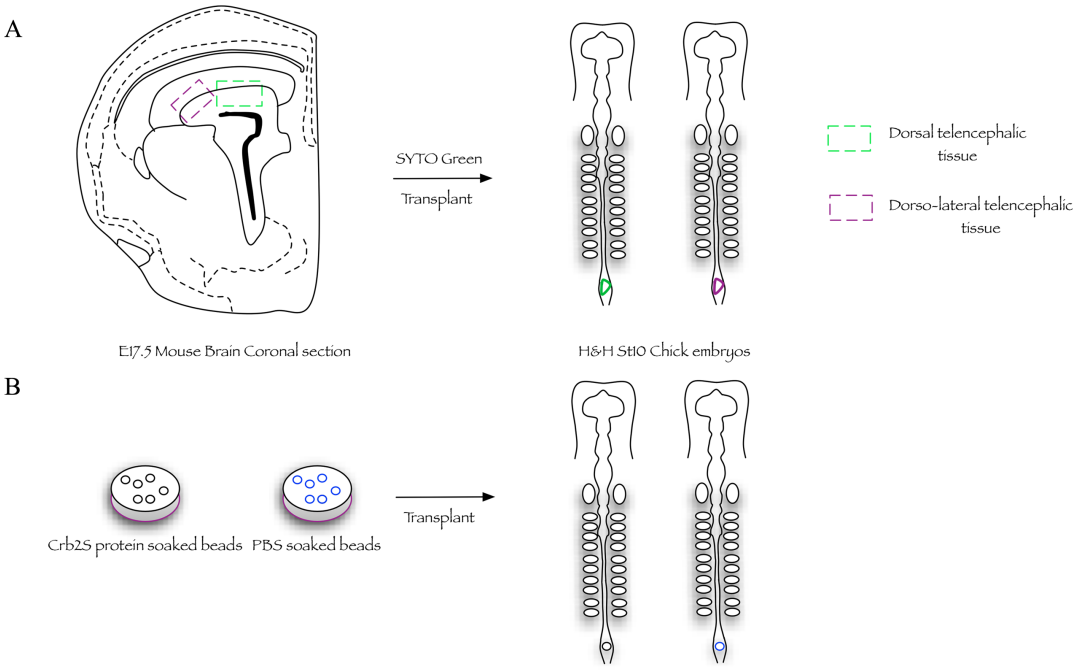


Fig 9.2 **Schematic illustration of the *in ovo* transplantation approaches.** A. Coronal section of an E17.5 brain is sub-dissected into dorsal telencephalic tissue, dorso-lateral telencephalic tissue and labelled with SYTO green fluorescent dye. Labelled tissue is transplanted to the caudal neuropore of H&H stage 10 chick embryos for incubation. B. Crb2S protein soaked or PBS soaked beads are transplanted to the caudal neuropore of H&H stage10 chick embryos

express Crb2 was used as a control for the transplantation experiments. In the control-transplanted embryos, Pax6 is expressed in the dorsal and intermediate progenitors of the neural tube (Fig 9.3A). Interestingly, in DT transplanted embryos Pax6 expression is downregulated within the neural tube and Pax6 positive cells are detected outside the neural tube (Fig 9.3 B). Moreover, in the control-transplanted embryos, Nkx6.1 expression is detected in the progenitors of the ventral neural tube (Fig 9.3 C). Similar to Pax6 expression in the DT transplanted embryos, Nkx6.1 positive cells were detected at ectopic locations outside the neural tube in a similar fashion as the altered Pax6 expression (Fig 9.3 D). In the control, Sox2 marks all the progenitors with the neural tube (Fig 9.3 E). Within the neural tube of DT transplanted embryos, there is a remarkable downregulation of Sox2 and a few Sox2 labelled cells are also detected outside the neural tube (Fig 9.3 F). Shh expression is detected in the ventral floor plate and notochord of control embryos (Fig 9.3G). In the DT transplanted embryos, Shh is detected in the notochord and floor plate but additionally Shh expression is also present in the intermediate neural tube region (Fig 9.3H). Ectopic Shh positive tissue is also detected near the notochord. The expression of 3B9, a notochord marker is also significantly altered in the DT transplanted embryos (Fig 9.3 J) compared to the control embryos (Fig 9.3 I).

