p57^{kip2} regulates timely cell cycle exit and differentiation in the developing vertebrate spinal cord

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SUMMARY

During the development of central nervous system, cell proliferation and neuronal differentiation must be tightly regulated to maintain progenitor pool and generate correct number and types of mature neurons. Cyclin-dependent kinase inhibitors (CKIs) provide negative control on cell cycle by inhibiting activities of cyclin-dependent kinases. In the developing vertebrate spinal cord, CKIs displayed distinct expression patterns in complementary regions. $p21^{cip1}$ is restricted in V2 interneurons, $p27^{kip1}$ and $p19^{INK4d}$ are mostly expressed in mature neurons. Whereas $p57^{kip2}$ is expressed in nascent interneurons at G1/G0 phase, preventing them from re-entering cell cycle. Loss of p57 leads to inappropriate entry into S phase and increased number of mature neurons were generated in the null mice. $p27^{kip1}$ does not appear to compensate for the loss of p57 during neurogenesis. Overexpression of Cip/Kip family in chick embryos indicates that CKI domain conserved among family members is sufficient and required to force cell cycle exit in dividing cells. Loss of function and gain of function studies also suggest that $p57^{kip2}$ is not required for cell cycle exit, rather for timely cell cycle withdrawal. Its C-terminal QT domain inhibits the transcription of homeodomain factors while inducing general neuronal differentiation program. Therefore $p57^{kip2}$ has dual functions of regulating proliferation and timely neuronal differentiation in vertebrate spinal cord.

INTRODUCTION

Development of vertebrate central nervous system (CNS) is a complex process that involves with multiple steps and sequential order of gene expressions. Neural induction and patterning are the two important steps that both require precise cell cycle control to maintain stem cell pool and generate certain amount of mature neuron in CNS (Bally-Cuif *et al.*, 2003). Timely regulated cell cycle exit and cellular differentiation determines appropriate number of post-mitotic cells produced at specific time window, which ensures normal organogenesis and morphogenesis during embryonic development (de Nooij, *et al.* 1996; Cremisi *et al.*, 2003). Extracellular signals and intrinsic factors exert controls on cell cycle by affecting the activities of cyclin-dependent kinases (Cdks). Cdks are the core elements of cell cycle machinery that integrate growth regulatory signals and intracellular signaling. Their activities require association with cognate cyclins and are regulated positively or negatively with different sites phosphorylated. In addition, cyclin-dependent kinase inhibitors (CKIs) bind and inhibit cyclin/Cdk complex (Sherr et al, 1999). Two classes of CKIs have been found in mammals based on their structure and target Cdks. INK4 family including p16 (Serrano *et al.* 1993), p15 (Hannon and Beach 1994), p18 (Guan *et al.* 1994; Hirai *et al.* 1995) and p19 (Chan *et al.* 1995; Hirai *et al.* 1995) share ankyrin repeats and specifically bind to Cdk4/6. Cip/kip family has three members, $p21^{cip1}$ (Harper *et al.* 1993), $p27^{kip1}$ (Polyak *et al.* 1994; Toyoshima and Hunter 1994) and $p57^{kip2}$ (Lee *et al.* 1995; Matsuoka *et al.* 1995) with common and unique domains, whose actions affect activities of cyclin D-, E- and A-dependent kinases.

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Previous work in retinae and ocular lens fiber development demonstrated that p57 is not critical for cell

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Abnormal cell cycling leads to ectopic cell division and cell death in p57*kip2* **null mice**

Proliferative cells in the spinal cord have their nuclei moving back and forth between apical and basal membrane, which is termed interkinetic nuclear migration. The nuclei are located laterally when cells incorporate DNA in S phase, move back close to central canal during G2 and divide at basal membrane (Fig 3). However interkinetic nuclear migration was disturbed in the mice lacking of p57 as we observed ectopic M phase cell laterally (Fig. 3G). Increased number of dividing cells seen in p57 null mice could be derived from maintained progenitor pool and/or delayed differentiation for immature neurons having extra round of cell cycle. Our data did not support the possibility of increased precursor cells in the spinal cord of p57 null mice (See the discussion). To address the latter possibility, first we exclude ectopic M phase cells in mutants and quantified M phase cell close to the central canal. We found no difference of basal mitotic cells between wild type and mutant (Fig.3J). This suggested ectopic M phase cells

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cycle exit (Zhang et al., 1998; Dyer and Cepko, 2001). To determine whether it is required for p57 to control neural development, we examine spinal cord defects in knockout mice. We focused on the embryos at e10.5d to e13.5d when most neurons are born and mature during this time window. To verify the hypothesis that p57 is expressed in differentiating neurons preventing inappropriate S entry, we first examined in p57 null mice S phase cells labeled with BrdU. One third of dorsal spinal cord was used to quantify BrdU+ cells (Fig. 3D). More BrdU-positive cells were found in mutant embryos at E10.5d (Fig.3A, B) and E11.5d (Fig.3C-D), and the significance is more evident in longer BrdU pulsed embryos (Fig. 3E). This result suggested that more cells entered cell cycle in mutant compared to those in wild type littermates within given time. The increased number of S phase cells could be accounted for by the inappropriate cell cycle entry of differentiating neurons in the absence p57 control on G1/S transition. In addition to S phase cells, we also examined dividing cells with phh3, which labels mitotic phase cells located close to central canal in wild type embryos. More M phase cells were observed in p57 null mice as well (Fig. 3F,G, I). These results proved that increased number of cells enter S phase and divide in the absence of p57 control on cell cycle.

