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COVER PAGE

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Grant Title: QTL mapping and phenotypic analysis of dreher suppressing modifying loci

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Summary of Research:
The research funded by the New Jersey Commission on Spinal Cord Research was focused on the roof plate, a signaling center located on the dorsal (top) midline of the developing spinal cord. Our previous research demonstrated that the roof plate is crucial for normal spinal cord development (Millonig et al., 2000). By elucidating the roof plate signals required for spinal cord development, new hypotheses will be generated as to how stem cells can be employed to treat spinal cord injury and disease.

We have investigated the role of the roof plate in dorsal spinal cord development by studying a mouse mutant called *dreher*. Our previous analysis has determined that no detectable roof plate cells are observed in the mutant. This is due to mutations in a gene, *Lmx1a*, necessary for the generation of this important signaling center.

The roof plate and neighboring dorsal midline cells secrete proteins that then direct dorsal progenitor neurons through different phases of their development: proliferation, cell cycle exit, migration and differentiation. The roof plate produces Bmps, proteins important for cell cycle exit, while roof plate plus flanking dorsal midline cells secrete another family of proteins, Wnts, necessary for neuronal proliferation.

In *dreher* because roof plate is not generated, no Bmps are detectable while Wnts are produced normally (Fig 1), providing an important resource for evaluating the differential effects of these signaling molecules during dorsal spinal cord development.

The *dreher* mutant is on a mixed genetic background between two strains, C57BL6 and C3He/J. Interestingly on this mixed background, the severity of the *dreher* phenotype varies considerably suggesting that unlinked genes can modify the effect of the mutation. These unlinked genes are likely to be important for roof plate development and/or signaling.

Our research goals were 2-fold: one, to characterize the effect of the *dreher* mutation of dorsal neuronal development; and two, to determine whether genetic background is responsible for the variability in the *dreher* phenotype.

Research update
Characterize the effect of the dreher mutation of dorsal neuronal development
Due to the differential expression of Bmps and Wnts in the *dreher* mutant, we hypothesized that proliferation and cell cycle exit would be affected in the mutant. We then investigated the ventricular zone (VZ), which is the area of the developing spinal cord where neural progenitors divide. An increase in the width, area and number of neural progenitors was observed in E12.5 *dr/dr* compared to +/+ embryos.

Once neural progenitors exit the cell cycle they migrate out of the VZ into a lateral region called the mantle zone (MZ). Interestingly, a concomitant decrease in the number of post-mitotic E12.5 neurons was observed in *dr/dr* compared to +/+, suggesting that the absence of roof plate in dreher embryos may be affecting cell cycle control. BrdU labeling was then performed which measures the number of proliferating neural progenitors. In support of our hypothesis an increase in the number of BrdU+ cells was detected in dreher embryos. When a BrdU pulse experiment was performed to investigate the number of progenitor cells that exit the cell cycle and migrate laterally out of the VZ into the MZ, an expected decrease was observed. These data are consistent with our hypothesis that the dreher mouse affects cell cycle exit.

To further investigate this possibility, we performed western analysis using a series of important cell cycle protein regulators (Cyclins A, D1, E, Cdk2, 4, p27, p21, Rb). For these experiments protein was isolated from dorsal and ventral E12.5 spinal cords dissected from +/+ and *dr/dr* embryos. Levels for

most of these proteins were not affected but a significant decrease was observed for both Rb and p21 (Fig 2). Both proteins are important in directing cycling progenitors out of cell cycle and into the differentiation pathway, consistent with the histological phenotype we have described.

In summary, our data has demonstrated that the *dreher* mutation exhibits a cell cycle defect, which is likely due to the imbalance of pro-mitotic Wnt proteins and pro-differentiative Bmps. These findings have led to the hypothesis that either increasing Wnt or decreasing Bmp activity could increase the efficiency of future stem cell therapies. By affecting Bmp or Wnt activity in this way the number of neural progenitors is likely to increase and this could have potential beneficial effects in the number of cells that differentiate into spinal cord neurons.

