IDENTIFICATION OF THE ROLE OF THE NUCLEAR MATRIX PROTEIN C1D IN DNA REPAIR AND RECOMBINATION

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BY
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ABSTRACT

IDENTIFICATION OF THE ROLE OF THE NUCLEAR MATRIX PROTEIN C1D IN DNA REPAIR AND RECOMBINATION

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P.hD in Molecular Biology and Genetics

Supervisor: Dr. Uğur Yavuzer

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The nuclear matrix protein C1D is an activator of the DNA dependent protein kinase (DNA-PK), which is essential for the repair of DNA double-strand breaks (DNA-DSBs). C1D is phosphorylated very efficiently by DNA-PK, and its mRNA and protein levels are induced upon γ-irradiation, suggesting that C1D may play a role in repair of DNA-DSBs *in vivo*. In an attempt to identify the possible biological functions of C1D and the nuclear matrix, two approaches were employed. One of the strategies was to apply yeast-two hybrid system to screen for polypeptides that interact with C1D. This screening revealed a number of cDNA clones that encode mainly proteins involved in recombination, DNA repair and transcription. Among the identified proteins, TRAX (Translin Associated protein X) was chosen for further analysis. Although, the biological function of TRAX remains unknown, its bipartite nuclear targeting sequences suggest a role for TRAX in the movement of associated proteins including Translin, into nucleus. After cloning the full-length TRAX ORF into bacterial and mammalian expression vectors, the specificity of the interaction between C1D and TRAX was confirmed both *in vivo* and *in vitro* conditions. Notably, it was shown that C1D and TRAX interact in mammalian cells only after γ-irradiation. In addition, it was demonstrated that the induced interaction of TRAX and C1D is not due to the alterations in their subcellular localizations but possibly through post-translational modifications in response to γ-irradiation.

The second strategy was identification of the *S. cerevisiae* C1D homolog. Because of the high homology between yeast and mammalian C1D proteins, *S. cerevisiae* was used as a model organism to reveal the function of Yc1d. *YC1D* gene was knocked-out in yeast and the phenotypic and functional consequences of disruption of the *YC1D* gene was analyzed. It was found that *yc1d* mutant strain was slightly sensitive to γ-irradiation and was defective in DNA-DSB repair, thus, raising the possibility that yeast C1D and human C1D might be functional homologs.

ÖZET

NÜKLEER MATRİKS PROTEİNİ C1D'NİN DNA TAMİR VE REKOMBİNASYONDAKİ ROLÜNÜN BELİRLENMESİ

TUBA ERDEMIR

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Nükleer matriks proteini C1D, DNA çift sarmal kırıklarının (DNA-DSBs) tamiri için gerekli olan, DNA-PK'nın aktivatörüdür. C1D'nin DNA-PK tarafından fosforlanması ve mRNA ile protein miktarının γ-radyasyonu sonucunda artması, *in vivo* koşullarda C1D'nin DNA-DSB'in tamirinde rol alabileceğini göstermektedir. C1D'nin ve nükleer matriksin olası biyolojik fonksiyonlarını tanımlamak amacıyla iki yaklaşımda bulunuldu. Birinci strateji, C1D ile etkileşen polipeptidleri maya-ikili hibrid sistemini uygulayarak taramaktı. Bu tarama sonucunda rekombinasyon, DNA tamiri ve transkripsiyonda rol alan proteinleri kodlayan bir grup cDNA klonu açığa çıkarıldı. Bulunan proteinler arasında TRAX (Translinle etkileşen X proteini), daha detaylı olarak incelenmek üzere seçildi. TRAX'ın biyojik fonksiyonu bilinmemesine rağmen, içerdiği ikili çekirdek hedefleme dizini sayesinde, aralarında Translin'in de bulunduğu proteinleri, hücre çekirdeğine taşıdığı düşünülmektedir. TRAX cDNAsının proteine kodlanan kısmının tümü, bakteri ve memeli ekspresyon vektörlerine klonlandıktan sonra, TRAX ve C1D arasındaki etkileşimin özgün olup olmadığı hem *in vivo* hem de *in vitro* koşullarda doğrulandı. Özellikle, memeli hücrelerinde C1D ve TRAX'ın ancak γ-radyasyonu sonrasında etkileşimde bulundukları gösterildi. Buna ek olarak, γ-radyasyonuna bağlı olarak oluşan TRAX ve C1D arasındaki bu etkileşimin, bu proteinlerin hücre içi lokasyonlarını değiştirmelerine değil, fakat oluşan olası bir post-translasyonel degişimden kaynaklandığı gösterildi.

İkinci strateji C1D'nin *S.cerevisiae* homoloğunu tanımlamaktı. Maya ve memeli C1D'si arasındaki yüksek benzerlikten ötürü, Yc1d'nin fonksiyonunu tanımlamak amacıyla, *S.cerevisiae* model organizma olarak kullanıldı. *YC1D* geni maya genomundan yok edilerek, bunun fenotipik ve fonksiyonel sonuçları araştırıldı. *yc1d* mutant maya neslinin γ-radyasyona karşı hafif bir duyarlılık göstermesi ve DNA-DSB tamirinde kusurlu olması, maya ve insan C1D'sinin fonksiyonel olarak da homolog olabileceklerini düşündürmektedir.

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ABBREVIATIONS

AD	Activation domain
APS	Ammonium persulfate
BME	beta mercaptoethanol
bp	Base pair
BS	Bloom Syndrome
BUR	Base unpairing region
cDNA	Complementary DNA
CFP	Cyan florescence protein
CPD	Cyclobutane pyrimidine dimers
C-Terminus	Carboxyl terminus
DBD	DNA binding domain
ddH_2O	Deionised distilled water
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxynucleic acid
DNA-PK	DNA dependent protein kinase
$DNA\text{-}PK_{cs}$	DNA dependent protein kinase catalytic subunit
dNTP	deoxynucleotide triphosphate
ds DNA	double stranded DNA
DSB	Double strand break
DTT	Dithiothretiol
EDTA	Diaminoethane tetra-acetic acid
EtBr	Ethidium Bromide
FCS	Fetal calf serum
g	gram
GFP	Green fluorescence protein
GST	Glutathione S-Transferase
HA	Heamaglutinin

hC1D	human C1D
hnRNA	Heterogenous nuclear RNA
HR	Homologous recombination
HRP	Horse Radish Peroxidase
Ig	Immunoglobulin
IR	Ionizing radiation
IVTT	In vitro transcription and translation
kDa	kilo Dalton
LacZ	β-galactosidase
LB	Luria-Bertoni media
Log	Logarithmic
LZ	Leucine zipper
MARs	Matrix attachment regions
MBR	Major breakpoint region
ml	milliliter
mM	millimolar
µg	microgram
µl	microliter
µM	micromolar
MMS	Methyl methane sulfate
mRNA	messenger RNA
MutLZ-TRAX	Leucine zipper motif mutant TRAX
NER	Nucleotide excision repair
ng	nanogram
NHEJ	Non-homologous end joining
NM	Nuclear matrix
N-terminus	Amino terminus
OD	Optical density
ONPG	o-nitrophenyl β-D-galactopyranoside
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phospate buffered saline
PCR	Polymerase chain reaction

PI3K	Phosphotidyl inositol-3 kinase
pmole	picomole
RFP	Red fluorescence protein
RMX	RAD50, MRE11, XRS2 complex
RNA	Ribonucleic acid
RNAse	Ribonuclease
RPA	Replication protein A
rpm	Revolutions per minute
Scid	Severe combined immunodeficiency
SDS	Sodium Dodecyl Sulfate
ss DNA	Single stranded DNA
SSA	Single-strand annealing
TBE	Tris-Borate-EDTA solution
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween 20
TE	Tris-EDTA solution
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
TRAX	Translin associated factor X
v	volt
WS	Werner syndrome
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
yc1d	Mutant yeast C1D gene
YC1D	Yeast C1D gene
Yc1d	Yeast C1D protein
YFP	Yellow fluorescence protein

INTRODUCTION

CHAPTER 1

1.1. DNA DAMAGE

DNA damage can be defined as any modification of DNA that alters its coding properties and leads to blockage of essential cellular processes such as transcripton and replication. Cellular responses to unrepaired DNA damage may include acute cytotoxicity, programmed cell death, mutation fixation, and increased recombination leading to genomic instability. Damage to genomic DNA occurs spontaneously and can be further enhanced by environmental mutagens such as ultraviolet (u.v.) light from the sun, inhaled cigarette smoke, or incompletely defined dietary factors. Large proportions of DNA alterations are caused by spontaneous damage, which occur with an estimated rate of $1\text{-}3x10^4$ per cell per day. DNA damaging agents of different types produce different kinds of lesions in DNA. Types of DNA damage can be divided into two main groups;

- *Single base modifications:* These are some minor base modifications or base replacements that might simply alter the coding sequence. This simple base substitution includes deamination, depurination, reaction of reactive oxygen species (ROSs), nucleotide analog incorporation, alkylation, insertion and deletions.

- *Damage that involve more than one base or, result in damage to the DNA backbone:* These are types of DNA damage that may distort DNA structure and interfere with replication or transcription. Ultraviolet light-induced thymine dimers, intrastrand and interstrand cross-linking between two bases, single strand or double strand breaks are also examples for this type of DNA damage.

If left unrepaired, DNA damage can have serious consequences for cells, causing mutations, which may lead to cancer formation or improper functioning of the encoded proteins. Therefore, for the faithful reproduction and preservation of the genomic integrity, damaged DNA must be repaired. Since there are many different types of DNA damage, it is not surprising that the repair system employs many different protein complexes. Approximately more than 100 genes participate in various aspects of DNA repair, even in organisms with very small genomes. As a result of analysis of the human genome sequence, considering evolutionary relationships and common sequence motifs in DNA repair genes, 130 human DNA repair genes have been identified (Wood et al., 2001) .

According to the mechanisms they employ in repairing different types of damage, they can be grouped into three.

- ***Direct reversal of DNA damage***: It is the simplest way to correct the damage. There are two well-studied proteins in this type of repair. One of them is cyclobutane pyrimidine dimers (CPD) photolyase, which reverts the covalent linkage of pyrimidine dimers in a light dependent reaction. CPD photolyases are found in bacteria, fungi, plants, and many vertebrates, but not in placental mammals. The other one is alkyl transferase that removes the methyl group from O^6-methylguanine. Alkyl transferase is present in all living organisms.

- ***Excision repair***: In cases where damage is present in just one strand, the damage can be accurately repaired by cutting the damaged DNA and replacing it with newly synthesized DNA using the complementary strand as a template. There are three types of excision repair. First one is mismatch repair, which repairs mismatched nucleotides and small loops. Second one is base excision repair that removes incorrect bases such as uracil or damaged bases like 3-methyladenine and the last one is nucleotide excision repair which removes bulky DNA adducts such as thymine dimers and 6-4 photoproducts. After the removal of the damaged region; DNA is repaired and re-ligated by proteins common to all three types of excision repair mechanisms.

- ***Double strand break repair:*** Among the other forms of DNA damage, double strand breaks (DSBs) are potentially the most dangerous, since they can lead to broken rearranged chromosomes, cell death, or cancer. Eukaryotes have evolved specific mechanisms to repair DSBs by either homologous recombination (HR), which requires the presence of homolog sequences or non-homologous end joining (NHEJ) by which the two ends of a DSB are joined by a process that is independent of DNA sequence homology (Nickoloff and Hoekstra, 1998).

1.2. DNA DOUBLE-STRAND BREAKS (DNA-DSBs)

DSBs are generated by exogenous agents such as ionizing radiation (IR) and certain chemotherapeutic drugs, or by spontaneously generated ROSs and mechanical stress on the chromosomes. They can also be produced when DNA replication forks encounter DNA single-stranded breaks or other types of lesions. They can occur at the termini of chromosomes due to defective metabolism of telomers. Additionally, they are generated to initiate recombination between homologous chromosomes during meiosis and also during V(D)J recombination, which is a process necessary for lymphocyte development (Critchlow and Jackson, 1998; Jackson and Jeggo, 1995; Khanna et al., 2001).

The presence of an unrepaired DSB will activate the DNA-damage response systems of a cell to arrest its progression through the cell cycle thereby, preventing possible chromosome loss or transmission of damaged DNA to the next cellular generation. The cell cycle checkpoints are believed to prevent the replication of damaged DNA (G1/S and intra S checkpoints) or segregation of damaged chromosomes (G2/M checkpoint). Once the damage is repaired, the cell cycle resumes. However if the level of DNA damage is beyond repair, DSBs can lead to programmed cell death (apoptosis). If a cell with an unrepaired DSB persists to divide, the broken chromosome ends can lead to chromosomal fragmentations, translocations and deletions, all of which can lead to carcinogenesis through activation of oncogenes, inactivation of tumour suppressor genes or loss of heterozygosity (Critchlow and Jackson, 1998; Kanaar et al., 1998). Notably, a large

proportion of cancers of lymphoid origin exhibit chromosomal rearrangements involving the immunoglobulin or T-cell receptor loci, indicating that they have arisen through inappropriate resolution of DNA DSBs corresponding to V(D)J recombination intermediates (Khanna and Jackson, 2001) .

1.3. PATHWAYS OF DSB REPAIR

To overcome deleterious effects of DSBs eukaryotes have evolved two main repair pathways, nonhomologous end joining (also called illegitimate recombination) (NHEJ) and homologous recombination (HR). Both mechanisms operate in all eukaryotic cells but the relative contribution of each mechanism varies. HR pathway requires extensive regions of DNA homology and repairs DNA accurately using information on the undamaged sister chromatid or homologous chromosome. On the contrary, DNA end-joining pathway uses no, or extremely limited sequence homology to rejoin juxtaposed ends in a manner that may not be error free. It seems that mammalian cells prefer NHEJ whereas HR is more common in the budding yeast *Saccharomyces cerevisiae.* One possible explanation for this preference in mammalian cells might be misalignment of repetitive DNA sequences that could lead to deletions and translocations. In addition to these two main mechanisms, DSBs can also be repaired by single strand annealing (SSA), which, can be considered as a sub-pathway of HR. In SSA; resection and annealing of short regions of complementary sequence initiates repair by SSA pathway in which ligation is preceded by the trimming of non-complementary single strand DNA tails (Karran, 2000).

1.3.1. Homologous Recombination

The yeast *S.cerevisiae* has been very useful in identification of genes required for DSB repair by HR. In *S.cerevisiae,* HR is performed by RAD52 epistasis group of proteins which have been identified mainly by yeast genetics using ionizing radiation hypersensitive mutants (Orr-Weaver and Szostak, 1985). Most of the

functional homologues of them have been isolated from mammalian species. According to the proposed model for HR (Figure 1) (Kanaar et al., 1998; Khanna and Jackson, 2001); DSBs generated by DNA damaging agents is processed to a single stranded region with 3'-overhang by an exonuclease and then Rad51 protein associates with 3' ssDNA-end and searches for the homologous duplex DNA to catalyse strand exchange in a reaction, which generates a joint molecule between homologous damaged and undamaged duplex DNA. In addition to Rad51 this step requires the coordinated action of Rad52, Rad54, Rad55/57 and the single strand binding protein RPA. DNA synthesis, requiring a DNA polymerase, its accessory factors and a ligase, then restores the missing information (Karran, 2000) .

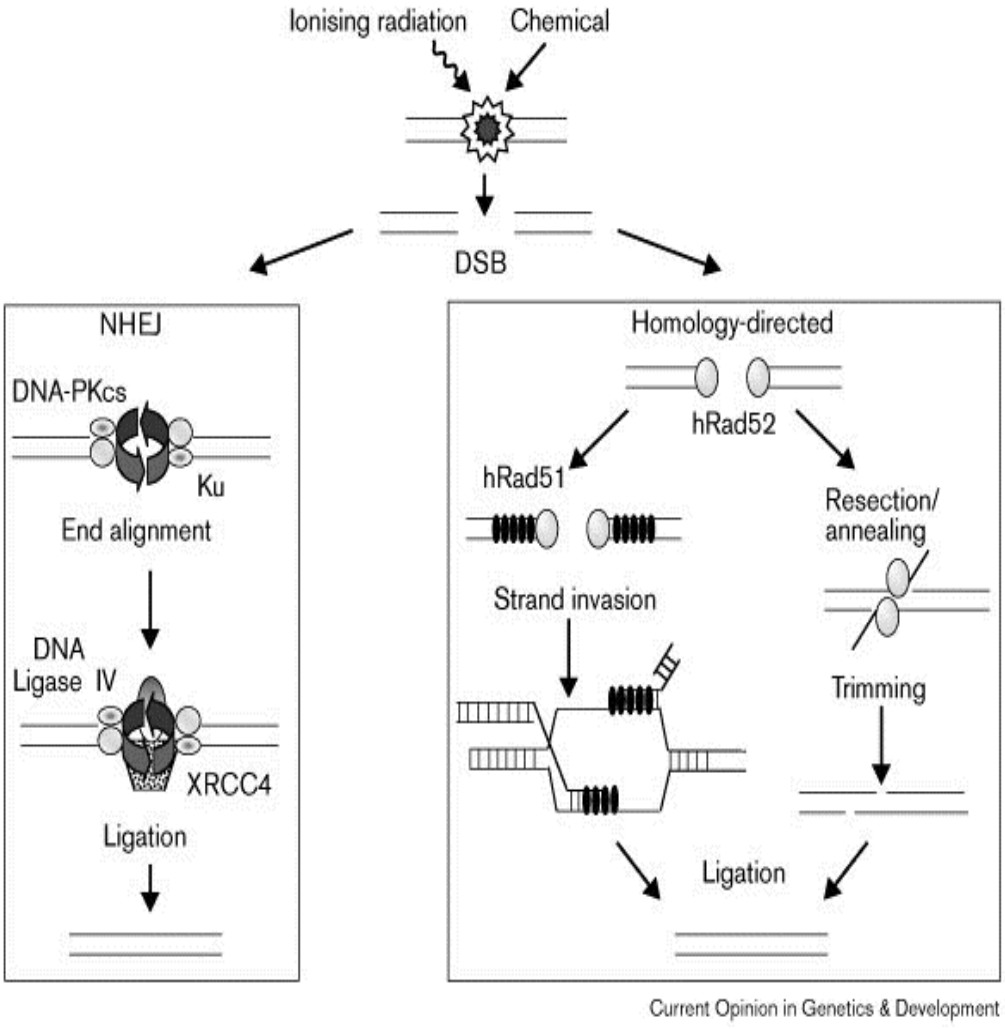


Figure 1: Pathways of DSB repair, (Karran, 2000)

Similar to yeast Rad51, mammalian Rad51 protein polymerizes on DNA to form nucleoprotein filaments that searches the homologous DNA and like yeast homologs, human Rad52 and Rad54 facilitate Rad51 strand invasion activity (Kanaar et al., 1998). Mammalian Rad51 also interacts with Brca1 and Brca2 proteins (products of breast cancer genes) (Haber, 1999), which are supposed to act as protein scaffolds to coordinate the actions of the HR machinery (Khanna and Jackson, 2001). Another protein that has been linked to HR in mammals is the protein kinase ATM, which is deficient in the cancer prone, radiosensitive, and the neurodegenerative syndrome ataxia telangiectasia (A-T). Upon IR, ATM phosphorylates proteins involved in HR such as Brca1 and c-Abl besides its other targets. The c-Abl tyrosine kinase modulates Rad51 strand exchange activity through phosphorylation (Khanna and Jackson, 2001; Rotman and Shiloh, 1999). The helicases defective in the human cancer predisposition and developmental disorders, Werner syndrome (WS) and Bloom syndrome (BS) have also been implicated in regulating HR through their ability to suppress NHEJ (Karow et al., 2000)

1.3.2. Single-Strand Annealing (SSA)

This pathway can be considered as a subpathway of HR and it doesn't involve formation and resolution of Holliday junctions. However, SSA relies on regions of homology with which to align the strands of DNA to be joined. *S.cerevisiae* genes, which define SSA, belong to the Rad52 epistasis group of HR. The hRad50, hMre11 and Nbs1 define the human SSA pathway. Mre11 has several activities, the most important of which may be 3-5' exonuclease activity which may remove damaged DNA to expose short lengths of single-stranded DNA. If a sister chromatid is available, then single stranded overhangs may initiate rejoining by homologous recombination. Alternatively, sites of limited homology within the resected region may anneal to begin repair by SSA. The single-stranded tails created by resection and annealing of overlapping ends are trimmed before ligation, which leads to loss of one of the intervening DNA (Fig 1) (Karran, 2000).

1.3.3. Non-Homologous End Joining

In contrast to HR, NHEJ is achieved without the need for extensive homology between DNA ends to be joined (Figure1). Many of the proteins involved in NHEJ have been identified in several ways such as biochemical fractionation of extracts capable of end joining reactions, genetic and biochemical analysis of the proteins required for DSB repair during V(D)J recombination and genetic analysis in yeast, rodent, and human cell lines. According to these analyses, DNA dependent protein kinase (DNA-PK) is found to be a key component of the NHEJ (Jeggo, 1997; Lees-Miller, 1996). DNA-PK is an abundant nuclear serine/threonine kinase that is activated in vitro by DNA double strand breaks and certain other discontinuities in the DNA double helix (Dvir et al., 1993; Gottlieb and Jackson, 1993).

Recently, several other yeast NHEJ components have been identified including the nuclease complex containing Rad50p, Mre11p, and Xrs2p (Boulton and Jackson, 1998; Milne et al., 1996). Most DNA DSBs are not blunt ended but have single-strand overhangs, which must be trimmed by exonucleases and/or endoucleases before rejoining. In yeast, Mre11p, Rad50p and Xrs2p complex seems to be involved in this trimming process. Mammalian cells have homologues of Rad50p and Mre11p but a different protein, Nbs1, seems to replace Xrs2p (Maser et al., 1997). Nbs1 (also called Nibrin) is a protein whose deficiency leads to a rare human genetic disorder called Nijmegan breakage syndrome (NBS) (Baumann and West, 1998; Carney et al., 1998). It is supposed that Nbs1 regulates function of Rad50p and Xrs2p through signaling the existence of DNA damage to the cell cycle checkpoint machinery. Repair by NHEJ is completed by the DNA ligase IV/ XRCC4 complex, of which yeast homologous are Lig4p and Lif1, respectively (Baumann and West, 1998; Featherstone and Jackson, 1999).

1.4. ROLE OF DNA-PK AND ITS COMPONENTS IN NHEJ

DNA-PK consists of the heterodimeric Ku proteins, of 70- and 80- kDa and a large catalytic domain of 470-kDa, termed DNA-PK_{CS} (catalytic subunit).

According to biochemical studies, the ser/thr kinase catalytic activity of DNA-PK was shown to be triggered upon association with DNA ends through its DNA targeting component, Ku 70/80 (Dvir et al., 1993; Gottlieb and Jackson, 1993). Although weak, DNA-PK_{CS} possesses an intrinsic DNA-end binding activity that is greatly stimulated and stabilized by Ku complex (Hammarsten and Chu, 1998).

DNA-PK_{CS} doesn't appear to have significant homology to other characterized proteins. However, the C-terminal region of DNA-PK_{CS} falls into phosphatidylinositol-3 kinase (PI3K) family and more specifically, DNA-PK_{CS} is most related to a subgroup of proteins in this family, which includes ATM, ATR, and TRRAP. These proteins have been shown to be involved in controlling transcription, cell cycle, and/or genome stability in organisms ranging from yeast to man (Hartley et al., 1995; Poltoratsky et al., 1995). Interestingly, they all have serine/threonine kinase activity rather than lipid kinase activity (Smith et al., 1999). DNA-PK also has an intrinsic phosphorylation activity. Autophosphorylation has an important role in regulation of DNA-PK activity by affecting dissociation of DNA-PK_{CS} from the Ku complex and results in inhibition of DNA-PK catalytic function (Chan and Lees-Miller, 1996). Upon activation, DNA-PK_{CS} phosphorylates a variety of proteins *in vitro* including itself, Ku complex, Sp1, c-Jun, c-Myc, p53, Hsp90, the RNA polymerase II subunit, RPA, XRCC4 and a recently identified nuclear matrix protein C1D (Anderson and Lees-Miller, 1992; Jackson, 1997; Leber et al., 1998; Lees-Miller, 1996; Smith et al., 1999; Yavuzer et al., 1998).

Ku was initially characterized as an antigen present in the sera of patients with autoimmune diseases (Mimori et al., 1981). Ku70 and Ku80 heterodimer binds, without sequence specificity, to a variety of DNA structures such as double stranded DNA ends, nicks, gaps, and hairpins, however, it doesn't bind to closed circular DNA (Dynan and Yoo, 1998). Another characteristic of Ku protein is its ability to translocate along the DNA molecules in an ATP independent manner (Paillard and Strauss, 1991). This ability of Ku is supposed to function transporting DNA-PK_{CS} to its physiologically relevant substrates. The cloning of cDNAs and genes for Ku subunits from a variety of species indicates that both Ku70 and Ku80 exist in organisms ranging from yeast to man (Dynan and Yoo, 1998).

The role of DNA-PK complex in NHEJ became evident through genetic complementation studies of rodent mutants that were shown to be sensitive to ionizing radiation and defective in repair of DNA DSBs and site-specific V(D)J recombination (Jeggo, 1997). Among the 11-complementation groups of IR-sensitive cells that were initially defined, 4 were defective in NHEJ (IR4, IR5, IR6, and IR7). It has been established that IR7 cells are deficient in DNA-PK_{CS}, whereas IR5 and IR6 cells are deficient in the Ku80 and Ku70 respectively. Subsequent work showed that IR4 cells are defective in Xrcc4, a small protein that forms a tight and specific complex with DNA ligase IV (Smith et al., 1999). In light of the above reports, proposed model is that Ku/DNA-PK_{CS} complex binds to DSBs and facilitates the recruitment and activation of other NHEJ components such as Xrcc4 and DNA ligase IV (Figure 1).

