UNIVERSITY OF INSUBRIA

DEPARTMENT OF THEORETICAL AND APPLIED SCIENCES

Doctorate of Research in Neurobiology

Ph.D. Thesis

Meeting at the midbody: unraveling a novel localization of CDKL5

Coordinator: Prof. Daniela Parolaro

Personal Investigator: Prof. Nicoletta Landsberger

Chetan Chandola

No. 716003

Cycle XXVI

2010-2013

DECLARATION BY THE CANDIDATE

I, **Chetan Chandola**, hereby declare that the work presented in the form of this thesis was carried out by me under the guidance of **Prof. Nicoletta Landsberger** at the Laboratory of Genetic and Epigenetic Control of Gene Expression, Department of Theoretical and Applied Sciences, University of Insubria, Via Manara 7, Busto Arsizio (VA), Italy.

I also declare that no part of this thesis has been previously submitted for the award of any degree or diploma at the University of Insubria or any other university.

Place: Busto Arsizio (VA), Italy (Chetan Chandola)

Date: 3-11-2013

ACKNOWLEDGEMENT

At the outset I would like to thank my thesis supervisor, **Prof. Nicoletta Landsbeger** and co-supervisor **Prof. Charlotte Kilstrup Nielsen** who gave me all the freedom I needed to carry out my projects and ideas, while always being around to keep me from going off track. I am grateful to them for their support throughout my Ph.D and helping me to become an independent researcher.

I would like to thank **Prof. Daniela Parolaro**, Head of the PhD program in Neurobiology, DISTA, University of Insubria, Busto Arsizio, Italy, for providing a stimulating and conducive research environment.

I've had the pleasure of working alongside a great bunch of colleagues in the lab and want to thank all of them, particularly, Paolo with whom I had one of the most enjoyable moments in the lab and teach me whatever little Italian I know, Anna & Laura for having patience to answer all my questions. I am glad that I met Gilda Stefanelli, one of the most fun-loving person that I have ever known in my life, Marta and Isabella for their jovial company and also helping me in my experiments. I also want to thank Francesco Bedogni, Francesco Galli, Anna Gandaglia, Clementina Cobolli and my previous lab members (Martina Gri, Dalila, Gigio and Elisa) for their great co-operation and friendliness. Finally, Sarfaraz Nawaz for not only being around throughout the PhD and helping me in my experiments and little doubts, but also for his wonderful company in all our sojourns across this country.

Lastly, I want to thank my family for being the pillars of my strength. They have always motivated me in all my endeavours and all I can say to them is a heartfelt thank you.

Thank you, all!

CONTENTS

INTRODUCTION

Rett Syndrome

Historical overview

Rett syndrome (RTT) was first identified by Dr. Andreas Rett in 1966 after he observed 22 patients with similar unique symptoms (Rett 1966). Further studies by Dr. Hagberg allowed Rett syndrome to be considered as a neurodevelopmental disorder (Hagberg et al., 1983). It is considered to be the second most common cause of intellectual disability in females, after Down's syndrome (Hagberg 1995), with a frequency of 1:10,000 live female births. Though it is predominantly found in females, few cases of RTT have also been found in males. The disorder is clinically diagnosed based on the internationally accepted diagnostic criteria that were developed and adapted over the years as a useful tool for clinicians, involved in the diagnostic work-up, and for researchers, dealing with RTT (Hagberg et al., 2002; Neul et al., 2010).
The *MECP2* gene was discovered by Amir et al., (Amir et al., 1999) as the main genetic cause of RTT; in fact, we now recognize that *MECP2* is responsible for >95% cases of classical RTT (Amir et al., 1999; Neul et al., 2008; Zoghbi 2005). On the contrary, some of the variant forms of RTT are caused by genes other than *MECP2*. Indeed, we recognize several variant forms of RTT, depending on the phenotype and the involved gene. The congenital variant is often caused by mutations in *FOXG1* whereas the infantile seizure onset variant (Hanefeld variant) is mainly associated with *CDKL5* mutations.

Clinical Features and Diagnosis

RTT syndrome has been broadly classified into two types: classical (typical) RTT and variant (atypical) RTT. Classic RTT patients appear to develop normally from 6-18 months of age. During this developmental period they appear to develop normal motor functions and social communication skills. The head-circumference of a RTT girl is normal at birth; however, its growth decelerates at 2-3 months of age (Schultz et al., 1993). The disorder is characterized by early neurological regression that severely affects motor, cognitive and communication skills, often leading to microcephaly, loss of acquired skills, absence of speech, emergence of autistic features, loss of

purposeful hand skills, replaced by stereotyped hand movements, other motor abnormalities including abnormal muscle tone, ataxia and apraxia, and often a seizure disorder. Many girls with RTT have autonomic perturbations, including irregular breathing. This includes hypoventilation or hyperventilation while awake, breath holding, aerophagia, forced expulsion of air and saliva, and apnea.

The RTT Cascade of Clinical Symptoms in a Staging System

To segregate the non-specific developmental profile in early stages of life from a more specific profile of the disorder in the later stages, clinicians developed a staging system, where they delineated loss of acquired skills and communicative and motor dysfunctions (Engerstrom 1990). The four clinical stages of RTT are described as follows:

The Early-Onset Stagnation Period (Stage I)

This occurs between 6 months and 1.5 years of age. A sudden change in the interactive behavior of the girl may appear. Others may be irritable and restless and parents may relate frequent crying and teething. At this moment postural development is under progress but delayed (Dan and Cheron 2008). The child may learn to sit upright but not to crawl or to stand up and bottom shuffling is very common. Babbling of new words appears but remains poor. In short, the overall developmental pattern looks grossly normal at this stage.

The Rapid Developmental Regression Period (Stage II)

This stage appears between 1 and 4 years of age and is characterized by rapid and specific regression of acquired abilities. This may happen either suddenly, sometimes with pseudo-toxic symptoms (high pitched crying, fever and apathy suggesting meningoencephalitis), or with a gradual decline in the ability to communicate and in motor response. Though eye contact is still present, the child shows lack of interest in people and surrounding environment. Previously acquired words and motor skills are lost, and mental deficiency is now evident. Crying at night, recurrent infections or bouts of unexplained fever are common in this stage and often accepted as an explanation for the developmental delay. Febrile seizures may be present and questions about epileptic paroxysms may arise. A decline in head growth is also observed in most cases.

The Pseudo-Stationary Stage (Stage III)

This stage starts when the regression stage is over. If walking was previously acquired, the child may still be able to do so, and if not, they may still learn it.

But the loss of purposeful hand use is obvious. The typical hand stereotypies become prominent at this stage and constitute the hallmark of the disorder,

showing hand wringing, hand washing and clapping as soon as the child is awake. The visual contact behavior returns and the child is more alert. Breathing abnormalities that were present in stage II may become more prominent in this stage. Many have overt clinical epilepsy requiring treatment, but many also become seizure free after some time. The feet and lower limbs are cold, with or without color change, and with or without atrophic changes. Motor regression slowly progresses in this stage whereas the ability to communicate still persists, mainly with the eyes. Though this stage may last for decades, the girls and women are able to learn about new things, situations and persons.

Late Motor Deterioration (Stage IVA)

This stage begins when the individual is unable to walk and becomes completely dependent on the wheel chair. Some individuals with severe RTT syndrome pass directly from stage II to Stage IVB, which is characterized by severe neurological impairment with pronounced muscle wasting and distortion of distal limbs. The feet are cold and hand stereotypies become less intense with age. In these quadriplegic females the lack of motor activity finally leads to a state of frozen rigidity. However, significant eye-contact and eye pointing behavior is still present even in the most severe situations.

Diagnostic criteria

In spite of molecular genetics, diagnosis of Rett syndrome remains mainly clinical. For this purpose a set of clinical observations are made to classify the disorder (Table 1). Variant RTT can be identified with slightly different set of clinical symptoms (Table 2): at least 2 out of 4 main criteria (a clinical profile characterized by a regression stage followed by a recovery of interaction while neuromotor regression continues, reduced hand skills, hand stereotypies, reduced babble speech, reduced communication skills) have to be met together with at least 5 of the 11 supportive criteria (breathing irregularities, air swallowing bruxism, kypho-scoliosis, abnormal gait, lower limb amyotrophy cold, purplish feet, diminished response to pain, sleep disturbances, laughing/screaming spells and intense eye contact).

Table1. Rett syndrome diagnostic criteria 2010 (Neul et al., 2010)

Consider RTT diagnosis when postnatal deceleration of head growth is observed

Required for typical or classic RTT

A period of regression followed by recovery or stabilization

1. All main and all exclusive criteria
2. Supportive criteria are not required, although often present in typical RTT

Required for atypical or variant RTT

1. A period of regression followed by recovery or stabilization
2. At least 2 of 4 main criteria
3. 5 out of 11 supportive criteria

Main criteria

1. Partial or complete loss of acquired purposeful hand skills
2. Partial or complete loss of acquired spoken language
3. Gait abnormalities: impaired (dyspraxia) or absence of ability (apraxia)
4. Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing and washing/rubbing automatisms

Exclusion criteria for typical RTT

1. Brain injury secondary to trauma (peri- or postnatally), neurometabolic disease or severe infection that cause neurological problems
2. Grossly abnormal psychomotor development in the first 6 months of life

Supportive criteria for atypical RTT

1. Breathing disturbances when awake
2. Bruxism when awake
3. Impaired sleep pattern
4. Abnormal muscle tone
5. Peripheral vasomotor disturbances
6. Scoliosis/kyphosis
7. Growth retardation
8. Small cold hands and feet
9. Inappropriate laughing/screaming spells
10. Diminished sensitivity to pain
11. Intense eye communication and eye-pointing behaviour

Table 2. The cardinal clinical features of Rett syndrome in relation to pathology (Julu et al., 2008)

Affected part	Reported pathology	Clinical observations
Cortical	Decreased dendritic arborization and smaller than normal brain.	Severe intellectual disability
Cortical	Epilepsy	Seizures
Extrapyramidal	Monoaminergic dysfunction	Dystonia, incoordination of motor activities, secondary orthopedic deformities, and muscle wasting with contractures
Brainstem	Monoaminergic dysfunction	Dyspraxia, agitation and sleep disturbances
Brainstem	Immaturity with incompetence inhibitory neural networks	Abnormal breathing rhythm and lack of integrative inhibitions are likely causes of sudden deaths
Brainstem	Dysautonomia	Cold and blue extremities and sympatho-vagal imbalance

RTT Variants and Differential Diagnosis

The variant or atypical RTT in *MeCP2*-related disorder, in general, is referred to as "forme fruste" based on a more protracted clinical course with more preserved communicative and motor abilities. Other variant forms include: (a) the rare congenital variant (Rolando variant); the most severe form of atypical RTT, with onset of classic RTT features during the first three months of life. This variant is often caused by mutations in the *FOXG1* gene (14q11-q13). (b) The late childhood regression form is characterized by a normal head circumference and by a more gradual and later onset (late childhood) regression of language and motor skills. (c) The preserved speech variant (PSD or Zappella variant) is marked by recovery of some verbal and manual skills and is often caused by mutations in *MECP2*. (d) The early onset seizure type (Hanefeld syndrome), characterized by seizures in the first months of life with subsequent development of RTT features. It is frequently caused by mutations in the X-linked *CDKL5* gene (Xp22). CDKL5-associated clinical features can be briefly summarized in. (a) normal prenatal history; (b) irritability, drowsiness and poor sucking in the

perinatal period before seizure onset; (c) early onset epilepsy before 5 months of age; (d) Rett like features consisting of deceleration of head growth, stereotypies, poor to absent voluntary hand use, and sleep disturbances, and (e) severe intellectual disability with poor eye contact and language (Bahi-Buisson et al., 2008; Hagberg & Skjeldal 1994; Neul et al., 2010).

MeCP2 and RTT

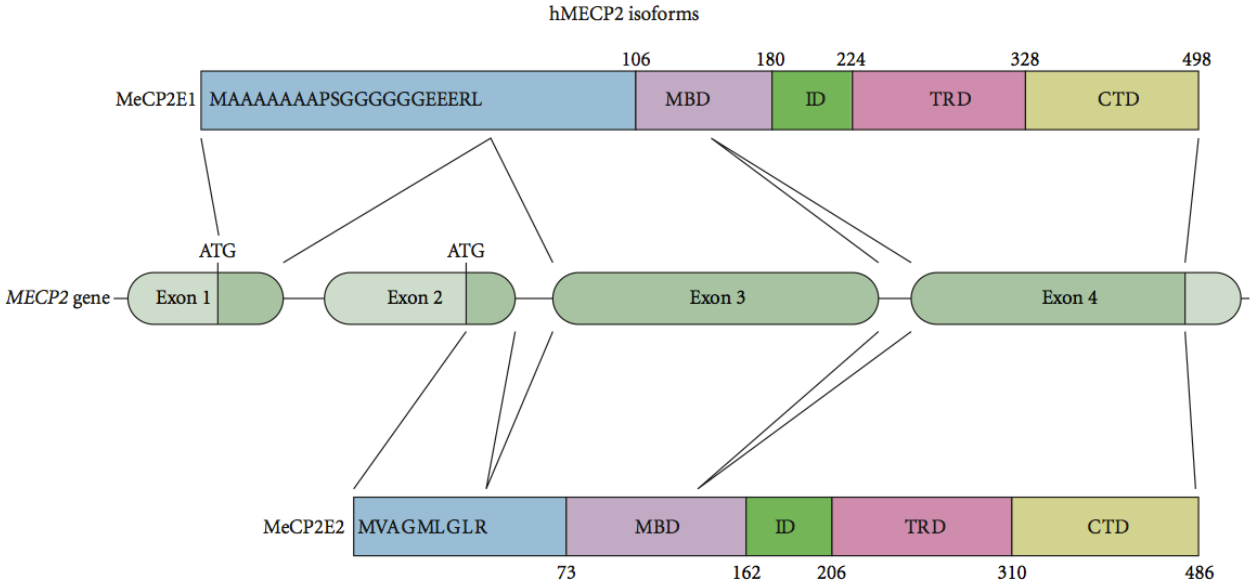


Figure 1: *MECP2* gene and protein isoforms. Schematic illustration of the gene structure of *MECP2* and the different domains of the two protein isoforms, MeCP2E1 and MeCP2E2. The primary amino acid composition of the N-terminus of MeCP2E1 and MeCP2E2 is depicted. (Zachariah & Rastegar 2012)

The MECP2 Gene

Classic RTT is a monogenic disorder caused by mutations in *MECP2* encoding methyl-CpG-binding protein MeCP2 (Amir et al., 1999). This gene is located in the Xq28 region, and is the founding member of the MBP (methyl binding protein) family of proteins(Lewis et al., 1992; Meehan et al., 1992). Mutations in the coding region of this gene can be identified in 80% of patients with the clinical diagnosis. The genomic locus of MECP2 spans approximately 76 kb and consists of 4 exons encoding two different isoforms (MECP2E1 and MECP2E2), due to alternate splicing of exons (Figure 1). *MECP2E1* isoform is expressed 10X more in the brain as compared to the *MECP2E2*. MECP2 has a long highly conserved 3'UTR that has multiple polyadenylation sites. Alternative 3' splicing leads to three different transcripts, short 1.8 kb and long 10 kb transcripts, with

the latter including a highly conserved (8.5 kb) 3'UTR, and a third additional low abundance transcript of 5-7 kb length (Pelka et al., 2005). MeCP2 is a nuclear protein that mainly colocalizes with densely methylated heterochromatin in mouse cells. Mecp2/*MECP2* transcripts are expressed differentially in different tissues and during different stages of development. In the brain, differential expression patterns are observed for the two MeCP2 isoforms (Dragich et al., 2007). The transcript levels are high during embryogenesis and decrease postnatally, but again increases during adulthood. On the contrary, protein levels are low during embryogenesis and increase postnatally upon neuronal maturation (Shahbazian et al., 2002b).

MeCP2E1 and E2 isoforms differ only in their N-terminal sequences, and have common Methyl Binding Domain (MBD) and Transcriptional Repression Domain (TRD). Though it seems that they have considerably overlapping functional properties but subtle etiologically relevant nonredundant functions of each transcript can't be ruled out. Differential expression pattern of the two transcripts has been observed in the developing mouse brain (Dragich et al., 2007) but it is yet to be confirmed that whether a similar variation is observed at the protein level or not. Mutation analysis of RTT patients has shown that exon 1 mutations can lead to severe RTT phenotypes. Some of these mutations do not seem to affect the transcription of MeCP2E2, suggesting that MeCP2E2 alone might not be able to compensate the loss of MeCP2E1. This idea was tested by a group that studied the RTT phenotype rescue capability by each isoform. They showed that MeCP2E1 alone is capable of compensating for overall MeCP2 deficiency in mice, in a dose dependent manner. Though MeCP2E2 also achieved phenotypic rescue, the degree of rescue was significantly higher for MeCP2E1, even at lower dosage levels (Kerr et al., 2012). The results of this study suggest that two isoforms have both redundant and nonredundant functions.

MeCP2 protein, interacting protein partners and posttranslational modifications

MeCP2 protein has three functional domains, viz., the methyl binding domain (MBD), the transcriptional repression domain (TRD) and the C-Terminal Domain (CTD). The MBD of MeCP2 consists of 63 residues with 4 anti-parallel beta-sheets that generate a wedge shaped structure. The MBD facilitates binding to the methylated CpG dinucleotides with a preference for adjacent A/T rich motifs (Klose et al., 2005). MeCP2 can also bind to non-methylated sequences such as the four-way DNA junctions (Galvao & Thomas 2005). However, the role of MeCP2 as a transcriptional repressor is mostly mediated through its TRD domain. The TRD interacts with a corepressor mSin3A, which further recruits

HDAC1 and HDAC2 causing transcriptional repression. Thus MeCP2 acts as a link between DNA methylation and chromatin remodeling (Nan et al., 1998). The TRD domain also helps in the interaction of MeCP2 with c-SKI (Kokura et al., 2001), YY1 (Forlani et al., 2010) and YB1 (Young et al., 2005). The CTD of MeCP2 is believed to have important functions, as transgenic mice lacking this domain display many RTT phenotypes (Shahbazian et al., 2002a). This C terminal region has a WW binding domain important for the interaction of MeCP2 with splicing factors (Buschdorf & Stratling 2004). WW binding domain recognizes the proline residues of interacting ligands, and is characterized by the presence of 2 tryptophan residues (W) that are separated by 20-22 amino acids.
MeCP2 is expressed in a wide variety of tissues but with the highest expression level in brain. Expression studies in rodents, macaque, and humans have revealed a similar pattern of heterogeneous MeCP2 expression in brain (Zachariah & Rastegar 2012). The MeCP2 expression patterns in different brain regions follows the developmental maturation of the CNS, being expressed initially in the earliest developing structures such as brainstem and thalamus (Shahbazian et al., 2002b); (Braunschweig et al., 2004; Mullaney et al., 2004). In rodents, MeCP2 expression in the olfactory bulb precedes synaptogenesis (Cassel et al., 2004; Cohen et al., 2003). MeCP2 expression is highest in neurons, with lower level of expression in glia (Ballas et al., 2009). Within neurons, MeCP2 expression is lower in immature neurons and highest in postmitotic neurons (Kishi & Macklis 2004). This elevated level of MeCP2 in mature neurons is maintained throughout the adulthood, implying its role in postmitotic function. In fact, MeCP2 deficiency in neurons is shown to cause RTT-like neurological phenotype in mouse (Chen et al., 2001). Recently, the expression of MeCP2 in glial cells has been shown to be necessary for neuronal maintenance, and in another study, it rescued RTT phenotype in a mouse model (Lioy et al., 2011).