Investigate whether genetic background is responsible for *dreher* phenotypic variability
To identify other genetic loci that control roof plate and dorsal spinal cord development, we took advantage of the variability in the *dreher* phenotype observed on a mixed B6 and C3H background. Congenic strains were generated for both B6 and C3H such that the mutation was placed on either an isogenic B6 or C3H background. This was accomplished by backcrossing the *dreher* mutation for 8-10 generations until the entire genome was B6 or C3H. This was determined by genotyping these mice for 64 markers that are polymorphic between B6 and C3H and spanned the genome in 20cM intervals.

Once these congenic lines were generated, a number of phenotypes were compared between lines. On the mixed B6/C3H background, ~7% of *dr/dr* die post-natally (total number of mice=53). Interestingly, this lethality is completely rescued for the *dreher* C3H congenic. In +/*dr* x +/*dr* crosses expected 25% of F2 progeny were dr/dr (total number of F2 mice=51). The opposite result was observed in the B6 *dreher* congenics: zero *dr/dr* mice survived past P0 (total number of F2 mice=51). These results indicate that unlinked modifier genes affect the penetrance of the *dreher* lethality phenotype.

We then initiated a cross to map the modifiers responsible for the phenotypic difference in lethality. $+/dr^{C3H}$ x $+/dr^{B6}$ mice were mated to each other, F1 +/*dr* C3H/B6 progeny were generated and then mated to each other to produce F2 mice. The day at which the *dr/dr* mice die is then determined. The cross is ongoing with the future plan to generate ~100 F2 *dr/dr* mice and then genotype them for unlinked genetic markers to map the genomic position of modifier genes using QTL analysis.

Project challenges: None out of the ordinary

Implications for future research: Our Aim 1 data support the current model that Wnts are pro-mitotic and Bmps are pro-differentiative during spinal cord development. These results have led to hypotheses concerning the modulation of Bmp/Wnt levels to increase the efficiency of stem cells differentiating into spinal cord neurons as a potential cell based therapy to treat spinal cord injury and disease.

Our Aim 2 analysis has demonstrated that the *dreher* phenotypes are modified by genetic background. Future research will be focused on mapping the genomic positions of these modifiers and identifying the genes responsible these effects. This will lead to a greater understanding of roof plate and spinal cord development.

Publications: Aim 1 is currently being prepared as a manuscript for submission

FIGURES

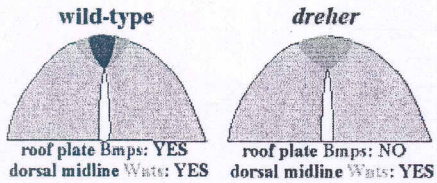

Fig 1. The roof plate secretes two classes of morphogenetic proteins: Bmps and Wnts. Bmps are produced exclusively by roof plate cells (blue) while Wnts (aqua) are secreted by roof plate plus adjacent dorsal midline cells. The *dreher* mutant specifically affects the production of Bmps without significantly affecting Wnt production.