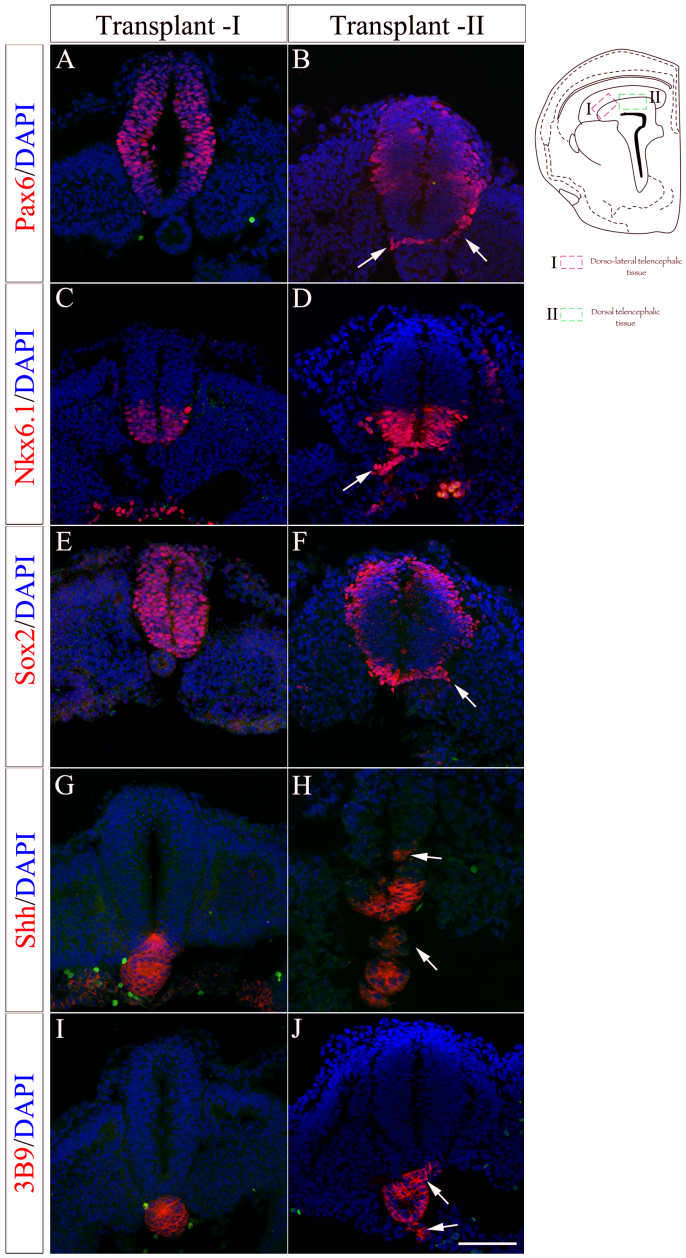
Overall, this shows that transplantation of DT tissue affects the localization and expression of neural progenitors. It also leads to the formation of ectopic notochord and floor plate-like structures.

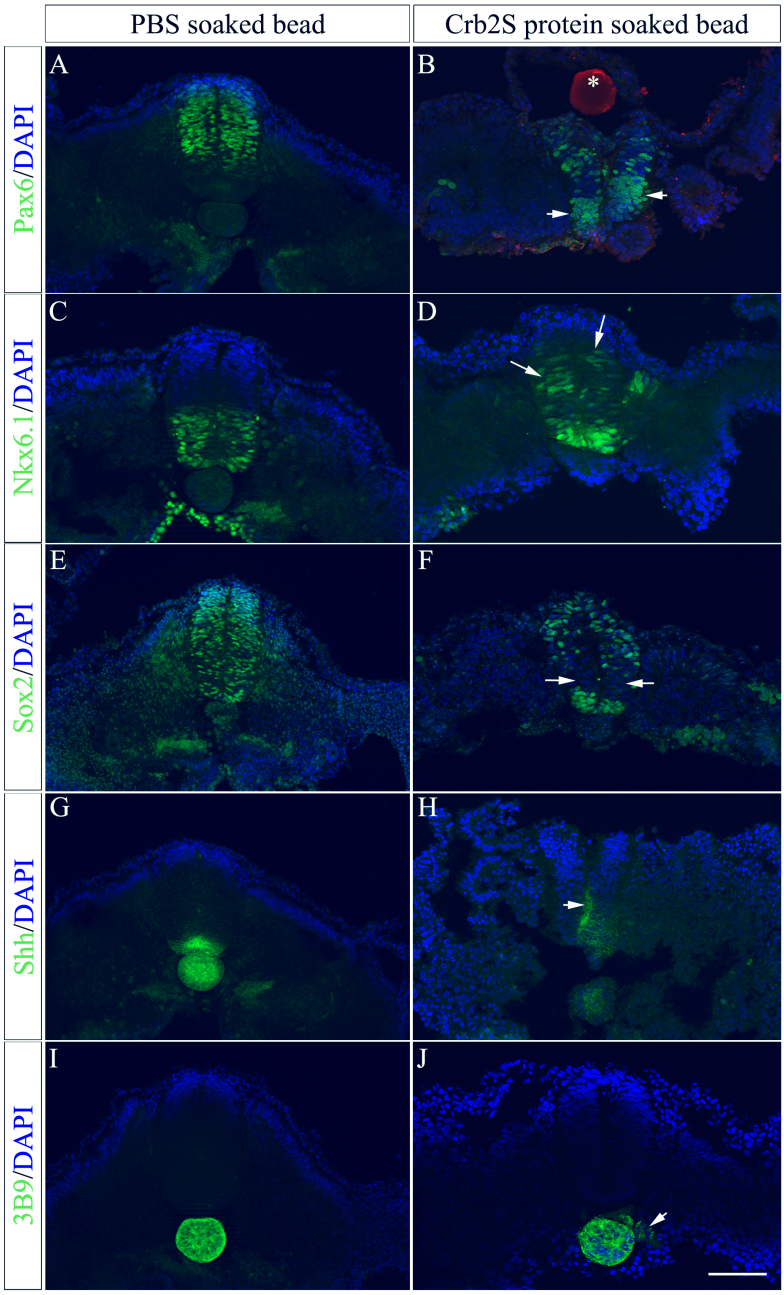
To study if Crb2 could mediate part of or all of this effect, we took advantage of *in ovo* bead transplantation approaches wherein Crb2S protein soaked beads were transplanted into the caudal neuropore of chick embryos and analysed after 24 hours. PBS soaked beads were used as controls. In the control embryos, Pax6 is expressed in the dorsal and intermediate progenitors and Nkx6.1 expression is restricted to the ventral neural tube progenitors. Analysis of embryos transplanted with