The post-translational modification of MeCP2 is known to modulate its gene regulatory activity (Tao et al., 2009; Zhou et al., 2006). Phosphorylation of the serine 421 (S421) residue was the first post-translational modification (PTM) to be described in MeCP2 and was found to be induced by increased neuronal activity associated with influx of calcium ions. This phosphorylation event was therefore believed to be triggered by a CaMKII (Ca^{++}/calmodulin-dependent protein kinase II)-reliant mechanism. Out of total 12 tissues tested, this PTM was found only in brain tissue, suggesting S421 phosphorylation to be solely a neuronal event (Zhou et al., 2006). This study also showed that MeCP2 S421 phosphorylation alleviates the repression of the BDNF gene. Another study demonstrated MeCP2 pS80 to be dependent on neuronal calcium influx, similar to S421 (Tao et al., 2009). However, in contrast to S421, the S80

phosphorylation was negatively regulated by neuronal activity, suggesting alternative signaling between the residues in resting or depolarized neurons. Additionally, the phosphorylation of both S80 and S421 was found to decrease the association of MeCP2 at specific loci.

MeCP2 functions

MeCP2 has dual functions, working both as a transcriptional activator via interaction with CREB (Chahrour et al., 2008), and as a repressor observed *in vitro* caused by chromatin compaction and nucleosome clustering (Georgel et al., 2003; Muotri et al., 2010; Skene et al., 2010). MeCP2 also functions as a linker histone in neurons, as its levels are as abundant as histone proteins (Skene et al., 2010). This study showed that in a MeCP2 mutant brain the transcription level of repetitive elements in neurons is increased. Thus, it suggests that instead of acting as a gene-specific transcriptional repressor, MeCP2 suppresses spurious transcription of repetitive elements across the genome in a DNA methylation dependent manner, thereby reducing "transcriptional noise" (Muotri et al., 2010; Skene et al., 2010).

MeCP2 has been classically described as a global transcriptional repressor (Nan et al., 1997). The ability of MeCP2 to bind with methylated DNA and its localization in murine pericentromeric heterochromatin led the researchers to conclude MeCP2 to be a methylated DNA binding transcriptional repressor (Lewis et al., 1992). MeCP2 is 100 times more abundant than MeCP1 in the nucleus, and it could bind both to the methylated and unmethylated DNA to repress transcription (Meehan et al., 1992). In contrast, another in vitro study showed that MeCP2 bound to the methylated promoters caused repression of target genes whereas it caused slightly enhanced transcription level when bound to the unmethylated promoters (Nan et al., 1997).

MeCP2 also plays a role in RNA processing (Young et al., 2005). Role of MeCP2 in RNA splicing was indicated by its interaction with a RNA binding protein YB1 (Y-box binding protein) (Young et al., 2005). The same study demonstrated that MeCP2 itself can bind to RNA and regulate splicing *in vivo*. In addition, MeCP2 interaction with the transcriptional activator CREB1 has shed a light on its involvement in active transcription (Chahrour et al., 2008; Yasui et al., 2007).

The role of MeCP2 in the CNS has been facilitated by the development of various mouse models and the study of known phenotypic effects of the disorder. In humans, neuronal development and synaptogenesis occur in the embryonic week 12 and 20 respectively (Marsh et al., 2008). The absence of MeCP2 during

this key stage of development may be responsible for the decrease in neuronal and overall brain size in RTT patients. Disruption in MeCP2 function might therefore interfere with neuronal maturation and synaptogenesis, culminating in abnormal development of CNS. Several studies have also confirmed the role of MeCP2 in the development of dendritic spines and dendritic arborization, eventually affecting the synaptic connectivity. These defects in dendritic morphology in MeCP2 mutants are likely to cause the cognitive impairments observed in RTT patients.

MeCP2 RTT mutations

The location of MeCP2 in the Xq28 region of X chromosome is an important aspect in the etiology of RTT (Quaderi et al., 1994). MeCP2 mutations in males cause severe infantile encephalopathy because of the complete absence of a functional MeCP2. In contrast, females being heterozygous for the mutation, because of the presence of Mecp2 in X chromosome, show less severe symptoms than males, since half of the cells express the mutant MeCP2 allele and the other half expressing the functional allele. Therefore, RTT syndrome is found almost exclusively in females.

Genetic analysis has identified 218 mutations linked to RTT (Miltenberger-Miltenyi & Laccone 2003). These mutations are spread in all the three domains, indicating that each domain has an important function. Usually these mutations are single point mutations that give rise to a missense or nonsense mutant resulting in either amino acid change or a truncated protein respectively.

MeCP2 mutations are mostly sporadic, occurring preferentially as C>T transitions of CpG dinucleotides and mostly on the paternal X-chromosome (Girard et al., 2001; Trappe et al., 2001). MeCP2 mutations have been detected in more than 90% of classic RTT patients. Approximately 65% of MECP2 mutations causing RTT are attributed to 8 recurrent missense or nonsense mutations within the MBD (R106W, R133C, T158M and R168X) or TRD (R255X, R270X, R294X and R306C) (Bienvenu et al., 2000; Schanen et al., 2004)(94, 95). These mutations have been termed mutational “hotspots” (Kriaucionis & Bird 2004) and interestingly, most of these mutations occur at arginine residues. Though methylation specificity of MeCP2 is said to be important in the etiology of RTT syndrome, the two mutations in the MBD, i.e., T158M and R168X that have a weak or no affect on methylation affinity of the protein respectively, and other pathogenic non-MBD mutations support the idea that MeCP2 functions other than methyl-CpG binding are also crucial for a normal neurodevelopment.

At present, there is no effective treatment for RTT syndrome. However, recent studies in MeCP2 knockout mice showing RTT phenotype showed rescue symptoms after reactivation of MeCP2 (Giacometti et al., 2007; Guy et al., 2007). This demonstrates that delivering MeCP2 in neurons might be an effective gene therapy method for alleviating the RTT phenotype to some extent.

CDKL5 and RTT

The CDKL5 Gene

The *CDKL5* gene was identified by positional cloning study and mapped on the short (p) arm of the X-chromosome at location 22, i.e., Xp22 region. Sequence analysis studies revealed its homology to several other serine-threonine kinase genes and identified one protein signature specific to this subgroup of kinases, and hence, the gene was called *STK9* (Serine Threonine Kinase; ref). By genomic analysis Montini et al., initially demonstrated that *CDKL5* consists of 20 coding exons (Montini et al., 1998). Later, three more exons were discovered, taking the total count of exons to 23 (Figure 2). The first three exons (1, 1a and 1b) are untranslated and probably represent 2 transcription start sites of the gene. (Kalscheuer et al., 2003). The initiation codon is located within exon 2.

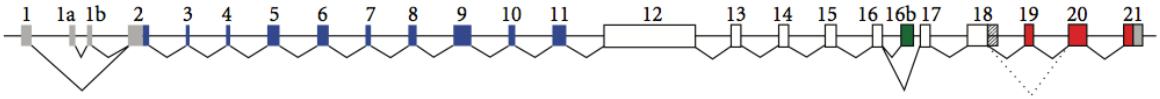


Figure 2: The human *CDKL5* gene with non-translated sequence in grey and exons encoding the catalytic domain in the blue. Exons encoding the common C-terminal region appears in white, whereas isoform specific sequences are shown in red, green and as hatched..
(Kilstrup-Nielsen et al., 2012)

Recently, an additional 123 bp exon was identified between exon 16 and 17; thus, by alternative splicing the two isoforms called *CDKL5* exon 16b (Fichou et al., 2011) and *CDKL5* exon 16a (Rademacher et al., 2011) are formed**.** Exon 16b has a very high degree of similarity between species, suggesting a functional role that has been maintained through evolution. Furthermore, transcript analysis studies have shown that the exon-16b containing mRNA isoform is specifically found in the brain (Fichou et al., 2011)**.** In rats, two splicing isoforms of *CDKL5* named *CDKL5a* and *CDKL5b* have been identified, each having a length of 934 and 877 amino acids respectively (Figure 3). *CDKL5a* isoform is expressed more

in pure neuronal cultures, and *CDKL5b* is the only isoform expressed in pure glial cultures, suggesting a role in gliogenesis (Chen et al., 2010). Moreover, the expression of a CDKL5 isoform with an alternative C-terminus that terminates at intron 18 has also been described by aligning the human and mouse CDKL5 proteins to the orthologs of other species (Williamson et al., 2012). This novel isoform, i.e. $CDKL5_{107}$, is the major CDKL5 transcript in the human brain and all other tissues investigated except the testis.

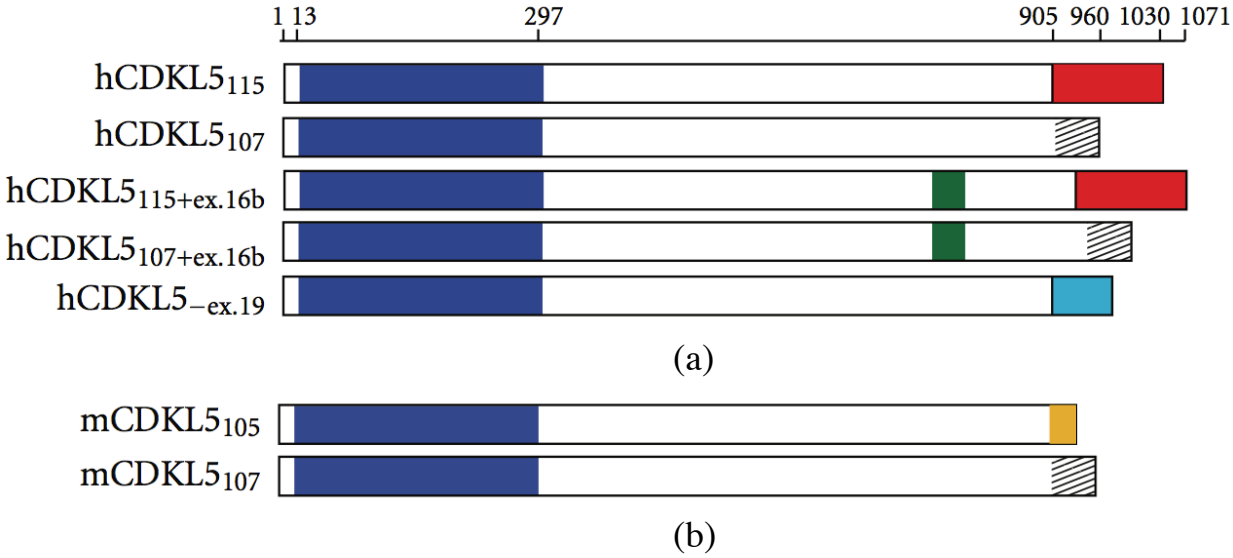


Figure 3. Splice variants of CDKL5. (a) hCDKL5 protein isoforms differing in the C-terminal region. $CDKL5_{115}$ contains the primate specific exons 19-21. In $CDKL5_{107}$, intron 18 is retained. The inclusion of exon 16b would generate $CDKL5_{115+ex.\ 16b}$ and/or $CDKL5_{107+ex.\ 16b}$. $hCDKL5_{-ex19}$ is a hypothetical splice variant in which exon 19 is excluded generating an alternative C-terminus. (b) The murine CDKL5 isoforms. $mCDKL5_{105}$ harbors a distinct C-terminal region encoded by a mouse specific exon 19 (orange). As in humans, the retention of intron 18 generates the common $CDKL5_{107}$ isoform.

(Kilstrup-Nielsen et al., 2012)

The CDKL5 Protein

As already mentioned, CDKL5 protein has different splice variants (Figure 3), the original transcript being 1030 aa long with an estimated molecular mass of 115 kDa (Bertani et al., 2006). CDKL5 belongs to the CMGC family of serine/threonine kinases (including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK), and CDK-like kinases) and is characterized by an N-terminal catalytic domain (13-297 aa), homologous to that of CDKL family members. CDKL5 is a unique member of this family because it has a remarkably long COOH-terminus, with more than 600 aa, usually not found in CDKL proteins, and evolutionarily conserved in different orthologs.

Initially, sequence analysis indicated that CDKL5 is a proline directed serine/threonine kinase closely related to p56 KKIAMRE (the protein encoded by CDKL2) and p42 KKIALRE (the protein encoded by CDKL1), which share homology with the members of mitogen-activated protein (MAP) kinase and cyclin-dependent kinase-like (CDKL) protein families (Montini et al., 1998). The sequence alignment of CDKL5 with these homologs showed 2 kinase signatures in the catalytic domain: (a) an ATP binding site (aa 14-47) and (b) a serine-threonine protein kinase active site (aa 127-144) (Figure 4). In addition, the catalytic domain has a conserved Thr-X-Tyr (TXY) sequence that corresponds to the TXY activation motifs of classic MAP kinases such as ERK2. Dual phosphorylation of this TEY motif is involved in activating, among others, kinases of the MAP kinase family.

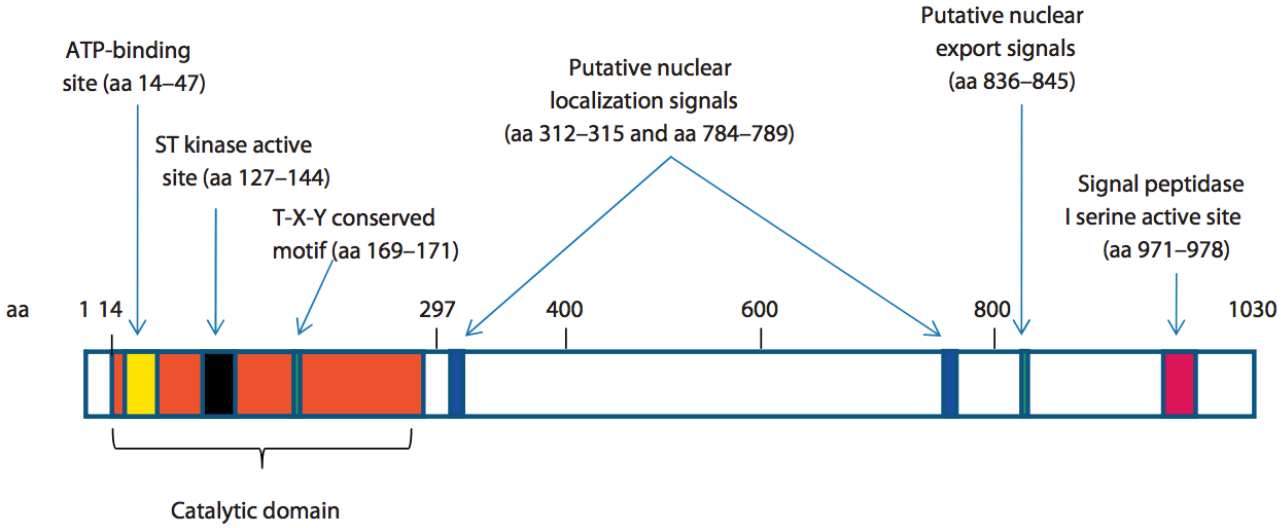
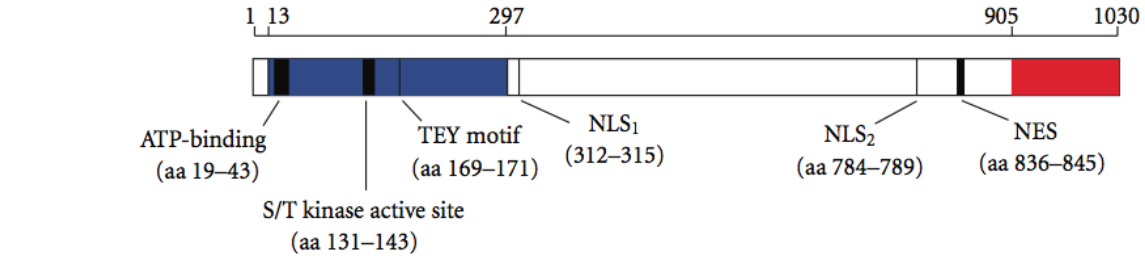


Figure 4: Schematic illustration of CDKL5. Full length human $CDKL5_{115}$ is 1030 aa long and contains an ATP binding site, the serine-threonine (ST) kinase active site and the conserved Thr-Xaa-Tyr (T-X-Y) motif within the catalytic domain. A putative signal peptidase I serine active site, 2 supposed nuclear localization signals and one supposed nuclear export signal are indicated. The number at the top refers to the amino acid positions.