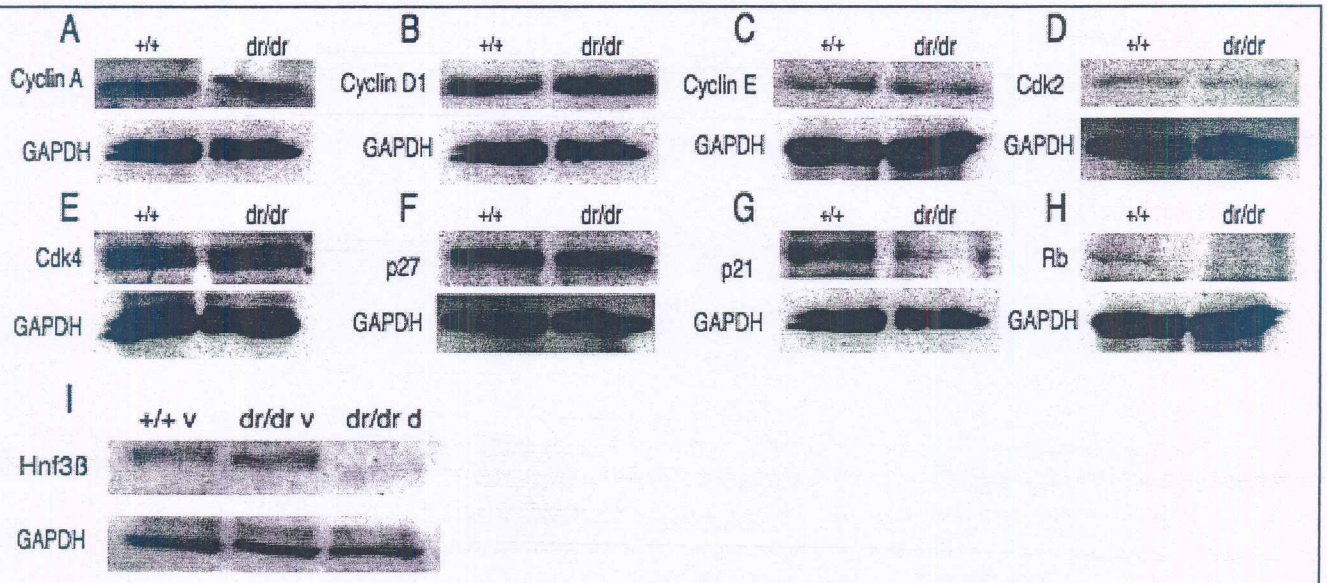

Fig 2. Cell cycle protein analysis in wild type and *dreher* E12.5 dorsal spinal cords. (A-H) Western Blot analysis for Cyclin A (A), Cyclin D1 (B), Cyclin E (C), Cdk2 (D), Cdk4 (E), p27 (F), p21 (G) and Rb (H). The analysis demonstrated a significant reduction in p21 (G) and Rb (H) levels in dreher dorsal E12.5 spinal cord compared to wild type controls. GAPDH was utilized as a loading control. (I) Western blot analysis was performed for a ventral spinal cord marker, Hnf3ß. Signal was detected in both wild type and dreher ventral but not dorsal lysates indicating that the dissections successfully divided the spinal cord into dorsal and ventral halves. GAPDH was used as a loading control.

RESULTS

Ventricular zone *dreher* phenotype

Due to the differential expression of Bmps and Wnts in the *dreher* mutant, we hypothesized that the ventricular zone (VZ) of the developing spinal cord would be affected by the mutation. Immunostaining for dorsal ventricular markers supported this possibility, uncovering a wider dorsal VZ in E12.5 *dr/dr* compared to +/+ littermate controls (Fig 1A). To investigate this further, immunohistochemistry for Pax7 (a dorsal VZ marker) was performed at E10.5 and E12.5 and the width of the dorsal ventricular zone was measured in +/+ and *dr/dr* embryos. For reproducibility the same region of the developing spinal cord was always examined. At E10.5 measurements were taken dorsally (position 1), which was defined by the dorsal limit of the ventricle. At E12.5, width measurements were taken both at position 1 and a more central position along the D-V axis (position 2), defined as the ventral limit of Pax7 immunostaining (Fig 1B). Give reason No difference in the width of the dorsal ventricular zone was observed at E10.5. However, by E12.5 a significant 12% and 25% increase in width was observed at positions 1 and 2, respectively (Fig 1C). VZ area measurements for E12.5 +/+ and *dr/dr* embryos also demonstrated in a significant increase, indicating a potential VZ phenotype in *dreher* (Fig 1D).

To investigate whether the increased ventricular width and area were due to an increase in the number of progenitors, the number of $Pax7^+$ cells was counted at E10.5 and E12.5. No difference between +/+ and *dr/dr* littermates was observed at E10.5. At E12.5 a significant increase in the number of $Pax7^+$ cells was detected (Fig 1D). To examine

whether the increased width and area could also be due to decreased packing density of progenitors, the number of $Pax7^+$ cells per unit volume was counted. No difference was observed, indicating that the increased width and area of the VZ is due to elevated number of cells and not decreased packing density (Fig 1F). did we do this?