Abnormal cell cycling leads to ectopic cell division and cell death in p57*kip2* **null mice**

Proliferative cells in the spinal cord have their nuclei moving back and forth between apical and basal membrane, which is termed interkinetic nuclear migration. The nuclei are located laterally when cells incorporate DNA in S phase, move back close to central canal during G2 and divide at basal membrane (Fig 3). However interkinetic nuclear migration was disturbed in the mice lacking of p57 as we observed ectopic M phase cell laterally (Fig. 3G). Increased number of dividing cells seen in p57 null mice could be derived from maintained progenitor pool and/or delayed differentiation for immature neurons having extra round of cell cycle. Our data did not support the possibility of increased precursor cells in the spinal cord of p57 null mice (See the discussion). To address the latter possibility, first we exclude ectopic M phase cells in mutants and quantified M phase cell close to the central canal. We found no difference of basal mitotic cells between wild type and mutant (Fig.3J). This suggested ectopic M phase cells

contribute to the increase of mitotic cells in null mice. Second, we wanted to show whether the differentiating neurons could enter another cell cycle incorporating BrdU in the absence of p57. To mark the p57 expressing region in null mice at VZ^L, we stained with transcription factor prox1, which colocalizes with p57 in most interneurons. BrdU-labeled cells are located medially to VZ^L in wild type embryos (Fig.3B). In mutant mice, BrdU cells are expanded laterally and colabeled with prox1 (Fig.4C). These findings support the hypothesis that in the absence of p57, nascent neurons may re-enter cell cycle but are unable to translocate back to basal membrane, and divide ectopically while they migrate laterally to MZ. To further support this theory, we labeled dividing cells with short BrdU pulse. Normally pax2 and lim1/2 expressing cells are not labeled with short pulse of BrdU (Fig.4F, H), indicating that these homeodomain factors are induced in G0 phase hours after the last cell division. In the absence of p57, pax2 and lim1/2 cells incorporated BrdU with 2 hours pulsed (Fig. 4G, I). This result directly proved that differentiating neurons are able to re-enter cell cycle when the checkpoint at G1/S is lost (Fig. J, K). It further supported that the differentiating neurons with cyclin D1 in high level retained the ability of entering cell cycle. It is also consistent with the Drosophila model that abundance of cyclin and Dacapo cooperatively control cell cycle exit.

During retina and lens development, loss of p57 results in abnormal cell division and cell death. We observed more mature neurons in mutants, which peaks at E11.5d and declines at later stage. To determine whether cell death compensates for the increase, we examined caspase 3 expression and found more cells labeled with caspase 3 in p57 null mice compared to wild type. It indicated that cell death may partially compensate for the increased number of neurons in p57 mutant.

More mature neurons were generated in p57 mutant

Since we observed increased number of dividing cells in p57 null mice, we asked next whether there is change of mature neurons as well. We examined p57-expressing interneurons from dN5 to V1 and $p57^-$ V2 interneurons. Increased number of interneurons from dN5 to V1 but not V2 was observed in mutants compared to wild type embryos (Fig.5A, B, D). We further looked at V1 interneurons at different stages.

From e10.5d to e13.5d, more en-1 neurons (V1) were generated in p57 knockout mice (Fig.5D). Most significantly, two-fold increase is seen in e11.5d mutant embryos. In p27/p57 double knockout mice, no significantly more cells were found compared to the single p57 null mice (Fig. 5C, G). Increased number of both dividing cells and differentiated neurons indicates that there is no limiting control over the transition from progenitor cells to mature neurons. $p27^{kip1}$ does not appear to compensate for the loss of p57. More mature neurons generated in double null mice also suggested that p27 and p57 seemed not be required for cell cycle exit. As we examined the increase of mature neurons, more cells appeared both in VZ and MZ of mutant detected with en-l (Fig. 5B) and pax2 (Fig.5F) antibodies compared to wild type littermates. We proposed that p57 prevented premature induction of homeodomain factors during cell fate acquisition and neuronal differentiation. In the absence of p57, they initiated precociously in the VZ and therefore generated more cells labeled with interneurons markers. To verify this theory, we detected pax2 mRNA and found them located in the VZ of mutant mice. This result uncovered novel function of p57 other than CKI in regulating differentiation during neurogenesis.

$p57^{kip2}$ regulates timely cell cycle exit and differentiation

Increased number of proliferating and differentiated neurons in p57 null mice raised the possibility that $p57^{kip2}$ might have control on differentiation in addition to inhibition on proliferation. Increased mature neurons in double null mice also indicated p27 and p57 are not required for cell cycle exit. To further understand the mechanisms that controls cell cycle exit and differentiation, we performed gain-of-function analysis in chick embryo. First we overexpressed mouse p57 in stage 12-14 chick embryos. In VZ cells overexpressed with p57, cellular proliferation was forced to cessation assayed by BrdU staining (Fig.6E), whereas proliferation was not perturbed by vector alone (Fig.6A). Overexpression of p21 and p27, which also have conserved N-terminal CDK inhibiting domain, arrests cell cycle progression as well (Fig.6C, D). The inhibitory domain alone including 3-10 helix structure (Hashimoto *et al.* 1998) is able to drive cell cycle withdrawal (Fig. 6F). In contrast, p57 with N-terminal inhibiting domain deleted did

not affect cell division (Fig. 6G). The chick EST sequence with conserved N-terminal CKI domain has the same ability of arresting cell cycle progression (data not shown). Therefore the CDK inhibitory domain in all Cip/kip family is sufficient and required to stop proliferation in transfected precursor cells.