Mice deficient in various components of NHEJ have been generated to study NHEJ in mammals. According to these experiments, deficiency of any components of DNA-PK leads to multiple defects, including growth retardation, hypersensitivity to IR and severe combined immunodeficiency (Scid) due to severely impaired V(D)J recombination, which is an essential process during mammalian lymphoid cell development (Smith et al., 1999).

V(D)J recombination is a well characterized site-specific recombination in higher eukaryotes which occurs during the development of B and T cells. In this process, an enormous repertoire of immnunoglobulin and T-cell receptor genes is produced by bringing together variable (V), diversity (D), and joining (J) sub-exonic gene segments in various combinations (Oettinger, 1999). V(D)J recombination is initiated by the generation of blunt DNA-DSBs between recombination signal sequences and coding gene segments. Subsequently, these intermediates are resolved by the ligation of pairs of coding ends to form coding joints, and pairs of recombination signal sequence ends to yield recombination signal sequence joints (Jeggo et al., 1995). Since site specific DNA-DSBs are introduced during the process of V(D)J recombination, there is a mechanistic link between the repair of IR induced DNA damage and DNA DSBs formed during V(D)J recombination (Smith and Jackson 1999). Indeed, DNA DSB repair mutants of IR4, IR5, IR6 and IR7 can not perform V(D)J recombination effectively (Jackson and Jeggo, 1995).

So far, no mutations for DNA-PK_{cs} have been identified in any congenital human diseases, suggesting that defects in NHEJ are rare or cause embryonic lethality in humans. However, DNA-PK deficiency leads to increased rates of neoplastic transformation in mice. Mice mutant for DNA-PK_{CS} and/or Ku70 have a high incidence of T-cell lymphomas and especially Ku70 knockout mice have increased rates of fibroblast transformation (Jhappan et al., 1997). Fibroblasts from Ku80 knockout mice show chromosomal instability associated with chromosome aberrations, including breakage, translocations and aneuploidy. Notably, loss of tumour suppressor p53 in Ku80 -/- mice induces early onset of lymphomas (Difilippantonio et al., 2000). Tumors obtained from Ku80 and p53 double mutants display a specific set of translocations including the immunoglobulin heavy chain IgH/Myc locus that is reminiscent of Burkitt Lymphoma (Difilippantonio et al., 2000). Combined DNA-PK_{CS} and p53 deficiency also promotes similar chromosomal translocations (Vanasse et al., 1999). These reports suggest that chromosomal rearrangements in the double mutant mice primarily result from an impairment in the normal NHEJ pathway for resolving V(D)J recombination intermediates, which leads to chromosomal translocations with oncogenic potential. Interestingly, a radiosensitive human cell line, MO59J, derived from glioma was found to be defective in DNA-PK_{CS} expression and DNA-PK activity, indicating a role for DNA-PK in cancer formation (Lees-Miller et al., 1995).

Besides being a DNA-damage activated protein kinase, DNA-PK could have an important role in DNA damage signalling. It is hypothesised that DNA-PK activation in response to DNA damaging agents could trigger signalling pathways that result in apoptosis and cell cycle arrest. Consistent with this idea, a gatekeeper protein p53 that controls cellular proliferation and apoptosis in response to perturbations in genomic DNA, is an in *vitro* substrate of DNA-PK. DNA-PK phosphorylates p53 at serine 37 and 15 residues, and this has been reported to destabilise interactions between p53 and Mdm2, a protein that negatively regulates p53 in vivo by targeting it to ubiquitin mediated proteolysis (Kubbutat et al., 1997; Lees-Miller et al., 1990; Lees-Miller et al., 1992; Smith and Jackson, 1999). Two relatives of DNA-PK_{CS}, ATM and ATR, proteins that have been reported to have a role in DNA damage signalling (Durocher and Jackson, 2001), also phosphorylate

p53 on serine 15 *in vitro* (Canman et al., 1998; Khanna et al., 1998). All these reports suggest that DNA-PK may be involved in DNA damage signalling process through p53 phosphorylation that results in p53 protein stabilization.

1.5. NUCLEAR MATRIX INVOLVEMENT IN REGULATION OF DNA-PK AND DNA-DSB REPAIR

It is clear that DNA-PK plays a key role in DNA DSB repair, however, the molecular mechanism it employs during this process is not precisely understood. Although several proteins have been found to act as DNA-PK substrates *in vitro,* physiological targets of DNA-PK and factors that regulate its activity are not yet known.

In an attempt to identify these mechanisms, the yeast two-hybrid system was applied to screen a cDNA library to identify DNA-PK_{CS} interacting proteins. As a result of this screen, the nuclear matrix protein C1D was identified to interact specifically with DNA-PK_{CS} (Yavuzer et al., 1998). Moreover C1D mRNA and protein levels were found to be induced specifically upon γ-irradiation. C1D can be phosphorylated by DNA-PK but is different from all other DNA-PK substrates in the sense that, it can activate DNA-PK in the absence of free DNA ends. These findings suggested that C1D might act as an accessory protein to target DNA-PK to nuclear matrix constitutively. In support of this model, Ku80 deficient xrs5 cells were reported to have nuclear matrix and envelope alterations compared to their wild type controls (Korte and Yasui, 1993; Yasui et al., 1991). Recently, DNA-PK was shown to bind specific regions within matrix attachment regions (MARs) that exhibit a high binding affinity to nuclear matrix (Galande and Kohwi-Shigematsu, 2000). These specific regions are called base unpairing regions (BURs) that represent a specialized DNA context with an unusually high propensity for base un-pairing under negative superhelical strain. In addition to DNA-PK_{CS}, poly (ADP-ribose) polymerase (PARP), Ku70/80, and HMG1 that has been shown to stimulate DNA-PK activity *in vitro* (Watanabe et al., 1994), were identified as BURs binding proteins (Galande and Kohwi-Shigematsu, 2000). BURs were also found at major breakpoint region

(MBR) of the untranslated region of BCL2 gene, suggesting that the nuclear matrix plays a role in DSB repair and/or recombination (Ramakrishnan et al., 2000). Taken together, there is enough evidence to support the idea that the activity of DNA-PK *in vivo* could be influenced by the nuclear matrix. As being a nuclear matrix protein, C1D stands as a good candidate to regulate the *in vivo* function of DNA-PK.

1.6. NUCLEAR MATRIX PROTEIN C1D

The ~30,000 to 40,000 genes of a human being, encoded in 6x 10^9 base pairs of DNA of a total length of almost 2 meters, are packaged into a nucleus 6-8 micrometers in diameter (Varga-Weisz and Becker, 1998). Several levels of DNA compaction exist in eukaryotic nucleus (Figure 2). The DNA is packed into nucleosomes, which consist of a core of histones with 166 DNA base pairs wrapped around the core, and resulting chromatin is further compacted into 30 nm fibers (solenoids) and DNA loop domains. These loop domains are anchored to the proteinaceous nucleoskeleton, also called nuclear matrix or scaffold through matrix attachment regions (MARs) (Laemmli et al., 1978; Paulson and Laemmli, 1977). Therefore, nuclear matrix organizes DNA into a dynamic conformation to perform complex nuclear processes such as heterogenous nuclear RNA (hnRNA) synthesis and processing, replication, DNA repair and recombination (Nelson et al., 1986).

Nucleus is a complex organelle containing subnuclear domains that compartmentalizes different nuclear processes. The nuclear matrix is the skeletal framework of the nucleus consisting of the nuclear-pore lamina, residual nucleoli, a network of proteins, DNA and RNA (Nickerson et al., 1995) (Figure 3). NM proteins are non-histone proteins that comprise of 10-25 % of total nuclear protein (Berezney et al., 1995). They are insoluble proteins with strong DNA affinity and they remain associated with genomic DNA, especially with highly repetitive DNA sequences, even after treatment of harsh denaturants (Werner and Neuer-Nitsche, 1989). Phosphodiester bonds between NM proteins and DNA was found to be responsible for this strong interaction (Juodka et al., 1991; Neuer et al., 1983).

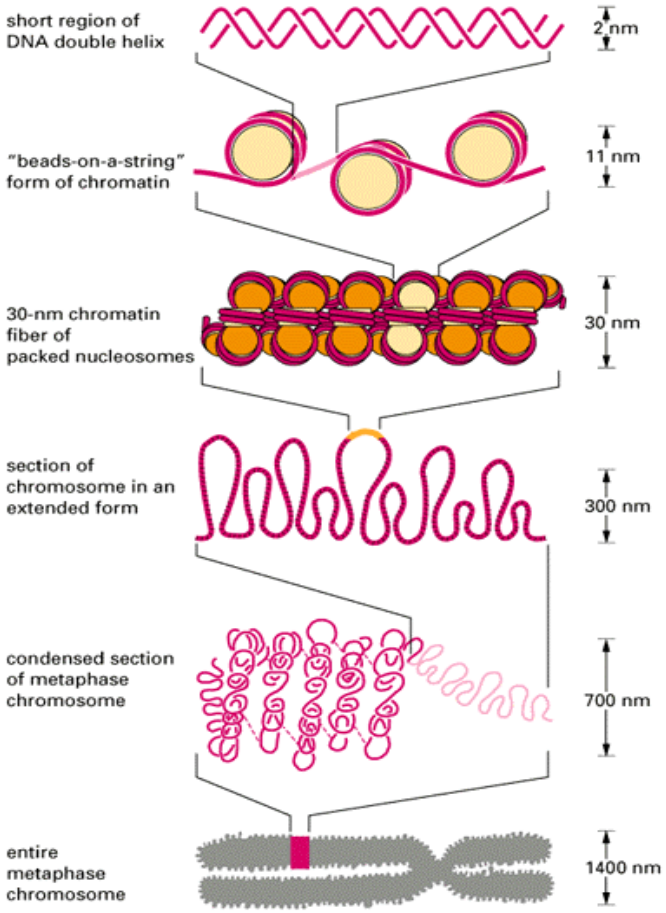


Figure 2: The levels of DNA organization in eukaryotic nucleus

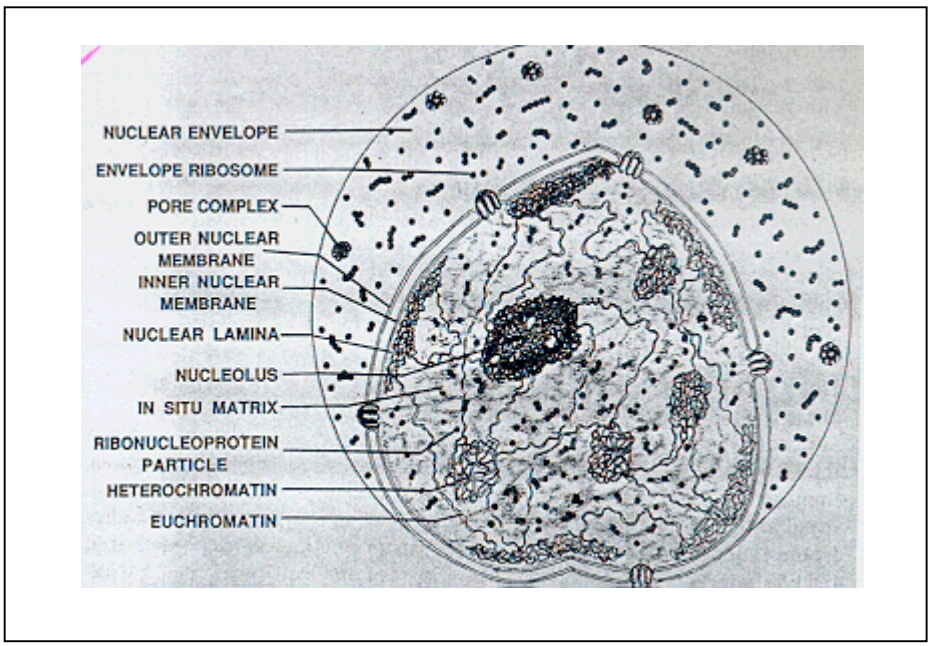


Figure 3: Schemetic view of nucleus

C1D is the first nuclear matrix protein characterized at the sequence level. The cDNA encoding C1D (human HC1D, X95592; 1995) was initially characterized in an assay to identify polypeptides released from rigorously extracted and nuclease treated DNA (Nehls et al., 1998).

In an independent research, C1D was found to be associated with the transcriptional repressor RevErb and the nuclear corepressors N-cor and SMRT (Zamir et al., 1997) suggesting that it could also be involved in transcriptional repression, possibly by recruiting the receptors and repressors to the nuclear matrix

C1D is physiologically expressed in 50 human cell tissues tested that points out its basic cellular function. However, cellular C1D expression is tightly regulated since elevated levels of C1D protein, which was induced by transient vector dependent expression, results in apoptotic cell death in cell lines with functional p53 (Rothbarth et al., 1999).

All these findings suggest that C1D is a multifunctional protein required for the maintenance of the genomic integrity by regulating proteins involved in DNA

repair, recombination or transcription. If the damage is beyond repair, C1D then induces apoptosis thereby protecting the organism from the consequences of unrepaired DNA damage.

Cellular proliferation and growth must be controlled tightly since loss of this control can lead to cell death or tumorigenesis. Any changes in uncontrolled cellular proliferation are reflected by changes in complex nuclear processes such as transcription, DNA replication, DNA repair-recombination and apoptosis. As nuclear matrix provides a platform for the assembly of protein machines involved in complex nuclear processes, components of the nuclear matrix could be important targets of tumorigenesis. Aberrant expression of nuclear matrix proteins in cancer cells (Yanagisawa et al., 1996) and tight association of nuclear oncoproteins with nuclear matrix (Deppert et al., 2000; Galande and Kohwi-Shigematsu, 2000) are the recent reports that postulate the importance of nuclear matrix in epigenesis of cancer.

The nuclear matrix protein C1D has been identified rather recently and the precise biological functions of this protein are not known yet. However, recent findings suggest that C1D may play a role in maintenance of genomic integrity. We believe that the identification of biological function of C1D will provide important clues in understanding the role of nuclear matrix in etiology of cancer. Therefore, the scope of this project is to identify the biological role of C1D in preservation of genomic integrity. To achieve this aim, two strategies were employed;

First strategy was to identify C1D interacting proteins through yeast two-hybrid screen and characterize the biological significance of these interactions. The involvement of C1D in several nuclear processes indicates that it may be a multi-functional protein. Since protein-protein interactions are critical to virtually every cellular process and also for nuclear matrix associated processes, it is highly possible that C1D performs its functions through associating with other proteins apart from DNA-PK. Identification of these proteins will not only provide important clues about the biological function of C1D protein, but also clarify functions of nuclear matrix as well.

Second strategy was identification of the function of *S.cerevisiae* C1D by deleting the C1D gene and analysing the effects of this deletion. Because most of the

important genes and cellular events are highly conserved throughout evolution, the results obtained using *S.cerevisiae* will be relevant to the functions of human C1D.

MATERIALS AND METHODS

CHAPTER 2

2.1. BACTERIAL STRAINS

Strain	Genotype	Usage	Source
M15 (pREP4)	F^-, Nal^S, Str^S, rif^8, lac^-, ara^-, gal^-, mtl^-, $recA^+$, uvr^+	purification of recombinant proteins with pQE vectors	QIAGEN
DH5α	F- supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1 deoR lambda-	a $recA^-$ host for propagation and storage of plasmids	Cold Spring Harbour Labs.
HB101	F- supE44 lacY1 ara-14 galK2 xyl-5 mtl-1 leuB6 proA2 delta(mcrC-mrr) recA13 rpsL20 thi-1 lambda	leu deficient strain used to rescue library plasmids	D.E. Ish Horowicz

Table 1: List of *E.coli* strains used during the course of this study

M15 stain contains multiple copies of pREP4 (kanamycin resistance marker) plasmid, which carries the *lacI* gene encoding the lac-repressor (details could be obtained from QIAGEN catalogue).

2.1.1. Growth and Maintenance of Bacteria

Bacterial strains were stored in 50% glycerol at -70^0C for long-term storage. Strains grown in LB (with or without antibiotic) to mid-log phase or saturation was mixed with sterile glycerol with a ratio of 1:1, mixed to homogeneity, and stored at -70^0C until required. Bacteria was recovered by scraping a small amount of cells from the frozen stock with an inoculation loop and streaking onto a LB-agar plate (supplemented with the appropriate antibiotics if necessary).

Liquid culture of plasmid carrying *E.coli* was performed in LB (5 g NaCl, 10 g Bacto-tryptone, 5 g Yeast Extract, 1 ml of 1 M NaOH, ddH_2O added to 1 L) with appropriate antibiotic selection. Liquid cultures were constantly agitated in a rotary shaking incubator (~200 rpm) at 37^0C. The specialised components of all media were obtained from Difco Laboratories Ltd.

2.2. YEAST STRAINS

Strain/Acc. No.	Genotype	Usage	Source
FY1679	Mata/α;ura3-52/ura3-52; trp1Δ63/TRP1;leu2Δ1/LEU2; his3Δ200/HIS3; GAL2/GAL2	Diploid wild type used to analyse function of Yc1d	**EUROSCARF**
FY1679-08A	Mata;ura3-52;leu2Δ1;trp1Δ63; his3Δ200; GAL2	Haploid wild type used to analyse function of Yc1d	**EUROSCARF**
YO1909	BY4741;Mata;his3Δ1;leu2Δ0; met15Δ0;ura3Δ0; YHR081w::kanMX4	Haploid *yc1d* mutant used to analyse function of Yc1d	**EUROSCARF**

Strain/Acc. No.	Genotype	Usage	Source
Y11909	BY4742;Matα;his3Δ1;leu2Δ0; lys2Δ0;ura3Δ0; YHR081w:kanMX4	Haploid *yc1d* mutant used to analyse function of Yc1d	**EUROSCARF**
Y21909	BY4743;Mata/α;his3Δ1/his3Δ1; leu2Δ0/leu2Δ0;lys2Δ0/LYS2; MET15/met15Δ0;ura3Δ0/ura30; YHR081w::kanMX4/YHR08w	Heterozygous diploid (*YC1D/yc1d*) mutant used to analyse function of Yc1d	**EUROSCARF**
Y31909	BY4743;Mata/α;his3Δ1/his3Δ1; leu2Δ0/leu2Δ0;lys2Δ0/LYS2; MET15/met15Δ0;ura3Δ0/ura30; YHR081w::kanMX4/YHR08w::kanMX4	Homozygote diploid mutant (*yc1d/yc1d*); used to analyse function of Yc1d	**EUROSCARF**
Y00870	BY4741;Mata;his3D1;leu2D0; met15D0;ura3ymr284w::kanX4	Haploid *yku70* mutant used as a control for various yeast assays.	**EUROSCARF**
Y00540	BY4741;Mata;his3D1;leu2D0; met15D0;ura3YML032c::kanM4	Haploid *rad52* mutant used as a control for various yeast assays.	**EUROSCARF**
W303	Matα;ade2-1;his3-11,15;leu2-3,112;trp1-1;ura3	Haploid wild type; used to knockout *YC1D*	**Soo-Hwang Teo**

Strain/Acc. No.	Genotype	Usage	Source
yc1d	Matα;ade2-1;yc1d::HIS3;leu2-3,112;trp1-1;ura3	Haploid *yc1d* mutant; used to analyse function of YC1D	**Constructed for this study**
***Yc1d-yku70* double mutant**	BY4741;Mata;yc1d::HIS3; leu2D0;met15D0; ura3ymr284w::kanMX4	Haploid *yc1d/yku70* double mutant; used to analyse function of Yc1d	**Constructed for this study**
AMR70	Matα;his3;lys2;leu2;URA3:: $(lexAop)_8$-lacZ GAL4 gal80	Yeast Two Hybrid Assay	**Hollenberg et al., 1995**
L40	Mata;his3Δ200;trp1-910; leu2-3,112; ade2;LYS2::$(lexAop)_4$-HIS3;URA3::$(lexAop)_8$-lacZ GAL4 gal80	Yeast Two Hybrid Assay	**Hollenberg et al., 1995**

Table 2: List of *S.cerevisiae* strains used during the course of this study

2.2.1. Growth And Maintenance Of Yeast

Yeast strains were stored in YPAD (10 g Yeast Extract, 20 g Peptone and 0.1g Adenine dissolved in 900 ml ddH_2O, sterilized by autoclaving, cooled to ~ 55^0C and 100 ml of 20% filter-sterilized glucose was added) rich medium with 50% glycerol and kept at -70^0C for long-term storage. Transformed yeast strains were stored in the appropriate minimal YC (1.2 g Yeast Nitrogen Base without amino acids, 5 g Ammonium Sulfate, 10 g Succinic Acid, 6 g NaOH and 0.1 g of all amino acids apart from Histidine, Uracil, Tryptophane and Leucine dissolved in 900 ml ddH_2O, and autoclaved) medium to keep selective pressure on plasmid. Yeast cells

were recovered by scraping a small amount of cells from the frozen stock with an inoculation loop and streaking them onto YPAD- or (appropriate YC) agar plates. Transformed yeast strains were plated on selective YC-agar (20 g agar was added to 900 ml YC medium and sterilized by autoclaving) plates lacking specific amino acids that were expressed by yeast plasmid. Yeast extract, Yeast Nitrogen Base and Peptone were purchased from DIFCO Laboratories Ltd. and the rest of the components of yeast media were purchased from SIGMA.

Liquid cultures of yeast were prepared by inoculating one large colony per 5-ml medium and incubating the culture at 30^0C for 16-18 hr with shaking at 230-270 rpm. For mid-log phase liquid culture, enough volume of overnight culture was inoculated into fresh medium to give an OD_{600} of 0.2-0.3 and the culture was incubated at 30^0C for 3-5 hours with shaking till it reached to an OD_{600} of 0.5-0.8. The density of cells in a culture was determined by measuring its optical density (OD) at 600 nm using spectrophotometer (Beckman Instruments Inc., CA, USA). 0.1 OD_{600} value corresponds to $0.5x10^7$ and $0.25x10^7$ cells/ml for haploid and diploids strains, respectively.

2.3. MAMMALIAN CELLS

COS7 cells derived from kidney of SV40 transformed African green monkey was used (ATCC CRL-1651). They were maintained at DMEM (SIGMA) supplemented with 10% Foetal Calf Serum, 1% Streptomycin/Penicillin (both from SIGMA) and 2% Fungiozone (GIBCO). The cells were kept in air supplemented with 5% CO_2 in a humidified incubator at 37^0C.

2.4. OLIGONUCLEOTIDES

2.4.1. Synthesis of Oligonucleotides

The oligonucleotides used for cloning and sequencing purposes were synthesized at the Beckman Oligo 1000M DNA Synthesiser (Beckman Instruments Inc. CA, USA) at Bilkent University, Department of Molecular Biology and Genetics (Ankara, Turkey). The longer oligonucleotides used in yeast assays and knockout experiments were either purchased from IONTEK (Bursa, Turkey) or were provided by MCRI (Oxted, U.K.)

2.4.2. List of Oligonucleotides Used in This Study

- ***Sequencing Primers:***

TC187 pACT Forward Primer
5' TATCTATTCGATGATGAAGAT 3'

TC 238 T7-myc-pLink Forward Primer
5′ CAG AAG CTG ATC TCC GAG 3′

TC239 pGEXTK2 Forward Primer
5' CCAAAATCGGATCTGGTT 3'

TC241 pQE30 Forward Primer
5′ GAA TTC ATT AAA GAG GAG AAA 3′

TC240 pCMV-HA Forward Primer
5' GACGTCCCTGATTATGCA 3'

- ***Primer pairs used for PCR and cloning***

Full-Length TRAX
TC234 **F** 5′ AGACGTCGACAAGGATCC ATG AGC AAC AAA GAA GGA TCA 3′
SalI **BamHI**

TC235 **R** 5′ AGACGTCGACGGATCC CTA AGA AAT GCC CTC TTC 3′
SalI **BamHI**

N-Terminal TRAX
UY29 **R** 5' AGAC**GGATCCCTCGAG**TTAAGCTTCCACATATTCCTG 3'
BamHI **XhoI**

MutLZ TRAX

UY30 **R** 5' AGACGGATCCCTCGAGTTAAGATCTAATTTCTGATTCAGTAAATAT 3'
BamHI XhoI Bgl II

UY31 **F** 5' AGACAGATCTGATGGTGTCAGACAAAAGATA 3'
Bgl II

Full-Length Translin

TC236 **F** 5' AGACGTCGACAAGGATCC ATG TCT GTG AGC GAG ATC TTC 3'
SalI BamHI

TC237 **R** 5' AGACGTCGACGGATCC CTA TTT TTC AAC ACA AGC 3'
SalI BamHI

Full-Length Human C1D

UY48 **F** 5' AGACAGATCTATGGCAGGTGAAGAAATTAATGAAGAC 3'
Bgl II

UY49 **R** 5' AGACAGATCTTTAACTTTTACTTTTTCCTTTATTGGCAAC 3'
Bgl II

- ***Primers Used in Yeast Based In-vivo Recombination Assay***

Oligonucleotide used for "GV256 Dead"

5' CTGACTGAGTGAAGATCTTCACTCAGTCAGGAGCT 3'
STOP STOP STOP Bgl II SacI

Primer pair used to amplify 800 bp region of LacZ

F 5' AGACGGATCCTCCTTTGCGAATACGCCCAC 3'
BamHI

R 5' AGACGAATTCTGTGAAAGAAAGCCTGACTG 3'
Eco RI

- ***Primers Designed to Knockout YC1D***

***YC1D* Disruption Cassette and PCR confirmation**

F[5']CAAAGCGGCAACGTCATAACCTTGGTATTTATTGGGCAA**CGTTTTAAGAGCT TGGTGAGC**[3']

R[5']CAAAAGTGTTCACTGCCAACTACAAGAATAGCATATACACAT**TCGAGTTCAA GAGAAAAA**[3']

R [5']CGGATCCCGTAGAAATGCTTTTGCCAAGG [3']

2.5. RECOMBINANT DNA TECHNIQUES

2.5.1. Polymerase Chain Reaction

Target DNA sequences between a pair of oligonucleotide primers were amplified by using either thermostable DNA Polymerase of *T. aquaticus* (Taq)(MBI) or *Pfu* DNA polymerase (recombinant enzyme expressed and purified in bacteria, was a gift from Emine Ercikan Abali). A typical 50 µl PCR reaction contained 10-100 ng of target DNA, 16 pmol of each primer, 2mM of each dNTP (200mM) (MBI Fermentes; Cat. No.R0181), 3 mM $MgCl_2$ (25 mM) (SIGMA), 1X Taq Pol buffer (SIGMA), and 0.5-1 units *Taq* DNA polymerase (SIGMA; Cat. No. D-4545). In cases, where longer DNA segments (>1kb) were amplified, 1 µl *Pfu* DNA polymerase was used with 1X Pfu Buffer (10x; 20mM Tris-HCl (pH: 8.0, 100mM KCl, 60 mM $(NH_4)_2SO_4$, 1 % Triton X-100, 100µg/ml nuclease free BSA). The reaction was preheated to 94^{o}C for 4 minutes and then subjected to 25-30 cycles of denaturation (0.5 min. at 95^{0}C), annealing (1-2 min. at 5^{0}C below the estimated T_m of the primers, which is generally between 48-55^{0}C) and synthesis (1-2 min. at 72^{0}C), in an automated thermal cycler (Perkin Elmer Gene Amp PCR System 9600). The product was assessed by gel electrophoresis and EtBr staining.