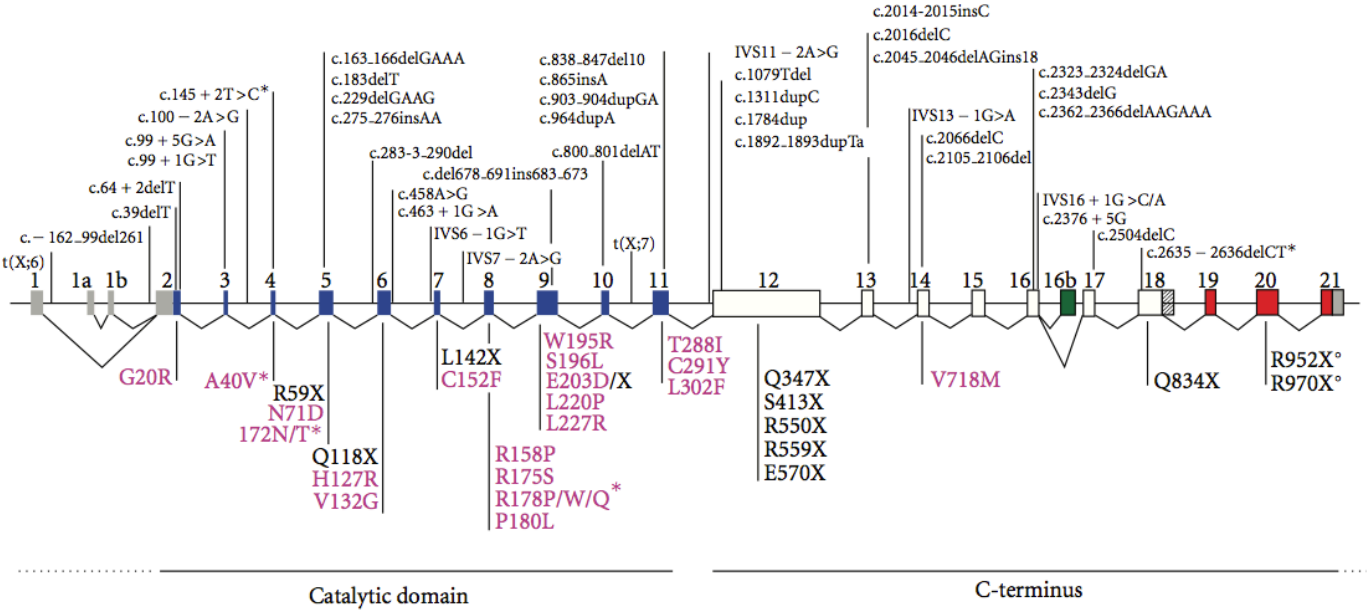
The long C-terminal (297-1030 aa) of $CDKL5_{115}$ has regulatory functions. In fact, this tail acts as a negative regulator of the catalytic activity of CDKL5, and through its putative signals for nuclear import (NLS) and export (NES) it modulates the subcellular distribution of CDKL5 (Bertani et al., 2006). A putative signal peptidase I serine active site (GTSMCTPL) is located between amino acids 971 and 978.

CDKL5 has two splice variants with distinct 5'UTRs: isoform I, containing exon 1, is transcribed by a wide range of tissues, whereas isoform II, including exons 1a and 1b, is expressed only in testis and fetal brain (Kalscheuer et al., 2003)

(Williamson et al., 2012). Alternative splicing gives rise to at least three hCDKL5 isoforms. The original CDKL5 transcript is 1030 aa long ($CDKL5_{115}$; 115 kDa). While $CDKL5_{115}$ is mainly expressed in testis, two recently identified transcripts are likely to be relevant for CDKL5 brain functions (Williamson et al., 2012).



(a)



(b)

Figure 5: CDKL5 molecular structure and its pathogenic mutations. (a) Schematic representation of $CDKL5_{115}$ with its functional domains and signatures. NLS: nuclear localization signal; NES: nuclear export signal. (b) All mutations reported in *CDKL5* to date are indicated. Mutations shown above *CDKL5* generate deletion and frameshift derivatives, as well as splice variants indicated with the cDNA nomenclature. Missense and nonsense mutations (fuchsia and black, respectively) are represented with the amino acid nomenclature below the *CDKL5* gene. *: recurrent mutations, °: uncertain pathogenicity.

(Kilstrup-Nielsen et al., 2012)

Fichou et al., 2011). This $CDKL5_{107}$ isoform, with alternative C-terminus and terminating at intron 18, is the most predominant form expressed in the brain, and is expressed in many species, along with human and mouse, rendering

CDKL5 mouse model of relevance for studying CDKL5 functions (Williamson et al., 2012). Interestingly, an alternative splice variant, containing another distinct C-terminus, has been predicted through bioinformatics simulation (EC gene analysis).

Cdkl5 is expressed in many tissues but with the highest level of expression in brain (cerebral cortex, hippocampus, cerebellum, striatum and brain stem), followed by testes and thymus (Lin et al., 2005). Its expression levels and subcellular distribution vary during the different developmental stages and in different brain areas (Rusconi et al., 2008). CDKL5 expression is low in embryonic cortex and is strongly increased during the perinatal and postnatal stages in maturing neurons in both cerebral cortex and hippocampus suggesting a role in neuronal maturation (Rusconi et al., 2008). Significant amounts of CDKL5 have also been found in the dendrites of cultured primary cortical neurons. The subcellular distribution on CDKL5 is controlled by an active cytoplasmic export mechanism together with and a nuclear import mechanism (Rusconi et al., 2008). The entry of CDKL5 into the nucleus depends on two NLS-like stretches (aa 312-315 and aa 784-789) of basic amino acids within the tail of the protein, whereas the cytoplasmic localization depends on an active nuclear export mediated by CRM1 nuclear export receptor (Rusconi et al., 2008).

CDKL5 mutations

The overall frequency of *CDKL5* mutations in females with the early-onset seizure variant of RTT is around 8-16% (Bahi-Buisson et al., 2008b; Nemos et al., 2009 (Mei et al., 2010). In females having both early onset seizure and infantile spasms this mutation rate raises to 28% (Bahi-Buisson et al., 2008b). Till now almost 100 patients have been described with different *CDKL5* mutations, including missense and nonsense mutations, small and large deletions, and frameshift and aberrant splicing alterations (Fig. 5). Although a small number of cases have been described so far, still hot-spots have been suggested for few mutations (indicated with an asterisk in Fig. 5b).

Missense mutations are mainly localized in the catalytic domain, thus confirming the relevance of the kinase activity of CDKL5 for proper brain function and/or development. These missense mutations in the catalytic domain can cause impairment in auto-phosphorylation and phosphorylation of target proteins, such as MeCP2 (Bertani et al., 2006; Tao et al., 2004). On the contrary, truncating mutations have been found all across the gene, both in the catalytic domain and the C-terminal tail, possibly leading to CDKL5 derivatives of various lengths. Though, so far, no report has shown the existence of truncated CDKL5 protein in patients' cells, the molecular effects of some of these pathogenic mutations

(missense and truncating derivatives in the background of $CDKL5_{115}$ isoform) have been shown by overexpressing these mutated derivatives in non-neuronal cell lines (Bertani et al., 2006; Lin et al., 2005; Rosas-Vargas et al., 2008). From these studies C-terminus appeared to act as a regulator of CDKL5 functions. Indeed, its truncations led to increased catalytic activity and also controlled its sub-cellular localization (Bertani et al., 2006; (Rusconi et al., 2008). Truncated CDKL5 derivatives had, in fact, been found to localize more in the nucleus; in particular, the pathogenic derivatives L879X and R781X appeared exclusively confined to the nucleus, whereas the full-length protein is equally distributed between the two compartments. Interestingly, as the leucine-rich Nuclear Export Signal-like motif, shown in figure 5a, is preserved in the L879X derivative, such motif seems to be insufficient for driving CDKL5 into the cytoplasm. Whether truncated CDKL5 acts as a loss- or gain-of function protein still remains to be understood; in fact, if expressed, they would be mislocalized hyperfunctional derivatives and we still do not know whether the lack of a functional CDKL5 in the cytoplasm or the presence of an hyperactive kinase in the nucleus, or both, result in the pathogenic phenotype. It is however important to recall that pathogenic duplications of the X-chromosome, including *CDKL5*, have also been reported showing RTT like features (ref). In the future it will be thus important to address whether an optimal CDKL5 level is important for the development and functioning of the central nervous system.

Genotype-phenotype correlation

At present, there are no conclusive data suggesting the existence of a genotype-phenotype correlation for CDKL5 mutations. Some reports suggest that mutations affecting the N-terminal catalytic domain are associated with more severe phenotype consisting of early onset and intractable infantile spasms followed by late onset multifocal myoclonic epilepsy (Bahi-Buisson et al., 2008a), with respect to mutations in the COOH-terminus resulting in a milder phenotype (Russo et al., 2009). However, these reports are debated, and generally the nature of mutations seems to not correlate with the clinical heterogeneity. This idea appears supported by a report in which two genetically identical *CDKL5*–mutated twin girls showed a significantly discordant phenotype (Weaving et al., 2004). In fact, one of the twins showed a clinical phenotype overlapping with RTT, while the other showed an autistic disorder with mild-to-moderate intellectual disability. Since both girls were characterized by random X-inactivation, it is thought that their phenotypic differences can be attributed to modifier genes that have been differentially induced by environmental and/or epigenetic factors.

Functions of CDKL5 protein

Although we still do not know which and how many functions harbors CDKL5 and which ones are relevant in the CDKL5-related pathologies, in the last years few publications have started to unravel the molecular activities of the kinase and the phenotypic consequences of its malfunctions in neurons. Herein, I will summarize the most relevant data.

CDKL5 expression correlates with neuronal maturation and maintenance

As already mentioned it is evident from earlier studies that CDKL5 expression correlates, both *in vitro* and *in vivo*, with neuronal maturation, reaching the highest level of expression when neurons acquire a mature phenotype, thus suggesting a role for CDKL5 in neuronal differentiation and arborization (Chen et al., 2010; Rusconi et al., 2011). Interestingly, even though CDKL5 expression levels decrease slightly in adult brain, they are significantly higher than in non-neuronal tissues. Hence, CDKL5 might have a role in maintaining neuronal functions in addition to maturation. Furthermore, since, CDKL5 intracellular distribution changes upon neuronal maturation and its nuclear fraction peaks in adult brain, such fraction may be involved in adult brain synaptic plasticity.

CDKL5 affects neuronal morphogenesis through cytoskeletal rearrangements

In brain disorders, such as RTT and Fragile X, arborization defects disrupt experience dependent neuronal maturation and plasticity (Kaufmann & Moser 2000). In vitro, by RNAi and overexpression of CDKL5 in cultured rat neurons, Chen et al., showed that CDKL5 is a critical regulator of neuronal morphogenesis and dendritic arborization. *In vivo*, CDKL5 shows overlapping functions and affects neuronal migration (Chen et al., 2010). Interestingly, CDKL5 colocalizes with F-actin in the growth cone and interacts with Rac1. Rac1 is a protein that belongs to the Rho GTPase family and it promotes formation and/or maturation of spines by remodeling the actin cytoskeleton of neuronal spines (Tolias et al., 2011). Functional experiments suggested that CDKL5 influences neuronal morphogenesis by acting upstream of Rac1, and CDKL5 itself is activated by transient phosphorylation from brain-derived neurotrophic factor (BDNF). In the absence of CDKL5, BDNF is unable to activate Rac1 (Chen et al., 2010).

Neuronal stimuli affect CDKL5 function

The knowledge of stimuli affecting CDKL5 expression might help in understanding how CDKL5 deficiency might impact brain functions. As already mentioned, the activity-regulated BDNF gene induces a transient phosphorylation of CDKL5 and this post-translational modification is required for Rac1 activation by BDNF (Chen et al., 2010). Furthermore, it has been demonstrated that CDKL5 is transported from the nucleus to the cytoplasm in response to the activation of extrasynaptic NMDA receptors (NMDA-R). After translocation, the kinase gets degraded (Rusconi et al., 2011). Recent publications suggest that extrasynaptic NMDA-R have a role in LTD and dephosphorylation of CREB; alterations in the cross-talk between synaptic and extrasynaptic receptor activities may play an important role in seizures (Hardingham & Bading 2010; Hardingham et al., 2002; Sierra-Paredes & Sierra-Marcuno 2007).

CDKL5 might regulate the functions of epigenetic factors and transcriptional regulators

There is some evidence suggesting a role of CDKL5 in regulating gene expression. Previous studies have shown that the transcriptional repressor MeCP2 and CDKL5 work in the same molecular pathway, sharing overlapping temporal and spatial expression pattern in the brain, and both being activated simultaneously during neuronal maturation. According to the current understanding, CDKL5 works upstream of MeCP2 and causes its phosphorylation either directly or indirectly, thereby influencing its function (Bertani et al., 2006; Mari et al., 2005). Since several studies have shown that, in neurons, MeCP2 acts as a dynamic epigenetic factor, regulating gene transcription during learning and memory through activity–dependent phosphorylation of specific serine residues (Chen et al., 2003; Martinowich et al., 2003; Tao et al., 2009; Zhou et al., 2006), it is possible to assume that in the absence of CDKL5, a specific phosphorylation dependent activity of MeCP2 might be altered causing a subset of Rett symptoms.

The connection of CDKL5 to epigenetics and gene expression appears strengthened by an article showing its interaction with DNMT1; it is interesting to recall that inhibition of DNA methyltransferase I (DNMTI) in the hippocampus affects contextual fear memories and LTP (Day & Sweatt 2011). Eventually, CDKL5 expression is induced by MeCP2 ablation, DNMT inhibition, and histone deacetylase inhibition (Carouge et al., 2010).

CDKL5 controls signal transduction pathways and event-related potentials whose disruption leads to austistic features

A recently developed CDKL5 KO mouse model has reaffirmed the role of CDKL5 for proper brain functioning by showing autistic like features (Wang et al., 2012). Furthermore, exploiting this mouse model, the authors have performed a serine/threonine kinome study and have revealed that many signal transduction pathways are disrupted. Interestingly, many of these pathways, including the AKT-mTOR pathway that appears down-regulated in the *Cdkl5*-null brain, have been implicated in the etiology of ASDs (Jeste et al., 2008; Tsai et al., 2012). Since mTOR is known to regulate cell growth, proliferation, motility and neural plasticity (Zoncu et al., 2011), any reduction in the AKT-mTOR activity, due to the absence of CDKL5, may lead to disruption of neuronal development. As mentioned, RNAi mediated knockdown of CDKL5 results in impaired dendritic outgrowth, neuronal migration (Chen et al., 2010), and spine maturation (Ricciardi et al., 2012); in the future it will be interesting to study whether the observed phenotype is linked to the down-regulated pathway.
Furthermore, *Cdkl5*$^{-/y}$ mice showed decreased phosphorylation profile of kinases involved in synaptic plasticity, including PKA, PKC, and protein kinase D (PKD), as well as kinases involved in cellular metabolism, including AMPK, ATM/ATR, and casein kinase (CK) (Wang et al., 2012).

CDKL5 influences dendritic spine development

Very rcently CDKL5 has been found to co-localize with the post synaptic density (PSD) of excitatory synapses both *in vivo* and *in vitro* (Ricciardi et al., 2012). Depletion of CDKL5 in rat hippocampal neurons indicated that CDKL5 is required for ensuring a correct number of well-shaped spines. These morphological alterations were associated with a reduction in the number of excitatory synapses and a significant decrese in spontaneous miniature excitatory postsynaptic currents (mEPSCs). However, there was no significant effect on inhibitory synapse density or any significant changes in miniature inhibitory postsynaptic currents (mEPSCs) (Ricciardi et al., 2012). CDKL5 also interacts with and phosphorylates Netrin G1 receptor (NGL-1). NGL-1 is a synaptic cell adhesion molecule which plays an important role in early synapse formation and subsequent maturation. NGL-1 binds with PSD95, a protein that plays an important role in learning and memory, and this binding is stabilised by phosphorylation of NGL-1 at Ser631 by CDKL5. This stabilisation of NGL-1/PSD95 helps in the targeting of PSD95 to new forming dendritic protrusions (Ricciardi et al., 2012). Summarizing the data, at least two important pathways

are associated with the cytoplasmic functions of CDKL5: Rac-1 and BDNF and, PSD95 with the NGL-1 complex.

CDKL5 binds with and phosphorylates amphiphysin 1

Amphiphysin is a novel substrate that has been found to interact with and get phosphorylated by CDKL5 at Ser-293. Again this mechanims occurs in the cytoplasm highlighting the relevance of the cytoplasmic portion of CDKL5; importantly, this phosphorylation is disrupted by mutations in the catalytic domain of CDKL5 (Sekiguchi et al., 2013). Phosphorylation of Amph1 by CDKL5 is found to be significantly higher than those of MeCP2 and Dnmt1. Amph1 is abundantly present in the cytoplasm and is highly expressed in neurons. Amph1, a mutlifunctional adaptor protein, plays an important role in neurotransmission and synaptic vesicle recycling through clathrin-mediated endocytosis (Wigge & McMahon 1998); accordingly, Amph1 deficient mice show major learning difficulties and irreversible seizures, suggesting its role in neural development and transmission (Di Paolo et al., 2002).
To conclude, although the identification of novel interactors and phosphorylation targets of CDKL5 will help in defining the neuronal functions of this kinase and the consequences of its deficiency, from the data obtained so far we can assume that the protein has a major role in synaptic formation, activity and plasticity.

Midbody

As we will show in the Results section, we have found that, in dividing Hela cells, CDKL5 localizes at the midbody, a transient structure that is formed during the late stages of mitosis. In the recent years the midbody has gained attention as new pathways and functions have been attributed to this structure, mainly the cytokinetic functions and some post-mitotic functions. In accordance with its role in abscission, mass spectrometry assays have shown that the midbody contains cytoskeleton proteins as well as other proteins involved in lipid rafts and vesicle trafficking (Skop et al., 2004). The midbody in animal cells is analogous to the cell plate present in plant cell division (Flemming 1891, Paweletz 2001) as both are composed of antiparallel microtubules with vesicles and amorphous electron-dense material centrally positioned.

Midbody structure and formation

Midbodies (MBs) were first studied and reported by Walther Fleming using histochemical methods (Flemming, 1891). Since its first description, it has been studied sporadically by cytologists (Buck & Tisdale 1962; Mullins & Biesele

1977; Mullins & McIntosh 1982; Saxton & McIntosh 1987). Its formation begins when the midzone – a bipolar antiparallel microtubule array that assembles between separating sister chromatids during anaphase (also called the central spindle), undergoes compaction during the final stages of cytokinesis, giving rise to a tightly packed structure with a central bulge and adjacent filaments, covered by a membrane. There is a positive correlation between the conversion of midzones to midbodies and cytokinetic furrow ingression This is evident from the fact that blocking furrow ingression results in accumulation of cells that are stuck in midzone assembly (Straight et al., 2003). Hence, furrow is believed to compact the antiparallel midzone MTs into a single large bundle that comprises the core of the MB (Fig 6a).