Next we asked whether other domains in p57 affect neuronal differentiation. Since full-length p57 forces cell cycle exit and reduces number of precursor cells, p57 sequence with N-terminal CKI domain deleted was transfected. Fewer pax2 and lim1/2 neurons were found in transfected side compared to the control one (Fig. 7B, C). In the C-termius, p57 is distinguished from p21/p27 with its unique domains following CDK inhibitory region, including proline rich domain, acidic region and QT domain (Fig.7A). p57 with CKI domain truncated had decreased percentage of pax2+ and lim1/2+ neurons similar to QT domain transfection, while the region 280-335aa is less efficient in reducing pax2 and lim1/2 interneurons. Interestingly, both ΔCKI and QT do not have the same inhibitory effect on generic neuronal markers TuJ and p27 expression. Rather it seemed to increase the expression of these markers in the transfected side (Fig 7D, E, I and J). Therefore it indicates that region at N-terminus of QT interfere with the induction of cell fate determinants and has distinct affect on the aspect of neuronal differentiation. This result supported the hypothesis that p57 has other function on differentiation than cell cycle control. It controls the timing expression of cell fate determinants and the generic neuronal markers during neuronal differentiation.

Discussion:

Multiple CKIs provide levels of control on cell cycle progression in the early development. However it has not been fully characterized for CKIs regulating proliferation and neuronal differentiation in the developing vertebrate spinal cord. In this paper, we examined CKIs expression and their function during neurogenesis of the vertebrate spinal cord. CKIs are overlapped but complementarily expressed. They have unique and redundant roles in regulating cell proliferation. $p57^{kip2}$ has dual functions in neurogenesis, with CKI domain inhibiting cell cycle re-entry and QT domain on timing differentiation.

p27 appears not able to compensate for the loss of p57, and CKI might be dispensable for the cell cycle exit in the developing spinal cord.

CKIs control cell cycle progression in developing spinal cord

During neurogenesis, determined progenitor cells exit cell cycle after certain cycles and initiate differentiation programs. Studies showed that the timing of these two steps could vary in distinct neuronal subtypes. For example in vertebrate spinal cord, cell fate specification in motor neuron and other interneurons is not identical regarding to their cell cycle states. To explore the expression pattern and functions of CKIs, we might shed some lights on the difference of integrated proliferation and differentiation along the spinal cord axis. CKIs have proposed functions of arresting cell cycle progression at G1/S and G2/M transition points. In particular, $p57^{kip2}$ of Cip/kip family has critical role in inhibiting cyclin E-CDK2 complex, executing the checkpoint at G1 to S entry. Studies have showed distinct distributions of CKIs in diverse contexts. In the spinal cord, we showed that Cip/Kip family members are all expressed in overlapped and complementary regions during neurogenesis. p21 is restricted in V2 domain and p27 is mainly expressed in mature neurons along dorsoventral axis in MZ. BrdU pulsing study indicated that $p57^{kip2}$ is expressed in newly born interneurons that are exiting from cell cycle. Reduced level of pax6/pax7 and diverse overlapping with mature neuronal markers in p57+ cells further supported that these cells are at the transition from proliferation to differentiation. Colocalization of p57, cyclin D1 and homeodomain transcription factors indicated that differentiating neurons have the capacity of cell cycling, requiring CKIs to inhibit the residual Cdk activities. Loss of p57 in immature neurons, therefore leads to inappropriate S phase entry, probably with remained cell cycle machinery as we observed low level of pax6 and high level of cyclin D1 in p57 expressing cells.

Our results in p57 mutant mice showed that ectopic S and M phase cells contribute to the increase of dividing cells in the absence of p57. Evidence argued against alternative possibility that the progenitor pool was increased in the absence of p57. First with longer BrdU pulsed, more BrdU+ cells and higher

significance of increased number were observed in mutant mice, indicating accumulating cells re-enter the cell cycle. Second in null mice and gain-of-function studies, there is no evident change of expression for Notch target genes Hes1 and Hes5 and related repressor Groucho/TLE (Data not showed). Third we verified that there is increased activity of CDK phosphorating pRB in the absence of $p57^{kip2}$ (supplement data). pRB is the downstream mediator of CKIs that regulates both proliferation and differentiation (Zhang, 1999). However there is still possibility that some of the differentiating neurons might translocate half-way back to the VZ.

Gain-of-function data suggested that CKI domain is required and sufficient to force cell cycle exit in progenitor cells. However increased number of mature neurons were observed in $p57^{kip2}$ and p27;p57 null mice, indicating that progenitor cells will ultimately exit cell cycle and differentiate lacking the cell cycle inhibitors. p27 and p19 are predominantly expressed in mature neurons. Low level of p27 in vivo is less likely to be released from CDK4/6 sequestration to inhibit cyclin E-CDK2 complex. And INK4 function depends on the availability of Cip/kip family. (Sherr and Roberts, 1999). We also found mouse p19, which inhibits exclusively CDK4/6 is not as efficient as Cip/Kip in arresting cell cycle progression when over-expressed in chick embryo (Data not shown). Therefore other CKIs than p27 and p57 might act redundantly in controlling cell cycle withdrawal. Alternatively, as observed in Drosophila (de Nooij, *et al.* 1996; Lane *et al.*1996) and other vertebrate systems, CKI cooperates with reduction of cell cycle machinery in regulating cell cycle arrest before cells differentiate. The expanded dividing cells predominantly observed ventral spinal cord of p57 null mice (Fig.3B) is consistent with the finding of highly expressed cyclin D1 in p57+ cells. No obvious change of cyclin D1 in null mice explained the inappropriate S phase re-entry of immature neurons in the absence of G1/S checkpoint control.

