The study of differential expressions of MCPH and Seckel syndrome genes and their paralogues

Running title: Spatio-temporal expression of genes

Authors: Mohsin M[1], Riaz S[1], Imran M[1], Cox JJ[2] and Khurshid M[1,3]

Affiliations: 1- Department of Biochemistry & Molecular Biology, the Islamia University of Bahawalpur, Pakistan

2- Wolfson Institute for Biomedical Research, University College London, UK

3- Corresponding author: maryam.khursheed@iub.edu.pk

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Abstract

Seckel syndrome and MCPH are single gene neurodevelopmental disorders with or without dwarfism. The genes are parts of essential cellular pathways but are regulated differently in both forms of microcephaly. These genes have diverged from single ancestral genes in to related but different gene paralogues during evolution. It is suggested that the paralogues might be redundant in other body organs and might rescue any abnormality in case of MCPH but not in Seckel syndrome. The study uncovers the spatio-temporal dynamics of these genes together with their paralogues at mRNA level where the different tissues of embryos might exhibit different transcript variants or different levels of the various transcript variants to find the complementation in case of the MCPH but divergence in case of the Seckel syndrome. Here, we studied Cdk5Rap2-Pde4dip, Phc1-Phc3 (for MCPH) and Cep63-Ccdc67 (for Seckel) in the embryonic mice tissues via RT-PCR throughout the peak of neurogenesis from E12.5 to E18.5. We found already known spatio-temporal differential expression. For MCPH, Cdk5Rap2-Pde4dip had similar profiles and seemed to be redundant whereas Phc1-Phc3 seem to have complementary roles in brain development. The expression profiles of Cep63-Ccdc67 were very different indicative of their diverged roles in development.

Summary Statement

The reverse transcription and relative quantitation in spatio-temporal embryonic mice tissues suggest, the paralogues in MCPH (primary microcephaly, PM) can rescue other organs but Seckel (PM with dwarfism) paralogues can't.

Introduction

Microcephaly both prenatal and postnatal is reported in humans. It might be due to genetic factors or genes that might cause non-syndromic microcephaly(Thornton and Woods, 2009) where only head size is reduced or syndromic forms where all organs are small together with craniofacial and other abnormalities(Asif *et al.*, 2023; Khetarpal *et al.*, 2016). Autosomal recessive primary microcephaly or MCPH is a non-syndromic condition in which affected have brain size reduction together with mild to moderate mental retardation and rest of the organs in the body are of normal size(Zaqout and Kaindl, 2022). Seckel syndrome on the other hand, is syndromic microcephaly which occurs in utero but is progressive and shows severe microcephaly with dwarfism and other

anomalies(Khetarpal *et al.*, 2016; Nano and Basto, 2017). Both MCPH and Seckel syndrome are heterogeneous and hence, indicate the different mechanisms of pathogenesis resulting from the gene mutations which might overlap sometimes such as MCPH6 gene CENPJ which is also involved in the Seckel syndrome(Asif *et al.*, 2023; Cuccurullo *et al.*, 2022). Both are heterogeneous and single genes are causative agents of both, the agents which are inherited in autosomal recessive manner so there must be some differential regulation done by the genes in each category and must control development in different manner(Asif *et al.*, 2023; Kaindl *et al.*, 2010). These genes are targeted to several core pathways such as centriole biogenesis, centrosome/spindle pole, kinetochore-microtubular pathway, nuclear pathways including the condensation-decondensation and nuclear envelope dynamics and other sub-cellular pathways(Bakircioglu *et al.*, 2011; Nicholas *et al.*, 2010; Sir *et al.*, 2011; Thornton and Woods, 2009).

During evolution genes diverge from ancestral genes in to different molecular entities with different acquired functions that may be related as these retain a considerable similarity termed as gene paralogues(Castellanos *et al.*, 2024). Primary microcephaly genes have been studied in the past decade for expression and by gene knock out in animal models which have given a large insight in to the disease but the data suggests ubiquitous roles of these proteins in all body cells and mice knockout are not true models of the disease with small to no reduction of organs(Perlman, 2016). Furthermore, the comparative expression analysis of genes and their paralogues might answer the question. Some studies are done with a double knockout of more than one PM gene and found a severe phenotype indicative of involvement in one master pathway of the cell incorporating all dynamics such as cell cycle, centrosomal/spindle pole, centriole biogenesis, nuclear and others where these genes have established roles(Farcy *et al.*, 2023; Jayaraman *et al.*, 2016; Morris-Rosendahl and Kaindl, 2015).

The one aspect that was missing was the differential expression patterns of microcephaly genes and their paralogues that can be explored for spatio-temporal expression analysis together with their established paralogues in both forms of microcephaly(Duan *et al.*, 2022a; Hettige and Ernst, 2019). Here we selected *CDK5RAP2-PDE4DIP* and *PHC1-PHC3* genes and their respective paralogues duos for MCPH and *CEP63-CCDC67* genes from Seckel syndrome(Awad *et al.*, 2013; Kraemer *et al.*, 2015; Ma *et al.*, 2014; Mani, 2022; Sir *et al.*, 2011; Tonkin *et al.*, 2002; Zhao *et*

al., 2013). These genes were selected because of their targeting was to different pathways that might be differentially important at different embryonic days. We used Swiss albino mice for the study and did mating via timed mating protocol and got staged embryos(Byers *et al.*, 2012). Later, we employed RT-PCR using gene specific primers to understand if there is some variations in transcripts or/and level of expression of the transcripts.

CDK5RAP2 at MCPH5 is involved in centrosome dynamics whereas *PHC1* at MCP11 is involved in nuclear dynamics and Seckel gene, *CEP63* at SCKL6 is a core centrioler gene and is involved in centriole duplication and their respective paralogues have related functions in the same or different pathways(Zaqout and Kaindl, 2022). In order to see if genes and their paralogues complement each other or if they replace each other's function being redundant or/and completely diverged and have different roles we set out to understand via simple but informative RT-PCR analysis an established method to uncover differential transcript variants or/and levels of expression.

Results

CDK5RAP2* and *PDE4DIP

CDK5RAP2 at MCPH3(Zaqout and Kaindl, 2022) is one of the first few MCPH genes to be identified. CDK5RAP2 and PDE4DIP/Myomegalin (Mani, 2022) are identified and confirmed as paralogues of each other by Position specific iteration BLAST, PSI-BLAST program (see figure no 1). The multiple sequence alignment, MSA showed very high similarities between the two proteins throughout most regions of the two proteins (see figure no. 1 A). These were further subjected to spatio-temporal investigation (Duan *et al.*, 2022a; Vaid and Huttner, 2020) in mice staged embryos in order to see if they actually can act by replacing each other. Cdk5Rap2, a protein kinase involved in the centrosome dynamics is found be most highly expressed in the head of E14.5 mice and interestingly its paralogue PDE4DIP is also most high in the E14.5 mice (see figure no 2 and 3). At E14.5 the basal progenitors are abundant and it is the mid-neurogenesis peak and hence represent that both genes are essential most specifically to the basal progenitors. Furthermore, we observed expected transcript variants in the samples and also some unexpected bands for potential new transcript variants. We plotted the cumulative expression as well as only

111 the expected transcript variants' expression by intensity scoring and also the potential new
112 transcript variants were treated the same way. All of these different scenarios gave us the similar
113 profile where the expression of *Cdk5Rap2* gradually up-regulated after E12.5 and E13.5 and
114 became highest in the E14.5 head and stayed at the same level at E15.5 and E18.5 in the mice
115 embryonic head suggesting a very important role of Cdk5Rap2 in these stages when basal
116 progenitors are most abundant and SVZ is the major layer in neuroepithelium (see figure no. 2 B,
117 C & D). Its paralogue, *PDE4DIP*, the highest expression of all the transcript variant expected and
118 novel in the E13.5 was shown to be on top followed by E14.5. However, in the potentially new
119 variants the E14.5 was highest and the expression down regulated in all scenarios later in
120 neurogenesis (see figure no. 3D). This is very interesting as at E13.5 and E14.5 there are apical as
121 well as basal progenitors with later being abundant and it might be possible that the embryonic
122 development in mice is dependent on the expression of genes and there paralogues in the brain
123 where both are required for brain development and are complementary but the time window for
124 the paralogue expression is short compared with the MCPH gene, *CDK5RAP2* suggesting
125 prolonged role of this gene is required for normal brain development which is also the longest
126 stage in the human development.