MB attains final structure by late cytokinesis and has three components – (a) the central core (also called stem body), (b) flanking MTs and associated organelles, and (c) the surrounding plasma membrane (Figure 6a). The central core has two components, the 'MB matrix' and 'MB ridges'. The MB matrix is mainly composed of antiparallel MT bundles and interspersed electron-dense material (Brinkley & Cartwright 1971; Kuo et al., 2011; McIntosh & Landis 1971; Mullins & Biesele 1977; Paweletz 1967). MB ridges surrounding the matrix account for the bulges within the intercellular bridge (Figure 6b) and is mainly composed of electron-dense material, a specialized thickening under the plasma membrane, and a few MTs (Mullins & Biesele 1977). MB ridges are likely to correspond to MB rings (MRs), donut shaped structures.

Fate of post-mitotic midbodies

Post mitotic MBs may have different fate depending on the cell type and status. Immediately after the abscission the post-mitotic MB is retained by one of the two daughter cells, connected to the cell body through a thin bridge, also known as tether. This tether can retract, delivering the post-mitotic MB into the cytoplasm of the daughter cell where it undergoes degradation or normal retention (Gromley et al., 2005; Ettinger et al., 2011; Kuo et al., 2011; Pohl & Jentsch 2009). Alternatively, the tether can be severed by the cell, releasing the post-mitotic MB into the extracellular space (Guizetti et al., 2011; Dubreuil et al., 2007; Elia et al., 2011; Ettinger et al., 2011), where it remains or is engulfed by a cell. These findings indicate that the fate determination of post-mitotic MBs involves multiple steps that might be tightly regulated, and is far different from their fate as residual bodies as thought earlier (Guizetti et al., 2011; Mullins & Biesele 1977).

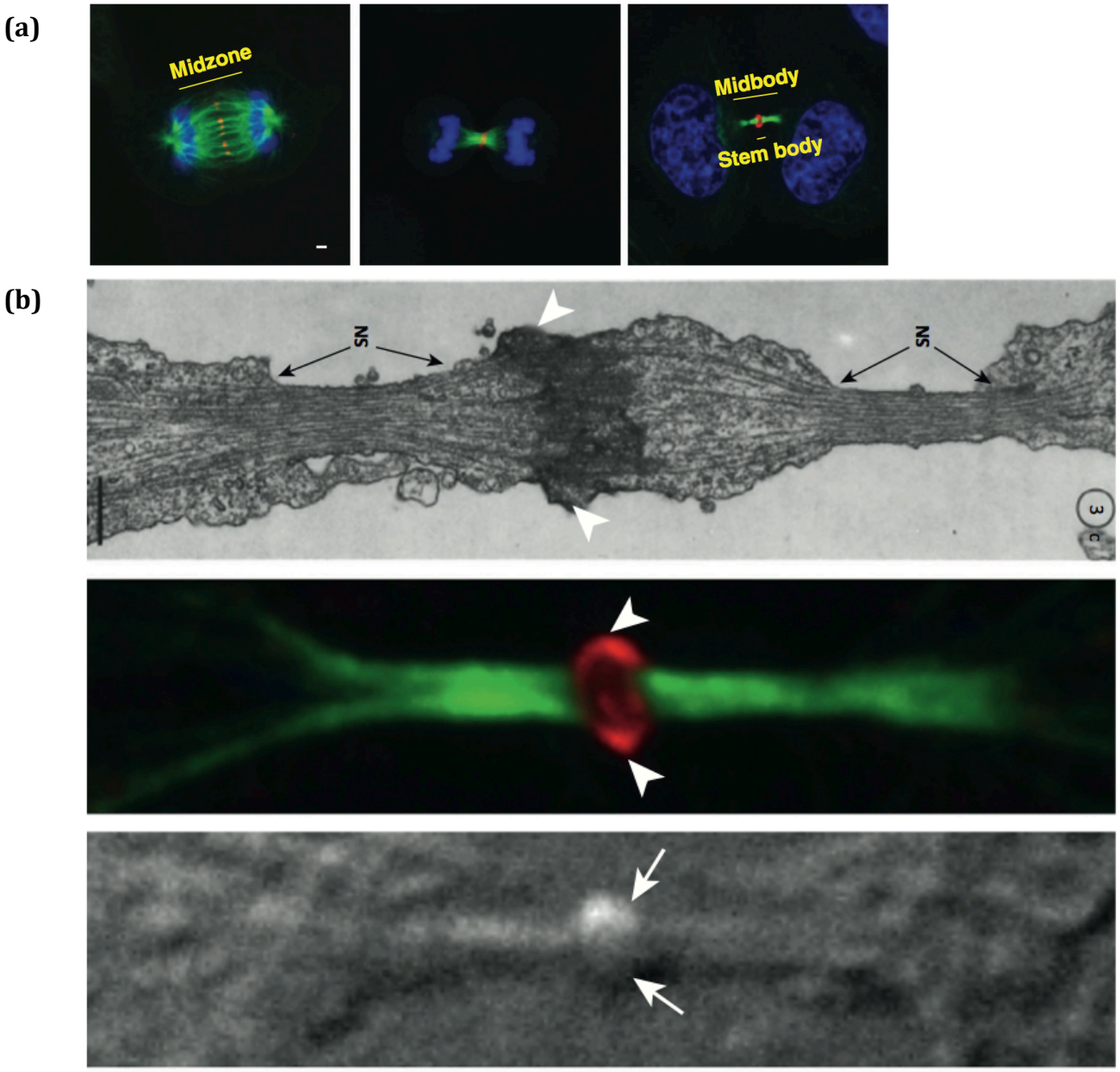


Figure 6: (a) Midzones between separated sister chromatids gradually convert into the midbody during furrow ingression. Individual midzone bundles are compacted into a single, large, midbody MT (microtubule) bundle. A bulge like structure named stembody is formed at the centre of midbody. The midzone, midbody and stembody are indicated in yellow. (b) Electron micrograph of MB shown in top panel. A comparable stage with corresponding differential-interference contrast (DIC) microscope image is shown in the middle and bottom panel, respectively. At the centre is the electron-dense MB core (top), which composes 'MB ridges' and 'MB matrix'. The MB ridges (arrowheads; top) correspond to the MB ring (MR) on immunofluorescence and bulge on DIC microscopy (arrows; bottom). The MB matrix, by contrast, contains many antiparallel microtubules and electron dense material.

(a) Chen et al., 2013 (b) Hu et al., 2012

MB functions

Abscission

The most important function attributed to the midbody is to localize abscission. Recently, significant progress has been made in understanding the mechanisms of both abscission and the subsequent midbody breakage. The development of midbody proteome (Skop et al., 2004) has lead to the discovery of a large number of molecules and pathways that contribure to abscission. These pathways include vesicle trafficking, membrane scission, MT severing and ubiquitination. Abscission may be carried out by the endosomal sorting complex required for transport (ESCRT) machinery and midbody breakage by the activity of microtububle-severing proteins (Elia et al., 2011; Gromley et al., 2005; Guizetti et al., 2011; Lee et al., 2008; Morita et al., 2007). However how ESCRT machinery and severing proteins are localized and temporally regulated is unknown. One problem in analysing the proteins in midbody assembly and abscission is that most of the midbody proteins are also required for furrow ingression; thus, their ablation causes earlier defects that preclude analysis of their subsequent specific roles.

Non-cytokinetic functions

Recent studies have suggested that midbodies may have non-cytokinetic functions too, such as polarity specification (Pollarolo et al., 2011; Wilcock et al., 2007), intercellular communication (Dubreuil et al., 2007; Marzesco et al., 2005) and cell fate determination. In relation to the role of midbody in polarity specification, it is important to mention here that neuronal polarization may lead to some neuropsychiatric disorders autism, epilepsy, mental retardation and scezophrenia (Li et al., 2011).

In the fly notum and chick spinal chord, post mitotic MBs of neurons were found to be present at the polarized/apical domain from where the future neurite or apical process sprouted (Pollarolo et al., 2011; Wilcock et al., 2007). Hence, this result suggested that post-mitotic MBs might specify neuronal polarity. Post-mitotic MBs have been implicated in intercellular communication, presumably to maintain a balance between differentiating cells and progenitors (Dubreuil et al., 2007; Marzesco et al., 2005). This hypothesis is based on the fact that MB release into the ventricle lumen increases significantly after neurogensis (Dubreuil et al., 2007; Marzesco et al., 2005).

Furthermore, recent studies have shown that experimental manipulation of MB clearance can cause cell fate conversion. For example, inhibition of MB clearance/release could sensitize oligopotent neural progenitors to differentiate

into neurons. On the contrary, terminally differentiated fibroblasts revert back to iPSCs on retention of MB (Ettinger et al., 2011; Kuo et al., 2011). Here, contradictarily MB retention results both in differentiation and dediferentiation of cells which may be because the modulator, presumably the MB, is itself modified in different ways.

EPERIMENTAL PROCEDURES

Plasmids

The three plasmids, viz., GFP-wt-CDKL5, GFP-CDKL5-N-terminal and GFP-CDKL5-C-Terminal used for transfection of HeLa cells were made as explained by Bertani et al (Bertani et al., 2006).

Antibodies

The following antibodies were used for western blotting and immunofluorescence experiments: polyclonal anti-GAPDH (Open Biosource), monoclonal anti-alpha-tubulin (Sigma), polyclonal anti-CDKL5 (Sigma), monoclonal anti-phospho aurora A/B/C (Cell Signalling), monoclonal anti-AKT (Cell Signalling), polyclonal anti-Tsg101 (Abcam), polyclonal anti-Cep55 (Abnova), polyclonal anti-Ect2 (Santa Crus), polyclonal anti-IQGAP1 (Santa Cruz), polyclonal ant-GFP (Molecular Probes).

Cell cultures and transfections

Cell cultures and transfections: human HeLa cells and MRC5 were maintained in Dulbecco's modified Eagle's medium (D-MeM) supplemented with 10% foetal bovine serum, 2mM L-Glutamine and 100 U/mL Penicillin-Streptomycin and grown at 37°C with 5% CO_2. Neuroblastoma Neuro-2a cell line was maintained in MeM supplemented with 10% foetal bovine serum, 1% non-essential aminoacids, 2mM L-Glutamine and 100 U/mL Penicillin-Streptomycin and grown at 37°C with 5% CO_2.

Low-density primary cultures of cortical neurons were prepared from brains of mouse embryos at 18 days (E18). Cortex was removed, centrifuged at 800g, washed twice in HBSS (GIBCO) and dissociated by a 15' incubation in 0.25% trypsin (Sigma) at 37°C. Suspended cells were plated at a density of 20'000-30'000 cells/cm^2 on poly-D-lysine (50g/mL, Sigma) coated glass coverslips in D-MeM with 10% horse serum and 2mM L-Glutamine. After 3-4 hours, coverslips were transferred in a dish containing a monolayer of cortical astrocytes without physical contact between neurons and glial cells. Neurons were grown in glial conditioned Neurobasal (Gibco) medium with B27 supplement (GIBCO) and 2mM L-Glutamine and incubated at 37°C with 5% CO_2. After 3 days in vitro

(div), cytosine-1-b-D-arabinofuranoside (Ara-C, Sigma) at the final concentration 2 mM was added to prevent astroglial proliferation.

Immunofluorescence

Cortical neurons were fixed at 4 days in vitro (DIV) by 4% PFA, cells were washed and subjected to immunofluorescence. After 10 minutes in permeabilization buffer (20mM HEPES pH 7.4; 300mM sucrose; 3mM $MgCl_2$; 0.2% Triton X-100 and 50mM NaCl), aspecific sites were saturated by a blocking solution (5% FBS in PBS) for 30 minutes and fixed neurons were incubated overnight at 4°C with primary antibodies in 5% FBS and 0.1% Triton X-100. The following day, cells were rinsed in PBS 3 times and incubated with the secondary antibodies (anti-rabbit Alexa Fluor 555 and anti-mouse Alexa Fluor 488, Invitrogen) in blocking solution for one hour. Finally, nuclei were stained with DAPI and the specific signals were analysed.

Untransfected cells: HeLa cells cultured on glass coverslips were washed in PBS and fixed with 2% PFA in PBS at room temperature for 10 minutes. After permeabilization with 0.2% Triton in PBS for 6 hours, cells were blocked in 5% BSA in PBS with 0.2% Triton X-100 for 1 hr. The fixed cells were stained with the indicated primary antibodies in the same blocking solution overnight at 4°C. Next day, cells were washed with PBS and incubated for 1 hour with the appropriated secondary antibodies at room temperature. After three PBS washes nuclei were stained with DAPI (Sigma) and mounted using Prolong Gold Antifade medium (Life Technologies). and the specific signals analysed with a Nikon Eclipse Ni fluorescence microscope.

Transfected HeLa cells: HeLa cells were seeded on glass coverslips and transiently transfected with GFP-tagged CDKL5 constructs using Lipofectamine 2000 (Invitrogen). 16-18 hours post-transfection, the cells were permeabilized (0.2% Triton in PBS for 2 minutes), fixed in 2% paraformaldehide (PFA) for 10 min, and blocked with blocking medium (5% BSA/ 0.2% Triton X-100/ PBS) for 1 hr. Then cells were stained with anti-GFP polyclonal and anti-alpha-tubulin monoclonal antibodies followed by secondary antibodies. Nuclei were stained with DAPI (Sigma) and the specific GFP-signals analysed with a Nikon Eclipse Ni fluorescence microscope.

Silencing experiments: HeLa cells were transfected with CDKL5 or scramble si-RNA 24 hours after plating using oligofectamine (Invitrogen) reagent following manufacturer's instructions. 72 hrs after the transfection, cells were scrapped for

immunoblot analysis and were subjected to immunofluorescence (refer IF protocol of untransfected cells).

siRNA

siRNA oligomers containing 19 nucleotides were synthesized using human CDKL5 sequence 5'-CUAUGGAGUUGUACUUAAA-3' (Thermo Scientific). The selected sequence was submitted to a BLAST search against the mouse genome, to ensure that only the CDKL5 gene was targeted. si-scramble non targeting siRNA (Thermo Scientific) was used as a control oligonucleotide.

Wound Healing assay

30,000 HeLa cells were seeded on a glass coverslip in a 6 well plate followed by transfection with si-scramble and si-CDKL5 oligos the next day. After 3 days of transfection, scratch wounds were made using a pipette tip and medium was changed to remove debris and mitotic cells. Cells were fixed at different time points, i.e., 0 and 24 hrs, followed by immunofluorescence.

Midbody isolation

HeLa cells were enriched in telophase by treatment with Nocodazole (100ng/ml for 4 hrs) followed by mitotic shake off, nocodazole washout and incubation of the collected cells for about 60 min. to reach telophase stage. Midbodies were isolated as described by Rinaldo et al (Rinaldo et al 2012) and extracted using an extraction buffer (50mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% NP-40, 5 mM EDTA) supplemented with protease- and phosphatase-inhibitor mix (Roche). TCEs (Total cell extracts) from asynchronized interphase cells were obtained in the same manner and analyzed by WB together with midbody extracts.

Coimmunoprecipitation assay

Midbody lysate was prepared from HeLa cells in lysis buffer (50mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% NP-40, 1 mM DTT, 5 mM EDTA) supplemented with protease- and phosphatase-inhibitor mix (Roche). The lysates was incubated with anti-CDKL5 polyclonal antibody (Invitrogen) overnight at 4ºC. The immunocomplex was then incubated with 50% rG-agarose beads (Invitrogen) blocked with 5% BSA for 1hr at 4ºC. The complexes bound to the beads were eluted by resuspending in Laemmli reducing sample buffer followed by heating at 90ºC for 5 min. The immunocomplex was subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies.

Results

CDKL5 localizes at the centrosome and midbody during mitosis in HeLa cells and in the centrosome of post-mitotic cortical neurons

While working on MeCP2 in our lab we found that MeCP2 localizes at the centrosome during early mitotic phase (Bergo et al., submitted for publication) in HeLa cells (Figure 7). Since it is known that MeCP2 and CDKL5 belong to the same molecular pathway (Mari et al 2005) and have a similar spatio-temporal localization pattern we decided to investigate the localization of CDKL5 during mitosis in dividing cells. We observed that similarly to MeCP2, also CDKL5 localizes at the spindle poles during early mitosis. However, in contrast to MeCP2 which remains present at the spindle poles throughout the mitosis, CDKL5 leaves the poles and starts localizing at the midzone during late anaphase, and localizes completely at the midbody during telophase till abscission (Figure 8a and b). As the cell enters interphase, CDKL5 acquires again its canonical nuclear punctate staining and does not show any co-localization with the centrosome.

Considering that MeCP2 deficiency mainly affects the CNS, we also studied whether MeCP2 localizes at the centrosome of mouse cortical neurons (DIV4). We found that similar to HeLa cells where MeCP2 is present at the centrosome during interphase in addition to mitosis, MeCP2 localizes at the centrosome of post-mitotic neurons (Figure 9a). Surprisingly, although CDKL5 does not localize at the centrosome of dividing cells, it was revealed at the centrosome of post-mitotic mouse cortical neurons (DIV4) (Figure 9b).
Next, we confirmed CDKL5 localization at the midbody by means of a biochemical approach. To this purpose we adapted a protocol from Rinaldo et al (Rinaldo et al 2012), as shown in the flowchart (Figure 10 a). Different markers were used to test the quality of the MB preparation. Phospho-aurora-A/B/C and alpha-tubulin confirmed enrichment of MB proteins whereas AKT and GAPDH were used to monitor the level of nuclear and cytoplasmic contamination respectively. From the same midbody fraction, both WB and immunofluorescence were performed (Figures 10b and c).

Exogenous CDKL5 is targeted to the midbody

To confirm CDKL5 localization at the midbody and to identify the involved protein domains, we transfected HeLa cells with GFP tagged wt-CDKL5 and performed an immunofluorescence, aimed at revealing whether the exogenous protein gets also localized at this cellular structure. As shown in Fig. 11a, exogenously expressed GFP-CDKL5 localizes at the midbody ring as the endogenous CDKL5. Further confirmation was obtained through the above described biochemical assay performed on GFP-CDKL5 transfected HeLa cells (Figure 11b). HeLa cells transfected with a GFP expressing plasmid was used as a negative control. A GFP positive signal of approx. 140 kDa confirmed the presence of GFP-CDKL5 in the midbody fraction of HeLa cells while the GFP alone showed no signal, confirming specificity of the result.
Altogether our data confirm the presence of endogenous and exogenously expressed CDKL5 at the midbody.

