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Identification of two novel mutations in the *CACNA1A* gene responsible for episodic ataxia type 2

EDITOR—Episodic ataxia type 2 (EA-2) (OMIM 108500) is an autosomal dominant neurological disorder. Affected subjects experience discrete episodes of cerebellar ataxia usually associated with migraine symptoms, interictal nystagmus, as well as residual mild and, in some cases, a progressive cerebellar incoordination. These attacks usually begin in childhood or adolescence, last a few hours, may be precipitated by stress, exercise, or fatigue, and respond to acetazolamide.[1–3]

As with other acetazolamide responsive diseases, EA-2 is a channelopathy.[4] It was first linked to chromosome 19p13[3 5–7] and subsequently shown to be allelic to familial hemiplegic migraine (FHM) when mutations for both disorders were identified in the P/Q type calcium channel α_{1A} subunit gene, *CACNA1A*.[8] Shortly thereafter, an intragenic expansion of a CAG repeat within *CACNA1A* was shown to cause spinocerebellar ataxia type 6 (SCA6).[9] To date, mutations causing EA-2 all appear to disrupt the translational reading frame of the α_{1A} subunit gene,[8 10–12] while those causing FHM all seem to be missense mutations.[8 13–15] A single missense mutation, however, in the *CACNA1A* gene has also been shown to cause severe progressive cerebellar ataxia.[16]

The α_{1A} subunit has been shown to be the pore forming unit of the P/Q type calcium channel[17 18] which is involved in controlling neurotransmitter release[19] and is expressed throughout the brain with abundant expression in the cerebellum.[20–22] This high voltage activated calcium channel consists of five subunits, α_{1A}, β_4, α_2, δ, and γ. The α_{1A} subunit is subdivided into four homologous domains (DI-DIV) that each contain six putative transmembrane regions (S1-S6) (fig 1).[19] The fourth transmembrane domain functions as the voltage sensor while the four loops between transmembrane domains S5-S6 compose the pore forming unit. Thus, the α_{1A} subunit of the P/Q type calcium channel is responsible for directing channel activity, while the other subunits appear to act as auxiliary regulators of the channel.[19 23]

Here, we describe two novel mutations in the *CACNA1A* gene that cause EA-2: a guanine insertion after nucleotide 3091 (insG3091) that is the first mutation identified to occur in an intracellular loop and a guanine deletion at nucleotide 5123 (delG5123) representing the most 3' mutation reported to date. Similar to previously reported EA-2 mutations, these nucleotide changes disrupt the *CACNA1A* translational reading frame and are predicted to result in proteins which prematurely truncate after domain I.

Blood samples were obtained with informed consent from 81 subjects: an apparently sporadic case of EA-2 and her sib, 29 members of a family segregating EA-2, and 50 unrelated, healthy controls. Genomic DNA was extracted from the blood samples using standard techniques. Probands were assessed to have EA-2 by a clinical neurologist and were referred for study.

Single stranded conformational polymorphism (SSCP) analysis was used to screen polymerase chain reaction (PCR) products of exons in the *CACNA1A* gene for molecular variants.[24 25] Published primers[8 16] and redesigned primers (table 1) were used to amplify all 47 exons from the intronic sequences flanking each exon. PCR amplification conditions were optimised for each primer pair and the products were labelled by incorporation of [α-^{32}P]dCTP into the amplification reaction. The labelled