Crb2S protein soaked beads show a remarkable alteration in dorsal and ventral expression domains of Pax6 (Fig 9.4 A) and Nkx6.1 (Fig 9.4 C). Pax6 expression is reduced in the dorsal and intermediate neural tube but is highly expressed in the ventral neural tube (arrows in Fig 9.4 B). Nkx6.1 expression domain is expanded and Nkx6.1 positive cells are detected in the dorsal and intermediate neural tube regions (arrows in Fig 9.4 D). The expression of Sox2 is also affected in Crb2S bead transplanted embryos. Sox2 expression is decreased in the Crb2S transplanted embryos and in particular from the intermediate region of the neural tube (Fig 9.4 arrow in F) compared to the control embryos where Sox2 is expressed in all progenitors of the neural tube (Fig 9.4 E). Shh is expressed in the ventral floor plate and notochord (Fig 9.4G) In Crb2S transplanted embryos, Shh is expressed in the notochord and also Shh expression domain is expanded (arrow in Fig 9.4 H). 3B9 is expressed in the notochord of both control (Fig 9.4 I) and Crb2S bead transplanted embryos additionally, in Crb2S embryos 3B9 positive cells are also detected outside the notochord (Fig 9.4, arrow in J).

Transplantation of Crb2S protein soaked beads gave a similar phenotype to that of DT transplanted embryos. However, compared to the DT transplanted embryos, the effect of Crb2S on localization of Pax6, Nkx6.1 and Sox2 neural progenitors outside the neural tube was subtle. However, Crb2S had a dramatic effect on the dorsal-ventral patterned progenitor domains within the neural tube. Both DT and Crb2S transplantations affected normal expression of Shh and 3B9. Taken together, this suggests that transplantation of either dorsal telencephalic tissue or Crb2S protein soaked beads affects the expression of dorsal-ventral patterning markers in the developing chick embryonic neural tube.

However, further experiments need to be carried out to enable conclusive interpretation of data and particularly to identify if there is any physiological relevance to the intriguing phenotypes observed upon transplantation of Crb2 positive tissue/beads.


Transplant -I
Transplant -II
Pax6/DAPI
Nkx6.1/DAPI
Sox2/DAPI
Shh/DAPI
3B9/DAPI
A
B
C
D
E
F
G
H
I
J
I
II
I Dorso-lateral telencephalic tissue
II Dorsal telencephalic tissue


PBS soaked bead
Crb2S protein soaked bead
Pax6/DAPI
Nkx6.1/DAPI
Sox2/DAPI
Shh/DAPI
3B9/DAPI
A
B
C
D
E
F
G
H
I
J

Appendix 2

Development of assays to assist with identification of proteins interacting with Crb2S

As mentioned in Chapter 1, Crumbs is the only known polarity protein to have an extracellular domain. Whilst the intracellular interacting partners of Crumbs are known, not much is known about the extracellular domain. Using Crb2S as a resource, we tried to develop assays to identify proteins potentially interacting with the extracellular domain of Crb2.

A. Stress fibre assay

DLD-1 (human colon adenocarcinoma cell line) stable cell lines overexpressing different Crb2 isoforms were previously made (Baijun Kou). The different clonal cells were stained for F-actin by Phalloidin staining. A difference in actin cytoskeleton was observed between cells overexpressing membrane-bound Crb2 and secreted Crb2 (Crb2S). In cells overexpressing Crb2S, contractile actin cytoskeletal structures-stress fibres were observed, whereas the stress fibre phenotype was not seen in cells expressing full length or altered start Crb2 or in cells where Crb2 expression was knocked down; as shown in Fig 9.5.

We had hoped to use the stress fibre phenotype as a scoreable readout for an RNAi screen to identify proteins interacting with Crb2S. Prior to this, the robustness of the screening assay had to be validated.

I carried out control experiments to validate stress fibre phenotype seen in cells overexpressing Crb2S. Initially, I analysed the previously made clonal DLD-1 cells overexpressing Crb2S. Unfortunately, I observed variations in the stress fibre phenotype between different Crb2S clonal cell lines (data not shown).

I designed a control vector described in Chapter 5, where the signal peptide of Crb2 was cloned into the same expression vector backbone used for Crb2S overexpression. I generated stable clonal cell lines for Crb2S and the control construct. Unfortunately, after Phalloidin staining I detected stress fibres in both Crb2S and control cell lines (Fig 9.6). This ruled out the use of the stress fibre phenotype as a useful assay to identify interacting proteins

B. Immunoprecipitation assay

As an alternative approach to identify proteins interacting with Crb2S, I carried out immunoprecipitation experiments (IPs). It has been reported previously that Pals1 interacts with Crb2 (Kim et al., 2010). I used this known interaction as a positive control for the IPs as there is currently no known binding partner for Crb2S (Fig 9.7A).

