

# A yeast model for the study of human *DFNA5*, a gene mutated in nonsyndromic hearing impairment

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Received 30 January 2003; received in revised form 8 May 2003; accepted 14 May 2003

## Abstract

A mutation in human *DFNA5* is associated with autosomal dominant nonsyndromic hearing impairment. The function of *DFNA5* protein remains unknown and no experimental model has been described so far. Here we describe fission yeast *Schizosaccharomyces pombe* as a model organism for studying the function of heterologously expressed *DFNA5*. We have expressed wild-type as well as mutant *DFNA5* alleles under control of regulatable *nmt1* promoter. Yeast cells tolerated expression of wild-type *DFNA5*, while expression of the mutant *DFNA5* allele, which is responsible for nonsyndromic autosomal dominant hearing impairment, led to cell cycle arrest. We identified new rat and horse *DFNA5* homologues and we describe a domain of homology shared between *DFNA5* and the Mcm10 family of DNA replication proteins. Genetic interactions between heterologously expressed *DFNA5* and a fission yeast *cdc23* (*mcm10*) mutant support a possible link between *DFNA5* and Mcm10 proteins.

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**Keywords:** Cell cycle; MCM10; *Schizosaccharomyces pombe*; Hearing impairment; *DFNA5*; Yeast

## 1. Introduction

Hearing impairment is the most frequent sensory handicap. Most of the childhood to early adulthood onset forms of nonsyndromic hereditary hearing impairment exhibit an autosomal dominant inheritance pattern and are indicated by *DFNA*. To date, more than 90 chromosomal loci for nonsyndromic hearing impairment have been mapped to the human genome (Hereditary Hearing Loss Homepage, Van Camp and Smith, World Wide Web URL: <http://www.uia.ac.be/dnalab/hhh>).

Human *DFNA5* (*ICERE-1*) is one of the genes associated with nonsyndromic autosomal dominant hearing impairment. An insertion/deletion mutation in intron 7 of this gene causes skipping of exon 8, resulting in premature termination

of the open reading frame [1]. In addition to its relevance to hearing impairment, the expression pattern of human *DFNA5* indicated that this gene might participate in the tumor biology of breast and skin cancers [2,3]. The physiological function of the *DFNA5* protein remains unknown and computational analysis did not give any information on the protein function [1]. In addition, no significant homology with any other protein could be detected. Here we report that *DFNA5* shares sequence similarity with the Mcm10 family of DNA replication proteins.

The *Mcm10* (*dna43*) gene was first identified in yeast *Saccharomyces cerevisiae* in two genetic screens for genes required for chromosomal DNA replication and mini-chromosome maintenance [4,5]. Mcm10 is required for efficient initiation of DNA replication. Mcm10 may also be required for the elongation step of DNA replication [6]. Recent observations showed that Mcm10 performs its function after prereplicative complex assembly and is required for loading of Cdc45 onto chromatin [7] (J. Gregan and S.E. Kearsey, unpublished data). All known members of the Mcm10 family share a conserved zinc-finger-like motif, referred to as the CCCH domain [8], which is essential for the Mcm10

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	: . . : * * . : *
mcm10_human	VLIMGEALDLGTCK-AKKNKNGEPCQTQTVNLRD-CEYCQYHVQAQYKKLSA-KRADLQ (377-430)
mcm10_Xenopus	VLLMGDAVDLGTCK-ARKKNGDPCTQMVLNLD-CEYCQYHVQAQYKKVSS-KRADLQ (369-422)
mcm10_Drosophila	VMILGQSKDLGTCR-ATKKNGDKTSVNVNLT-CDYCFHVKQYEGKMS--RRSELQ (124-176)
mcm10_Anopheles	VMVLGQSRDLGTCR-SRKNKNGDRCTSIIVNLGK-CEYCVYHIKQYENKAS--NRGGLL (196-249)
mcm10_S.cerevisiae	I LEIGSSRD LGWCPIV NKKTHKKCGSPINISL-HKCCDYHREVQFRGTS-A-KRIELN (297-351)
mcm10_C.elegans	I VEIGQS AHFGTCKGIRQQDQGRCSNFVNSSL-SEFCVPHVMSAARKLSA-KRGVFN (254-308)
Dfna5_human	EMPDSAAALLGTCC-KLQIIPTLCHLLRALSD-DGVSDLEDPTLTPKDT-ERFGIV (395-448)
Dfna5_horse	EMPDNAALLGTCC-KLQIIIPALCHLLHAMSH-DGVCDELDPALAPLKDT-ERFGVA (396-449)
Dfna5h_mouse	EMPDNATVFLGTCC-KLHVIVSSLCCLLHALSD-DSVCDPHNPTLAPLRDT-ERFGIV (398-451)
Dfna5_rat	EMPDNATVFLGTCC-KLHVIVPSLCHLLHALSD-DSVCDPQDPTLAPLRDT-ERFGIV
mcm10_S.pombe	LLEIGRSKHLGYCS-SRRKSGELCKHWLDKRA-GDVCEYHVVDLAVQRMS-TRTEFA (287-340)
mcm10_Arabidopsis	MVKLGVSAADYGVCT-AKRKDGTTCTSVVNKRLRTAFRDLKSGQIYTVPEPPADRSNGK (198-253)
consensus/90%	. h . . stsh . hGhCp . . hph . sp . Cp . hhshp . . sths . hc . . . h . h . s . . pR . th .