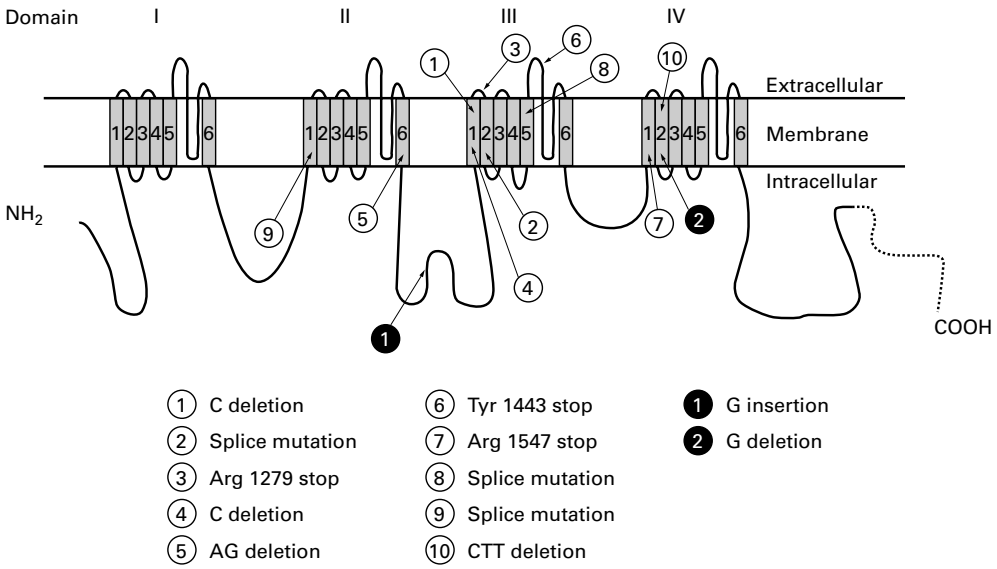

Figure 1 Schematic diagram of the P/Q type calcium channel α_{1A} subunit illustrating the location of EA-2 mutations. The two EA-2 causing mutations identified in this study are represented in black. The 10 previously reported EA-2 causing mutations[8 10–12] are in white.

PCR product was diluted with 50 µl loading dye (98% formamide, 10 mmol/l EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol FF) and denatured at 95°C for five minutes. An aliquot of the denatured PCR product was then loaded immediately onto a non-denaturing gel (55 cm × 30 cm) consisting of 7.5% acrylamide (49:1 acrylamide:bisacrylamide) and 0.5 × TBE. Gel electrophoresis was performed at 4°C and 50 W. Gels were transferred to filter paper and exposed to *x* ray film (AGFA Curix Ortho HT-G Ecopac) at −80°C overnight. Abnormally migrating conformers were detected by visual inspection.

PCR products were initially gel purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech). DNA sequence analysis of the purified PCR products was then performed manually using the Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech) according to the manufacturer's recommended conditions. Denatured sequencing reactions were immediately loaded and electrophoresed through a 6% acrylamide gel (SequaGel-6, National Diagnostics). Sequencing gels were exposed to *x* ray film (AGFA Curix Ortho HT-G Ecopac) for 18 hours at −80°C and autoradiographs were examined for sequence changes. All nucleotide, codon, and exon numbering corresponds to GenBank accession number X99897 (http://www.ncbi.nlm.nih.gov/).

Allele specific oligonucleotide hybridisation (ASOH) was performed to look for segregation of mutations in a family and to rule out the possibility that these mutations are common polymorphisms. Exon 30 was amplified from 200 ng of genomic DNA from case 1, her sib, and 50 normal controls. Exon 19b was amplified from 200 ng of genomic DNA from patient IV.7 (case 2), 28 of his family members, and 50 normal controls. PCR products were electrophoresed through 1.5% agarose gels and transferred onto Hybond-N membranes (Amersham Pharmacia Biotech) for three hours under denaturing conditions (0.5 N NaOH, 1.5 mol/l NaCl). The blots were then neutralised (1 mol/l Tris-Cl, pH 7.5, 1.5 mol/l NaCl) and the DNA was UV cross linked to the membranes. Membranes were prehybridised (5 × SSPE, 5 × Denhardt's, and 1% SDS) for 30 minutes and the respective blots were then probed for one hour with either wild type exon 30 ASO (5' - TGGAATGTGTGCTGAAAG - 3') at 52°C, mutant exon 30 ASO (5' - TGGAATGTGTGCTAAAG - 3') at 48°C, wild type exon 19 ASO (5' - CGGCAGGGGGGCAG CAG - 3') at 62°C, or mutant exon 19 ASO (5' - CGGCAGGGGGGGCAGCAG - 3') at 66°C. Oligonucleotides were end labelled using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP. Unincorporated phosphates were removed with a G-25 Sephadex spin column (Boehringer Mannheim) according to the manufacturer's instructions. The activity of the probes was determined by scintilation counting and $0.6\text{-}0.8 \times 10^6$ cpm of denatured probe was added per ml of hybridisation solution. Membranes were washed twice with 2 × SSPE/0.1% SDS for 15 minutes at the hybridisation temperature and then exposed to *x* ray film.