N-terminal domain of CDKL5 is responsible for the midbody localization

After the confirmation of exogenously expressed CDKL5 in the midbody, we decided to investigate the domain of CDKL5 responsible for this localization. For this purpose we used two different truncation derivatives, respectively characterized by the lack of the kinase catalytic domain (GFP-CDKL5-C-ter) or the long C-terminal domain (GFP-CDKL5-N-ter), along with the wt control, GFP-CDKL5 (Figure 12). These constructs were transiently overexpressed in HeLa cells and their midbody localization was analyzed by a biochemical assay. Midbody fraction along with total cell extract from interphase cells of each transfected plasmid were prepared and analyzed by western blot. We observed a GFP-CDKL5 signal in the midbody fraction of GFP-CDKL5 and GFP-CDKL5-Nter but not in the GFP-CDKL5-Cter sample (Figure 13). The absence of signal in the MB fraction of GFP transfected cells confirmed the specificity of the result. Thus, we suggest that the N-terminal portion of CDKL5 is required for its MB localization.

CDKL5 depletion causes mitotic defects and micronuclei formation

We proceeded addressing whether a deficiency of CDKL5 causes any phenotype usually associated to the midbody. To this purpose, we silenced HeLa cells with si-CDKL5 and a si-scramble as a control and analyzed progression through mitosis. On silencing the CDKL5 gene we found mitotic defects in proliferating HeLa cells. The mitotic spindles of the CDKL5 silenced cells were elongated and “comet shaped” with irregular spindle arrangements (Figure 14a). Accordingly,

we measured a significant increase in the number of mitotic (Figure 14b) and multipolar cells (Figure 14c).

It is known that mitotic spindle is essential for correct chromosome segregation, and centrosome abnormalities are often associated with chromosome instability. In some cases, centrosome impairment can lead to aberrant mitosis that leads to cell death following mitotic checkpoints; however we did not observe any significant increase in apoptosis in CDKL5 silenced HeLa cells (data not shown). In other cases, although cytokinesis occurs and daughter cells are viable, they fail cytokinesis and become binucleated (Figure 14d), as we found in CDKL5 silenced HeLa cells.

Due to the improper attachment of microtubules to chromosomes small, extranuclear bodies known as micronuclei are formed from lagging chromosomes during mitosis. Since we observed a defective spindle formation in CDKL5 silenced HeLa cells we decided to analyze the micronuclei formation by a quantitative analysis. As expected, we found an approximately 2-fold increase in the micronuclei formation (Figure 15a,b) in CDKL5 silenced HeLa cells as compared to the control.

CDKL5-depletion causes defect in cell migration

Since centrosomes and microtubule assembly and dynamics are important for cell motility (Kodani and Sutterlin, 2008; Watanabe et al, 2005), we also analyzed the affect of CDKL5 depletion on cell migration. As above, HeLa cells were transfected with CDKL5 siRNA and scramble siRNA as a control. After 72 hours of silencing, a wound was made on the evenly confluent monolayer and the kinetics of its healing was observed. After 24 hours of making the wound, HeLa cells transfected with the scramble-siRNA filled the wound by 67%. In contrast, CDKL5 silenced cells showed a significant delay in migration towards the wound (37% wound healing; Figure 16a,b). CDKL5 silencing was confirmed by western blotting (Figure 16c)

CDKL5 interacts with IQGAP1, Cep55 and Tsg101 in the midbody

Having demonstrated CDKL5 localization at the midbody, we consider of high relevance understanding which factor can target CDKL5 at the midbody or depend on CDKL5 for its specific localization. Interestingly, through a previously performed yeast two-hybrid screening we found that CDKL5 interacts with IQGAP1 (IQ-motif-containing GTPase activating protein), a scaffolding protein localized at the midbody (Skop et al 2004). Hence, we decided to confirm

IQGAP1 interaction with CDKL5. A coimmunoprecipitation assay from the midbody fraction of HeLa cells using an anti-CDKL5 antibody followed by immunoblotting was thus performed. As expected, we observed the interaction of CDKL5 with IQGAP1 in the midbody fraction. In the future we will have to confirm that IQGAP1 acts as a platform for the localization of CDKL5 at the midbody.

Furthermore, considering that CDKL5 deficiency determines an increase in the number of multinucleated cells and defects in cytokinetic abscission, we analyzed whether a set of proteins involved in abscission interact with CDKL5 at the midbody. Using the same experimental setup as described above we performed a coimmunoprecipitation assay using the anti-CDKL5 Ab. Among the various proteins analyzed, we found Cep55 and Tsg101 to be interactors of CDKL5 at the midbody, whereas another protein Ect2 showed no coprecipitation (Figure 17). The presence of Cep55 and Tsg101 has already been published at the midbody; importantly, their absence has shown defects in abscission (Morita et al., 2007). We speculate that CDKL5 interaction with Cep55 and Tsg101 at the midbody might have some role in facilitating abscission.

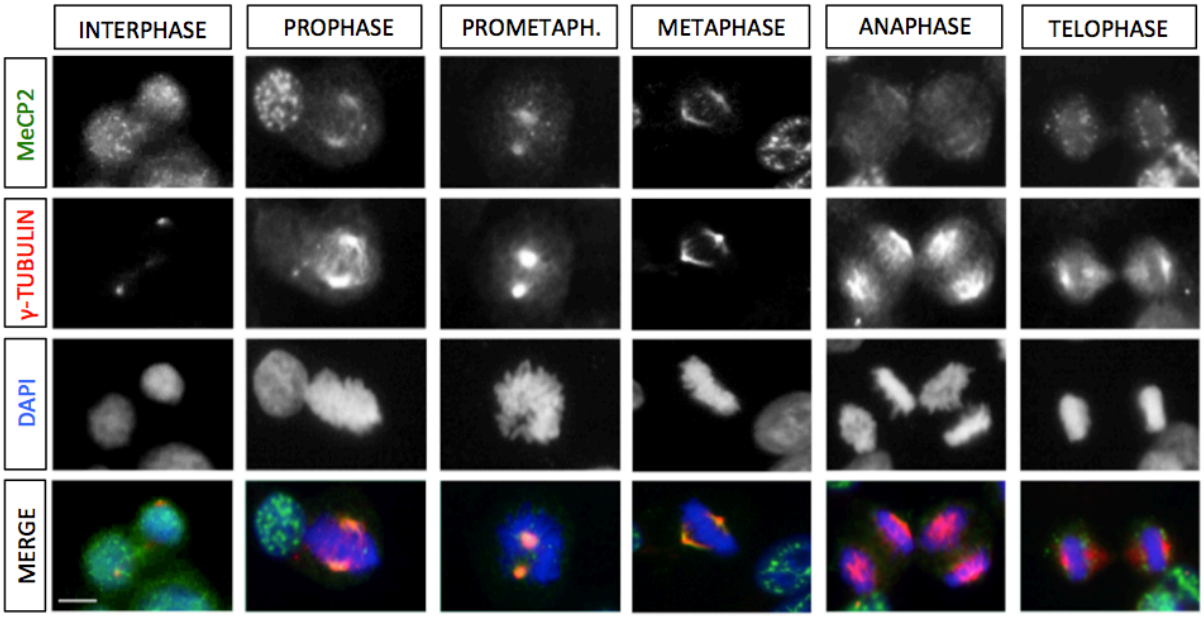


Figure 7. In HeLa cells MeCP2 (green) localizes at the spindle poles during early stages of mitosis, i.e., till anaphase as shown by gamma-tubulin staining (red), and then leaves the spindle poles. During interphase it shows a punctate staining in the nucleus.

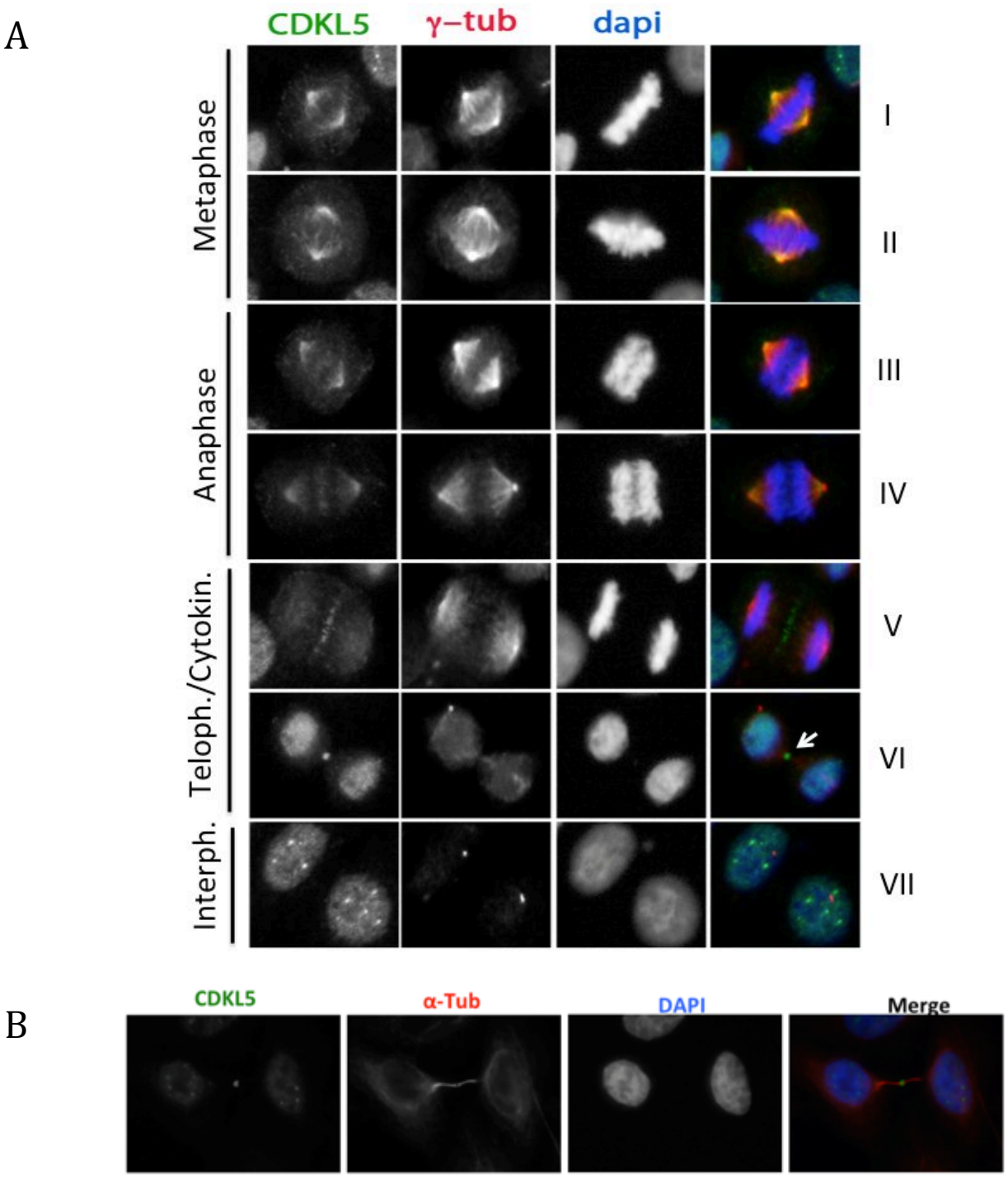


Figure 8: (A) CDKL5 is present at the spindle poles, stained with y-tubulin (red), during early mitotic stages (I, II, III). From anaphase (IV), it starts localizing at the midzone. At telophase (VI) it is completely localized at the midbody. Finally, at interphase (VII) it forms speckles in the nucleus. (B) Immunofluorescence showing CDKL5 (green) at the midbody along with α-tubulin staining the midbody filaments.

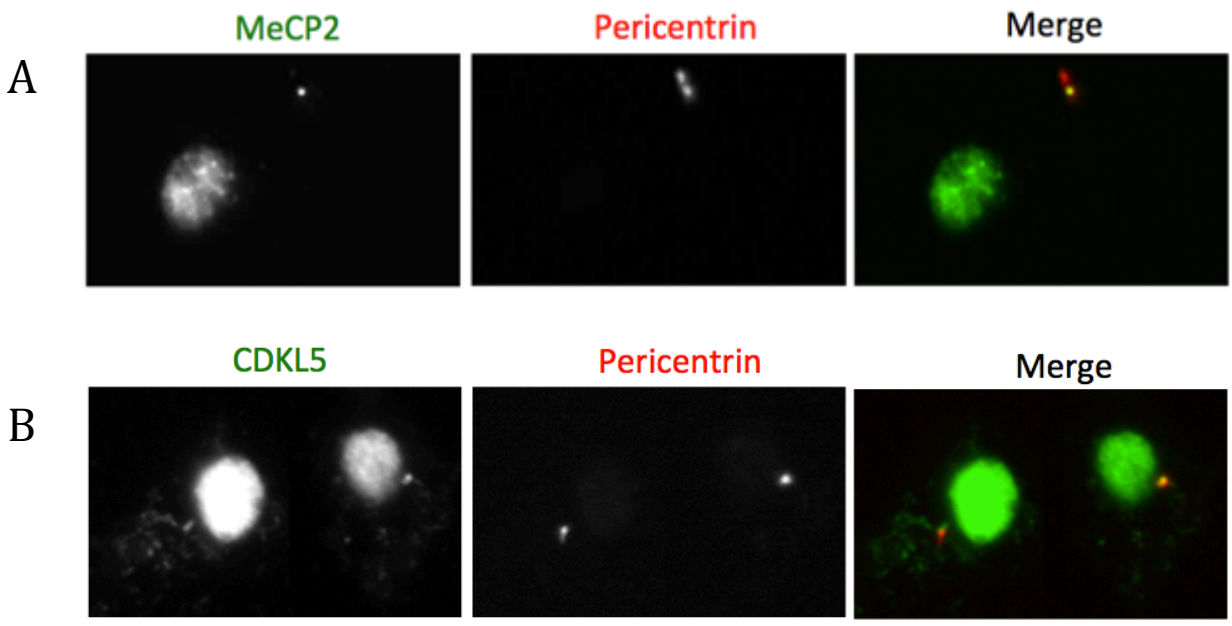
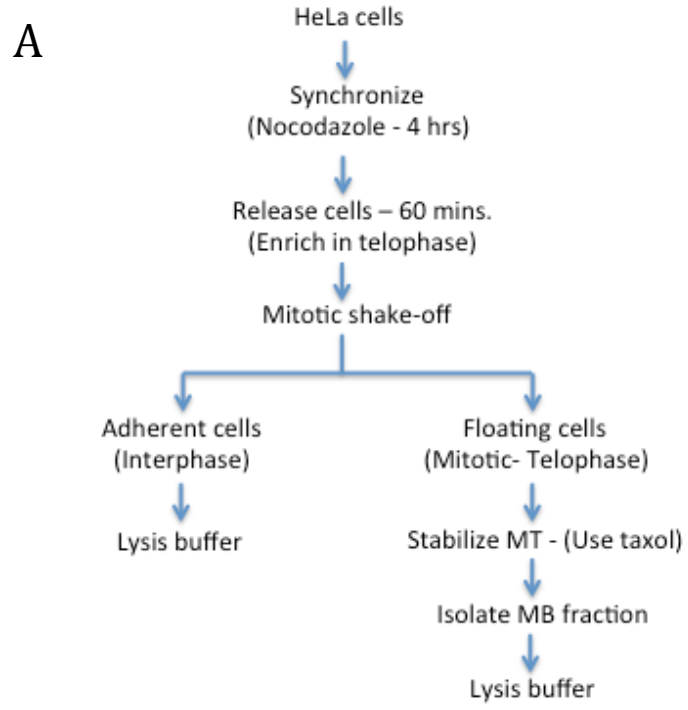
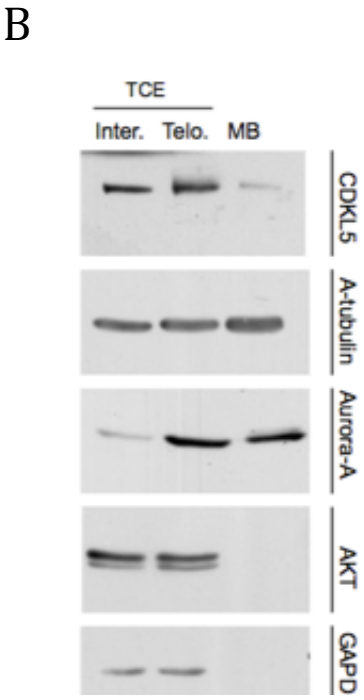


Figure 9: Immunofluorescence staining of murine cortical neurons (DIV 4): In green, MeCP2 (upper left panels) or CDKL5 (lower left panels); in red pericentrin (central panels); on the right merged images.