The PCR product was purified either by phenol/chloroform extraction and ethanol precipitation (Ausubel et al., 1987) or by gel extraction kit (Nucleospin-Macherey-Nagel; Cat.No. 740590) according to manufacturer's instructions. The PCR products were digested with the appropriate restriction enzymes overnight and ligated into plasmids accordingly.

2.5.2. DNA Purification From Agarose Gel

The desired DNA bands were cut from the agarose gel under UV illumination and DNA was extracted from agarose gel slices by Gel extraction kit, Nucleospin extract (MN 740590), according to the manufacturer's instructions.

2.5.3. Quantification of DNA

Concentrations and purity of nucleic acids were determined by measuring absorbency at 260nm and 280nm in a spectrophotometer (Beckmann PU640 Instruments Inc., CA, USA). The ratio between absorbance values at OD_{260} and OD_{280} was taken. Nucleic acid samples displaying OD_{260} and OD_{280} values in the range of 1.8 to 2.0 are regarded as highly pure. The concentration of DNA was determined automatically by using oligo/DNA quantitation program of BECKMAN spectrophotometer.

2.5.4. DNA Sequencing

The automated sequencing of the constructs was carried out on a ABI Prism 377 sequencer by Birsen Cevher at Bilkent University, Department of Molecular Biology and Genetics (Ankara, Turkey).

2.5.5. Enzymatic Manipulation of DNA

2.5.5.1. Restriction Enzyme Digestion of DNA

Restriction enzyme digests of DNA were performed in 20-50 μl reactions that contained 5-10 units of enzyme/1 μg of DNA with the appropriate buffer. The digests were conducted at 37^0C for 1-5 hours, depending to the amount of DNA to be restricted. Restriction enzymes were purchased from MBI, ROCHE and GIBCO.

2.5.5.2. Dephosphorylation of DNA Ends

Shrimp Alkaline phosphatase (SAP- Roche, Cat. No. 1 758 250) was used for dephosphorylation of either staggered or blunt-ended DNA ends to prevent religation of plasmid DNA. After digestion of vector DNA with appropriate restriction enzymes, up to 1pmol 5'-terminal phosphorylated DNA fragments (either 5' protruding or 5' recessive ends) were incubated with 1 unit of SAP at 37^oC for 1 hr. SAP was inactivated at 65 ^{o}C for 15 min and used for ligation.

2.5.5.3. DNA Ligation

The DNA to be ligated was purified by phenol extraction, ethanol precipitated and resuspended in ddH_2O. The vector was dephosphorylated with SAP as mentioned above. Prior to ligation, vector and insert concentrations were checked by agarose gel electrophoresis, and a 1:4 vector:insert ratio was maintained in the ligation reactions. Ligations were performed in 15 μl reaction volumes containing 0.1 μg plasmid DNA and a molar excess of insert DNA in the presence of 1-4 Weiss units of T4 DNA ligase (Roche: Cat. No. 481220) with standard ligation buffer supplied by the manufacturer. The reactions were incubated either at room temperature for 4-6 hours or at 16^oC overnight.

2.5.5.4. Repairing of 5'-Overhanging Ends to Generate Blunt Ends

Klenow fragment was applied to convert 5'-extensions to blunt ends that were required in cloning experiments. 0.1-4 μg DNA was digested with restriction enzymes that produced 5'-overhangs, and was purified with phenol/chloroform

extraction followed by ethanol precipitation. A 20 µl reaction contained 2 µl dNTP (0.5 mM each), 2 µl Klenow 10x buffer, 2 µl Klenow enzyme (STRATAGENE; Cat. No. 600071), 0.5 µl DTT (1 M stock), 0.5 µl BSA (10 mg/ml). The reaction was incubated at 37^0C for 1 hour and stopped by heating at 75^0C for 15 minutes.

2.5.6. Preparation of Plasmid DNA

2.5.6.1. Introduction of Plasmid DNA into E.Coli Cells Using $Cacl_2$

A single colony of appropriate *E.Coli* strain was inoculated into 5 ml LB and the culture was incubated at 37^0C, 250 rpm overnight. The saturated culture was diluted in 50 ml LB to an OD_{600} of 0.2-0.3 and grown at 37^0C incubator with shaking at 250 rpm until the OD_{600} reached to 0.6-0.7. The cells were pelleted by centrifugation at 2500 g, at 4^0C for 10 minutes, resuspended in 40 ml ice cold 100 mM $CaCl_2$ and incubated on ice for 1 hour. The cells were pelleted as above and resuspended in 2 ml ice cold $CaCl_2$ (Maniatis et al., 1982). The competent cells prepared by this method were either used immediately or stored at 4^0C overnight.

DNA (less than 100 ng) was added to 150 µl competent cells in round bottom tubes, and incubated on ice for 30 minutes with occasional flicking. The cells were then exposed to a 90 second heat shock at 42^0C, chilled on ice for 1-2 minutes, 800 µl LB was added and incubation continued at 37^0C for one hour. The culture was then pelleted, resuspended in 100 µl LB, and was spread on L-agar plates supplemented with 50 µg/ml ampicillin and/or 25 µg/ml kanamaycin and incubated overnight at 37^0C.

2.5.6.2. High Efficiency Transformation with Quick and Simple Method

A single colony of appropriate *E.coli* strain was inoculated into 5 ml LB and culture was incubated at 37^0C shaker at 250-rpm overnight. 250 ml of SOB (0.5% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, sterilised by autoclaving) was inoculated with appropriate *E.coli* strain, grown to an OD_{600} of 0.6 at 18-25^0C shaker at 250 rpm. The culture was chilled on ice for 10 minutes, centrifuged at 2500 g for 10 minutes at 4^0C. The pellet was then

resuspended in 80 ml of ice-cold TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7, sterilised by filtration through 0.45 μm filter, stored at 4^0C), and incubated on ice for 10 minutes. The mixture was pelleted as above, resuspended gently in 20 ml TB and 7% DMSO was added, mixed gently and incubated on ice for 10 minutes. Aliquots of this mixture were then immediately chilled in liquid nitrogen, and stored in liquid nitrogen for up to 6 months without a significant loss in transformation efficiency.

For transformation with supercompetent cells, an aliquot was thawed at room temperature. 200 μl of the cells was dispersed to round bottom tubes and mixed with plasmid DNA (less than 100 ng) and the mixture was incubated on ice for 30 minutes. The cells were then exposed to a 30-second heat shock at 42°C, chilled on ice for 1-2 minutes, 800 μl SOC (SOB with 20 mM glucose) was added and incubation continued at 37°C for one hour with vigorous shaking at 250 rpm. The culture was then pelleted, washed with 1 ml LB and centrifuged again, resuspended in 100 μl SOC, and was spread on L-agar plates supplemented with 50 μg/ml ampicillin and/or 25 μg/ml kanamaycin and incubated overnight at 37°C (Inoue et al., 1990).

2.5.7. Plasmid DNA Purification

2.5.7.1. Small Scale Isolation (Alkaline Lysis Miniprep)

Small-scale preparation of plasmid DNA was performed by standard methods based on NaOH/SDS cell lysis and potassium acetate precipitation of cellular debris (Maniatis et al., 1982).

Cells were harvested by centrifugation (13,000 rpm, 1 minutes, at room temperature) from a 1.5 ml overnight culture of bacteria carrying the plasmid of interest. Following resuspension of the bacterial pellet in 100 μl solution I (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA), 200 μl solution II (200 mM NaOH, 1% SDS) and 150 μl solution III (3M potassium acetate, 11.5% acetic acid glacial) was added, mixed and then protein was extracted with an equal volume of

phenol:chloroform 1:1. The phases were separated by centrifugation (13,000 rpm, 5 minutes at room temperature) and the DNA was precipitated from the aqueous phase by addition of 1 ml cold absolute ethanol. After washing once with 70% ethanol, the pellet was dried and resuspended in 30 µl ddH_2O including 10µg/ml DNase-free RNase A to destroy the RNA in the product.

2.5.7.2. Medium Scale Isolation

Cells were grown in 30-50 ml LB (containing appropriate antibiotic) overnight to saturation and plasmid DNA was isolated by using Nucleobond Ax100 Midi Prep kit (MN 740573), according to the manufacturer's instructions.

2.5.8. Expression Vectors and Cloning Strategy

The way the constructs that were prepared and used during this study have been summarized in the table below. The maps of plasmids used in cloning are available in Appendix section.

Vector Digest	Insert Digest	Construct	Usage
pQE30-*XhoI*	C1D-*SalI*	pQE30-C1D	Bacterial expression, purification
pBTM116-*XhoI*	C1D-*SalI*	pBTM116-C1D	Yeast expression
	TRAX-*SalI*	pBTM116-TRAX	Yeast expression
	Translin-*SalI*	pBTM116-Translin	Yeast expression
	mutLZ-TRAX-*SalI*	pBTM116-mutLZ-TRAX	Yeast expression
	N-TRAX-*XhoI –SalI*	pBTM116-N-TRAX	Yeast expression

Vector Digest	Insert Digest	Construct	Usage
pACT2-*XhoI*	C1D-*SalI*	pACT-C1D	Yeast expression
	TRAX-*SalI*	pACT2-TRAX	Yeast expression
	Translin-*SalI*	pACT2-Translin	Yeast expression
	MutLZ-TRAX- *SalI*	pACT2-mutLZ-TRAX	Yeast expression
pACT2-*BamHI*	C-TRAX-*BamHI*	pACT2-C-TRAX	Yeast expression
pEYFP-*BamHI*	TRAX-*BamHI*	pEYFP-TRAX	Subcellular localization
pECFP-*BamHI*	Translin-*BamHI*	pECFP-Translin	Subcellular localization
pdsRed1-*BglII*	C1D-*BglII*	pDsRead1-C1D	Subcellular localization
T7-myc-pLink-*BamHI*	TRAX-*BamHI*	T7-myc-pLink-TRAX	in vitro transcription and translation
	Translin-*BamHI*	T7-myc-pLink-Translin	in vitro transcription and translation
T7-pLink-*BamHI*	TRAX-*BamHI*	T7-pLink-TRAX	in vitro transcription and translation
	Translin-*BamHI*	T7-pLinkTranslin	in vitro transcription and translation
T7-myc-pLink-*BamHI*	mutLZ-TRAX-*BamHI*	T7-pLink-mutLZ-TRAX	in vitro transcription and translation
pCMV5'2N3-*XhoI*	C1D-*SalI*	pCMV5'1N3-C1D	Expression in mammalian cells
pEGFP-N2-*EcoRI-BamHI*	TRAX-*EcoRI-BamHI*	pEGFP-N2-TRAX	Expression in mammalian cells
pGV256 'Live' - *Sac I*	Double stranded oligonucleotide with 'STOP' codons-*SacI*	pGV256-'Dead'	Expression in yeast cells for recombination assay

Table 3: The way the constructs that were prepared during the course of this study

2.5.8.1. Linker Scan and Deletion Mutagenesis

Linker Scan Mutagenesis method (Ausubel et al., 1987) was applied to introduce mutations into the putative leucine zipper motif of TRAX within an otherwise intact protein. MutLZ-TRAX was generated in two steps. First, the region between amino acids 1-194 was isolated by PCR with mismatch primers (TC234/ UY30) placing Sal I-Bam HI sites at the 5' end and a Bgl II site at the 3' end. The region between amino acids 194-272 was then amplified via PCR, using primers (UY 31/TC235) that place a Bgl II site at the 5' end and Sal I-Bam HI sites at the 3' end. The two PCR fragments were digested with Sal I-Bgl II and cloned in a triple ligation into the Xho I site of pACT. This construct produced a mutant TRAX where Bgl II site is inserted, which changes the 194th amino acid leucine to arginine and preceding lysine to serine. The whole fragment was then cut out with BamHI and cloned into T7-Myc-plink, it was transcribed and translated in vitro using TnT Coupled Reticulocyte Lysate System (PROMEGA; Cat. No. L4610) to confirm that it gives a similar size product to wild type TRAX.

N- terminal region of TRAX between nucleotides 131-550 (including LZ region) was isolated by PCR with TC234 and UY29 primers placing *Sal I* to 5' end and *Xho I* to 3'end. Then, this construct was cloned into *XhoI* site of pACT2.

2.5.8.2. Construction of "pGV256 Dead"

The oligonucleotide that contains a unique restriction enzyme site (Bgl II) in the middle, which is preceded by 'STOP' codons ("TGA" in three different reading frames, see section 2.4.2) was designed as a self-annealing primer and a Sac I restriction half-site was placed at the 3'-end. To obtain double-stranded oligonucleotide, 10 ng of primer was incubated at 95^{0}C for 2 minutes, 70^{0}C for 10 minutes, followed by at 37^{0}C for 10 minutes and finally, incubated at room temperature for an additional 10 minutes. As shown below, after hybridization the double-stranded oligonucleotide has a Bgl II site in the middle, flanked by "STOP" codons (indicated in bold) in different reading frames, with a Sac I site at both ends mimicking the digested sequence.

Bgl II *Sac I*

5’ C**TGACTGAGTGA**AGATCTTCACTCAGTCAG GAGCT 3’
3’ TCGAGG**ACTGACTCACT**TCTAGA**AGTGAGTCAGT**C 5’

Sac I

Nascent synthetic oligonucleotides lack a 5’ phosphate group and thus cannot be directly ligated to other DNA, such as plasmids or other oligonucleotides, therefore it is necessary to phosphorylate with T4 polynucleotide kinase. In a typical reaction 100 ng of oligonucleotide was phosphorylated in a 50 µl volume containing 5 µl 10X kinase buffer, 2 mM ATP, and 1 µl T4 polynucleotide kinase. The reaction was incubated at 37^0C for 1 hour and phosphorylated oligonucleotide was recovered by phenol:chloroform extraction and ethanol precipitation. Next, the pGV256’Live’plasmid was digested from the unique *Sac I* site within the *LacZ* coding sequences and the phoshorylated oligonucleotide was ligated as described in 2.5.5.3. The ligation product was then transformed into the bacterial strain DH5α and transformed cells were plated on X-gal (25 µg/ml), IPTG (0.1 mM) and ampicillin (50 µg/ml) containing LB agar plates to select white colonies harbouring plasmid with a disrupted *LacZ* (pGV256 Dead). The clones were further confirmed by isolating the DNA and digesting with *Bgl II* and *Bam HI* restriction enzymes, which give a ~2250 bp fragment.

2.6. YEAST TECHNIQUES

2.6.1. Yeast Two-Hybrid Screen

The yeast two-hybrid screen was performed as described previously using a B-lymphocyte cDNA library in pACT (Harper et al., 1993) and full-length C1D in fusion with the DNA-binding domain of bacterial LexA protein (DBD-C1D) (Yavuzer et al., 1998). The yeast strains and the control plasmids (DBD-Lamin and

DBD-Daughterless) were kindly provided by S. Hollenberg. *S. cerevisiae* L40 (*MAT**a** trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) was grown at 30^0C in YPAD medium and was transformed with DBD-C1D. Human B-lymphocyte cDNA library was then transformed into this strain using large scale transformation (lithium acetate method). Double transformants were plated on selective medium lacking Trp, Leu, His, Lys and Ura and were grown at 30^0C for 3 days. Colonies were transferred on Whatman 40 filters to test for β-galactosidase activity using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Positive clones were rescued and tested for specificity by mating analysis or retransforming into L40 together with the control plasmids mentioned above. For the mating assay, DNA from positive clones was extracted using glass beads and then transformed by electroporation into the bacterial strain HB101, which has a defect in the leuB gene that can be complemented by LEU2 from yeast and therefore provides selection of the library plasmids. The transformants were plated onto M9 medium containing ampicillin and proline and incubated at 37^0C for 24 hours. DNAs of 5 colonies from each plate were then isolated by the alkaline lysis method and transformed into the yeast strain AMR70 (*MAT* α, *his3 lys2 trp1 leu2 URA3::lexAop)$_8$-lacZ*) and were mated with the L40 strain carrying the bait plasmid. The details of this screen including large-scale library transformation, elimination of false positives and database search has been written in MSc thesis of Bleda Bilican (Bilican, 1999).

2.6.2. Two-Hybrid Assay

S.cerevisiae L40 strain was used in yeast two-hybrid assays. L40 can detect weak LexA activators as histidine prototrophs without the use of 3-aminotriazole (3-AT). The expression of the *LacZ* and *HIS3* coding sequences are driven respectively, by minimal GAL1 and HIS3 promoters fused to multimerized LexA binding sites. The LexA DNA binding domain fusion vector pBTM116 poly-stop, was modified by U. Yavuzer from the original plasmid constructed by S. Fields. It carries the TRP1 gene and has a polylinker downstream of LexA coding sequence.

The Gal4 activation domain fusion vector pACT (Kindly provided by S. Elledge), carries the LEU2 gene and contains NLS-Gal4-linker unit driven by the ADH promoter. The cDNAs of proteins to be analysed are cloned into one of these plasmids in fusion with DBD or AD. They were then transformed into yeast as described below and β-galactosidase activity was measured as in 2.6.4 or 2.6.5.

2.6.3. Small Scale Yeast Transformation

10 ml YPAD was inoculated with a yeast colony and culture was incubated overnight at 30^0 C. Then saturated culture was diluted to an OD_{600} of around 0.2-0.3 in 50 ml YPAD and incubated an additional 2-4 hours till mid-log phase. Cells were centrifuged at 2500 rpm for 5 minutes at room temperature and cell pellet was resuspended in 40 ml TE. Cells were re-centrifuged at the same speed and cell pellet was resuspended in 2 ml of 100 mM LiAc (LiAc/ 0.5XTE, filter sterilized) and incubated at room temperature for 10 minute. 1 μg plasmid DNA and 100 μg denatured salmon sperm DNA (SIGMA) were distributed to tubes and 200 μl yeast suspension was added on them and all the ingredients of tube were mixed well. 700 μl of 100 mM LiAc/40% PEG-3350/ 1XTE was added to tubes and tubes were incubated at 30^0 C for 30 minutes on a rotating wheel. After incubation, 88 μl DMSO was added, heat shock was applied at 42^0 C for 7 minutes. Cells were centrifuged for 10 sec, supernatant was discarded and pellet was resuspended in 1 ml TE. One more time, cells were collected by centrifuge and pellet was resuspended in 50-100 μl TE. Cells were plated on selective plates and incubated at 30^0C incubator for 3 days till colonies were detected.

2.6.4. Colony -Lift β-Galactosidase Assay Using X-gal as a substrate

Colonies were transferred on to nitrocellulose filter (HYBOND C; Amersham). Filter was removed and was placed, colony side-up, in an aluminium boat floating in liquid nitrogen and then was immersed into the liquid nitrogen for 30

seconds. After allowing enough time for the filter to thaw at room temperature, it was transferred to a petri dish containing one Whatman filter circle (100mm) soaked in 1.5 ml Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and β-Mercaptoethanol (2.7 ml/lt) that was added freshly to the buffer) and 150 µl X-gal (SIGMA, 50 mg/ml). The dish was covered, fixed and plates were incubated in a 30^0C incubator for 2-3 hours till blue colour appeared. Depending to the strength of protein-protein interactions, the time for the blue colonies appearing varied from 30 minutes-3 hours.

2.6.5. Liquid Culture Assay Using ONPG as a Substrate

For quantification purposes, liquid cultures were assayed for β-Galactosidase activity using ONPG (o-nitrophenyl β-D-galactopyronoside) as a substrate. The detailed protocol for this assay can be found in CLONTECH- Matchmaker library manual. Briefly, a yeast colony was grown at 30^0C to mid-log phase (OD_{600}) and the exact OD_{600} was recorded. 1.5 ml of culture was placed to three eppendorf tubes and cells were centrifuged at max-speed for 30 seconds, supernatant was removed and cell pellet was resuspended in 1 ml Z buffer. 0.1 ml of suspension was removed to a new tube and the cells were lysed by 3 freeze-thaw cycles. 700 µl of Z buffer and 160 µl of ONPG (4 mg/ml) was added to 100 µl of lysate and incubated at 30^0C till a yellow colour appeared. The reaction was stopped by addition of 400 µl 1 M Na_2CO_3 and OD_{420} of the samples were read. β-galactosidase units were calculated according to the formula: $1000 \times OD_{420} / (t \times V \times OD_{600})$ where "t" is elapsed time (in minutes) of incubation and "V" is 0.1 ml x concentration factor.

2.6.6. Extraction of Yeast Proteins

5 ml of appropriate yeast medium was inoculated with a very small mass of yeast colony from a plate and incubated overnight to give an OD_{600} of 0.5-2.0. 1 OD_{600} units of cells were transferred to a tube containing 1 ml of 50 mM Tris (pH

7.5) on ice. Cells were centrifuged and cell pellet was suspended in 15 µl ESB (2 % SDS, 80 mM Tris pH:6.8, 10 % Glycerol, 1.5 % DTT and 0.1 mg/ml Bromophenol blue). After boiling of cell suspension at 100^0C for 3 minutes, the glass beads (425-600 microns, Acid washed SIGMA, Cat. No.G-8772) were added and cell-glass bead suspension was mixed by vigorous vortexing for 2 minutes. Finally 35 µl of ESB was added to the sample, briefly vortexed and boiled for additional 1 minute. Tubes were briefly centrifuged and 5-20 µl of supernatant was loaded on a SDS gel to analyse protein expression.

2.6.7. Colony- PCR

A small glob of yeast cells were suspended in 30 µl of sterile ddH_2O (fewer cells is better than too many). 10 µl of yeast cell suspension was added to the 40 µl PCR reaction mix including 50 pmol of each primers, 2 mM of each dNTP (200 µM), 2 mM $MgCl_2$ (25 mM), 1xTaq Buffer and 2 units of Taq Polymerase. PCR was carried out for 30 cycles under the following conditions; denaturation at 94^0C for 4 minutes, annealing at 50^0C 1 minute, extension at 72^0C for 2 minutes and final extension at 72^0C for 10 minutes. An aliquot of PCR product was analysed by agarose gel electrophoresis to verify amplification.

2.6.8. *In vivo* Plasmid-based Repair Assay

3 µg of *S.cerevisiae-E.coli* shuttle plasmid, pRS313 (HIS3 reporter) was linearized with different restriction endonucleases (10 unit from each) to produce 5'- (*BamHI* digest), 3'- overhanging ends (*SacI* and *PstI* digests) or blunt ends (*SmaI* digest). Linearized plasmid was then purified from the agarose gel using gel extraction kit (see section 2.5.2) and 100 ng of the linearized and supercoiled versions of the plasmids were transformed into yeast strains in parallel by using small scale yeast transformation (see section 2.6.3). Transformed yeast cells were plated on histidine deficient YC agar plates and incubated at 30^0C for 3-4 days until

yeast colonies appeared. NHEJ efficiency of yeast strain was determined by counting the number of colonies harbouring re-ligated plasmid on each plate and normalizing the potential minor differences in transformation efficiencies between various yeast strains with the supercoiled version of the same plasmid. For NHEJ accuracy assays, *SacI*-linearized and supercoiled forms of "pGV256 Live" was used.

2.6.9. *In vivo* plasmid-based Homologous Recombination Assay

Initially, 5 μg 'pGV256 Dead' plasmid (URA3) was linearized with *Bgl II* and was purified from the agarose gel by gel extraction kit. A 800 bp region of LacZ coding sequences encompassing the region where the 'STOP' codons were integrated was amplified by PCR from the 'pGV256 Live' plasmid using 'LacZ forward and reverse primer pair. Amplified LacZ fragment (10 ng) was transformed into the recipient yeast strain together with the *Bgl II*-digested linear 'pGV256 Dead' plasmid (90 ng) by using small yeast transformation. Transformed yeast cells were plated on uracil deficient YC agar plates and incubated at 30^0C for 3-4 days. After, the colonies appeared, colony lift β-Galactosidase assay using X-gal as a substrate was performed and number of colonies were counted. The ratio of blue colonies over the total number of colonies on plates reflects HR for that particular yeast strain.