Table 1 Redesigned primers for SSCP analysis of the CACNA1A gene

Exon	*Forward primer*	*Reverse primer*
14	cag aaa gtt ggg aaa gtg agc	Same as ref 8
19a	atc cat cca agc tac agt gc	ctt gtt ggt gtt gtt gtt gc
22	ctg aac ctg cgc tac ttt gag atg t	atc cca ccc tac cta tga gca ttt t
23	Same as ref 8	tgt gtt ctc act tat aat ctg c
24	gag agg cta gag agt ggg tgg cag c	caa gag gaa acc ctt gcg aag aga c
28	tct ccc tgc ccc att cct tg	agt ttt taa agg aca gat gg
29	Same as ref 8	ttt atc agg gta gag gca gg
30	gac ccc tgc tac tcc tgc ttc	ttc caa gcc cat agc tgt agc
31	cct ctg ttg gca tct ctg tc	ttc tcc ctc tct ggt cat gg
42	ttc tgg aag ctg ccc aag cg	ttc ctt cca ccg caa cca cc
44	tct gtg tgc acc atc cat g	Same as ref 8
45	ctt ggt gct agc tgc tga c	Same as ref 8

Case 1 is a 43 year old woman who has had episodes of loss of equilibrium and loss of coordination of the limbs, to the point where she may be unable to walk. These episodes were usually precipitated by physical activity or by sudden emotional excitement and lasted for four to five minutes to several hours. On examination she was noted to have horizontal and vertical nystagmus and she complained of diplopia on lateral gaze. She had some incoordination on finger to nose testing and tandem gait was possible. Deep tendon reflexes varied from brisk to sluggish on different examinations. Diamox proved effective in preventing attacks. She was seen originally at the age of 14 when she presented with a five month history of intermittent headaches, double vision, dizziness, and faintness. Family history was negative for episodic ataxia. Both parents are dead, but her sib gave a blood sample for mutation analysis.

Case 1 was evaluated for a mutation in *CACNA1A*. SSCP analysis unveiled a unique conformer in exon 30 (fig 2A1, lane 8) which was further evaluated by PCR and direct sequencing. Sequence analysis showed a single guanine deletion at nucleotide position 5123 (codon 1616) in one allele, as illustrated by the start of a double banding pattern on the autoradiograph at this site (fig 2B1). This deletion disrupts the translational reading frame and alters the subsequent amino acid sequence resulting in the formation of a stop codon at the junction of exons 30 and 31, codon 1624 (fig 2C1). ASOH was then performed using PCR amplified DNA from the patient, her sib, and 50 unrelated controls. The mutant oligonucleotide hybridised with only the patient sample (data not shown).

Case 2 is the proband of a 39 member white family spanning four generations (IV.7, fig 3A). The inheritance pattern is autosomal dominant and the disorder has been previously linked to the EA-2 locus on 19p13.[3] The proband, aged 29 years, began experiencing episodes at 18 months. Attacks were stereotyped and heralded by lightheadedness and a sense of leg weakness. This was quickly followed by progressive truncal ataxia with an inability to stand or sit unaided. There were striking head titubations and large amplitude dysmetric limb movements. Speech was scanning and markedly dysarthric. Ocular movements were impaired with notable difficulty in initiating volitional saccades, saccadic pursuit, ocular dysmetria, and coarse nystagmus. During an attack, the subject was slow to respond and appeared dazed. Headache and nausea occasionally developed towards the end of an attack. True vertigo or vomiting was rare. The most consistent precipitating factors were physical and emotional stress. On examination, the patient showed nystagmus on lateral and upward gaze, saccadic pursuit, impaired optokinetic responses, and mild cerebellar limb and gait ataxia.