Dual function of $p57^{kip2}$ in neurogenesis

In vertebrate retina, p57 was detected at different stages and its role in regulating neuronal differentiation at late stages was suggested (Dyer and Cepko, 2000). In the midbrain, p57 was proposed to cooperate with nuclear orphan receptor Nurr1 in developing dopamine cells (Joseph *et al.*, 2003). We provided

evidence that p57 has dual functions in regulating proliferation and differentiation in the developing spinal cord. In the absence of p57, immature neurons re-entered S phase and increased number of dividing cells were seen. Supposedly there is no inhibition on differentiation, reduced post-mitotic cells and/or delayed neuronal differentiation would be expected due to the extra round of cell cycle for the differentiating neurons. In contrast, increased number of mature neurons were observed in null mice, indicating normal or increased output from the pool of dividing cells. In-situ result of pax2 supported that differentiation program is initiated earlier in null mice (Fig. 5). The onset of differentiation also occurred earlier along rostrocaudal axis of spinal cord in the knockout mice (Data not shown). These data prompted us to the hypothesis that $p57^{kip2}$ inhibits differentiation as well as proliferation. The proposed novel function of p57 was consistent with its restricted expression in immature neurons, then down-regulated in mature neurons. This hypothesis was further supported by the gain-of-function in chick embryos. $p57^{kip2}$ with CKI domain truncated and its QT domain were both able to inhibit expression of homeodomain transcription factors. Therefore the QT domain in the C-terminus of p57 act in controlling timely cell fate specification independent of cell cycle regulation. Taken together, we proposed that p57 controls proliferation with CKI domain and timely differentiation with its QT domain.

Cell autonomous and non-autonomous activity of $p57^{kip2}$

$p57^{kip2}$ is found to be expressed in the interneurons, but not in V2 and MN regions. In p57 null mice, V0 and V1 but not V2 interneurons were increased, indicating its cell autonomous activity. However we observed robust apoptotic cells in MN as well as in interneurons regions. This non-autonomous change could result from defective neighboring interneurons or muscular abnormalities during motor neuron axon pathfinding. The exact mechanism needs to be determined.

The roles of QT domain in neuronal differentiation

It has been long proposed for unique function of QT domain of p57 (Matsuoka *et al.*, 1995; Lee *et al.*, 1995). Recent studies showed activities of other domains in p57 (Reynaud *et al.*, 2000; Yokoo *et al.*,

2003) and other functions of CKIs than cell cycle inhibitors (Zezula *et al.*, 2001; Coqueret, 2003). We proposed opposite regulation of differentiation pathways by p57 QT domain in the vertebrate spinal cord. It inhibited the expression of homeodomain transcription factors but not changing the cell fates. At the meantime QT domain alone can induce generic neuronal differentiation, including β-tubulin. Our results strongly supported recent study in xenopus tropicalis that depletion of p27(Xic1), the only known Cip/kip family caused an increase in proliferation and a suppression of the neuronal differentiation marker, N-tubulin (Carruthers et al., 2003). Although p27 also has QT domain in C-terminus, it shares only 44% identity with p57 QT. Overexpression p27 QT has much less efficiency in inhibiting HD factors than p57 QT (Data not shown). No DNA binding region was identified on QT domain, therefore it is likely that its function is operated through protein-protein interactions. In the QT domain, it contains nuclear localization signal and CDK phosphorylation site (Matsuoka et al., 1995; Lee et al.,1995). Therefore it is attracting to speculate that recruitment of activator or repressor on HD or neuronal promoters by QT domain might be regulated by the N-terminal CKI domain. In this mechanism, proliferation and differentiation during neurogenesis might be integrated and coordinated by one molecule.

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Figure Legends

Fig.1. CKIs display distinct spatial expression patterns in the developing vertebrate spinal cord. (A)It showed that $p27^{cip1}$ is highly expressed in V2 interneuron region in the spinal cord of E11d mouse embryo. (B) By contrast, $p27^{kip1}$ is widely expressed in mature neurons in mantle zone (MZ) at the same stage. (C) $p57^{kip2}$ is initiated early at E9.5-E10d. (D) showed at E11d the unique pattern of $p57^{kip2}$ along dorsoventral axis of the spinal cord, absent from V2 and motor neuron (MN) regions. (E) and (F) displayed the similar pattern of p57 mRNA in E11d mouse and E4d chick embryos. (G) summarized the unique and complementary expression of CKIs during neurogenesis.

