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# **Of rings and spines: the multiple facets of Citron proteins in neural development.**

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## **Abstract**

The Citron protein was originally identified for its capability to specifically bind the active form of RhoA small GTPase, leading to the simplistic hypothesis that it may work as a RhoA downstream effector in actin remodeling. More than two decades later, a much more complex picture has emerged. In particular, it has become clear that in animals, and especially in mammals, the functions of the Citron gene (*CIT*) are intimately linked to many aspects of central nervous system (CNS) development and function, although the gene is broadly expressed. More specifically, *CIT* encodes two main isoforms, Citron-kinase (CIT-K) and Citron-N (CIT-N), characterized by complementary expression pattern and different functions. Moreover, in many of their activities, *CIT* proteins act more as upstream regulators than as downstream effectors of RhoA. Finally it has been found that, besides working through actin, *CIT* proteins have many crucial functional interactions with the microtubule cytoskeleton and may directly affect genome stability. In this review, we will summarize these advances and illustrate their actual or potential relevance for CNS diseases, including microcephaly and psychiatric disorders.

## **Introduction**

During the last few years, many studies have highlighted the complex biological roles of *CIT* proteins in different cells and experimental models. The purpose of this review is to summarize the studies that relate CIT-K and CIT-N to the development and function of the CNS, as well as to the connected pathologic disorders. The role of CIT-K in cytokinesis and its possible relevance in cancer have already been reviewed in detail.<sup>1</sup>

### **Structure and phylogenetic history of *CIT* products.**

CIT-K is the largest product of mammalian *CIT*, with a molecular mass of 230 kD.<sup>2,3</sup> The protein displays a modular organization very similar to other members of the Myotonic Dystrophy subfamily of AGC-kinases, such as Rho-kinases (ROCKs) and CDC42BPA/CDC42BPB (also known as MRCKs), which are the closest CIT-K relatives.<sup>4,5</sup> CIT-K shares with MRCKs an amino-terminal kinase domain, followed by an extended coiled-coil region, a phorbol-ester/DAG-type zinc finger, a Pleckstrin Homology domain (PH) and a Citron-Nik1 homology (CNH) domain.<sup>3</sup> In addition, CIT-K is characterized at its C-terminus by a putative SH3 partner domain, and a PDZ partner domain.<sup>3</sup> By analyzing public sequence databases, we have derived a phylogenetic history of these proteins (unpublished data). In particular, we have found that the basic modular organization of CIT-K and MRCKs and their phylogenetic separation from ROCKs can be clearly recognized in Choanoflagellates, which encode a putative MRCK orthologue distinct from ROCK. In contrast, CIT-K, MRCK or ROCK orthologues cannot be found in yeasts, which only show partial homologies to these proteins. The duplication that led to the phylogenetic separation between MRCK and CIT-K may date back to early metazoans, since CIT-K putative orthologues can be found in Hydras and in Sponges (unpublished data). Besides CIT-K, the *CIT* gene encodes CIT-N, a shorter variant that differs from CIT-K only for the absence of the kinase domain.<sup>6-8</sup> CIT-N is produced from an alternative transcription start

site, located in the 10<sup>th</sup> CIT-K intron in mammals.<sup>9</sup> It is at the moment unknown whether CIT-N is a mammal-specific protein or equivalent isoforms are also expressed in other organisms.

### **Expression and localization of *CIT* proteins.**

The expression of CIT-K and CIT-N in mammalian tissues is remarkably different. CIT-K is ubiquitously expressed in proliferating cells,<sup>2,3,10</sup> while CIT-N is expressed only in the Central Nervous System (CNS), starting when the cells become post-mitotic and reaching highest levels in fully differentiated neurons.<sup>7,10-12</sup> Studies in non-neuronal cells have revealed that the expression and localization of CIT-K are dynamically modulated in different cell cycle phases. Protein levels are low in G1 cells, increase during cell cycle progression and peak in mitosis.<sup>12</sup> CIT-K is localized to the nucleus in interphase<sup>12</sup> and becomes dispersed in the cytoplasm during prophase, concomitantly with increased protein levels.<sup>12,13</sup> Removal of the soluble pool by mild detergent extraction revealed that, from metaphase to anaphase, CIT-K is associated with mitotic spindle and spindle poles.<sup>13</sup> At anaphase, CIT-K concentrates at the cleavage furrow and at the midbody,<sup>3</sup> and remains detectable in midbody remnants.<sup>14</sup> In contrast to CIT-K, CIT-N displays in both neurons and astrocytes a specific association with the endomembrane system, in particular with the Golgi apparatus. In neurons, this association is already apparent since early post-mitotic stage and increases with time.<sup>15</sup> As neuronal differentiation proceeds, CIT-N is found on dendritic Golgi outposts and on TGN38-positive membranes associated with dendritic spines.<sup>9</sup> Finally, in differentiated neurons, CIT-N is enriched in synapses, and in particular in post-synaptic densities (PSD), electron dense structures involved in associative learning.<sup>7,8</sup>

### **Phenotypes linked to CIT genetic variants.**

Based on expression pattern, localization and mutants' overexpression studies, it was first hypothesized that CIT-K could be a ubiquitous effector of RhoA involved in cytokinesis.<sup>3</sup>