Fig. 1. DFNA5 and Mcm10 proteins share a domain of homology. Multiple alignment of the Mcm10-CCCH domains of human (Swiss-Prot: Q9H3P9), *Xenopus laevis* (Swiss-Prot: Q9DEX0), *Drosophila melanogaster* (GenBank: NP\_610097), *Caenorhabditis elegans* (Swiss-Prot: Q9U2C2), *Anopheles gambiae* (GenBank: EAA10332), *Arabidopsis thaliana* (GenBank: NP\_179694), *Schizosaccharomyces pombe* (Swiss-Prot: O42709) and *Saccharomyces cerevisiae* (Swiss-Prot: P32354) and of DFNA5 proteins from human (Swiss-Prot: O60443), mouse (Swiss-Prot: Q9Z2D3), horse (GenBank: AY194290) and rat (GenBank: AY194291) aligned using CLUSTAL W [22]. Identical residues are indicated by an asterisk, strong and weak conservations based on CLUSTAL W are indicated by a double dot and single dot respectively. The first and the last aligned residues in each of the sequences are designated on the right. The 90% consensus shown below the alignment was calculated using Consensus (<http://www.bork.embl-heidelberg.de:8080/Alignment/consensus.html>) and the following amino acid groupings were used: hydrophobic (h; A,C,F,G,H,I,K,L,M,R,T,V,W,Y), small (s; A,C,D,G,N,P,S,T,V), turnlike (t; A,C,D,E,G,H,K,N,Q,R,S,T), polar (p; C,D,E,H,K,N,Q,R,S,T), charged (c; D,E,H,K,R).

function [9,10]. The hallmarks of the CCCH domain are conserved cysteine residues resembling a zinc-finger motif as well as conserved adjacent glycine and arginine residues (Fig. 1).

## 2. Materials and methods

### 2.1. Fission yeast methods

Media, growth conditions and genetic methods were as described by Moreno et al. [11]. Thiamine was used at 5 µg/ml to repress the *nmt1* promoter.

Flow cytometry analysis and microscopy were as described in Ref. [12].

### 2.2. Plasmid constructs

#### 2.2.1. Horse and rat homologues of DFNA5

A horse skin library in λ ZAP Express [13] and a rat brain library in λ TriplEx (Clontech) were plated using standard techniques. A 1500-bp mouse DFNA5 hybridization probe was PCR-generated using primers 5'-GTTGATGCTGGAG-GAGACC-3' and 5'-GTCTTGACCTGTAGCATGTCC-3' and the previously cloned mouse *Dfna5h* as template [1]. The probe was purified with the QIAquick Gel Extraction Kit (Qiagen). Membranes resulting from plaque lifting were hybridized with the <sup>32</sup>P-labelled probe using standard techniques. Positive clones were rescreened to purity and phagemid was excised according to the manufacturer's guidelines. Inserts were sequenced using the Big-Dye Dye-Terminator Cycle Sequencing Kit (ABI). Fragments were separated on an ABI 3100 automated DNA sequencer (ABI).

After isolation of the partial rat cDNA, a rat-specific probe was generated using PCR primers 5'-GGAGCTTCC-

GTTCTTTGC-3' and 5'-CAGCAGTGTGCTGGTGTGC-3'. In addition, 5'-RACE experiments were performed on the rat library using a rat DFNA5-specific primer 5'-CCCAGCATTGTACAGCATGTCCAAAGG-3' and a TriplEx vector primer 5'-CTCGGGAAGCGCGCCATTGTG-TTGG-3'.

#### 2.2.2. Cloning of human full-length and mutant DFNA5

To obtain a human full-length DFNA5 clone, a PCR reaction was performed on Human Leukocyte Marathon-Ready cDNA (Clontech) using the Advantage cDNA PCR kit (Clontech) and the following primers: 5'-CCG-ACATCTCCCGGATAATCTGG-3' and 5'-GGTCAACTTT-TAACGTGCATATGACC-3'. The PCR reaction consisted of 35 cycles of 30-s denaturation at 94 °C and 4-min extension at 68 °C. The PCR product was cloned into the pT-Adv vector using the AdvanTage PCR cloning kit (Clontech).

