Mutation or inactivation of CDKL5 kinase is associated with a human neurodevelopmental condition commonly referred to as CDKL5 deficiency disorder.6 Two recent phosphoproteomics studies identify the first physiological substrates of mammalian CDKL5 and evaluate functional consequences of their phosphorylation and its loss in cells lacking functional CDKL5, highlighting potential roles for this kinase in regulating neuronal microtubule dynamics.

The EMBO Journal (2018) 37: e100848
See also: IM Muñoz et al (December 2018) and LL Baltussen et al (December 2018)

Protein phosphorylation plays a fundamental role in basic cell biology, and the protein kinase superfamily has become a goldmine for potential drug-intervention strategies due to kinase mutation or dysregulation in human diseases. However, it is often (conveniently) forgotten that mechanistic information is lacking for much of the human kinome, and this includes an absence of basic knowledge pertaining to regulatory mechanisms and substrate specificity (Wilson et al, 2018). A case-in-point are the cyclin-dependent kinase-like (CDKL) kinases, a small family of five poorly characterised and structurally distinct Ser/Thr protein kinases from the CGMC evolutionary branch of the kinome, which contains GSK3α, MAPK and CDK families (Canning et al, 2018). Mammalian CDKL5 (also known as STK9) is widely expressed in cells, where it is targeted to a variety of subcellular structures (Barbiero et al, 2017; Oi et al, 2017). Of particular interest, inactivation of the X-linked CDKL5 gene, or disease-associated mutations commonly found in the N-terminal CDKL5 catalytic domain, causes a neurodevelopmental disorder termed CDKL5 deficiency disorder (CDD; Tao et al, 2004; Weaving et al, 2004), which has some overlapping features with Rett syndrome (Scala et al, 2005) and West syndrome (Kalscheuer et al, 2003).6

Writing in The EMBO Journal, two groups simultaneously reveal the first physiological substrates for CDKL5, including potential new biomarkers for reporting cellular CDKL5 activity. Using distinct, but highly complementary mass spectrometry (MS)-based phosphoproteomics approaches, the laboratories of John Rouse and Matthias Trost (Muñoz et al, 2018), and Sila Ullanir (Baltussen et al, 2018) evaluate functional consequences of CDKL5-catalysed substrate phosphorylation and its loss in cells lacking functional CDKL5. These findings will be critical for understanding microtubule dynamics and cilium-based signalling in CDD cell models, including neurons from patients with pathological CDKL5 mutations.

Both teams exploited MS to establish, in as unambiguous a way as is possible, the direct substrates of CDKL5 in cellular models. Although the approaches taken were different, they (happily) converge on a subset of substrates related both through shared subcellular function(s) and conserved consensus site(s) of CDKL5 phosphorylation. Muñoz and colleagues initially employed a clean CRISPR knockout/in approach in human U2OS cells ± CDKL5, which was coupled to a quantitative (6-plex TMT) phosphoproteomic workflow (Muñoz et al, 2018). This high-quality, internally controlled experimental strategy revealed nearly 200 sites of intracellular protein phosphorylation (including CDKL5 autophosphorylation) whose increase was statistically relevant when CDKL5 was added back to CDKL5 genome-edited cells. In a distinct, no-less impressive approach, Baltussen and colleagues developed CDKL5 chemical proteomics, employing purified catalytically active CDKL5-F89A/C152A, an analogue-sensitive (as) version selectively using the bulky ATP analogue benzyl-ATP/βS, to thiophosphorylate potential substrates in a mouse brain lysate (Baltussen et al, 2018). After affinity-based purification of phosphorylated substrate-derived peptides, mass spectrometry identified multiple potential CDKL5 substrates, including murine microtubule-associated targets MAP1S (two sites of phosphorylation), EB2 and ARHGEF2 (Baltussen et al, 2018) and, independently, human MAP1S, CEP131 and DLG5 (Muñoz et al, 2018). At a single stroke, these consolidating approaches reveal, for the very first time, new signalling functions of CDKL5 that are relevant to microtubule assembly, cilia-based signalling and perhaps polarity-based cellular networks (Fig 1A).