2.6.10. HO Endonuclease Sensitivity Assay

The pCHOL(LEU2), that expresses homothallic switching (HO) endonuclease under the control of GAL I promoter was kindly provided by Allison Rattray, Fred Hutchinson Cancer Research Centre, U.S.A. This plasmid was transformed into the appropriate yeast strains and the transformants were grown to mid log phase (OD_{600}: 0.5-0.8), washed two times with ddH_2O and resuspended in YC-Leu minus liquid medium containing either glucose (2%) or galactose (2%) as carbon source. Samples were taken at various time points, the number of cell determined by measuring OD_{600} and cells were plated on non-selective YPAD plates.

The survival rate was calculated as the proportion of colony forming units to total cells and was normalized to the number of colonies before galactose induction (Downs et al., 2000).

2.6.11. Phenotype analyses

Mid-Log phase liquid yeast cultures were prepared by inoculating one large colony per 5-ml medium and incubating the culture at 30^0C for 16-18 hr with shaking at 230-270 rpm. The culture was then diluted in TE six times by serial 10-fold dilutions. Aliquots (5 μl) of each dilution was spotted on YPAD plates with or without MMS (0.01 %, SIGMA) and were then incubated at 30^0C for 3-4 days to analyse sensitivity towards MMS. To analyse temperature sensitivity of yeast strains, serial dilutions of yeast strains spotted on YPAD plates were incubated either at 30^0C or 39^0C for 3 days. For u.v. and γ-irradiation sensitivity, serial dilutions of yeast strains spotted on YPAD plates in duplicates were exposed to either u.v. irradiation at 100 j/m^2 (UV Stratalinker 1800, STRATAGENE) or 50 Gy of gamma (Cs^{137}) source, followed by incubation at 30^0C for 3-4 days. The photos of the plates were captured with Biorad Multi Analyst Software. As we don't have a gamma (Cs^{137}) source at Bilkent University, irradiation experiment was performed at Marie Curie Research Institute by U.Yavuzer.

2.7. CELL CULTURE TECHNIQUES

2.7.1. Maintenance and Subculturing of Cells

COS7 cells were grown as monolayers in culture flasks or petri dishes in a medium mentioned in section 2.3. Cells were subcultured twice a week using Trypsin/EDTA (BICHROM, Cat. No. L2143) for detachment. The medium was aspirated and Trypsin/EDTA solution was added to the petri dish or culture flask and

was incubated at 37^0C for 30 seconds. After removal of Trypsin/EDTA solution, the flask was kept at 37^0C incubator for an additional 10 minutes. Then cells were detached by tapping flask/petri dish gently and the cells were collected in 10 ml growth medium. Cell suspension was centrifuged at 2000 rpm for 5 minutes at 4^0C and the pellet was resuspended in fresh medium and diluted to a concentration of $2x10^5$cells/ml and placed into a new culture dish.

2.7.1.1. Cryopreservation of Cells

Pre-confluent cells were trypsinized, pelleted and resuspended in freezing medium (DMEM including 10 % DMSO and 5% FCS). 1 ml of cells (approximately $3\text{-}5x10^6$ cells) was delivered into each freezing vial and vials were placed into -80^0C freezer overnight. Next day, vials were transferred into a liquid nitrogen chamber quickly.

2.7.1.2. Thawing of Frozen Cells

Frozen cells were thawed quickly in a 37^0C water bath and directly transferred into growth medium. Cells were collected at 2000 rpm for 5 minutes at 4^0C. Pellet was resuspended in growth medium and transferred to medium in flask.

2.7.1.3. Estimation of Cell Number by haemocytometer Counting

Cells were trypsinized and resuspended in growth medium. Using pasteur pipette, 1 ml from cell suspension was taken into an eppendorf and 10 µl was delivered into each side of the cover-slipped haemocytometer by capillary action. Cells in the middle square were counted in both chambers (up and down), mean value of number of cells was taken and multiplied by $1x10^4$ to give the number of cells per millilitre in the sample counted (Spector et al., 1997)

2.7.2. Transient Transfections

2.7.2.1. Transfection Using FuGENE™ 6

FuGENE 6 Transfection Reagent (Roche, Cat.No. 1814443) is a multi-component lipid based transfection reagent that complexes with and transports DNA into the cell during transfection. It provides very high transfection efficiency, demonstrates no cytotoxicity and requires minimal optimization compared to other methods employed for mammalian cell transfections.

The day before transfection, COS7 cells were plated into 35 mm 6-well plates at a density of $3x10^4$. Transfections were performed using FuGENE 6 transfection reagent with 0.5 μg of DNA according to the manufacturer's instructions. Cells were harvested 24 hours after transfection for the following immunoprecipitation experiment.

2.8. BIOCHEMICAL TECHNIQUES

2.8.1. Immunoprecipitation

This technique was used to analyse the protein/protein interactions in mammalian cells. The plasmids expressing the desired proteins were initially transfected into the mammalian cells as described above and the protein complexes were purified by the aid of antibodies and interactions were detected by immunoblotting using the corresponding antibodies.

2.8.1.1. Cell Lysis and Sample Preparation

24 hours following transfection, tissue culture plates were placed on ice, growth medium was removed, and cell monolayer was washed twice with ice cold PBS. Cell lysis buffer, NET-N, [150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.5% NP40, 10 % Glycerol, and 1x Complete™ protease inhibitor (ROCHE, Cat. No. 1873580) was added to the monolayer (0.3 ml for a 35-mm dish, 0.6 ml for

a 60-mm dish, or 1 ml for 100-mm dish) and then cells were incubated on ice for 30 minutes with occasional agitation. The cells were scraped to one side of the dish with a rubber policeman and transferred into a fresh eppendorf. Samples were centrifuged for 10 minutes 12,000xg at 4^0C and supernatants were transferred to a fresh tube.

2.8.1.2. Antibody Incubation and Purification of Cellular Proteins

The supernatant, which is the total cell lysate, was removed into a new tube and was incubated with either anti-GFP (Clontech) or anti-HA monoclonal antibody (Roche) for 2 hours on a rotating wheel. Meanwhile, 50 µl of the homogenous protein A/G agarose suspension (25 µl bed volume) (Protein A-Agarose, Roche, Cat. No. 1719408; Protein G-Agarose, Roche, Cat. No.1719416) per sample was washed three times with 0.5 ml of NET-N lysis Buffer (10 minutes washing followed by 5 minutes centrifugation at 2500 rpm to remove supernatant). After 2 hours incubation period, the cell lysate/antibody mix was added onto the equilibrated beads and was incubated for further 2 hours. The beads were collected at the end of the incubation by centrifugation at 2500 rpm for 5 minutes and the supernatant was removed. Then the beads were washed four times with 0.5 ml of Lysis buffer as described before. After the last wash, supernatant was discarded and the beads were resuspended in 20 µl of 2x SDS Loading Buffer. The samples were boiled for 5 minutes, centrifuged at full speed for 5 minutes and then loaded on to SDS-PAGE gel.

2.8.2. Immunoblotting

Following SDS-PAGE, proteins were transferred from the gel onto a nitrocellulose membrane by Mini Trans-Blot Electrophoretic Transfer Cell (BIO RAD, Cat. No. 170-3930). Nitrocellulose membrane (Schleicher & Schuell, Protran BA 85 Cellulosenitrate, Cat. No. BB0451-1) and the filter paper (3MM Whatman) were cut according to the dimensions of the gel. The nitrocellulose paper was soaked in ddH_2O and wetted by capillary action from the bottom. The gel, nitrocellulose membrane, filter papers and filter pads were equilibrated in transfer buffer (For

proteins with a molecular weight 80 kDa-400 kDa; 50mM Tris, 380 mM Glycine, 20% Methanol, 0.1% SDS (10% stock), and for proteins ≤80 kDa; 25 mM Tris, 192 mM Glycine, 20% Methanol) for 15 minutes to 1 hour depending on gel thickness. Then the transfer stack was prepared in the following order starting from the side that would face the cathode: one pre-wetted fiber pad, one sheet of 3 MM Whatman paper, the gel, pre-wetted nitrocellulose membrane, one sheet of Whatman paper and finally the second fiber pad. The transfer stack was then placed into the tank filled with transfer buffer and transfer was performed at 100V for 1 hour for proteins with molecular weights smaller than 150 kD and at 40V for 3 hours for bigger proteins.

After the transfer, the membrane was washed twice with TBS-T (TBS + 0.5% Tween-20), and incubated in 50 ml of blocking solution (10% non-fat dry milk in TBS-T) with continuous shaking for 1 hour at room temperature or overnight at 4^0C. The membrane was briefly rinsed using two changes of washing buffer then washed once for 15 minutes and twice for 5 minutes with fresh changes of the washing buffer at room temperature. While membrane was being washed, primary antibody was diluted to 1/1000-5000 in 2.5% non-fat dry milk in TBST (TBS with 0.2% Tween 20). The membrane was incubated in diluted primary antibody for 1 hour at room temperature or overnight at 4^0C. Membrane was washed as indicated previously. HRP (Horse raddish peroxidase) conjugated secondary antibody was diluted to 1/10,000 in 2.5 % non-fat dry milk in TBST and the membrane was incubated in diluted secondary antibody for 1 hour at room temperature. Membrane was washed as indicated previously.

For detection ECL (**E**nhanced **C**hemi**L**uminescence; Amersham Pharmacia Biotech, Cat. No. RPN2106) was carried out according to the manufacturer's instructions.

The complete removal of primary and secondary antibodies from membrane is possible. The membrane could be stripped of bound antibodies and re-probed several times using different antibodies. For this purpose, the membrane was submerged in stripping buffer (100 mM 2-mercaproethanol, 2% SDS, 62.5 mM Tris-HCL pH 6.7) and incubated at 50^0C for 30 minutes (For more stringent conditions temperature can be increased up to 70^0C). The membrane was washed twice for 10 minutes in TBST at room temperature using large volumes wash buffer. The

membrane was blocked in blocking reagent for 1 hour and immunoblotting was performed as described above.

2.8.3. In Vitro Transcription and Translation

The *in vitro* transcription and translation of target proteins were carried out with the TnT Coupled Reticulocyte Lysate Kit (Promega), according to the manufacturer's instructions using radioactively labelled methionine (S^{35}). Samples were resolved on a SDS-PAGE gel. Following electrophoresis, the proteins were fixed by using a fixing solution [isopropanol:water:acetic acid (25:65:10)] for 30 minutes and then the gel was soaked in Amplify solution (Amersham) for 15-30 minutes. The gel was then dried under vacuum at 70^0C for 1 hour. Dried gel was exposed to X-ray film and stored at -80^0C overnight. After exposure, the film was developed using Hyper Processor (Amersham).

2.8.4. Ni-NTA Pull Down Assays

His-tagged C1D (pQE-C1D) and His-tagged hepatitis-C virus core protein, p16 (kindly provided by Mehmet Ozturk) were expressed in *E.coli*, purified under denaturing conditions and immobilized on Ni-NTA beads according to the manufacture's instructions (Qiagen). The details of these protocols can be found in MSc thesis of Dilhan Oncel (Oncel, 2001). 10 μg of radiolabelled in vitro transcribed and translated protein product and the immobilized fusion proteins were incubated in 200 μl binding buffer (20% Glycerol, 100 mM NaCl, 0.1xTE pH 8.0, 0.1% NP-40, 1 mM DTT, 30 μg BSA) at room temperature for 20 minutes followed by four washes using wash buffer (20% Glycerol, 100 mM NaCl, 0.1xTE pH 8.0 and 0.5% NP-40). The beads were then resuspended in 20 μl of 2xSDS sample buffer, boiled, centrifuged at 13,000 rpm for 5 minutes and loaded on 10% SDS-PAGE. The gel was dried and visualized by autoradiography.

2.9. GEL ELECTROPHORESIS

2.9.1. Agarose Gel Electrophoresis

DNA fragments were separated by gel electrophoresis using agarose at concentrations of 0.8-2.0% w/v in 1x TBE buffer. Samples were mixed with loading dye (8% deionised formamide, 1xTBE and 0.1% bromophenol blue) and the samples were loaded onto the gel and electrophoresis was performed at 100V. When the run was complete, the gel was stained in 1x TBE containing 0.1% EtBr for 5 minutes and rinsed with dH_20. DNA fragments were visualised by using UV transilluminator. The gel photos were captured with BioRad Multi-Analyst Software running on a PC and a hardcopy was produced by Lexmark Optra laser printer.

2.9.2. Denaturing Discontinuous Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoretic separation of proteins under denaturing conditions was performed as described in Current protocols (Ausubel et al., 1987). Resolving gels were made up to the required concentration, 5-15%, from a 40% acrylamide stock solution (38% acrylamide, 2% bisacrylamide, Severn laboratories). The electrophoresis apparatus (Mini-Protean 3, BioRad) was assembled according to manufacturer's instructions. Acrylamide stock solution (to the desired gel percentage) and 1x Lower Buffer solution (4xTris-Cl/SDS pH 8.8; 1.5 M Tris-HCl containing 0.4 % SDS) were mixed in ddH_2O and 10% ammonium persulphate and TEMED (20 µl and 5 µl, respectively, for a 5 ml solution) were added and stirred gently. Immediately the mixture was poured into the gap between the glass plates so that sufficient space is left for the stacking gel and water saturated isobutanol was added slowly to cover the top with a layer. After the polymerisation, the isobutanol layer was removed, rinsed with water several times. The stacking gel was prepared by mixing 3.9% acrylamide, 1xUpper Buffer (4x Tris-Cl/SDS pH 6.8; 0.5 M Tris-Cl

containing 0.4 % SDS), 20µl of 10% ammonium persulphate, and finally 5 µl of TEMED (for 5 ml) in ddH_2O and was poured onto the top of the resolving gel. The comb was immediately inserted into the stacking gel avoiding the formation of bubbles. After the stacking gel polymerised, the comb was carefully removed and the wells were rinsed with the SDS running buffer (25 mM Tris, 192 mM Glycine 0.1 % SDS) remove any unpolymerised acrylamide. The samples were mixed with appropriate volume of 2x SDS loading buffer (For 10 ml final volume; 2.5 ml 4x Tris.Cl/SDS, pH 6.8, 2 ml glycerol, 0.4 g SDS, 0.2 ml 2-BME or 0.31 g DTT, 0.1 mg bromphenol blue), boiled at 100^oC for 5 minutes to denature proteins, centrifuged at full speed for 5 minutes, and then loaded on to gel. Gels were run at 70V until the samples left the stacking gel. As the samples entered to the resolving gel the voltage was raised to 100V. After the electrophoresis, gel was carefully removed and stained in Coomassie Brilliant Blue (0.25% Coomassie Brilliant Blue G250, 45% methanol, 10% acetic acid) for 20 minutes, and destained in a solution of 30% methanol, 10% acetic acid, and 60% deionised distilled water for 1-4 hours. Alternatively gels were subjected to electrophoresis for the purpose of immunoblotting.

2.10. MICROSCOPY AND MICROPHOTOGRAPHY

Vectors expressing C1D, TRAX, and Translin in fusion with GFP and various GFP variants were transiently transfected into COS7 cells as described in section 2.8.4. 24 hours after transfection, the expression of the fusion proteins were examined in the control and γ-irradiated living cells for 24 hours. Florescence microscopy was performed on a Zeiss Axiovert 135TV microscope equipped with a CCD camera (Hitachi Denshi, Ltd) and appropriate filter sets, and the images were processed by using OpenLab software (Improvision). All the expression plasmids were constructed at Bilkent University, however, as we do not have a flourescence microscope equipped with different filter sets and also a γ-irradiation source, the microscopic analyses and γ-irradiation experiments were performed at Marie Curie Research Institude by U.Yavuzer and B. Bilican.

2.11. RADIOCHEMICALS

[^{35}S]methionine and [γ-^{32}P]ATP were supplied by Amersham International and DuPont NEN.

2.12. EQUIPMENT

The following equiments were routinely used during this study: Automatic pipettes (Rainin), filter and 3MM paper (Whatman), GeneAmp PCR System 9600 (Perkin Elmer), heating blocks (Stuarts Scientific), benchtop centrifuge (Heraus Instruments), Avanti J-25I centrifuge (Beckman), gel tanks for agarose (E-C Apparatus Corporation), Mini-PROTEAN 3 Cell (BioRad), spectrophotometer DU640 (Beckman), power supply PAC 300(BioRad), pH meter (Beckman), UV transilluminator (Herolab), Slab Gel Drier (Savant), Mini Trans-Blot (Biorad).

2.13. STANDARD SOLUTIONS AND BUFFERS

Dithiothreitol (DTT), 1 M

1.545 g DTT was dissolved in 10 ml ddH_2O, store at –20^{o}C

EDTA (ethylenediamine tetraacetic acid), 0.5 M pH 8.0

186.1 g $Na_2EDTA.2H_2O$ was dissolved in 700 ml ddH_2O, pH adjusted to 8.0 with 10 M NaOH, volume completed to 1 liter

Ethidium Bromide, 10 mg/ml

0.2 g was dissolved in 20 ml ddH_2O

NaCl, 5 M

29.2 g was dissolved in 100 ml ddH_2O

NaOH, 10 M

200 g NaOH was dissolved in 500 ml ddH_2O

Sodium Acetate, 3 M

40.8 g sodium acetate.3H_2O was dissolved in 60 ml ddH_2O. After pH adjustment to 4.8-5.2 with 3 M acetic acid, volume was completed to 100 ml.

Tris-Cl, pH 8.0

121 g Tris base was dissolved in 800 ml ddH_2O. After pH was adjusted to 8.0 by adding 58.4 ml 0.1 M HCl, volume was completed to 1 liter.

Phosphate-Buffered Saline (PBS)

Working Solution, ~pH 7.3:	10x Stock Solution (1lt):
137 mM NaCl	80 g NaCl
2.7 mM KCl	2 g KCl
4.3 mM $Na_2HPO_4.7H_2O$	11.5 g $Na_2HPO_4.7H_2O$
1.4 mM KH_2PO_4	2 g KH_2PO_4

Tris Buffered Saline (TBS) pH 8.0, 1 lt

2.42 g Tris base (20 mM)

8 g NaCl (137 mM)

3.8 ml HCl

TBE Buffer:

Working Solution:	10x Stock Solution (1lt):
45 mM Tris-borate	108 g Tris Base
1 mM EDTA.	55 g Boric Acid
	40 ml 0.5 M EDTA, pH 8.0

TAE Buffer:

Working Solution	50x Stock Solution (1lt)
0.4 M Tris acetate	2 M Tris Base (242 g)
0.001 M EDTA	57.1 ml Glacial Acetic Acid
	50 mM EDTA

1x TE:

10 mM Tris, pH 8.0

1 mM EDTA, pH 8.0

RESULTS

CHAPTER 3

IDENTIFICATION OF C1D INTERACTING PROTEINS

3.1. IDENTIFICATION OF C1D INTERACTING PROTEINS USING YEAST TWO HYBRID SCREEN

This assay relies on two fundamental properties of eukaryotic transcription factors. They typically have modular structure with at least two discrete domains; a DNA binding domain (DBD) and an activator domain (AD). These two domains need not to be covalently linked and can be brought together by interaction of any two polypeptides. The application of this system requires two constructs: a DBD fused to a bait protein, X, and AD domain fused to a prey protein, Y. These two hybrids are expressed in a yeast recipient cell containing one or more reporter genes. If the X and Y proteins interact, they built a functional activator by bringing the activation domain in close proximity with the DNA binding domain which can be detected and/or selected by expression of reporter genes (Fields and Song, 1989). The yeast two hybrid assay is explained schematically in figure 4.

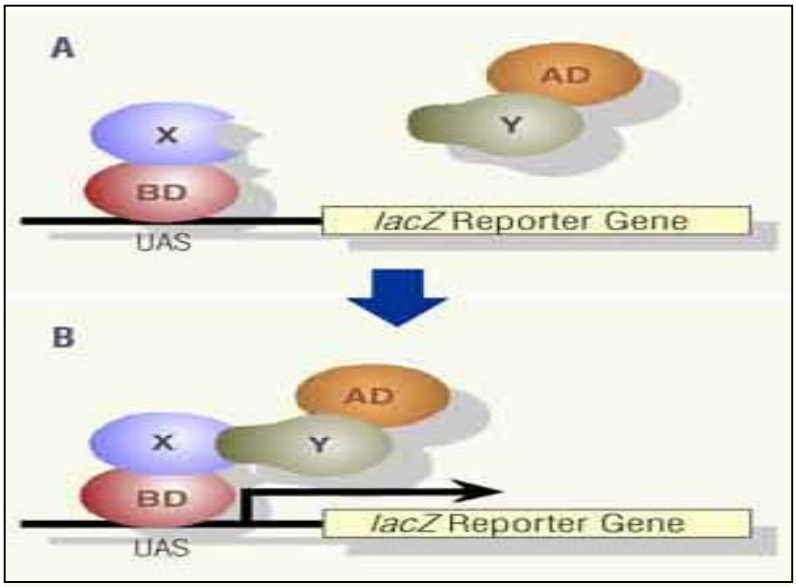


Figure 4: Yeast Two Hybrid Assay

In order to identify the interacting proteins with C1D, yeast two hybrid screen was performed. For this purpose, a human B-lymphocyte cDNA library in fusion with an AD was screened using C1D expressed in yeast in fusion with a DBD. As a consequence we identified 80 positive cDNAs that were grouped into five. One group was comprised of C1D itself that can be accepted as a positive control for this screen since C1D homodimerisation has been reported previously (Yavuzer et al., 1998). The second group of cDNAs encode the most intriguing protein in our screen, Translin associated factor X, TRAX (Aoki et al., 1997). TRAX has been shown to form a stable complex with Translin protein that is supposed to be a recombination hot spot binding protein seen in many chromosomal translocations. Other identified C1D interacting proteins were; ERCC1 that is involved in nucleotide excision repair, SL1 which is a transcription factor of RNA polymerase 1, and eIFp40 protein that is involved in translational initiation. The summary of yeast two hybrid results is presented in Figure 5. The detail of how this screen was performed has been written in MSc thesis of Bleda Bilican (Bilican, 1999).

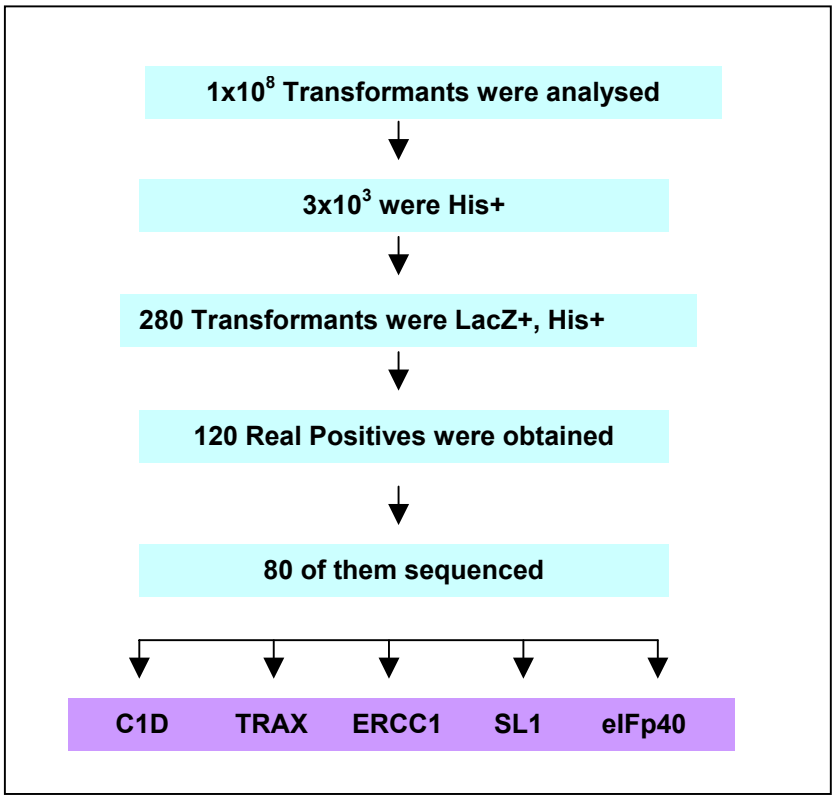


Figure 5: Summary of Yeast Two Hybrid Screen using C1D as bait

3.2. FEATURES OF TRAX

TRAX stands for Translin Associated protein X, and it was identified as an interacting protein with Translin in a yeast two hybrid screen (GenBank accession no. X95073,(Aoki et al., 1997)). TRAX encodes a 33 kDa protein and exhibits 28 % homology to Translin. The biological functions for either of the proteins are not known yet. Interestingly, Translin was identified as binding to consensus sequences, ATGCAG and GCC(A/T)(G/C)(G/C)(A/T), found at the breakpoint junctions of various chromosomal translocations seen in many lymphoid malignancy and solid tumours (Aoki et al., 1995; Chalk et al., 1997). Both Trax and Translin are highly conserved throughout evolution and beside mammals, they are found in various species such as *Xenopus laevis*, *Arabidopsis thaliana*, *Drosophila*, and *S.pombe*

(Devon et al., 2000). All these evidence suggest that TRAX and Translin should have important biological functions and possibly play a role in genesis of cancer.

Therefore, it was decided to analyse the TRAX/C1D interaction further and decipher the biological consequences of this interaction. Although, Translin was not amongst the proteins identified that were found to interact with C1D in our yeast two-hybrid screen, it was included in some of the assays as it binds to TRAX with a very strong affinity.

3.3. C1D AND FULL LENGTH TRAX INTERACT IN YEAST SPECIFICALLY

Clone 41, the longest cDNA clone that was found to interact with C1D specifically, was first aligned against the full-length TRAX protein using ClustalW program. As seen in figure 6, this cDNA encodes only a partial region of TRAX protein.