To obtain a human mutant DFNA5 clone, total RNA was isolated from EBV-transformed lymphoblastoid cell lines derived from DFNA5 patients [1] using Trizol (Life Technologies). cDNA was prepared using random hexamers from the SuperScript Preamplification System (Life Technologies). A similar PCR reaction as described above was performed. The resulting PCR fragments were cloned into the pCR4-TOPO vector using the TOPO TA Cloning kit (Invitrogen), and colonies that contained mutant DFNA5 were selected.

Both the human full-length and the mutant DFNA5 clones were completely bidirectionally sequenced in order to exclude the introduction of cloning errors.

Mouse *Dfna5h* was PCR amplified from the pBluescript-Dfna5h using oligonucleotide primers 5'XhoI mouseDFNA5 (ATATCTCGAGATGTTTGCCAAAGCAACTC) and 3'SmaI mouseDFNA5 (ATATCCGGGCTAGTCTTGACC-

TGTAG) and cloned into *XhoI* and *SmaI* sites of the plasmid pREP3x, resulting in pREP3x-mDFNA5.

Human *DFNA5* was excised from the plasmid pT-Adv-hDFNA5 by *BamHI* and *SmaI* and cloned into *BamHI* and *SmaI* sites of the plasmid pREP3x, resulting in pREP3x-hDFNA5. Mutant *DFNA5* was excised from the plasmid pCR4-hDFNA5<sup>mut</sup> by *EcoRI* and cloned into the *EcoRI* site of the plasmid pT-Adv-hDFNA5, replacing the wild-type *DFNA5* allele and resulting in pT-Adv-hDFNA5<sup>mut</sup> plasmid. Mutant *DFNA5* was excised from the plasmid pT-Adv-hDFNA5<sup>mut</sup> by *BamHI* and *HincII* and cloned into the *BamHI* and *SmaI* sites of the plasmid pREP3x, resulting in pREP3x-hDFNA5<sup>mut</sup> plasmid. All constructs were verified by sequencing.

### 2.3. Yeast strains

Degron-*cdc23-M36* strain P1146 (*cdc23-M36-td*) was constructed by *BglIII* linearization of the plasmid pSMRG2+degron+*cdc23* (J. Gregan and S.E. Kearsey, unpublished data) and integration at the *cdc23* locus of the *Schizosaccharomyces pombe* strain P155 (*cdc23-M36 h<sup>-</sup>*), which contained *cdc23-M36* temperature-sensitive allele. A similar strategy was used to construct degron-*cdc23-IE2* strain P1145 (*cdc23-IE2-td*), where pSMRG2+degron+*cdc23* plasmid was integrated at the *cdc23* locus of the *S. pombe* strain P893 (*cdc23-IE2 leu1-32 h<sup>-</sup>*), which contained the *cdc23-IE2* temperature-sensitive allele.