127 Basal progenitors in humans and in the higher primates are largest in number and contribute
128 neurons to all six layers of the cerebral cortex and thought of as the evolving factor between the
129 highly convoluted gyrencephalic brain in higher mammals and lissencephalic brain as is in the
130 mice. Our data suggest roles of CDK5RAP2 and PDE4DIP more critically in these progenitors.
131 Both *CDK5RAP2* and *PDE4DIP* are shown to be expressed in similar locations such as
132 centrosomes and involved in microtubule and spindle dynamics furthermore, the *PDE4DIP* if
133 mutated cause anomalies of the cardiac system suggested the diverged roles of these proteins in
134 different organ development. We suggest both of the protein are essential for development where
135 CDK5RAP2 specifically controls the brain development by prolonged expression through
136 neurogenesis whereas *PDE4DIP* expresses in small time window that might be essential for the
137 other organs development

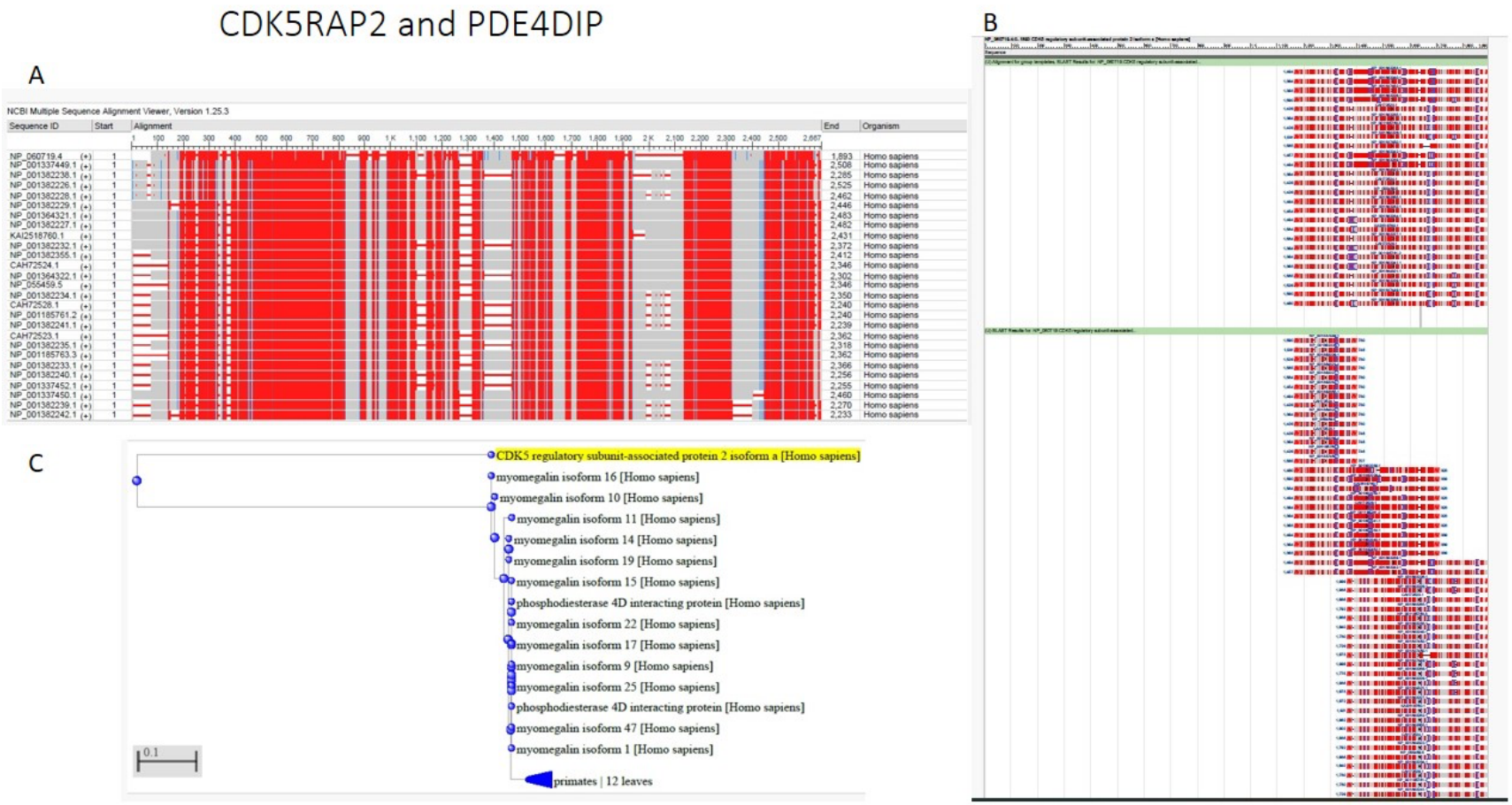

Figure 1: PSI BLAST was run to identify and confirm the ancestry, similarity and overall similarity between CDK5RAP2 and PDE4DIP/Myomegalin in Humans. A) Multiple Sequence Alignment between the CDK5RAP2 and Myomegalin/PDE4DIP is shown and is indicative of very high similarity especially at the N-terminus and other domains, B) BLAST between Isoform 1 of CDK5RAP2 was done and came up with similarities in various regions with almost all isoforms of Myomegalin/PDE4DIP, C) Ancestry of both CDK5RAP2 and PDE4DIP was confirmed by Gene tree where both genes split in a single evolution event and myomegalin showed a lot of further splits indicative of active evolution.

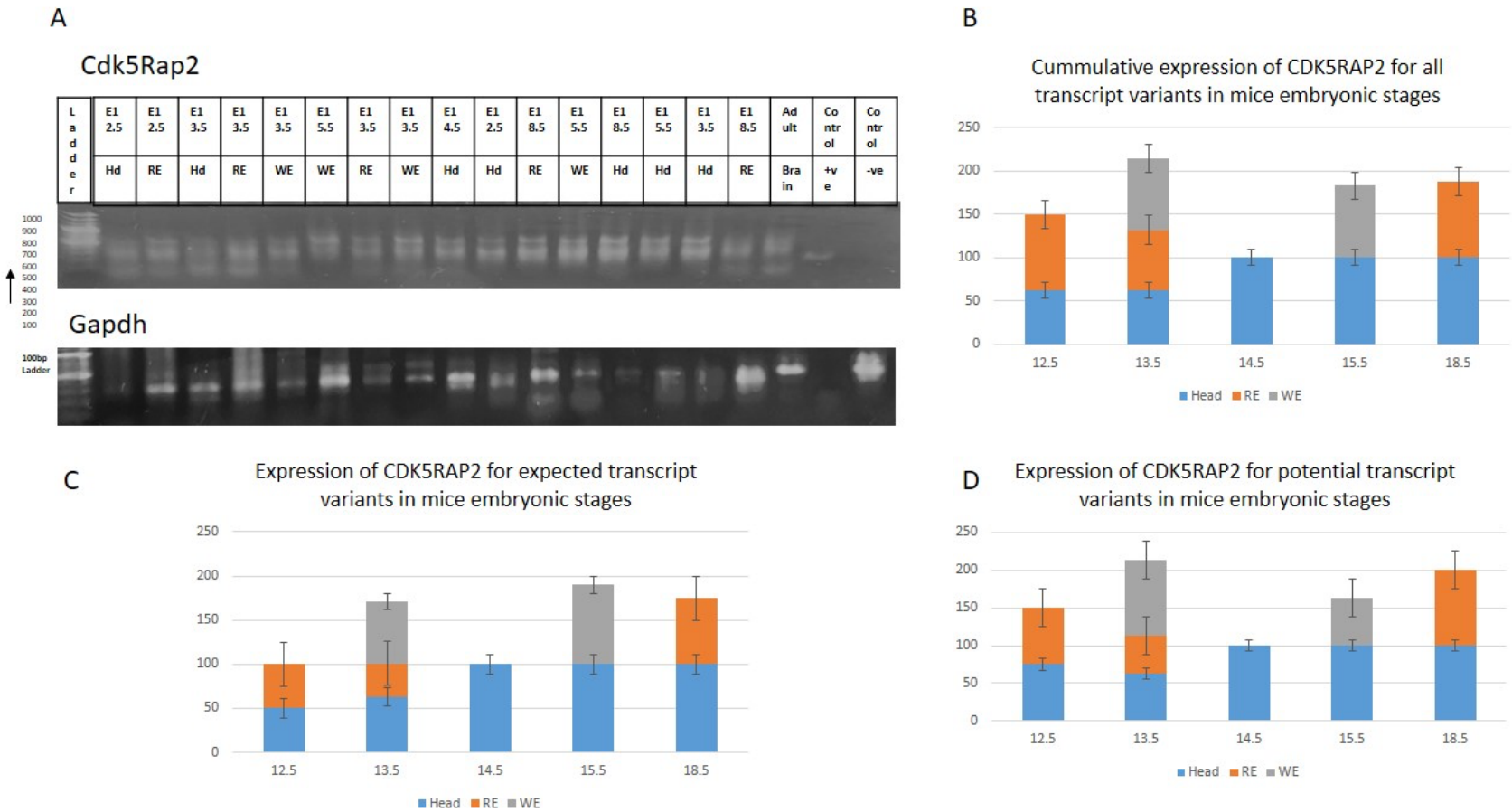

Figure 2: *Cdk5Rap2* an MCPH gene was subjected to transcriptional analysis by RT-PCR using gene specific primers. The *Gapdh* was used as a control. The results have shown expected band sizes and some potential new variants. The analysis was done by relative quantification of level of expression by band density and by given it a score from 4(100%) to 0(0%). The values for all different stages and embryonic regions were plotted in the graphs. A) Gel pictures, B) Cumulative expression at spatio-temporal dynamics C) Expected transcript variants scores D) New potential variants were plotted for each embryonic stage and region.

