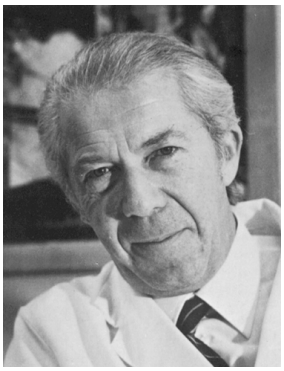
Discussion



Christian de Duve (°1917) shared the 1974 Nobel Prize in Physiology or Medicine with Albert Claude and George E. Palade 'for their discoveries concerning the structural and functional organisation of the cell'.

'... keep your eyes open for the odd or unexpected and never dismiss it simply because it does not fit within your program. If chance offers you a clue, follow the trail. You may not discover what you were looking for, but what you discover may be more interesting than what you were looking for. In retrospect, I find that my greatest luck came from the unforeseen: insulin that wasn't and mitochondria that weren't.
My last recommendation is for the powers that be. Fund the investigator, not the investigation. Do please remember, I beseech you, this self-evident yet rarely recognized truth that science, at least its spearhead called basic research, explores the unknown and is therefore unable, by definition, to predict how useful or profitable its discoveries will be. Rather than demanding assurances on this account that cannot possibly be honestly provided, put your trust in the investigator's skill, instinct, curiosity, and motivation. This will produce the best research contributing best to the advancement of knowledge, the true aim of science...'

de Duve, C. (2004). *J Biol Chem* 279, 21679-88.

How the human mind works has intrigued people for ages. Contemporary science is looking for answers in a variety of ways, employing, for example, evolutionary biology, (experimental) psychology and cognitive neuroscience. Molecular biologists try to find an answer by attempting to define a molecular basis for human cognition.

A. Current state of knowledge

At least 50% of the co-variation among measures of cognitive ability is genetic. Molecular characterisation of *de novo* X;A balanced translocations in patients with mild, NS-MR is a powerful starting point in unravelling these genetics of intelligence.

In his seminal paper on intelligence, Spearman postulated the existence of a general intrinsic intelligence, which he called '*g*' factor[828]. Even though a consensus definition of what precisely *g* is has not been settled yet[829], a method to quantify *g* has been developed. So-called factor analysis creates a composite score for *g* that represents what diverse measures of cognitive abilities have in common. In other words, each test assessing cognition is weighted by its overall correlation with all other tests, resulting in a measure for an individual's *g*. This is different in IQ tests, which consider each test equally rather than weighting these tests by their contribution to *g*. However, since similar abilities are measured, IQ scores correlate highly with *g* scores derived from factor analysis (r ~0.80)[830]. In what follows, the word 'intelligence' refers to this *g* score; that is, the substantial covariation among diverse measures of cognitive ability.

Analyses of the world's accumulated data from family, adoptive and twin studies have firmly established a genetic component of at least 50% for intelligence[12,831]. Because of the presumably large number of genes involved in mental ability and the presence of many alleles per gene, resulting in the continuous nature of intelligence, it is believed that a vast number of QTLs represents the genetic component of cognition[830,832]. Identifying such QTLs will be an extremely arduous task, due to the individually small contribution of each QTL to the genetics of *g*. However, investigation of genetic defects in mentally retarded patients is a manageable approach to single out intelligence genes. This is so because (i) a genetic defect represents a QTL with an exceptionally large contribution, i.e. mutation of a gene involved in

human cognition will lead to MR, and (ii) multivariate genetic analyses have found that genetic correlations among specific cognitive abilities are close to one, i.e. the same genes affect diverse cognitive abilities[833,834].

With regard to gene hunting in mentally retarded patients, it is essential to realise that mild and severe MR are two different entities: whereas mild MR is familial, severe MR is not (Fig. I-1c). In other words, mild MR is mostly hereditable, and hence genetic, whereas severe MR is frequently – but not exclusively – due to non-genetic factors such as pre-, peri- and post-natal injury, trauma of the head or infections[11,17,835-837].

MR has also been classified in syndromic and non-syndromic forms. Even though it turned out that such categories often could not be maintained at the molecular level[60,61,75], it should be pointed out that, in syndromic forms of MR, causality between gene defect and MR is less straightforward than in non-syndromic cases. This is so because, in S-MR, (i) it is unclear whether the gene defect is responsible for the MR, for the accompanying *consistent* somatic features or for both and (ii) it is possible that the MR is a secondary effect of the associated phenotypic characteristics.

Taken together, it is therefore preferable to search for genes involved in cognition in patients with mild, NS-MR.

A battery of methodologies has been developed to facilitate the search for gene defects in patients. One approach uses chromosomal aberrations as pointers to putatively interesting genomic loci. *De novo* reciprocal translocations, one such group of aberrations that has been instrumental in the elucidation of several congenital disorders[666], have a prevalence of approximately 1 in 2000 live births with a frequency of congenital malformations of 6.1%, which is about double that of the usual estimate at birth[838]. This observation suggests that, in disease-associated balanced translocations, there exists a causal link between the aberrant karyotype and the disorder in ~50% of cases. Due to the low gene content of the human genome[19], it may be assumed that disruption of a gene or its regulatory elements by a translocation BP significantly increases the concordance between genotype and phenotype. Disrupted X-chromosomal genes are even more promising in this respect as they often represent null alleles. This is not only true for single copy non-pseudoautosomal genes in males but also holds true in females, since the unaffected X chromosome is preferentially inactivated in ~80% of female carriers of X;A translocations. Such skewed XCI is believed to assure normal transcription of the translocated autosome[35].

With the observation of a 20 – 45 % male excess among MR patients[42,44,80,839,840], the occurrence of pedigrees with an X-linked inheritance of MR[45,56,841] and the cloning of 72 genes involved in XLMR to date (Tables I-4 – 6), an involvement of X-chromosomal genes in the aetiology of MR has been proven beyond the shadow of a doubt.

In conclusion, positional cloning of genes through the molecular characterisation of BPs from *de novo* X;A balanced translocations in patients with mild, NS-MR is a powerful starting point in the identification of genes important in human cognition.

B. Hypothesis: the t(X;8) translocation probably causes the patient's mental retardation

We postulate that the patient's MR most likely arose from disruption of either or both of the genes affected by the translocation BPs.

The delineation of a genotype – phenotype correlation in a mildly mentally retarded carrier of a balanced X;A translocation uniquely combines handles on gene locus as well as on *g*, and thus creates an excellent starting point for the search for genes with a role in cognitive ability.

We postulate that the patient's MR arose from disruption of either or both of the genes, *hFBXO25* and *hKIAA1202*, affected by the translocation BPs. Both genes were previously uncharacterised, which precluded a continuation of the genotype – phenotype analysis based on *a priori* knowledge of their function. Therefore, we decided to investigate both genes in parallel, but with emphasis on the X-chromosomal gene, as this was amenable to mutation screening in a large cohort of patients with suspected XLMR. Moreover, disruption of the X-linked gene would likely result in a complete absence of gene product, as has been outlined in the previous section, whereas the intact copy of the autosomal gene may well suffice to guarantee unimpeded cellular function.

C. It is highly unlikely that the idiopathic seizures of the t(X;8) patient correlate with her mild mental retardation

The patient carries a balanced *de novo* 46,X,t(X;8)(p11.2;p22.3) translocation affecting *hFBXO25* and *hKIAA1202*. She is mildly mentally retarded and suffers from idiopathic epilepsy. Co-morbidity of such epilepsy and mild MR is low, all but the severest seizures do not typically interfere with IQ, and the correlation of age at seizure onset and severity of MR is disputed. Epilepsy and MR both affect dendritic spines, but the molecular basis of epilepsy *per se* differs from that of MR. However, epilepsy associated with MR can have genetic aetiologies common to both disorders. We argue that the patient's MR is not the consequence of her epilepsy and we consider it to be non-syndromic with regard to finding genes involved in human cognition.

With the intention of finding genes that are involved in human mental ability, we set out to characterise the BPs of a translocation in a mentally retarded patient.

Karyotyping of metaphase spreads from the patient showed that she carries an apparently balanced *de novo* 46,X,t(X;8)(p11.2;p22.3) translocation. Cloning of the X;8 junction fragment indicated that two genes were disrupted by the BPs. While the autosomal BP disrupts *hFBXO25*, the X-chromosomal BP affects *hKIAA1202*.

Although no IQ test scores were available for the patient, several observations support a diagnosis of mild MR:

- Delivery was uneventful.
- Growth parameters at birth were all within the 75th percentile. At the age of 11 years, they were still normal.
- Early motor development (< 9 months) was normal; unassisted walking was possible at the age of 15 months.
- The attending physician described the patient as a quiet, anxious girl presenting with mild to moderate MR. The physician noted that she could not perform complex requests and that her fine motor skills were poor.
- Gross neurological examination and brain MRI scan were normal.
- The patient attended a school for children with learning disabilities until age 10 and one for children with MR until age 20. As of 2006, she has been employed in a sheltered workplace for the mentally handicapped.

Apart from being mentally retarded, the patient also suffers from idiopatic epilepsy, which might have had an influence on the development of her MR and therefore needs to be discussed.

By the age of 2 years, the weight of the neonatal brain has tripled and reached ~75% of the expected adult weight[842,843]. Extensive dendritic branching with synapse formation and

myelination parallel this remarkable expansion[843,844]. Not surprisingly, epileptic episodes, defined as *recurrent* seizures and affecting ~1% of the population[845], upsetting this critical phase of complex, dynamic, and adaptive post-natal brain development traditionally have been believed to have deleterious effects on cognition, implying that epilepsy causes MR. However, this was a premature conclusion as the available data failed to prove causality of the epilepsy – MR correlation.

Indeed, more recent studies pointed out several inconsistencies in results and methodologies of the precedent investigations[846-848], and drew a more balanced picture of the relationship between MR and epilepsy[17,849-852]. The anecdotal evidence that many epileptics have become famous for their achievements supports the idea that seizures do not inevitably produce lasting harm to the brain[853].

Co-morbidity estimates for epilepsy and MR come from (i) studies establishing the incidence of MR among epileptics and (ii) investigations assessing the prevalence of epilepsy among the mentally retarded.

The first type of study has the intrinsic problem that the onset of MR, by definition in early childhood, is difficult to monitor in young children, as the ability to assess higher cortical functions is still very limited. The later recognition of retardation does not necessarily indicate that the retardation followed the onset of seizures. Co-morbidity estimates range from 8 – 10% for mild MR and 20 – 30% for severe MR in epidemiologic studies not selecting for seizure characteristics[854,855].

The second type of study estimates the co-morbidity between MR and epilepsy to be 10 – 16% for mild MR[835,856-858] and 27 – 36% for severe MR[835-837,856,859] but, due to the cross-sectional nature of these investigations, which underestimates the incidence of MR, includes relatively more patients with associated disabilities and comprises also single-seizure events, epilepsy prevalence rates – especially in mildly retarded patients – were almost certainly gauged too high. More recent longitudinal studies indicate that the cumulative frequency of epilepsy with mild MR, is low, hovering around 3% at age 10 years[849,860].

The presumption of epileptic seizures having adverse effects on mental development has been amended in recent years. Although the most severe forms of intractable epilepsy do seem to affect IQ negatively[861-866], this has been proven different for milder idiopathic forms. One problem was the use of random members of the general population as controls in earlier studies, whereas more recent investigations compared probands with their seizure-free siblings[850,851], with a control group of mentally retarded children born to consanguineous par-

ents[17], or with mentally retarded populations co-morbid with a different psychopathology[867]. Such studies consistently showed no significant difference in cognitive ability between patients and controls[850,851,867]. Also, IQ scores did not change appreciably with time, and there was no significant difference in IQ before and after the onset of seizures[850,851].

There is, as of yet, no consensus on the correlation between age at seizure onset and severity of MR. Several studies suggest that younger ages at seizure onset are associated with lower IQ scores, affecting all[868] or only subsets of the battery of cognitive tests[869-871], but these investigations are cross-sectional studies concentrating on severe forms of epilepsy. In addition, some researchers provide evidence for a significant difference between the median age of seizure onset and patients with mild or severe MR (3.1 years for mild MR vs. 0.8 years for severe MR)[872], whereas several other authors do not find such significant differences[860,873,874].