Mouse embryonic stem cells were differentiated to neural progenitors *in vitro.* Previous work from our lab has shown that Crb2 is expressed in these progenitors (Boroviak & Rashbass, 2011). I collected neural progenitor cell lysates and incubated them with Crb2S V5 His tagged fusion protein (Crb2S). The lysates were immunoprecipitated with an antibody against full length Crb2 and blotted for V5 tag antibody to detect Crb2S (Fig 9.7B). This showed that endogenously expressed Crb2 isoform is capable of binding with the recombinant Crb2S protein. Although, the IP data is fairly preliminary it is a good starting point to identify Crb2S interacting proteins by following a candidate approach.

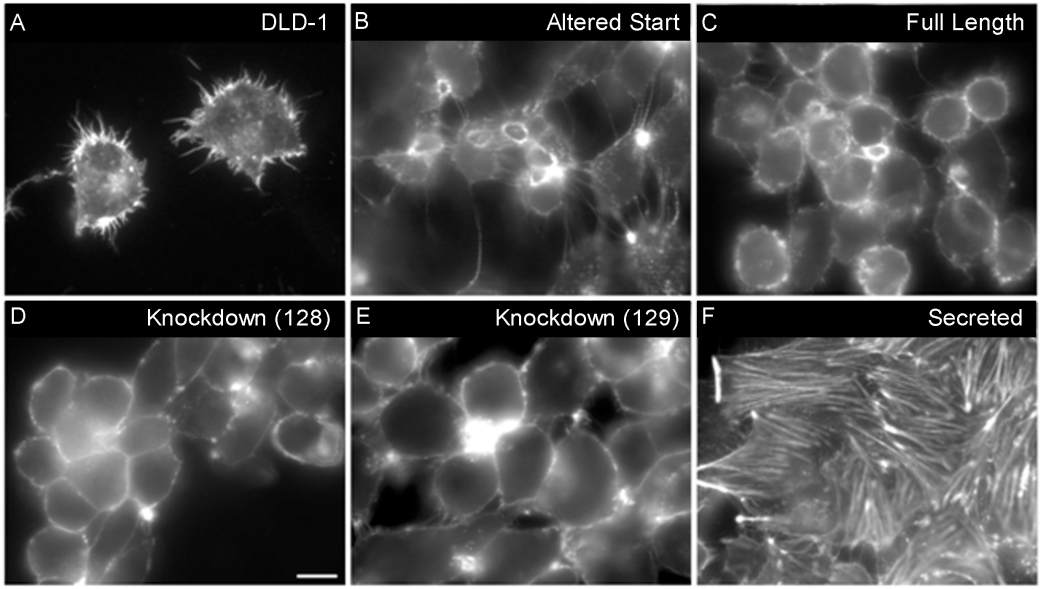


Fig 9.5 **Overexpression of Crb2S isoform induces the formation of stress fibres.** DLD-1 cells (A) and stable clonal cell lines of the different Crb2 isoforms - altered start (B), full length (C) and secreted (F) and Crb2 knockdown cells (D,E) were labelled with Phalloidin to detect F-Actin. Only in stable clonal cell lines overexpressing the secreted Crb2 isoform actin stress fibres are detected. Scale bar = 20 μm Image from Baijun Kou

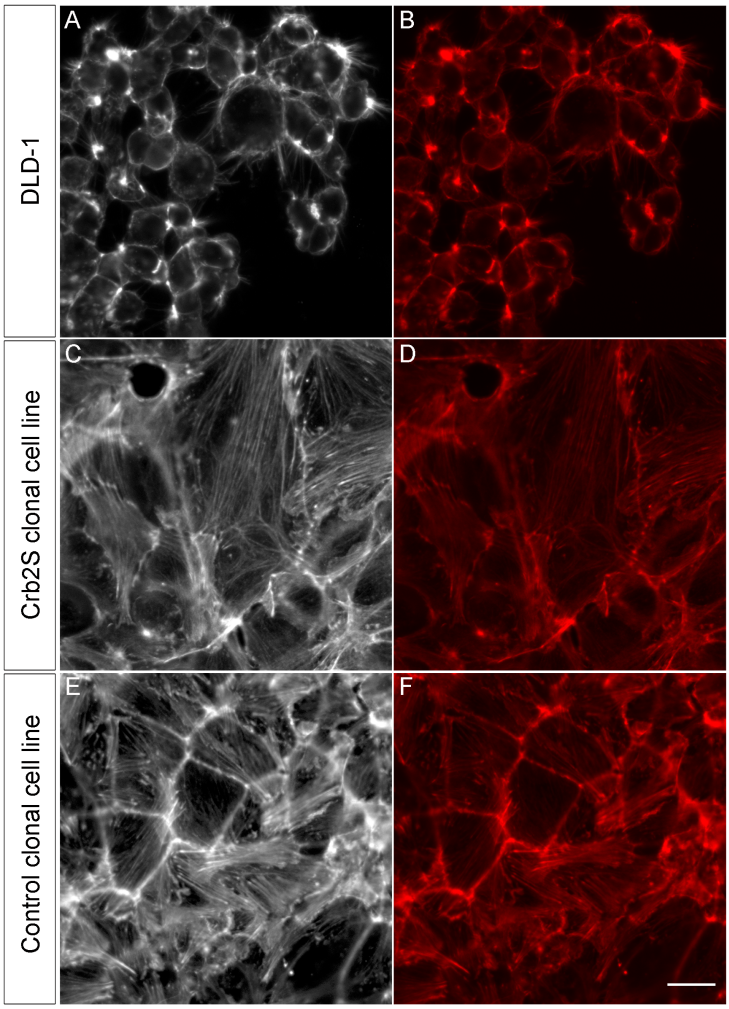


Fig 9.6 **Formation of stress fibres in stable clonal cell lines is not induced by Crb2S**. DLD-1 cells (A) stable clonal cell line overexpressing Crb2S isoform (B) and a control clonal cell line expressing only signal peptide of Crb2 (C) were labelled with Phalloidin. Stress fibres were detected in both Crb2S and control cell lines.
Scale bar = 20 μm