Exon 19 was shown to contain a number of different conformers that migrate in a very similar pattern (fig 2A2). Exon 19, from patient IV.7 and control samples (fig 2A2, lanes 1, 2, 5, and 7, respectively), each representing a different conformer, was PCR amplified and sequenced. Patient IV.7 was discovered to have a guanine insertion after nucleotide 3091 (codon 939) in one allele, which is shown by the double banding pattern in the sequence starting after base 3091 (fig 2B2). The guanine insertion disrupts the translational reading frame thereby altering downstream amino acids until a premature stop codon is encountered at codon 1067 in the middle of exon 20 (fig 2C2). DNA from 28 family members along with patient IV.7 was subjected to ASOH. All of the affected, and none of the unaffected, subjects carried the guanine insertion after position 3091, showing that the nucleotide insertion cosegregated with the disease phenotype (fig 3A, B). ASOH was also performed using genomic DNA from 50 controls in which none of them hybridised to the mutant allele, indicating that this change was not present in the 100 chromosomes tested (data not shown). DNA sequence

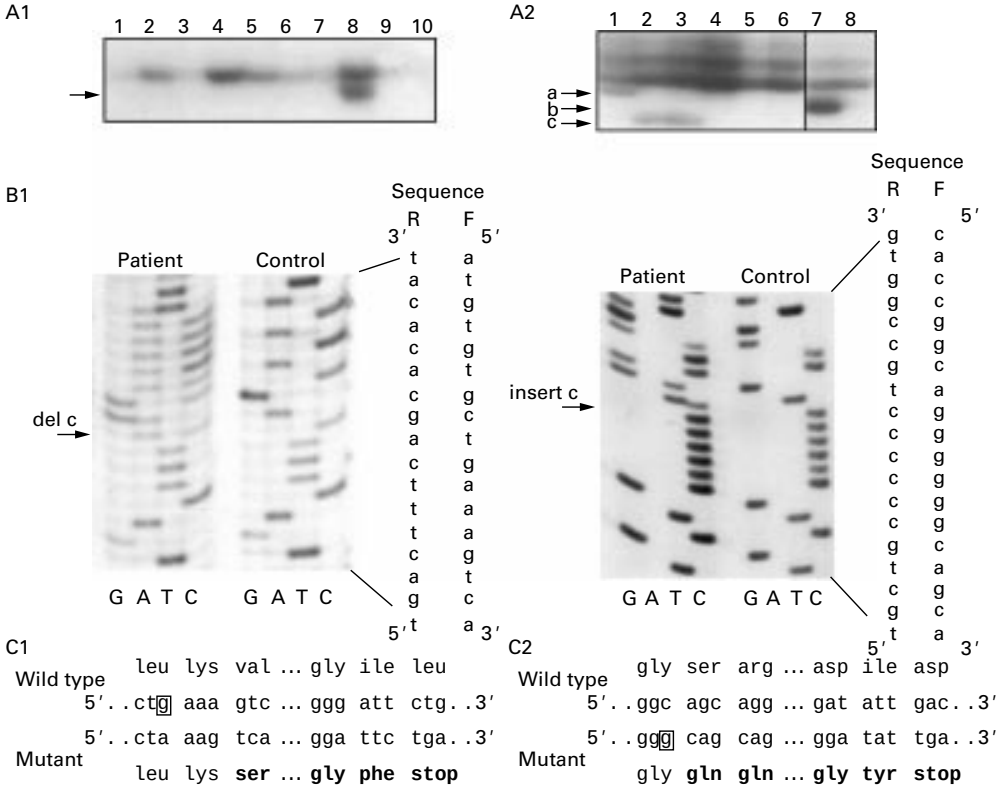

Figure 2 Detection of aberrantly migrating conformers (panel A) and the resultant nucleotide changes (panels B and C) in the CACNA1A gene. Panel A1: SSCP analysis of exon 30 of the CACNA1A gene showing an aberrantly migrating conformer (arrow) in case 1 with EA-2 (lane 8). Panel A2: a composite of SSCP results of exon 19 of the CACNA1A gene depicting the presence of four different banding patterns. An aberrantly migrating conformer (arrow a) is present in EA-2 patient IV.7 (lane 1). Aberrantly migrating conformers (arrows b and c) in lanes 2, 3, and 7 represent polymorphisms in unrelated subjects. Lanes 4, 5, 6, and 8 represent the wild type banding pattern. Panel B1 and B2: DNA sequence analysis of the reverse strand of exon 30 and exon 19 of the CACNA1A gene displays a cytosine deletion and a cytosine insertion in one allele (arrow) of case 1 and patient IV.7, respectively, which corresponds to a guanine deletion at nucleotide 5123 and a guanine insertion after nucleotide 3091 in the coding strand. DNA sequence from the autoradiograph is given in both the reverse (R) and forward (F) orientations starting at nucleotide 5114 or nucleotide 3078 of the coding strand. Panel C1 and C2: nucleotide and amino acid comparison of wild type and mutant sequences as a result of the guanine deletion at position 5123 and the guanine insertion after position 3091. The guanine deletion and guanine insertion are boxed with the subsequent amino acid changes in bold.