This scenario is consistent with data obtained in mammalian cultured cell lines.<sup>14,16</sup> In addition, *Drosophila Cit-K* mutants show cytokinesis failure in different districts and a late embryonic lethal phenotype, which is consistent with an essential mitotic function.<sup>5,17,18</sup> Conversely, converging genetic evidence show that in mammals the functional requirement for CIT-K is restricted to the developing CNS.<sup>10,19</sup> Indeed, mutant mice totally lacking CIT-K but expressing normal levels of CIT-N are affected by microcephaly and cerebellar hypoplasia, associated with ataxia and lethal seizures.<sup>10</sup> During development, the affected tissues display high levels of apoptosis and high frequency of binucleated and multinucleated cells, deriving from cytokinesis failure.<sup>10</sup> In contrast, most of the other tissues are spared from these phenotypes, with the notable exception of the male germ line<sup>20</sup> and of slightly increased apoptosis levels in developing liver.<sup>12</sup> A similar spontaneous mutation in rats leads to an almost identical phenotype, which was named *Flathead*.<sup>19,21</sup> Further analysis of this model showed that CIT-K is also essential for forebrain post-natal neurogenesis.<sup>22</sup> The discrepancy between the restricted knockout phenotypes and the general effects in rodent and *Drosophila* cells was initially ascribed to species-specific differences.<sup>14</sup> However, four independent studies have described 20 patients affected by microcephaly caused by mutations in CIT-K.<sup>23-26</sup> The similarity between human and rodent phenotypes is further supported by the neuropathological findings of multinucleated cells and apoptosis in a patient,<sup>23</sup> as well as in patients-derived neural progenitors.<sup>24</sup> Consequently, *CIT* is now classified as one of the causal genes of autosomal recessive primary microcephaly (MCPH), a medical condition characterized by significantly smaller head circumference, compared to other infants of the same sex and age, and has been named *MCPH17* (OMIM : 617090). *MCPH17* patients share particularly severe microcephaly with simplified gyral pattern, cerebellar hypoplasia, corpus callosum agenesis, short stature, intellectual disability and spasticity. Notably, the severe drug-resistant epilepsy observed in mice and rats is not found in most of the *MCPH17* patients,

who only in one case showed pharmacologically tractable seizures.<sup>26</sup> Other aspects of the mammalian phenotype can be species-specific. Indeed, at least three patients showed renal hypoplasia or agenesis and one is also showing heart defects.<sup>23,26</sup> Moreover, *Flathead* rats have strongly disorganized retina,<sup>21,27</sup> while no gross eye abnormalities were found in patients and mice. Despite these differences, the specific alteration of brain development in mammals versus the embryonic lethal phenotype in *Drosophila* suggest that the CNS-specific requirement for CIT-K is a relatively recent phylogenetic acquisition. Importantly, besides to protein-truncating variants,<sup>23,24</sup> many of the mutations identified in patients are missense substitutions leading to kinase domain inactivation<sup>24</sup> (Table 1), indicating that CIT-K catalytic activity is essential for function. It is worth noting that patients bearing homozygous kinase-dead mutations show milder phenotype than the patients with homozygous null variants (Table 1). In future, it will be very important to define whether the observed differences were only due to genetic background or to the difference between total protein loss and kinase inactivation.

In addition to the dramatic genetic disease produced by homozygous inactivating mutations, *CIT* variants have been associated with psychiatric disorders. Indeed, *CIT* SNPs were associated with bipolar disorder<sup>28</sup> and schizophrenia<sup>29</sup>.

De novo gain-of-copy number of *CIT* was found in at least two cases of sporadic schizophrenia.<sup>30,31</sup> Although the latter results need further support, all the available genetic data strongly point to a crucial role of *CIT* in mammalian brain development, which may impact on the pathogenesis of different human brain disorders.

### **CIT-K in proliferating neural progenitors: many roads leading to microcephaly.**

Overexpression of mutant CIT-K constructs in HeLa cells leads to cleavage furrow instability during early stages of cytokinesis.<sup>3</sup> Moreover, CIT-K efficiently phosphorylates in vitro myosin regulatory light chain (MRLC) on both Ser-19 and Thr-18, a modification that accumulates at the cleavage furrow of dividing cells.<sup>32</sup> On this basis, CIT-K was

considered the crucial downstream effector of the ECT2 (Pbl in *Drosophila*)/RhoA axis that drives acto/myosin ring contraction during cytokinesis.<sup>18</sup> However, loss of function studies revealed that CIT-K is dispensable for furrow ingression, while it is required at late cytokinesis stages for abscission<sup>33,34</sup> (Figure 1). At this stage, active RhoA is not required for the midbody localization of CIT-K, while CIT-K is essential to maintain active RhoA at midbody. Indeed, CIT-K can regulate the activation state of RhoA most likely through a scaffolding function, which could be mediated by its RhoA binding domain.<sup>33</sup>

Electron microscopy analysis of human HeLa cells showed that CIT-K is particularly important to organize midbody structure.<sup>35</sup> Moreover in CIT-K-deficient cells the highly ordered arrangement of midbody proteins and the connection between the cortex and the central spindle microtubules are lost, which in turn leads to abscission failure.<sup>35-37</sup> Importantly, analysis of cell division in cells obtained from mutant mice and flies confirmed the physiological relevance of these observations.<sup>5,33</sup>

Live cell imaging of developing *Flathead* cortical slices suggested that CIT-K loss may lead to additional mitotic phenotypes, such as metaphase arrest and abnormal spindle function.<sup>38</sup> In agreement with this possibility, it was found that CIT-K loss is recruited by the microcephaly protein ASPM to the spindle and spindle poles, where it promotes the correct organization of astral MT, thus allowing the anchorage to cell cortex required for proper spindle orientation.<sup>13</sup> CIT-K-depleted HeLa cells, mouse and *Drosophila* CIT-K knockout neural progenitors show increased ratio of cells dividing with tilted cleavage angles, which in knockout mice correlated with increased frequency of cell cycle exit.<sup>13</sup>