Mantle zone *dreher* phenotype

The above VZ results supported our hypothesis that the *dreher* mutation affects the number of dorsal progenitors. If this were the case we might expect to observe a decrease in the width, area and number of dorsal post-mitotic neurons in the E12.5 dorsal spinal cord. To investigate this possibility immunostaining for NeuN (Fig 2A), a marker of differentiated mantle zone (MZ) neurons, was performed and similar measurements as described above were taken. No difference in MZ width measurements was observed at E10.5 (+/+: 16.20 +/- 8.68; *dr/dr*: 12.55 +/- 9.56; *P*=.22) microns? but at E12.5 a significantly smaller mantle zone was observed in *dr/dr* at dorsal positions 1 and 2. However, this effect was limited only to the dorsal spinal cord, as ventral measurements revealed no difference in the width of the MZ (Fig 2B, C). The area of the dorsal MZ was also significantly smaller in *dr/dr* compared to +/+ littermates (Fig 2D).

To investigate whether these differences could be simply explained by a decrease in the number of $NeuN^+$ cells, positive cells were counted in the E12.5 developing dorsal horn – put specifics into VZ. A 25% reduction in the number of $NeuN^+$ cells was observed in the *dreher* (Fig 2E). The packing density was also investigated but no difference was detected (Fig 2F), indicating that the smaller mantle zone in *dr/dr* was due to decreased

cell number and not increased cell packing. Finally, apoptosis was investigated as a contributing factor to this difference in cell number by anti-activated Caspase 3 immunostaining and TUNEL labeling and no difference was observed (data not shown). Together these results indicate that by E12.5, the *dreher* mutation results in a significant increase in the number of dorsal progenitors and a decrease in the number of dorsal post-mitotic neurons.

Header

Given that Wnt signaling is pro-mitotic and Bmps are pro-differentiative, we explored whether the *dreher* mutation affects the number of replicating cells by performing BrdU immunohistochemistry at E12.5 (Fig 3A). To identify $BrdU^+$ cells in the dorsal VZ, adjacent sections were immunostained with anti-BrdU and then overlaid with Pax7. We observed a significant increase in the number of $BrdU^+$ cells in the E12.5 *dreher* spinal cord (Fig 3B). To examine whether this increase was due simply to a total increase in the number of ventricular zone cells, the BrdU labeling index was calculated for the dorsal ventricular zone at both E10.5 and E12.5 (specifics in MM). At E10.5, no difference in the BrdU labeling index was observed, but at E12.5 a significant increase was detected (data not shown, Fig 3C).

The increase in the number of VZ and $BrdU^+$ cells in *dreher*, coupled with the reduction in MZ cells, could be indicative of a cell cycle phenotype whereby fewer cells are exiting the cell cycle and migrating laterally out of the VZ into the MZ. To investigate this possibility, a BrdU pulse experiment was performed at E12.5 where pregnant *+/dr* females were injected with BrdU at E12.0 and then sacrificed 12 hours later at E12.5.

This 12 hour period allows for the visualization of VZ cells that have exited the cell cycle and migrated laterally into the MZ. Double immunohistochemistry was then performed with antibodies against BrdU, as well as Ki67, a proliferative cell marker that labels all proliferating cells in the VZ. Ki67/BrdU double-labeled cells were delineated as VZ cells, while BrdU singly labeled cells situated in the MZ were identified as being post-mitotic (Fig 4A). The number of singly labeled $BrdU^+$ cells was counted dorsally and in a more central position–how was this determined? (Fig 4B). A significant decrease in the number of singly labeled $BrdU^+$ cells in *dr/dr* at position 1 but not at position 2 was observed (Fig 4C). **need #s for graph- do we really want to do this-NO? What happens when combine the data** These data are consistent with the *dreher* mutation causing a decrease in cell cycle exit and lateral migration of VZ cells, an effect that reduces as distance increases from the dorsal midline.

Mechanisms Underlying Cell Cycle Phenotypes Observed in *d/dr*

To examine the underlying mechanism affecting the cell cycle exit in dorsal VZ cells, the expression levels of key cell cycle proteins expressed in the developing spinal cord were examined by Western analysis. For accurate measurements on dorsal cell cycle component expression, E12.5 spinal cords were dissected from +/+ and *dr/dr* embryos and then divided into dorsal and ventral portions. Western blots for HNF3β, which is expressed solely in the ventral neural tube (Monaghan et al., 1993; Hu et al., 2004), was used as a control to ensure that the spinal cords were properly divided into dorsal and ventral portions. This analysis revealed no difference in HNF3β levels between +/+ and

dr/dr ventral lysates and no expression in dorsal lysates (Fig 5A), indicating that the dissections to separate dorsal and ventral halves of the spinal cord were successful.