Fig. 2. $p57^{kip2}$ is expressed in differentiating neurons at G1/G0 phase. (A) to (E) are the E11d embryos pulsed with diverse lengths of BrdU prior to collection. When pulsed with 2 to 8hr, p57+ cells were rarely labeled with BrdU ((A) to (D)). $p57^{kip2}$ is located laterally outside the ventricular zone (VZ), which was designated as VZ^L. Double stained cells with p57 and BrdU were observed in embryos pulsed with 12 hours (E). (F) is the schema of p57 showing its expression initiated about 8 hours after last cell cycle. (G) to (P) p57+ cells are the nascent interneurons that have reduced progenitor markers and increasing level of differentiating markers. (G) and (H) showed p57 expression in relating to progenitor cell factors pax7/pax6. In the intermediate spinal cord, low level of pax6 existed in p57+ cells (I). bHLH factor Ngn2 also overlapped with p57 at VZ^L (J). (K) p57 is expressed in the interneurons that express cell fate specification factor Lhx1. (L) Interneurons from dN6 to V2 have high level of cyclin D1 expression, overlapped with p57 from dN6 to V1 regions. (M) to (P) are the E11.5d mouse embryos showing that mature neuronal markers p27(M), NeuN(N), TuJ(O), Neurofilament (P) were coexpressed with p57 to the variable degrees. (Q) Schematic diagram of p57 expression in regarding to different markers in the developing vertebrate spinal cord.

Fig.3. Loss of p57 resulted in abnormal cell cycling. The proliferative zone was evidently expanded laterally in the cord (B,D). Increased number of dividing cells labeled with BrdU was observed in p57 null mice at E10.5d (B) and E11.5d (D) compared to the wild type embryos (A) and (C). One third of dorsal spinal cord delineated with square brackets in (C) and (D) was used to quantify the BrdU+ cells in wild type and mutant mice. (E) Significantly increased number of BrdU+ cells were observed in mutant compared to wild type littermates ($p=0.0287$) when pulsed with BrdU for 4 hours. Highly significance was seen in longer pulse with 8 hours ($p=0.0018$). (F)-(J) M phase cells stained with phh3 were increased in mutants at E11.5d (J). Ectopic M phase cells were observed laterally in VZL and MZ in mutants (G, I). † $p<0.05$, * denotes $p<0.01$.

Fig. 4. Abnormal cell cycling in p57 null mice leads to ectopic cell division and cell death. (A) and (A') showed the diagram of interkinetic nuclear migration in the spinal cord. (B) Normally dividing cells are located in VZ and prox1, which is highly expressed in VZ^L, marked the outside boundary of proliferating cells. (C) In p57 null mice, dividing cells were observed in VZ^L, labeled with prox-1 and BrdU as indicated by the arrowhead. More cell death was seen in p57 mutants (E) compared to wild type (D) Immature neurons may enter extra round of cell cycle in the absence of p57. 2 hours BrdU pulse at E10.5d embryos showed that differentiating neurons (G, I) incorporated BrdU in the absence of p57. The inset in (G) clearly exhibited pax2 cells stained with BrdU. (J) and (K) displayed the model that loss of p57 in the nascent neurons at G1/S transition resulted in S phase re-entry and BrdU incorporation.

Fig. 5. More mature neurons were generated in p57 mutant. Signifcantly increased number of en-1 cells were observed in p57 single (B) and p27;p57 double mutant (C) compared to wild type (A). V0 and V1 but not V2 were increased (D). No more increase was seen in double mutant compared to p57 single knockout mice. At different stages, more en-1 cells were found in p57 mutant. Highest significance were seen in E11.5d with 2-fold of increase in the null mice (I) ($p<0.001$). Differentiating neurons appeared in

VZ (D, E) of mutant mice, indicating premature induction of HD factors. In situ hybridization of pax2 proved mRNA present close to central canal (H) in mutant compared to wild type (G).

Fig. 6. CKI domain conserved in Cip/kip family is sufficient and required for arresting cell cycle in proliferating cells. (A) showed four important domains in p57, CKI domain followed by proline domain, acidic domain and QT rich domain. Constructs used belowed were compared to the full length sequence. (B) pCIG vector alone did not disturb cell division assayed by BrdU. (C), (D) and (E) were the embryos transfected with mouse p21, p27 and p57, respectively. Dividing cells were forced to exit cell cycle by overexpression of cip/kip. CKI is sufficient to arrest cell cycle (F). With CKI domain truncated, proliferation was not altered, suggesting that it is required for its function of cell cycle control.

Fig.7. QT domain regulates the timing of neuronal differentiation. (A) _CKI and QT domain (F) did not affect cell division but it inhibited the transcription of HD domains pax2 (B, G) and Lim1/2 (C, H). However they are both able to increase the expression of p27 (D, I) and TuJ (E, J). (K) Zebra fish _-tubulin enhancer reporter showed the activation in mature neurons. Cotransfected with p57QT ectopically induced the reporter in VZ (L).