In fact, some laboratories provide evidence that epilepsy may be a consequence of the brain abnormalities causing mental deficit, thereby challenging the prevailing view that epilepsy causes MR. Ellenberg *et al.* recorded no significant differences when comparing IQ scores from epileptic children with those from their non-epileptic siblings. They found, however, that variability in IQ and rate of MR were higher in children with seizures. Interestingly, such differences disappeared when children who had neurologic abnormalities *before* seizure onset were excluded[850]. Although the number of hemiplegic children in Huttenlocher and Hapke's cohort of patients with severe epilepsy is small, they reported a clear tendency for lower intelligence levels in children with post-natally acquired brain lesions compared to those with congenital hemiplegia, irrespective of seizure history. The authors noted that 'the occurrence of mental defect may be related to the aetiology of the seizure more than it is related to the age of the child at the time of seizure occurrence, or to seizure severity or frequency'[875]. Goulden and colleagues support this hypothesis. They measured a cumulative frequency of developing epilepsy during the first 22 years of life in 5% of mentally retarded patients without associated features, but reaching 75% if the MR was associated with postnatal brain injury[849].

Anatomical evidence supports the finding that brain abnormalities may be an aetiology common to epilepsy *and* MR. Comparison of dendritic anomalies observed in Golgi preparations from cortical pyramidal neurons of mentally retarded patients and patients with long-

standing epilepsy shows a striking similarity (Figs. I-4c'' and IV-1), suggesting that dendritic spines may constitute an anatomical substrate for both MR and epilepsy[273,274,844,876-879].

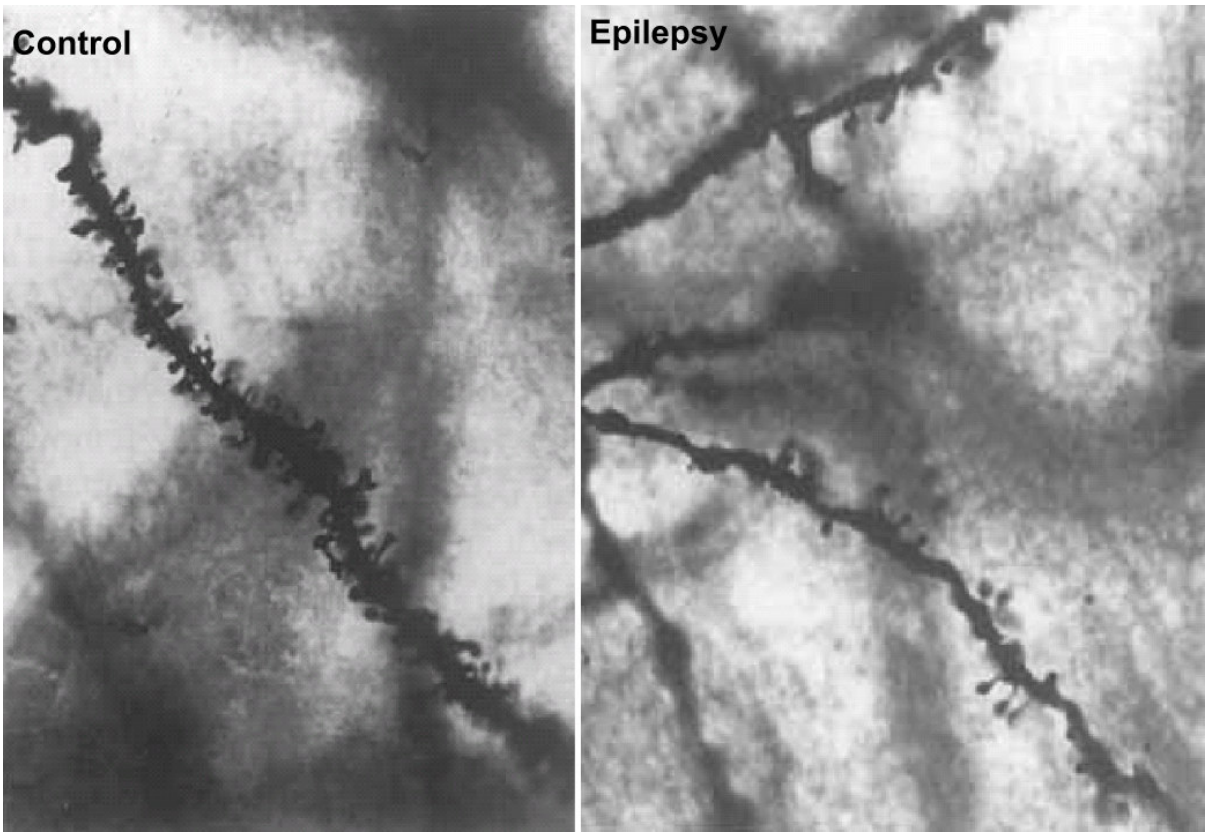


Fig. IV-1 | **Microanatomical features of dendritic anomalies in epileptic patients.**

Close-ups of Golgi-impregnated dendritic branches of pyramidal neurons obtained from the temporal cortices of a neurologically normal adult (left panel, Control) and of a patient with long-standing epilepsy (right panel, Epilepsy). Note the sparsity of spines. Comparison with Fig. I-4c'', which shows abnormalities in the dendritic spine morphology of a mentally impaired individual, reveals a striking similarity between the microanatomical aberrations in spine morphology of epileptic and mentally retarded patients. Original magnification 1000×.

Pictures adapted from *877*.

Spine dysgenesis, characterised by a marked sparsity of dendritic arborisation and a reduction in spine density, was initially described in post-mortem specimens of cerebral cortex from individuals with isolated MR[273,274], but has also been observed in the genetic Down, Rett and Fragile X syndromes[277]. The recent characterisation of several (XL)MR genes regulating the Rho family of GTPases[70], which in turn modulate dendritic spine morphology through remodelling of the Actin cytoskeleton[880-882], is strong evidence for a causal role of spine dysgenesis in MR. Increases in dendritic spine density in the rat hippocampal CA1 region upon spatial learning[883] or in forebrain song nuclei of songbirds upon song learning[884], and changes in dendritic motility and morphology[885] support the importance of dendritic

spines in cognition. These findings have been replicated during LTP[886-888], which is considered to be the cellular basis of learning and memory[542], and has been shown to also be dependent on changes in the Actin cytoskeleton (see IV.F.3)[288].

In contrast to patients with severe forms of epilepsy[889,890], the majority of epileptics show no clear evidence of neuronal death, suggesting that more subtle mechanisms of seizure-induced brain injury may exist. Investigations of resected brain tissue from patients suggest that such subtle epileptogenic brain injury is present as dendritic alterations in the neocortex and hippocampus[876,879,891]. In addition, animal models recapitulating different forms of epilepsy show reduction in spine density in the dentate gyrus[892], hippocampus[893-896], amygdala[897] and neocortex[898].

Intriguingly, the anatomical similarity in brains from mentally retarded and epileptic patients is not straightforwardly mirrored at the molecular level.

In vivo time-lapse studies assaying consequences of epileptiform activity in the murine hippocampus[899] and neocortex[900] showed a modest loss in dendritic spines over several hours. This is in contrast to time-lapse experiments assessing the outcome of LTP on dendritic spines. Such studies reveal the formation of new spines within minutes of LTP induction in hippocampal slice cultures[901,902], which is in line with a degree of spine motility on a time scale of seconds, as observed in hippocampal slice culture[288,903,904], as well as in the neocortex of living mice[905,906]. Thus, the molecular mechanisms by which seizures and other patterns of electrical activity, such as LTP, influence dendritic spines might be different.

Although the genetics underlying common forms of epilepsy are complex and most of the susceptibility genes remain unknown[907,908], it has been shown that those genetic defects underlying idiopathic epilepsy and familial epilepsy syndromes – that is, epilepsy in the absence of MR – upset ion channel functioning in the vast majority of cases. The combined data from the epilepsy field suggest that ion channel mutations are a common cause of idiopathic epilepsy, but are rare causes of common epilepsy syndromes[909-916]. So-called channelopathies have not been detected as a *direct* cause of MR[a] yet[70], suggesting that certain forms of epilepsy have aetiologies distinct from those discovered for MR. On the other hand, mutations in several genes (e.g. *ARX*, *ATRX*, *CDKL5*, *DCX*, *SYN1*, *SLC6A8*, *PHF6*, *E6-AP* and *ATP6AP2*)

[a] It should be noted, however, that *indirect* links between MR and ion channels have been reported.
SAP102, encoded by *DLG3*[917], interacts with the NR2 subunit of the NMDA receptor[100,918]. Disruption of NMDA receptor targeting or signalling, due to mutations in *DLG3*, is thought to underly the intellectual impairment observed in individuals with pathologic *DLG3* variants[99].
Interaction of Cereblon, encoded by *CRBN*, a gene that is mutated in autosomal recessive MR[87], with the cytosolic C-terminus of the α subunit of the large-conductance Ca^{2+}-activated K^+ channels has been shown to reduce their functionality at the cell surface[919].

have been identified that give rise to MR and associated forms of epilepsy[62,166,176,472,474]. It should be noted that inter- and even intrafamilial phenotypes, with regard to both MR and epilepsy for different, but also for identical, mutations in some of these genes, frequently vary considerably. Such phenotypic variability is difficult to explain, but may be a result of – unidentified – QTLs[553,920-924].

In conclusion, it seems that (i) epilepsy and MR affect the same anatomical brain structures, and (ii) whereas epilepsy *per se* has a molecular aetiology distinct from that of MR, forms of epilepsy associated with MR have genetic aetiologies common to both the convulsive disorder and mental disability.

Based on the low co-morbidity of idiopathic epilepsy and mild MR, the stability of IQ during epileptic activity, and the disputed importance of early age at seizure onset with regard to the degree of MR later in life, we believe it to be unlikely that our patient's idiopathic epilepsy caused her mild mental deficit. Should this nevertheless be the case, the evidence outlined above argues for the existence of genetic defects that result in MR with associated epilepsy. Therefore, we consider the MR in our patient to be non-syndromic with regard to finding genes involved in mental ability.

D. *hFBXO25* encodes an F-box protein and hKIAA1202 associates with the Actin cytoskeleton

The t(X;8) translocation investigated during this study disrupts the uncharacterised *hFBXO25* and *hKIAA1202* genes. This work shows that they encode proteins involved in regulated protein breakdown and associated with the F-actin cytoskeleton, respectively.

D.1. Brain-expressed *hFBXO25* encodes an F-box protein, which is part of the SCF^{hFBXO25} E3 Ubiquitin ligase

hFBXO25, encoding a *de facto* FBP, is expressed in foetal and adult brain and forms part of a functional SCF E3 ligase of which the function relies on S244, possibly through hydrogen bonding with Skp1 K128.

The chromosome 8 BP of the translocation under study disrupts the uncharacterised *hFBXO25* gene. Conventional northern hybridisations showed that *hFBXO25* is ubiquitously transcribed, including expression in foetal and adult brain. Spatial expression patterns of the murine *hFBXO25* homologue showed almost exclusive neuronal expression at E14.5. *hFBXO25* was first mentioned as a partial *in silico* protein in a paper identifying a

family of human FBPs[765]. FBPs are a part of the SCF E3 Ubiquitin ligases involved in the regulation of concerted protein breakdown[592,594].