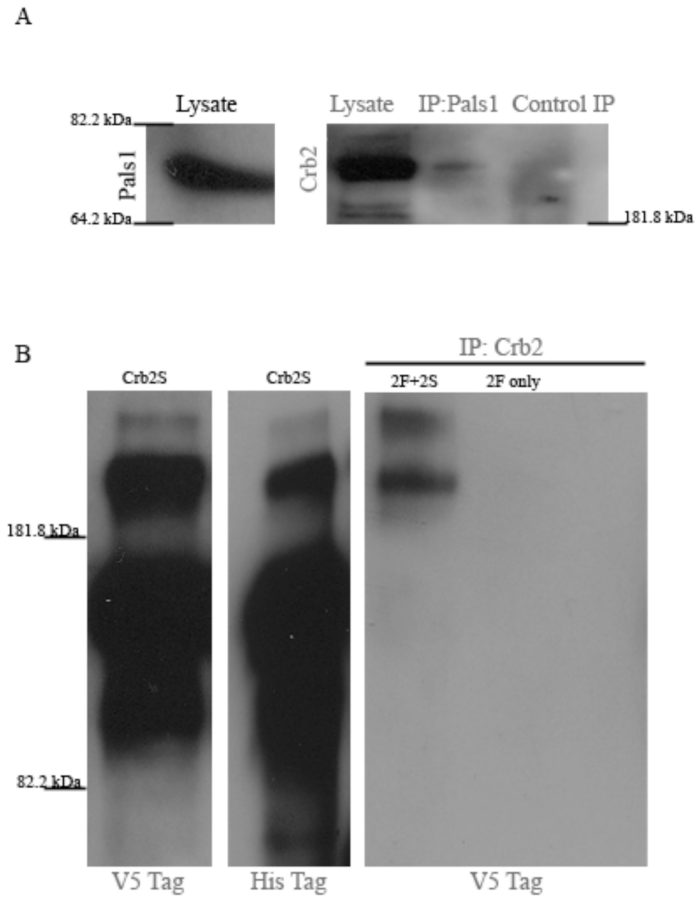


Fig 9.7 A - **Pals1 immunoprecipitates Crb2 from mouse embryonic stem cell (ESC) derived neural progenitor lysates.** Lysate panels show Pals1 and Crb2F protein expression in the ESC derived neural progenitor samples. Crb2F protein is detected in lysates immunoprecipitated with Pals1 antibody and probed for Crb2F. Rabbit pre-immune serum was used for control immunoprecipitations. B- **Crb2F immunoprecipitates Crb2S** from ESC derived neural progenitor lysates incubated with Crb2S-V5 His tag protein. ESC derived neural progenitors expressing Crb2F were incubated with Crb2S V5 His tagged protein. The first two panels represent Crb2S protein probed for V5 tag Ab and His tag Ab. Crb2S is detected in the sample immunoprecipitated with Crb2F antibody and probed for Crb2S-V5 tagged protein . For control, immunoprecipitation using Crb2F antibody was carried out on ESC derived neural cell lysates.

Appendix 3

Crb2S protein sequencing data

In this section I have included data from the LC-ESI mass spectrometry sequencing analysis of Crb2S. The description of the analysed samples is included in Chapter 5 (Fig 5.4 C and Fig 5.5 A). Sample I (Intense) and Sample III (Intense and Weak) were identified as Crb2. Sample II and IV correspond to spectrin alpha chain and heat shock protein respectively.

Database searches restricted to the mouse taxonomy provided significant identification scores. The matched peptide sequences hits are shown in red and MASCOT identification scores are also shown.

I- Intense

Match to: **CRUM2_MOUSE** Score: **209 Crumbs homolog 2**
OS=Mus musculus Matched peptides shown in **Bold Red**