Westerns were then performed on +/+ and *dr/dr* E12.5 dorsal hindlimb spinal cords using antibodies against the major cell cycle components, Cyclin A, Cyclin E, Cyclin B1, Cyclin D1, Cdk2, Cdk4, Cdk6, as well as 3 cell cycle regulatory proteins: p21, p27 and Rb. Antibodies against GAPDH were used as internal loading controls. No significant difference in the protein levels of Cyclin A, Cyclin E, Cyclin B1, Cyclin D1, Cdk2, Cdk4, Cdk6 and p27 was observed in *dr/dr* as compared to +/+. However, an approximate 40-50% reduction in p21 and Rb levels was observed in *dr/dr* at this age (Fig 5B). These results indicate that the major cell cycle machinery components governing G_1/S transition are disrupted in *dreher*. Together with BrdU pulse results, these data indicate that the *dreher* mutation results in cell cycle deregulation and causes a portion of progenitor cells to re-enter the cell cycle, rather than exiting and terminally differentiating.

FIGURE LEGENDS

Figure 1. Dreher dorsal ventricular zone (VZ) phenotype. (A) Pax7 immunohistochemistry counterstained with DAPI is consistent with a dreher dorsal VZ phenotype. Explain more? (B) Schematic diagrams of E10.5 (left panel) and E12.5 spinal cord (right panel) demonstrating ventricular zone measurement positions (solid lines) a and b along the dorsal-ventral axis. The position where ventricular cell density was counted is also illustrated (hatched box). Abbreviation: sl, sulcans limitans. (C) The effect of the dreher mutation on ventricular zone width is represented by a bar graph illustrating the differences at position a in E10.5, and positions a and b in E12.5, +/+ (white bars) and dr/dr (grey bars) embryos (D) The increase in ventricular zone cell density in dreher (grey bar) as opposed to wild type (white bar) is illustrated.*, $P<0.05$; ***, $P<0.001$

Figure 2. The dreher mutation decreases the width of the dorsal post-mitotic zone at E12.5. (A) Immunohistochemistry for NeuN on transverse sections of E12.5 spinal cord demonstrates a significant decrease in the width of the dorsal mantle zone in dr/dr embryos (right panel) as compared to +/+ embryos (left panel). White line denotes the width of the mantle zone. (B) A schematic diagram illustrating the locations (positions a, b and c) along the dorsal-ventral axis that mantle zone widths (solid lines) were measured. The position in which post-mitotic cell density was counted is also illustrated (hatched box). Abbreviation: sl, sulcans limitans. (C) The effect of the dreher mutation on mantle zone width is represented by a bar graph illustrating the differences at positions a, b and c in E12.5 +/+ (white bars) and dr/dr embryos (grey bars).

Figure 3. The dreher mutation causes an increase in the number of S phase cells in the dorsal ventricular zone at E12.5. (A) Confocal microscopy for BrdU immunohistochemistry on transverse sections of E12.5 spinal cord illustrates an increase in the number of BrdU+ cells in the dorsal spinal cord of dr/dr embryos (right panel) as compared to +/+ littermates (left panel). (B) The effect of the dreher mutation on the total number of dorsal BrdU+ cells is represented by a bar graph illustrating the difference between E12.5 +/+ (white bars) and dr/dr (grey bars) embryos. (C) The effect of the dreher mutation on the BrdU labeling index is represented by bar graphs illustrating the differences in E10.5 (left panel) and E12.5 (right panel) +/+ (white bars) and dr/dr (grey bars) embryos.