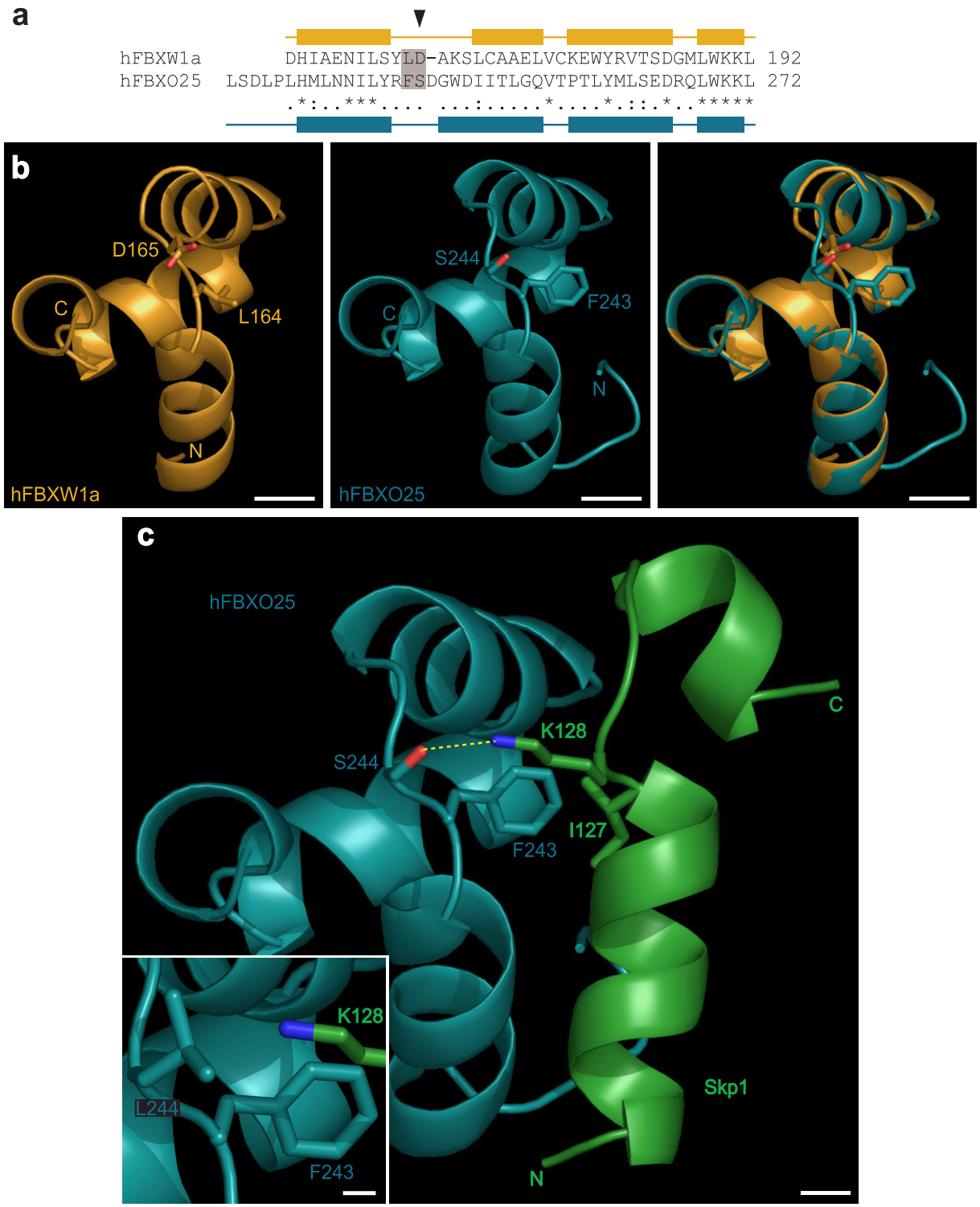
In silico analyses confirmed the presence of an F-box and co-IP experiments showed binding between hFBXO25 and Skp1, Cul1 and Roc1, establishing hFBXO25 as a *bona fide* FBP, which is part of the $SCF^{hFBXO25}$ E3 Ubiquitin ligase complex. Another set of co-IPs showed the functionality of this complex, which we found to be dependent on an uncommon serine residue at position 244 within the F-box. The fact that exchanging this serine for a much more prevalent leucine residue abolished the hFBXO25 – Skp1 binding is surprising as even conservative leucine-to-alanine F-box mutants do not longer bind Skp1[610,765]. A plausible reason for the latter observation could be that the leucine residue contacts Skp1 I127 (Fig. I-7c)[641,643] and contributes to packing of the F-box helices[643]. The rationale for the serine-to-leucine exchange stemmed from the multiple sequence alignment shown in Fig. III-16a. However, structural non-equivalence could possibly explain the unexpected importance of hFBXO25 S244 in the F-box – Skp1 interaction. Automated structural homology modelling of the hFBXO25 F-box yielded the hFBXW1a F-box as the most similar structure; after manual refinement, both structures could be superposed with a root mean SD of 0.52 Å for 152 common backbone atoms, which is well within the error of resolution. This analysis supports the idea of structural non-equivalence: hFBXO25 S244 seems to be equivalent with hFBXW1a D165 rather than with L164, which itself appears to correspond to hFBXO25 F243 (Figs. IV-2a – b). hFBXW1a L164 contacts Skp1 I127 (Fig. I-7c)[641], a role that may be

Fig. IV-2 | *Next page.* **hFBXO25 S244 is structurally equivalent with hFBXW1a D165.**

a. Structure-based sequence alignment comparing the F-boxes of hFBXW1a and hFBXO25. Rectangles represent helices. An asterisk marks identities, a colon conserved substitutions and a dot semi-conserved substitutions. Those residues highlighted in panels b and c are shaded in grey. An arrowhead indicates hFBXO25 S244. Note the difference around position S244 when comparing with the multiple sequence alignment shown in Fig. III-16a (S244 ≡ D165 vs. S244 ≡ L164). Colour coding corresponds to the one used in panels b and c.

b. The left panel shows a rendering of the X-ray crystal structure of the human hFBXW1a F-box (bright orange), as reported by Wu *et al.*[641]. The middle panel is a model of the hFBXO25 F-box (deep teal), based on the hFBXW1a structure. The extent of homology between both structures can be appreciated from the overlay shown in the right panel. The overlay also illustrates the structural equivalence of hFBXO25 F243 and S244 with hFBXW1a L164 and D165, respectively. Side chain atoms, red (oxygen) and blue (nitrogen). N- and C-termini are indicated (N and C, respectively); scale bars, 5 Å.

c. Possible hydrogen bonding (dashed line) between hFBXO25 S244 (deep teal) and Skp1 K128 (forest) is upset in a hFBXO25 S244L mutant (inset). A hFBXO25 F243 – Skp1 I127 interaction may correspond to the reported hFBXW1a L164 – Skp1 I127 interaction (Fig. I-7c). Relative position of Skp1 based on the hFBXW1a – Skp1 crystal structure[641]. Side chain atoms, red (oxygen) and blue (nitrogen). N- and C-termini are indicated (N and C, respectively); scale bars, 2 Å (main) and 1.5 Å (inset).



mirrored by hFBXO25 F243 rather than S244. The modelled structure also offers an insight in the molecular environment of the latter. It is plausible that its electronegative hydroxyl group interacts with the positively charged NH_3^+ of Skp1 K128, possibly through means of a hydrogen bond (Fig. IV-2c). The distance between the S244 hydroxyl and K128 ammonium groups is in good agreement with that published for hydrogen bonding between such groups[925]. An alternative idea would be that the S244 hydroxyl group acquires a negative

charge through phosphorylation that would interact with Skp1 K128. However, this scenario is unlikely for two reasons. First, owing to steric hindrance, addition of a phosphate group, which adds a Van der Waals volume of ~51 $Å^3$ (calculated according to Zhao *et al.*[926]), on S244 is unlikely. Second, the structurally equivalent hFBXW1a D165 could, similar to S244, interact with Skp1 K128 through its electronegativity but cannot be phosphorylated. In any case, the S244L mutant F-box loses its ability to interact with Skp1 K128 (Fig. IV-2c, inset), which may contribute to elimination of the hFBXO25 – Skp1 binding.

D.2. Brain-expressed hKIAA1202 colocalises with filamentous Actin at cellular sites of rapid Actin remodelling and is able to direct its subcellular distribution

hKIAA1202 is expressed in foetal and adult brain. hKIAA1202-V5 has the ability to redirect F-actin's subcellular localisation and the endogenous protein co-localises with F-actin at sites of rapid Actin remodelling.

The X-chromosomal BP of the t(X;8) translocation disrupts the uncharacterised *hKIAA1202* gene, which was initially recovered in a large screen for long ORFs expressed in human adult brain[757]. Alternative splicing at the *hKIAA1202* locus results in several transcripts. Interestingly, we recovered a considerable number of scrambled transcripts (Table III-7). Exon scrambling has been reported for six genes[767,768,927-931] and, even though possible mechanisms have been described[932,933], it remains controversial[934]. Several mechanisms, outlined in Table IV-1, could result in artifactual disruption of sequence linearity. Although none of these mechanisms played a role in our experiments (Table IV-1), more extensive studies are required to validate *hKIAA1202* exon scrambling.

Northern hybridisations indicated ubiquitous *hKIAA1202* expression, including transcription in foetal and adult brain. Isoforms I – IV are expressed in fibroblasts but not in lymphoblastoid cell lines. This is unfortunate, as only a lymphoblastoid cell line of the patient is available and, hence, the absence of those *hKIAA1202* transcripts disrupted by the translocation could not be verified experimentally. Several observations indicate very low levels of *Kiaa1202* expression. First, *hKIAA1202* signals on the northern hybridisation presented in Fig. III-21a needed ~10-fold longer exposures as compared to the *β-actin* signals on the same membranes. Second, with regard to conventional protocols, a ~50-fold larger amount of total RNA was used in *hKIAA1202* RT reactions[935]. Third, databases of human spliced ESTs contain ~13 times more entries for *hFBXO25* than for *hKIAA1202* (145 vs. 11). Fourth, PCR reactions with up to 50 amplification cycles were necessary to amplify *hKIAA1202* from cDNA

preparations. In addition, while *mFbxo25* was linearly amplified up to 27 cycles (see III.B.1.2.2.2), *mKiaa1202* amplification only plateaued after 31 cycles (see III.C.1.2.2). Fifth, *mKiaa1202* ISH on E14.5 sections never yielded a specific signal, despite intense efforts as outlined under III.C.1.2.2. This is surprising as at least one of the *mKiaa1202* probes yielded distinct signals in northern hybridisations and RT-PCR studies show *mKiaa1202* expression from E8.5 – E16.5[199]. Taken together, it is unlikely that the absence of specific *mKiaa1202* hybridisation signals can be attributed to technical problems. Rather, we believe it to be a consequence of *mKiaa1202*'s low levels of expression.

At the cellular level, over-expressed hKIAA1202 ORF I localised in a punctuated pattern at the cytosolic side of the cell membrane, a pattern distinctly different from that observed with an antibody raised against an antigenic hKIAA1202 peptide and presumably recognising endogenous hKIAA1202. Endogenous hKIAA1202 also localised to the neurites of neuronal cells and to the leading edge of fibroblasts, which are both sites of rapid Actin remodelling[936].

Based on computational analyses, it turned out that *hKIAA1202* encodes one of four founding members of the novel Shrm protein family[200]. As outlined below, several members of this family are cytoskeletal proteins interacting with Actin. Due to its connection with Shrm family members and to the cytoskeletal localisation of over-expressed ORF I, we investigated the possibility of a hKIAA1202 – F-actin interaction. Several lines of evidence suggest that an *in vivo* interaction between hKIAA1202 and F-actin indeed exists:

- Over-expressed hKIAA1202 partially co-localises with F-actin.
- Upon disruption of F-actin, hKIAA1202-V5 expression significantly overlaps with the localisation of F-actin remnants.
- Endogenous hKIAA1202 co-localises with F-actin at sites of rapid Actin remodelling.
- hKIAA1202 is able to direct the subcellular distribution of F-actin.

Preliminary *in vitro* results support an interaction between hKIAA1202 and the F-actin cytoskeleton.