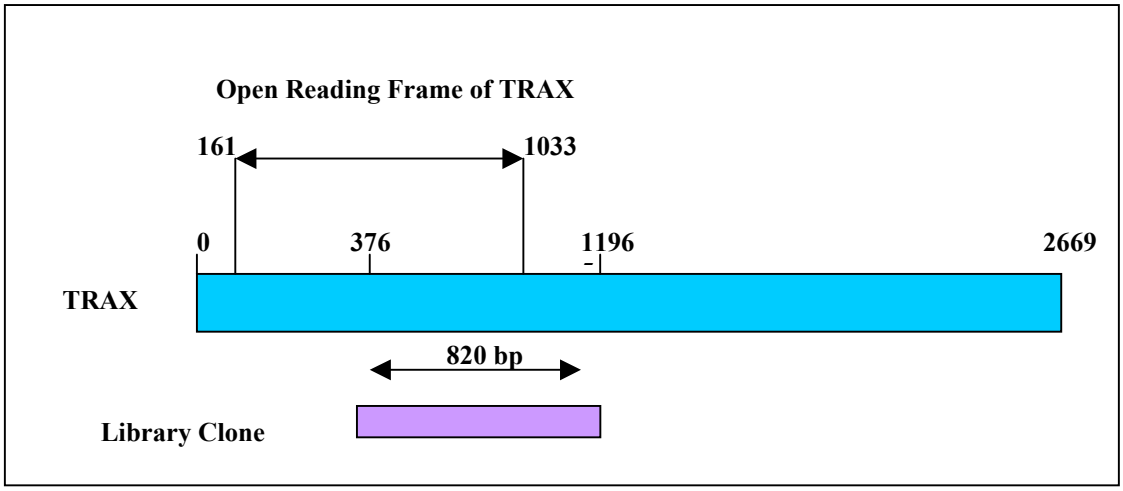


Figure 6: Alignment of Clone41 Against full length TRAX

In order to confirm that the full length TRAX also interacts specifically with C1D as does the library clone, the full-length open reading frame of TRAX was isolated by RT-PCR and cloned into the yeast expression plasmid, pACT. These plasmid enables TRAX to be expressed in yeast as an activation domain tagged fusion protein (AD-TRAX). AD-TRAX was then transformed into L40 together with plasmids expressing the lexA DNA binding domain (DBD) alone, DBD-Lamin, DBD-Daughterless, or DBD-C1D and the level of activity from the integrated *LacZ* reporter was determined using the ONPG assay. As seen in figure 7, TRAX interacts specifically with DBD-C1D since no interaction is detected with DBD-alone and DBD fused to heterologous proteins such as DBD-Lamin and DBD-Daughterless.

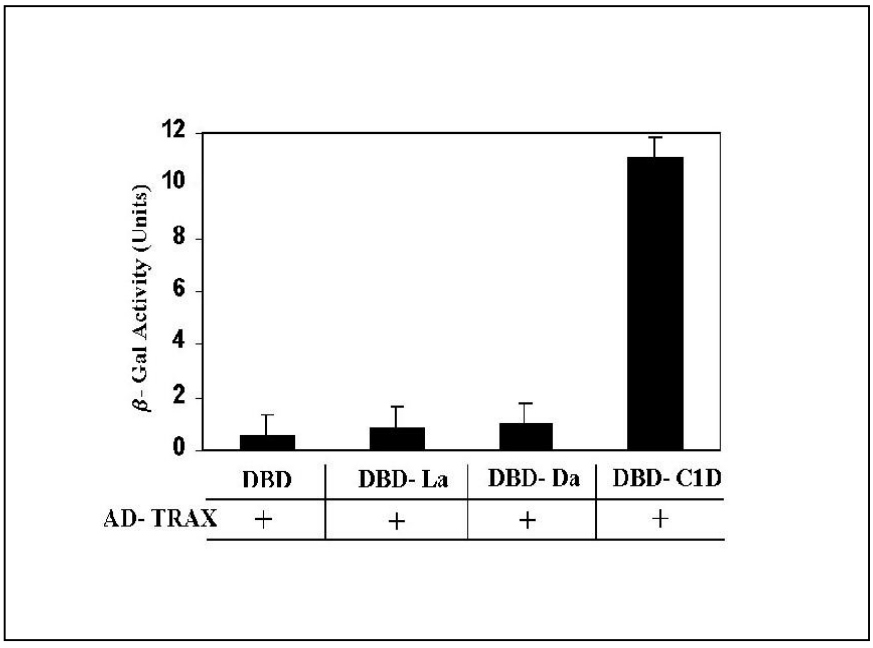


Figure 7: C1D and TRAX interact specifically in yeast

3.4. ANALYSIS OF TRAX AND C1D INTERACTION *IN VITRO*

For *in vitro* studies, cDNA encoding the full-length C1D protein (16 kDa) was cloned into bacterial expression plasmid pQE and was expressed in *E.coli* as a His-tagged fusion protein. Hepatitis C virus core protein, HCV-core (kindly provided by M. Ozturk), which has a similar size to C1D, was also expressed in bacteria and used as a control to verify the specificity of interaction. Both C1D and HCV-core proteins were purified from bacteria under the same denaturing conditions using 6-1 M urea gradient and were immobililized on Ni-NTA column. The immobilized fusion proteins were then incubated with the *in vitro* transcribed and translated TRAX and pull-down assays were performed. As shown in Figure 8, a substantial proportion of input TRAX ($1/10^{th}$) is bound by C1D (Lane 3), whereas no interaction is observed between TRAX and pQE alone (Lane 2) or HCV-core protein (Lane 4), demonstrating that TRAX and C1D also interact specifically *in vitro*.

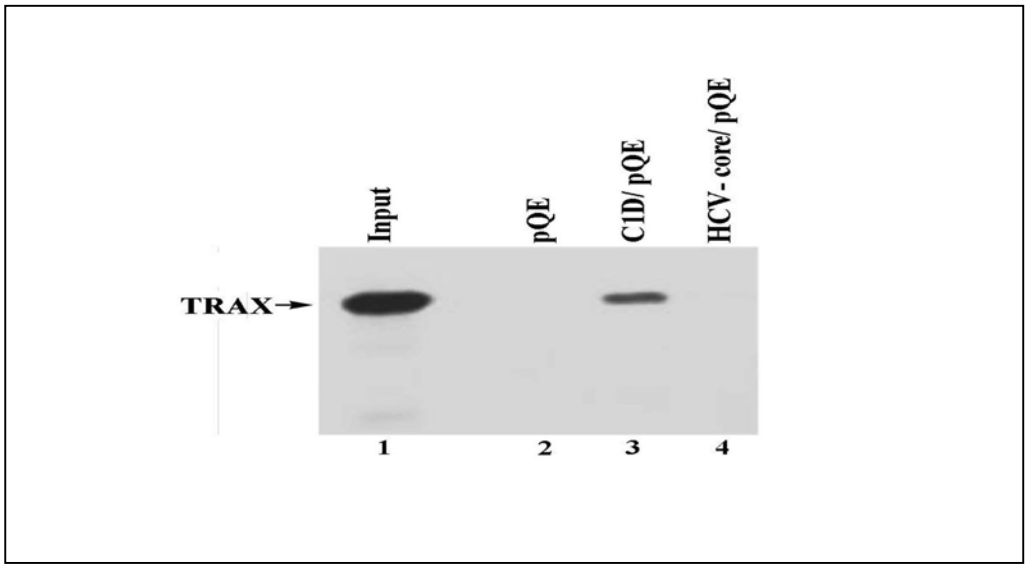


Figure 8: C1D and TRAX interact *in vitro*. IVTT TRAX was incubated with the indicated fusion proteins immobilized on Ni-NTA coulmn

3.5. MAPPING THE INTERACTION DOMAINS OF TRAX

3.5.1. Constructing the LZ Mutant Form of TRAX and in vivo/in vitro expression studies

TRAX encodes a 33 kDa protein (290 amino acid) that contains a bipartitate nuclear targeting motif between the amino acids 11-27 and a putative leucine zipper (LZ) motif between the amino acids 73-108 in its N-terminal region. Translin and TRAX share 28% identity at the protein level with a conservation of 38% in the C-terminal region of TRAX (Aoki et al., 1997). The leucine zipper motifs are involved in protein-protein interactions. In order to analyse the importance of LZ motif of TRAX in protein-protein interactions, a mutant TRAX cDNA was constructed (mutLZ-TRAX) where the LZ motif has been mutated within otherwise intact protein (Figure 9).

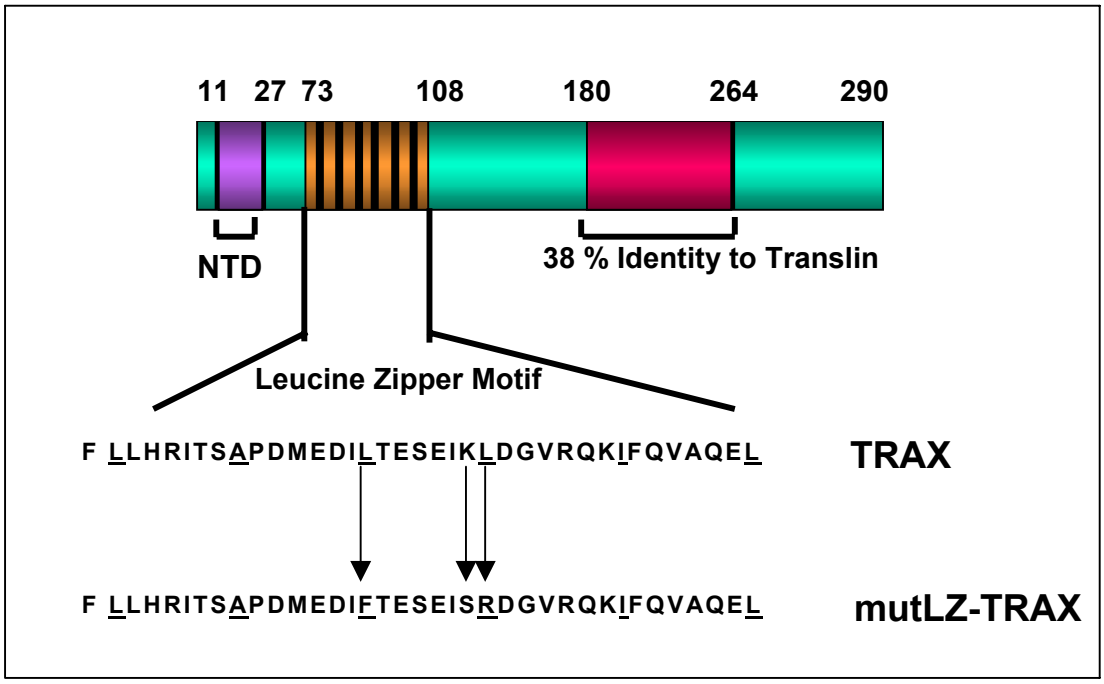


Figure 9. Domains of TRAX. N-terminus of TRAX contains a Nuclear Targeting Domain (NTD) and putative Leucine Zipper (LZ) motif and C-terminus shows high identity to Translin. In order to obtain LZ motif mutant TRAX (mutLZ-TRAX), by linker scan mutagenesis, the third leucine (L) was replaced by phenyalanine (F) and fourth leucine and preceding lysine (K) were replaced by arginine (R) and serine (S), respectively.

The mutLZ-TRAX was initially cloned into T7pLink and in vitro transcribed and translated under the control of T7 promoter. As shown in figure 10, mutLZ-TRAX (Lane 3) exhibits a similar size to the wild type TRAX (Lane 2), indicating that the mutations introduced into a certain region within an otherwise intact protein did not cause truncations.

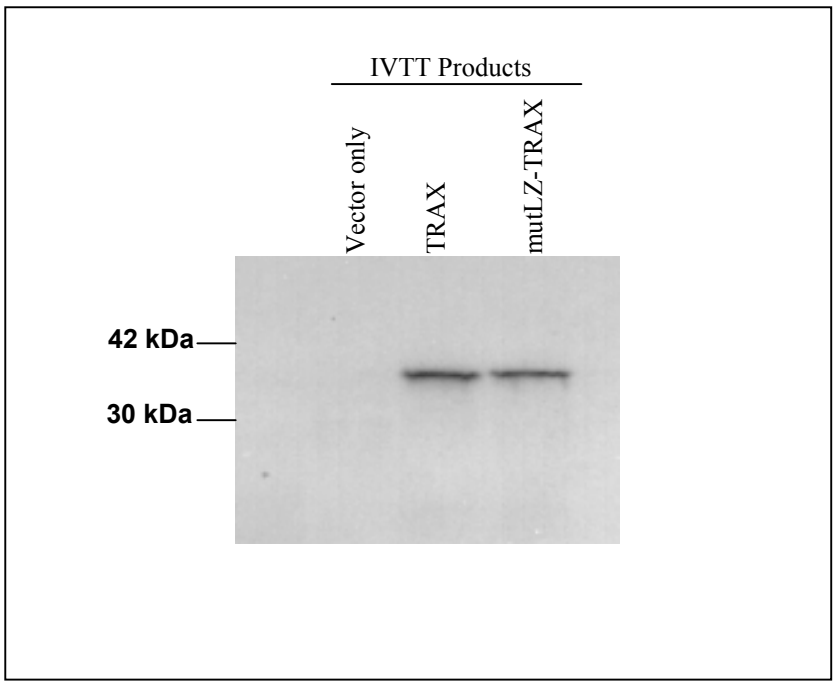


Figure 10: The IVTT of mutLZ-TRAX gives a similar size protein product to the wild type protein

mutLZ-TRAX was then subcloned into the yeast expression plasmid, pACT2 and expressed in yeast in fusion with an activation domain. This particular plasmid also contains an HA epitope enabling the detection of expressed proteins in yeast. As controls, wild type TRAX (AD-TRAX) and pACT2 plasmid on its own were also introduced into yeast in parallel with mutLZ-TRAX. The lysates from each transformed strain was prepared and subjected to SDS-PAGE followed by probing with an antibody raised against HA-epitope. As expected, mutLZ-TRAX was detected as a protein with a similar size to the wild type TRAX (Figure 11, Lanes 1 and 2) and no such protein was detected in lysates obtained from pACT2 transformed

or non-transformed yeast strains (Lanes 3 and 4). Therefore, mutLZ-TRAX is expressed *in vivo* as well and the mutations we have introduced did not have any effect in overall structure of the protein.

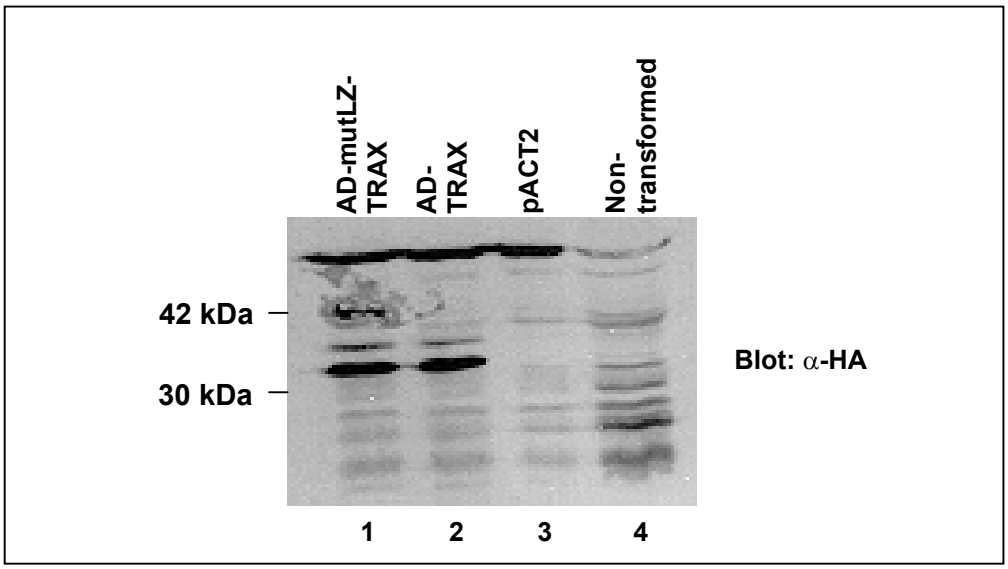


Figure 11: Verification of mutLZ-TRAX and TRAX expression in the yeast strain L40 by immunobloting.

3.5.2. TRAX Interacts with C1D through Its Putative Leucine Zipper Region

For mapping the interaction domains of TRAX, yeast two-hybrid assay was performed. To this end, plasmids expressing the wild type and two mutant forms of TRAX (N-terminal region TRAX containing the LZ motif and mutLZ-TRAX) in fusion with an activation domain were introduced into the yeast strain L40 together with the plasmid expressing DBD-C1D and β-Galactosidase activity was determined using ONPG as a substrate.

In accordance with the previous yeast two hybrid assay result (Figure 7), a significant activity was detected between TRAX and C1D, whereas a rather low level of β-galactosidase activity was detected with mutLZ-TRAX and C1D (Figure 12,

Lane 1 and 3), demonstrating that the putative leucine zipper domain of TRAX is essential for its interaction with C1D. Interestingly, the N-terminal region of TRAX containing an intact LZ region (N-Ter) interacted with C1D as strongly as the wild type TRAX (Lane 2), further confirming the requirement of the putative leucine zipper region for interaction between TRAX and C1D.

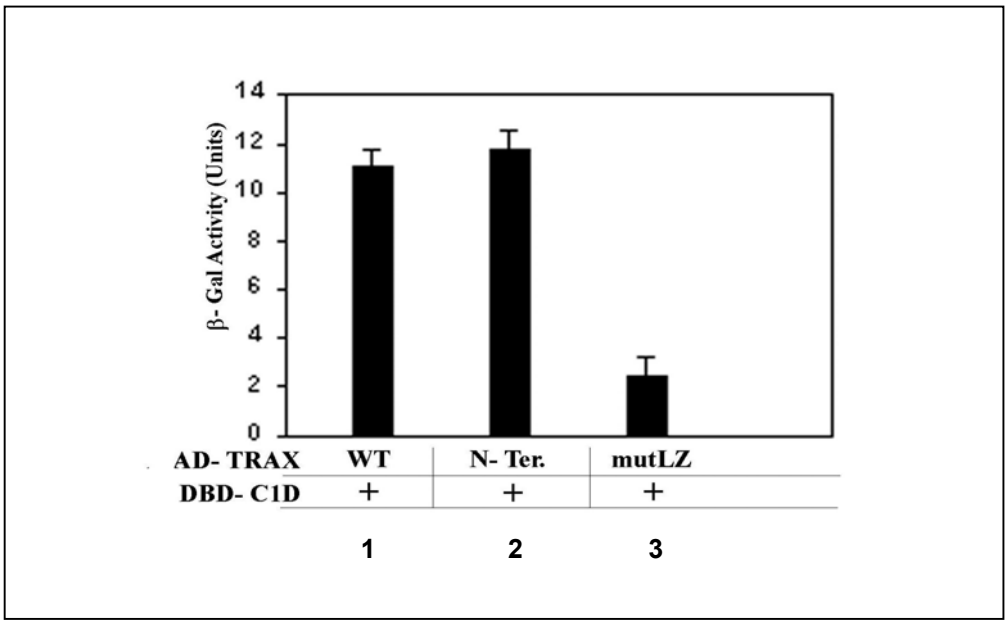


Figure 12: The putative Leucine zipper region of TRAX is essential for TRAX/C1D interaction

3.5.3. LZ Region of TRAX is Also Important For Interaction With Translin

Although TRAX was initially identified as a Translin interacting protein, the interaction domains are not known yet. According to our results (Figure 12), LZ motif of TRAX is particularly important for TRAX/C1D interaction. Since TRAX also interacts with Translin, it was important to determine whether LZ motif is also critical for TRAX/Translin interaction. In order to determine the regions of TRAX that are responsible for TRAX/Translin interaction, AD fusions of TRAX, mutLZ-TRAX, and N-TRAX were expressed together ywith DBD fusion of Translin in the yeast strain L40 and the level of interaction among them was determined by

β-galactosidase activity. As seen in figure 13 (Lane 1), TRAX/Translin interacted very strongly, (almost 10-fold stronger than TRAX/C1D interaction), however the disruption of LZ region of TRAX abolished this interaction (Lane 2). Unlike the C1D/TRAX interaction which is mainly governed through the N-terminal region of TRAX containing an intact LZ, TRAX/Translin interaction is likely to require the full-length TRAX protein as the N-terminal mutant TRAX did not interact with Translin (Lanes 3).

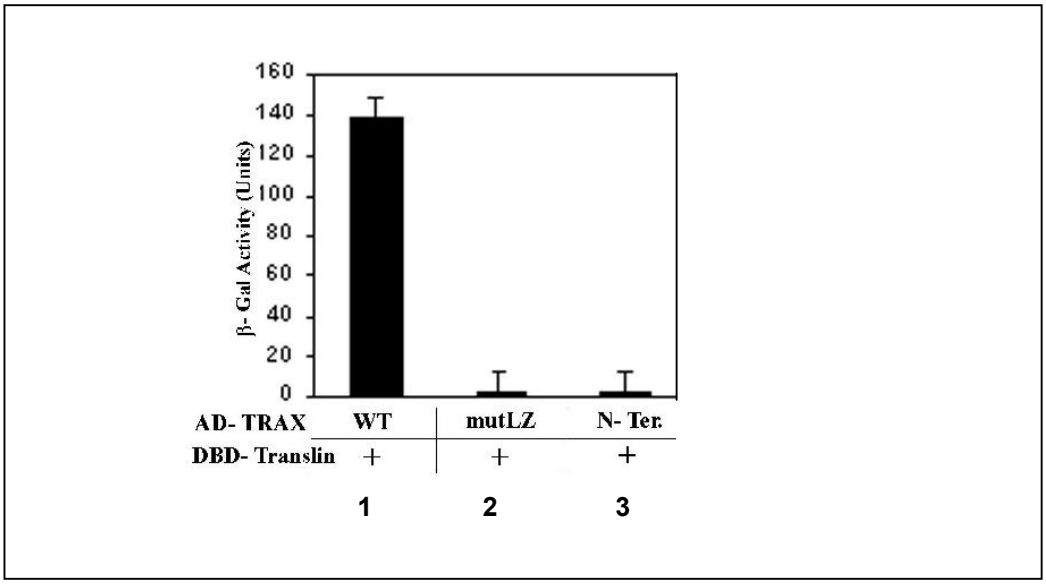


Figure 13: Translin requires the full-length TRAX with intact LZ for interaction.

3.5.4. C1D and Translin Compete with Each Other In Binding to TRAX

According to our yeast two hybrid results (Figure 12 and Figure 13), TRAX interacts with both C1D and Translin through its LZ region. It was important to determine whether these three proteins form a complex or C1D and Translin would compete with each other to bind to TRAX. To determine the relative interaction among them, His-tagged C1D was immobilized on Ni-NTA beads and incubated with the *in vitro* transcribed and translated (IVTT) TRAX and Translin either alone (Figure 14, Lanes 3 and 4) or together (Lanes 5,6, and7).

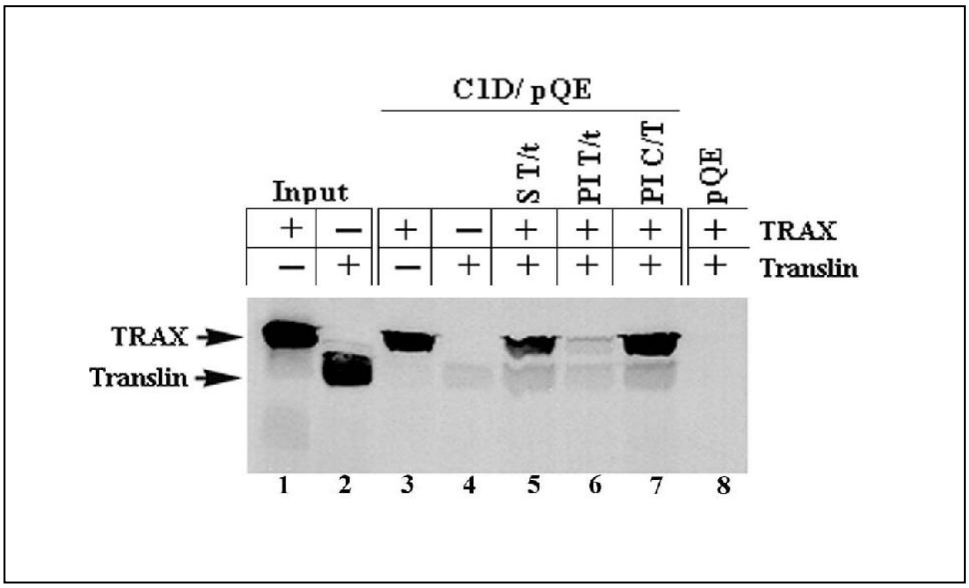


Figure 14: In vitro competition assay
Lane 1: TRAX input
Lane 2: Translin input
Lane 3: TRAX incubated with immobilized C1D
Lane 4: Translin incubated with immobilized C1D
Lane 5: Simultaneous (S) incubation of immobilized C1D with TRAX (T) and Translin (t)
Lane 6: Pre-incubation of TRAX (T) and Translin (t) before addition on to immobilized C1D
Lane 7: Pre-incubation of immobilized C1D (C) with TRAX (T) before addition of Translin (t)
Lane 8: Incubation TRAX and Translin with The plasmid expressing His only (pQE) as a negative control

Similar to our previous Ni-NTA pull down assay result (Figure 8), a substantial amount of TRAX was found to bind C1D (Figure 14, Lane 3), whereas binding of Translin to C1D was barely detectable (Lane 4). When TRAX and Translin were incubated simultaneously with immobilized C1D, the binding of TRAX to C1D was similar to the levels obtained when TRAX was incubated with C1D alone (Lane 5). By contrast, when TRAX and Translin were pre-incubated for 1 hour and then added to immobilized C1D, only small amount of TRAX was found to bind C1D (Lane 6). When TRAX was incubated with C1D for 1 hour prior to the addition of Translin,

binding of TRAX to C1D was increased almost twofold with respect to its binding without pre-incubation in the absence of Translin (compare lanes 3 and 7). As both C1D and Translin require LZ region of TRAX to bind (Figure 12 and 13), these results would suggest that C1D and Translin bind to TRAX in a mutually exclusive manner.