The simultaneous presence of cytokinesis failure and apoptosis in CIT-K knockout mammals raised the question of whether, in this context, programmed cell death is a secondary consequence of cytokinesis failure. This point was controversial, since it has generally been found that cytokinesis failure leads to cell cycle arrest, rather than to apoptosis.<sup>39-41</sup> A different interpretation has recently been provided by a study showing



that CIT-K loss leads to high levels of spontaneous DNA double strand breaks and strongly activates P53<sup>42</sup> (Figure 1). Apoptosis, microcephaly and neurological phenotypes that characterize CIT-K null mice, including early post-natal lethality, can be significantly reverted by P53 inactivation. DNA double strand breaks (DSB) accumulation and increased sensitivity to ionizing radiations were also observed in *Drosophila* and in immortal cell lines. Importantly, in all these cases increased DSB occurred not only in tetraploid cells, but also in cells with normal DNA content, indicating that CIT-K is necessary to maintain genome stability independently of its action in cytokinesis.<sup>42</sup> On this basis, the microcephaly caused by CIT-K loss is very likely to result from a combination of massive apoptosis and increased cell cycle exit. The former is produced by P53 activation, which could be the end point of DNA damage accumulation.<sup>42</sup> The latter could be caused by P53 activation at sub-apoptotic levels, but also by P53-independent mechanisms. Indeed, despite the elimination of the apoptosis, CIT-K/P53 double knockout mice show only a partial rescue of brain size and display a P53-independent growth inhibitory signature during development.<sup>42</sup> A decrease of the ratio between proliferative and differentiative divisions of neural progenitors could be one of the possible P53-independent events. This mechanism has been proposed in the case of other microcephaly genes, such as ASPM, WDR62 or MCPH1.<sup>43</sup> Moreover, P53-independent pathways could be engaged by cytokinesis failure, which may trigger senescence<sup>39</sup> or activate the Hippo pathway.<sup>40</sup> The altered microtubule dynamics documented in CIT-K-deficient cells<sup>13,44</sup> could be the common cause of all these phenotypes, since it has recently been found that a modest increase of mitotic length due to microtubule instability is sufficient to produce in neural progenitors both DNA-damage and apoptosis.<sup>45</sup> The multiple pathogenetic mechanisms activated by CIT-K loss are probably the reason why MCPH17 is one of the most severe forms of primary microcephaly.<sup>23,24</sup>

### ***CIT* proteins in neuronal differentiation and function.**

The transition from neurogenesis to neural differentiation is accompanied by the dramatic switch in *CIT* isoforms, with downregulation of CIT-K and upregulation of CIT-N,<sup>7,10</sup> suggesting that these proteins play complementary roles in neuronal differentiation and function. However, relatively high levels of CIT-K mRNA are still present in post-mitotic neurons within the neocortex and in the thalamus. In situ hybridization experiments, using a kinase domain-specific probe, showed that CIT-K expression in the cortex is regionally restricted and becomes much stronger in the most superficial layers, suggesting an involvement in the differentiation of specific neuronal populations. In support of these observation, in neuroblastoma cell lines, neurite outgrowth is reduced by CIT-K overexpression and increased by CIT-K kinase dead mutants.<sup>11</sup> In dorsal root ganglia neurons (DRGN), CIT-K expression is induced in vivo by traumatic injury and CIT-K knockdown stimulates neurite outgrowth in vitro, even in presence of the inhibitory signals given by myelin extracts.<sup>46</sup> Similarly, siRNAs directed to both isoforms increased axon extension in cultured primary hippocampal neurons, which express almost exclusively CIT-N.<sup>47</sup> Altogether, these studies suggest that the two isoforms may physiologically cooperate to limit neurite extension during neuronal differentiation. In young neurons, CIT-N may also exert complex time-dependent effects on the architecture of the Golgi, by locally modulating actin polymerization through RhoA anchoring<sup>15,47</sup> (Figure 1). In mature neurons, the localization of CIT-N to spine-associated Golgi compartments and to the PSD is functionally relevant for spine maturation and maintenance.<sup>9,48</sup> CIT-N-depleted neurons in culture show an absolute reduction of spines density and a relative increase of immature spines<sup>9,48</sup> (Figure 1). Importantly, the requirement of CIT-N for spine maturation was validated in vivo, by comparing CIT-K knockout mice with a total loss of function mutant, lacking both CIT-K and CIT-N.<sup>9</sup> Although spine density was similarly reduced in both lines, mice lacking both isoforms have an increased ratio of morphologically immature spines. Even for this activity, CIT-N was found to act more as an anchoring protein for active RhoA

than as a downstream effector.<sup>9</sup> Due to the early neonatal lethality by epilepsy associated with both alleles, it is still unclear whether these difference may have neurophysiological and behavioral consequences.

### **Molecular complexes and mechanisms of *CIT* proteins, at the interface between actin and microtubules.**

Considering the different functions of *CIT* proteins in proliferating cells and in post-mitotic neurons, it is not surprising that they may physically and functionally interact with many different partners (Figure 1). In dividing cells, the interaction with active RhoA<sup>49</sup> and the association with actin filaments<sup>34</sup> are both involved in localizing CIT-K at the cleavage furrow, where it can also interact with the septin- and actin-binding protein Anillin.<sup>33,34</sup> Moreover, CIT-K interacts directly through its CNH region with Aurora B, INCENP and Borealin, three components of the Chromosomal Passenger Complex (CPC), which localize at different sites and regulates key mitotic events, including the organization of contractile apparatus and midbody.<sup>1,35</sup>

CIT-K also interacts, through its coiled-coil region, with the kinesins KIF14 and KIF23/MKLP1. KIF14 is recruited to the midbody by CIT-K, and both proteins cooperate to focus KIF23 and the microtubule-bundling protein PRC1 at the midbody.<sup>14,36,37</sup> Conversely, it has been proposed that KIF14 may be crucial to localize CIT-K to the midzone.<sup>14,50</sup>

Although the inter-dependence between CIT-K and KIF14 for their correct localization to the midbody may be context-dependent, a strong functional link between the two proteins is suggested by the finding that KIF14 knockout mice seem to phenocopy CIT-K knockout.<sup>51</sup> Thus, CIT-K may contribute to the stability of midbody by bridging CPC proteins with midbody ring kinesins MKLP1 and KIF14.<sup>1</sup> Additionally, it has recently been proposed that CIT-K may regulate midbody stability by increasing indirectly the phosphorylation of TUBB3 on S444, through its association with CK2a.<sup>44</sup> This mechanism could partially explain the tissue specificity of CIT-K sensitivity, since high levels of TUBB3

are expressed by cells that are more sensitive to CIT-K loss (such as committed neuronal progenitors), while modulation of TUBB3 expression levels modifies the sensitivity of cells to CIT-K depletion.<sup>44</sup> Finally, CIT-K may strengthen the structure of the midbody by promoting cortical anchorage of the intercellular bridge, through its interactions with both actin and microtubule-associated proteins.<sup>1</sup> Altogether, these mechanisms justify the master role of CIT-K in midbody maturation, and may represent a basis to further assess why it is critically important in neural progenitor but can be compensated in other cell types.

