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CDKL5 REGULATES NEURONAL POLARIZATION OF HIPPOCAMPAL NEURONS THROUGH ITS INTERACTION WITH SHOOTIN1

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SUMMARY

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder that represents the second cause of mental retardation in females. Symptoms manifest after a period of apparently normal development and are characterized by stereotypic hand movements, mental retardation, epileptic crisis, hyperventilation, constipation and cardiovascular abnormalities. The vast majority of patients affected by the classical form of RTT carries mutations in the methyl-CpG binding protein 2 (MeCP2), a ubiquitous protein that binds to methylated promoters and represses the transcription of downstream genes. Moreover, according to the fact that RTT is exclusively a neurological disease, MeCP2 has been shown to dynamically modulate the transcription of specific neuronal genes, such as *Bdnf*. Importantly, the phosphorylation of MeCP2 has been demonstrated to be required for *Bdnf* transcription upon neuronal activity. Previously, MeCP2 was described only as a repressor, but recently, it has also been indicated as an activator.

Besides the classical form of RTT, also a number of variants have been reported: some of them cause a milder clinical picture than the classical form, while others display a more severe phenotype with earlier onset. Among the latter, the Hanefeld variant is characterized by the absence of an initial asymptomatic period and by the early onset of pharmacologically untreatable seizures. Several patients affected by this variant have been found to carry mutations in the cyclin-dependent kinase like 5 gene (*CDKL5*), a serine/threonine kinase containing an N-terminal catalytic domain and a long C-terminal tail with regulatory functions. Previous publications demonstrated that CDKL5 and MeCP2 interact physically and that the kinase is able to autophosphorylate and to mediate the phosphorylation of recombinant MeCP2 *in*

vitro. Recently, a novel and unexpected role of CDKL5 in the structural organization of nuclear speckles and the dynamics of their components has been described. CDKL5 is highly enriched in the nuclear speckles, and co-localizes with SC35, a non-snRNP splicing factor of the serine-rich family of proteins. The localization of CDKL5 is not mediated through binding to RNA, but depends on protein-protein interactions.
The aim of this work was to elucidate new molecular pathways involving CDKL5 that could be the cause of some clinical features correlating with CDKL5 mutations. The C-terminus of CDKL5 was used as bait in a yeast two-hybrid screening of a human adult brain library. The nature of the identified interactors places CDKL5 in different molecular networks involved in regulating distinct aspects of neuronal functions. Mainly, four molecular pathways could be identified: neuronal polarization, cytoskeleton organization, transport and axonal transport and cell signalling and degradation. The various interactions were assigned different degrees of confidence with only shootin1, a protein that is involved in neuronal polarization, having obtained the highest confidence score. Thus, we decided to analyze in more details the interaction between shootin1 and CDKL5 and to investigate the putative role of CDKL5 for neuronal polarization. In this contest, we compared the expression pattern of CDKL5 and shootin1, and found that during brain development the two proteins are both present between post-natal stages P4 and P14. We confirmed the interaction of endogenous CDKL5 and shootin1 by co-immunoprecipitation experiments, whereas no association could be detected between the exogenous proteins upon over-expression in HEK293 cells. The levels of CDKL5 increase significantly during maturation of primary hippocampal neurons and importantly, we found a co-localization of CDKL5 and shootin1 in the neurites and axonal growth cone up to 48 h after plating. Altogether these data confirm shootin1 as a novel CDKL5 interacting protein and suggest a role of the kinase for neuronal specification.
To verify our hypothesis we performed loss-of-function experiments, where CDKL5 silencing was obtained using shRNAs and at the same time GFP protein. CDKL5 expression was silenced in primary hippocampal neurons and neuronal morphology of GFP positive neurons analyzed together with neuronal polarization using the axonal specific marker Tau1. As expected, the control neurons, expressing a shRNA against LacZ, showed an increase in the percentage of polarized cells from 48 h to 96 h. In contrast to that, the suppression of CDKL5 decreased the polarized phenotype at 48 and 72 h whereas this effect was less strong at 96 h indicating that a

deficiency of CDKL5 causes a delay in neuronal polarization, similar to the effect caused by loss of shootin1. Interestingly, we found that the strong accumulation of shootin1 in the newly formed axon at 72 h could not be observed in neurons interfered for CDKL5. Taken together, these results indicate that CDKL5 may affect neuronal polarization in a shootin1-dependent manner, with CDKL5 working upstream of shootin1. In according with this hypothesis, our preliminary experiments indicate that shootin1 phosphorylation is altered in neurons devoid of CDKL5. In fact, the shootin1 isoforms that can be detected in two-dimensional gel electrophoresis analysis, shift slightly towards a more basic pH. All the above data are in accordance with CDKL5 regulating axon specification in a pathway that involves also shootin1. However, besides a defect in neuronal polarization the loss of CDKL5 also appears to influence neuronal morphology. The neurons devoid of the kinase appear disorganized with an overall reduction in neurite length and a loss of the asymmetrical shape characterizing the polarized cells

Taken together, the results showed in this work indicate that the RTT pathological state may be correlated to an altered capability of neurons to connect each other. This is indicated from (i) CDKL5 is expressed during neuronal development, (ii) CDKL5 interacts with shootin1, a protein with a key role in neuronal polarization and axon specification, (iii) the ablation of CDKL5 alters the neuronal morphogenesis suggesting a role of the kinase in neuronal maturation. Further work will be aimed at investigating in more in details this interesting issue.