In collaboration with Dr. U. Stelzl at the MDC in Berlin, a Y2H screen with clones approximately encompassing half of the hKIAA1202 ORF uncovered other possible binding partners. In light of hKIAA1202's connection to the cytoskeleton, we considered the intermediate filament protein Vimentin, which formed a high-confidence interaction with

Table IV-1 | **Mechanisms possibly generating artificial exon scrambling and their applicability in explaining putative *hKIAA1202* exon scrambling**

Cells → Cell culture → RNA isolation → Reverse transcription → PCR → TA-ligation in vector → Transformation → DNA sequencing → Sequence alignment

Experimental procedure	**Mechanisms possibly generating artificial exon scrambling (Ref.)**	**Explanation for putative *hKIAA1202* exon scrambling?**
Cell culturing	Illegitimate recombination in the cell.	No. Non-consensus splicing also occurs in tissue.
RNA isolation	-	NA
Reverse transcription	Reverse transcriptase has been shown to promote homologous recombination by template-switching during RNA synthesis[937].	No. (i) Only homologous recombination is detected, requiring 900 bp of homology between template molecules. No such homology within *hKIAA1202*. (ii) RNAseH$^-$ reverse transcriptases, such as the one used in this study, cannot promote recombination.
PCR	Template-switching during PCR has been shown in the absence[938] and presence[939] of DNA damage.	No. (i) Homology between template molecules required. No such homology within *hKIAA1202*. (ii) Insertion of adenosine residues occurs at sites of template jumping. Such illegitimate insertions were never observed in *hKIAA1202* amplicons. (iii) Template jumping occurs at sites of DNA damage. Freshly prepared cDNA is not damaged.
TA-ligation in vector	Formation of concatenates.	No. Only unique sequences were recovered, no concatenates.
Transformation	Bacterial recombination.	No. Sequence of PCR products is identical to that of plasmids.
DNA sequencing	-	NA
Sequence alignment	Computational misalignment may generate *in silico* exon scrambling.	No. All alignments were inspected manually.

hKIAA1202 in the Y2H screen, an interesting candidate for further analyses. Pilot experiments, including co-localisation studies and co-IPs, indeed suggested an interaction between hKIAA1202 and Vimentin. In addition to the putative interaction partners listed in Table III-12, auto-activation of one of the Y2H constructs, co-IP experiments and cross-linking studies indicated the presence of an oligomerisation domain in hKIAA1202.

E. The Ubiquitin – proteasome pathway is important in brain function, but involvement of hFBXO25 in mental retardation is unlikely

Not surprisingly, aberration of a cellular system as widespread and as important as the regulated breakdown of proteins, which involves their modification with Ubiquitin and subsequent proteasomal degradation, has been implicated in the pathogenesis of many diseases[577,591,940,941]. These include neurodegenerative disorders and a range of malignancies. Although initial molecular characterisation of *hFBXO25* and its transcripts supports a role for it in neuronal tissues, an involvement in mental ability is improbable.

E.1. The Ubiquitin – proteasome pathway in neurodegenerative disease and mental retardation

Anomalies in the UPP underlie multiple neurodegenerative disorders. Mutations in enzymes and their substrates cause PD, a frameshift in the Ubiquitin transcript is selectively observed in the brains of AD patients, and the proteasome degrades mutant forms of poly-glutamine proteins slower than the WT variant and these aggregate in inclusion bodies, which contain accumulated Ubiquitin conjugates. Only a few links between neurodegeneration and MR involving the UPP have been reported.

PD (OMIM 168600), first described by James Parkinson (1755 – 1824) in 1817[942], is one of the most common neurologic disorders, with an incidence of 1 in 500 and affecting approximately 1% of the population over age 50[943]. The disorder is characterised by progressive deterioration of dopaminergic neurons in the substantia nigra[944]. Symptoms include tremor, muscle rigidity, bradykinesia and slow speech.

Mutations in *PARK2,* the gene encoding Parkin, appear to be responsible for the pathogenesis of AR-JP (OMIM 600116)[945], one of the most common familial forms of PD. Strikingly, AR-JP is characterised by the absence of Lewy bodies[946], the neuropathological hallmark of idiopathic PD. Parkin is a component of the SCF^{hFBXW7}-like E3 complex composed of Cullin 1 and the FBP hFBXW7. Skp1 is not required for E3 activity in this complex. *hFBXW7* co-expression specifically potentiates Parkin's (auto-)ubiquitinylation activity[763],

and mutations recovered in AR-JP patients abolish the Ubiquitin-protein ligase activity[947]. Cyclin E, a regulatory subunit of CDK2[948], is a substrate of SCF^{hFBXW7} [763]. Accumulation of Cyclin E has been implicated in the regulation of apoptosis in post-mitotic neurons[949,950] and hence, AR-JP-associated Parkin mutations may also cause neuronal loss because of increased Cyclin E-mediated apoptosis. Another possible mechanism leading to AR-JP is suggested by the observation that CDCrel-1, an SV-associated protein[951], is also a target of the Parkin E3 complex[952]. Over-expression of CDCrel-1 in the substantia nigra of rats resulted in a progressive loss of dopaminergic neurons followed by a decline of dopamine levels. In cell culture, it inhibited dopamine release[953]. Together, these results suggest that misregulation of CDCrel-1 levels may contribute to the development of AR-JP.

Mutations in α-synuclein[954], a protein that may regulate dopamine release[955], are implicated in PD[956]. α-synuclein is a major component of Lewy bodies[957]. It is degraded by the proteasome, whereby degradation of mutant protein is about half as slow as that of the WT form[958]. Interestingly, Parkin ubiquitinylates Synphilin1, which interacts with α-synuclein[959].

Mutations in *UCH-L1*, which encodes C-terminal hydrolase-L1 responsible for degrading poly-ubiquitin chains back to Ubiquitin monomers[960,961], underlie a familial form of PD. The mutant protein is impaired in its enzymatic function, which may result in accumulation of proteins that become toxic to the cell[962]. It has been proposed that certain UCH-L1 variants have a protective effect in the pathogenesis of PD[963], and Liu *et al.* provided evidence that UCH-L1 exhibits a dimerisation-dependent Ubiquitin ligase activity which correlates to PD susceptibility[964]. UCH-L1 is neuron-specific[965,966] and has been shown to be a constituent of Lewy bodies in some PD patients[967]. In mice, mutated UCH-L1 leads to gracile axonal dystrophy, an autosomal recessive condition that manifests with sensory ataxia at an early age and motor ataxia at a later stage. These mice also show axonal degeneration and Ubiquitin-positive aggregates in the gracile neurons[968,969].

AD (OMIM 104300) is by far the most common cause of dementia and usually has an onset after age 65, although early onset AD is not uncommon. The prevalence of the disease increases with age. In approximately 20% of cases, the first signs are personality changes, such as withdrawal and apathy, or agitation and suspiciousness. The common symptoms in all cases are the gradual decline in intellectual ability and memory function, with a loss of recent memories and retention of older ones.

A direct connection between AD and Ubiquitin-dependent degradation was identified with the recovery of a frameshift mutation in the Ubiquitin transcript, termed Ub(+1), that is

selectively observed in the brains of AD patients[970]. Although the mutated Ubiquitin molecules are efficient substrates for poly-ubiquitinylation, the resulting poly-Ubiquitin chains are resistant to disassembly by DUBs, potentially hampering the Ubiquitin – proteasome machinery[971].

Expansion of CAG repeats, which encode glutamine residues, in the *ataxin1 – 3*, *huntingtin* and *androgen receptor* genes is known to be causative for spinocerebellar ataxias 1 – 3 (OMIM 164400, 183090 and 109150, respectively)[972-974], HD (OMIM 143100)[975] and spinobulbar muscular atrophy (OMIM 313200)[976], respectively. The poly-glutamine-containing proteins aggregate in inclusion bodies, which have been found to enclose accumulated Ubiquitin conjugates and/or are associated with Ubiquitin and proteasomal subunits[977,978]. These aggregates impair the functioning of the proteasome system[979] and poly-glutamine-expanded N-terminal Huntingtin fragments induce apoptosis[980].

Although WT (2 Qs) and mutant (92 Qs) poly-glutamine Ataxin1 are equally well ubiquitinylated, the mutant form is degraded slower *in vitro* than the WT, possibly underlying its accumulation and subsequent aggregation[981]. This may hold true for Huntingtin as well, as it, too, is likely targeted by the Ubiquitin system[583].

Although neurodegenerative disorders and MR are both pathologies affecting the brain, they are two distinct clinical entities, as emphasised by their different ages of onset. Still, a few connections between neurodegeneration and MR at the molecular level have been demonstrated. The Ub(+1) Ubiquitin transcript that was observed in AD brains was also found in the brains of patients with Down syndrome[970]. Down syndrome represents the most frequent genetic cause of MR. The poly-glutamine stretches in Huntingtin, the Androgen receptor[240] and Ataxin1[238] were shown to interact with PQBP1, an important player in XLMR[58]. These interactions link late-onset neurodegeneration with MR.

E.2. The Ubiquitin – proteasome pathway in synaptic function and mental retardation

The UPP regulates protein levels at the synapse, effecting its growth, function and connectivity. Degradation of MR proteins at the synapse and mutations in E3s important in synaptic function have been reported.

Accurate interconnecting of developing neurons is a prerequisite for correct brain function. Growth cones, flat structures at the tip of outgrowing neurites, convert environmental cues into a direction of growth and, upon reaching their targets, form the synaptic

terminal. Neuronal activity then shapes the synapse and determines its use; for example, high-frequency stimulation of afferent pathways results in LTP. Since growth cones and synaptic terminals are distant from their cell bodies, local mechanisms that control growth cone behaviour and synaptic development are of specific significance in regulating synaptic function. A plethora of studies have established the Ubiquitin pathway as one such local mechanism[982,983]; extensive research has shown that the UPP is required for regulation of protein levels at the synapse, effecting its growth, function and connectivity.

Measuring across the *Drosophila* neuromuscular junction, DiAntonio and colleagues found that over-expression of the DUB Faf[984] in the developing nervous system led to synaptic overgrowth, as well as to impaired synaptic transmission[985]. Loss-of-function alleles of *hiw*, a gene regulating synaptic growth in *Drosophila*[986], enhance a *faf* over-expressing genetic background to lethality. Hiw is a RING finger protein, which may be an indication that it is an E3 Ubiquitin ligase[987]. The reciprocal interactions between a Ubiquitin ligase and a DUB indicate that balanced ubiquitinylation is needed to regulate synaptic growth.

Also employing the *Drosophila* neuromuscular junction as a model system, Speese *et al.* showed that blocking the proteasome resulted in an increase of about 50% in excitatory junctional current amplitudes[988]. In *Aplysia*, the UPP has been demonstrated to regulate levels of the TF C/EBP, which is required for the induction of synaptic plasticity[989]. The DUB Ap-uch underlies long-term facilitation[990], the non-associative counterpart of LTP, and is another link between the Ubiquitin pathway and synaptic function in *Aplysia*. But also in vertebrates, synaptic functionality relies on concerted protein degradation. In rat hippocampal synapses, the transition from short-term potentiation to LTP requires a functional Ubiquitin degradation pathway[991].

Cloning and characterisation of the *Drosophila* mutant *bendless*, which affects neuronal connectivity and growth cone physiology and encodes a protein homologous to the Ubiquitin-conjugating enzymes, suggested that targeted protein degradation regulates neural processes such as axon guidance[992,993]. Campbell and Holt showed that Netrin 1[a] and lysophosphatidic acid, two extracellular molecules that act as guidance cues[996,997], induce ubiquitinylation in the growth cone and blocking of the proteasome affects the growth cones' response to both of these cues[998]. Therefore, establishment of proper neuronal connectivity relies on the rapid ubiquitinylation and degradation of target molecules.

[a] *NetrinG1*, proposed as a candidate disease gene for Rett syndrome (OMIM 312750) [994], a progressive neurodevelopmental disorder that is a leading cause of neurological dysfunction in females, is functionally divergent from other Netrins [995].

The proper development and function of synapses in the brain, which is dependent on proteasomal protein breakdown as outlined above, is a condition *sine qua non* for good mental health. Thus, it should not come as a surprise that some proteins involved in mental ability have been linked to the UPP.

Several lines of evidence suggest that the protein kinase LIMK1 is involved in modulation of axon formation and dendritogenesis through Actin reorganisation triggered by extracellular signals[315-319]. *LIMK1* hemizygosity has been proposed as a cause for the severely impaired visuospatial construction characteristic of WBS[312], a developmental disorder featuring general cognitive disability but adequate performance in face processing, and relatively unaffected language abilities[999,1000]. Tursun *et al.* found that the Ubiquitin ligase Rnf6 binds to, poly-ubiquitinylates and targets LIMK1 for proteasomal degradation in growth cones of primary hippocampal neurons. Their findings reveal a function for the UPP in regulating local growth cone Actin dynamics[1001].