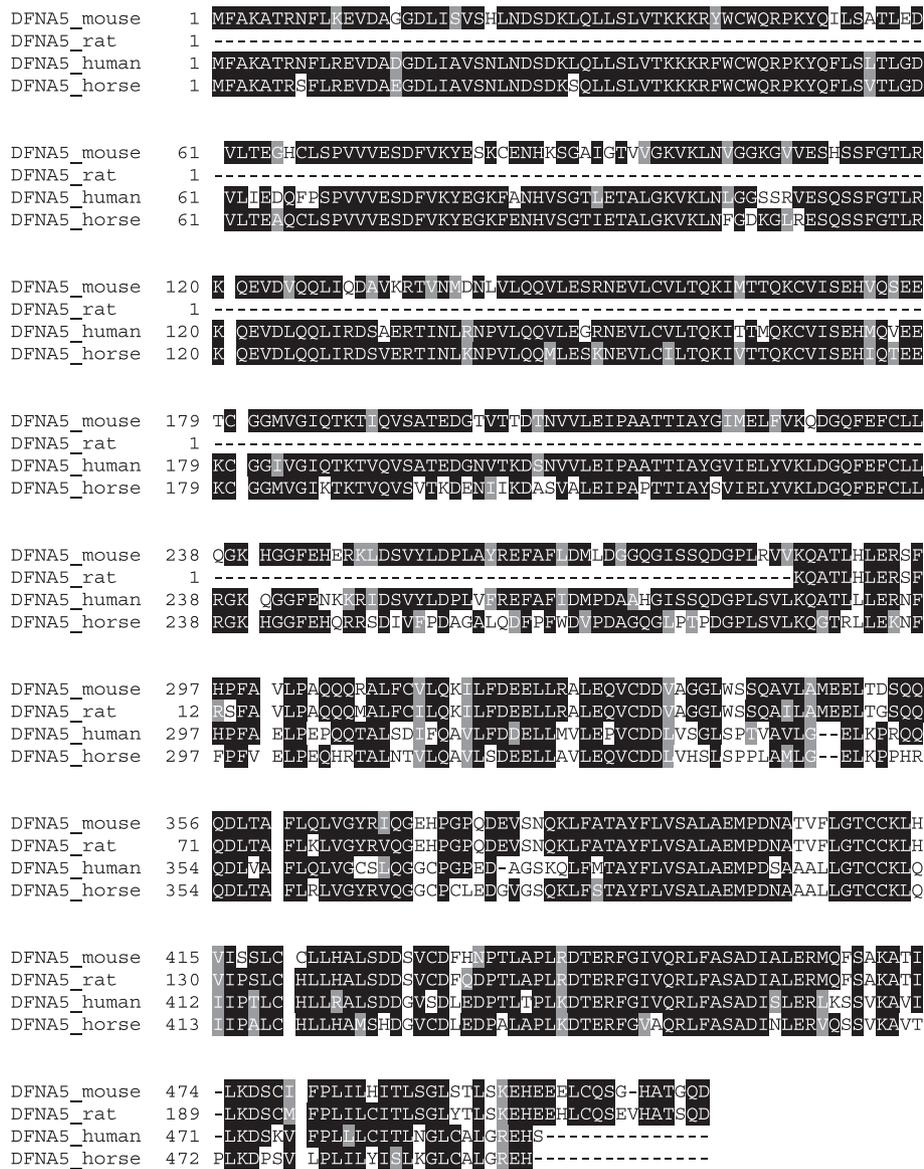


Fig. 2. Multiple alignment of DFNA5 proteins. DFNA5 homologues from human (Swiss-Prot: O60443), mouse (Swiss-Prot: Q9Z2D3), horse (GenBank: AY194290) and partial sequence of the rat (GenBank: AY194291) were aligned using CLUSTAL W [22]. Identical amino acid residues are shaded in black and similar amino acid residues are in gray shades.

DFNA5 proteins were heterologously expressed in a wild-type *S. pombe* strain P138 (*ade6-M210 leu1-32 ura4-D18 h<sup>-</sup>*).

#### 2.4. Reverse transcription-polymerase chain reaction (RT-PCR) assay

RT-PCR were performed with total cellular RNA, avian myeloblastosis virus reverse transcriptase and Tfl DNA polymerase (Promega) according to the manufacturer's protocol.

Two oligonucleotide primers, DFNA5 5' (atgtttgccaag-caaccagg) and DFNA5 3' (tcagagatcacaccttctgc), were used to amplify a 515-bp RT-PCR product corresponding to the human *DFNA5* mRNA. The identity of the *DFNA5* RT-PCR product was confirmed by sequencing.

### 3. Results

#### 3.1. Sequence analysis

We used PROSITE [14] to search SWISS-PROT, TrEMBL and PDB databases using a consensus sequence of the CCCH domains of Mcm10 proteins as a PROSITE entry. Apart from known Mcm10 homologues, we identified the human *DFNA5* protein and its mouse homologue *Dfna5h* (Fig. 1). Interestingly, the mutation associated with nonsyndromic hearing impairment results in the premature termination of the *DFNA5* open reading frame, so that the truncated protein is lacking a region containing the CCCH domain [1]. Iterative PSI-BLAST searches [15] with the CCCH domains did not detect any significant homologies other than *DFNA5*, *Dfna5h* and members of the Mcm10 family.

To further characterize the *DFNA5* proteins, we identified additional *DFNA5* homologues. Screening one million plaques from a horse skin cDNA library resulted in eight positive clones. Three of these clones were chimeric, four contained partial horse *DFNA5* mRNA and one contained the complete horse *DFNA5* mRNA (2219 bp; GenBank accession number AY194290). The horse *DFNA5* protein

showed 75.0% similarity with human and 66.6% similarity with mouse *DFNA5*.