3.6. TRAX-C1D INTERACT IN MAMMALIAN CELLS

After demonstrating the interaction between C1D and TRAX in yeast and *in vitro*, mammalian cells were tested for interaction between these two molecules. C1D is an insoluble protein and it remains associated with DNA even after treatment with harsh denaturants (Nehls et al., 1998), rendering it impossible to perform immunoprecipitations using an antibody raised against the endogenous C1D protein. To overcome this limitation, COS7 cells were transiently transfected with a vector expressing HA epitope-tagged version of C1D protein (HA-C1D). Transfection of cells with C1D-expressing plasmids enables the production of sufficient soluble C1D and thus allows co-immunoprecipitation assays to be performed (Yavuzer et al., 1998). In agreement with this, when lysates from HA-C1D transfected cells were immunoprecipitated using an anti-HA antibody, followed by immunoblotting with an antibody against C1D, both the SDS-resistant dimeric (32 kDa) and monomeric (16 kDa) forms of C1D were readily detectable (Figure 15, Lane 1). To analyse the C1D/TRAX interaction, COS7 cells were co-transfected with vectors expressing HA-C1D and YFP-tagged TRAX (YPP-TRAX) and the lysates were immunoprecipitated with an anti-GFP antibody, which recognizes the YFP peptide. The immunoprecipitates were then subjected to immunoblotting with anti-C1D antibody. However, it was observed that C1D did not interact with TRAX under these conditions (Figure 15, Lane 2).

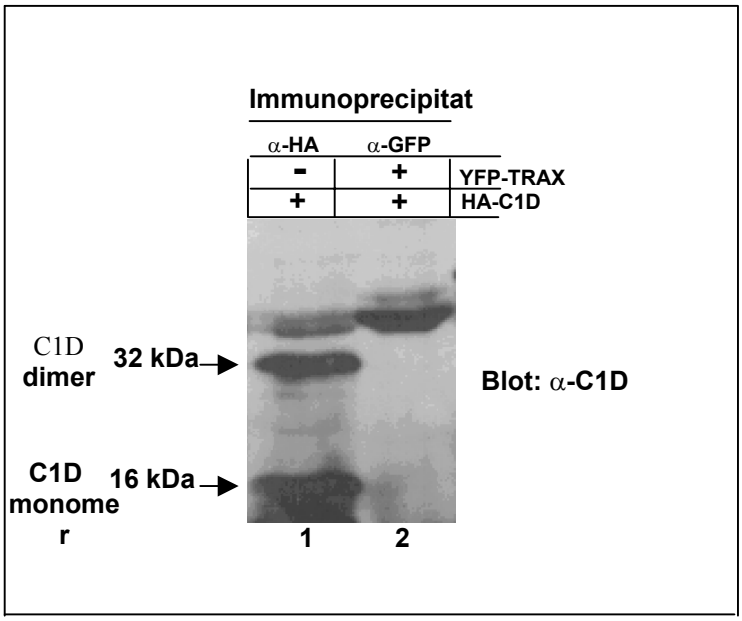


Figure 15: In mammalian cells TRAX and C1D don't interact under normal conditions. Plasmids used in transfections are indicated on the right hand side. Antibodies used for immunoprecipitation are as indicated. Western blot was performed by anti-C1D antibody. The upper band detected above C1D dimer is as a non-specific band.

3.6.1. TRAX and C1D Interaction is γ-Irradiation Inducible In Mammalian Cells

It was previously shown that mRNA and protein levels of C1D are induced upon γ-irradiation, a type of DNA-damage that induces double strand breaks (DSBs) in the DNA (Yavuzer et al., 1998). It was possible therefore, that C1D could perform its functions after being activated by such a stimulus. To demonstrate this, COS7 cells were transiently transfected with vectors expressing HA-C1D, YFP-TRAX or both. Untransfected cells were also included as controls. 24 hour following transfections, 20 Gy of γ-irradiation was applied to a set of transiently transfected cells and after incubating for an additional hour, lysates were prepared from unirradiated (Figure 16, lanes 1,2) and irradiated cells (lanes 3-6). In both

unirradiated and irradiated cells, C1D monomers and dimers were detected in immunoprecipitations using anti-HA antibody followed by immunoblotting with anti-C1D (Figure 16, lanes 1 and 4). As expected and in agreement with previous experiments (Figure 15, lane 2), when HA-C1D and YFP-TRAX were co-transfected and immunoprecipitated with anti-GFP antibody and probed with anti-C1D antibody, C1D did not interact with TRAX in unirradiated cells (Lane 2). However, in lysates obtained from irradiated cells, 32 kDa SDS-resistant homodimer, but not the 16kDa monomer of C1D, was detectable (Lane 3). Since, C1D was not detected in lysates from untransfected or YFP-TRAX expressing cells (Lanes 6 and 5), it was concluded that C1D and TRAX interact in mammalian cells as well, however only in response to γ-irradiation.

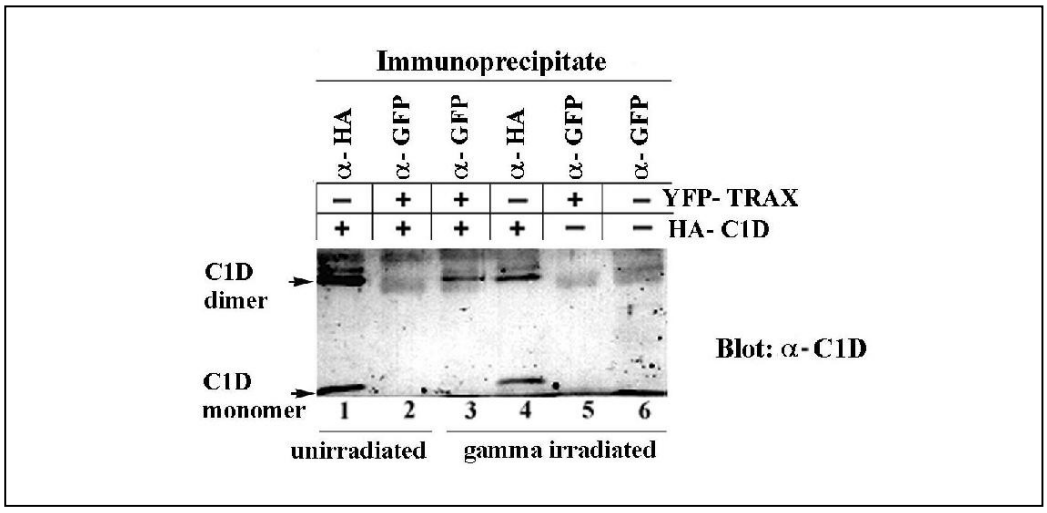


Figure 16: C1D and TRAX interact in mammalian cells in response to γ-irradiation. Plasmids used in transfections are indicated on the right hand side. Antibodies used for immunoprecipitations are as indicated. Western blot was performed by anti-C1D antibody.

3.6.2. C1D and TRAX Interact Only In Response to DNA Damaging Agents Inducing DNA-DSBs

Since γ-irradiation specifically induces DSBs formation in the DNA, It was important to determine whether C1D/TRAX interaction would be induced by other DNA damaging agents, such as ultra violet (u.v.) irradiation that induces thymine dimers which are mainly repaired by nucleotide excision repair (NER) pathway. To test this, COS7 cells were again transiently transfected with vectors expressing HA-C1D, GFP-TRAX or both and 50 j/m^2 u.v. irradiation was applied to a set of transfected cells 1 hour before harvesting. The lysates were prepared from unirradiated (Figure 17, lanes 1 and 2) and u.v. irradiated cells (Lanes 3-7) and were immunoprecipitated using antibodies against GFP or HA-epitope. As controls, lysates from untransfected and HA-C1D or GFP-TRAX transfected cells were also immunoprecipitated using the same antibodies (Fig. 17, lanes 4-7). The immunoprecipitates were then blotted with an anti-C1D antibody (Figure 17, upper panel). As expected, anti-HA antibody immunoprecipitated the 32 kDa dimeric form of C1D in cell lysates from HA-C1D transfected cells (Lane 1). Due to usage of a low percentage gel, the 16 kDa monomeric form was not visible in this particular experiment. However, C1D was not detectable when cell lysates from GFP-TRAX and HA-C1D transfected cells were immunoprecipitated with an anti-GFP antibody (Lane 2). In contrast to γ-irradiation, treatment of these double transfected cells with u.v. irradiation did not induce interaction of TRAX with C1D (Lane 3).

To confirm that the lack of interaction between C1D and TRAX upon u.v. irradiation is not due to absence of TRAX expression, the blot was stripped and re-probed with an antibody raised against the GFP peptide (Figure 17, lower panel). A protein of ~60-kDa, which is the expected size for GFP-TRAX protein, was detected in all cell lysates from GFP-TRAX transfected cells (Lanes 2, 3, and 7) but not in cell lysates from untransfected or HA-C1D transfected cells (Lanes 1,5, and 6).

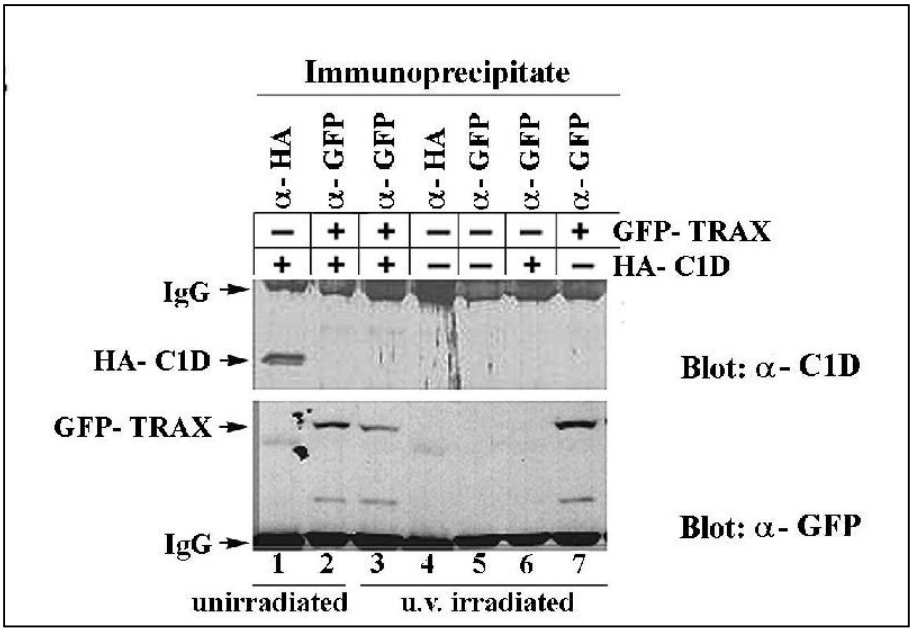


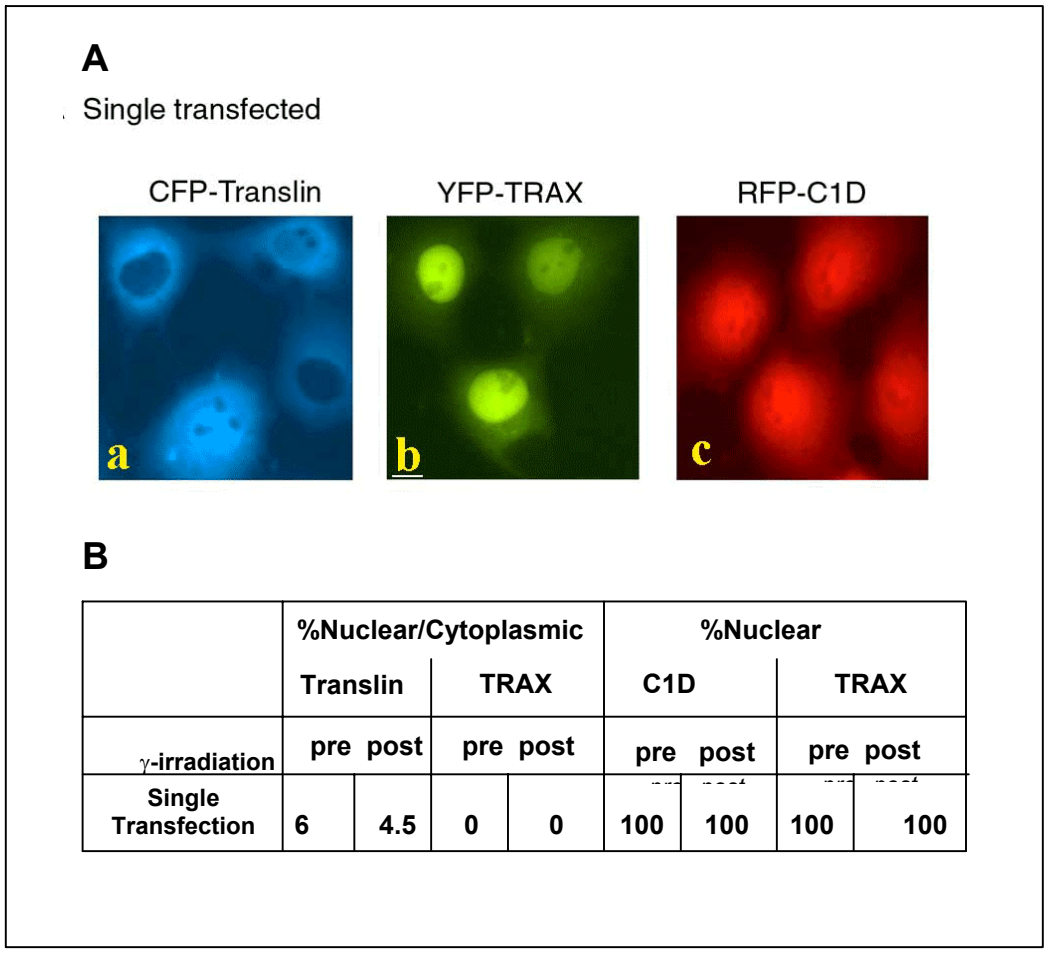
Figure 17: TRAX and C1D don't interact in mammalian cells in response to u.v. irradiation

3.7. SUBCELLULAR LOCALIZATION OF C1D, TRAX AND TRANSLIN IN MAMMALIAN CELLS

The data presented so far demonstrates that TRAX interacts specifically with both C1D and Translin, however C1D and Translin compete with each other for binding to TRAX. Thus, the three molecules can not form a complex, and in mammalian cells TRAX interacts with C1D only in response to γ-irradiation. In order to demonstrate the biological reason for this, the subcellular localizations of these three molecules were examined and the effects of γ-irradiation on the subcellular distribution of TRAX, C1D and Translin were analysed. To this end, these proteins were expressed in COS7 cells as fusions with different GFP variants either alone or combination and the expression patterns were determined in the living cells. Thus, Translin was expressed as a CFP fusion protein (CFP-Translin), TRAX as YFP-TRAX and C1D as RFP-C1D giving cyan, green or red colours with the appropriate filters. All the expression plasmids were constructed at Bilkent

University, however, as we do not have a fluorescence microscopy equipped with different filter sets, the microscopic analyses were performed at Marie Curie Research Institute by U.Yavuzer and B. Bilican.

First the subcellular localization of each protein was analysed. In agreement with previous results (Chennathukuzhi et al., 2001; Kasai et al., 1997). CFP-Translin was found to be cytoplasmic in over 90 % of the cells examined (Figure 18Aa, B). In around 6 % of the cells however, CFP-Translin exhibited both nuclear and cytoplasmic localization (Figure 18B). γ-Irradiation or other DNA damaging agents did not cause any alteration in its subcellular localization in agreement with previous observations (Chennathukuzhi et al., 2001). In contrast, YFP-TRAX was localized to nucleus in all cells examined, whereas C1D was observed in a diffuse nuclear (predominantly)/cytoplasmic staining pattern as observed using anti-C1D antibody (U. Yavuzer unpublished data) (Figure 18A b and c respectively and figure 18B). As with Translin, localization of TRAX and C1D did not change following γ-irradiation (Figure 18B).



B

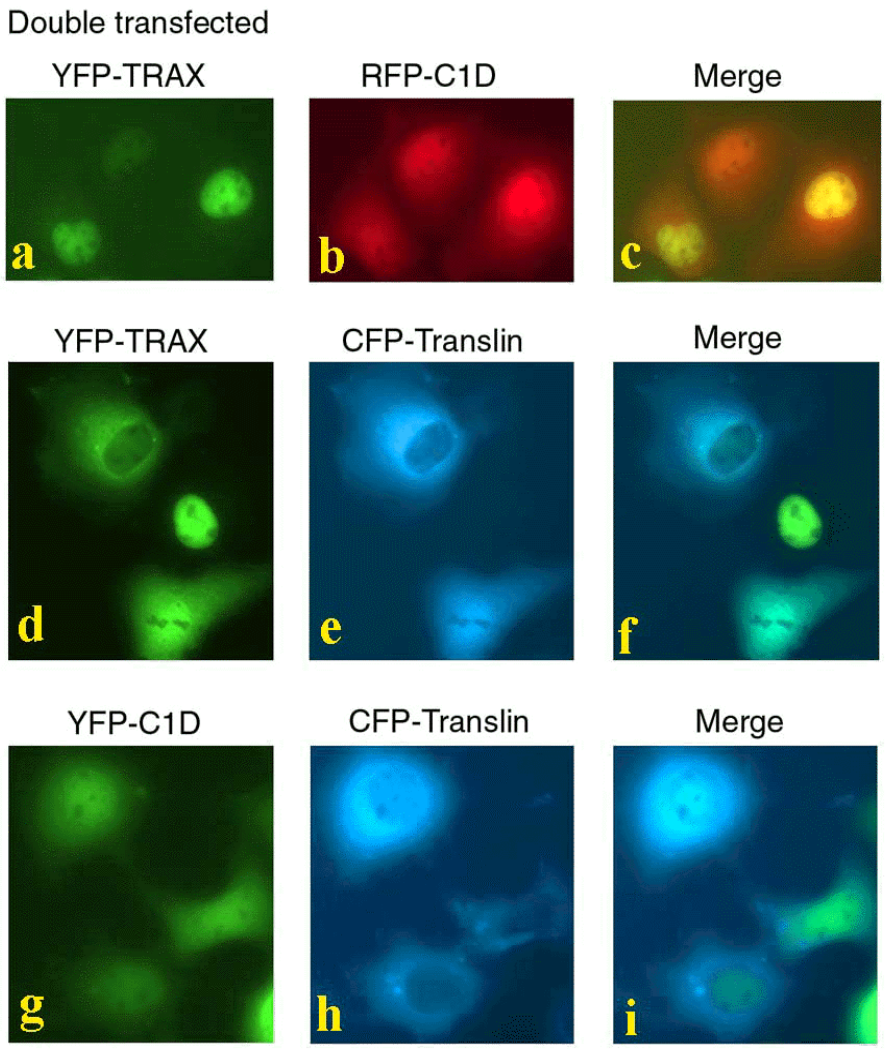
	%Nuclear/Cytoplasmic				%Nuclear			
	Translin		TRAX		C1D		TRAX	
γ-irradiation	pre	post	pre	post	pre	post	pre	post
Single Transfection	6	4.5	0	0	100	100	100	100

Figure 18: (A) Subcellular localization of Translin, TRAX and C1D when they were expressed alone. (B) Summary of the results demonstrating the percentage of nuclear/cytoplasmic and nuclear localizations of Translin, TRAX and C1D before and after γ-irradiation

Since, in 6% of the cells Translin exhibited nuclear/cytoplasmic localization when expressed alone, we wanted to see whether an alteration would occur in its nuclear localization when expressed together with C1D or TRAX. To constitute a basal level for the percentage of cells exhibiting nuclear Translin in double transfections, Translin was co-expressed with a plasmid expressing YFP only and was found to show nuclear/cytoplasmic localization in around 10 % of the cells (Figure 19B). A different picture emerged with TRAX or C1D double transfections: Co-expression of Translin with TRAX resulted in almost 3.5-fold increase in the proportion of cells in which Translin was observed to be nuclear/cytoplasmic (34 %)

co-localizing with TRAX, and a striking alteration in the location of TRAX, with around 58 % of cells expressing TRAX both in cytoplasm and nucleus (compared to 100 % when expressed alone). In 42% of the cells TRAX was solely cytoplasmic and colocalized with Translin. Importantly, in these cells where TRAX was solely cytoplasmic, Translin was never found to be nuclear. In Figure 19A d-f, a representative area where three cells are shown, of which two of the cells contain both TRAX and Translin. In one of the cells, TRAX and Translin are co-localized to cytoplasm whereas in the other, a diffuse nuclear/cytoplasmic staining pattern is observed. The third cell expresses TRAX only and as expected, when Translin is not co-expressed, TRAX is solely nuclear. This single TRAX expressing cell in this particular area also acts as an internal control demonstrating that the filters used to detect YFP or CFP are highly specific. These alterations in the compartmentalization of TRAX and Translin presumably reflect interactions between these two proteins and indicates that the relative expression levels of these proteins is important in determining their subcellular localization. γ-irradiation did not cause a significant alteration in the subcellular localizations of TRAX and Translin. On the contrary when TRAX was expressed with C1D, neither C1D nor TRAX changed their localizations either before or after γ-irradiation and TRAX stayed in the nucleus (Figure 19Aa) whereas C1D showed its usual nuclear/cytoplasmic staining pattern (b), co-localizing with TRAX in the nucleus (c). Co-expression of Translin and C1D (Figure 19A g-i) resulted in a slight increase in the proportion of cells containing nuclear Translin (19%) however; once again γ-irradiation did not have any effect on the subcellular localizations of either Translin or C1D. These results demonstrate that γ-irradiation does not effect the localization of the TRAX, Translin or C1D, however, the otherwise nuclear TRAX changes its localization when Translin is co-expressed and stays in the nucleus when C1D is co-expressed.

A

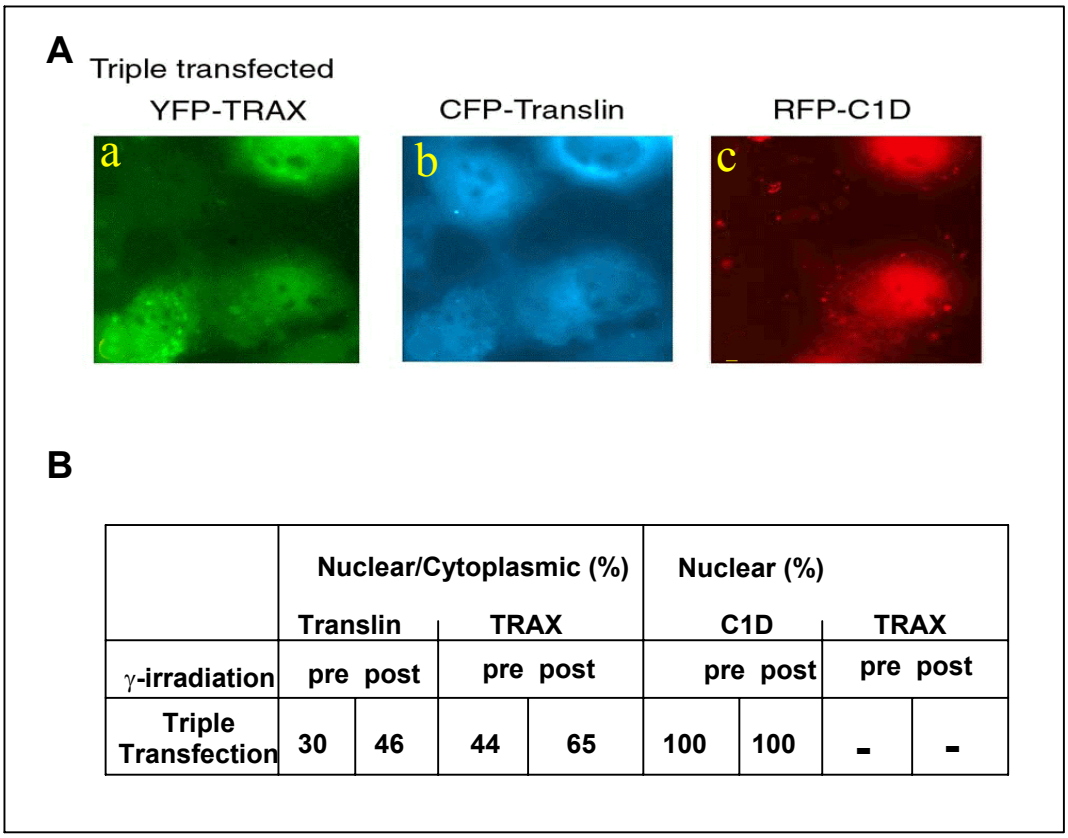


B

γ-irradiation	% Nuclear / Cytoplasmic				% Nuclear			
	Translin		TRAX		C1D		TRAX	
	pre		pre		pre		pre	
Double Transfection	10	11	-	-	-	-	-	-
	34	43	58	61	-	-	-	-
	19	15	-	-	100	100	-	-
	-	-	-	-	100	100	100	100

Figure 19: (A) Effect of TRAX-C1D, TRAX-Translin and C1D-Translin coexpression on expression pattern of TRAX, C1D and Translin (B) Summary of the results demonstrating the percentage of % Nuclear/Cytoplasmic and % Nuclear localizations of Translin, TRAX and C1D before and after γ-irradiation

In cells triple transfected with YFP-TRAX, CFP-Translin and RFP-C1D expression vectors, we were not able to detect any significant alteration in the subcellular localizations of TRAX or Translin compared to TRAX/Translin double transfected cells (Figure 20B). In other words, C1D over expression did not cause an overall alteration in the localization of TRAX or Translin. In figure 20A a-c, a group of cells of which two were triple transfected are shown. C1D again exhibits a nuclear (predominantly)/cytoplasmic staining (c), whereas TRAX is solely nuclear in one of these cells (a, upper cell) and nuclear/cytoplasmic in lower cell. Translin on the other hand, is also nuclear/cytoplasmic in the lower cell, but is mainly cytoplasmic in the upper cell and therefore does not co-localize with TRAX. In the bottom, left hand side a third cell (a and b) which only expresses nuclear/cytoplasmic TRAX and Translin, and fourth cell (b) expressing Translin only, again acting as internal controls demonstrating that the different filter used for GFP, YFP or DsRed kare specific. Although in a few cells expression of C1D appears to maintain TRAX in the nucleus while Translin was mainly cytoplasmic, the majority of cells showed a nuclear/cytoplasmic staining, making it difficult to determine whether TRAX was complexed with Translin or C1D. However, we noted a slight increase in the percentage of cells experessing nuclear TRAX or Translin after irradiation in the presence of C1D compared to no increase in the double transfected cells (Figure 20B).