In the case of the microtubule-binding protein MAP1S, a biological function for phosphorylation was confirmed in elegant murine follow-up experiments, exploiting phospho-specific MAP1S antibodies to demonstrate that phosphorylation on Ser812 (equivalent to human Ser900) was impaired in microtubule binding in vitro, consistent with a significant reduction in microtubule dynamics in living cortical neuron dendrites...
derived from CDKL5 knockout mice (Baltussen et al., 2018). In order to probe signalling with additional reagents, both studies made use of additional phosphospecific antibodies. For example, the phosphorylation of MAP1S at Ser812 and EB2 at Ser222 is both markedly reduced in CDKL5 knockout mice regardless of age, validating phosphoproteomics data and establishing CDKL5 activity markers in cells based around proteins that modulate microtubule trafficking and dynamics (Fig 1B). Perhaps the icing on the cake, however, is the demonstration that endogenous EB2 phosphorylation (as judged by a phospho-specific EB2 antibody able to recognise an identical phospho-epitope in human and mouse EB2) is severely reduced (by ~80%) in neurons derived from CDD patient fibroblasts after reprogramming into iSPCs (Baltussen et al., 2018). Importantly, many of the antibodies validated in the two studies can now be employed, or re-engineered as higher-affinity antibodies, to serve as diagnostic tools for other CDKL orthologues. Further studies have confirmed biochemically with phosphoproteomics data sets, which demonstrate CDKL5 autophosphorylation sites in Ser/Pro motifs alongside a dominant general Arg-Pro-X-Ser-Ala phosphorylation consensus emerging from both papers is the amino acid sequence Arg-Pro-X-Ser/Thr-Ala/Pro, in which the Ser or Thr residue (heavily biased 85:15% Ser:Thr) is phosphorylated, adjacent to a C-terminal residue (commonly Ala or Pro). These latter findings, confirmed biochemically with model peptide substrates, agree with phosphoproteomics data sets, which demonstrated CDKL5 autophosphorylation sites in Ser/Pro motifs alongside a dominant general Arg-X-X-Ser/Thr-Ala/Pro motif (Muñoz et al., 2018). Although the CDKL5 consensus overlaps to some extent with known sites of phosphorylation for other “basophilic” kinases (e.g. Arg-X-X-Ser-Ala for several AGC kinases) and MAPK/CDKs (minimally Ser/Thr-Pro), the absolute conservation of a Pro at –2, and a specific requirement for Arg at –3, may well be diagnostic for CDKL5 (and perhaps other CDKL orthologues). Further studies employing active CDKL1/2/3/4 proteins (which, somewhat surprisingly, do not appear to phosphorylate any of the physiological CDKL5 substrates identified in this study) should open up these unstudied human kinases to careful scrutiny using similar chemical genetic or genome editing approaches.

The functional consequences of patholog-ical CDKL5 mutations were previously uncertain, with evidence for inhibition and preservation of activity, alongside Tyr phosphorylation in the TEY activation segment (Bertani et al., 2006). By quantifying the reduction of CDKL5-dependent MAP1S and CEP131 phosphorylation in cells expressing specific CDD mutants, and by using a new MAP1S S900 peptide assay to assess immunoprecipitated CDKL5 activity, Muñoz and colleagues now unequivocally identify them as loss-of-function mutations that inactivate CDKL5.