MALVGPRIWG PRRDIYPLLL LLLLLLLLLL PWVPAGLVPP ETPSVCASDP **51**
CAPGTKCQAT ESGGYTCEPS ELGGCATQPC HHGALCVPQG PDPNSFRCYC **101**
VPGFQGPHCE LDIDECASRP CQHGGTCQNL ADHYECHCPL GYAGVTCEAE **151**
VDECSSAPCL HGGSCLDGVG SYRCVCAPGY AGANCQLDVD ECQSQPCAHG **201**
GVCHDLVNGF R**CDCADTGYE GAR**CEQEVLE CASAPCAHNA SCLDGFRSFR **251**
CLCWPGFSGE RCEVDEDECA SGPCQNGGQC LQRSDPTLYG GVQAIFPGAF **301**
SFSHAAGFLC SCPLGFAGND CSMDVDECAS GPCLNGGSCQ DLPNGFQCYC **351**
QDGYTGLTCQ EDMDECQSEP CLHGGTCSDT VAGYICQCPE AWGGHDCSVQ **401**
LTGCQGHTCP LAATCIPTFK **SGLHGYFCR**C PPGTYGPFCG QNTTFSVVSG **451**
SSVWGLVPAA ASLGLALRFR **TTLLAGTLAT LK**DTRDSLEL VLVGAVLQAT **501**
LSRHGTAVLI LTLPDLALND GHWHQVEVTL HLGTLELRLW HEGCPGQLCV **551**
ASGPVATGPT ASVASGPPGS YSIYLGGGVF AGCFQDVR**VE GHLLLPEELK** **601**
GTVLLGCERR EPCQPLPCAH GGACVDLWTH FRCDCPRPYR GATCTDEVPA **651**
ATFGLGGATS SASFLLHQLG PNLTVSFFLR TREPAGLLLQ FANDSVASLT **701**
VFLSEGQIR**A EGLGHPAVVL PGR**WDDGLPH LVMLSFGPDQ LQDLGQRLYV **751**
GGR**FYPDDTQ LWGGPFR**GCL QDLQLNSIHL PFFSSPMENS SWPSELEAGQ **801**
SSNLTQGCVS EDTCNPNPCF NGGTCHVTWN DFYCTCSENF TGPTCAQQRW **851**
CPRQPCLPPA TCEEVPDGFV CVAEATFREG PPAVFTGHNV SSSLSGLTLA **901**
FRTRDSEAGL LRAVSAAGAH SNIWLAVRNG SLAGDVAGSV LPAPGPRVAD **951**
GAWHRVRLAR EFPQAAASRW LLWLDGAATP VALHGLGGDL GFLQGPGAVP **1001**
LLLAENFTGC LGRVALGDFP LPLAPPRSGT VSGAREHFVA WPGSPAVSLG **1051**
CRGGPVCSPS PCLHGGACRD LFDAFACSCG PAWEGPRCEI RADPCRSTPC **1101**
VRGQCHARPD GRFECRCPPG FSGPRCRLPV LPQGCNLNST CKDGAPCEGG **1151**
PLGTNCSCQE GLAGLRCQSL DKPCEASPCL NGGTCRVASG IFECTCSAGF **1201**
SGQFCEVVKT LPLPLPFPLL EVAVPAACAC LLLLLLGLLS GILAARKRRQ **1251**
SEGTYSPSQQ EVAGARLEMD SVLKVPPEER LI

I-Weak

Match to: **SPTA2_MOUSE** Score: **349 Spectrin alpha chain, brain OS=Mus musculus**

MDPSGVKVLE TAEDIQERRQ QVLDRYHRFK ELSTLRRQKL EDSYRFQFFQ **51**
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KLDETGNLMI SEGHFASETI RTRLMELHRQ WELLLEKMRE KGIKLLQAQK **151**
LVQYLRECED VMDWINDKEA IVTSEELGQD LEHVEVLQKK FEEFQTDLAA **201**
HEERVNEVSQ FAAKLIQEQH PEEELIKTKQ DEVNAAWQRL KGLALQRQGK **251**
LFGAAEVQRF NRDVDETIGW IKEKEQLMAS DDFGRDLASV QALLRKHEGL **301**
ERDLAALEDK VKALCAEADR LQQSHPLSAS QIQVKREELI TNWEQIRTLA **351**
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DRHQEHKGEI DAHEDSFKSA DESGQALLAA SHYASDEVRE KLSILSEERT **451**
ALLELWELRR QQYEQCMDLQ LFYRDTEQVD NWMSKQEAFL LNEDLGDSLD **501**
SVEALLKKHE DFEKSLSAQE EKITALDEFA TKLIQNNHYA MEDVATRRDA **551**
LLSRRNALHE RAMHRRAQLA DSFHLQQFFR DSDELKSWVN EKMKTATDEA **601**
YKDPSNLQGK VQKHQAFEAE LSANQSRIDA LEKAGQKLID VNHYAKEEVA **651**
ARMNEVISLW KKLLEATELK GIKLREANQQ QQFNRNVEDI ELWLYEVEGH **701**
LASDDYGKDL TNVQNLQKKH ALLEADVAAH QDRIDGITIQ ARQFQDAGHF **751**
DAENIKKKQE ALVARYEALK EPMVARKQKL ADSLRLQQLF RDVEDEETWI **801**
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EAESWMREKE PIVGSTDYGK DEDSAEALLK KHEALMSDLS AYGSSIQALR **951**
EQAQSCRQQV APMDDETGKE LVLALYDYQE KSPREVTMKK GDILTLLNST **1001**
NKDWWKVEVN DRQGFVPAAY VKKLDPAQSA SRENLLEEQG SIALRQGQID **1051**
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NKVAEDLESE GLMAEEVQAV QQQEVYGAMP RDEADSKTAS PWKSARLMVH **1201**
TVATFNSIKE LNERWRSLQQ LAEERSQLLG SAHEVQRFHR DADETKEWIE **1251**
EKNQALNTDN YGHDLASVQA LQRKHEGFER DLAALGDKVN SLGETAQRLI **1301**
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LMSWINGIRG LVSSDELAKD VTGAEALLER HQEHRTEIDA RAGTFQAFEQ **1401**
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II

Match to: **SPTA2_MOUSE** Score: **295 Spectrin alpha chain, brain OS=Mus musculus**