Mutations in the *MID1* gene have been shown to underlie the X-linked form of Opitz syndrome (OMIM 300000)[388], which is characterised by midline abnormalities such as hypertelorism, hypospadias, cleft lip, heart defects and agenesis of the corpus callosum. Between 40 and 50% of these patients are developmentally delayed[1002,1003]. MID1 is a Ubiquitin ligase that targets Protein phosphatase 2A for proteasomal degradation[1004,1005] by binding α4, its regulatory subunit[1006].

Mutations in the imprinted and brain-expressed *UBE3A* gene[1007], mapping to the Angelman's syndrome critical region on 15q11-q13[1008], have been implicated in Angelman's syndrome (OMIM 105830)[1009,1010], a severe form of mental and motor retardation with seizures, inappropriate smiling and laughter, and abnormal gait. The gene encodes the HECT-type E3 ligase E6-AP. Generation of an *mUbe3a* KO showed that E6-AP is crucial for long-term synaptic plasticity[1011].

Two other possible links between the UPP and MR have been reported, namely the degradation of Synaptophysin and Fmr2. Synaptophysin is an integral membrane protein of SVs[1012], which has been implicated in neurotransmitter release[1013,1014], synaptic plasticity[1015] and LTP[1016]. Wheeler *et al.* demonstrated that Siah-1a and Siah-2, homologues of the *Drosophila* E3 ligase Seven in Absentia[1017], associate with Synaptophysin, facilitate its ubiquitinylation and promote its proteasomal degradation[1018]. In their search for the cause of neurodegeneration in the robotic mouse, a mouse model of autosomal dominant cerebellar ataxia due to mutated Af4 characterised by ataxia and adult-onset Purkinje cell loss[1019], Oliver and

colleagues found that Af4 and Fmr2, another member of the ALF family of proline- and serine-rich proteins implied in transcriptional regulation, are also degraded in a Siah-dependent fashion[1020]. Transcriptional silencing of *FMR2* results in mild MR[107,108] and study of the *Fmr2* KO mouse suggests a role for Fmr2 in regulation of synaptic plasticity[106].

For the sake of completeness, it should be noted that both Ubiquitin-independent and Ubiquitin-dependent, but proteasome-independent proteolysis are mechanisms which have recently been implied in (putative) aetiologies of MR.

Mutations in *PRSS12*, which encodes the Trypsin-like serine protease Neurotrypsin[1021], and in *CRBN*, which codes for the ATP-dependent lon protease Cereblon[87], cause autosomal MR[87,88], suggesting that Ubiquitin-independent proteolysis is required for normal synaptic function, memory and learning.

A large body of evidence shows that the cell uses mono-ubiquitinylation as a post-translational modification[575,983,1022,1023] to, for example, internalise membrane proteins by endocytosis[1024,1025] and sort them into early endosomes[1026,1027], resulting in their lysosomal degradation. In the nervous system, mono-ubiquitinylation tags the glutamate[1028] and glycine[1029] neurotransmitter receptors for internalisation and subsequent lysosomal proteolysis, connecting regulation of neurotransmitter signalling to proteasome-independent protein breakdown.

E.3. In pathology, F-box proteins are mainly linked to malignancies

In line with their function in cell cycle control, mutations in FBPs and aberrations in their regulation most frequently cause cancer.

To date, FBPs, the interchangeable components transferring substrate specificity to the SCF complexes, have been involved in muscle wasting (Fbxo32)[762,1030], Desmin-related myopathy (hFBXO4)[1031], cardiovascular development (mFbxw7)[1032] and the autosomal dominant disorder splithand/foot malformation (mFbxw4)[1033].

However, mutations in FBPs and aberrations in their regulation have been most frequently described in association with several different malignancies, which is in line with the principal function of SCF complexes in controlling cell proliferation through degradation of cyclins, CDKIs and TFs[574,592,594]. For example, misregulation and mutation of Fbxw7, which regulates cellular levels of Cyclin E[1034], have been implicated in breast[1035] and endometrial[1036] cancers, epithelial tumours[1037], and ovarian carcinomas[1034]. Another example is the observation that irregularities in levels of hFBXL1, which is required for degradation of

the CDKI p27[1038-1040], are associated with a large number of malignancies, such as acute myelogenous leukaemia[1041], lymphomas[1042,1043], cancer of the prostate[1044,1045] and colon[1046], soft tissue[1047] and Kaposi's sarcoma[1048], and laryngeal[1049] and oral[1050,1051] squamous cell carcinomas.

Only a single connection between an FBP and neurodegeneration has been reported. Mutations in the presenilin genes *PS1* and *PS2* have been linked to autosomal dominant, early onset AD[1052-1055]. These genes are involved in cleavage of the amyloid protein precursor to the amyloid β-peptide[1056-1059], which forms the main constituent of amyloid plaques, one of the pathological hallmarks of AD[1060]. Studies in *C. elegans* and human cell culture demonstrated an interaction between Fbxw7 and PS1, resulting in its enhanced ubiquitinylation and alteration of amyloid β-peptide production[1061,1062].

E.4. Involvement of hFBXO25 in mental retardation is unlikely

Due to its expression in the brain, it is possible that mutations in *hFBXO25* underlie certain neuronal phenotypes but involvement in MR seems improbable, as evidence linking FBPs to cognition is, at best, circumstantial.

The expression of *Fbxo25* in the brain, but especially the neuronal specificity of *mFbxo25* expression in embryonic mouse, suggests a role for *Fbxo25* in neuronal development.

Mizukami and colleagues have shown an altered Ubiquitin immunoreactivity in the rat hippocampus after severing the perforant path[1063], which connects the hippocampus with the entorhinal cortex and represents the main excitatory pathway to the hippocampus. Ubiquitin immunohistochemistry in cases of atypical Pick's disease (that is, without Pick inclusion bodies), one of the frontotemporal dementias in which this connection is damaged, revealed Ubiquitin-positive intraneuronal inclusions in the dentate gyrus and Ubiquitin-positive neurites in the cerebral cortex[1064]. Similar results were obtained in patients with atypical Pick's disease in whom the perforant path was not affected[1065]. In addition, the Ub(+1) frameshift mutation that has been observed in patients with AD and Down syndrome has very recently also been detected in the brains of patients with typical Pick's disease (that is, with Pick bodies)[1066]. The specific *mFbxo25* expression in adult brain, confined to the hippocampus, the cerebral cortex and the Purkinje cell layer, might point to an involvement of *hFBXO25*/*mFbxo25* in the function or establishment of the perforant path or in the aetiology of Pick's disease.

In addition, disruption of *hFBXO25* could be involved in the aetiology of our patient's epilepsy, as seizures have been documented to involve each of the brain areas in which we found *mFbxo25* expression. First, electrical stimulation of the perforant path in the rat, damaging the hippocampal CA1 and CA3 pyramidal cell layers and leading to a loss of certain subsets of interneurons, has been developed as an epilepsy model[895,1067-1069]. Second, a large percentage of intractable epilepsy is caused by malformations of the cerebral cortex[1070-1072]. And third, several different animal models[1073-1075], as well as studies in patients[1076,1077], have shown a loss of Purkinje cells due to seizure activity. Characterisation of a t(X;19) translocation disrupting the same X-chromosomal gene in an unrelated, mentally retarded patient who is not suffering from epileptic seizures (see IV.F.1) supports a putative involvement of hFBXO25 in epilepsy[759]. Although not formally tested, we assume that the t(X;8) patient has one WT copy of *hFBXO25* and, hence, her phenotype would arise from haploinsufficiency. However, at present, we cannot exclude that the difference in epileptic status seen between both translocation carriers may arise from individual variations in genetic background and/or environmental disparities. One way to test specific involvement of hFBXO25 in epilepsy would be to undertake *hFBXO25* mutation screening. Such a screen would be hampered considerably by the idiopathic nature of the patients' seizures and by *hFBXO25*'s autosomal location, requiring time- and cost-intensive linkage studies in order to group patients according to putatively affected loci.

To date none of the epilepsies has been linked to unidentified mutations in 8p22, the chromosomal region harbouring *hFBXO25*. Linkage to 8p22 has been reported for colorectal, prostate and breast cancer[1078-1081], ovarian adenocarcinoma[1082], schizophrenia[1083], depression spectrum disorder[1084], psychosis[1085], adult onset distal myopathy[1086], obesity[1087], end stage renal disease with increased body mass index[1088] and keratolytic winter erythema[1089,1090].

As outlined under IV.E.1 – IV.E.3, the evidence for involvement of FBPs in cognition is, at best, circumstantial. First, the UPP mostly seems to play a role in neurodegenerative disorders, which are clinically distinct from MR. Second, mainly substrates or invariant components of the pathway, such as E3s, DUBs and Ubiquitin itself, seem to be involved in human pathology. Third, when interchangeable components, such as the FBPs, are associated with disease, the vast majority of the evidence points to a role in cancer. Fourth, should FBPs be important in human cognition, it is surprising that no publication directly implicating an FBP in MR could be found, given the fact that, since the appearance of FBPs on the molecular scene ten years ago[610], ~70 human FBPs have been identified[645] and mental (dis)ability is one of the most researched topics in the life sciences.

Taken together, it is possible that mutations in the *hFBXO25* gene underlie (yet unresolved) neuronal phenotypes, including Pick's disease or certain forms of epilepsy, but involvement in MR seems improbable.

F. Several *hKIAA1202* patient-specific variants, its putative interaction partners, including Actin, and its domain structure suggest a function for it in human cognition

Several lines of evidence, such as the recovery of *hKIAA1202* sequence variants in mentally retarded patients and the identification of some of its interaction partners, support a role for *hKIAA1202* as a gene involved in cognition.

F.1. Patient-specific *hKIAA1202* variants

Mutation screening among mentally impaired individuals recovered a second translocation disrupting *hKIAA1202* and several patient-specific sequence variants.

In collaboration with the group of Dr. A. Hanauer (Strasbourg, France), a second mildly mentally retarded patient, who is not related to the t(X;8) patient described in this study but also carries a translocation that disrupts *hKIAA1202*, has been identified[199]. This patient carries an apparently balanced *de novo* 46,X,t(X;19)(p11.2;p13.3) translocation, which disrupts *hKIAA1202* in intron 2a strongly supporting a role for *hKIAA1202* in mental ability. Two genes are located in the BP region on chromosome 19, but it is unclear whether these genes are affected by the translocation.

The proband was examined at the age of 19. She was the daughter of healthy, Algerian parents who were first cousins, and had five healthy sisters and three healthy brothers, suggesting that the consanguinity of her parents was unlikely to be responsible for (part of) her phenotypic manifestations. There was no family history of MR or related illnesses. The patient had a history of intra-uterine growth delay with a birth weight of 2570 g (< 10^{th} percentile) and length of 45 cm (< 5^{th} percentile), and presented with short stature (139 cm at 15 years, < 3^{rd} percentile) and hypotrophic limbs since birth. Although it was not possible to formally assess her intellectual abilities, the attending physician stated that she displayed obvious evidence of mental disability. She attended a normal primary school with poor results until the age of 9, after which she was admitted to a special school until the age of 16. She had poor socialisation and needed some assistance to cope with the demands of everyday life. She had a poor vocabulary, poor comprehension and had difficulties articulating. Between

1999 and 2001, she underwent several hospitalisations due to a right cardiac ventricular dysplasia of unknown origin and died after heart transplantation in 2001. It should be noted that *hKIAA1202* is expressed at high levels in heart (Fig. III-21a).

There was no evidence for a cardiac defect in the t(X;8) patient (Dr. G. Barbi, personal communication). Apart from the epileptic seizures in the t(X;8) patient and the cardiac condition of the t(X;19) patient, the phenotypic differences between the two patients is relatively small and most likely arises from the different autosomal BPs or from more general disparities in genetic and/or environmental backgrounds.

Through collaborations with the Greenwood Genetic Center (Greenwood, USA), and the Euro-MRX Consortium (Paris and Tours, France; Leuven, Belgium; Nijmegen, The Netherlands; Adelaide, Australia and Berlin, Germany), a large patient panel and a substantial cohort of controls were available for *hKIAA1202* mutation screening. Several sequence alterations, summarised in Table IV-2, were discovered[199].