Table 2 Polymorphisms in exon 19 of the CACNA1A gene

Location	*Sequence change*	*Consequence*	*Frequency*
Exon 19	2965 C-G	Pro896Arg	0.014
Exon 19	3029 G-C	Glu917Asp	0.021
Exon 19	3125 G-A	Thr949Thr	0.146

analysis showed that the other conformers detected in exon 19 were rare polymorphisms in patient DNA samples (table 2).

In the present study, we report the identification of two novel mutations in the *CACNA1A* gene that are responsible for EA-2, as well as the discovery of three new polymorphisms. These mutations are consistent with those previously published as they alter the translational reading frame of the α_{1A} subunit gene and are predicted to result in the production of truncated proteins (fig 1).[8 10 11]

The guanine deletion in exon 30 occurs in domain IV S2 and is predicted to result in the premature truncation of the protein following this transmembrane region. Interestingly, delG5123 represents the most C-terminal mutation published to date. If the mutant allele is translated, we would predict the complete formation of domains I, II, and III.

The guanine insertion in exon 19 is also a unique EA-2 mutation as it represents the first reported mutation to occur in an intracellular loop. The ASOH results for exon 19 showed that the mutation cosegregated with the disease allele in all of the affected family members (7/7). Previous screening of this family failed to detect a mutation in exon 19, which may have been because of other polymorphic conformers which migrated with similar banding patterns. We were able to decipher the different conformers as three polymorphisms and a mutation by extending the electrophoresis time of the SSCP gels. The need for long migration times for specific exons may partially explain why SSCP analysis may only detect 85% of all aberrantly migrating bands.[26]

This single nucleotide insertion in exon 19 occurs at codon 939 between domains II and III and is predicted to change 128 amino acids before forming a truncated protein. The synaptic protein interaction site of the α_{1A} subunit of the P/Q type calcium channel encompasses amino acids 722-1036 within the domain II-III linker.[27] Thus, the guanine insertion and subsequent shift in the translational reading frame may affect the normal function of the channel, not only through the formation of a truncated protein, but also through the disruption of the direct interaction of the intracellular loop with proteins of the synaptic vesicle docking/fusion machinery as well as altering G protein and protein kinase C dependent modulation.[28–30] Disruption of these intracellular interactions may contribute to the disease mechanism and could explain subtle differences observed in EA-2 phenotypes. This synaptic protein interaction site is also subject to alternative splicing which may lead to the formation of isoforms with varying functions.[19 30] The guanine insertion mutation will have been translated in some isoforms and spliced out of others, supporting our prediction that a mutation in this region may lead to varying EA-2 phenotypes and as such may explain the very early age of onset in patient IV.7.

To date, all of the known EA-2 mutations are unique and have resulted in premature truncations of *CACNA1A* beyond domain I. We predict that a truncation mutation in domain I would result in an unstable protein, leading to haploinsufficiency, and not an EA-2 phenotype, whereas a truncation mutation beyond domain I could be expected to produce a stable truncated protein resulting in episodic ataxia. Thus, the severity of the disease is most likely affected by the location of the mutation and the identification of new EA-2 causing mutations is therefore important