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LVQYLRECED VMDWINDKEA IVTSEELGQD LEHVEVLQKK FEEFQTDLAA **201**
HEERVNEVSQ FAAKLIQEQH PEEELIKTKQ DEVNAAWQRL KGLALQRQGK **251**
LFGAAEVQRF NRDVDETIGW IKEKEQLMAS DDFGRDLASV QALLRKHEGL **301**
ERDLAALEDK VKALCAEADR LQQSHPLSAS QIQVKREELI TNWEQIRTLA **351**
AERHARLDDS YRLQRFLADF RDLTSWVTEM KALINADELA NDVAGAEALL **401**
DRHQEHKGEI DAHEDSFKSA DESGQALLAA SHYASDEVRE KLSILSEERT **451**
ALLELWELRR QQYEQCMDLQ LFYRDTEQVD NWMSKQEAFL LNEDLGDSLD **501**
SVEALLKKHE DFEKSLSAQE EKITALDEFA TKLIQNNHYA MEDVATRRDA **551**
LLSRRNALHE RAMHRRAQLA DSFHLQQFFR DSDELKSWVN EKMKTATDEA **601**
YKDPSNLQGK VQKHQAFEAE LSANQSRIDA LEKAGQKLID VNHYAKEEVA **651**
ARMNEVISLW KKLLEATELK GIKLREANQQ QQFNRNVEDI ELWLYEVEGH **701**
LASDDYGKDL TNVQNLQKKH ALLEADVAAH QDRIDGITIQ ARQFQDAGHF **751**
DAENIKKKQE ALVARYEALK EPMVARKQKL ADSLRLQQLF RDVEDEETWI **801**
REKEPIAAST NRGKDLIGVQ NLLKKHQALQ AEIAGHEPRI KAVTQKGNAM **851**
VEEGHFAAED VKAKLSELNQ KWEALKAKAS QRRQDLEDSL QAQQYFADAN **901**
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GMRMQHNLEQ QIQARNTTGV TEEALKEFSM MFKHFDKDKS GRLNHQEFKS **2351**
CLRSLGYDLP MVEEGEPDPE FEAILDTVDP NRDGHVSLQE YMAFMISRET **2401**
ENVKSSEEIE SAFRALSSEG KPYVTKEELY QNLTREQADY CVSHMKPYVD **2451**
GKGRELPTAF DYVEFTRSLF VN

III Intense

Match to: **CRUM2_MOUSE** Score: **315 Crumbs homolog 2 OS=Mus musculus**

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CAPGTKCQAT ESGGYTCEPS ELGGCATQPC HHGALCVPQG PDPNSFRCYC **101**
VPGFQGPHCE LDIDECASRP CQHGGTCQNL ADHYECHCPL GYAGVTCEAE **151**
VDECSSAPCL HGGSCLDGVG SYRCVCAPGY AGANCQLDVD ECQSQPCAHG **201**

GVCHDLVNGF RCDCADTGYE GARCEQEVLE CASAPCAHNA SCLDGFRSFR 251
CLCWPGFSGE RCEVDEDECA SGPCQNGGQC LQRSDPTLYG GVQAIFPGAF 301
SFSHAAGFLC SCPLGFAGND CSMDVDECAS GPCLNGGSCQ DLPNGFQCYC 351
QDGYTGLTCQ EDMDECQSEP CLHGGTCSDT VAGYICQCPE AWGGHDCSVQ 401
LTGCQGHTCP LAATCIPTFK SGLHGYFCRC PPGTYGPFCG QNTTFSVVSG 451
SSVWGLVPAA ASLGLALRFR TTLLAGTLAT LKDTRDSLEL VLVGAVLQAT 501
LSRHGTAVLI LTLPDLALND GHWHQVEVTL HLGTLELRLW HEGCPGQLCV 551
ASGPVATGPT ASVASGPPGS YSIYLGGGVF AGCFQDVRVE GHLLLPEELK 601
GTVLLGCERR EPCQPLPCAH GGACVDLWTH FRCDCPRPYR GATCTDEVPA 651
ATFGLGGATS SASFLLHQLG PNLTVSFFLR TREPAGLLLQ FANDSVASLT 701
VFLSEGQIRA EGLGHPAVVL PGRWDDGLPH LVMLSFGPDQ LQDLGQRLYV 751
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SSNLTQGCVS EDTCNPNPCF NGGTCHVTWN DFYCTCSENF TGPTCAQQRW 851
CPRQPCLPPA TCEEVPDGFV CVAEATFREG PPAVFTGHNV SSSLSGLTLA 901
FRTRDSEAGL LRAVSAAGAH SNIWLAVRNG SLAGDVAGSV LPAPGPRVAD 951
GAWHRVRLAR EFPQAAASRW LLWLDGAATP VALHGLGGDL GFLQGPGAVP 1001
LLLAENFTGC LGRVALGDFP LPLAPPRSGT VSGAREHFVA WPGSPAVSLG 1051
CRGGPVCSPS PCLHGGACRD LFDAFACSCG PAWEGPRCEI RADPCRSTPC 1101
VRGQCHARPD GRFECRCPPG FSGPRCRLPV LPQGCNLNST CKDGAPCEGG 1151
PLGTNCSCQE GLAGLRCQSL DKPCEASPCL NGGTCRVASG IFECTCSAGF 1201
SGQFCEVVKT LPLPLPFPLL EVAVPAACAC LLLLLLGLLS GILAARKRRQ 1251
SEGTYSPSQQ EVAGARLEMD SVLKVPPEER LI