The novel silent change p.E474E (c.1422A>G) was found to segregate in family K8140, which consists of a mother and two affected boys[199]. This alteration was not detected in 1266 control X chromosomes (Table IV-2). The c.1422A>G transition could be a very rare polymorphism. Alternatively, this alteration may affect an ESE site. The RESCUE-ESE algorithm predicted the presence of at least six possible ESE hexamers involving the A at position 1422. A point mutation in one of these putative ESE sites could result in exon skipping[721], a possibility that needs pursuing (see V.A).

We found a missense exchange, c.4116G>T leading to p.L1372F, which is located in the ASD2 domain and affects a semi-conserved amino acid just N-terminal of a predicted leucine zipper motif, which itself is highly conserved in vertebrate Kiaa1202 homologues. The exchange was found in a Dutch family and in a family of Central American origin, but was absent in 488 control X chromosomes. In both instances, this transversion did not segregate perfectly with the disease. In the Dutch family, the putative mutation was found in two affected brothers, but also in a brother considered to be within the lower range of normal intelligence. In the family from Central America, four affected males, two brothers and two half-brothers, carried the exchange, and two obligate carrier females and the affected brothers' sister were heterozygous for the alteration. However, one affected cousin did not carry the exchange and the grandfather, never examined but reported to be unaffected, carried the transversion. The continuous nature of intelligence, the absence of the c.4116G>T exchange in controls and its occurrence in two unrelated families with a history of MR may warrant further follow-up. In-

complete penetrance could explain a shift from mild MR to the lower ranges of normal intelligence. A very similar situation has been reported in which a c.515C>A exchange in the *TM4SF2* gene, leading to p.P172H, was found in affected members of two unrelated MR families. The substitution was not recovered in control chromosomes, but it was also present in a presumably unaffected carrier of one of the families[137]. The fact that the supposedly unaffected individual[136] was considered to be of borderline intelligence in a previous publication[134] supports the idea of incomplete penetrance shifting intelligence across the artificial MR threshold. In addition, several examples of considerable phenotypic variation within MR families have been reported[58,921]. Modifier loci and genetic interactions are two models that can explain penetrance levels and phenotypic variability of sequence variants[924,1091,1092]. Indeed, genetic modifiers have been reported for autism[553], and the Fragile X[920] and Prader-Willi[922] syndromes, which include a delay in mental development.

Of particular interest, a c.3266C>T transition, which leads to a p.S1089L missense exchange, was recovered. The substituted serine residue is located 10 amino acids N-terminal of an EVH1-BS and resides in a stretch of 60 residues that is 83% identical to the mouse and rat hKIAA1202 homologues. The c.3266C>T exchange segregated with the clinical manifestations of the Stocco dos Santos XLMR syndrome observed in a large four-generation Brazilian family of Spanish – Portuguese descent (Fig. IV-3a), in which the linkage interval included the *hKIAA1202* gene. Linkage was observed at the c.3266C>T substitution with a LOD score of 3.02 at $\theta = 0$. The c.3266C>T sequence alteration was absent in > 1000 control X chromosomes of different ethnic backgrounds[199]. All family members investigated had normal karyotypes and tested negative for the Fragile X syndrome. Among other phenotypic characteristics, the affected males in the Stocco dos Santos family (Fig. IV-3b) presented with severe MR, growth retardation, delayed or no speech, (very frequent) seizures, hyperactivity and abnormal serotonin levels. Their mothers' serotonin levels were also outside the normal range and they too experienced seizures and periods of depression[1093,1094]. A large number of spontaneous abortions in carrier females and a high frequency of mortality among boys, who were reported to have similar appearances to the patients, within the first year of life are apparent from the pedigree shown in Fig. IV-3a[1093]. Unfortunately, no significant structural homologies are present in the PDB for hKIAA1202, precluding molecular modelling of the p.L1372F and p.S1089L exchanges.

Table IV-2 | **Overview of sequence alterations in *hKIAA1202***

ID[§]	DNA variant[†]	Amino acid variant[†]	Method of detection	Patients[‡]	Control chromo-somes[‡, ¿]	Novel/Known[¶] Comment
1	**c.270-8268(AC)$_{31}$**	Intron 2a	Direct se-quencing	1/21 (T50)	0/46	Novel
2	c.1422A>G	p.E474E	SSCP	1/220 (K8140)	0/1266	Novel Putative disease-causing (SNP ss35032395)
3	**c.2957+21T>C**	3' of exon 5	DHPLC	2/220 (MRX45, N61)	2/46	Novel (SNP ss35032396)
4	c.3266C>T	p.S1089L	SSCP	1/220 (K8885)	0/1021	Novel Putative disease-causing Stocco dos Santos XLMR syndrome
5	**c.3426A>G**	p.E1142E	Direct se-quencing	1/45 (N61)	0/29	Known (SNP rs6614552)
6	...(CAG)$_3$(CAA)...(GAG)$_6$...(GAG)$_6$	...Q$_4$...E$_6$...E$_6$	Direct se-quencing	1/45 (P15)	0/29	Novel
7	**...(CAG)$_3$(CAA)...(GAG)$_7$...(GAG)$_6$**	...Q$_4$...E$_7$...E$_6$	Direct se-quencing	14/45 (MRX17/26/31/45, N39, T50, WTS, SHS, HA, P5/6/12/13/19)	4/29	Novel
8	**... ---------- ...(GAG)$_6$...(GAG)$_3$(GAA)(GAG)$_2$**	...---- ...E$_6$...E$_6$	Direct se-quencing	29/45 (MRX1/12/15/44/65, N9/17/40/42/45, T3/40, L38, D2, 411-99, 4291, 842-02, P1 – 4/7 – 11/16 – 18)	25/29	Novel
9	**...(CAG)$_3$(CAA)...(GAG)$_7$...(GAG)$_3$(GAA)(GAG)$_2$**	...Q$_4$...E$_7$...E$_6$	Direct se-quencing	1/45 (N61)	0/29	Novel

Table IV-2 | **Overview of sequence alterations in *hKIAA1202***

ID[§]	DNA variant[†]	Amino acid variant[†]	Method of detection	Patients[‡]	Control chromo-somes[‡, ¿]	Novel/Known[¶] Comment
10	c.3483G>A	p.E1161E	SSCP	6/220 (K8360, K8600, K8923, K8745, K8949, K9008)	ND	Known (SNP rs3747282)
11	c.3749C>T	p.S1250L	SSCP	1/220 (K8949)	0/200 54/181	Known (SNP rs12689863)
12	**c.4116G>T**	p.L1372F	SSCP	2/220 (42/98, K8440)	0/488	Novel No full segregation with the disease within both probands' families (SNP ss35032397)
13	**c.*333G>A**	3'UTR	DHPLC	23/43 (MRX44, N9/42/45, T3/40, K8140, K8440, K8600, K8949, K8956, K8964, K9008, K8065, K8070, K8835, K8360, K8900, K8820, K8885, K8745, K8923, K8825)	ND	Novel (SNP ss35032398)
14	**$(AAAG)_7$...$(AAAG)_5$**	3'UTR	Direct se-quencing	ND	58/83	Novel
15	**$(AAAG)_7$...$(AAAG)_6$**	3'UTR	Direct se-quencing	ND	1/83	Novel
16	**$(AAAG)_8$...$(AAAG)_5$**	3'UTR	Direct se-quencing	ND	20/83	Novel
17	**$(AAAG)_9$...$(AAAG)_5$**	3'UTR	Direct se-quencing	ND	3/83	Novel
18	**$(AAAG)_{14}$...$(AAAG)_5$**	3'UTR	Direct se-quencing	ND	1/83	Novel

Table IV-2 | **Overview of sequence alterations in *hKIAA1202***

ID[§]	DNA variant[†]	Amino acid variant[†]	Method of detection	Patients[‡]	Control chromo-somes[‡, ¿]	Novel/Known[¶] Comment
19	**$(AAAG)_{14}$...$(AAAG)_4$ [c.*1408$(AAAG)_4$]**	3'UTR	Direct se-quencing	1/21 (N61)	0/83	Novel Putative disease-causing
20	**46,X,t(X;8)(p11.2;p22.3)**	NA	Positional cloning	NA	NA	Novel Putative disease-causing
21	46,X,t(X;19)(p11.2;p13.3)	NA	Positional cloning	NA	NA	Novel Putative disease-causing

[§] Identifiers correspond to those in Fig. III-20b.
[†] Sequence alterations found in this study are in bold face. Where applicable, nomenclature as proposed in den Dunnen and Antonarakis[755]. Also see http://www.hgvs.org/mutnomen/.
[‡] For those variants also investigated by collaborators, the total number of patients and control chromosomes screened, is given.
[¿] Absence of the sequence alteration is presented with regard to the number of chromosomes tested, presence of the alteration is listed as a number of individuals.
[¶] In case of novel sequence variations, SNP identifiers are provided as published in Hagens *et al.*[199].

An intriguing issue is the relatively mild phenotype in both translocation patients compared to that of the affected Stocco dos Santos family members. Several observations could explain such phenotypic variation.

An XCI assay showed that one X-chromosome is inactivated in 95% [t(X;8) patient] and 89% [t(X;19) patient] of cultured lymphoblastoids. This incomplete XCI in both translocation patients might explain their relatively mild phenotypes. Also, the XCI analysis was perfor-

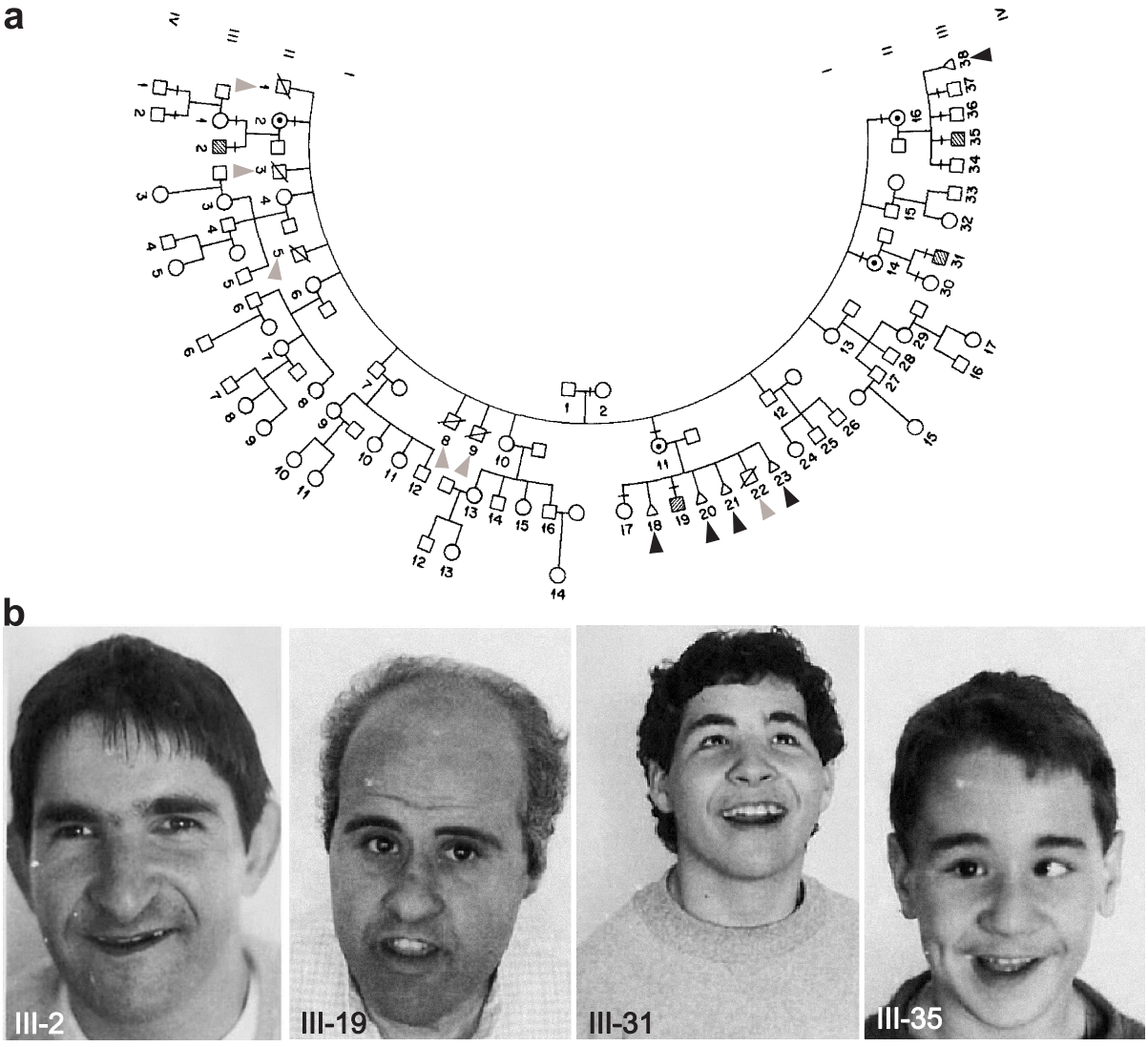


Fig. IV-3 | **Stocco dos Santos X-linked mental retardation syndrome.**

a. Pedigree of the Stocco dos Santos family. Note the high incidences of infant mortality (grey arrowheads) in the sibship of obligate carrier II-2 and miscarriages (black arrowheads) in the sibship of patient III-19.
b. Affected members of the Stocco dos Santos family at ages 28 (III-2), 30 (III-19), 16 (III-31) and 10 (III-35) years.