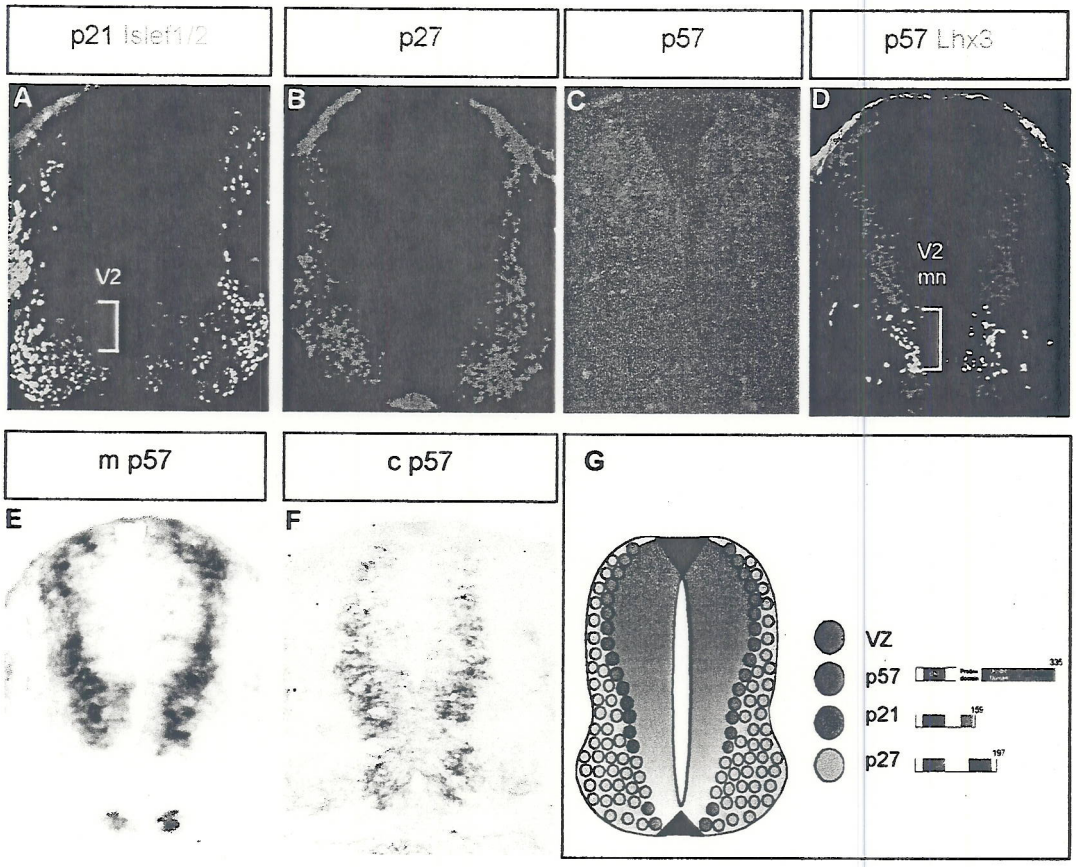
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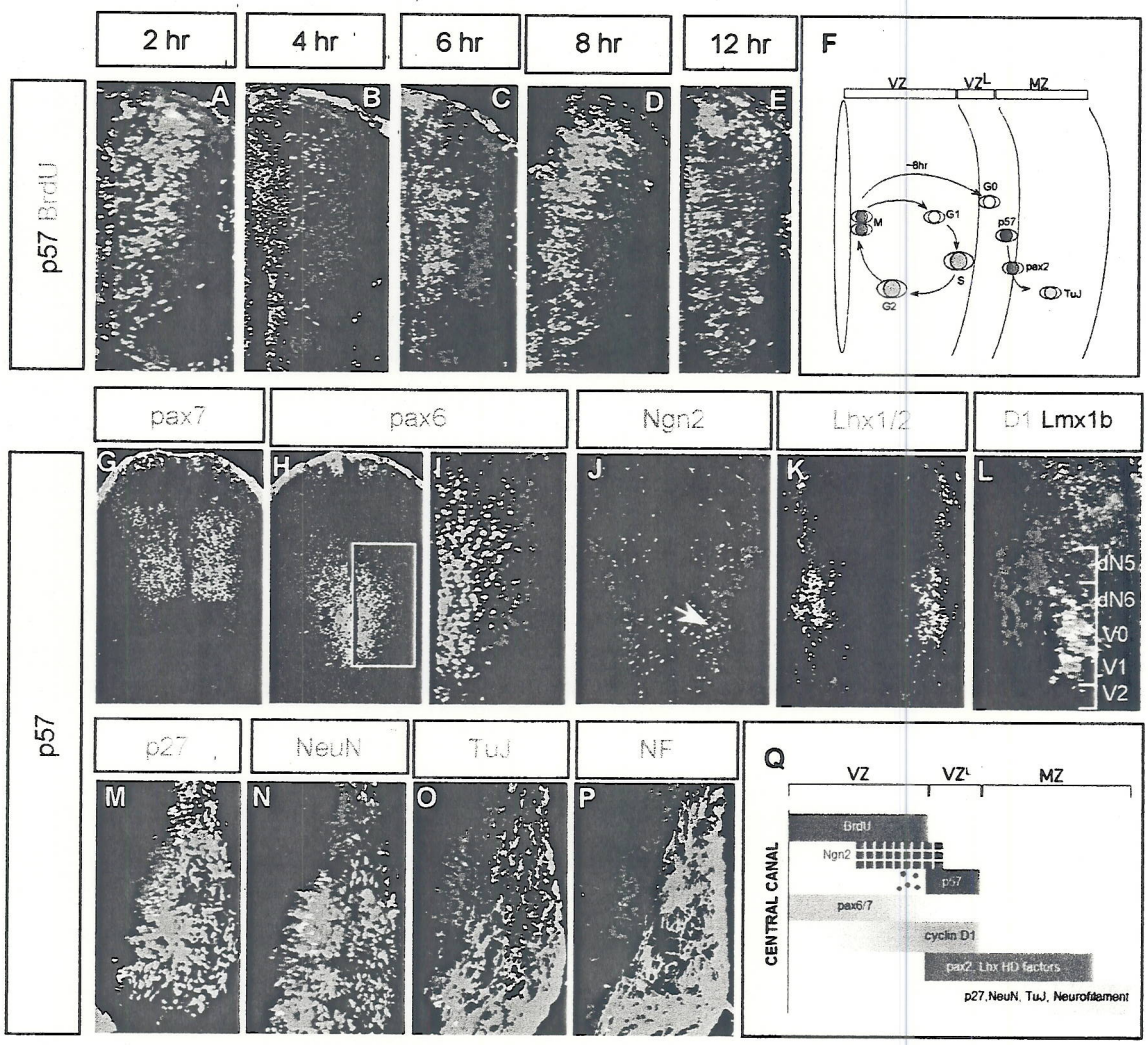
Figure 1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p57
p27
NeuN
TuJ
NF
M
N
O
P
Q
VZ
VZL
MZ
BrdU
Ngn2
p57
pax6/7
cyclin D1
pax2, Lhx HD factors
p27,NeuN, TuJ, Neurofilament
CENTRAL CANAL