III Weak

Match to: **CRUM2_MOUSE** Score: **183 Crumbs homolog 2 OS=Mus musculus**

MALVGPRIWG PRRDIYPLLL LLLLLLLLLL PWVPAGLVPP ETPSVCASDP 51
CAPGTKCQAT ESGGYTCEPS ELGGCATQPC HHGALCVPQG PDPNSFRCYC 101
VPGFQGPHCE LDIDECASRP CQHGGTCQNL ADHYECHCPL GYAGVTCEAE 151
VDECSSAPCL HGGSCLDGVG SYRCVCAPGY AGANCQLDVD ECQSQPCAHG 201
GVCHDLVNGF RCDCADTGYE GARCEQEVLE CASAPCAHNA SCLDGFRSFR 251
CLCWPGFSGE RCEVDEDECA SGPCQNGGQC LQRSDPTLYG GVQAIFPGAF 301
SFSHAAGFLC SCPLGFAGND CSMDVDECAS GPCLNGGSCQ DLPNGFQCYC 351
QDGYTGLTCQ EDMDECQSEP CLHGGTCSDT VAGYICQCPE AWGGHDCSVQ 401
LTGCQGHTCP LAATCIPTFK SGLHGYFCRC PPGTYGPFCG QNTTFSVVSG 451
SSVWGLVPAA ASLGLALRFR TTLLAGTLAT LKDTRDSLEL VLVGAVLQAT 501
LSRHGTAVLI LTLPDLALND GHWHQVEVTL HLGTLELRLW HEGCPGQLCV 551
ASGPVATGPT ASVASGPPGS YSIYLGGGVF AGCFQDVRVE GHLLLPEELK 601
GTVLLGCERR EPCQPLPCAH GGACVDLWTH FRCDCPRPYR GATCTDEVPA 651
ATFGLGGATS SASFLLHQLG PNLTVSFFLR TREPAGLLLQ FANDSVASLT 701
VFLSEGQIRA EGLGHPAVVL PGRWDDGLPH LVMLSFGPDQ LQDLGQRLYV 751
GGRFYPDDTQ LWGGPFRGCL QDLQLNSIHL PFFSSPMENS SWPSELEAGQ 801
SSNLTQGCVS EDTCNPNPCF NGGTCHVTWN DFYCTCSENF TGPTCAQQRW 851
CPRQPCLPPA TCEEVPDGFV CVAEATFREG PPAVFTGHNV SSSLSGLTLA 901
FRTRDSEAGL LRAVSAAGAH SNIWLAVRNG SLAGDVAGSV LPAPGPRVAD 951
GAWHRVRLAR EFPQAAASRW LLWLDGAATP VALHGLGGDL GFLQGPGAVP 1001
LLLAENFTGC LGRVALGDFP LPLAPPRSGT VSGAREHFVA WPGSPAVSLG 1051
CRGGPVCSPS PCLHGGACRD LFDAFACSCG PAWEGPRCEI RADPCRSTPC 1101
VRGQCHARPD GRFECRCPPG FSGPRCRLPV LPQGCNLNST CKDGAPCEGG 1151
PLGTNCSCQE GLAGLRCQSL DKPCEASPCL NGGTCRVASG IFECTCSAGF 1201
SGQFCEVVKT LPLPLPFPLL EVAVPAACAC LLLLLLGLLS GILAARKRRQ 1251
SEGTYSPSQQ EVAGARLEMD SVLKVPPEER LI

IV

Match to: **HS71A_MOUSE** Score:**350 Heat shock 70kDa protein 1A OS=Mus musculus**

MAKNTAIGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL 51
IGDAAKNQVA LNPQNTVFDA KRLIGRKFGD AVVQSDMKHW PFQVVNDGDK 101
PKVQVNYKGE SRSFFPEEIS SMVLTKMKEI AEAYLGHPVT NAVITVPAYF 151
NDSQRQATKD AGVIAGLNVL RIINEPTAAA IAYGLDRTGK GERNVLIFDL 201
GGGTFDVSIL TIDDGIFEVK ATAGDTHLGG EDFDNRLVSH FVEEFKRKHK 251

KDISQNKRAV RRLRTACERA KRTLSSSTQA SLEIDSLFEG IDFYTSITRA **301**
RFEELCSDLF RGTLEPVEKA LRDAKMDK**AQ IHDLVLVGGS TR**IPKVQK**LL** **351**
QDFFNGRDLN KSINPDEAVA YGAAVQAAIL MGDKSENVQD LLLLDVAPLS **401**
LGLETAGGVM TALIKRNSTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT **451**
RDNNLLGRFE LSGIPPAPRG VPQIEVTFDI DANGILNVTA TDKSTGKANK **501**
ITITNDKGRL SKEEIERMVQ EAERYKAEDE VQRDRVAAK**N ALESYAFNMK** **551**
SAVEDEGLKG KLSEADKKKV LDKCQEVISW LDSNTLADKE EFVHKREELE **601**
RVCSPIISGL YQGAGAPGAG GFGAQAPK**GA SGSGPTIEEV D**

CHAPTER 10

References

10. REFERENCES

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