	Nuclear/Cytoplasmic (%)				Nuclear (%)			
	Translin		TRAX		C1D		TRAX	
γ-irradiation	pre	post	pre	post	pre	post	pre	post
Triple Transfection	30	46	44	65	100	100	-	-

Figure 20: (A) Subcellular Localizations of TRAX, Translin and C1D in triple transfected cells (B) Summary of results demonstrating the percentage of nuclear/cytoplasmic and nuclear localizations of TRAX, Translin and C1D before and after γ-irradiation.

In conclusion, *in vitro* and *in vivo* experiments in yeast and mammalian cells suggest that the nuclear matrix protein C1D interacts with TRAX in response to DNA damage and possibly could inhibit TRAX/Translin complex formation by masking the Translin binding site. This, as a consequence may inhibit binding of TRAX/Translin to DNA and prevent any unwanted TRAX/Translin based recombination events, which may lead to translocations seen in many types of cancer (for details please see the 'Discussion' section). Although, these experiments give important clues about the biological function of C1D, they do not provide any direct evidence. Therefore, it was decided to use *S. cerevisiae*, since this organism has served as a useful tool for analyzing mammalian gene function and deducing biological processes in human cells.

CHAPTER 4

4.1. IDENTIFICATION OF *S.CEREVISIAE* HOMOLOG OF C1D

In light of the evidence presented in the previous chapter and also the published work on C1D show that C1D is regulated in response to γ-irradation and suggest that C1D might play a role in DNA repair and recombination. The easiest way of demonstrating this directly would be via the usage of *S. cerevisiae*, since it is rather cheap and easy to combine genetics and biochemistry to identify the function of a given gene using this organism. Indeed, many disease genes and most of the genes involved in the repair of damaged DNA in mammalian cells were initially characterized in yeast.

There are at least two distinct pathways in eukaryotes to repair DNA-DSBs; HR and NHEJ (Haber, 1999). In mammals, the NHEJ pathway appears to be the major pathway, whereas yeast cells mostly rely on HR.

In eukaryotic organisms, RAD52 epistasis group of genes is identified to encode proteins that participate in HR. Many members of this group were initially identified in *S.cerevisiae* as mutants sensitive to γ-irradiation. They include *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *MRE11* and *XRS2*. Mutants in this group have defects in the repair of DNA-DSBs by HR (Paques and Haber, 1999).

Genetic and biochemical studies have shown that end-joining in yeast and mammals requires the same core set of proteins, the DNA end binding subunits of DNA-PK, Ku70 and Ku80 as well as DNA Ligase IV and its associated factor XRCC4 (called *YKU70*, *YKU80*, LIG4 *and LIF*, respectively, in *S.cerevisiae*) (Boulton and Jackson, 1998; Schar et al., 1997; Teo and Jackson, 1997; Wilson et al., 1997). *RAD50*, *MRE11* and *XRS2* gene products have also been implicated in NHEJ pathway. Interestingly, this complex has been shown to play important roles both in NHEJ and HR (Boulton and Jackson, 1998; Moreau et al., 1999).

In this section, I will present the data towards identification of the yeast homolog of C1D and the assays that were used to decipher the biological function of yeast C1D in DNA repair and recombination. It is important to keep in mind that, although many human genes may have homologs in yeast, this doesn't mean that they are functionally similar. Therefore, it was important to determine whether human and yeast C1D perform the same functions in an organism.

Initially, a BLASTP search was performed and it was seen that the *S.cerevisiae YHR081W* gene encodes a hypothetical protein exhibiting 25% and 27% identity to mouse (acc. no. CAA648444) and human (acc. no. CAA64845) C1D, respectively. This gene is referred to as *YC1D* from now on. Since the role of *YC1D* in HR and NHEJ was decided to be analysed, it was important also to obtain mutant strains for *RAD52* and *YKU70* as both genes are the best-studied examples for each type of DSB repair. Therefore, haploid and diploid mutant strains for *YC1D*, *YKU70* and *RAD52* genes were purchased from EUROSCARF (description of the strains can be found in Chapter 2) and both phenotypic and functional assays were performed.

4.2. PHENOTYPIC ANALYSIS OF *YC1D* MUTANT STRAIN

4.2.1. *yc1d* Mutant Strain is Temperature Sensitive

To address the function of Yc1d, the phenotypic consequences of deleting the *YC1D* gene in haploid and diploid strains were examined. Previous findings about the function of human C1D protein suggest that C1D could be a multi functional protein involved in preservation of genomic integrity by assisting factors involved in DNA repair, recombination, transcription and apoptosis. Therefore, if yeast C1D and human C1D are orthologs, absence of Yc1d could result in deficiency of preservation of genomic integrity, which would be manifested by some phenotypic features, such as growth retardation, high sensitivity to increased temperature and DNA damaging agents. These types of phenotypic analyses are routinely applied for the identification of the yeast proteins involved in chromosome metabolism, cell cycle checkpoint,

DNA repair/recombination, DNA replication and chromatin remodelling (Bennett et al., 2001).

The *yc1d* haploid and homozygote or heterozygote diploid mutant strains were initially analysed for temperature sensitivity. Both *MATa* and *MAT*α type haploid and homozygote diploid strains deleted for *YC1D* gene were temperature-sensitive for growth at 39^0C (Figure 21B). On the contrary, the heterozygous diploid strain, *YC1D/yc1d*, did not show any defect for growth at the restricted temperature, suggesting that the temperature sensitive phenotype is a direct consequence of Yc1d deficiency.

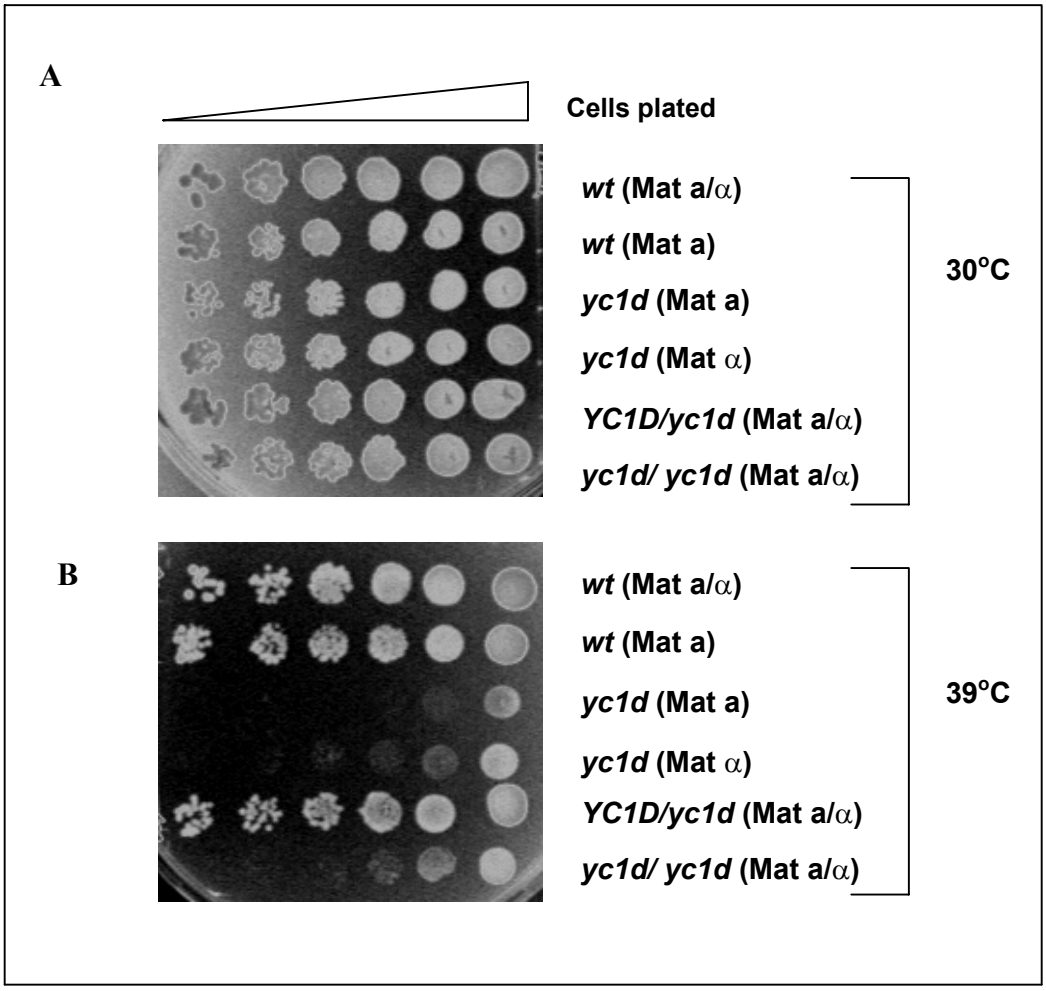


Figure 21: *yc1d* mutant strain is temperature sensitive**.** Aliquots (5µl) of serial 10-fold dilutions of yeast cultures were spotted onto YPED medium and were grown for 3 days at 30^0C or 39^0C, as indicated.

4.2.2. *yc1d* Mutant Strains are Slightly Sensitive to γ-Irradiation

Next, the sensitivity of *yc1d* mutant strains to variuos physical and chemical DNA damaging agents, such as u.v. irradiation, methyl methane sulfonate (MMS) and γ-irradiation were examined. As mentioned before, u.v. irradiation induces thymidine dimers which are mainly repaired by nucleotide excision repair (NER), whereas MMS and γ-irradiation, induce DNA-DSBs that are repaired by either homologous recombination (HR) or non-homologous end joining(NHEJ). Although previously it was reported that the *yku70* mutant strain is resistant towards MMS, u.v. and γ-irradiation (Boulton and Jackson, 1996), in our experimental conditions we were able to detect a very slight sensitivity of *yku70* mutant strain towards MMS (Figure 22A). Interestingly, *yc1d* haploid mutant strain was resistant to both u.v. and MMS (Figure 22A and B). However, unlike the *yku70* mutant strain, both mating type *yc1d* mutants were slightly sensitive towards γ-irradiation (Figure 22C). As a control, a *rad52* mutant strain was also included in these assays and as reported before, it was found to be sensitive to MMS and γ-irradiation, however only slightly sensitive towards u.v. treatment (Bennett et al., 2001).

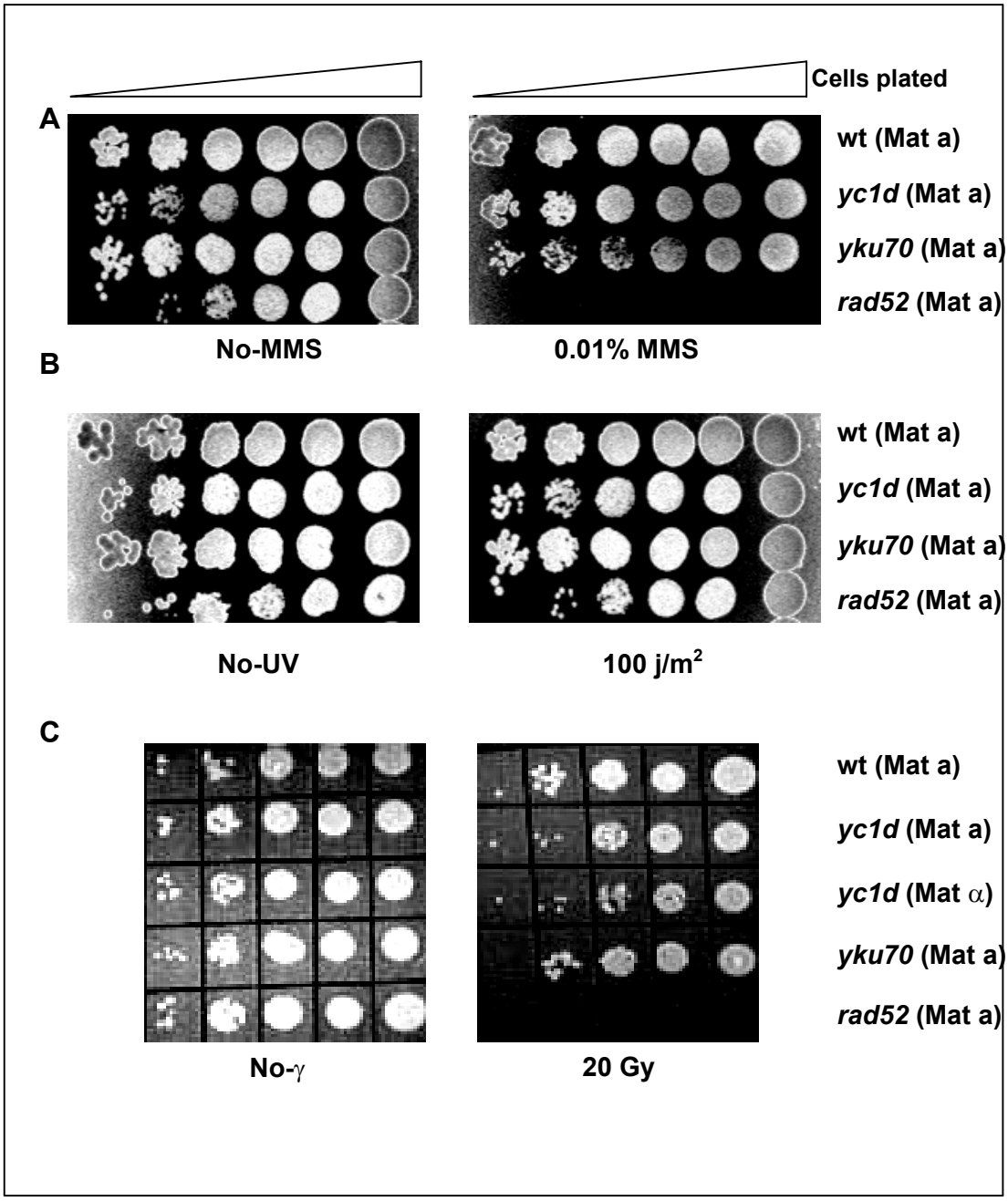


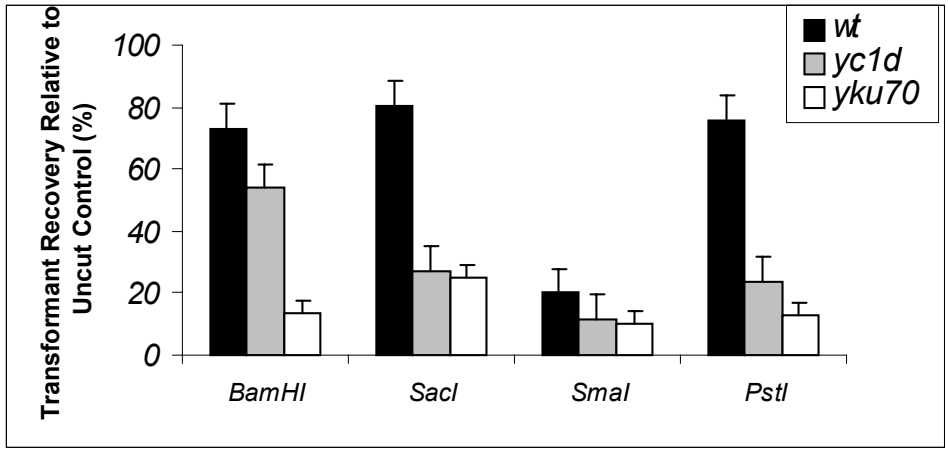
Figure 22: *yc1d* mutant strain is not sensitive to either MMS or UV radiation, however is slightly sensitive to γ-irradiation

4.3. IDENTIFICATION OF THE FUNCTION OF *YC1D* GENE

4.3.1. *YC1D* Is Involved In DSB Repair

Human C1D has been found to interact with proteins involved in DNA DSB repair and recombination. In addition, *YC1D* deletion causes *S. cerevisiae* cells sensitive to high temperatures and to a slight extent, to γ-irradiation. Therefore, it is likely that Yc1d may be involved in repair of DNA double-strand breaks. In order to examine this, a plasmid-based repair assay was employed to assess the efficiency of DNA DSB repair. This assay was previously used to analyse the role of Yku70 in NHEJ (Boulton and Jackson, 1996). Briefly, a *S. cerevisiae-E.Coli* shuttle plasmid was linearized within its multiple cloning site using restriction enzymes that produce 5'- and 3'- overhanging or blunt-ends. The linearized and the supercoiled versions of the same plasmid were then introduced into yeast strains in parallel and the transformants were plated onto selective medium. Linearized plasmids can only be propagated in *S.cerevisiae* after they have been recircularized and ligated. Therefore, by counting the number of colonies harbouring religated plasmid on each plate and normalizing to the efficiency of transformation with the supercoiled plasmid, the efficiency of ligation, thus NHEJ, can be measured. In wild type strains both the 5'- and 3'-overhanging ends were religated efficiently, whereas as reported previously, the plasmids bearing two blunt termini (as generated by *SmaI* digest) were not (Boulton and Jackson, 1996). We observed that *yc1d* mutant strain was defective in rejoining the 3'-overhanging (*SacI* and *PstI* digests) and blunt-ended (*SmaI* digest) plasmid DNA, however unlike *yku70* mutant strains, *yc1d* mutant strains exhibited only a slight defect in repair of the 5'-overhanging ends (Figure 23A). Moreover, introduction of a plasmid expressing wild type Yc1d into the *yc1d* mutant strain complemented the defect seen in the repair of 3'-overhanging ends in these mutant strains (Figure 23B).

A



B

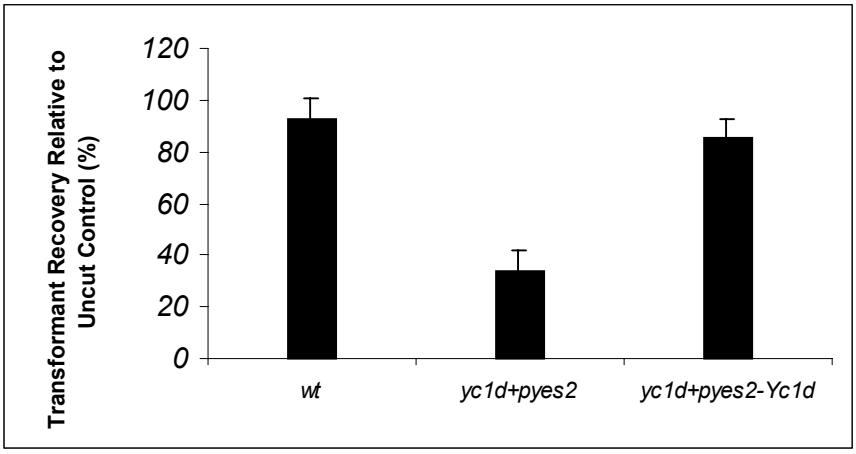


Figure 23: ***yc1d*** **mutant strain is defective in NHEJ as demonstrated by a plasmid-based** ***in vivo*** **DNA end joining assay. A)** Transformant yield of linearized plasmids with the indicated restriction enzymes in wild type and different mutant backgrounds. **B)** Transformant yield of SacI or PstI-linearized plasmid in yc1d mutant strain is restored to wild type levels when a plasmid expressing Yc1d is introduced.

4.3.2. In The Absence of *YC1D*, Accurate End-joining Is Impaired

Although the *yc1d* mutant strain is deficient in repair of 3'-overhanging ends, there is almost 20 % residual repair activity (Figure 23A). It was important therefore, to analyse the transformants and determine whether the NHEJ has been performed accurately in the absence of *YC1D*. For this purpose, again the plasmid-based repair assay was performed with some modifications. In this modified assay, a yeast episomal plasmid (pGV256 Live) expressing *LacZ* under the control of the constitutively active *CYC* (Cytochrome C) promoter was used. When this plasmid is introduced into yeast, the colonies produce a blue colour in the presence of the substrate X-gal, due to the expression of β-galactosidase. pGV256 Live was linearized using the unique *SacI* site that is found in the middle of the *LacZ* ORF, introduced into the wild type, *yc1d* and *yku70* mutant strains and plated onto selective medium. As described in the previous section, only the transformants harboring recirculized and ligated plasmids can grow on selective medium. In this case, because the plasmid is linearized within the coding sequence of *LacZ*, re-ligation will produce transformants, but only the colonies harbouring accurately re-ligated plasmid will give blue colour in the presence of X-Gal. Consequently, the ratio of number of blue colonies to the total number of transformants will give the efficiency of accurate repair.

The result showed that, *yc1d* mutant strain is defective in accurate repair of DNA ends, since only 50% of the transformants were accurately repaired, thus giving blue colour in the presence of X-Gal (Figure 24). The other half of the residual repair activity was governed by an error-prone pathway. In agreement with a previous report, absence of Yku70 leads to error prone repair of DNA ends (Boulton and Jackson, 1996) and only 25% of the transformants were repaired accurately (Figure 24). Therefore, although not to the extent of Yku70, Yc1d also acts as a barrier to error-prone DNA repair pathways.

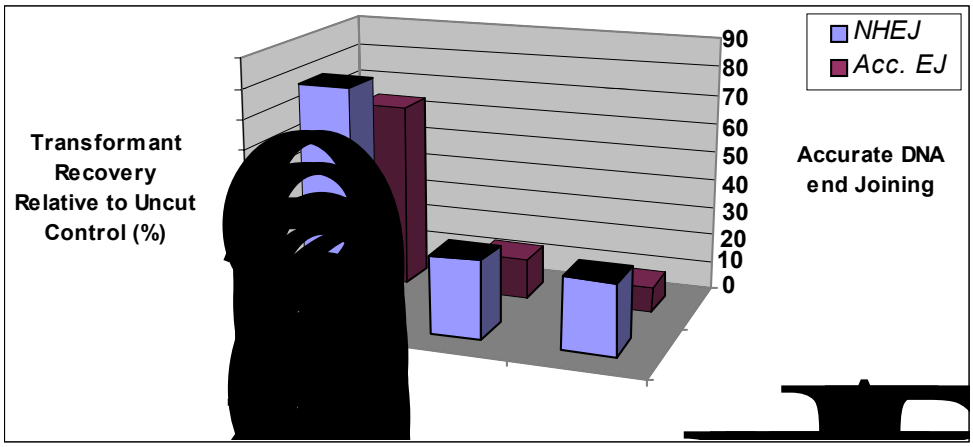


Figure 24: Accurate DNA-end joining is impaired in *yc1d* and *yku70* mutant strains.

4.3.3. Role of Yc1d in Homologous Recombination

Since the repair of DSBs may also be performed by homologous recombination, the role of *YC1D* gene in recombination was assayed. For this purpose a novel in vivo recombination assay was developed using the "pGV256 Live" plasmid. The schematic representation of this assay is shown in Figure 25. First, "pGV256 Live" was modified by inserting a double-stranded oligonucleotide into the unique *SacI* site within the LacZ coding sequences. This 21- bp oligonucleotide contains a unique restriction enzyme site (*Bgl II*) in the middle flanked by 'STOP' codons in different reading frames, thus disrupting the expression of LacZ (Figure 25B). We termed this plasmid "pGV256 Dead" as no β-galactosidase activity is produced by the yeast transformants harbouring this plasmid. A 800 bp region of LacZ coding sequences encompassing the region where the 'STOP' codons were integrated was amplified by PCR and introduced into the recipient yeast strain together with the *BglII*-linearized plasmid pGV256 Dead. Since there is no homology between the *LacZ* fragment and *S.cerevisiae*

chromosomal sequences, the only way the plasmid can be propagated is either by re-circularization and ligation of the plasmid, in which case there would be transformants but none of them will produce blue colour in the presence of X-gal (Figure 25C). Alternatively, the 800-bp LacZ PCR fragment could be integrated into the plasmid by homologous recombination, which will manifest itself by production of blue colour (Figure 25D). Therefore, the ratio of blue colonies over to the total number of colonies on a selective plate will directly reflect the efficiency of homologous recombination for those particular yeast strains.