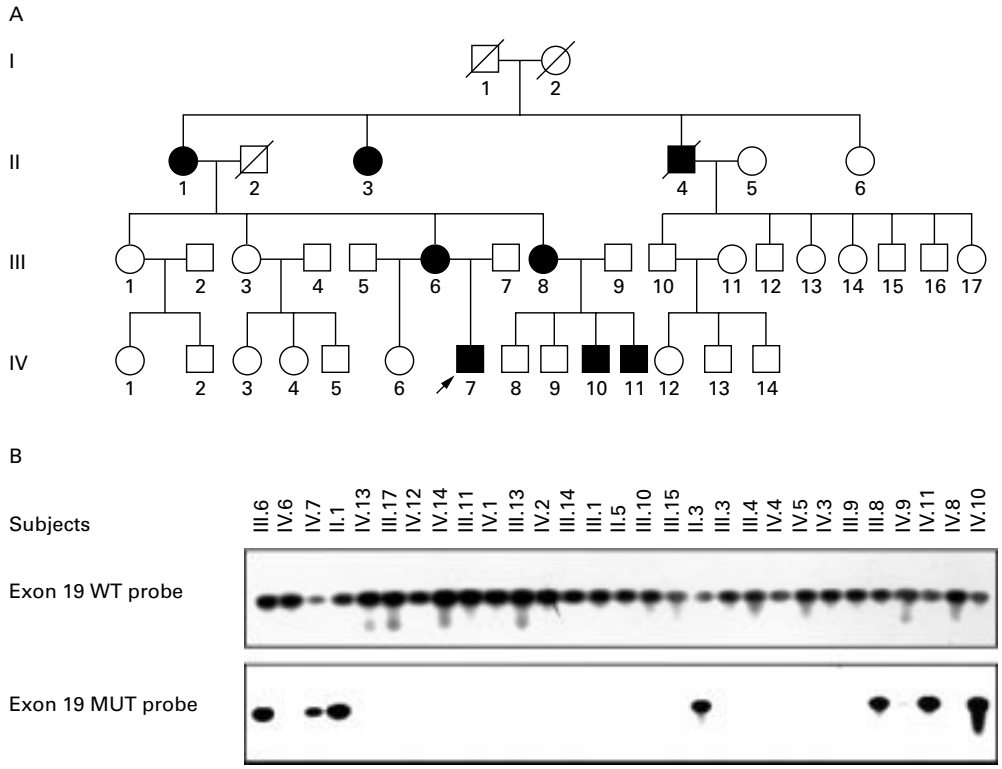

Figure 3 The guanine insertion in exon 19 of the CACNA1A gene cosegregated with all affected family members. Panel A: pedigree of EA-2 segregating kindred where the proband is indicated by an arrow. Panel B: ASOH was performed using a wild type oligonucleotide, which hybridised to DNA from all family members tested, and a mutant oligonucleotide, which only hybridised to DNA from the affected subjects.

for elucidating the disease mechanism and for providing more accurate disease diagnosis.

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Detection of 11 germline inactivating *TP53* mutations and absence of *TP63* and *HCHK2* mutations in 17 French families with Li-Fraumeni or Li-Fraumeni-like syndrome

EDITOR—The Li-Fraumeni syndrome (LFS) represents one of the most devastating genetic predispositions to cancers. This rare syndrome, affecting children and young adults, is characterised by a wide spectrum of early onset malignancies including bone and soft tissue sarcomas, brain tumours, adrenocortical tumours, and premenopausal breast cancers.[1] LFS was initially defined using stringent criteria[2]: (1) a proband with a sarcoma diagnosed before the age of 45, (2) a first degree relative with cancer before the age of 45, and (3) another first or second degree relative with either a sarcoma diagnosed at any age or any cancer diagnosed under the age of 45. Subsequently, Birch *et al*[3] defined Li-Fraumeni-like (LFL) syndrome as a proband with any childhood tumour or sarcoma, brain tumour, or adrenocortical tumour under 45 years, plus a first or second degree relative with a typical LFS tumour at any age and another first or second degree relative with any cancer under the age of 60. Eeles[4] proposed more relaxed criteria for LFL: a clustering of two typical LFS tumours in subjects who are first or second degree relatives at any age. Since the original reports of germline mutations of the tumour suppressor gene *TP53* in LFS,[5 6] numerous studies have shown that germline *TP53* mutations can be detected in approximately 70% of LFS families and 20% of LFL families,[1] suggesting the possible involvement of other genes in LFS. This hypothesis was recently confirmed by the detection, in one LFS family and one family suggestive of LFS, of germline mutations of *hCHK2*, the human homologue of the *Saccharomyces cerevisiae RAD53* gene, located on chromosome 22q12.[7 8] *hCHK2* encodes a kinase, which is able to phosphorylate, in response to DNA