Figure 2

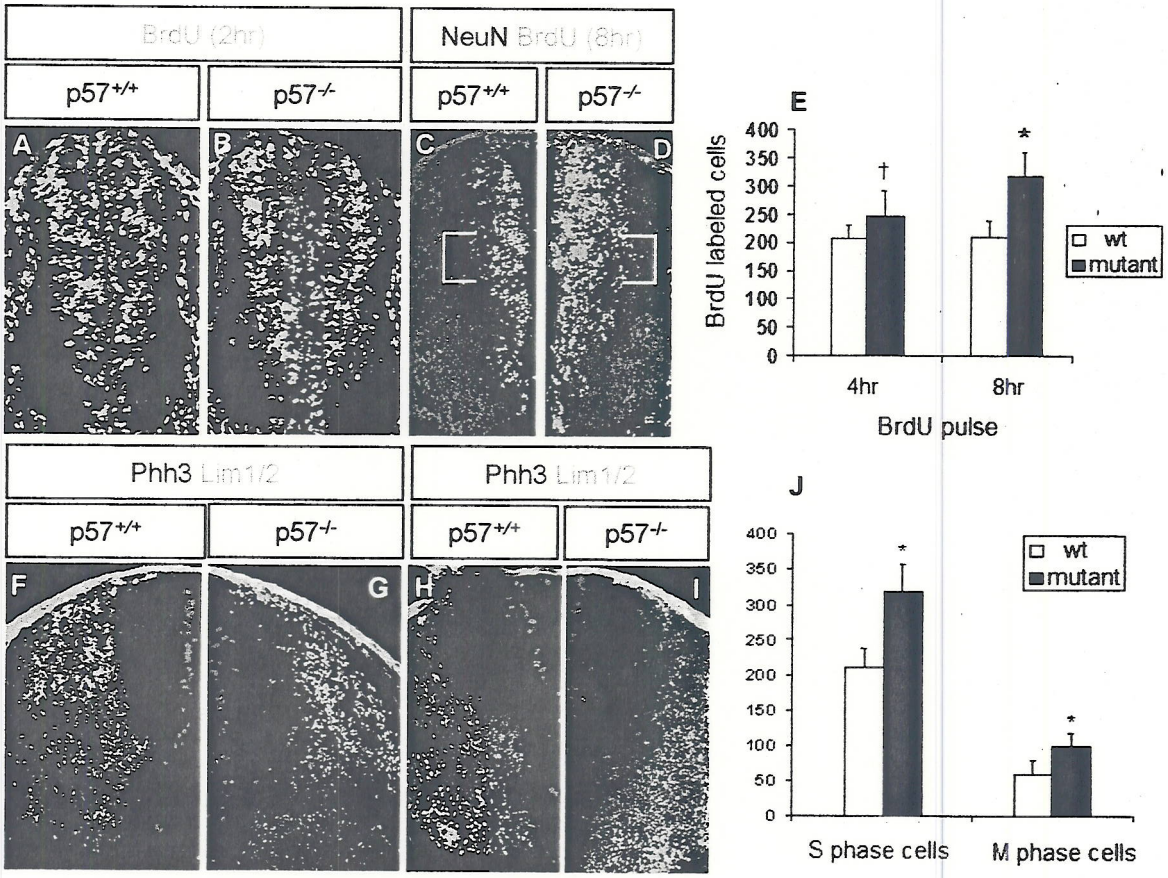
BrdU (2hr)
NeuN BrdU (8hr)
p57+/+
p57-/-
p57+/+
p57-/-
A
B
C
D
Phh3 Lim1/2
Phh3 Lim1/2
p57+/+
p57-/-
p57+/+
p57-/-
F
G
H
I
E
BrdU labeled cells
400
350
300
250
200
150
100
50
0
†
*
wt
mutant
4hr
8hr
BrdU pulse
J
400
350
300
250
200
150
100
50
0
*
*
wt
mutant
S phase cells
M phase cells