Figure 4. The number of cells migrating laterally out of the ventricular zone is decreased in dreher at E12.5. (A) Schematic demonstrating cell count area a and b (hatched boxes). (B, C) Immunohistochemistry with BrdU and Ki67 showing a reduced number of cells migrating laterally out of the ventricular zone in dreher (C) as comapred to +/+ (B). White boxes deleniate post-mitotic zone.

Figure 5. Cell cycle protein analysis in wild type and *dreher* E12.5 dorsal spinal cords. (A-H) Western Blot analysis for Cyclin A (A), Cyclin D1 (B), Cyclin E (C), Cdk2 (D), Cdk4 (E), p27 (F), p21 (G) and Rb (H). The analysis demonstrated a significant reduction in p21 (G) and Rb (H) levels in dreher dorsal E12.5 spinal cord compared to wild type controls. GAPDH was utilized as a loading control. (I) Western blot analysis was

performed for a ventral spinal cord marker, Hnf3ß. Signal was detected in both wild type and dreher ventral but not dorsal lysates indicating that the dissections successfully divided the spinal cord into dorsal and ventral halves. GAPDH was used as a loading control.

Figure 1

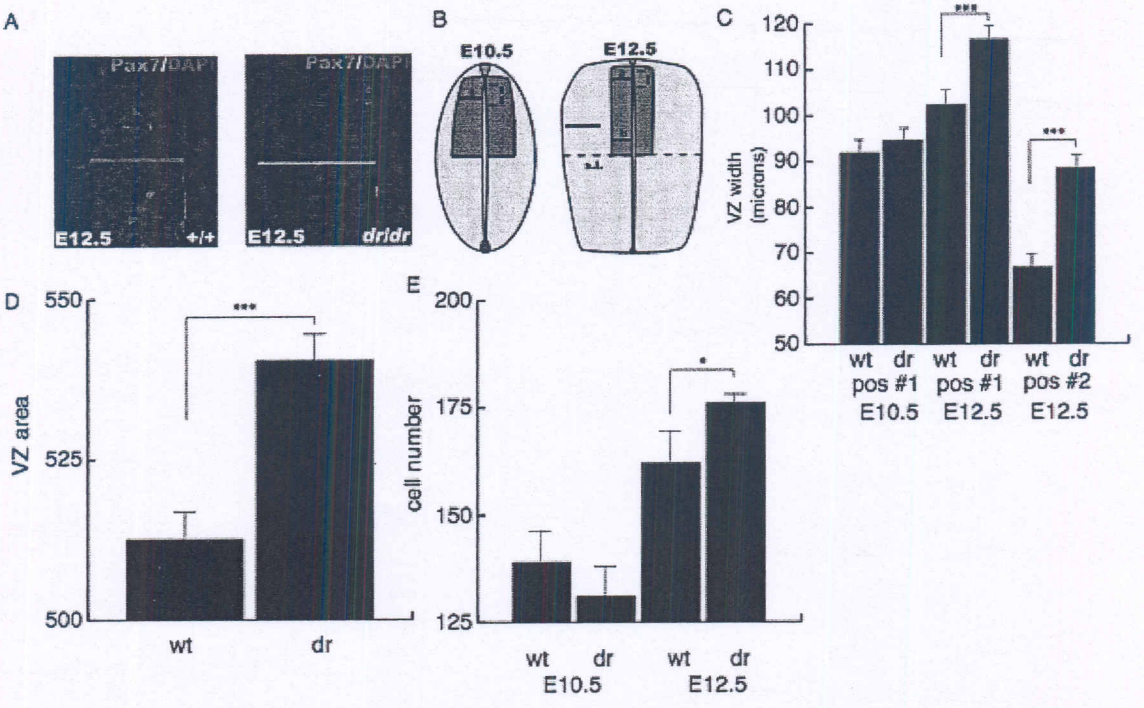

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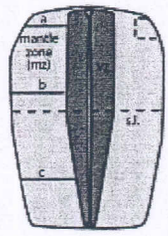
Figure 2



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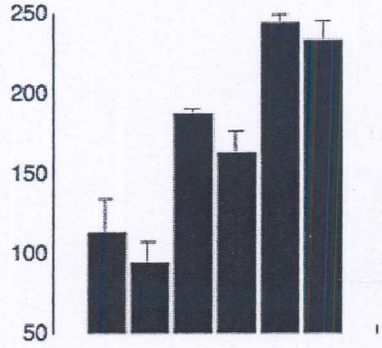