C

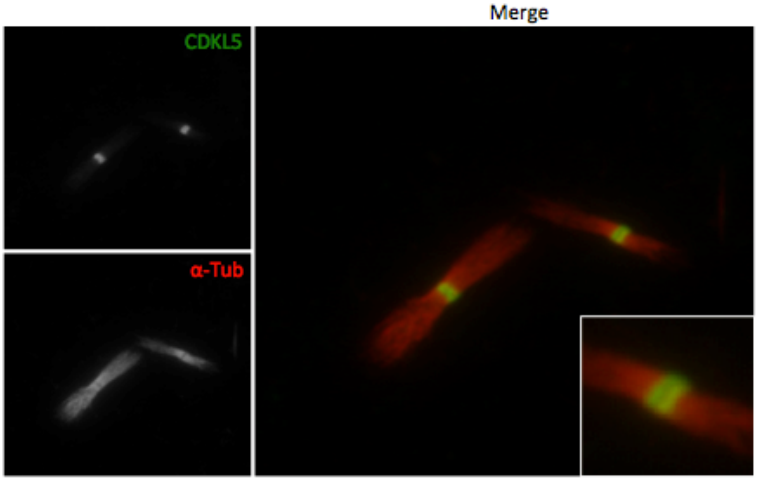


Figure 10. (A) The schematic diagram shows the protocol for isolation of midbody from HeLa cells (B) MB lysate from HeLa cells was loaded along with total cell extracts from interphase and telophase enriched cells respectively. (C) IF from the same midbody fraction used for the WB. CDKL5 (green) and alpha-tubulin (red).
TCE: Total cell extract; Inter-Interphase; Telo-Telophase; MB-Midbody

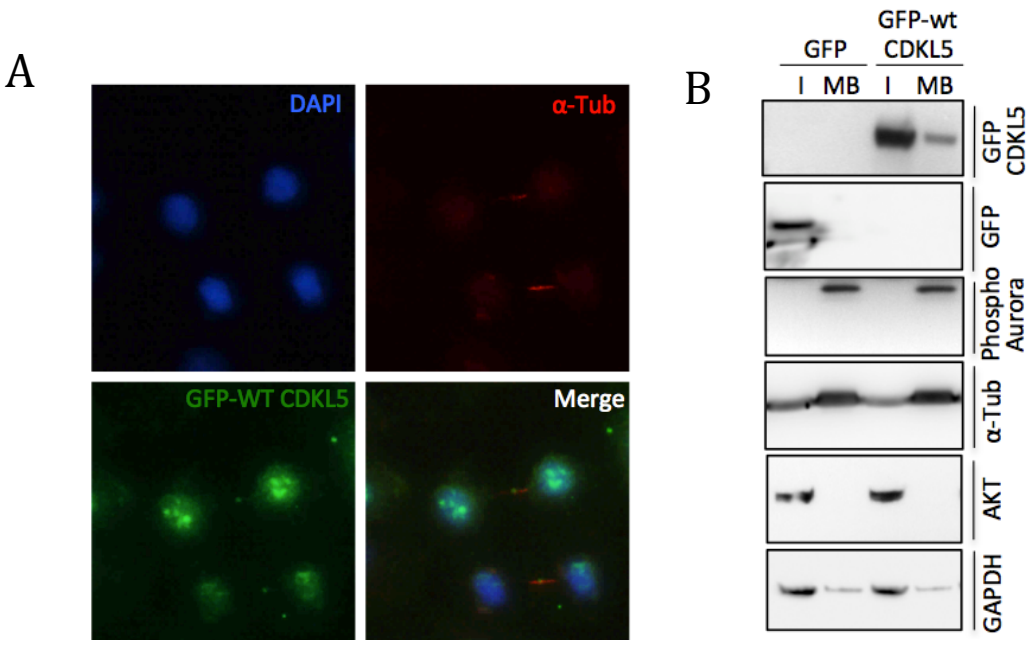


Figure 11. Expression of exogenous CDKL5 at the MB of HeLa cells. (A) GFP-CDKL5 was transiently overexpressed in HeLa cells. Exogenously expressed CDKL5 (green) can be observed in the MB along with α-tubulin (red) staining the MB filaments. Nuclei are stained with DAPI (blue). (B) MB fractions from HeLa cells transfected with GFP and GFP-CDKL5 expressing plasmids along with TCE from interphase cells of each transfected sample were loaded for biochemical analysis. I - Interphase; TCE - Total cell extract; MB - Midbody

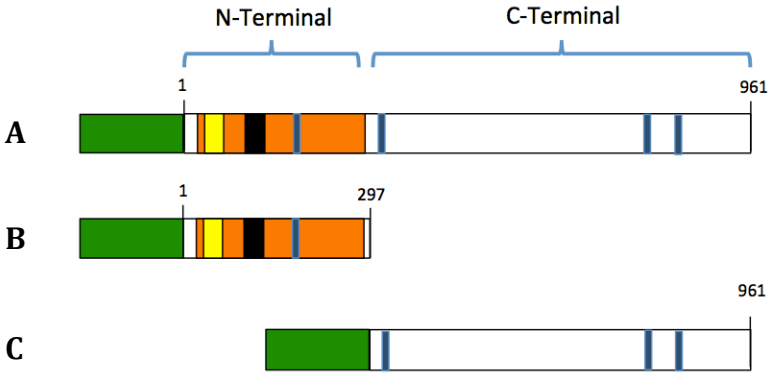


Figure 12. GFP-CDKL5 truncation derivatives. (A) GFP-wt-CDKL5 (B) GFP-CDKL5-N terminal (C) GFP-CDKL5 C-terminal.

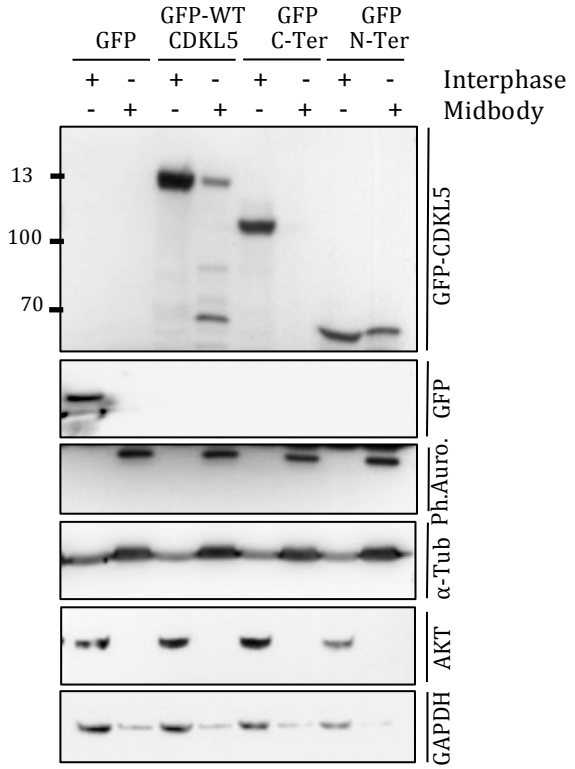


Figure 13. The N-terminal portion of CDKL5 is responsible for the midbody localization.
Total cell extracts of interphase cells and midbody fractions obtained from HeLa cells transfected with GFP, GFP-CDKL5, GFP-CDKL5-C term and GFP-CDKL5-N term were analyzed by western blot.
Enrichment of midbody fraction was determined by staining with α-tubulin and phospho-aurora antibodies. Nuclear and cytoplasmic contaminations were verified by AKT and GAPDH respectively.

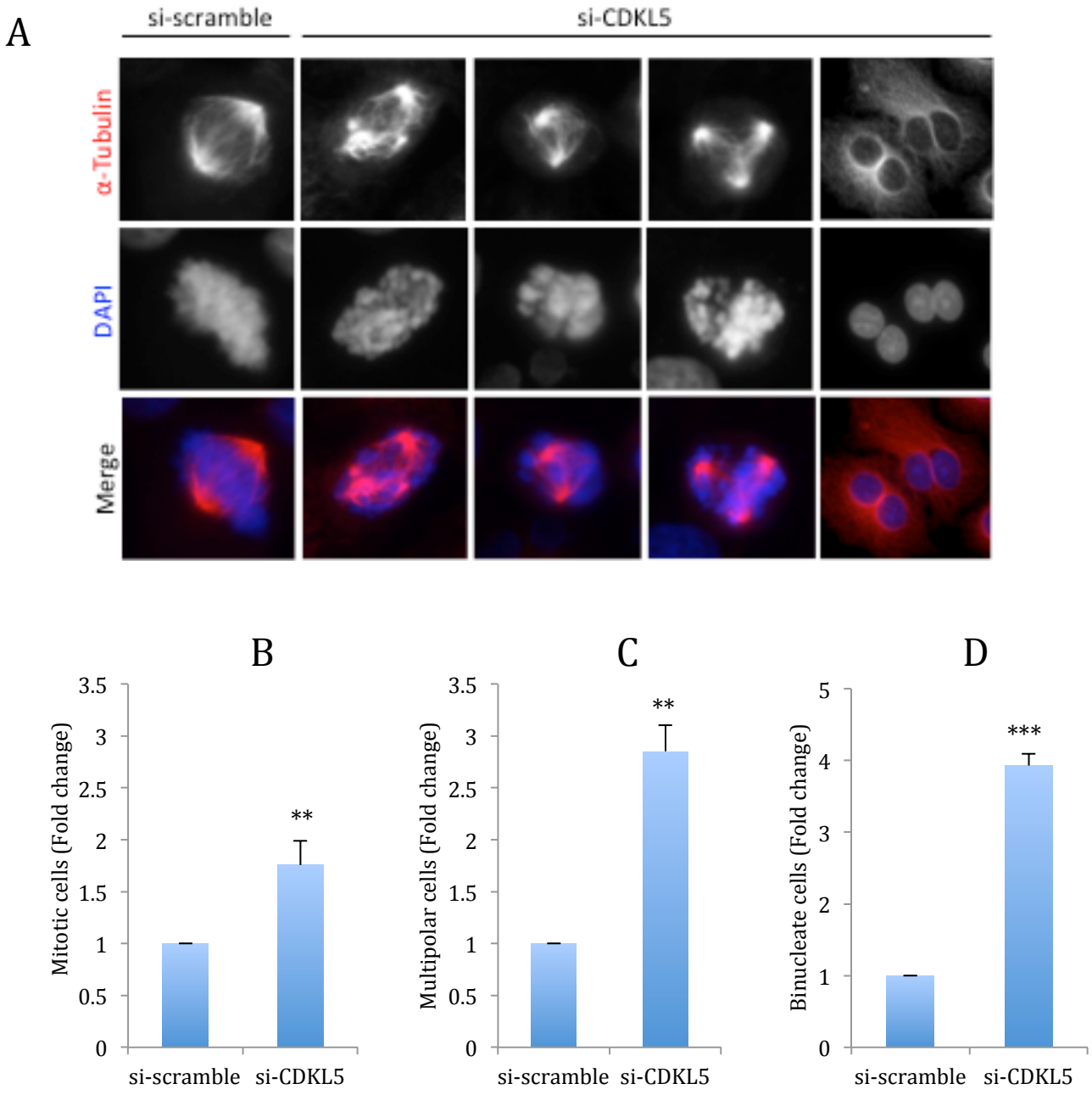
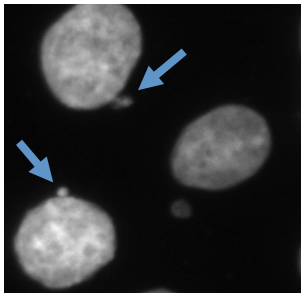


Figure 14: Efficient mitotic spindle formation requires CDKL5. (A) HeLa cells were silenced with si-CDKL5 and a si-scramble as a control and stained with α-tubulin (red) to monitor the microtubule organization. DNA was stained with DAPI (blue). The image shows defects in mitotic spindle organization. (B) CDKL5 silenced HeLa cells show 1.8 fold increase in the number of mitotic cells. (C) CDKL5 silenced HeLa cells show 2.85 fold increase in the number of multipolar cells. (D) CDKL5 silenced HeLa cells show 3.93 fold increase in the number of binucleate cells. Values are mean ± s.e.m. n=3, $p<0.05$

A



B

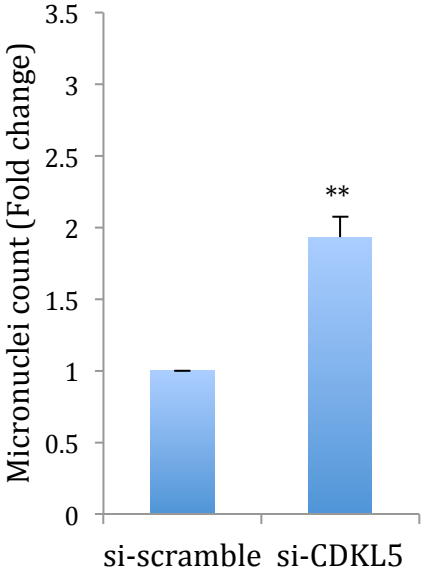


Figure 15: CDKL5 depletion causes micronuclei formation: (A) HeLa cells silenced by si-CDKL5 were stained with DAPI. Micronuclei are indicated with arrows. (B) Quantitative analysis of micronuclei count was done from control and CDKL5-silenced HeLa cells using ImageJ software. Values are means ±s.e.m. n =3, $p<0.05$

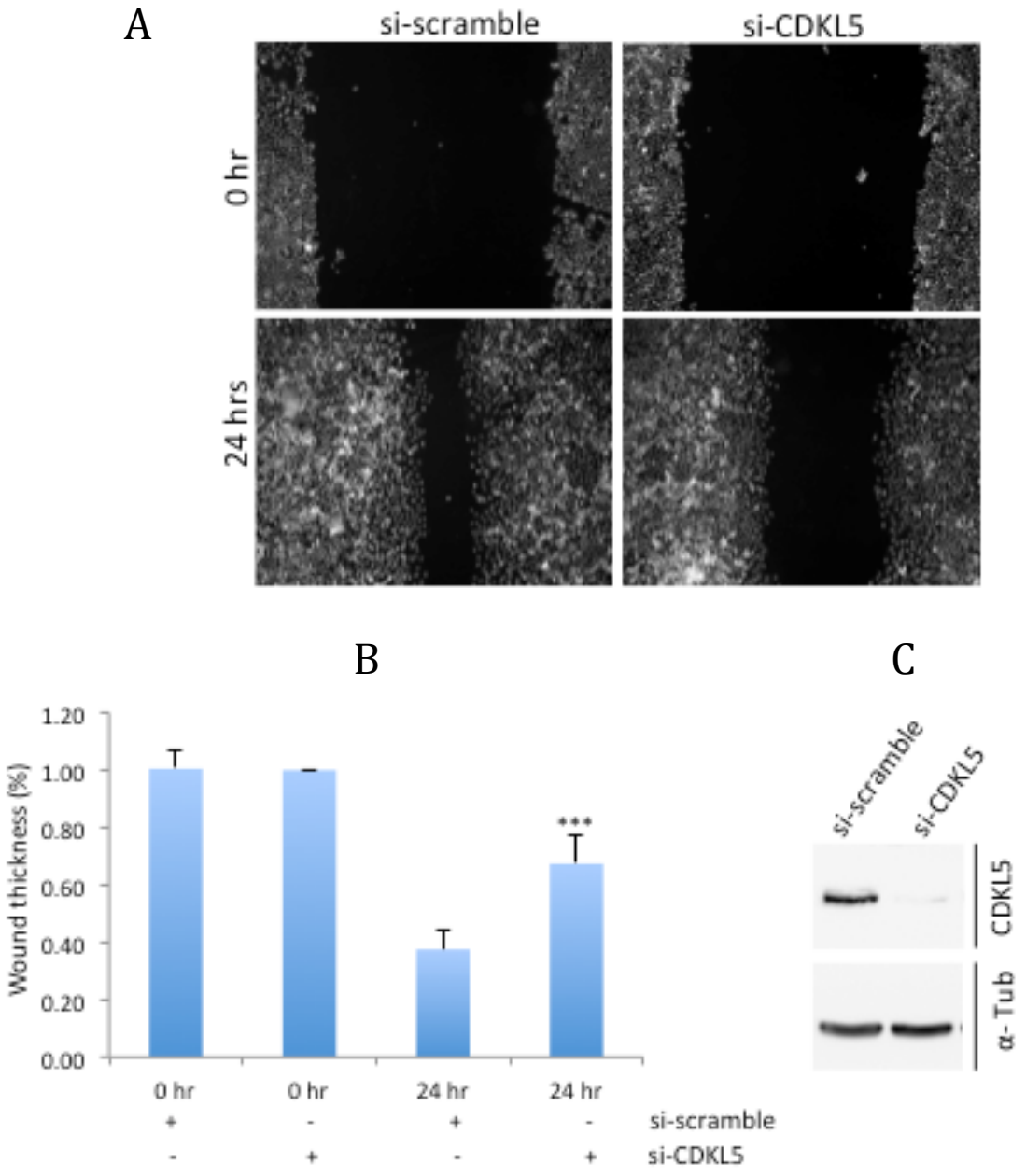


Figure 16: CDKL5 deficiency reduces migration efficiency in HeLa cells. (A) Migration of control and CDKL5 silenced HeLa cells into a scratch wound was monitored by following a time-course immunofluorescence. Cells were fixed and stained with DAPI immediately after the scratch and 24 hrs later to evaluate the migration ability of HeLa into the wound. (B) A quantitative analysis of the migration assay was done using the ImageJ software. n=3, $p<0.005$ (C) HeLa cells transfected with scramble and CDKL5 si-RNA were silenced for three days and endogenous CDKL5 level was analyzed by western blot.

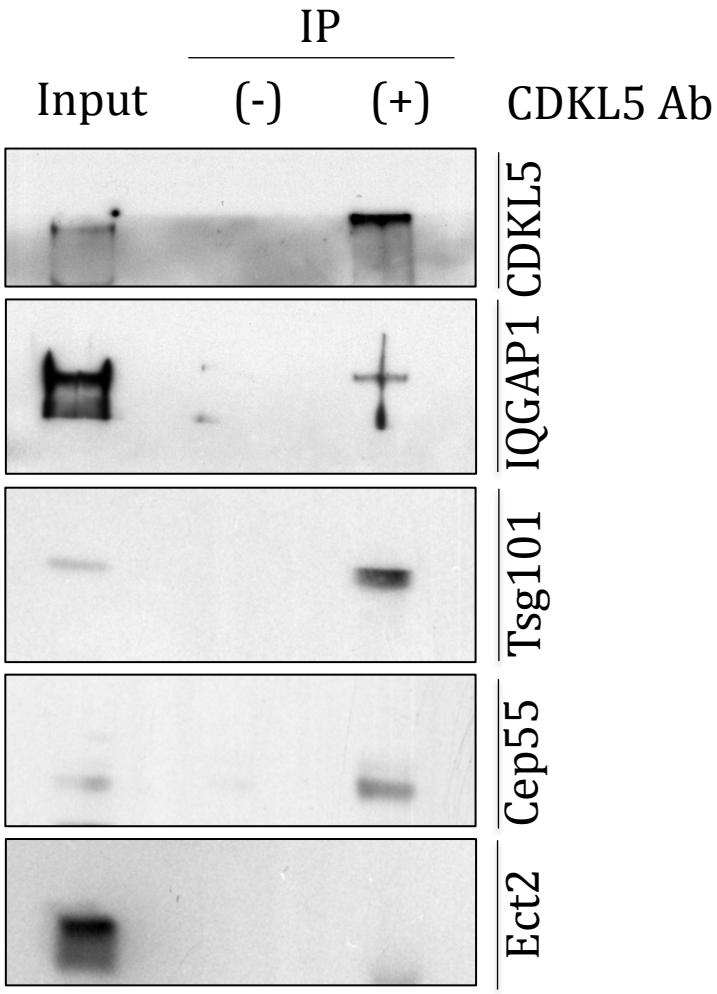


Figure 17. CDKL5 interacts with the scaffold protein IQGAP1 and the abscission proteins Tsg101 and Cep55 at the MB. CDKL5 was immunoprecipitated from MB fraction of HeLa cells using anti-CDKL5 Ab. CDKL5 interaction with IQGAP1, Tsg101, Cep55 and Ect2 was then studied by immunoblotting. Immunoblotting of western blot was done with each Ab as labelled. IP-Immunoprecipitation; (-) – without Ab; (+) – with Ab.