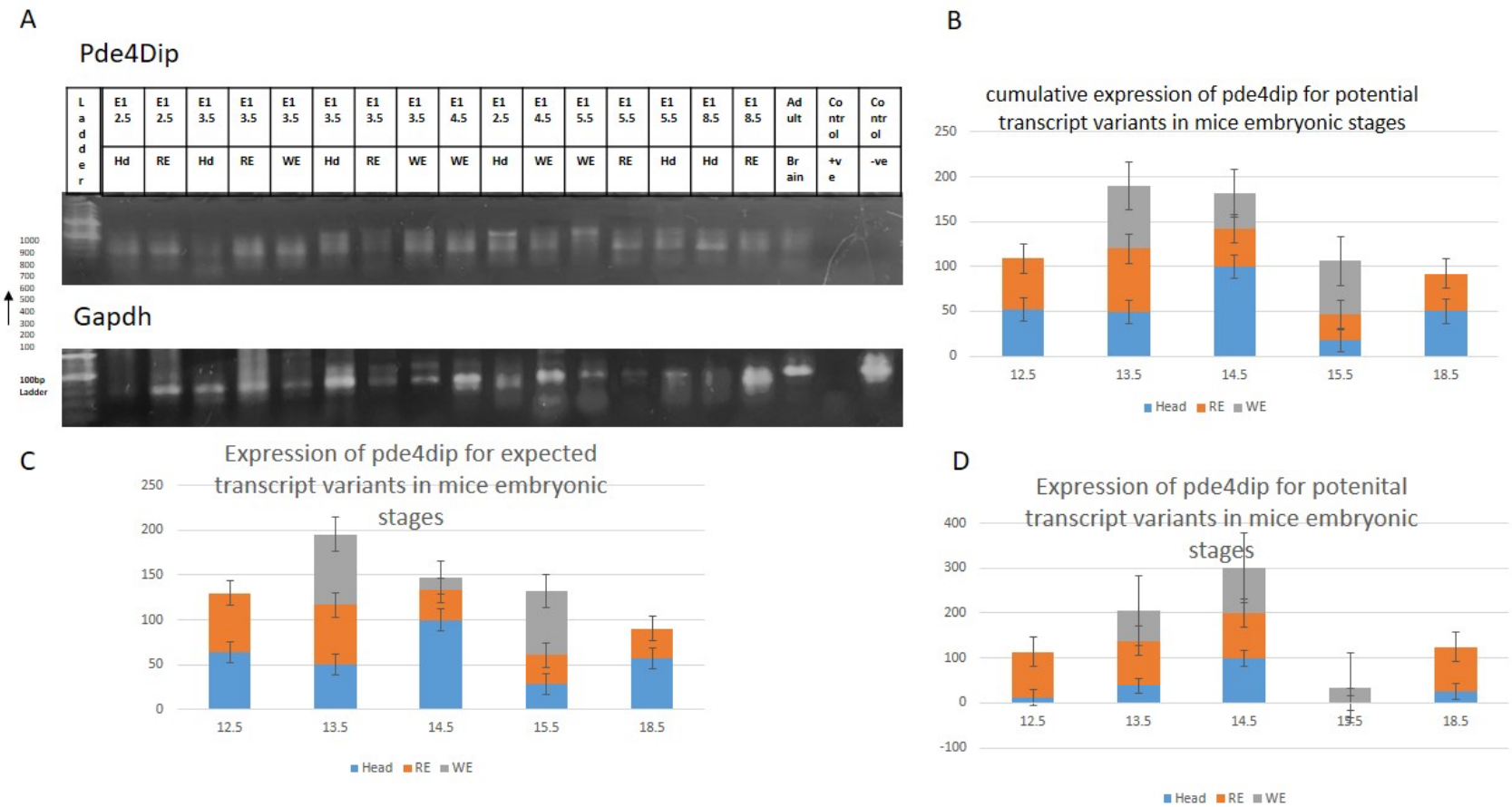

Figure 3: *Pde4dip/Myomegalin* was subjected to RT-PCR analysis with mRNA being by oligodT primers and dsDNA was made by gene specific primers. A) Gel pictures of *Pde4Dip* and *Gapdh* are shown at various embryonic days in mice in various embryonic regions (i.e., Head, rest of the embryo and whole embryos). B) The relative quantifications data for cumulative scores of all transcript variants is shown in various stages of neurogenesis in mice in various regions. C) Expected transcript variants data.

156 ***CEP63* and *CCDC67***

157 *CEP63* at SCKL6 on chromosome 3q22(Ma *et al.*, 2014; Morris-Rosendahl and Kaindl, 2015; Sir
158 *et al.*, 2011) is a gene involved in the broad development involving different organs and organ
159 systems. The autosomal recessive mutation in *CEP63* gene cause overall reduction leading to
160 dwarfism and severe microcephaly. The CEP63 protein as a core centrosomal protein involved in
161 the centriole duplication and recruitment of CEP152 at MCPH9 codes for a protein that also targets
162 to the centrosome. Centriole duplication is an important part of centrioler/centrosomal/spindle pole
163 pathway which is required for faithful mitosis and cell cycle progression. Its paralogue
164 CCDC67/DEUP1(Zhao *et al.*, 2013) is also studied to be involved in the centriole duplication and
165 de novo centriole assembly and hence thought to compliment the roles of CEP63 in development.
166 *DEUP1* mutations reported show the multi-organ affected with the disease including retinopathy,
167 cardiomyopathy, hypogonadism and others however, the reduction in size of the brain is not
168 reported and mental retardation is not common. Hence, although both proteins are important for
169 development CEP63 seem to have brain specific roles. The two proteins were subjected to PSI-
170 BLAST and MSA showed some smaller similar regions which were coiled coiled regions essential
171 to centriole/centrosome targeting (see figure no. 4 A, B & C). We then started to dig in to spatio-
172 temporal expression analysis of both Seckel gene and the paralogue. We found that cumulative
173 expression of all the transcript variants for *Cep63* in the various embryonic samples at all stages
174 of neurogenesis was more or less consistent from E12.5 to E18.5 with highest expressions in E18.5
175 head (see figure no. 5B). The novel transcript variants were expressed in embryonic head at similar
176 levels at E12.5 then down-regulated at E13.5 and then again up-regulated and remained at the same
177 highest level as in E12.5 from E14.5 to E18.5 suggesting important functional relevance to the
178 early and late progenitors (see figure no. 5CD). Whereas *DEUP1* cumulative expression gradually
179 up-regulated from E12.5 to E18.5 in a consistent way (see figure no. 6B). The potential novel
180 variants also showed the same trend with gradual up-regulation from E12.5 to E18.5. The rest of
181 the embryo where we separated embryonic head from rest of the body showed gradual increase
182 later from E15.5 to E18.5 (see figure no. 6D). The *Cep63* in the rest of the embryo samples also
183 showed gradual up-regulation from E15.5 to E18.5 with highest being at E18.5. The data suggests
184 that the role of DEUP1 to be more general and is required in large copies at the end of neurogenesis
185 and development, on the other hand CEP63 seem to be highly essential at early to mid and late
186 neurogenesis with highest cumulative scores at E12.5, E15.5 and E18.5.

Both genes being highly up-regulated at mid to late gestation points shows their potential co-relation with the SVZ progenitors which are abundant at these stages. There are also small proportion of OSVZ progenitors in mice which are detected in late neurogenesis and are of high relevance in humans and both genes seem to be also contributing to their neurogenesis. The rest of the embryo gave some up and down regulation in *Cep63* but remained stable in Deup1 suggesting a more controlled and consistent expression of *Ccdc67*. These results suggest that at the time of development Cep63 and Ccdc67 essentially control the brain development but the other embryonic tissues require the maintenance of Ccdc67 throughout embryonic development. Hence the broad-spectrum disorders of organs and organ systems are found in both *Cep63* as well as *Ccdc67* in mutated individuals.

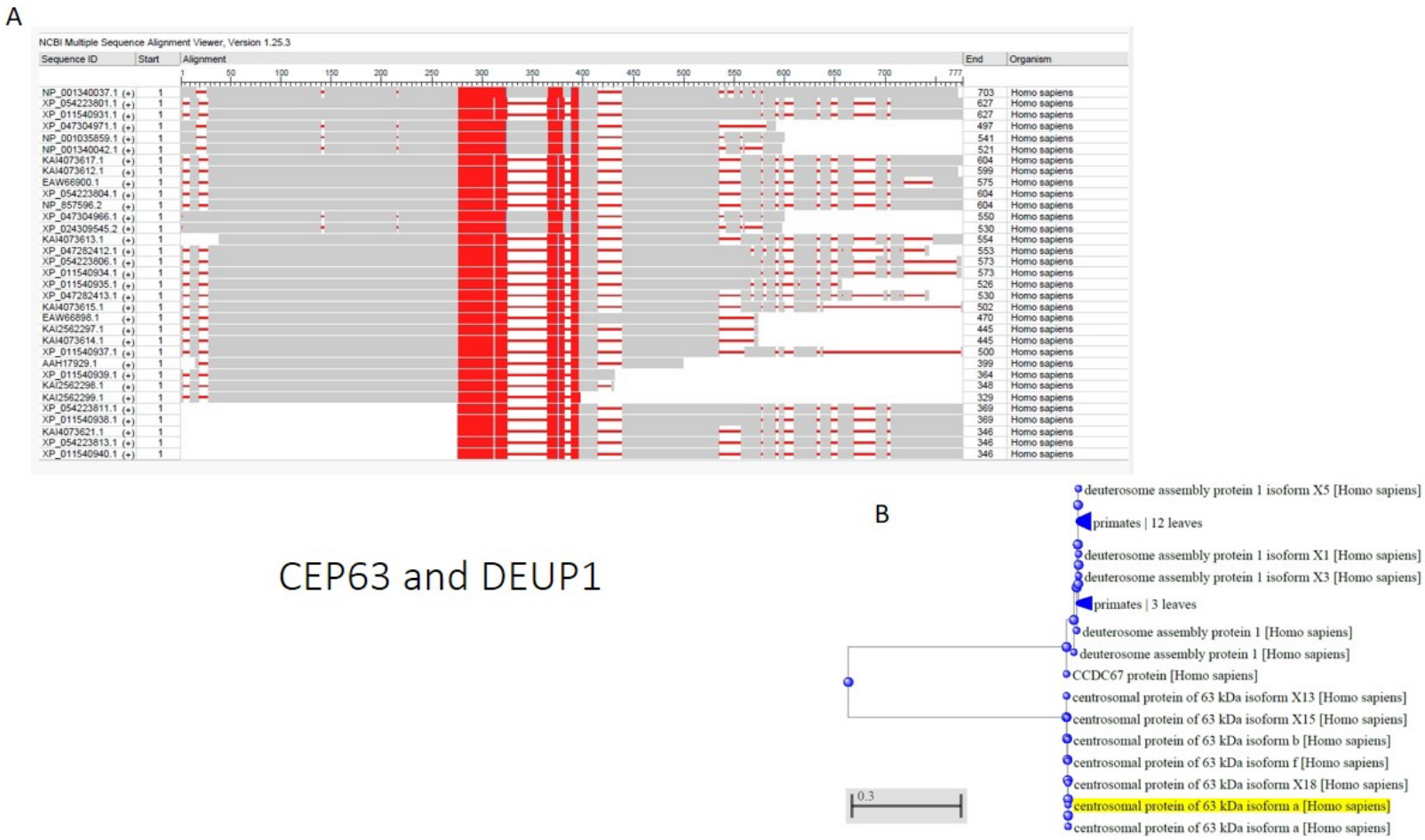

Figure 4: *CEP63* and *DEUP1/CCDC67* are two genes which are both undergoing active evolution and got split from a same ancestral gene. A) The Multiple Sequence Alignment of CEP63 is shown with various isoforms of DEUP1 and shows regions of high similarities scattered throughout but mostly in the center. Both proteins are targeted to the centrioles and the shared regions have coiled coiled domains important to the centrioler targeting. In addition to this, they also harbor a lot of varying regions where the two proteins are clearly showing divergence and new acquired functions B) Gene tree of both gene is shown where they have split in a single evolution event and undergoing active evolution.