CIT-K interacts physically with RanBPM, a scaffolding protein that localizes to the plasma membrane and adherens junctions of polarized epithelial cells.<sup>52</sup> Correct CIT-K localization during mitosis and normal progression through M-phase of neural progenitors depend on RanBPM expression, suggesting that RanBPM could facilitate progression through mitosis and into cytokinesis through CIT-K.<sup>52</sup>

Another crucial microtubule-related partner of CIT-K is the microcephaly protein ASPM, which is required to focus the minus ends of spindle microtubules and is responsible for CIT-K localization to spindle poles.<sup>13,53</sup> The two proteins act in a pathway that regulates nucleation and stability of astral microtubules, thus promoting the interaction between mitotic spindle and cortex.<sup>13</sup> Spindle-associated CIT-K may promote the stability of astral microtubules through the CK2a/TUBB3 axis (unpublished data), while it is not clear how it could mediate microtubule nucleation. It has recently been demonstrated that ASPM forms a physiological complex with Katanin,<sup>54</sup> a conserved microtubule-severing protein complex that is also linked to microcephaly.<sup>55,56</sup> ASPM–katanin complex is required for correct spindle orientation, probably through a combination of microtubule-severing and minus-end blocking activities.<sup>54</sup> Therefore, it would be very interesting to analyze whether CIT-K is involved in these activities.

The significance of the ASPM/CIT-K interaction is potentially more widespread, since ASPM can also localize at the midbody and influence cytokinesis.<sup>53,57</sup> Moreover, as in the case of CIT-K knockdown, ASPM loss leads to the accumulation of DNA damage.<sup>58</sup> Since ASPM is the most commonly mutated microcephaly gene (MCPH5), unraveling the details of this interaction could be very important for the definition of common microcephaly mechanisms.

Initial characterization of the mechanisms leading to DSB accumulation after CIT-K loss have shown that it could derive, at least in part, by decreased efficiency of the Homologous Recombination (HR) pathway, due to reduced recruitment of RAD51 to DNA damage foci.<sup>42</sup> This view is further supported by the identification of physical interactions of CIT-K with KIF4A<sup>59</sup> and CDKN1B (P27KIP1),<sup>60</sup> which have both been implicated in RAD51 loading at DNA repair foci.

However, since increased accumulation of DSB after IR was found also in cells in G1,<sup>42</sup> in which RAD51 is weakly expressed and HR is not very relevant, it is possible that CIT-K may also be involved in Non-Homologous End Joining.

In the case of differentiating and mature neurons, the activity of *CIT* proteins has been associated more with the actin cytoskeleton than with microtubules. Indeed, in mature brain, CIT-N interacts with many actin-remodeling proteins implicated in the transduction pathway of Rho small GTPases, including ROCK-II, LIMK, P116Rip and PIIa.<sup>15</sup> In addition, CIT-K and CIT-N interact with TTC3, one of the proteins encoded by the Down syndrome critical region, which has been found to increase active RhoA levels and potently inhibits neuronal differentiation.<sup>61</sup> Consistent with this scenario, in neuronal cells CIT-N locally promotes RhoA recruitment and actin polymerization at Golgi and spines.<sup>9,15</sup> Moreover, the inhibitory effects of *CIT* proteins on neuronal differentiation can be reverted to a large extent by decreasing RhoA activity and actin polymerization.<sup>47</sup> However, recent studies have shown that also CIT-N could act at the interface between microtubules and actin.

Indeed, CIT-N promotes actin polymerization at spines downstream of p140Cap, a Src-interacting protein associated to microtubule plus-tip protein EB3.<sup>48</sup> To this regard, it is also interesting to notice that CIT-K has been described as a direct substrate of Src, in an Eph-initiated signal transduction pathway leading to cytokinesis inhibition.<sup>62</sup> Together, these results suggest that phosphorylation by Src could be a common mechanisms to regulate the interaction of *CIT* proteins with their partners. Finally, CIT-K was identified in a Two Hybrid screen as one of the interactors of the protein encoded by the *Disrupted In Schizophrenia 1* gene (*DISC1*), which is one of the most recurrent genetic risk factor for bipolar disorder, schizophrenia and other psychiatric disorders.<sup>29</sup> *DISC1* has important interactions with both actin and microtubule cytoskeleton, and may affect neural cell proliferation as well as dendritic spine dynamics.<sup>63</sup> This interaction could be crucial to understand how *CIT* proteins may play a role in psychiatric disorders. The finding that the *CIT* variants more closely associated with psychiatric phenotypes map close to the exons encoding the *DISC1* interacting region seems to support this scenario.<sup>29</sup> However, more studies are certainly required to clarify this point.

### **Concluding remarks**

Work performed in the last few years has significantly extended the current knowledge about the complex biological functions of *CIT* proteins, especially with regard to their role in cytokinesis and mammalian CNS development. Nevertheless, some outstanding questions still remain. An important point will be to better define the relative contribution of CIT-K and CIT-N to post-natal brain function. Indeed, although the higher expression of CIT-N suggests that this is the pivotal *CIT* isoform in adult brain functions, most of the studies so far performed made use of reagents directed against common *CIT* regions. Addressing this point in relation to higher CNS functions will be particularly critical, especially because psychiatric phenotypes are related to alterations that may increase the expression of CIT-K, whose effects are potentially drugable.

A second important point will be to better understand to what extent the mechanisms that lead to microcephaly after CIT-K loss are shared by other microcephaly syndromes of genetic or non genetic origin, in order to identify possible common therapeutic strategies. Finally, it will be very interesting to better elucidate why developing and adult mammalian tissue have a restricted requirement for CIT-K, while tumor cells and lower organisms show a general requirement.