Another exciting outcome from these studies is the finding that catalytically active CDKL5 phosphorylates Ser/Thr in a sharply defined, specific amino acid consensus sequence in its target substrates (Fig 1A), as has been documented for many other protein kinases. A harmonious minimal CDKL5 phosphorylation consensus emerging from both papers is the amino acid sequence Arg-Pro-X-Ser-Thr-Ala/Pro, in which the Ser or Thr residue (heavily biased 85:15% Ser:Thr) is phosphorylated, adjacent to a C-terminal residue (commonly Ala or Pro). These latter findings, confirmed biochemically with model peptide substrates, agree with phosphoproteomics data sets, which demonstrated CDKL5 autophosphorylation sites in Ser/Pro motifs alongside a dominant general Arg-X-X-Ser/Thr-Ala/Pro motif (Muñoz et al., 2018). Although the CDKL5 consensus overlaps to some extent with known sites of phosphorylation for other “basophilic” kinases (e.g. Arg-X-X-Ser-Ala for several AGC kinases) and MAPK/CDKs (minimally Ser/Thr-Pro), the absolute conservation of a Pro at –2, and a specific requirement for Arg at –3, may well be diagnostic for CDKL5 (and perhaps other CDKL orthologues). Further studies employing active CDKL1/2/3/4 proteins (which, somewhat surprisingly, do not appear to phosphorylate any of the physiological CDKL5 substrates identified in this study) should open up these unstudied human kinases to careful scrutiny using similar chemical genetic or genome editing approaches.

The functional consequences of patholog-ical CDKL5 mutations were previously uncertain, with evidence for inhibition and preservation of activity, alongside Tyr phosphorylation in the TEY activation segment (Bertani et al., 2006). By quantifying the reduction of CDKL5-dependent MAP1S and CEP131 phosphorylation in cells expressing specific CDD mutants, and by using a new MAP1S S900 peptide assay to assess immunoprecipitated CDKL5 activity, Muñoz and colleagues now unequivocally identify them as loss-of-function mutations that inactivate CDKL5.

Intriguingly, biochemical analysis demonstrates that CDKL5 also possesses an inherent ability to phosphorylate itself on a conserved regulatory residue (Tyr171) in the activation segment Thr-Glu-Tyr motif. Although the CDKL5 consensus overlaps to some extent with known sites of phosphorylation for other “basophilic” kinases (e.g. Arg-X-X-Ser-Ala for several AGC kinases) and MAPK/CDKs (minimally Ser/Thr-Pro), the absolute conservation of a Pro at –2, and a specific requirement for Arg at –3, may well be diagnostic for CDKL5 (and perhaps other CDKL orthologues). Further studies employing active CDKL1/2/3/4 proteins (which, somewhat surprisingly, do not appear to phosphorylate any of the physiological CDKL5 substrates identified in this study) should open up these unstudied human kinases to careful scrutiny using similar chemical genetic or genome editing approaches.

The functional consequences of patholog-ical CDKL5 mutations were previously uncertain, with evidence for inhibition and preservation of activity, alongside Tyr phosphorylation in the TEY activation segment (Bertani et al., 2006). By quantifying the reduction of CDKL5-dependent MAP1S and CEP131 phosphorylation in cells expressing specific CDD mutants, and by using a new MAP1S S900 peptide assay to assess immunoprecipitated CDKL5 activity, Muñoz and colleagues now unequivocally identify them as loss-of-function mutations that inactivate CDKL5.
might also be relevant for generating active CDKL5 in cells (Bertani et al., 2006). Interestingly, a Thr-X-Tyr motif is conserved in all five human CDKL protein kinases; its mutation to an Asp-X-Glu phosphomimetic version in CDKL1-5 prior to crystallographic studies (Canning et al., 2018) provides supporting evidence of the importance of this motif for driving structural dynamics. However, how (or even whether) CDKL5 phosphorylation is regulated at this motif in cells remains to be established, since the mechanism through which it is triggered is currently unknown. It is intriguing to speculate that CDKL5 is not only able to control its own activity, but that mutations in CDD patients might also lead to a loss in CDKL5 catalytic output through a lack of activation-loop Tyr phosphorylation. The best-known “dual-specificity” protein kinases are the MAPKK/MEK family of MAPK activators, which catalyse dual phosphorylation of a Thr-X-Tyr motif (Thr-Glu-Tyr for MEK1/2) in the activation segment of cognate MAPK substrates. A concerted autoactivation mechanism in CDKL5 suggests that it possesses (biochemical) properties of a dual-specificity protein kinase, and opens up this mechanistic event in CDKL kinases for further analysis. In the related MAPKs, dual phosphorylation of the Thr-X-Tyr motif massively increases catalytic activity, whereas Tyr15 phosphorylation is strongly associated with inhibition at a motif distinct from the activation segment in CDKs. It will therefore be interesting to evaluate whether CDKL5 autophosphorylation on Tyr (and/or Thr) also occurs on the other four CDKL proteins (where the human sequence is Thr-Asp-Tyr, rather than Thr-Glu-Tyr), or whether Tyr is also phosphorylated on cellular proteins distinct from CDKL5. Finally, CDKL5 Tyr autophosphorylation does not rule out the presence of distinct Tyr or dual-specificity kinases lying “upstream” of CDKL5; putative activators include the interacting dual-specificity tyrosine phosphorylation-regulated kinase DYRK1A (Oi et al., 2017), which was previously shown to contribute to CDKL5 subcellular targeting.