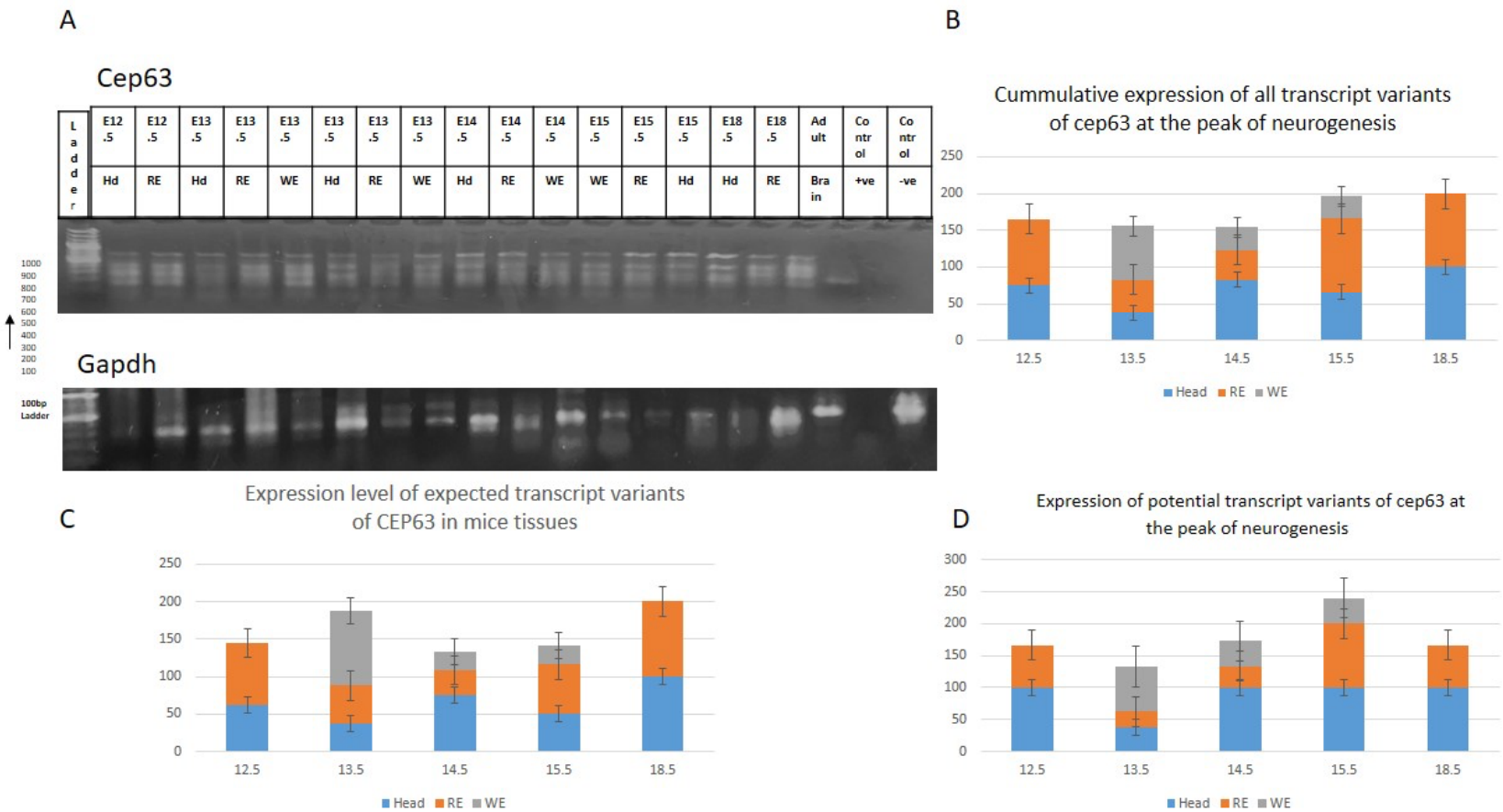

Figure 5: RT-PCR was done for *Cep63* throughout the peak of neurogenesis in mice i.e, E12.5 to E15.5 and E18.5. At each stage the mice embryos were divided in to the head regions, rest of the embryo and some whole embryos were also processed. Gene specific primers were utilized to do RT-PCR. A) Gel picture of RT-PCR in *Cep63* and Control *Gapdh* B) The cumulative expression of all the transcript variants showing highest expression at E12.5 but is showing quite consistent expression whereas head of E18.5 shows highest cumulative expression the time of OSVZ cell abundance. B) Expected transcript variants shows highest expression at E18.5 C) the potential new transcript variants shown expression at earlier and later stages.

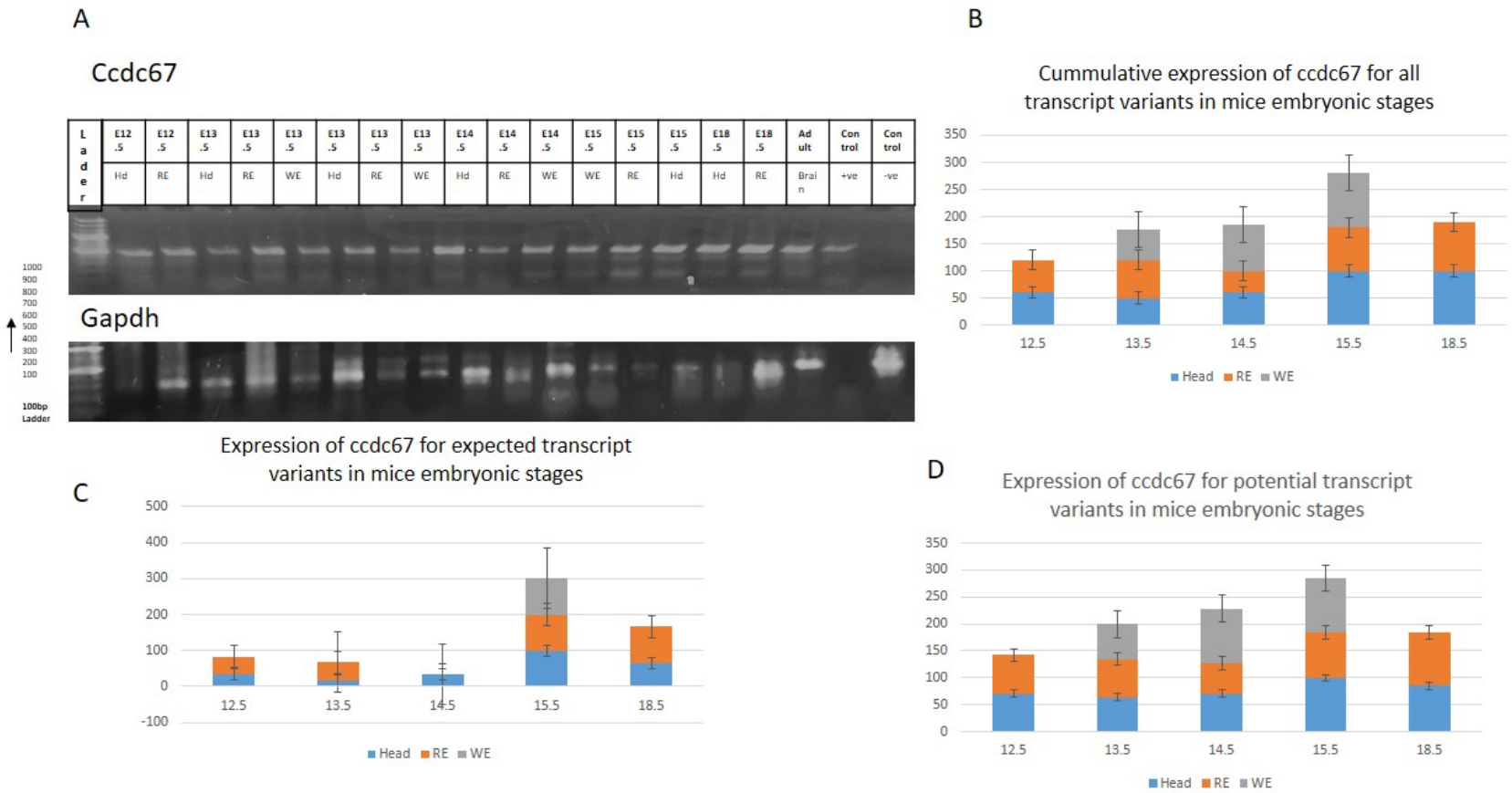

Figure 6: DEUP1 /CCDC67 a centriole targeted protein also a paralogue of Cep63 which also targets to centrioles and is a core component is subjected to transcriptional analysis by RT-PCR. A) Gels of *Ccdc67* and control *Gapdh* B) *Ccdc67* gradually increased from E12.5 to E18.5 reaching highest level gradually at later stages in cumulative scores B) The expected transcript variant also gradually increased C) The potential new variants showed same trend with quite consistent expression in the head region but collectively up-regulated from E12.5 to E18.5