Discussion

Rett syndrome is a progressive neurodevelopmental disorder affecting almost exclusively females. The classical form of Rett syndrome is characterized by the appearance of neurodevelopment arrest and regression after several months of apparently normal development, (Hagberg, 1983). Symptoms that appear during this period of infantile life will persist throughout the whole adulthood, preventing affected individuals from leading a normal and independent physical and social life.

The classical form of RS is often associated with mutations in the *MECP2* gene. Besides the classical form, also atypical cases of RS have been reported; these can be characterized both by milder phenotypes, as is the case for the "preserved speech variant", and by more severe symptoms, as it occurs in the Hanefeld variant. Children affected by the Hanefeld variant develop seizures soon after birth and the epileptic crisis often become pharmacologically intractable (Hanefeld, 1985). Patients affected by the Hanefeld variant often carry mutation in the cyclin-dependent kinase-like 5 (*CDKL5*) gene. CDKL5 mutations have also been found in patients affected by the West syndrome, intellectual disability and infantile spasms; generally, the common features of all these patients are the occurrence of early onset seizures and a delayed neurodevelopment.

CDKL5 is a serine/threonine kinase characterized by a catalytic domain at the N-terminus, which shares a high homology with that of MAPK and CDK family members, and a long C-terminal tail certainly involved in regulating the kinase activity, turnover and subcellular localization. In fact, the C-terminal tail is known to negatively regulate the catalytic activity of the kinase and is also responsible for its localization in the cytoplasm. The presence of putative NLS (Nuclear Localization Signal) and NES (Nuclear Export Signal) motifs in the COOH domain of CDKL5 influences, in fact, its nucleo-cytolasmic localization (Rusconi et al., 2008). The relevance of the COOH terminal in controlling CDKL5 localization is strengthened by the pathogenic truncation mutations R781X and L879X that appear sequestered in the nucleus (Bertani et al., 2008). Eventually, the COOH terminal domain affects the stability of CDKL5. In fact, the $CDKL5_{107}$ isoform is found to be less susceptible to the proteasomal degradation as compared to the long $CDKL5_{115}$ isoform, thus indicating that the

very C-terminal region, from amino acid 905, contains signals responsible for this degradation (Williamson et al., 2012).

So far, the majority of the research studies on CDKL5 functions have been focused to neurodevelopment. However, a recent work assayed whether the kinase affects cell proliferation/differentiation. In particular, this study has shown that CDKL5 expression induces differentiation and inhibits the proliferation of the SH-SY5Y neuroblastoma cells under the negative regulation of the transcription factor MYCN (Valli et al., 2012).

Our work starts from the observation that MeCP2 localizes at the spindle poles during early mitotic stages (Bergo et al., submitted for publication) in HeLa cells. Since it is known that MeCP2 and CDKL5 belong to the same molecular pathway (Mari et al., 2005) we decided to investigate the localization of CDKL5 in mitotic cells. Our observation that CDKL5 localizes at the spindle poles during early mitotic phase, followed by its localization at the midzone and then midbody during telophase, suggests a role of the kinase at this transient organelle. We also found that both, MeCP2 and CDKL5, localize at the centrosome in post-mitotic (DIV4) neurons. This observation is quite remarkable with respect to CDKL5 because we were unable to find it in the interphase centrosome of dividing cells; it seems that this localization is specific of post mitotic neurons, or possibly, and more general, of postmitotic cells. This phenomenon might suggest that CDKL5 plays different roles in the interphase of diverse cells. Though CDKL5 localization in the centrosome of post-mitotic neurons is open to a wide range of possible functions, its role in neuronal migration is more plausible. In fact, our data clearly demonstrate that cycling cells devoid of CDKL5 are defective in wound healing, and Chen et al. (2010) have demonstrated that CDKL5 deficiency in rat brain causes a delay in neuronal migration. In the future it will be quite interesting to explore whether CDKL5 affects neuronal migration through its centrosomal localization.

Having demonstrated that CDKL5 localizes at the midbody, we decided to analyze which domain is responsible for this localization. Using a biochemical assay we showed that a CDKL5 construct devoid of the catalytic domain doesn't localize at the midbody. Hence, we conclude that the catalytic domain, i.e., the N-terminal domain of CDKL5 is responsible for the midbody localization. But we were unable to conclude whether the catalytic activity of the kinase is required for this localization: its role remains to be ascertained. Furthermore, in the future it will be important to address whether CDKL5 affects the phosphorylation state of proteins that localize either at the centrosome or the midbody. To this regards we want to make the point that so far all certain

missense pathogenic mutations fall into the catalytic domain and appears to affect the enzymatic activity of CDKL5.

Focusing on dividing cells, we found that CDKL5 deficiency impairs basic cellular processes such as spindle pole formation, mitosis and cell migration. Defects in normal spindle formation in the absence of CDKL5 are understandable considering its presence at the centrosome during early mitotic phase. During this study we also observed that CDKL5 deficiency causes an increase of mitotic cells. A possible explanation of this phenotype could be that a higher number of cells are stuck in mitosis due to the spindle checkpoint activation occurring during aberrant mitosis (Wells & Murray 1996). We would like to further investigate the role of CDKL5 silencing on the activation of spindle checkpoint by analyzing by western blot and immunofluorescence proteins that are known for their role in spindle checkpoint activation, such as viz., Bub1, BubR1, Mps1 and MAD proteins (Burke 2000).

Eventually, since micronuclei represent a mechanism by which errors in chromosome segregation and DNA breaks are eliminated from the nucleus of the cell and spindle aberrations can cause chromosome instability, we investigated whether CDKL5 deficiency causes an increase in the number of micronuclei (Fukasawa 2007). In accordance with our hypothesis, we observed an approximately two-fold increase in the number of micronuclei in CDKL5 silenced HeLa cells as compared to the control, which is obvious due to the defective spindle formation. In addition, an increase in binucleate cells after CDKL5 silencing suggests a defect in abscission. The observed phenotype appears well justified by CDKL5 localization at the midbody during late mitosis; in fact, it is very well known that the midbody localizes at the site of abscission, where it affects cytokinesis.

Considering that CDKL5 deficiency causes micronuclei formation and binucleated cells, we suggest a role of CDKL5 in genetic stability and cancer risk. Importantly, a possible association of CDKL5 and leukemia has already been suggested (Kawahara et al., 2007) Zang ZJ, Ong CK, Cutcutache I, Yu W, Zhang SL, Huang D, Ler LD, Dykema K, Gan A, Tao J, Lim S, Liu Y, Futreal PA, Grabsch H, Furge KA, Goh LK, Rozen S, Teh BT, Tan P., 2011). Eventually, as mentioned, through a wound-healing assay, we have reported a functional role of CDKL5 in cell migration.

Having demonstrated that CDKL5 localizes at the midbody and that CDKL5 depletion causes several defects in cycling cells that can be linked to the midbody, we decided to focus our future research on the mechanisms bringing

the kinase to the organelle and the consequence, at the biochemical levels, of CDKL5 deficiency. Because of that we interrogated a previously performed two hybrid screening, to address whether CDKL5 might have been found interacting with protein normally localized in this organelle. In this way we found that IQGAP1 (IQ motif containing GTPase activating protein) was proposed as a possible interactor of the C-terminal tail of CDKL5. In accordance with the genetic screening, by coimmunoprecipitation we found that CDKL5 interacts with IQGAP1. IQGAP1 is a multifunctional scaffolding protein that has ability to interact with a wide variety of proteins that result in a wide array of functions. It is known to affect cell proliferation and migration in NIH3T3 cell line (Wang et al., 2009). In neurons it facilitates neuronal migration (Kholmanskikh et al., 2006), dendrite formation (Swiech et al., 2011), spine development (Gao et al., 2011), synaptic plasticity (Gao et al., 2011), memory formation (Schrick et al., 2007) and neurite outgrowth as well as polarization as reviewed by Jausoro et al., (Jausoro et al., 2012). A review has highlighted that IQGAP1 is overexpressed in many human malignancies at both mRNA and protein level (White et al., 2009), and a direct evidence of IQGAP1 involvement in cancer progression has been proved both *in vitro* and *in vivo* by Jadeski et al. (Jadeski et al., 2008). Importantly, since the C-terminal domain seems to be involved in the identified interaction, whereas CDKL5 N-terminal domain appears to be required for its localization at the midbody, we suggest that CDKL5 might be important for IQGAP localization at the midbody and/or might regulate its activity/localization through phosphorylation. In the future work, mainly through immunofluorescence assays coupled to specific shRNAs we will test these possibilities.

Eventually, we have started to address whether other proteins of the midbody interact with CDKL5. Although we are aware that a much more exhaustive analysis is required, for the time being we have found that CDKL5 interacts with two proteins Cep55 and Tsg101. Cep55 is a coiled-coil protein that is required for the establishment and proper function of the midbody structure. In Cep55 knockdown cells there is an abrogation of midbody formation, and the structural and regulatory components of the midbody are either absent or mislocalized. In addition, it facilitates membrane fusion during the terminal stage of cytokinesis that results in abscission (Zhao et al., 2006). The other interactor of CDKL5 in the midbody, Tsg101, is a subunit of human ESCRT-1 complex, a regulator of vesicle trafficking process. It helps in the formation of multivesicular bodies (MVBs) and also retroviral budding which is an event similar to the cytokinesis of eukaryotic cells (Carlton & Martin-Serrano 2007). It is interesting to note that

Tsg101 interacts with Cep55 and IQGAP1 along with other proteins to facilitate cytokinesis (Morita et al., 2007).

On the contrary we didn't observe any interaction of CDKL5 with another abscission protein Ect2. Ect2 is a guanine nucleotide exchange factor and transforming protein that is related to Rho-specific exchange factors and yeast cell cycle regulators. The expression of this gene is elevated with the onset of DNA synthesis and remains elevated during G2 and M phases. This protein plays an important role in the regulation of cytokinesis (Tatsumoto et al., 1999), epithelial cell polarity (Liu et al., 2004), tumor cell proliferation (Justilien & Fields 2009) and it controls the formation of mitotic spindle assembly (Tatsumoto et al., 2003).

Taking cues from our observations we would like to address the following questions in the near future:

a. Determine the domain of CDKL5 that interacts with Cep55 and Tsg101 at the midbody.
b. Determine if CDKL5 is involved in the correct localization of IQGAP1, Cep55 and Tsg101 at the midbody.
c. Does CDKL5 phosphorylate IQGAP1, Cepp55 and Tsg101 at the midbody?
d. Since CDKL5 localizes at the centrosome during prophase and its absence causes defective spindle formation we would like to know whether CKL5 depletion cause impairment of microtubule nucleation?
e. Determine if there is a role of centrosome in delayed migration in CDKL5 depleted HeLa cells.

BIBLIOGRAPHY

Adachi H, Takahashi Y, Hasebe T, Shirouzu M, Yokoyama S, Sutoh K. 1997. Dictyostelium IQGAP-related protein specifically involved in the completion of cytokinesis. *J Cell Biol* 137:891-8

Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. 1999. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet* 23:185-8

Bahi-Buisson N, Nectoux J, Rosas-Vargas H, Milh M, Boddaert N, et al. 2008. Key clinical features to identify girls with CDKL5 mutations. *Brain* 131:2647-61

Ballas N, Lioy DT, Grunseich C, Mandel G. 2009. Non-cell autonomous influence of MeCP2-deficient glia on neuronal dendritic morphology. *Nat Neurosci* 12:311-7

Bertani I, Rusconi L, Bolognese F, Forlani G, Conca B, et al. 2006. Functional consequences of mutations in CDKL5, an X-linked gene involved in infantile spasms and mental retardation. *J Biol Chem* 281:32048-56

Bienvenu T, Carrie A, de Roux N, Vinet MC, Jonveaux P, et al. 2000. MECP2 mutations account for most cases of typical forms of Rett syndrome. *Hum Mol Genet* 9:1377-84

Braunschweig D, Simcox T, Samaco RC, LaSalle JM. 2004. X-Chromosome inactivation ratios affect wild-type MeCP2 expression within mosaic Rett syndrome and Mecp2-/+ mouse brain. *Hum Mol Genet* 13:1275-86

Brinkley BR, Cartwright J, Jr. 1971. Ultrastructural analysis of mitotic spindle elongation in mammalian cells in vitro. Direct microtubule counts. *J Cell Biol* 50:416-31

Buck RC, Tisdale JM. 1962. The fine structure of the mid-body of the rat erythroblast. *J Cell Biol* 13:109-15

Burke DJ. 2000. Complexity in the spindle checkpoint. *Curr Opin Genet Dev* 10:26-31

Buschdorf JP, Stratling WH. 2004. A WW domain binding region in methyl-CpG-binding protein MeCP2: impact on Rett syndrome. *J Mol Med (Berl)* 82:135-43

Carlton JG, Martin-Serrano J. 2007. Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. *Science* 316:1908-12

Cassel S, Revel MO, Kelche C, Zwiller J. 2004. Expression of the methyl-CpG-binding protein MeCP2 in rat brain. An ontogenetic study. *Neurobiol Dis* 15:206-11

Chahrour M, Jung SY, Shaw C, Zhou X, Wong ST, et al. 2008. MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science* 320:1224-9

Chen Q, Zhu YC, Yu J, Miao S, Zheng J, et al. 2010. CDKL5, a protein associated with rett syndrome, regulates neuronal morphogenesis via Rac1 signaling. *J Neurosci* 30:12777-86

Chen RZ, Akbarian S, Tudor M, Jaenisch R. 2001. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet* 27:327-31

Chen WG, Chang Q, Lin Y, Meissner A, West AE, et al. 2003. Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science* 302:885-9

Cohen DR, Matarazzo V, Palmer AM, Tu Y, Jeon OH, et al. 2003. Expression of MeCP2 in olfactory receptor neurons is developmentally regulated and occurs before synaptogenesis. *Mol Cell Neurosci* 22:417-29

Day JJ, Sweatt JD. 2011. Epigenetic mechanisms in cognition. *Neuron* 70:813-29

Di Paolo G, Sankaranarayanan S, Wenk MR, Daniell L, Perucco E, et al. 2002. Decreased synaptic vesicle recycling efficiency and cognitive deficits in amphiphysin 1 knockout mice. *Neuron* 33:789-804

Dragich JM, Kim YH, Arnold AP, Schanen NC. 2007. Differential distribution of the MeCP2 splice variants in the postnatal mouse brain. *J Comp Neurol* 501:526-42

Dubreuil V, Marzesco AM, Corbeil D, Huttner WB, Wilsch-Brauninger M. 2007. Midbody and primary cilium of neural progenitors release extracellular membrane particles enriched in the stem cell marker prominin-1. *J Cell Biol* 176:483-95

Elia N, Sougrat R, Spurlin TA, Hurley JH, Lippincott-Schwartz J. 2011. Dynamics of endosomal sorting complex required for transport (ESCRT) machinery during cytokinesis and its role in abscission. *Proc Natl Acad Sci U S A* 108:4846-51

Engerstrom IW. 1990. Rett syndrome in Sweden. Neurodevelopment--disability--pathophysiology. *Acta Paediatr Scand Suppl* 369:1-60

Ettinger AW, Wilsch-Brauninger M, Marzesco AM, Bickle M, Lohmann A, et al. 2011. Proliferating versus differentiating stem and cancer cells exhibit distinct midbody-release behaviour. *Nat Commun* 2:503

Fichou Y, Nectoux J, Bahi-Buisson N, Chelly J, Bienvenu T. 2011. An isoform of the severe encephalopathy-related CDKL5 gene, including a novel exon with extremely high sequence conservation, is specifically expressed in brain. *J Hum Genet* 56:52-7

Forlani G, Giarda E, Ala U, Di Cunto F, Salani M, et al. 2010. The MeCP2/YY1 interaction regulates ANT1 expression at 4q35: novel hints for Rett syndrome pathogenesis. *Hum Mol Genet* 19:3114-23

Fukasawa K. 2007. Oncogenes and tumour suppressors take on centrosomes. *Nat Rev Cancer* 7:911-24

Galvao TC, Thomas JO. 2005. Structure-specific binding of MeCP2 to four-way junction DNA through its methyl CpG-binding domain. *Nucleic Acids Res* 33:6603-9

Gao C, Frausto SF, Guedea AL, Tronson NC, Jovasevic V, et al. 2011. IQGAP1 regulates NR2A signaling, spine density, and cognitive processes. *J Neurosci* 31:8533-42

Georgel PT, Horowitz-Scherer RA, Adkins N, Woodcock CL, Wade PA, Hansen JC. 2003. Chromatin compaction by human MeCP2. Assembly of novel secondary chromatin structures in the absence of DNA methylation. *J Biol Chem* 278:32181-8

Giacometti E, Luikenhuis S, Beard C, Jaenisch R. 2007. Partial rescue of MeCP2 deficiency by postnatal activation of MeCP2. *Proc Natl Acad Sci U S A* 104:1931-6

Girard M, Couvert P, Carrie A, Tardieu M, Chelly J, et al. 2001. Parental origin of de novo MECP2 mutations in Rett syndrome. *Eur J Hum Genet* 9:231-6

Gromley A, Yeaman C, Rosa J, Redick S, Chen CT, et al. 2005. Centriolin anchoring of exocyst and SNARE complexes at the midbody is required for secretory-vesicle-mediated abscission. *Cell* 123:75-87

Guizetti J, Schermelleh L, Mantler J, Maar S, Poser I, et al. 2011. Cortical constriction during abscission involves helices of ESCRT-III-dependent filaments. *Science* 331:1616-20

Guy J, Gan J, Selfridge J, Cobb S, Bird A. 2007. Reversal of neurological defects in a mouse model of Rett syndrome. *Science* 315:1143-7