A central impact from this work is the discovery of the very first physiological CDKL5 substrates and the emergence of a framework for the development of multiple approaches to study and measure (and eventually normalise) CDKL5 activity in systems relevant to CDD, such as the brain. Further detective work will be required to uncover the complete set of cellular CDKL5 substrates (many more being predicted in these phosphoproteomics studies) and to decipher regulatory networks associated with CDKL5 catalytic output that are central to physiological function and underlie CDKL5 loss-of-function in CDD. Unravelling the molecular mechanisms of CDKL5 activation and inactivation could also help link subset(s) of the CDKL5 network that contribute to disease phenotypes to potential therapeutics. In terms of targetable outputs, factors that control CDKL5 activation include pharmacological agents linked to activity of the NMDA receptor (Tramarin et al., 2018), whose inhibition by experimental neuronal depolarisation has a significant experimental effect on EB2 phosphorylation at pS222 (Baltussen et al., 2018). Several avenues might also be explored in related areas of cell biology, most notably any links between human CDKL5 and the cilipathy-associated protein CENP131, which is associated with centriolar stability and the DNA damage response. Centriolar and cilium-based analysis also provide fascinating new potential models for functional CDKL5 analysis. For example, human CDKL5 has recently been demonstrated to localise to the cilium, and effects of patient-derived mutations have been modelled in C. elegans, where CeCDKL1 (most similar to human CDKL1/4) regulates cilium length, and Chlamydomonas, where CrCDKL5 is also required for controlling aspects of ciliary dynamics (Canning et al., 2018).

Finally, very recent studies have begun to analyse small molecule kinase inhibitors, including the pre-clinical GSK3β inhibitor Tideglusib, which exhibits some promise for restoring memory function in immature CDKL5 knockout mice, although the specific intracellular target that mediates these effects is unknown (Fuchs et al., 2018). Further work is now required to link the specific subset(s) of phosphorylated CDKL5 targets that contribute to disease phenotypes, and to understand the function of the long C-terminal region, which is much longer in CDKL5 than other CDKL proteins, and presumably plays a key role in its physiological function. Rational drug design using recently available CDKL5 structures and small molecule screening data (Canning et al., 2018) could provide new ways to activate mutated (inactive) CDKL5 mutants, alongside the development of genetic therapies, or even protein-replacement therapies (Trazzi et al., 2018).