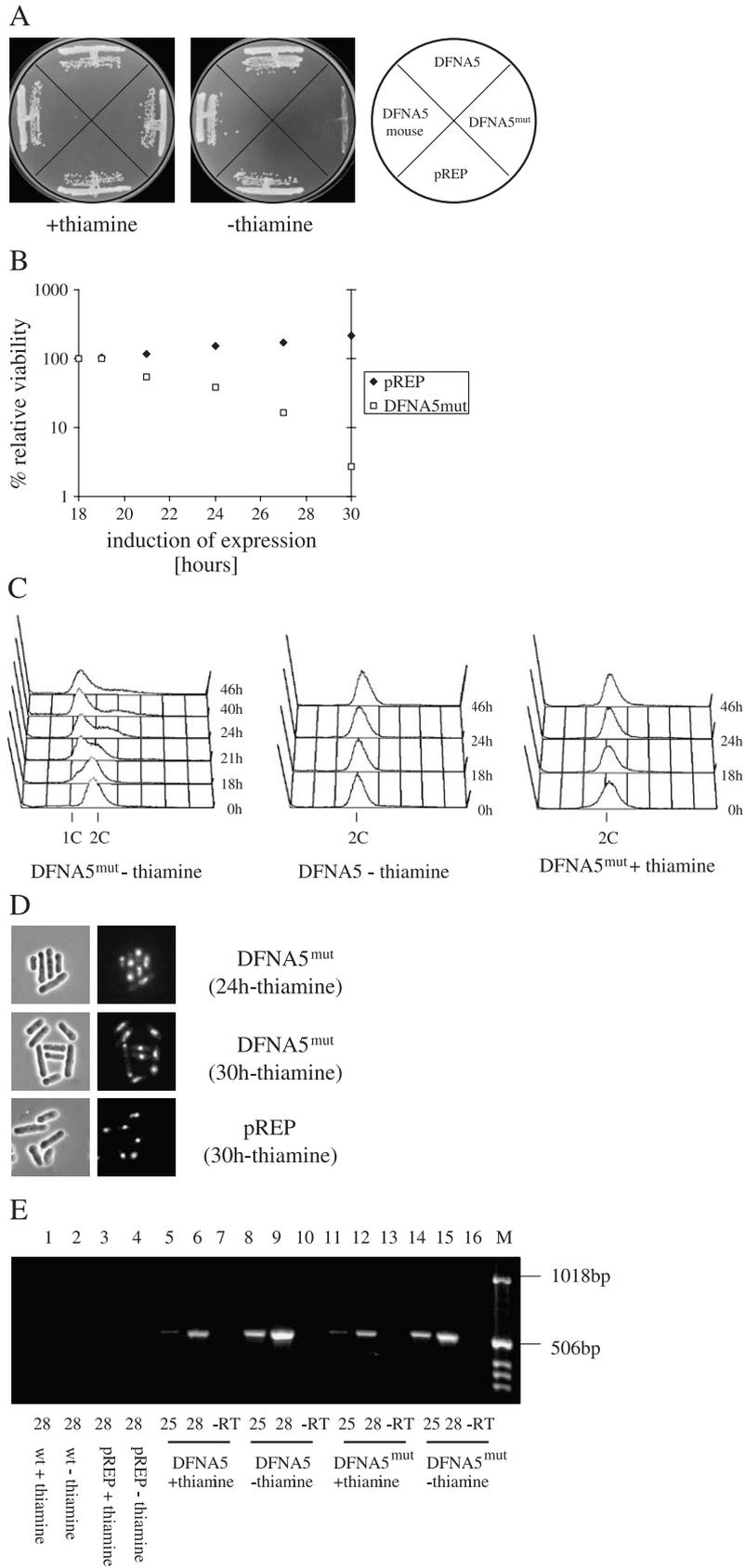
Only one partial cDNA clone resulted after screening 10<sup>6</sup> clones from a rat brain cDNA library (1515 bp; GenBank accession number AY194291). The rat brain library was rescreened using a rat-specific *DFNA5* probe. In addition, 5'-RACE experiments were performed on the rat brain library. However, we did not succeed in generating a full-length rat *DFNA5* clone. The partial rat *DFNA5* protein showed 90.7% similarity with mouse, 64.0% with human and 60.4% with horse *DFNA5* (Fig. 2). Both rat and horse *DFNA5* homologues share the domain of homology with the Mcm10 family (Fig. 1).

Nuclear localization has been shown experimentally for the human, *Xenopus*, *S. pombe* and *S. cerevisiae* Mcm10 proteins [16]. Subcellular localization of *DFNA5* proteins has not been determined yet, but both human *DFNA5* and mouse *Dfna5h* as well as horse *DFNA5* proteins share a stretch of positively charged amino acid residues (KKKR) near their N-termini, which resemble a basic core of the monopartite nuclear localization signal K(K/R)X(K/R) [17]. Although PSORT II prediction identified a "pat4" type of nuclear localization signal in all *DFNA5* homologues, it predicted the cytoplasmic localization of *DFNA5* proteins as the most likely [18]. Therefore, the subcellular localization of *DFNA5* proteins will need to be experimentally determined.