Hagberg B. 1995. Rett syndrome: clinical peculiarities and biological mysteries. *Acta Paediatr* 84:971-6

Hagberg B, Aicardi J, Dias K, Ramos O. 1983. A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. *Ann Neurol* 14:471-9

Hagberg B, Hanefeld F, Percy A, Skjeldal O. 2002. An update on clinically applicable diagnostic criteria in Rett syndrome. Comments to Rett Syndrome Clinical Criteria Consensus Panel Satellite to European

Paediatric Neurology Society Meeting, Baden Baden, Germany, 11 September 2001. *Eur J Paediatr Neurol* 6:293-7

Hagberg BA, Skjeldal OH. 1994. Rett variants: a suggested model for inclusion criteria. *Pediatr Neurol* 11:5-11

Hardingham GE, Bading H. 2010. Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat Rev Neurosci* 11:682-96

Hardingham GE, Fukunaga Y, Bading H. 2002. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci* 5:405-14

Hu CK, Coughlin M, Mitchison TJ. 2012. Midbody assembly and its regulation during cytokinesis. *Mol Biol Cell* 23:1024-34

Jadeski L, Mataraza JM, Jeong HW, Li Z, Sacks DB. 2008. IQGAP1 stimulates proliferation and enhances tumorigenesis of human breast epithelial cells. *J Biol Chem* 283:1008-17

Jausoro I, Mestres I, Remedi M, Sanchez M, Caceres A. 2012. IQGAP1: A microtubule-microfilament scaffolding protein with multiple roles in nerve cell development and synaptic plasticity. *Histol Histopathol* 27:1385-94

Jeste SS, Sahin M, Bolton P, Ploubidis GB, Humphrey A. 2008. Characterization of autism in young children with tuberous sclerosis complex. *J Child Neurol* 23:520-5

Justilien V, Fields AP. 2009. Ect2 links the PKCiota-Par6alpha complex to Rac1 activation and cellular transformation. *Oncogene* 28:3597-607

Kalscheuer VM, Tao J, Donnelly A, Hollway G, Schwinger E, et al. 2003. Disruption of the serine/threonine kinase 9 gene causes severe X-linked infantile spasms and mental retardation. *Am J Hum Genet* 72:1401-11

Kaufmann WE, Moser HW. 2000. Dendritic anomalies in disorders associated with mental retardation. *Cereb Cortex* 10:981-91

Kawahara M, Hori T, Matsubara Y, Okawa K, Uchiyama T. 2007. Cyclin-dependent kinaselike 5 is a novel target of immunotherapy in adult T-cell leukemia. *J Immunother* 30:499-505

Kerr B, Soto CJ, Saez M, Abrams A, Walz K, Young JI. 2012. Transgenic complementation of MeCP2 deficiency: phenotypic rescue of Mecp2-null mice by isoform-specific transgenes. *Eur J Hum Genet* 20:69-76

Kholmanskikh SS, Koeller HB, Wynshaw-Boris A, Gomez T, Letourneau PC, Ross ME. 2006. Calcium-dependent interaction of Lis1 with IQGAP1 and Cdc42 promotes neuronal motility. *Nat Neurosci* 9:50-7

Kilstrup-Nielsen C, Rusconi L, La Montanara P, Ciceri D, Bergo A, et al. 2012. What we know and would like to know about CDKL5 and its involvement in epileptic encephalopathy. *Neural Plast* 2012:728267

Kishi N, Macklis JD. 2004. MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions. *Mol Cell Neurosci* 27:306-21

Klose RJ, Sarraf SA, Schmiedeberg L, McDermott SM, Stancheva I, Bird AP. 2005. DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. *Mol Cell* 19:667-78

Kokura K, Kaul SC, Wadhwa R, Nomura T, Khan MM, et al. 2001. The Ski protein family is required for MeCP2-mediated transcriptional repression. *J Biol Chem* 276:34115-21

Kriaucionis S, Bird A. 2004. The major form of MeCP2 has a novel N-terminus generated by alternative splicing. *Nucleic Acids Res* 32:1818-23

Kuo TC, Chen CT, Baron D, Onder TT, Loewer S, et al. 2011. Midbody accumulation through evasion of autophagy contributes to cellular reprogramming and tumorigenicity. *Nat Cell Biol* 13:1214-23

Lee HH, Elia N, Ghirlando R, Lippincott-Schwartz J, Hurley JH. 2008. Midbody targeting of the ESCRT machinery by a noncanonical coiled coil in CEP55. *Science* 322:576-80

Lewis JD, Meehan RR, Henzel WJ, Maurer-Fogy I, Jeppesen P, et al. 1992. Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* 69:905-14

Li Z, He X, Feng J. 2013. 16p11.2 is required for neuronal polarity. *World Journal of neuroscience* 3:221-227

Lin C, Franco B, Rosner MR. 2005. CDKL5/Stk9 kinase inactivation is associated with neuronal developmental disorders. *Hum Mol Genet* 14:3775-86

Lioy DT, Garg SK, Monaghan CE, Raber J, Foust KD, et al. 2011. A role for glia in the progression of Rett's syndrome. *Nature* 475:497-500

Liu XF, Ishida H, Raziuddin R, Miki T. 2004. Nucleotide exchange factor ECT2 interacts with the polarity protein complex Par6/Par3/protein kinase Czeta (PKCzeta) and regulates PKCzeta activity. *Mol Cell Biol* 24:6665-75

Mari F, Azimonti S, Bertani I, Bolognese F, Colombo E, et al. 2005. CDKL5 belongs to the same molecular pathway of MeCP2 and it is responsible for the early-onset seizure variant of Rett syndrome. *Hum Mol Genet* 14:1935-46

Marsh R, Gerber AJ, Peterson BS. 2008. Neuroimaging studies of normal brain development and their relevance for understanding childhood neuropsychiatric disorders. *J Am Acad Child Adolesc Psychiatry* 47:1233-51

Martinowich K, Hattori D, Wu H, Fouse S, He F, et al. 2003. DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science* 302:890-3

Marzesco AM, Janich P, Wilsch-Brauninger M, Dubreuil V, Langenfeld K, et al. 2005. Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells. *J Cell Sci* 118:2849-58

McIntosh JR, Landis SC. 1971. The distribution of spindle microtubules during mitosis in cultured human cells. *J Cell Biol* 49:468-97

Meehan RR, Lewis JD, Bird AP. 1992. Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic Acids Res* 20:5085-92

Mei D, Marini C, Novara F, Bernardina BD, Granata T, et al. 2010. Xp22.3 genomic deletions involving the CDKL5 gene in girls with early onset epileptic encephalopathy. *Epilepsia* 51:647-54

Miltenberger-Miltenyi G, Laccone F. 2003. Mutations and polymorphisms in the human methyl CpG-binding protein MECP2. *Hum Mutat* 22:107-15

Montini E, Andolfi G, Caruso A, Buchner G, Walpole SM, et al. 1998. Identification and characterization of a novel serine-threonine kinase gene from the Xp22 region. *Genomics* 51:427-33

Morita E, Sandrin V, Chung HY, Morham SG, Gygi SP, et al. 2007. Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis. *EMBO J* 26:4215-27

Mullaney BC, Johnston MV, Blue ME. 2004. Developmental expression of methyl-CpG binding protein 2 is dynamically regulated in the rodent brain. *Neuroscience* 123:939-49

Mullins JM, Biesele JJ. 1977. Terminal phase of cytokinesis in D-98s cells. *J Cell Biol* 73:672-84

Mullins JM, McIntosh JR. 1982. Isolation and initial characterization of the mammalian midbody. *J Cell Biol* 94:654-61

Muotri AR, Marchetto MC, Coufal NG, Oefner R, Yeo G, et al. 2010. L1 retrotransposition in neurons is modulated by MeCP2. *Nature* 468:443-6

Nan X, Campoy FJ, Bird A. 1997. MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* 88:471-81

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, et al. 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393:386-9

Neul JL, Fang P, Barrish J, Lane J, Caeg EB, et al. 2008. Specific mutations in methyl-CpG-binding protein 2 confer different severity in Rett syndrome. *Neurology* 70:1313-21

Neul JL, Kaufmann WE, Glaze DG, Christodoulou J, Clarke AJ, et al. 2010. Rett syndrome: revised diagnostic criteria and nomenclature. *Ann Neurol* 68:944-50

Paweletz N. 1967. [On the function of the "Flemming body" during division of animal cells]. *Naturwissenschaften* 54:533-5

Pelka GJ, Watson CM, Christodoulou J, Tam PP. 2005. Distinct expression profiles of Mecp2 transcripts with different lengths of 3'UTR in the brain and visceral organs during mouse development. *Genomics* 85:441-52

Pohl C, Jentsch S. 2009. Midbody ring disposal by autophagy is a post-abscission event of cytokinesis. *Nat Cell Biol* 11:65-70

Pollarolo G, Schulz JG, Munck S, Dotti CG. 2011. Cytokinesis remnants define first neuronal asymmetry in vivo. *Nat Neurosci* 14:1525-33

Quaderi NA, Meehan RR, Tate PH, Cross SH, Bird AP, et al. 1994. Genetic and physical mapping of a gene encoding a methyl CpG binding protein, Mecp2, to the mouse X chromosome. *Genomics* 22:648-51

Rademacher N, Hambrock M, Fischer U, Moser B, Ceulemans B, et al. 2011. Identification of a novel CDKL5 exon and pathogenic mutations in patients with severe mental retardation, early-onset seizures and Rett-like features. *Neurogenetics* 12:165-7

Rett A. 1966. [On a unusual brain atrophy syndrome in hyperammonemia in childhood]. *Wien Med Wochenschr* 116:723-6

Ricciardi S, Ungaro F, Hambrock M, Rademacher N, Stefanelli G, et al. 2012. CDKL5 ensures excitatory synapse stability by reinforcing NGL-1-PSD95 interaction in the postsynaptic compartment and is impaired in patient iPSC-derived neurons. *Nat Cell Biol* 14:911-23

Rinaldo C, Moncada A, Gradi A, Ciuffini L, D'Eliseo D, et al. 2012. HIPK2 controls cytokinesis and prevents tetraploidization by phosphorylating histone H2B at the midbody. *Mol Cell* 47:87-98

Rosas-Vargas H, Bahi-Buisson N, Philippe C, Nectoux J, Girard B, et al. 2008. Impairment of CDKL5 nuclear localisation as a cause for severe infantile encephalopathy. *J Med Genet* 45:172-8

Rusconi L, Kilstrup-Nielsen C, Landsberger N. 2011. Extrasynaptic N-methyl-D-aspartate (NMDA) receptor stimulation induces cytoplasmic translocation of the CDKL5 kinase and its proteasomal degradation. *J Biol Chem* 286:36550-8

Rusconi L, Salvatoni L, Giudici L, Bertani I, Kilstrup-Nielsen C, et al. 2008. CDKL5 expression is modulated during neuronal development and its subcellular distribution is tightly regulated by the C-terminal tail. *J Biol Chem* 283:30101-11

Russo S, Marchi M, Cogliati F, Bonati MT, Pintaudi M, et al. 2009. Novel mutations in the CDKL5 gene, predicted effects and associated phenotypes. *Neurogenetics* 10:241-50

Saxton WM, McIntosh JR. 1987. Interzone microtubule behavior in late anaphase and telophase spindles. *J Cell Biol* 105:875-86

Schanen C, Houwink EJ, Dorrani N, Lane J, Everett R, et al. 2004. Phenotypic manifestations of MECP2 mutations in classical and atypical Rett syndrome. *Am J Med Genet A* 126A:129-40

Schrick C, Fischer A, Srivastava DP, Tronson NC, Penzes P, Radulovic J. 2007. N-cadherin regulates cytoskeletally associated IQGAP1/ERK signaling and memory formation. *Neuron* 55:786-98

Schultz RJ, Glaze DG, Motil KJ, Armstrong DD, del Junco DJ, et al. 1993. The pattern of growth failure in Rett syndrome. *Am J Dis Child* 147:633-7

Sekiguchi M, Katayama S, Hatano N, Shigeri Y, Sueyoshi N, Kameshita I. 2013. Identification of amphiphysin 1 as an endogenous substrate for CDKL5, a protein kinase associated with X-linked neurodevelopmental disorder. *Arch Biochem Biophys* 535:257-67

Shahbazian M, Young J, Yuva-Paylor L, Spencer C, Antalffy B, et al. 2002a. Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. *Neuron* 35:243-54

Shahbazian MD, Antalffy B, Armstrong DL, Zoghbi HY. 2002b. Insight into Rett syndrome: MeCP2 levels display tissue- and cell-specific differences and correlate with neuronal maturation. *Hum Mol Genet* 11:115-24

Sierra-Paredes G, Sierra-Marcuno G. 2007. Extrasynaptic GABA and glutamate receptors in epilepsy. *CNS Neurol Disord Drug Targets* 6:288-300

Skene PJ, Illingworth RS, Webb S, Kerr AR, James KD, et al. 2010. Neuronal MeCP2 is expressed at near histone-octamer levels and globally alters the chromatin state. *Mol Cell* 37:457-68

Skop AR, Liu H, Yates J, 3rd, Meyer BJ, Heald R. 2004. Dissection of the mammalian midbody proteome reveals conserved cytokinesis mechanisms. *Science* 305:61-6

Straight AF, Cheung A, Limouze J, Chen I, Westwood NJ, et al. 2003. Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. *Science* 299:1743-7

Swiech L, Blazejczyk M, Urbanska M, Pietruszka P, Dortland BR, et al. 2011. CLIP-170 and IQGAP1 cooperatively regulate dendrite morphology. *J Neurosci* 31:4555-68

Tao J, Hu K, Chang Q, Wu H, Sherman NE, et al. 2009. Phosphorylation of MeCP2 at Serine 80 regulates its chromatin association and neurological function. *Proc Natl Acad Sci U S A* 106:4882-7

Tatsumoto T, Sakata H, Dasso M, Miki T. 2003. Potential roles of the nucleotide exchange factor ECT2 and Cdc42 GTPase in spindle assembly in Xenopus egg cell-free extracts. *J Cell Biochem* 90:892-900

Tatsumoto T, Xie X, Blumenthal R, Okamoto I, Miki T. 1999. Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. *J Cell Biol* 147:921-8

Tao J, Van Esch H, Hagedorn-Greiwe M, Hoffmann K, Moser B, et al. 2004. Mutations in the X-linked cyclin-dependent kinase-like 5 (CDKL5/STK9) gene are associated with severe neurodevelopmental retardation. *Am J Hum Genet* 75:1149-54

Tolias KF, Duman JG, Um K. 2011. Control of synapse development and plasticity by Rho GTPase regulatory proteins. *Prog Neurobiol* 94:133-48

Trappe R, Laccone F, Cobilanschi J, Meins M, Huppke P, et al. 2001. MECP2 mutations in sporadic cases of Rett syndrome are almost exclusively of paternal origin. *Am J Hum Genet* 68:1093-101

Tsai PT, Hull C, Chu Y, Greene-Colozzi E, Sadowski AR, et al. 2012. Autistic-like behaviour and cerebellar dysfunction in Purkinje cell Tsc1 mutant mice. *Nature* 488:647-51

Valli E, Trazzi S, Fuchs C, Erriquez D, Bartesaghi R, et al. 2012. CDKL5, a novel MYCN-repressed gene, blocks cell cycle and promotes differentiation of neuronal cells. *Biochim Biophys Acta* 1819:1173-85

Wang IT, Allen M, Goffin D, Zhu X, Fairless AH, et al. 2012. Loss of CDKL5 disrupts kinome profile and event-related potentials leading to autistic-like phenotypes in mice. *Proc Natl Acad Sci U S A* 109:21516-21

Wang JB, Sonn R, Tekletsadik YK, Samorodnitsky D, Osman MA. 2009. IQGAP1 regulates cell proliferation through a novel CDC42-mTOR pathway. *J Cell Sci* 122:2024-33

Weaving LS, Christodoulou J, Williamson SL, Friend KL, McKenzie OL, et al. 2004. Mutations of CDKL5 cause a severe neurodevelopmental disorder with infantile spasms and mental retardation. *Am J Hum Genet* 75:1079-93

Wells WA, Murray AW. 1996. Aberrantly segregating centromeres activate the spindle assembly checkpoint in budding yeast. *J Cell Biol* 133:75-84

White CD, Brown MD, Sacks DB. 2009. IQGAPs in cancer: a family of scaffold proteins underlying tumorigenesis. *FEBS Lett* 583:1817-24

Wigge P, McMahon HT. 1998. The amphiphysin family of proteins and their role in endocytosis at the synapse. *Trends Neurosci* 21:339-44

Wilcock AC, Swedlow JR, Storey KG. 2007. Mitotic spindle orientation distinguishes stem cell and terminal modes of neuron production in the early spinal cord. *Development* 134:1943-54

Williamson SL, Giudici L, Kilstrup-Nielsen C, Gold W, Pelka GJ, et al. 2012. A novel transcript of cyclin-dependent kinase-like 5 (CDKL5) has an alternative C-terminus and is the predominant transcript in brain. *Hum Genet* 131:187-200

Yasui DH, Peddada S, Bieda MC, Vallero RO, Hogart A, et al. 2007. Integrated epigenomic analyses of neuronal MeCP2 reveal a role for long-range interaction with active genes. *Proc Natl Acad Sci U S A* 104:19416-21

Young JI, Hong EP, Castle JC, Crespo-Barreto J, Bowman AB, et al. 2005. Regulation of RNA splicing by the methylation-dependent transcriptional repressor methyl-CpG binding protein 2. *Proc Natl Acad Sci U S A* 102:17551-8

Zachariah RM, Rastegar M. 2012. Linking epigenetics to human disease and Rett syndrome: the emerging novel and challenging concepts in MeCP2 research. *Neural Plast* 2012:415825

Zhao WM, Seki A, Fang G. 2006. Cep55, a microtubule-bundling protein, associates with centralspindlin to control the midbody integrity and cell abscission during cytokinesis. *Mol Biol Cell* 17:3881-96

Zhou Z, Hong EJ, Cohen S, Zhao WN, Ho HY, et al. 2006. Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. *Neuron* 52:255-69

Zoghbi HY. 2005. MeCP2 dysfunction in humans and mice. *J Child Neurol* 20:736-40

Zoncu R, Efeyan A, Sabatini DM. 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 12:21-35