217 ***PHC1* and *PHC3***

218 *PHC1* and *PHC3*(Tonkin *et al.*, 2002) are the members of Polyhomeotic-Like Proteins family and
219 are targeted to the PcG- PRC1, Polycomb group of repressor complexes, and is involved in
220 maintenance of pluripotency during development by repressing the transcription of genes involved
221 in differentiation especially *HOX* genes in addition to other important roles in the nuclear
222 dynamics(Awad *et al.*, 2013). This might be of significance in relation to MCPH where a single
223 change in PHC1 can lead to considerable brain size reduction while leaving other organ systems
224 intact. The *PHC3* is one of the paralogues of *PHC1* and was selected for this study because they
225 split from the ancestral genes as two diverged genes in evolution as a single event and hence, could
226 indicate separate acquired functions. The PSI-BLAST showed that two paralogues are very similar
227 and MSA showed C-terminus conservation which are essential domains for chromatin remodeling
228 by PRC1 complex suggestive of complimentary roles (see figure no. 7 A, B and C). We set out to
229 study their spatio-temporal dynamics by RT-PCR in mice embryonic tissues at the times of
230 neurogenesis from E12.5 to E18.5. RT-PCR results show that the total expression of *Phc1* is
231 highest at E12.5 and E13.5 with gradual down regulation at E14.5 and E15.5 whereas it again up-
232 regulated at E18.5 (see figure no. 8B). The expression in the embryonic head was highest at E12.5
233 and later at E18.5. The expression of rest of embryonic tissues showed consistent expression from
234 E12.5 to E18.5 (see figure no. 8B). Hence indicating Phc1 to be essential in development in general
235 whereas a controlled expression patterns in the brain indicating its control in early and late
236 neurogenesis. Its paralogue, PHC3 which is thought to complement its function in the development
237 is also investigated for mRNA expression. Our results show that *Phc3* was highly expressed E12.5
238 and slightly down regulated at E13.5 to E14.5 and enhanced at E15.5 to E18.5 being highest at
239 these later stages (see figure no. 9 A& B). To dissect it further the embryonic heads and rest of
240 embryos of mice showed consistent *Phc3* expression which was highest at E12.5 and later at E15.5
241 to E18.5. The data indicate complementary roles of Phc1 and Phc3 in the brain whereas early and
242 late neurogenesis is controlled by these proteins (see figure no. 9). Interestingly, RT-PCR has
243 showed a novel variant of Phc3 in embryonic heads of early E12.5 and E13.5 in addition to
244 common variant which was expressed equally in all tissues suggesting its relevance to the brain
245 only (see figure no. 9, C). This suggest that in the brain Phc1 function is complemented by *Phc3*
246 whereas rest of the embryos the Phc3 and Phc1 play essential diverged roles which might not be
247 sensitive to single gene mutations.

At early and late neurogenesis, the radial glial cells and OSVZ cells are abundant and both have high importance in human brain development during neurogenesis. The radial glial cells being the initial progenitor cells and give rise to various progenitors and neuron lineages might be controlled by these two genes in a way that PHC1 comes first and then PHC3 complements and control the final brain size. At MCP11 *PHC1*, if mutated leads to MCPH and indicate only brain reduction and other body organs are normal in size and might involve these spatio-temporal pattern.

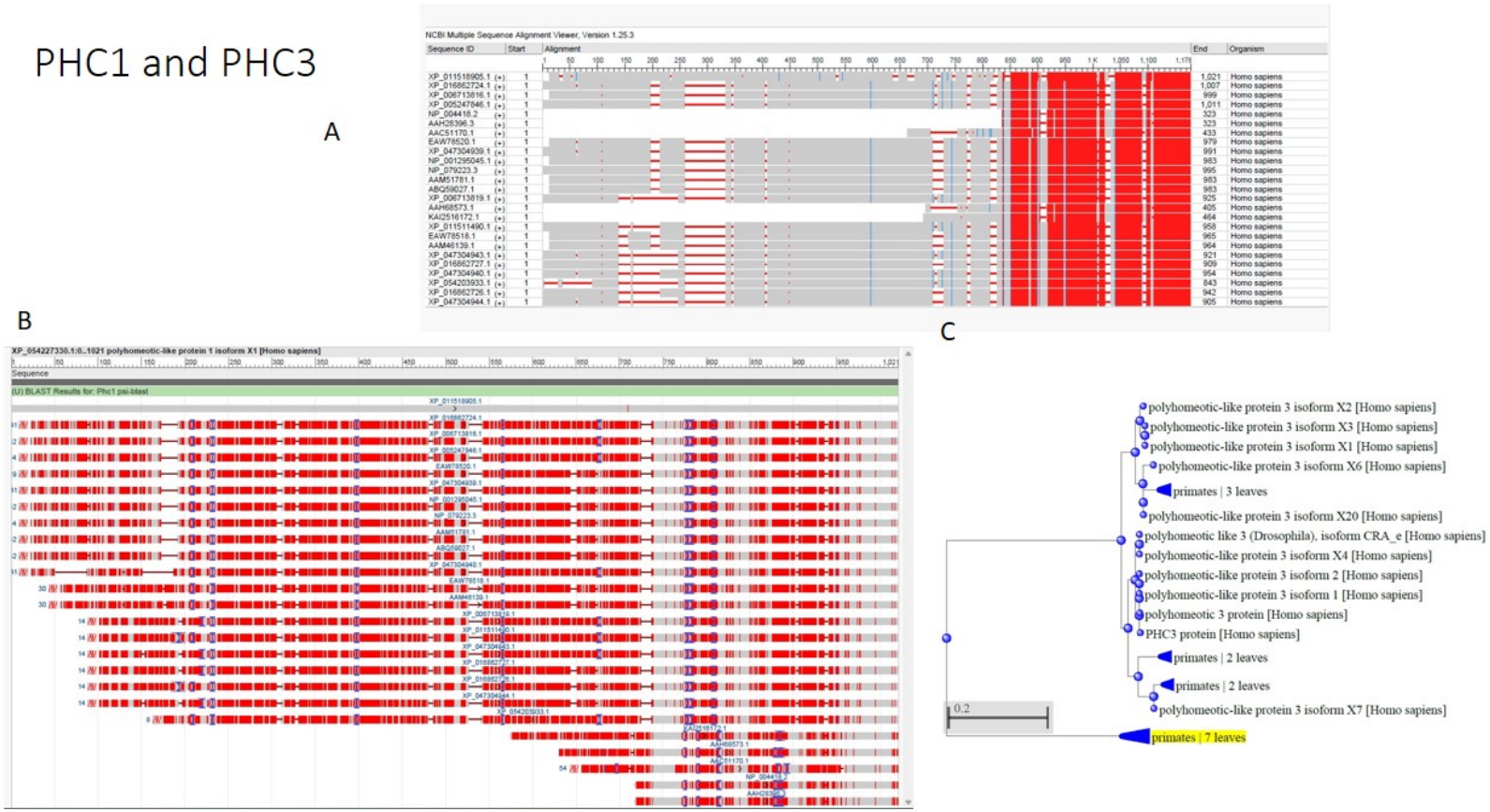

Figure 7: PSI-BLAST was used to identify, calculate similarity and ancestry of MCPH gene, *PHC1*'s paralogue, *PHC3*. Both genes are parts of PcG-PRC1 complex components and control pluripotency by chromatin remodeling. They seem to be targeted to the same pathway and were thought to be complementary to each other. A) PHC1 and PHC3 were aligned by Multiple Sequence Alignment and were shown to be highly similar in the C terminus regions and several other regions. The C terminus of both the proteins has FCS-Zinc finger domains and SAM domains essential for the chromatin remodeling B) The BLAST of Isoform 1 of PHC1 with all isoforms of PHC3 showing regions of similarity throughout in both proteins but very consistent C terminus C) The gene tree of *PHC1* and *PHC3* showing same ancestral gene and active evolution in PHC3.