#### 3.2. Heterologous expression of *DFNA5* genes in yeast

To further characterize *DFNA5*, we have cloned wild-type alleles of the mouse *Dfna5h* and human *DFNA5* as well as the mutant *DFNA5* allele from *DFNA5* patients and expressed them in the fission yeast *S. pombe* under a control of regulatable *nmt1* promoter [19]. The *nmt1* promoter can be repressed in the presence of thiamine in the growth medium and it can be induced by removing the thiamine. While heterologous expression of the human and mouse *DFNA5* proteins did not result in any apparent phenotype, expression of the mutant *DFNA5* allele caused loss of viability of the yeast cells as assessed from growth on plates lacking thiamine (Fig. 3A). We also observed a reduction in

Fig. 3. Heterologous expression of the *DFNA5* proteins in fission yeast. (A) Wild-type strain (P138) transformed with either empty vector pREP3x (pREP), or construct containing mouse *Dfna5h*—pREP3x-m*DFNA5* (*DFNA5*<sub>mouse</sub>), or human wild-type *DFNA5*—pREP3x-h*DFNA5* (*DFNA5*) or mutant allele of human *DFNA5*—pREP3x-h*DFNA5*<sup>mut</sup> (*DFNA5*<sup>mut</sup>) were grown on EMM plates with or without added thiamine for 3 days at 32 °C. (B) Wild-type strains (P138) transformed with either empty vector pREP3x (wt-pREP) or construct containing mutant *DFNA5* allele—pREP3x-h*DFNA5*<sup>mut</sup> (*DFNA5*<sup>mut</sup>) were grown in EMM media without thiamine to induce expression of the mutant *DFNA5* gene for the indicated time. Aliquots were taken and plated onto YE3S plates to determine viability. Percentage of relative viability was calculated as (number of colonies at time = *t* since thiamine withdrawal)/100/(number of colonies at *t* = 18 h since thiamine withdrawal). Values represent averages of two experiments. (C) Flow cytometry analysis of the strains described for panel A. Aliquots for flow cytometry were taken at indicated time since thiamine withdrawal. (D) Light microscopy images of ethanol-fixed cells as described for panel A taken at indicated time since thiamine withdrawal. Left-hand panels show phase images and right-hand panels show DAPI staining of DNA. (E) RT-PCR assays detecting expression of wild-type and mutant alleles of the human *DFNA5* heterologously expressed in the yeast cells. RT-PCR assays were performed with total RNA isolated from the wild-type yeast strain P138 (wt) (lanes 1–2) or the P138 strain transformed with either empty vector pREP3x (pREP) (lanes 3–4) or pREP3x-h*DFNA5* construct (*DFNA5*) (lanes 5–10) or pREP3x-*DFNA5*<sup>mut</sup> construct (*DFNA5*<sup>mut</sup>) (lanes 11–16). Strains were grown in EMM medium with or without thiamine for 20 h. RT-PCR products obtained after 25 or 28 cycles of PCR amplification are shown. RT-PCR products are also seen under +thiamine conditions (lanes 5,6, 11,22) as the *nmt1* promoter is known to be leaky when repressed with thiamine. Control reactions after 28 cycles of PCR amplification where no reverse transcriptase was added (–RT) to exclude DNA contamination in the samples are shown. DNA size marker (1-kb DNA ladder, Invitrogen) is given in lane 17 (M).



viability in exponentially growing cells after transfer to medium lacking thiamine (Fig. 3B). Flow cytometry of exponentially growing wild-type haploid fission yeast cells

shows only peak corresponding to 2C DNA content. This is because cells enter S phase before cytokinesis is completed. Flow cytometry analysis showed that yeast cells expressing