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### **References**

1. D'Avino PP. Citron kinase - renaissance of a neglected mitotic kinase. *J Cell Sci* 2017; 130:1701–8; PMID:28468989; <http://dx.doi.org/10.1242/jcs.200253>.
2. Di Cunto F, Calautti E, Hsiao J, Ong L, Topley G, Turco E, Dotto GP. Citron Rho-interacting kinase, a novel tissue-specific Ser/Thr kinase encompassing the Rho-Rac-binding protein citron. *J Biol Chem* 1998; 273:29706–11; PMID:9792683; <http://dx.doi.org/10.1074/jbc.273.45.29706>.
3. Madaule P, Eda M, Watanabe N, Fujisawa K, Matsuoka T, Bito H, Ishizaki T, Narumiya S. Role of citron kinase as a target of the small GTPase Rho in cytokinesis. *Nature* 1998; 394:491–4; PMID:9697773; <http://dx.doi.org/10.1038/28873>.
4. Leroux AE, Schulze JO, Biondi RM. AGC kinases, mechanisms of regulation and innovative drug development. *Semin Cancer Biol* 2017; S1044-579X(17)30085-8; PMID:28591657; <http://dx.doi.org/10.1016/j.semcancer.2017.05.011>.

5. Naim V, Imarisio S, Di Cunto F, Gatti M, Bonaccorsi S. *Drosophila* citron kinase is required for the final steps of cytokinesis. *Mol Biol Cell* 2004; 15:5053–63; PMID:15371536; <http://dx.doi.org/10.1091/mbc.E04-06-0536>.
6. Madaule P, Furuyashiki T, Reid T, Ishizaki T, Watanabe G, Morii N, Narumiya S. A novel partner for the GTP-bound forms of rho and rac. *FEBS Lett* 1995; 377:243–8; PMID:8543060; [http://dx.doi.org/10.1016/0014-5793\(95\)01351-2](http://dx.doi.org/10.1016/0014-5793(95)01351-2).
7. Furuyashiki T, Fujisawa K, Fujita A, Madaule P, Uchino S, Mishina M, Bito H, Narumiya S. Citron, a Rho-target, interacts with PSD-95/SAP-90 at glutamatergic synapses in the thalamus. *J Neurosci* 1999; 19:109–18; PMID:9870943.
8. Zhang W, Vazquez L, Apperson M, Kennedy MB. Citron binds to PSD-95 at glutamatergic synapses on inhibitory neurons in the hippocampus. *J Neurosci* 1999; 19:96–108; PMID:9870942.
9. Camera P, Schubert V, Pellegrino M, Berto G, Vercelli A, Muzzi P, Hirsch E, Altruda F, Dotti CG, Di Cunto F. The RhoA-associated protein Citron-N controls dendritic spine maintenance by interacting with spine-associated Golgi compartments. *EMBO Rep* 2008; 9(4):384-929; PMID:18309323; <http://dx.doi.org/10.1038/embor.2008.21>.
10. Di Cunto F, Imarisio S, Hirsch E, Broccoli V, Bulfone A, Migheli A, Atzori C, Turco E, Triolo R, Dotto GP, et al. Defective neurogenesis in citron kinase knockout mice by altered cytokinesis and massive apoptosis. *Neuron* 2000; 28:115–27; PMID:11086988; [http://dx.doi.org/10.1016/S0896-6273\(00\)00090-8](http://dx.doi.org/10.1016/S0896-6273(00)00090-8).
11. Di Cunto F, Ferrara L, Curtetti R, Imarisio S, Guazzone S, Broccoli V, Bulfone A, Altruda F, Vercelli A, Silengo L. Role of citron kinase in dendritic morphogenesis of cortical neurons. *Brain Res Bull* 2003; 60(4):319-27; PMID:12781320; [http://dx.doi.org/10.1016/S0361-9230\(03\)00058-3](http://dx.doi.org/10.1016/S0361-9230(03)00058-3).
12. Liu H, Di Cunto F, Imarisio S, Reid LM. Citron kinase is a cell cycle-dependent, nuclear protein required for G2/M transition of hepatocytes. *J Biol Chem* 2003;



278:2541–8; PMID:12411428; <http://dx.doi.org/10.1074/jbc.M210391200>.

13. Gai M, Bianchi FT, Vagnoni C, Verni F, Bonaccorsi S, Pasquero S, Berto GE, Sgrò F, Chiotto AMA, Annaratone L, et al. ASPM and CITK regulate spindle orientation by affecting the dynamics of astral microtubules. *EMBO Rep* 2016; 17(10):1396-1409; PMID:27562601; <http://dx.doi.org/10.15252/embr.201541823>.
14. Gruneberg U, Neef R, Li X, Chan EHY, Chalamalasetty RB, Nigg EA, Barr FA. KIF14 and citron kinase act together to promote efficient cytokinesis. *J Cell Biol* 2006; 172:363–72; PMID:16431929; <http://dx.doi.org/10.1083/jcb.200511061>.
15. Camera P, Santos Da Silva J, Griffiths G, Giuffrida MG, Ferrara L, Schubert V, Imarisio S, Silengo L, Dotti CG, Di Cunto F. Citron-N is a neuronal Rho-associated protein involved in Golgi organization through actin cytoskeleton regulation. *Nat Cell Biol* 2003; 5(12):1071-8; PMID:14595335; <http://dx.doi.org/10.1038/ncb1064>.
16. McKenzie C, D'Avino PP. Investigating cytokinesis failure as a strategy in cancer therapy. *Oncotarget* 2016; 7(52):87323-87341; PMID:27895316; <http://dx.doi.org/10.18632/oncotarget.13556>.
17. D'Avino PP, Savoian MS, Glover DM. Mutations in sticky lead to defective organization of the contractile ring during cytokinesis and are enhanced by Rho and suppressed by Rac. *J Cell Biol* 2004; 166:61–71; PMID:15240570; <http://dx.doi.org/10.1083/jcb.200402157>.
18. Shandala T, Gregory SL, Dalton HE, Smallhorn M, Saint R. Citron kinase is an essential effector of the Pbl-activated Rho signalling pathway in *Drosophila melanogaster*. *Development* 2004; 131:5053–63; PMID:15459099; <http://dx.doi.org/10.1242/dev.01382>.
19. Sarkisian MRMR, Li W, Di Cunto F, D'Mello SRSR, LoTurco JJJ. Citron-kinase, a protein essential to cytokinesis in neuronal progenitors, is deleted in the flathead mutant rat. *J Neurosci* 2002; 22:RC217; PMID:11932363.