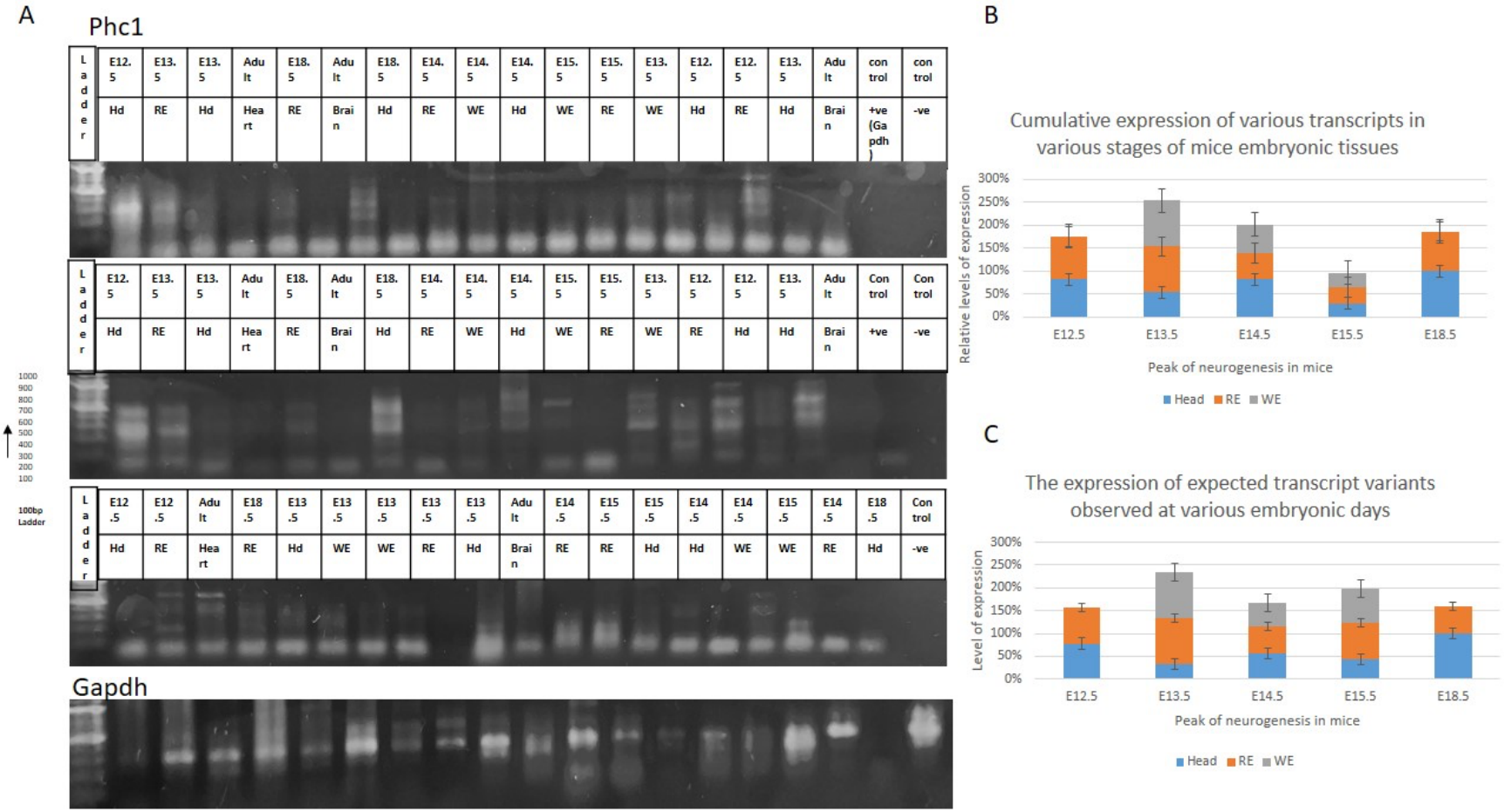

Figure 8: The RT-PCR data of Phc1 in various samples of mice embryos from peak of neurogenesis by gene specific primers. Three pairs of primers were used. A) Gel pictures of *Phc1* and Gapdh primers amplified products by ethidium bromide. B) Relative quantified band intensity for cumulative expression for all the transcript variants showing highest expression in the start, mid and end of neurogenesis but overall showing consistent expression which dips from E14.5 to E15.5 but again rises at E18.5 C) The potential new transcript shows similar trend but it relatively consistent at start and end.

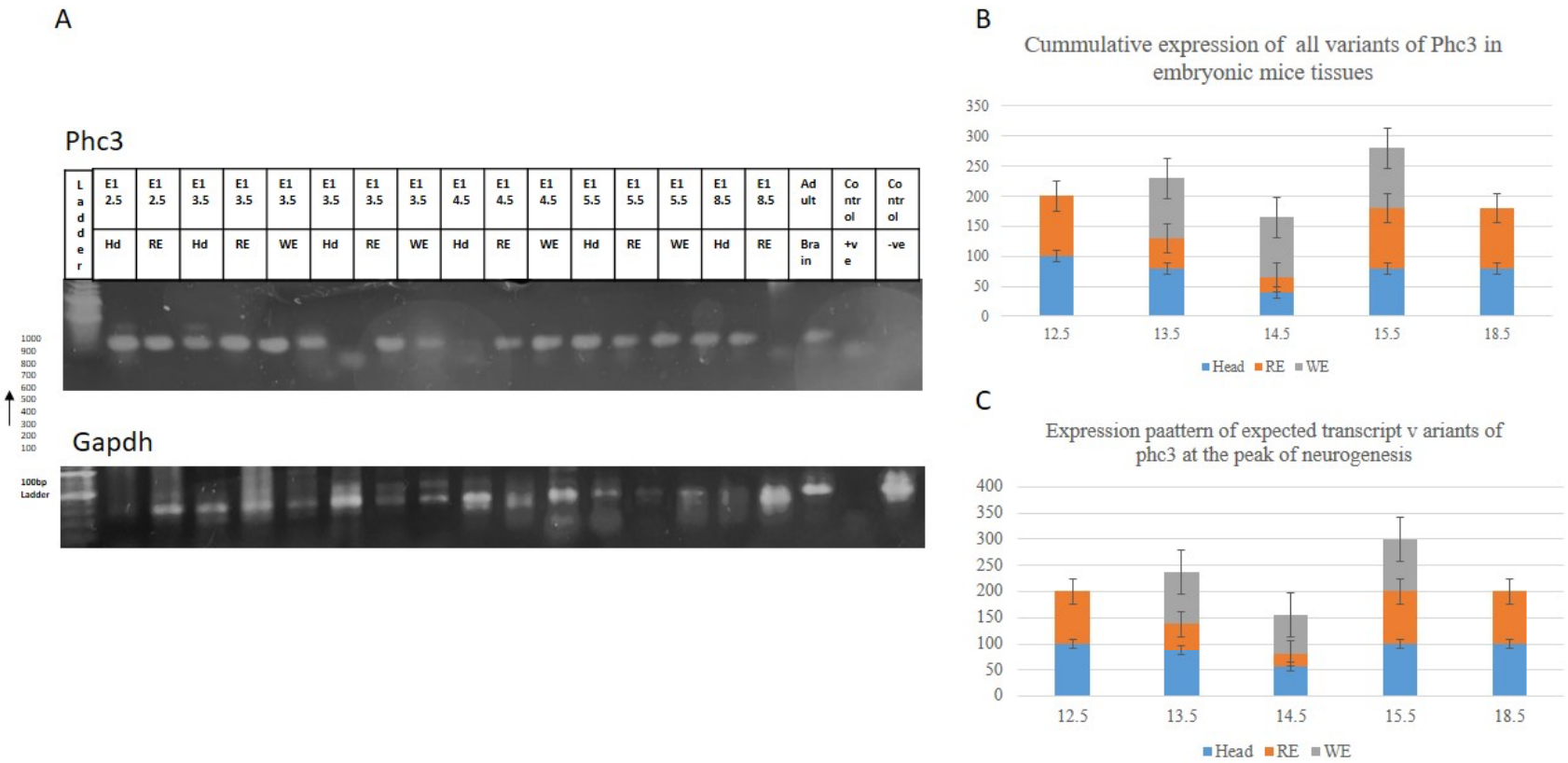

Figure 9: The RT-PCR data of PHC3 by gene specific primers. A) DNA Gel electrophoresis pictures of *Phc3* and control *Gapdh* showing the presence or absence and level of intensity of each sample bands. The peak of neurogenesis in mice was targeted by getting staged embryos from E12.5 to E15.5 and then E18.5 for late neurogenesis. The samples were prepared as head, Hd, rest of embryo, RE and Whole embryo, WE and are showing above each band B) The intensity level of each band was relatively quantified and given scores from 4(100%) to 0(0%) and were plotted for cumulative expression scores and found to be highest throughout neurogenesis with highest at earlier stage of E12.5 but otherwise consistent C) The potential new transcript variant was found to be present in E12.5 and E13.5 head and may be significant but all expected bands showed high trend in start and end of neurogenesis

Temporal dynamics of Genes and paralogues

Development is regulated by gene expression by transcription to RNA and then translation to proteins. The differential gene expression might be critical in order to regulate the normal development. The genetic neurodevelopmental disorders such as MCPH (microcephaly) and Seckel syndrome (microcephaly with dwarfism) seem to result due to faulty genes most of which reside in the centrosomes/spindle poles but also in other compartments. The single gene mutations are causative agents for both however with very different clinical profile and overlapping diagnosis, i.e., primary microcephaly. We have explored the idea of gene-paralogue pairs in both conditions by RT-PCR and did relative quantification and drew conclusions. Interestingly however, when we explored the same data separately for each gene in the head of embryo, Hd, and rest of embryo, RE (the embryonic body) we found the synchronized patterns in all three studied gene-paralogue pairs. In the head mRNA, one gene in each pair is having constant expression and other gene expression is going up and down with time and the synchronizing data is indicative of decisive proof of temporal dynamics during development. Furthermore, the MCPH gene, Cdk5Rap2 and Pde4Dip and Seckel gene, Cep63 and Ccdc67 pairs are showing opposite patterns (see Figure 10) with the target gene being constant and the paralogue changing in the first case whereas Cep63 is continuously changing in expression with ups and down but paralogue is pretty much constant. Interestingly, MCPH11 gene, Phc1 and its paralogue pair Phc3 seem to show very same profile as Cep63-Ccdc67 with identical Cep63 and Phc1 patterns (Fig 10). This might be seen as the two genes are complementary and regulate brain development as a pair and seem to regulate this process by continuous presence of one gene and changing behavior of the other gene in the pair. The genes have duplicated in evolution and might indicate separate roles of each gene. We were further intrigued to study individual gene expression in the rest of embryo, RE, in same time periods and again it was really fascinating that we were seeing temporal changes with synchronization in patterns of different genes. This proves that not only temporally gene expression at mRNA level is changing but spatially it is also modulating. Interestingly, MCPH genes, seem to show synchronized patterns in the gene-paralogue pairs of Cdk5Rap2-Pde4Dip and Phc1-Phc3 with Phc1 and Pde4Dip being identical (see Figure 11). The Phc1's paralogue, Phc3 has same profile in Hd and RE (See Figure 10 & 11) and seem to needed in same dosage throughout development. The Seckel pair, Cep63 and Ccdc67 seem to have same patterns and might indicate they can override each other during development. (see Figure 11)

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313

314 **Figure no. 10:** Temporal expression of gene-paralogue pairs in the heads of mice embryos at various embryonic days. The Cdk5Rap2,
315 Ccdc67 and Phc3 expression profiles were matching with their expression getting constant at later developmental stages in the head of
316 embryos whereas Pde4Dip, Cep63 and Phc1 seem to have constantly changing and similar curves indicative of differential temporal
317 gene expression.