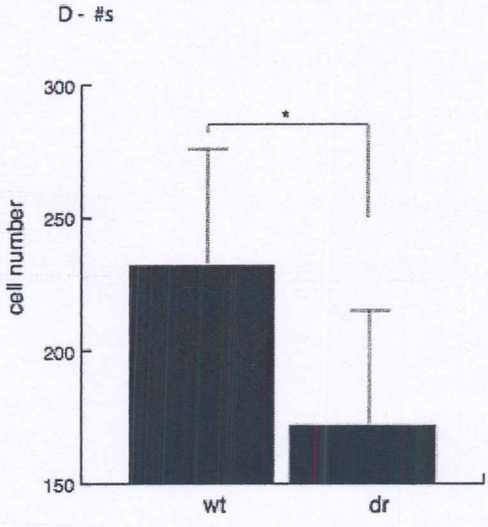

Figure 3

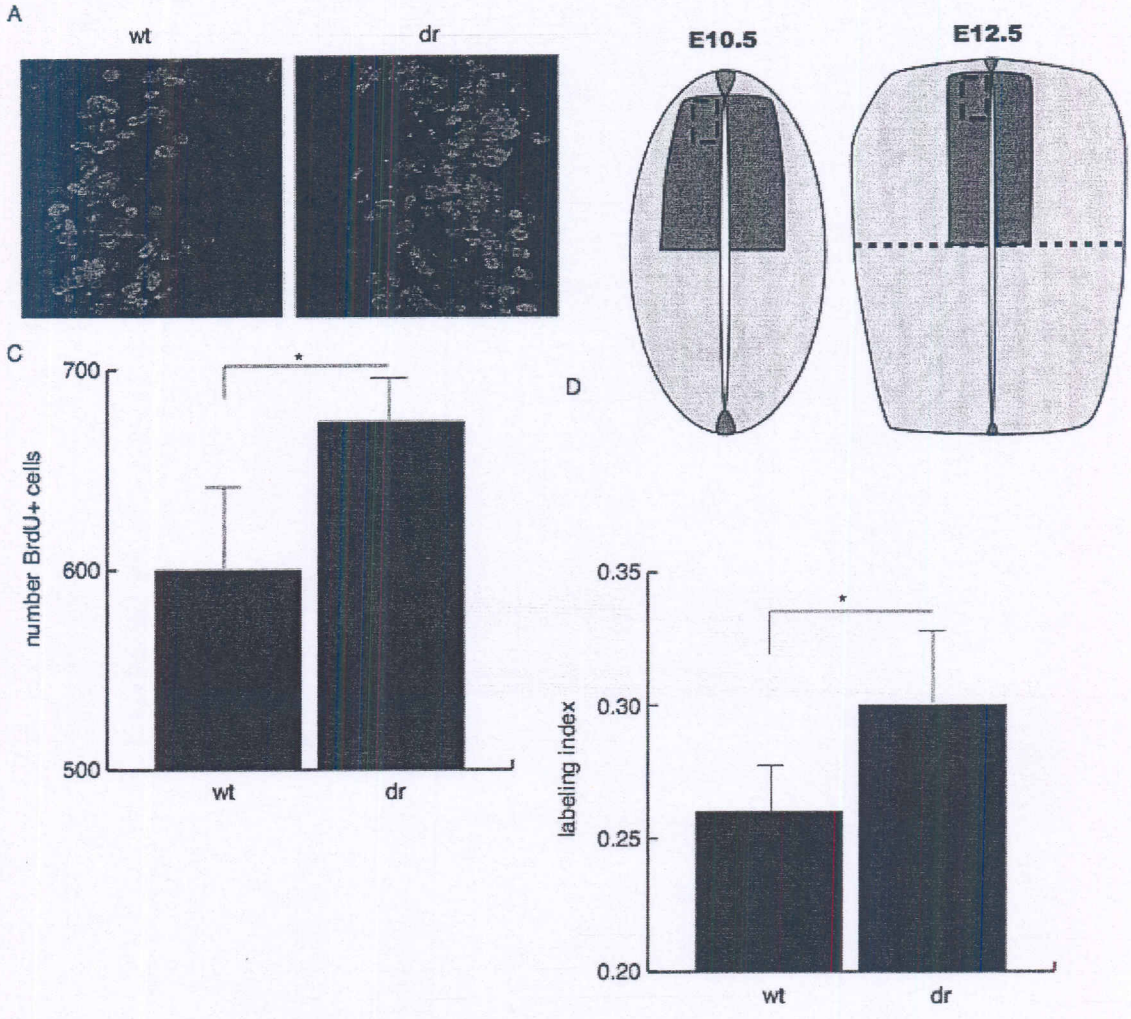

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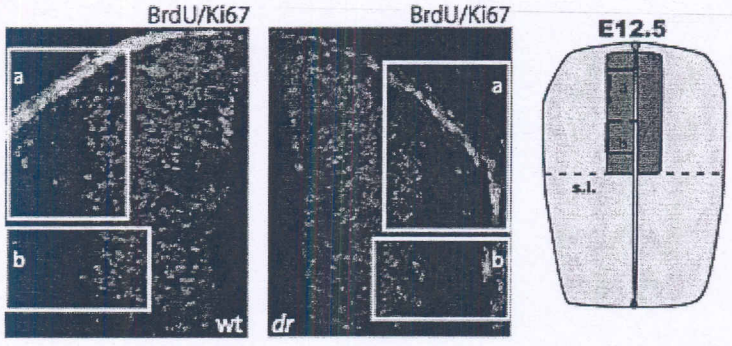
Figure 4