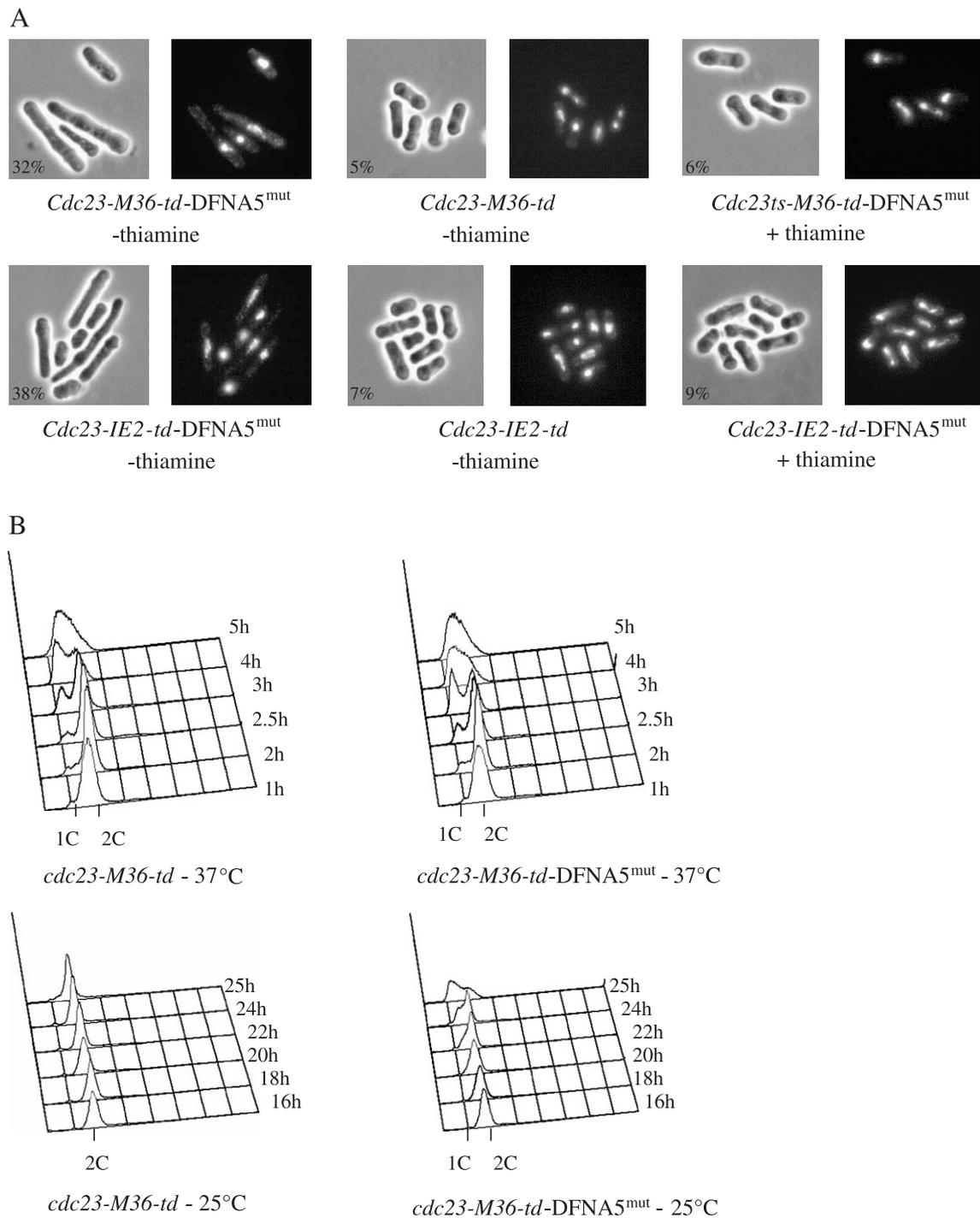


Fig. 4. Mutant *DFNA5* allele heterologously expressed in fission yeast has synthetic phenotype with *cdc23* mutant. (A) *cdc23-M36-td* and *cdc23-IE2-td* strains containing temperature-sensitive alleles of *cdc23* and transformed with either empty vector pREP3x (*cdc23-M36-td*, *cdc23-IE2-td*) or construct containing mutant *DFNA5* allele—pREP3x-h*DFNA5<sup>mut</sup>* (*cdc23ts-M36-td-DFNA5<sup>mut</sup>*, *cdc23-IE2-td-DFNA5<sup>mut</sup>*) were grown on EMM with or without thiamine at permissive temperature, 25 °C, for 24 h and fixed with ethanol. Left-hand panels show phase images and right-hand panels show DAPI staining of DNA. Percentage of elongated cells (longer than 15 μm) is shown. (B) Flow cytometry analysis of the *cdc23-M36-td* strain transformed with either empty vector pREP3x (*cdc23-M36-td*) or construct containing mutant *DFNA5* allele—pREP3x-h*DFNA5<sup>mut</sup>* (*cdc23ts-M36-td-DFNA5<sup>mut</sup>*). Strains were grown in EMM media without thiamine at permissive temperature (25 °C) for 16 h to induce expression of the mutant *DFNA5*. The cultures were then split and one half was shifted to nonpermissive temperature (37 °C) to inactivate the Cdc23 protein. Aliquots for flow cytometry were taken at indicated time.

the mutant *DFNA5* arrested in cell cycle with approximately 1C DNA content, suggesting that the cells were blocked in G1 or early S phase (Fig. 3C). Interestingly, this is the phenotype associated with *mcm10* mutants [5,8]. However, unlike many fission yeast mutants blocked in DNA replication, cells expressing mutant *DFNA5* did not elongate significantly (less than 5% of cells were elongated) and arrested cells did not show a “cut” phenotype where septation and/or cytokinesis occur in the absence of normal sister chromatid separation (Fig. 3D). We used an RT-PCR assay to confirm that both the wild-type and the mutant alleles of human *DFNA5* are expressed in the yeast cells upon induction (Fig. 3E).