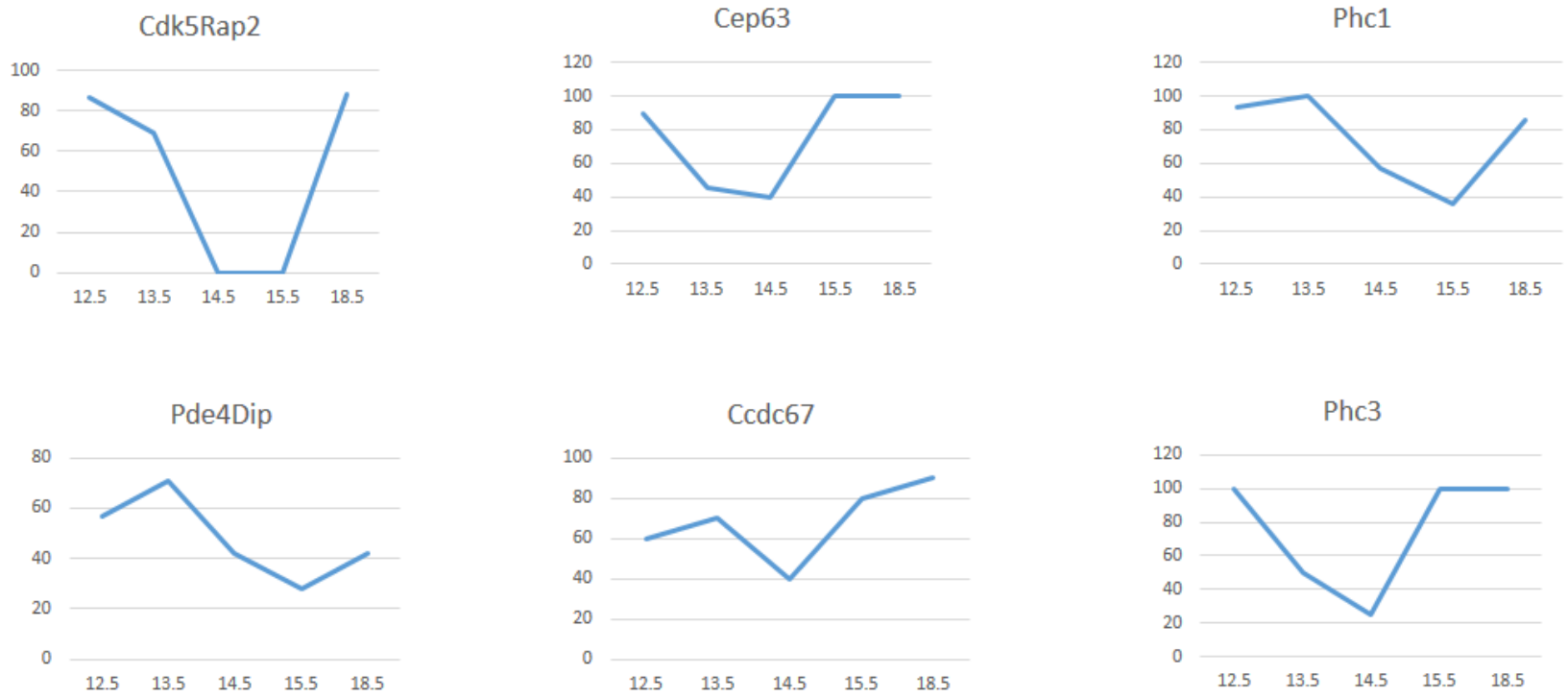
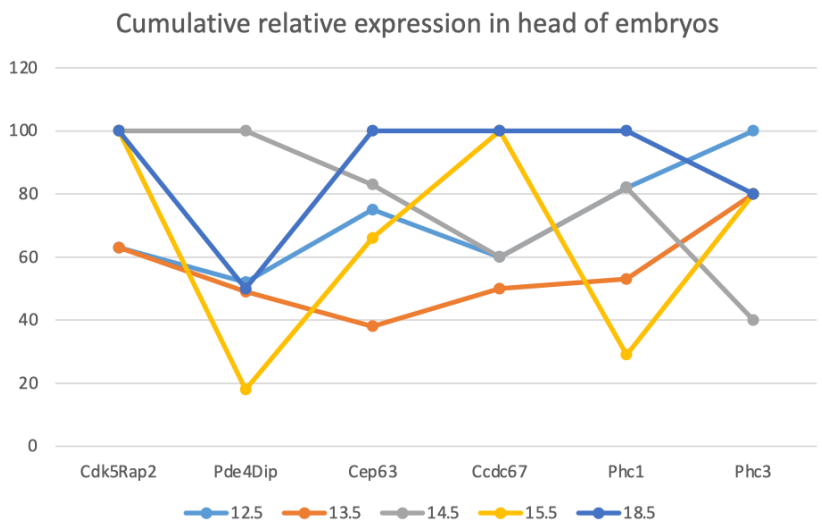


Figure no. 11: Temporal expression of genes and paralogues in the embryonic bodies (Rest of embryos, RE) of mice throughout neurogenesis. The Pde4Dip, Phc1 are identical in expression curves but total expression is different. The Cep63 and Phc3 curves are mating and Cdk5Rap2 curve is not definitive due to two missing samples of RE at E14.5 and E15.5 and must be repeated.

323 **The cumulative relative scores and total expression of each gene during neurogenesis**

324 We then asked whether these genes are sequentially upregulating or if they are required in dose
325 dependent fashion. We plotted the cumulative scores of each gene in stages of neurogenesis and
326 found that in the Hd, the genes are at high level in the start of neurogenesis and later stages. In the
327 RE, it is similar but genes seem to have more constant profiles for the genes. The total expression
328 of genes in both Hd and RE seem to suggest same profile with Phc3 being the most expressed
329 among the six genes studies and Pde4Dip being the least expressed (see Figure 12&13). This can
330 be viewed by keeping in mind the idea of single master pathway where, sequentially the genes are
331 upregulating but another possibility also exists that these genes might have differing roles in
332 development and different doses of each are required or both. Furthermore, in the RE, the total
333 expression profile of all three genes, Cdk5Rap2, Cep63 and Phc1 are more or less balanced with
334 one paralogue, Phc3 being highest and at similar level to its paralogue, Phc1 (see Figure 13). This
335 re-iterate our earlier suggestion that the microcephaly gene, Phc1 and its paralogue, Phc3 are
336 required by development together and are complementing each other and are targeted to same
337 pathway. In the Hd tissues, the changing doses of genes is required in order to control division of
338 various progenitors which are brain specific only and hence control its development through
339 temporal dynamics (see Figure 12). The other two gene-paralogue pairs have diverged and hence
340 control different functions especially the Seckel gene pair.



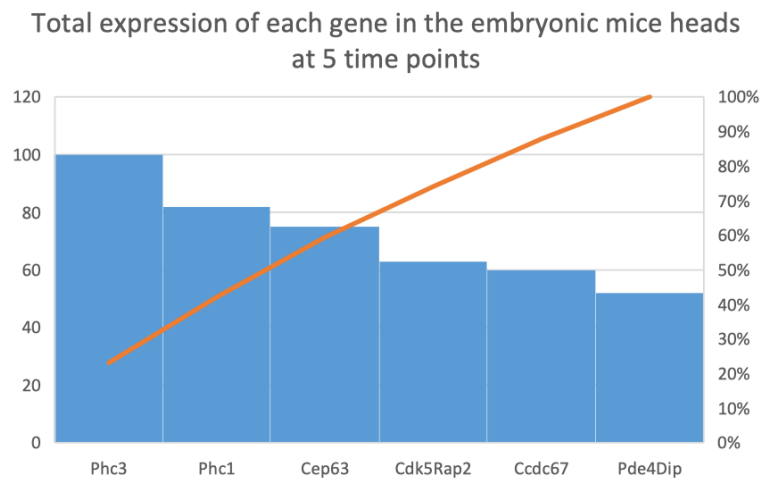


Figure no. 12: Relative quantification of all six genes at various time points throughout neurogenesis. A) Cumulative relative expression of genes in embryonic heads. B) Total expression of each gene in the embryonic heads in mice at E12.5, E13.5, E14.5, E15.5 and E18.5 and shows a linear curve.

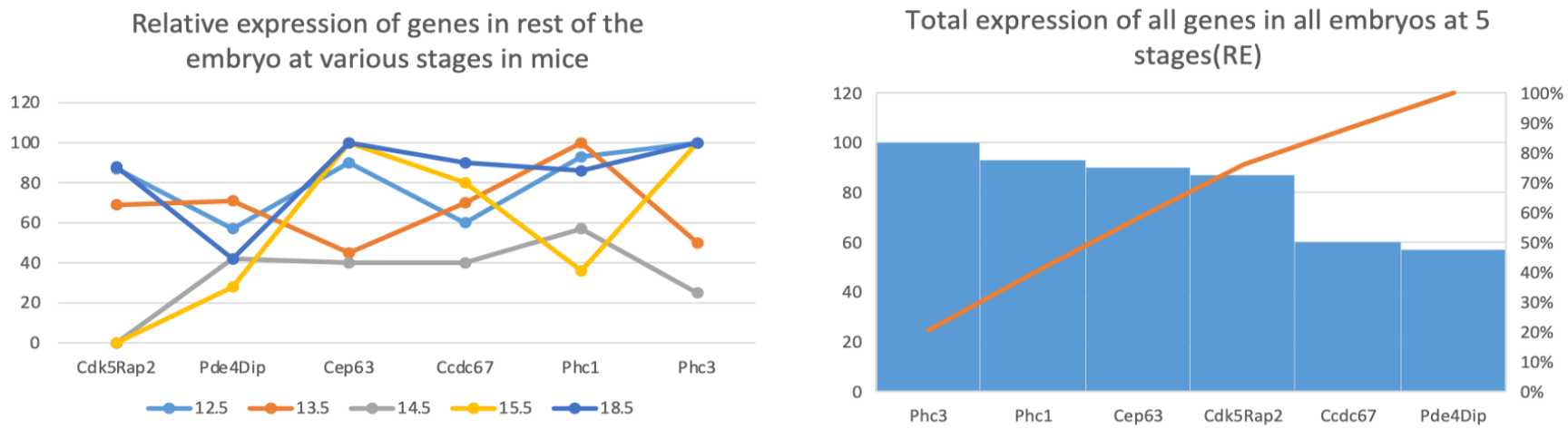


Figure no.13: Relative quantification of all six genes at various time points throughout neurogenesis. A) Cumulative relative expression of genes in embryonic bodies, RE. B) Total expression of each gene in the embryonic bodies, RE, in mice at E12.5, E13.5, E14.5, E15.5 and E18.5 and shows a linear curve.