20. Di Cunto F, Imarisio S, Camera P, Boitani C, Altruda F, Silengo L. Essential role of citron kinase in cytokinesis of spermatogenic precursors. *J Cell Sci* 2002; 115(Pt 24):4819-26; PMID: 12432070; <http://dx.doi.org/10.1242/jcs.00163>.
21. Roberts MR, Bittman K, Li WW, French R, Mitchell B, LoTurco JJ, D'Mello SR. The flathead mutation causes CNS-specific developmental abnormalities and apoptosis. *J Neurosci* 2000; 20:2295–306; PMID:10704505.
22. Ackman JB, Ramos RL, Sarkisian MR, Loturco JJ. Citron kinase is required for postnatal neurogenesis in the hippocampus. *Dev Neurosci* 2007; 29:113–23; PMID:17148954; <http://dx.doi.org/10.1159/000096216>.
23. Harding BN, Moccia A, Drunat S, Soukarieh O, Tubeuf H, Chitty LS, Verloes A, Gressens P, El Ghouzzi V, Joriot S, et al. Mutations in Citron Kinase Cause Recessive Microlissencephaly with Multinucleated Neurons. *Am J Hum Genet* 2016; 99:511–20; PMID:27453579; <http://dx.doi.org/10.1016/j.ajhg.2016.07.003>.
24. Li H, Bielas SL, Zaki MS, Ismail S, Farfara D, Um K, Rosti RO, Scott EC, Tu S, Chi NC, et al. Biallelic Mutations in Citron Kinase Link Mitotic Cytokinesis to Human Primary Microcephaly. *Am J Hum Genet* 2016; 99:501–10; PMID:27453578; <http://dx.doi.org/10.1016/j.ajhg.2016.07.004>.
25. Basit S, Al-Harbi KM, Alhijji SAM, Albalawi AM, Alharby E, Eldardear A, Samman MI. CIT, a gene involved in neurogenic cytokinesis, is mutated in human primary microcephaly. *Hum Genet* 2016; 135:1199–207; PMID:27519304; <http://dx.doi.org/10.1007/s00439-016-1724-0>.
26. Shaheen R, Hashem A, Abdel-Salam GMH, Al-Fadhli F, Ewida N, Alkuraya FS. Mutations in CIT, encoding citron rho-interacting serine/threonine kinase, cause severe primary microcephaly in humans. *Hum Genet* 2016; 135:1191–7; PMID:27503289; <http://dx.doi.org/10.1007/s00439-016-1722-2>.
27. Karunakaran DKP, Chhaya N, Lemoine C, Congdon S, Black A, Kanadia R. Loss of

- Citron Kinase Affects a Subset of Progenitor Cells That Alters Late but Not Early Neurogenesis in the Developing Rat Retina. *Invest Ophthalmol Vis Sci* 2015; 56:787–98; PMID:25593024; <http://dx.doi.org/10.1167/iovs.14-15272>.
28. Lyons-Warren A, Chang JJ, Balkissoon R, Kamiya A, Garant M, Nurnberger J, Scheftner W, Reich T, McMahon F, Kelsoe J, et al. Evidence of association between bipolar disorder and Citron on chromosome 12q24. *Mol Psychiatry* 2005; 10:807–9; PMID:15983625; <http://dx.doi.org/10.1038/sj.mp.4001703>.
29. Nicodemus KK, Callicott JH, Higier RG, Luna A, Nixon DC, Lipska BK, Vakkalanka R, Giegling I, Rujescu D, St Clair D, et al. Evidence of statistical epistasis between DISC1, CIT and NDEL1 impacting risk for schizophrenia: biological validation with functional neuroimaging. *Hum Genet* 2010; 127:441–52; PMID:20084519; <http://dx.doi.org/10.1007/s00439-009-0782-y>.
30. Xu B, Roos JL, Levy S, van Rensburg EJ, Gogos JA, Karayiorgou M. Strong association of de novo copy number mutations with sporadic schizophrenia. *Nat Genet* 2008; 40:880–5; PMID:18511947; <http://dx.doi.org/10.1038/ng.162>.
31. Johnstone M, Maclean A, Heyrman L, Lenaerts A-S, Nordin A, Nilsson L-G, De Rijk P, Goossens D, Adolfsson R, St Clair DM, et al. Copy Number Variations in DISC1 and DISC1-Interacting Partners in Major Mental Illness. *Mol neuropsychiatry* 2015; 1:175–90; PMID:27239468; <http://dx.doi.org/10.1159/000438788>.
32. Yamashiro S, Totsukawa G, Yamakita Y, Sasaki Y, Madaule P, Ishizaki T, Narumiya S, Matsumura F. Citron kinase, a Rho-dependent kinase, induces di-phosphorylation of regulatory light chain of myosin II. *Mol Biol Cell* 2003; 14:1745–56; PMID:12802051; <http://dx.doi.org/10.1091/mbc.E02-07-0427>.
33. Gai M, Camera P, Dema A, Bianchi F, Berto G, Scarpa E, Germena G, Di Cunto F. Citron kinase controls abscission through RhoA and anillin. *Mol Biol Cell* 2011; 22:3768–78; PMID:21849473; <http://dx.doi.org/10.1091/mbc.E10-12-0952>.