### 3.3. Genetic interactions between *DFNA5* and *mcm10*

Following a possible link between *DFNA5* and *Mcm10* proteins, we tested the effect of *DFNA5* expression in a fission yeast *mcm10* homologue, the *cdc23* mutant. Neither human nor mouse *DFNA5* proteins were able to complement a fission yeast *cdc23* mutant (not shown), which is not surprising since the proteins do not share significant similarity outside of the CCCH domain. When mutant *DFNA5* was expressed in a *cdc23* temperature-sensitive degron mutant strains (*cdc23-M36-td*, *cdc23-IE2-td*), even at the permissive temperature there was a significantly higher number of elongated cells compared to strains not expressing the mutant *DFNA5* (Fig. 4A). Elongated cells indicate a cell cycle block, which uncouples growth from cell cycle events (e.g. block in chromosomal DNA replication). Moreover, when mutant *DFNA5* expression was induced in a *cdc23-M36-td* mutant and subsequently shifted to nonpermissive temperature, cells arrested with unreplicated DNA earlier than strains not expressing mutant *DFNA5* allele (Fig. 4B). Therefore, in fission yeast, heterologous expression of the mutant *DFNA5* allele has a synthetic phenotype with the *cdc23*.

## 4. Discussion

Here we describe the first model system for studying molecular effects caused by the mutation in the *DFNA5* gene, which was found to be responsible for nonsyndromic hearing impairment. Comparison of the wild-type and the mutant *DFNA5* alleles heterologously expressed in yeast allowed us to examine a phenotype associated with a dominant negative or gain of function effect of the *DFNA5* mutant. We provide several lines of evidence suggesting a possible link between the *DFNA5* and *Mcm10* proteins. Firstly, we identified a domain of homology shared between the *Mcm10* family of replication proteins and members of the *DFNA5* family, including the newly described horse and rat *DFNA5* homologues. The function of the CCCH domain remains obscure, but its importance is underlined by the fact that it is essential for the function of *Mcm10* proteins. In addition, this domain is absent in the dominant *DFNA5*

mutant resulting in hearing impairment. Secondly, we found that heterologous expression of the mutant *DFNA5* in fission yeast leads to a block in G1 or early S phase of the cell cycle with approximately 1C DNA content. Furthermore, expression of the mutant *DFNA5* in the yeast *cdc23* mutant has a synthetic growth defect and the arrest caused by *cdc23* inactivation is faster when the mutant *DFNA5* is induced. The observed effect could be either direct or indirect and it is possible that there are other currently unidentified cellular processes affected by the mutant *DFNA5* allele.

Could *DFNA5* have a function related to the DNA replication function of *Mcm10*? This cannot be strongly predicted since the proteins do not share significant similarity outside of the CCCH domain and it is unlikely that *DFNA5* is a distant homologue of *Mcm10* proteins. However, it is of interest that the reduction in *DFNA5* expression can contribute to the etoposide resistance in human melanoma cells. The etoposide-resistant phenotype may be due to an increased cellular susceptibility to drug-induced apoptosis [3]. Etoposide is used as an antineoplastic agent and causes DNA strand breakage via inhibition of DNA topoisomerase II. This could implicate *DFNA5* in some aspect of the response to DNA damage. Indeed, alterations in DNA repair proteins have been observed in drug-resistant melanoma lines [20,21]. Alternatively, *DFNA5* and *Mcm10* proteins may have unrelated functions, but share the CCCH domain to perform an activity unrelated to DNA replication. Further experiments are required to clarify the function of *DFNA5*. Our fission yeast model now provides a tool to examine the molecular basis underlying the *DFNA5* hearing impairment in human.

## Acknowledgements

This work was supported by a grant from the Cancer Research UK.

LVL holds a research position with the Flemish FWO (Fonds voor Wetenschappelijk Onderzoek). This research was supported by a grant of the GSKE (Geneeskundige Stichting Koningin Elisabeth) to GVC and LVL, and by a grant of the University of Antwerp to LVL.

The construction of the horse cDNA library was funded in part by The United Professional Horsemen's Association, The American Saddlebred Horse Association and The Belgian Draft Horse Corporation.

We thank Lynne Larkman for technical assistance.

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