Discussion

The MCPH and Seckel are disorders involving a very tragic microcephaly condition which is further added upon by dwarfism in the syndromic later form. Scientists are also fascinated by this condition because the microcephaly is similar to the head sizes in our closest relative higher primates. Not only the size of head, which is most notably small in the cerebral cortex region that is exceptionally developed in *Homo Sapiens,* but it limits the children to learn language or do very complex tasks and moderately to severe mental retardation which might just reflect their inability to mimic human specific brain functions but are quite decent. It is a disorder but opens up new avenues in to studying evolution and human acquired traits. The most work has mostly focused on the uncovering of pathogenic genes which was really challenging and took a long time and was speedup by high throughput sequencing and computational analysis. Furthermore, some really important functional analysis was done on these genes and found to be highly informative but end at a dead point. All these genes are part of essential core pathways that control cells and neural progenitors as the type of cell also have it in all cells and seem to be ubiquitous more or less furthermore, their up-regulation or down-regulation is also reported in the many cancers and tumor cells indicating their regulated roles. Patient derived cell lines show reduced or absent expression but it is also not true reflection when we think of it as developmental disorders. Animal models have shown some promise but had varying results. We set out to the basic developmental principal where genes are differentially regulated with respect to stage and time and controls different specific roles that they play in the development. We set out to study three pairs of genes and their paralogues which are targeted to different pathways and both MCPH and Seckel syndromes. We used RT-PCR to check if there was any variation in their expression levels or presence of absence of transcripts at various stages throughout peak of neurogenesis and we numerically calculated the levels of presence or absence and sometimes used many primers and added up all scores and interestingly these have actually given us positive insight in to the disorders and it is the first report to actually do so. This will further open new chapters in to the exciting research on DNA/RNA and protein levels in future and combing high tech machines could give important insights in to this field of spatio-temporal dynamics (Duan *et al.*, 2022b; Kim and An, 2020; Roberson *et al.*, 2021; West *et al.*, 2022) during development.

380 Our data suggests that neural progenitor cell divisions in to neurons through symmetric and
381 asymmetric divisions are controlled by various cellular pathways and MCPH genes indicate series
382 of up-regulation at different embryonic days suggesting their overall hierarchy in the control of
383 neurogenesis. In addition to this, their respective paralogues seem to complement their roles in
384 neural development which might indicate their divergence in the other organ development apart
385 from the brain. *Cdk5Rap2-Pde4dip* showed up-regulation at mid-late neurogenesis and seem to
386 have some redundancy whereas *Cep63-Ccdc67* duo was very different and expression of *Cep63*
387 was consistent throughout neurogenesis, but *Ccdc67* gradually increased during development and
388 was highest at the late neurogenesis and development suggestive of diverged and essential roles of
389 the two genes which are both essential in development and lastly, *Phc1-Phc3* showed up-
390 regulation at early and late neurogenesis and might be complimentary for brain development.
391 Hence, suggesting the roles of different pathways are important for cell divisions at different times
392 and stages and might be possibly part of a higher single master pathway incorporating all the
393 pathways. All the genes studied showed spatio-temporal(Pang *et al.*, 2024) variation in expression
394 and when plotted on their own in term of temporal expression change at the studied stages in two
395 compartments, embryonic heads, Hd, and embryonic body, RE. The genes seem to distribute in
396 two common pairs of similar patterns of temporal expression changes consisting of actively
397 varying in Pde4Dip, Cep63 and Phc1 and of more increased expression at earlier stages and
398 constant in later stages in Cdk5Rap2, Ccdc67 and Phc3 which seem to suggest that these gene-
399 paralogue duos actually are similar and that's way result in neurodevelopmental disorders if any
400 is mutated. The Cep63 is Seckel syndrome causing gene and is more severe condition with overall
401 reduction in body size and other ailments so were interested to look at the individual gene profiles
402 in the RE samples from embryos and again data was really interesting regarding grouping of genes
403 in two categories. Pde4Dip and Phc1had same profile and was actively changing and was also
404 upregulating later at these stages whereas, Cep63 and Phc3 was same. Cdk5Rap2 cannot be fully
405 recapitulated due to two missing sample data for RE at E13.5 and E14.5 which may be repeated in
406 future. Ccdc67 was similar to Cep63 and seem to suggest both proteins to be essential for body
407 development and reason that Seckel syndrome results for missing Cep63. These dynamics need to
408 be studied for other MCPH and Seckel genes and their paralogues and the mRNA data must be
409 validated by protein expression analysis and work may be replicated in human based model or
410 single cells from mice (Brooks *et al.*, 2024). The mRNA has been shown to transcribe in bursts

411 and this phenomenon may also be studied (Pomp *et al.*, 2024) and our results may also be due to
412 this phenomenon but may be regulated differently due to unique nature of developing brain cells.

413

414 ## Study limitations

415 The study is only preliminary and doesn't fully recapitulate all the MCPH and Seckel genes which
416 exceed to more than 45. It is also required that this data should be verified by total gene expression
417 variation in each tissue and stage by qRT-PCR. Furthermore, single cell expression profiling must
418 be done to get definitive answers to spatio-temporal changes. High throughput mRNA and protein
419 analysis for these genes and paralogues must be done on single cell samples in order to know the
420 neural stem and progenitor cells for differential expression and then deduce and relate it to primary
421 microcephaly (Birk *et al.*, 2024; Winkler *et al.*, 2024). Human brain organoids might be able to
422 answer these questions in the start.

423

424 ## Materials and Methods

425 **Animal mating and tissue harvesting**

426 Swiss albino mice strain was used and the mice were purchased from the Department of Pharmacy
427 at Islamia University of Bahawalpur. The housing was done as per international standards. Timed
428 mating of mice (follow DOI: 10.1101/2023.12.16.57983) was done at proestrous-estrous stage and
429 staged embryos were collected later at specific required days in RNA later™ (Invitrogen) after
430 dissecting mice anesthetized by xylazine-ketamine in 1:10 combination and were subjected to
431 cervical dislocation. The mice work was approved by institutional ethical committee of IUB via
432 letter no. 3990/ORIC. For each embryonic day, we had 3 mice with average of 6 embryos.

433 **RNA Extraction**

434 The total RNA was extracted from 100mg of tissue using TRIzol (Ambion) method. The minced
435 tissue was incubated with 1mL of TRIzol reagent overnight at 37°C. Next day, the DNA and
436 Proteins were separated by phase separation and RNA was pelleted down. The pellet was washed

437 with 70% Ethanol and air dried at room temperature for 5 mins. Pellet was resuspended in 20µl of
438 RNase/DNase free water.

439 **First strand cDNA synthesis**

440 First strand cDNA was synthesized after calculating the RNA as 1µg from nanodrop method (Gen5
441 software). RevertAid (Thermo fisher scientific) first strand cDNA synthesis kit was used to do the
442 reverse transcription using the described protocol. OligodT primers supplied with the kit were used
443 to amplify the first strand.

444 **Second strand cDNA synthesis**

445 The first strand cDNA synthesized was amplified into double stranded DNA by using gene specific
446 primers and the estimated known sizes were upto 900bp so Taq DNA polymerase system (Thermo
447 fisher scientific/ Synbio) was used as a standard method.

448 **Gene specific primers**

449 The intron spanning gene specific primers targeting all known common transcript variants and
450 sometimes specific transcripts were used. Primer 3plus and primer blast was used for the primer
451 designing. The Insilico- PCR was done to calculate product sizes and targeted transcripts from
452 UCSC genome browser. The melting temperatures and GC content was also calculated. The
453 sequences are attached in the supplementary tables.

454 **PCR program**

455 The PCR was done at initial denaturation at 95°C for 5 mins, 35 cycles of denaturation at 95°C for
456 30 sec, annealing at 53/55/56°C for 30 sec and extension at 72°C for 1 min. Final extension was
457 done at 72°C for 10 mins. PCR machine was set at 4°C forever.

458 **Gel electrophoresis**

459 The 2% agarose gel was prepared and 3µl ethidium bromide was added for viewing in UV
460 transilluminator. The 100 bp DNA ladder was used with each primer to determine the size of
461 bands.

462 **Relative Intensity scoring**

The DNA bands were marked by presence and relative intensity. The highest intensity was scored as 4 (100%) and was further calculated down to 1(25%) as intensity in samples drop. The absence of band was numbered 0 (0%). Later, cumulative scores of all bands for each primer were added and relative percentage was calculated for each sample. Means were taken for more than one sample wherever applicable.

Excel software

Free Microsoft excel was used to plot the relative percentages for each sample. The error bars are also added.

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Competing interests

Authors declare no competing interests.

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Data and resource availability

The paper uses gene specific primers and it is included as supplementary data. Furthermore, a preprint of the paper with data is on open preprint server, Biorxiv.

Diversity and inclusion statement

We are don't discriminate anyone on basis of race, nationality, religion and gender.

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584

585 **Supplementary data**

586 The supporting data in form of calculation and primers sequence are provided with the article as
587 S1 Table and S2 Table.