References
Baltussen LL, Negraes PD, Silvestre M, Claxton S, Moeskops M, Christodoulou E, Flynn HR, Snijders AP, Muotri AR, Ulaner SK (2018) Chemical genetic identification of CDKL5 substrates reveals its role in neuronal microtubule dynamics. EMBO J 37: e99763
Barbiero I, Valente D, Chandola C, Magi F, Bergo A, Montonefriorio I, Tramarin M, Fazzari M, Saddou S, Landsberger N, Rinaldo C, Kilstup-Nielsen C (2017) CDKL5 localizes at the centrosome and midbody and is required for faithful cell division. Sci Rep 7: 6228
Bertani I, Rusconi L, Bolognese F, Forlani G, Conca B, de Monte L, Badaracco G, Landsberger N, Kilstup-Nielsen C (2006) Functional consequences of mutations in CDKL5, an X-linked gene involved in infantile spasms and mental retardation. J Biol Chem 281: 32048 – 32056
Canning P, Park K, Goncalves J, Li C, Howard CJ, Sharpe TD, Holt Lj, Pelletier L, Bullock AN, Leroux MR (2018) CDKL5 family kinases have evolved distinct structural features and ciliary function. Cell Rep 22: 885 – 894
Fuchs C, Fustini N, Trazzi S, Gennaccaro L, Rimondini R, Ciani E (2018) Treatment with the GSK3-beta inhibitor Tideglusib improves hippocampal development and memory performance in juvenile, but not adult, Cdkl5 knockout mice. Eur J Neurosci 47: 1054 – 1066
Kalscheuer VM, Tao J, Donnelly A, Holloway G, Schwinger E, Kubart S, Menzel C, Hoeltzenbein M, Tommerup N, Eyre H, Harbord M, Haan E, Sutherland GR, Ropers HH, Geck J (2003) Disruption of the serine/threonine kinase gene causes severe X-linked infantile spasms and mental retardation. Am J Hum Genet 72: 1401 – 1411
Muñoz IM, Morgan ME, Peltier J, Weiland F, Gregorczyk M, Brown FC, Macartney T, Toth R, Trost M, Rouse J (2018) Phosphoproteomic screening identifies physiological substrates of the CDKL5 kinase. EMBO J 37: e95539
Oi A, Katayama S, Hatan H, Sugiyama Y, Kameshita I, Sueyoshi N (2017) Subcellular distribution of cyclin-dependent kinase-like 5 (CDKL5) is regulated through phosphorylation by dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A). Biochem Biophys Res Comm 482: 239 – 245
Scala E, Ariani F, Mari F, Caselli R, Pescucci C, Longo I, Meloni I, Giachino D, Bruttini M, Hayek G, Zappella M, Renieri A (2005) CDKL5/STK9 is mutated in Rett syndrome variant with infantile spasms. J Med Genet 42: 103 – 107
Tao J, van Esch H, Hagedorn-Grewe M, Hoffmann K, Moser B, Raynaud M, Sperner J, Fryns JP, Schwingler E, Geicz J, Ropers HH, Kalscheuer VM (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: 1149–1154

Tramarin M, Rusconi L, Pizzamiglio L, Barbiero I, Peroni D, Scaramuzza L, Guilmis T, Cavalla D, Antonucci F, Klistrup-Nielsen C (2018) The antidepressant tianeptine reverts synaptic AMPA receptor defects caused by deficiency of CDKL5. Hum Mol Genet 27: 2052–2063

Trazzi S, De Franceschi M, Fuchs C, Bastianini S, Viggiano R, Lupori L, Mazzotti R, Medici G, Lo Martire V, Ren E, Rimondini R, Zoccoli G, Bartsaghi R, Pizzorusso T, Ciani E (2018) CDKL5 protein substitution therapy rescues neurological phenotypes of a mouse model of CDKL5 disorder. Hum Mol Genet 27: 1572–1592

Weaving LS, Christoudoulou J, Williamson SL, Friend KL, McKenzie OL, Archer H, Evans J, Clarke A, Pelka GJ, Tam PP, Watson C, Lahooti H, Ellaway CJ, Bennetts B, Leonard H, Gecz J (2004) Mutations of CDKL5 cause a severe neurodevelopmental disorder with infantile spasms and mental retardation. Am J Hum Genet 75: 1079–1093

Wilson LJ, Linley A, Hammond DE, Hood FE, Coulson JM, Macewan DJ, Ross SJ, Slupsly JR, Smith PD, Eyers PA, Prior IA (2018) New perspectives, opportunities, and challenges in exploring the human protein kinome. Can Res 78: 15–29