Diagram (a) adapted and pictures (b) taken from *1093*.

med in lymphoblastoid cell lines and the situation in the brain may be significantly different[1095-1097].

The hypothesis that *hKIAA1202* escapes XCI, another reason that may account for a difference in phenotypic expression, was tested in a fibroblast cell line from a female carrier of incontinentia pigmenti featuring skewed XCI and expressing a *hKIAA1202* polymorphism. The results indicated that *hKIAA1202* was subjected to XCI, at least in cultured fibroblasts[199], a finding that was later confirmed in a comprehensive XCI profile of the human X chromosome[24].

We cannot exclude the involvement of other factors, both genetic and environmental, in the phenotypic differences observed among *hKIAA1202* mutation carriers. Moreover, identical mutations in XLMR genes have been identified in patients with considerable clinical differences[58]. Also, the translocations may represent loss-of-function mutations, whereas the p.S1089L missense mutation could exert a dominant negative effect.

Alternatively, the p.S1089L exchange, despite not being identified in over 1000 control chromosomes, could represent a rare polymorphism that segregates independently from the Stocco dos Santos XLMR syndrome.

Still, the most likely explanation comes from the observation that certain uncharacterised hKIAA1202 isoforms are still expressed in a t(X;8) cell line, showing that not all *hKIAA1202* transcripts are affected by the translocation. However, since the p.S1089L exchange is located in exon 6, it seems reasonable to assume that not only the known transcripts are mutated, but that also some or all of the mRNAs transcribed from the unidentified promoter downstream of the translocation BPs, located in introns 1 and 2a, will be affected. XCI studies in peripheral blood of all obligate carrier women in the Stocco dos Santos family showed skewed inactivation in excess of 90:10, which at first was explained by a variability of the XCI pattern at the *hKIAA1202* locus during development or by tissue specificity of the XCI, as none of the known isoforms are expressed in blood[199]. However, the presence of putatively mutated *hKIAA1202* isoforms in lymphocytes offers the more straightforward explanation of secondary selection processes assuring maintenance of functional euploidy and resulting in preferential inactivation of the X chromosome expressing the deleterious mutation. Such mutations are often lethal to hemizygous male offspring who inherit them, resulting in increased frequencies of spontaneous abortion in carrier females[1098-1100]. Therefore, a putative *hKIAA1202* mutation might be the cause for the large numbers of spontaneous abortions and high rate of infant mortality in the Stocco dos Santos family. Possibly, a small remaining amount of mutant protein, owing to the incompleteness of XCI in the carriers, exerts its influ-

ence in a dominant negative way and leads to their moderate phenotype, which has been regarded as a reduced penetrance of the syndrome in these women[1094].

Although c.270-8268$(AC)_{31}$, ...$(CAG)_3(CAA)$...$(GAG)_6$...$(GAG)_6$, ...$(CAG)_3(CAA)$...$(GAG)_7$...$(GAG)_3(GAA)(GAG)_2$ and c.*1408$(AAAG)_4$ represent novel patient-specific variants of variable repeats, we did not investigate their putative role in MR as (i) differences to controls were small and (ii) many more controls would need to be screened to reach a degree of significance. However, it should be noted that repeat expansions in UTRs, such as c.*1408$(AAAG)_4$, have been shown to cause (neurodegenerative) disease[1101]. Presumably owing to the fact that interrupted trinucleotide repeats are stable[1102], we did not observe expansions of the imperfect CAG repeat in exon 6, which may have had the potential to impair cognition. Finally, involvement of sequence variations in MR that were also recovered in controls (c.2957+21T>C, c.3749C>T), represented established SNPs (c.3426A>G, c.3483G>A, c.3749C>T) and/or were present in a large number of patients (c.*333G>A), which is unusual for XLMR genes[70], is unlikely.

Taken together, some of the identified sequence alterations in *hKIAA1202*, especially the two translocations and the c.3266C>T exchange segregating with the Stocco dos Santos XLMR syndrome, suggest a role for *hKIAA1202* in human cognition.

F.2. Several putative hKIAA1202 binding partners link hKIAA1202 to possible mental retardation pathways

Y2H screening yielded hKIAA1202 interaction candidates that suggest its involvement in established MR pathways such as chromatin remodelling, transcriptional and translational control, and cytoskeletal rearrangement.

A screen for proteins interacting with hKIAA1202 suggested several candidate binding partners which may be involved in pathways that have emerged as contributing to (human) cognition, such as transcriptional regulation (through chromatin remodelling), Ubiquitin conjugation and cytoskeletal remodelling. Interestingly, all interaction candidates bind, though not exclusively, with the Y2H-4 construct. This part of the hKIAA1202 ORF also contains the putative oligomerisation domain. The most prominent feature of the Y2H-4 ORF is the presence of a CRR (PS50311) containing 21% Cys residues. CRRs are a very diverse class of motifs, represented in > 120 different entries in the Pfam database of common protein domains and families. They have been implicated in homo-dimerisation[1103-1105] and were also shown to be important in interactions between different proteins[1105-1110]. Finally, it is

worth noting that HTATIP, the catalytic subunit of the NuA4 histone acetyltransferase complex[806], which is one of the hKIAA1202 candidate interaction partners (Table III-12), specifically interacts with the CRR of the HIV Tat protein[1111], supporting the idea that hKIAA1202's CRR may be involved in its binding to putative binding partners.

The Group I interaction candidates regulate transcription through histone modification and/or chromatin remodelling. As outlined under I.A.4.4, chromatin remodelling is a well-established pathway involved in the aetiology of MR. Given hKIAA1202's association with the F-actin cytoskeleton, it may be surprising at first that it would interact with several nuclear proteins. However, from the subcellular localisation of endogenous hKIAA1202, it is apparent that hKIAA1202 is not exclusively located at the cell membrane, but rather shows a more general distribution, including some nuclear staining. A predicted bipartite NLS (PS00015)[1112] supports further a putative function for hKIAA1202 in the nucleus.

In light of the hKIAA1202 – Actin interaction, it is interesting that Ikura *et al.* found that the NuA4 histone acetyltransferase complex, which contains the possible Group I hKIAA1202 binding partner HTATIP (Table III-12), also includes β-actin as a structural component[806]. Furthermore, β-actin localisation at the leading edge of cells is important physiologically, as upsetting this localisation in fibroblasts leads to a decrease in cell motility[1113]. These combined observations might link hKIAA1202's localisation at the leading edge in fibroblasts to its presence in the nucleus.

The Group II interaction candidates are involved in post-translational modification through a Ubiquitin or Ubiquitin-like conjugation pathway. As described under I.A.4.6, regulation of targeted protein degradation has been shown to be important in neurodegenerative disorders and MR.

Of particular interest is the putative interaction of hKIAA1202 with the Ubiquitin-like SUMO (Table III-12). Several different functions for SUMOylation have been discovered[817,1114]. For example, through binding to lysine residues that can be ubiquitinylated, SUMO has been identified as a Ubiquitin antagonist which stabilises its target. Such an interplay between Ubiquitin and SUMO has been reported to regulate Huntingtin stability, thereby affecting the degree of neurodegeneration in a *Drosophila* model for HD[1115]. Another apparent function for SUMOylation is its role in regulating subcellular localisation. SUMO-dependent localisation has been described for several proteins[1116-1118]. Moreover, proteins in the cytoplasm are modified with SUMO, but experiments have shown that nuclear targeting is often required for SUMOylation to occur[1119]. This may be important with regard to

hKIAA1202's localisation in both the cytoplasm and the nucleus. Within the nucleus, SUMO has been identified as a modulator of chromatin structure through modification of histone tails[408,1120] and TFs[1121-1124], which, like the Group I putative binding partners, links hKIAA1202 to regulation of transcription.

Group III interaction candidates represent proteins of unknown function. One of them, hKIAA1377 (Table III-12), has been shown to interact with Huntingtin[818], possibly linking hKIAA1202 to neurodegeneration.

Group IV interaction candidates consist of proteins with different functions. Several of them represent interesting putative hKIAA1202 binding partners.

As already mentioned under III.C.3.4.3.4, Vimentin, an intermediate filament, is particularly interesting as a binding candidate given the association of hKIAA1202 with the cytoskeleton. In addition, Vimentin has been shown to be important in the early stages of neuritogenesis[801,802,1125] and over-expression of Vimentin in differentiated neuroblastoma cells enhances elongation of axonal neurites[1126]. Preliminary experiments have shown partial co-localisation with Vimentin, and co-IP experiments suggest an interaction between Vimentin and hKIAA1202, although these results need to be confirmed.

A possible binding of hKIAA1202 to the Importin α1 subunit, which functions as an adaptor protein in nuclear protein import[820], would be consistent with hKIAA1202's nuclear localisation. As shown in Fig. III-20c, hKIAA1202 contains a predicted bipartite NLS. The α1 subunit of Importin has been demonstrated to bind such a signal[819,820].

Although the physiological significance of EF-1γ remains elusive, it has been proposed that it organises the EF-1 subunits[1127]. Furthermore, GST activity of recombinant rice EF-1γ has been documented, but the function of this enzymatic activity needs elucidating[1128]. As many different functions for EF-1 have been documented[823], putative binding of hKIAA1202 to its γ subunit is intriguing (Table III-12). EF-1 facilitates translation by delivering aminoacyl-tRNAs to the ribosome[824,825]. Interestingly, several proteins that are implicated in MR, such as FTSJ1[113] and FMRP[53], have functions in translation. The latter is an RNA-binding protein that associates with poly-ribosomes[333]. Loss of FMRP causes the Fragile X syndrome[53], the most frequent form of inherited MR with a prevalence of 1:4000 males[76]. Several publications have reported on local translation of dendritic mRNAs that is modulated by synaptic activity, including translation of *FMRP*[1129,1130] and FMRP-dependent translation[1131,1132], implicating FMRP in synaptic plasticity through regulation of translation[334,1133]. Intriguingly, *EF-1* mRNA was shown to be highly enriched in the dendrites of hippocampal

pyramidal neurons when compared to their cell body mRNA content[1134]. Possibly, cytoskeletal structures function as sites for such localised translation of dendritic mRNAs, as ultrastructural studies have visualised mRNAs at Actin filament intersections[1135]. This observation is consistent with the finding that EF-1 binds F-actin[1136] and is implicated in Actin bundling[1137]. Phosphorylation of EF-1 by Rho-kinase reduces both its interaction with F-actin and its Actin bundling capacity[1138]. As already mentioned under I.A.4.3, remodelling of the Actin cytoskeleton through the Rho GTPase pathway has emerged as an important molecular mechanism underlying MR[880-882]. Another link between EF-1 and brain function appeared when Cho *et al.* identified a specific interaction between the EF-1γ subunit and the D_3 dopamine receptor[822]. This neurotransmitter receptor is involved in the regulation of behavioural activity[1139], anxiety[1140,1141], and stimulus-reward learning[1142]. Taken together, a possible interaction between hKIAA1202 and the γ subunit of EF-1 might indicate a role for hKIAA1202 in dendritic mRNA translation at the Actin cytoskeleton and/or regulation of dopamine levels. The former function is especially appealing with regard to the localisation of hKIAA1202 ORF I and its association with F-actin.