34. Bassi ZI, Verbrugge KJ, Capalbo L, Gregory S, Montembault E, Glover DM, D'Avino PP. Sticky/Citron kinase maintains proper RhoA localization at the cleavage site during cytokinesis. *J Cell Biol* 2011; 195:595–603; PMID:22084308; <http://dx.doi.org/10.1083/jcb.201105136>.
35. McKenzie C, Bassi ZI, Debski J, Gottardo M, Callaini G, Dadlez M, D'Avino PP. Cross-regulation between Aurora B and Citron kinase controls midbody architecture in cytokinesis. *Open Biol* 2016; 6:160019; PMID:27009191; <http://dx.doi.org/10.1098/rsob.160019>.
36. Bassi ZI, Audusseau M, Riparbelli MG, Callaini G, D'Avino PP. Citron kinase controls a molecular network required for midbody formation in cytokinesis. *Proc Natl Acad Sci* 2013; 110:9782–7; PMID:23716662; <http://dx.doi.org/10.1073/pnas.1301328110>.
37. Watanabe S, De Zan T, Ishizaki T, Narumiya S. Citron kinase mediates transition from constriction to abscission through its coiled-coil domain. *J Cell Sci* 2013; 126:1773–84; PMID:23444367; <http://dx.doi.org/10.1242/jcs.116608>.
38. LoTurco JJ, Sarkisian MR, Cosker L, Bai J. Citron kinase is a regulator of mitosis and neurogenic cytokinesis in the neocortical ventricular zone. *Cereb Cortex* 2003; 13:588–91; PMID:12764032; <http://dx.doi.org/10.1093/cercor/13.6.588>.
39. Panopoulos A, Pacios-Bras C, Choi J, Yenjerla M, Sussman MA, Fotedar R, Margolis RL. Failure of cell cleavage induces senescence in tetraploid primary cells. *Mol Biol Cell* 2014; 25:3105–18; PMID:25143403; <http://dx.doi.org/10.1091/mbc.E14-03-0844>.
40. Ganem N, Cornils H, Chiu S-Y, O'Rourke K, Arnaud J, Yimlamai D, Théry M, Camargo F, Pellman D. Cytokinesis Failure Triggers Hippo Tumor Suppressor Pathway Activation. *Cell* 2014; 158:833–48; PMID:25126788; <http://dx.doi.org/10.1016/j.cell.2014.06.029>.

41. Kuffer C, Kuznetsova AY, Storchova Z. Abnormal mitosis triggers p53-dependent cell cycle arrest in human tetraploid cells. *Chromosoma* 2013; 122:305–18; PMID:23624524; <http://dx.doi.org/10.1007/s00412-013-0414-0>.
42. Bianchi FT, Tocco C, Pallavicini G, Liu Y, Verni F, Merigliano C, Bonaccorsi S, El-Assawy N, Priano L, Gai M, et al. Citron Kinase Deficiency Leads to Chromosomal Instability and TP53-Sensitive Microcephaly. *Cell Rep* 2017; 18:1674–86; PMID:28199840; <http://dx.doi.org/10.1016/j.celrep.2017.01.054>.
43. Faheem M, Naseer MI, Rasool M, Chaudhary AG, Kumosani TA, Ilyas AM, Pushparaj PN, Ahmed F, Algahtani HA, Al-Qahtani MH, et al. Molecular genetics of human primary microcephaly: an overview. *BMC Med Genomics* 2015; 8:S4; PMID:25951892; PMID:25951892; <http://dx.doi.org/10.1186/1755-8794-8-S1-S4>.
44. Sgrò F, Bianchi FT, Falcone M, Pallavicini G, Gai M, Chiotto AMA, Berto GE, Turco E, Chang YJ, Huttner WB, et al. Tissue-specific control of midbody microtubule stability by Citron kinase through modulation of TUBB3 phosphorylation. *Cell Death Differ* 2016; 23:801–13; PMID:26586574; <http://dx.doi.org/10.1038/cdd.2015.142>.
45. Pilaz L-J, McMahon J, Miller E, Lennox A, Suzuki A, Salmon E, Silver D. Prolonged Mitosis of Neural Progenitors Alters Cell Fate in the Developing Brain. *Neuron* 2016; 89:83–99; PMID:26748089; <http://dx.doi.org/10.1016/j.neuron.2015.12.007>.
46. Ahmed Z, Douglas MR, Read ML, Berry M, Logan A. Citron kinase regulates axon growth through a pathway that converges on cofilin downstream of RhoA. *Neurobiol Dis* 2011; 41:421–9; PMID:20971191; <http://dx.doi.org/10.1016/j.nbd.2010.10.012>.
47. Berto GE, Iobbi C, Camera P, Scarpa E, Iampietro C, Bianchi F, Gai M, Sgrò F, Cristofani F, Gärtner A, et al. The DCR protein TTC3 affects differentiation and Golgi compactness in neurons through specific actin-regulating pathways. *PLoS One* 2014; 9(4):e93721. PMID:24695496 <http://dx.doi.org/10.1371/journal.pone.0093721>.
48. Repetto D, Camera P, Melani R, Morello N, Russo I, Calcagno E, Tomasoni R,