Figure 3

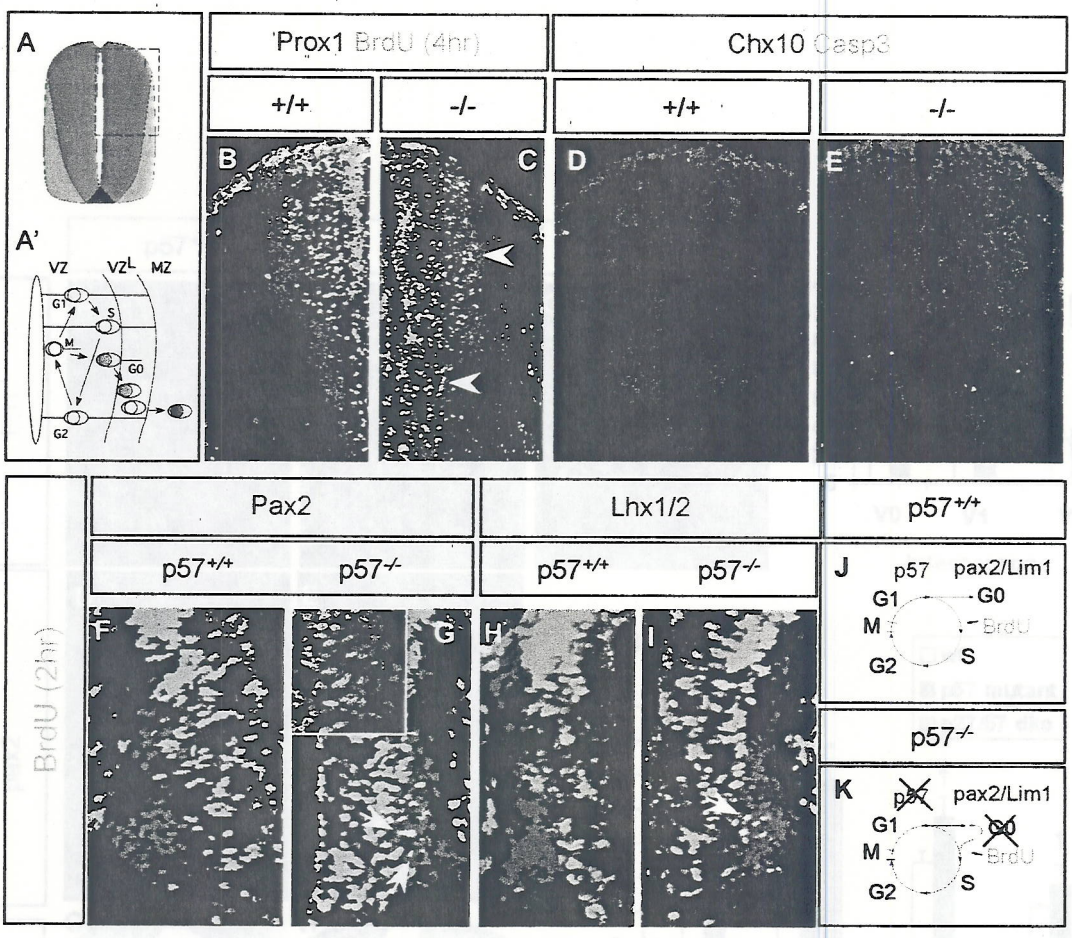
A
A'
VZ
VZ^L
MZ
G1
S
M
G0
G2
Prox1 BrdU (4hr)
Chx10 Casp3
+/+
-/-
+/+
-/-
B
C
D
E
Pax2
Lhx1/2
p57+/+
p57+/+
p57-/-
p57+/+
p57-/-
BrdU (2hr)
F
G
H
I
J
p57
pax2/Lim1
G1
G0
M
BrdU
G2
S
p57-/-
K
p57
pax2/Lim1
G1
G0
M
BrdU
G2
S

Figure 4

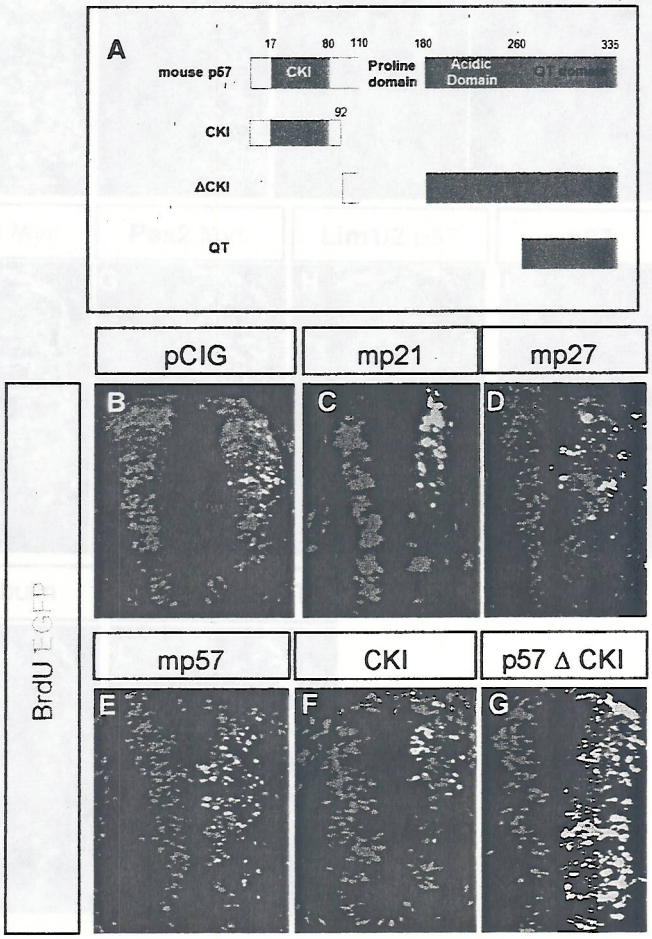
A
17
80
110
180
260
335
mouse p57
CKI
Proline domain
Acidic Domain
92
CKI
ΔCKI
QT
pCIG
mp21
mp27
B
C
D
BrdU EGFP
mp57
CKI
p57 Δ CKI
E
F
G

Figure 6