Finally, putative binding of hKIAA1202 with Pellino 1, a scaffold protein involved in the IL-1 signalling pathway, is worthy of note, as mutations in *IL1RAPL1*, which encodes the IL-1 receptor accessory protein-like 1, have been recovered in mentally retarded patients[123,535], possibly linking hKIAA1202 to MR through IL-1-mediated signalling.

In conclusion, Y2H screening with the partial hKIAA1202 ORF yielded several promising interaction candidates that could suggest involvement of hKIAA1202 in established MR pathways, such as chromatin remodelling, transcriptional and translational control, and cytoskeletal rearrangement.

F.3. hKIAA1202's domain structure and its interaction with filamentous Actin are compatible with a function in cognition

hKIAA1202 interacts with F-actin and belongs to the Shrm family of cytoskeleton-associated proteins. It contains an EVH1-BS, and PDZ and ASD2 domains. The EVH1-BS is involved in rapid Actin remodelling. PDZ domains allow assembly of protein complexes, such as at the post-synaptic density. ASD2 domains reorganise apical populations of F-actin. Dynamic Actin filaments play an essential role in the mechanisms underlying LTP, a molecular learning paradigm.

Based on its domain structure, consisting of a PDZ domain, an EVH1-BS, an ASD2 domain and a predicted PDZ-BS, hKIAA1202 (hShroom 4a) is one of four founding

members of the Shrm family of cytoskeletal proteins. xApx (xShroom 1), hAPXL (hShroom 2) and mShrm (mShroom 3a) are the other proteins that established this family[200].

xApx was shown to encode an apical protein in *Xenopus* A6 renal epithelial cells[779]; it associates with the membrane cytoskeleton via binding to α-spectrin, and may be involved in clustering and retaining the epithelial sodium channel ENaC at the microvillar domain of the apical membrane in Na^+-reabsorbing epithelia[790].

hAPXL was first characterised as a candidate gene for ocular albinism type 1, but the absence of mutations in patients suggested that it is not directly involved in the disease[780]. Raychowdhury *et al.* investigated porcine Apxl in a swine renal tubular epithelial cell line and showed that affinity-purified Actin complexes co-precipitated both Apxl and ENaC, suggestive for an association of Apxl with the cytoskeleton[789]. Very recently, such an association was confirmed when Dietz *et al.* showed direct binding between mApxl and F-actin. The authors demonstrated that mApxl localises to the apical junctional complex in most populations of epithelial cells, including gut and neural epithelium, to the apical plasma membrane in kidney tubules, and to cortical Actin at the plasma membrane in fibroblasts[1143].

mShrm was identified as the gene underlying the murine *shrm* neurological mutant characterised by defective neural tube closure[777]. Its encoded protein, mShrmL, localises to Actin stress fibres, causes them to form bundles[1143] and was shown to directly bind F-actin. mShrm has been implicated in cytoarchitectural changes of the cytoskeleton[777]. xShrm3, the *Xenopus* homologue of mShrmL, induces apical constriction that leads to the formation of hingepoints, and is thought to be a key player in the cytoarchitecture of the neuroepithelial sheet[1144]. Further studies demonstrated that mShrm regulates the cell shape changes eventually leading to closure of the neural tube via apical positioning of an actomyosin network in the neuroepithelium. This idea is consistent with the observation that mShrm is localised in the apical junctional complex of all neuroepithelial cells during neurulation and functions to facilitate the sub-apical distribution of non-muscle Myosin II-B[1145].

PDZ domains, first identified in the post-synaptic density protein PSD-95[775], are implicated in the formation of large protein complexes at the membranes of polarised cells such as neurons[1146,1147], and in the organisation of synaptic signalling pathways[1148,1149]. PDZ domains recognise specific short peptide sequences, called PDZ-BSs, most often at the C-terminus of their binding partners[778], such as ion channels[1150-1152]. As hKIAA1202 contains both a PDZ domain and a putative PDZ-BS, it may form part of a protein complex and function, for example, as a molecular scaffold or by clustering ion channels at the membrane. Genes encod-

ing proteins with a PDZ domain have been shown to be mutated in mentally impaired individuals. For example, mutations in *DLG3*, the gene coding for SAP102 that contains three N-terminal PDZ domains[917], were identified in four families with moderate to severe XLMR[99].

EVH1-BSs are proline-rich motifs binding the EVH1 domain present in the Ena/VASP family. Members of this family are important in rapid Actin remodelling[776,1153]. Proteins of the Homer family, which contain an EVH1 domain, influence dendritic spine morphogenesis and synaptic transmission[295,296,1154,1155]. Oligophrenin 1, which acts as a Rho-GAP[231], binds Homer 1 through its Homer-specific EVH1-BS[232]. Mutations in *OPHN1*, which encodes Oligophrenin 1, cause MR[231,234]. Together, these observations provide evidence for the involvement of EVH1-BSs in human cognition through cytoskeletal rearrangements necessary for morphological spine changes.

The ASD2 domain is unique to the Shrm protein family[200] and in mShrm, it has been shown to regulate apical constriction by reorganising apical populations of F-actin and forming a contractile actomyosin network[1145]. This finding is consistent with SBASE searches that reveal a similarity between hKIAA1202 residues 1327 – 1489, which coincide with the ASD2 domain, and the tropomyosin protein family (PF00261). Indeed, although mApxl, hKIAA1202 and dmCG8603, all members of the Shrm family, do not induce apical constriction, their ASD2 domains have the intrinsic capability of doing so; expression of chimeric proteins targeting the ASD2 domains to the apical junctional complex result in apical constriction[1143].

Several of the observations described above are interesting in light of hKIAA1202's submembranous localisation and its interaction with F-actin. We demonstrated that over-expressed hKIAA1202 co-localises partially with F-actin and almost entirely with F-actin remnants upon disruption of the Actin cytoskeleton. The subcellular distribution pattern of endogenous hKIAA1202 is different from that of the over-expressed protein, probably owing to the fact that the α-hKIAA1202 antibody recognises multiple protein species. Still, we found co-localisation of the endogenous protein with F-actin at the leading edge of fibroblasts and in the neurites of (differentiating) neuronal cells, both sites of rapid Actin remodelling[267,286,287,936]. These observations are consistent with our demonstration that hKIAA1202 is able to redirect the subcellular distribution of F-actin *in vivo*. Preliminary co-IP studies also indicate an interaction between F-actin and hKIAA1202. In addition, very recent *in vitro* co-sedementation assays have shown a direct interaction between mKiaa1202 and F-actin (Dr. J. Hildebrand, personal communication). A last hint of a connection between hKIAA1202 and

the cytoskeleton comes from the finding that Vimentin, another cytoskeletal protein, might be a hKIAA1202 interaction partner.

Apart from the large number of mutations affecting Actin remodelling and causing MR, as described under I.A.4.3.2, a vast body of evidence shows an involvement of cytoskeletal rearrangement as a molecular basis for learning and memory. With the introduction of technologically advanced direct time-lapse studies and the establishment of novel high-resolution imaging, an answer to Crick's question 'Do spines twitch?' has emerged[1156]. The answer is an overwhelming 'Yes, they do!'

Using hippocampal slice cultures, an increase in Actin filament bundles was observed after tetanus-induced LTP in the synaptic cytoskeleton as early as 1991[1157]. One of the first time-lapse studies monitoring spine motility revealed large Actin-dependent changes in dendritic spine shape occurring within seconds[288], supporting these observations. Investigations were taken a step further by recording (de)polymerisation of Actin during learning paradigms, such as LTP. Using pharmacological agents to stabilise or destabilise Actin fibres, LTP was shown to be dependent on Actin polymerisation. The Actin assembly inhibitors latrunculin A/B and cytochalasin B/D, and the Actin filament stabiliser phalloidin all specifically reduced synaptic response; the magnitude of LTP was decreased and the maintenance of LTP was impaired[1158,1159]. In line with these observations, Okamoto *et al.* demonstrated that the dynamic equilibrium between polymeric F-actin and monomeric G-actin in the dendritic spines of rat hippocampal neurons depends on synaptic activity. While LTP causes a continuing shift of the equilibrium towards F-actin, LTD shifts the equilibrium towards G-actin[1160]. Other laboratories showed that the increase in F-actin perseveres for up to five weeks and correlates with the persistence of LTP[1161]. Moreover, experiments revealed that the increase in F-actin content depends on NMDA receptor activation and involves the inactivation of Cofilin[1161], an Actin depolymerising factor[1162], and the targeting of Profilin, a stabiliser of dynamic Actin structures[1163,1164], to spine heads[1165].

These results are consistent with a model in which dynamic Actin filaments play an essential role in the molecular mechanisms underlying LTP and LTD.

Taken together, the domain structure of hKIAA1202 and its association with the F-actin cytoskeleton are compatible with a possible role in mental ability.

G. Conclusion: *hKIAA1202* is a candidate gene for X-linked mental retardation

We characterised the BPs of a t(X;8) translocation in a mentally retarded girl. *hFBXO25*, disrupted by the autosomal BP, encodes an FBP. No direct connection between FBPs and MR has been reported. Therefore, we believe that *hFBXO25* hemizygosity is not the cause of the patient's mental handicap. On the other hand, the absence of several *hKIAA1202* isoforms in our patient due to disruption by the X-chromosomal BP may play a role in her mental impairment. hKIAA1202's domain structure, the nature of some of its putative binding partners, its association with the cytoskeleton and the recovery of patient-specific sequence variants support this assumption. We propose *hKIAA1202* as a candidate XLMR gene.

In this study, we characterised two genes disrupted by the BPs of a t(X;8) translocation in a mentally retarded girl in order to identify genetic loci important in cognition.

The autosomal BP disrupts *hFBXO25*, which is expressed in the human foetal and adult brain. Moreover, its murine counterpart seems to be neuronal-specific in E14.5 embryos. hFBXO25 forms part of a functional $SCF^{hFBXO25}$ E3 complex and is therefore a *de facto* FBP. FBPs confer substrate specificity to the SCF E3 Ubiquitin ligases, which are important in cell cycle progression by poly-ubiquitinylating cell cycle regulators prior to proteasomal degradation. While several neurodegenerative disorders are linked to anomalies in the UPP, misregulation of, or mutations in, FBPs are mainly associated with malignancies. To date, no direct connection between FBPs and MR has been established. Combined with the fact that our patient has an intact copy of chromosome 8, we argue that it is unlikely for disruption of *hFBXO25* to be the cause of her mental handicap. It is understood that a more detailed functional analysis is required before any definite conclusions can be drawn. In addition, based on its expression pattern, it is possible that mutations in *hFBXO25* underlie unresolved neuronal phenotypes other than mental deficit.

The X-chromosomal BP disrupts *hKIAA1202*, which is also expressed in the foetal and adult brain. A second mentally retarded girl with a translocation disrupting *hKIAA1202* was identified. In addition, two nucleotide exchanges in *hKIAA1202* which segregated with MR were not found in large numbers of control chromosomes. An overwhelming amount of data has connected cognition with dendritic spine microanatomy and motility, which are dependent on rapid rearrangment of the F-actin cytoskeleton[880-882,1166,1167]. Although hKIAA1202's function remains elusive, its domain structure and affiliation with a family of cytoskeletal proteins, members of which are involved in regulation of cytoarchitecture, its submembranous localisation, also at sites of rapid Actin remodelling, its co-localisation with F-actin, its potential to redirect F-actin within the cell, the nature of some of its putative binding partners

and the absence of several *hKIAA1202* isoforms in our patient, suggest that disruption of *hKIAA1202* may play a role in the patient's mental impairment.

Evolution, patiently shaping nature, may support this conclusion: whereas hFBXO25 is one of only six FBPs with homologues in the fly and the worm[645], which seems consistent with a fundamental putative function such as cell cycle regulation, hKIAA1202 homologues are only present among vertebrates, which may be indicative of a more specialised function.

The evidence presented in this work supports a role for *hKIAA1202* as a gene important in human cognition.