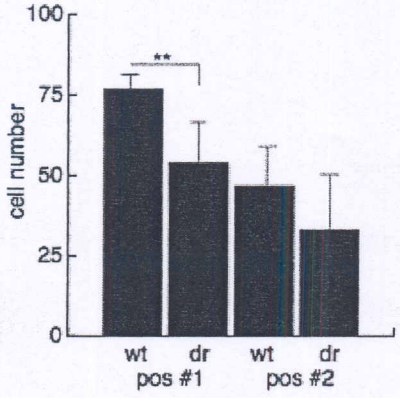


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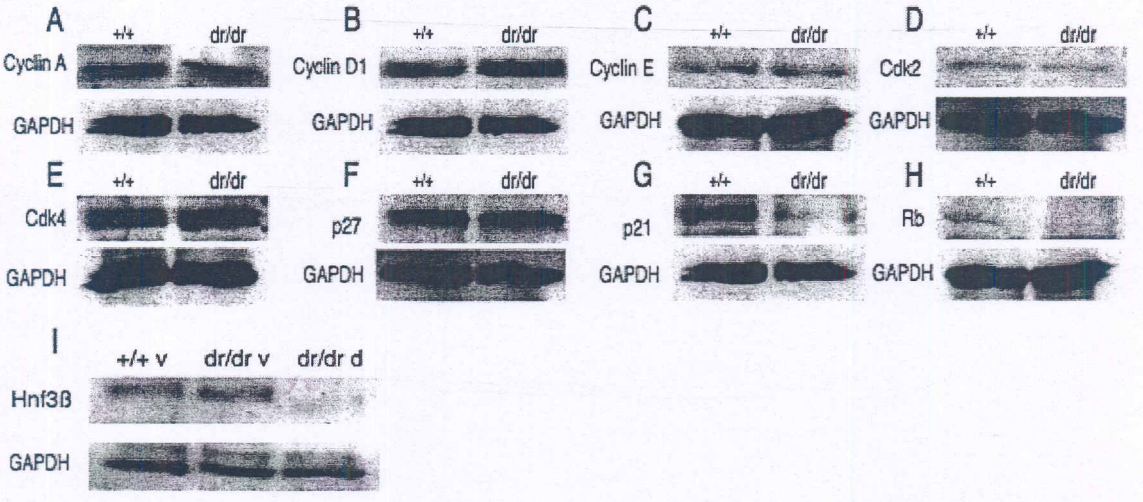
Figure 5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