- Bianchi F, Berto G, Giustetto M, et al. p140Cap regulates memory and synaptic plasticity through src-mediated and citron-N-mediated actin reorganization. *J Neurosci* 2014; 34(4):1542-1553; PMID:24453341; <http://dx.doi.org/10.1523/JNEUROSCI.2341-13.2014>.
49. Eda M, Yonemura S, Kato T, Watanabe N, Ishizaki T, Madaule P, Narumiya S. Rho-dependent transfer of Citron-kinase to the cleavage furrow of dividing cells. *J Cell Sci* 2001; 114:3273–84; PMID:11591816.
50. Cullati SN, Kabeche L, Kettenbach AN, Gerber SA. A bifurcated signaling cascade of NIMA-related kinases controls distinct kinesins in anaphase. *J Cell Biol* 2017; 216(8):2339-2354; PMID:28630147; <http://dx.doi.org/10.1083/jcb.201512055>.
51. Fujikura K, Setsu T, Tanigaki K, Abe T, Kiyonari H, Terashima T, Sakisaka T. Kif14 mutation causes severe brain malformation and hypomyelination. *PLoS One* 2013; 8:e53490; PMID:23308235; <http://dx.doi.org/10.1371/journal.pone.0053490>.
52. Chang Y, Paramasivam M, Girgenti MJ, Walikonis RS, Bianchi E, LoTurco JJ. RanBPM regulates the progression of neuronal precursors through M-phase at the surface of the neocortical ventricular zone. *Dev Neurobiol* 2009; 70(1):1-15; PMID:19790105; <http://dx.doi.org/10.1002/dneu.20750>.
53. Paramasivam M, Chang YJ, LoTurco JJ. ASPM and citron kinase co-localize to the midbody ring during cytokinesis. *Cell Cycle* 2007; 6:1605–12; PMID:17534152; <http://dx.doi.org/10.4161/cc.6.13.4356>.
54. Jiang K, Rezabkova L, Hua S, Liu Q, Capitani G, Maarten Altelaar AF, Heck AJR, Kammerer RA, Steinmetz MO, Akhmanova A. Microtubule minus-end regulation at spindle poles by an ASPM–katanin complex. *Nat Cell Biol* 2017; 19:480–92; PMID:28436967; <http://dx.doi.org/10.1038/ncb3511>.
55. Mishra-Gorur K, Çağlayan AO, Schaffer AE, Chabu C, Henegariu O, Vonhoff F, Akgümüş GT, Nishimura S, Han W, Tu S, et al. Mutations in KATNB1 Cause

- Complex Cerebral Malformations by Disrupting Asymmetrically Dividing Neural Progenitors. *Neuron* 2014; 84:1226–39; PMID:25521378; <http://dx.doi.org/10.1016/j.neuron.2014.12.014>.
56. Hu WF, Pomp O, Ben-Omran T, Kodani A, Henke K, Mochida GH, Yu TW, Woodworth MB, Bonnard C, Raj GS, et al. Katanin p80 Regulates Human Cortical Development by Limiting Centriole and Cilia Number. *Neuron* 2014; 84:1240–57; PMID:25521379; <http://dx.doi.org/10.1016/j.neuron.2014.12.017>.
57. Higgins J, Midgley C, Bergh A-M, Bell SM, Askham JM, Roberts E, Binns RK, Sharif SM, Bennett C, Glover DM, et al. Human ASPM participates in spindle organisation, spindle orientation and cytokinesis. *BMC Cell Biol* 2010; 11:85; PMID:21044324; <http://dx.doi.org/10.1186/1471-2121-11-85>.
58. Williams SE, Garcia I, Crowther AJ, Li S, Stewart A, Liu H, Lough KJ, O'Neill S, Veleta K, Oyarzabal EA, et al. Aspm sustains postnatal cerebellar neurogenesis and medulloblastoma growth in mice. *Development* 2015; 142:3921–32; PMID:26450969; <http://dx.doi.org/10.1242/dev.124271>.
59. Maliga Z, Junqueira M, Toyoda Y, Ettinger A, Mora-Bermudez F, Klemm RW, Vasilij A, Guhr E, Ibarlucea-Benitez I, Poser I, et al. A genomic toolkit to investigate kinesin and myosin motor function in cells. *Nat Cell Biol* 2013; 15:325–34; PMID:23417121; <http://dx.doi.org/10.1038/ncb2689>.
60. Serres MP, Kossatz U, Chi Y, Roberts JM, Malek NP, Besson A. p27(Kip1) controls cytokinesis via the regulation of citron kinase activation. *J Clin Invest* 2012; 122:844–58; PMID:22293177; <http://dx.doi.org/10.1172/JCI60376>.
61. Berto G, Camera P, Fusco C, Imarisio S, Ambrogio C, Chiarle R, Silengo L, Di Cunto F. The Down syndrome critical region protein TTC3 inhibits neuronal differentiation via RhoA and Citron kinase. *J Cell Sci* 2007; 120(Pt 11):1859-67; PMID:17488780; <http://dx.doi.org/10.1242/jcs.000703>.

62. Jungas T, Perchey RT, Fawal M, Callot C, Froment C, Burlet-Schiltz O, Besson A, Davy A. Eph-mediated tyrosine phosphorylation of citron kinase controls abscission. *J Cell Biol* 2016; 214:555–69; PMID:27551053; <http://dx.doi.org/10.1083/jcb.201602057>.
63. Narayan S, Nakajima K, Sawa A. DISC1: a key lead in studying cortical development and associated brain disorders. *Neurosci* 2013; 19:451–64; PMID:23300216.

## Figure legends

### Figure 1

**A)** Developmental abnormalities produced by CIT-K loss in mammalian cortex. During cortical development of CIT-K null mice a series of parallel events cause a reduction of the neural progenitors' pool and of the number of neurons produced, leading to microcephaly. The mitosis of apical progenitors mitosis is altered, with increased number of oblique divisions as well as delayed or disrupted anaphases. A high fraction of neurons and a lower fraction of basal and apical progenitors undergo apoptosis. Binucleated neurons accumulate, in consequence of cytokinesis failure in neural progenitors. Pyramidal neurons have less dendritic spines.

**B)** Altered processes resulting from CIT-K loss. 1. CIT-K regulates spindle orientation primarily by promoting the nucleation and stability of astral microtubules, thus allowing the spindle to cell cortex anchorage required for proper spindle orientation. 2. CIT-K regulates abscission by promoting midbody stability. Loss of CIT-K leads to cytokinesis failure, with consequent cell fusion and binucleation. 3. CIT-K loss impairs DNA repair and leads to DSB accumulation.

**C)** Altered processes resulting from CIT-N loss 1. CIT-N is required to maintain Golgi architecture by modulating local actin polymerization: loss of CIT-N leads to Golgi



fragmentation. 2. CIT-N works as a scaffold protein in neuronal dendritic spine organization, binding to Golgi membranes and affecting actin remodelling. Loss of CIT-N impairs maturation and maintenance of dendritic spines. 3. CIT-N and CIT-K may regulate axon extension.

In panels B and C the main partners of CIT proteins in the different processes are listed.