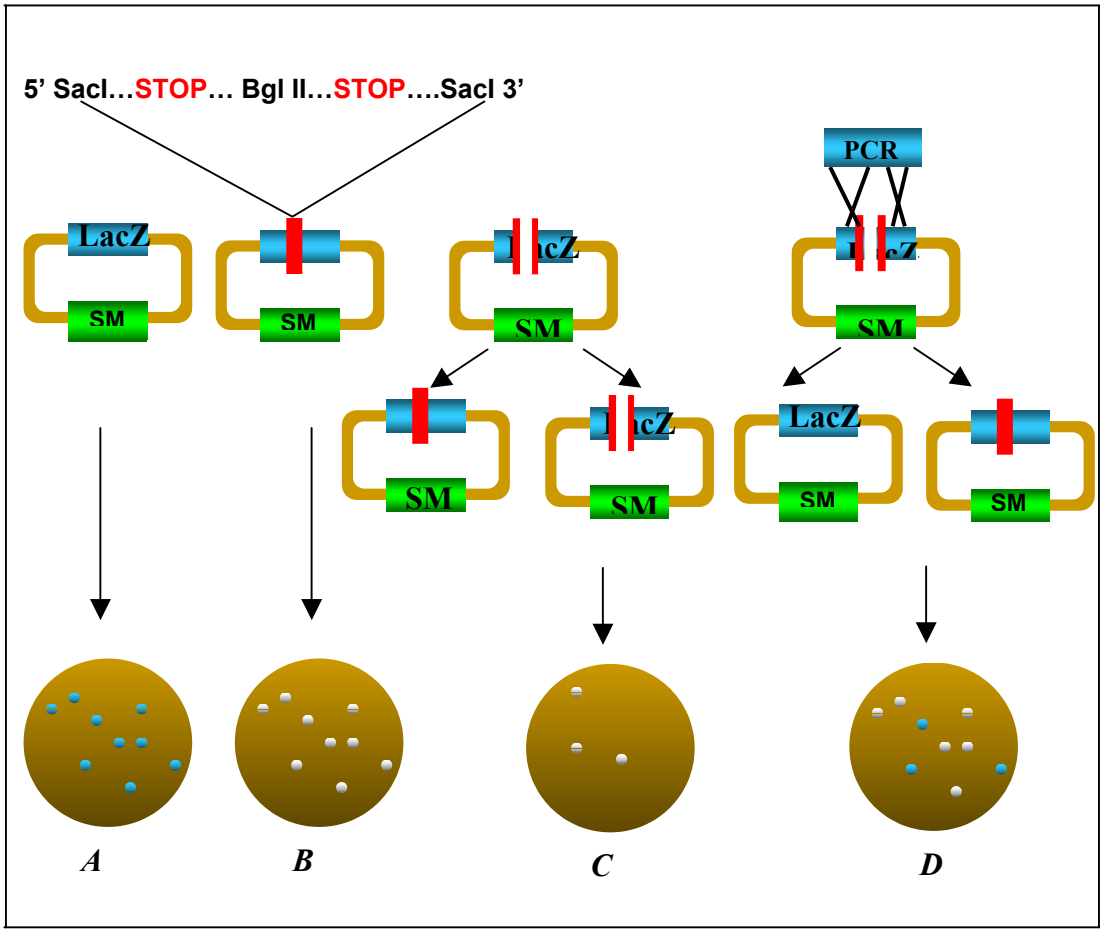


Figure 25: Schematic view of *in vivo* homologous recombination assay

To validate this novel in vivo recombination assay, the *rad52* mutant strain that was shown to be deficient in HR (Paques and Haber, 1999) and the *yku70* mutant strain which is mainly deficient in DNA end joining but not in HR (Lewis et al., 1999), were also analysed along with the wild type and *yc1d* mutant strains. As expected, the in vivo recombination assay demonstrated that the *yku70* mutant strain was able to perform HR as efficiently as the wild type strain, whereas the *rad52* mutant strain was completely defective in HR. Notably, *yc1d* mutant strains exhibited a twofold reduction in HR compared to wild type strain (Figure 26).

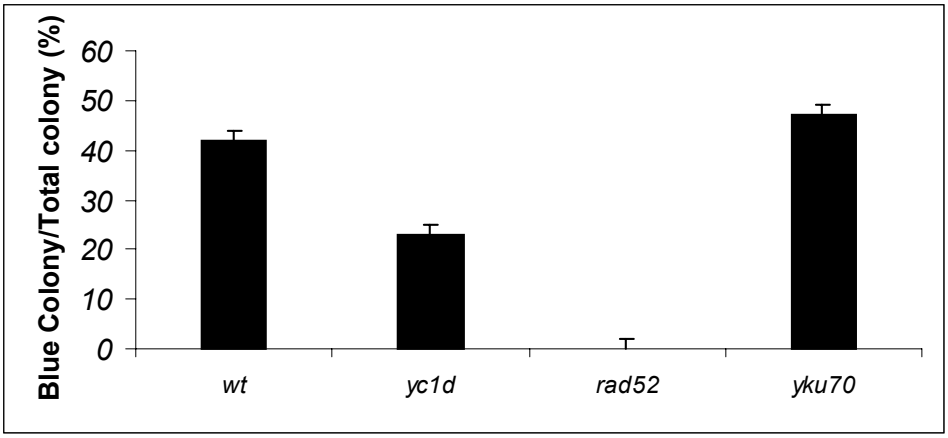


Figure 26: Analysis of *yc1d* role in HR recombination using our *in vivo* HR assay

In order to confirm the results obtained using this novel in vivo recombination assay, we also checked the same strains for growth in the presence of the homothallic switching (HO) endonuclease by using a galactose inducible construct. This assay is based on expression of HO endonuclease from a plasmid with a galactose inducible GAL1-10 promoter, which leads to a site specific DSB at the MAT locus (Moore and Haber, 1996). Since repair of HO endonuclease- induced DSBs is performed by HR, strains deficient in HR are not able to survive in growth

medium containing galactose if they are carrying this construct (Lewis et al., 1999). Therefore, the survival rates of WT, *rad52* and *yc1d* mutant strains harbouring the plasmid expressing galactose-inducible HO endonuclease were assessed by counting the number of colonies formed on galactose-containing medium. As seen in Figure 27, survival of *rad52* mutant strain was severely debilitated with respect to the wild type strain. In accordance with our in vivo plasmid-based recombination assay, the *yc1d* mutant strain exhibited 57% efficiency in survival rate with respect to the wild type strain, which had a 82% efficiency. Importantly, this reduction in survival rate was restored to wild type levels upon introducing into the *yc1d* mutant strain a plasmid expressing wild type Yc1d. Therefore, we concluded that the assay we have developed is as efficient as the other assays measuring the recombination efficiency and that Yc1d also plays a role in homologous recombination.

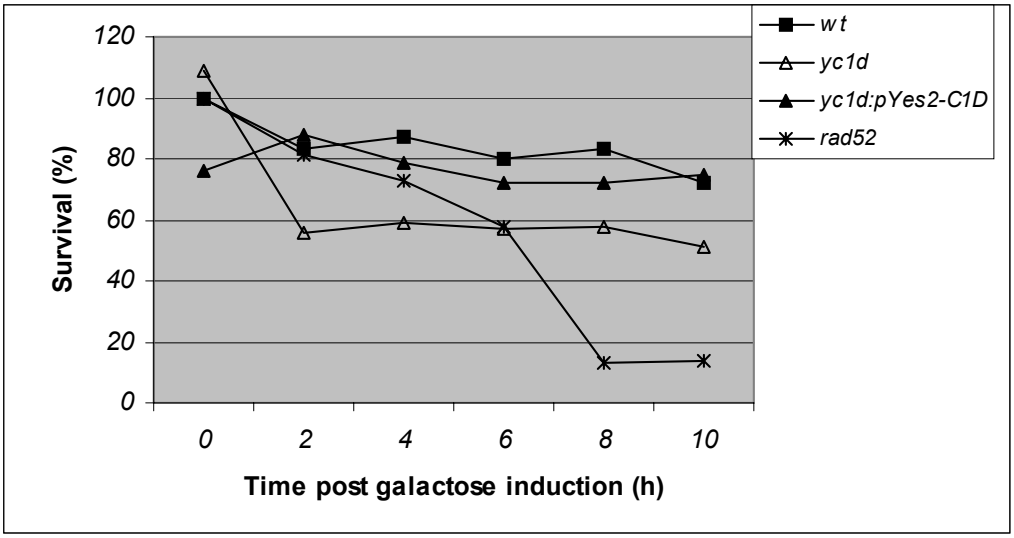


Figure 27: Survival of strains containing a plasmid with a galactose inducible HO-endonuclease expressed relative to survival time at zero

4.4. *YKU70* AND *YC1D* ARE IN THE SAME EPISTASIS GROUP

The data obtained so far demonstrates that both the *yku70* and *yc1d* mutant strains are not only phenotypically similar but both genes have similar effects in NHEJ. In addition and in contrast to *YKU70*, *YC1D* is also involved in HR. It was important therefore to determine whether Yc1d and Yku70 belong to the same epistasis group. In an attempt to analyse this possibility, the phenotypic and functional consequences of Yc1d deletion on a *yku70* mutant background were analysed.

4.4.1. Preparation of Yeast Strains Deleted for Both *YC1D* and *YKU70*

To this end, gene knock out strategy was employed, which relies on deletion of the certain coding DNA sequence by transforming host organism with genetically engineered DNA molecule carrying appropriate selective marker. Accordingly, DNA molecule used for this purpose is named gene disruption or gene replacement cassette. Knocking out the gene of interest is based on the homologous recombination between homologous DNA sequences present on the disruption cassette and the host genome. The employment of *S. cerevisiae* for gene knockout strategy is particularly easy and well developed since the rate of homologous recombination is very high and the whole genome has been sequenced in April 1996.

PCR mediated gene knockout strategy was applied to delete *YC1D* on a *yku70* mutant background. In this method, the PCR primers used had 5'-ends (~40 nucleotides) that correspond to a region 200 bp upstream (forward primer) or downstream (reverse primer) of the open reading frame Yc1d. The 3'-ends (~20 nucleotides) of these primers contained sequences that anneal and allow the amplification of HIS3 as selectable marker. Using these primers and a plasmid containing the open reading frame of his3 as a template, PCR was applied and the gene disruption cassette was obtained (Figure 28). The gene disruption cassette was then introduced into the recipient *yku70* mutant strain and transformed colonies were selected on histidine deficient selective plates.

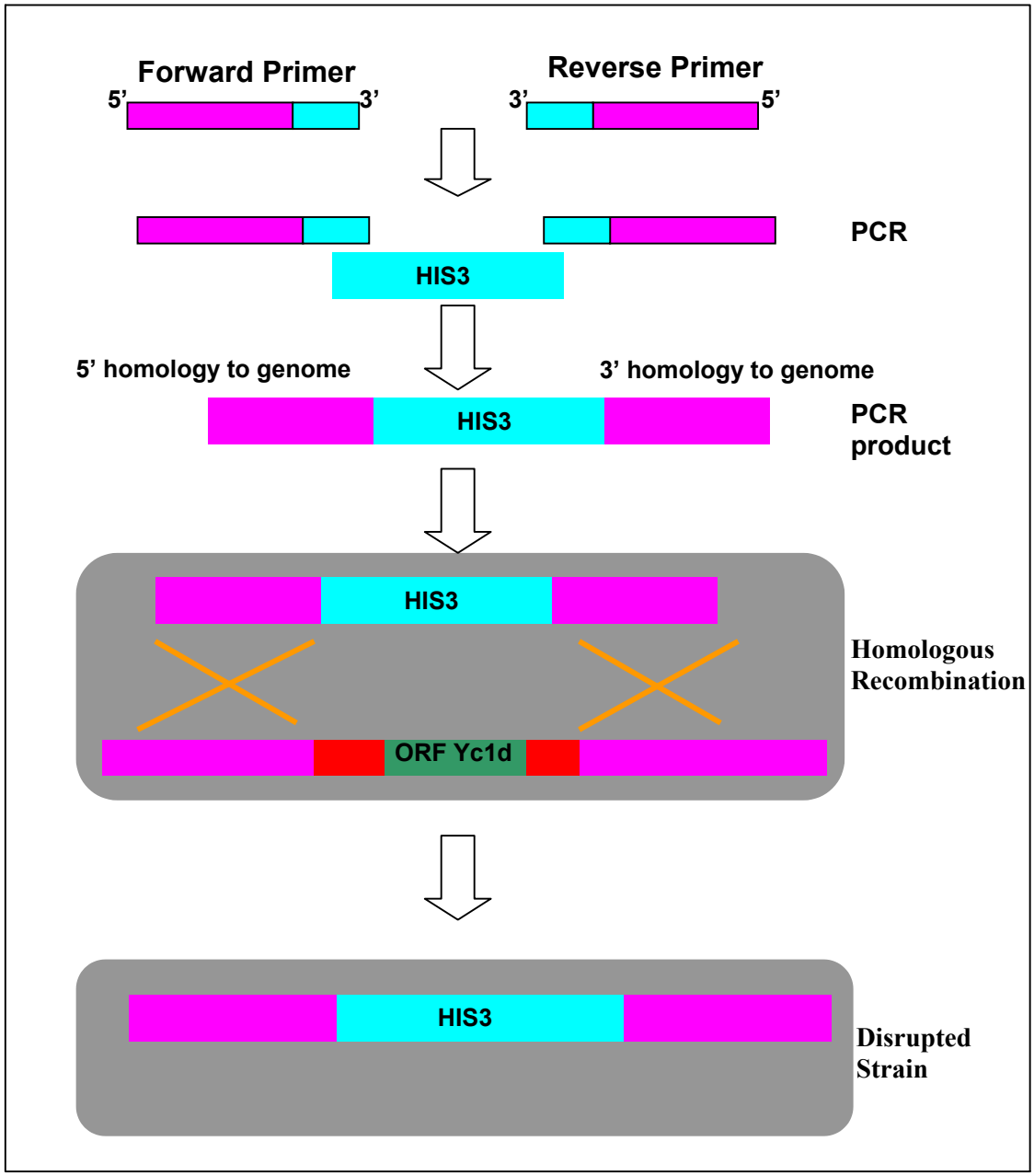


Figure 28: Steps of PCR mediated gene knockout strategy

Targeted integration of the cassette into the *YC1D* locus was confirmed by PCR using a forward primer that corresponds to a region 300 bp upstream of the open reading frame of Yc1d and a reverse primer (20 nucleotides) that corresponds to the integrated His3. By using these primers, the expected size PCR product will be obtained only if the disruption cassette has been integrated into the C1D locus. As anticipated, and shown in Figure 29, 1.02 Kb fragment was amplified from genomic DNA of *yc1d-yku70* double mutant strains (Figure 29, lanes 1-4, 6) but not from *yku70* mutant strain (Lane 5).

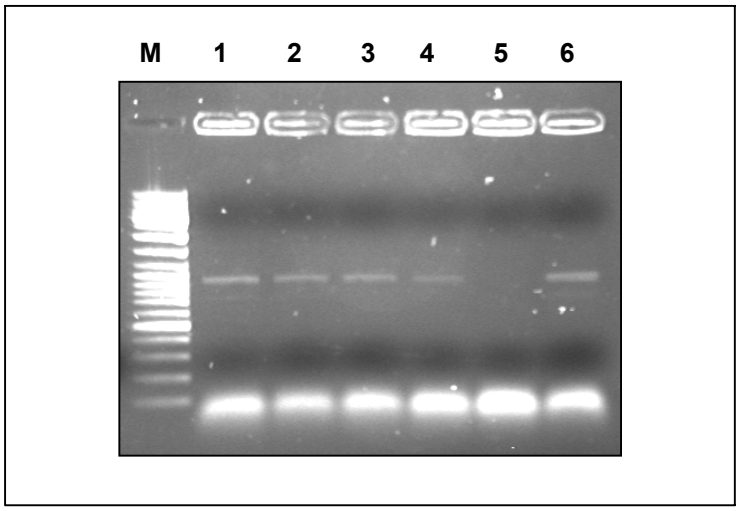


Figure 29: Confirmation of the integration of the disruption cassette into the *YC1D* gene loci in *yku70* mutant background

4.4.2. Phenotypic Analysis of *yc1d/yku70* Mutant Strain

4.4.2.1. yc1d/yku70 mutant strain is also temperature sensitive

After obtaining the *yc1d-yku70* double mutant strain, the same set of phenotypic and functional analyses, which were previously applied to *yc1d* mutant strain were employed. Initially temperature sensitivity of the *yc1d-yku70* strain was analysed and compared with parental *yc1d* and *yku70* mutant strains. In accordance with a previous report, *yku70* mutant strain shows high sensitivity for growth at 39^{o}C

(Boulton and Jackson, 1996) and as previously shown in Figure 21, *yc1d* mutant strain displays growth defect at this temperature (Figure 30). It was observed that the *yc1d-yku70* double mutant strain also displayed temperature sensitivity, exhibiting a growth arrest similar to *yc1d* or *yku70* mutant strains at high temperatures (Figure 30).

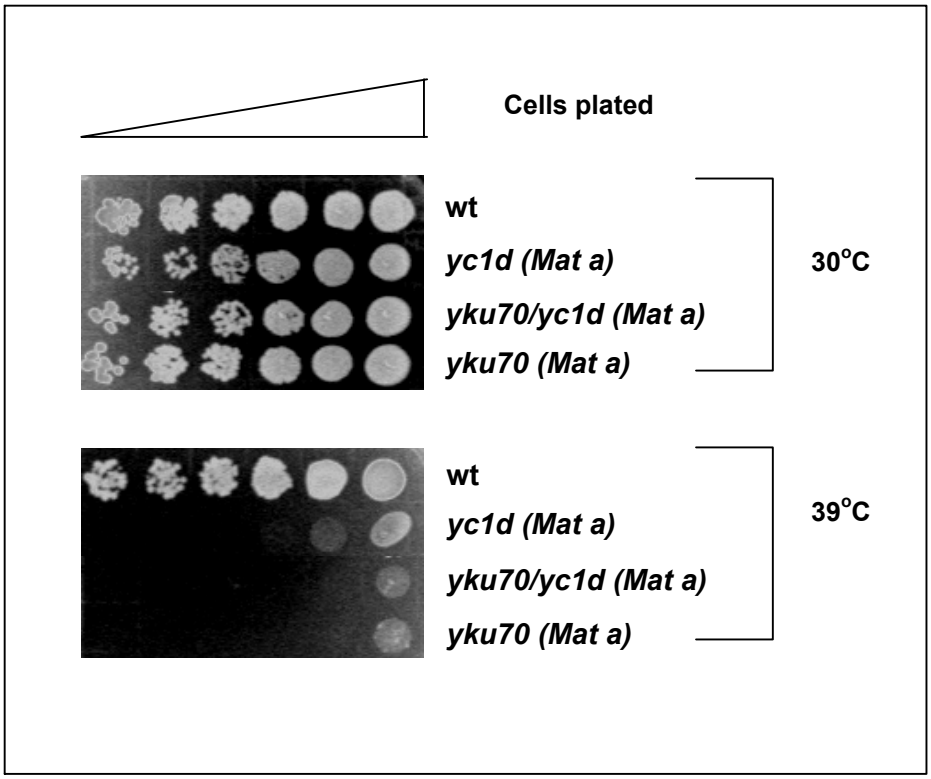


Figure 30: The *yc1d-yku70* double mutant strain is temperature sensitive

4.4.2.2. yc1d-yku70 double mutant strain displayed no obvious sensitivity to DNA damaging agents.

Then, we analysed the sensitivity of *yc1d-yku70* double mutant strain to DNA damaging agents such as MMS, u.v. irradiation and γ-irradiation and compared sensitivity of this strain to the wild type and parental strains. Neither *yku70* nor *yc1d* mutant strains were sensitive towards u.v. treatment and so neither were the double

knock-out mutant strains (Figure 31B). MMS treatment showed that the *yc1d* mutant strain was resistant to MMS, whereas the *yku70* mutant strain exhibited a slight sensitivity as has been presented earlier in Figure 22. The *yc1d-yku70* double mutant strains were also slightly sensitive, however not any more than *yku70* mutant strain (Figure 31A). The response of the strains to γ-irradiation was potentially interesting (Figure 31C). The *yc1d* haploid mutant strain exhibited a slight sensitivity to γ-irradiation with respect to the *yku70* mutant strain as demonstrated previously in Figure 22. Interestingly, the sensitivity of *yku70/yc1d* double mutants was similar to the *yku70* mutant strain, suggesting that *YKU70* gene might be involved in inhibition of homologous recombination. Thus, the absence of Yku70 restores the slight growth defect seen in Yc1d deficiency and when both genes are deleted, the mutant strain becomes resistant to γ-irradiation.

In conclusion, we were not able to detect dramatic alterations in responses of the yc1d/yku70 double mutants to different DNA damaging agents relative to the parental strains.

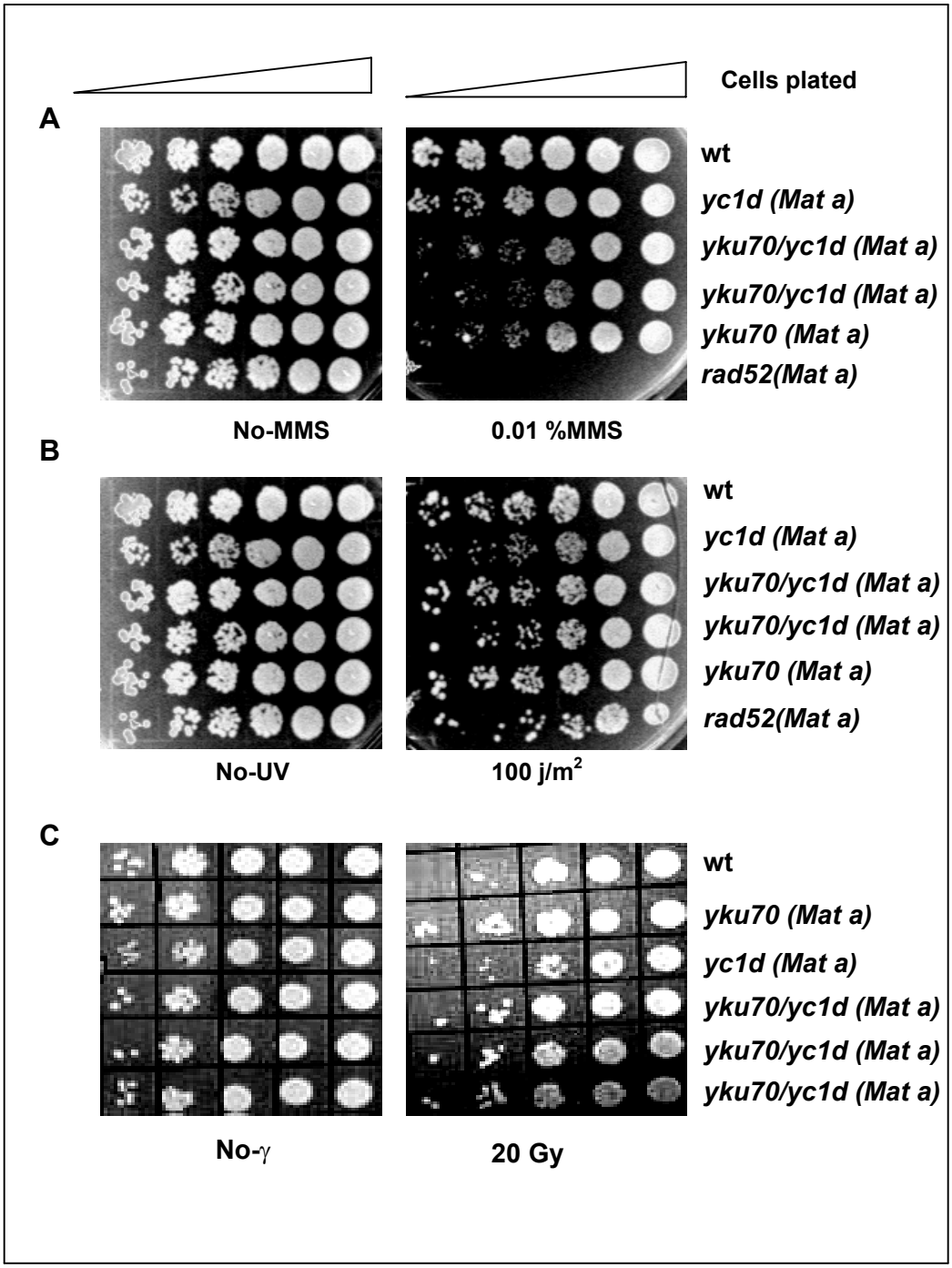


Figure 31: As *yku70* and *yc1d, yc1d-yku70* double mutant strain is not sensitive to MMS, u.v., and γ irradiation

4.4.3. Analysis of *yc1d-yku70* Double Mutant for NHEJ

Next, the *yku70-yc1d* double knockouts were analysed for their ability to join the DNA ends using the plasmid-based DNA repair assay. As presented in section 4.3.1, the efficiency of rejoining the 3'-overhanging DNA ends especially is severely debilitated in *yc1d* and *yku70* mutant strains. If *YC1D* and *YKU70* genes are on the same pathway, then the disruption of both genes will accentuate the efficiency of repair. Therefore, a plasmid that was digested with *SacI* or *PstI* to produce 3'-overhanging ends was transformed into the *yc1d*, *yku70* and *yku70/yc1d* mutant strains in parallel with the supercoiled form of the same plasmid and DNA end-joining efficiency was assessed as described previously. As shown in Figure 32, the double knockout mutant strains exhibited an even less efficiency of NHEJ with respect to the *yc1d* or *yku70* mutant strains. It was concluded therefore that, the *YC1D* and *YKU70* genes are in the same epistasis group and possibly take part in the same pathway in NHEJ.

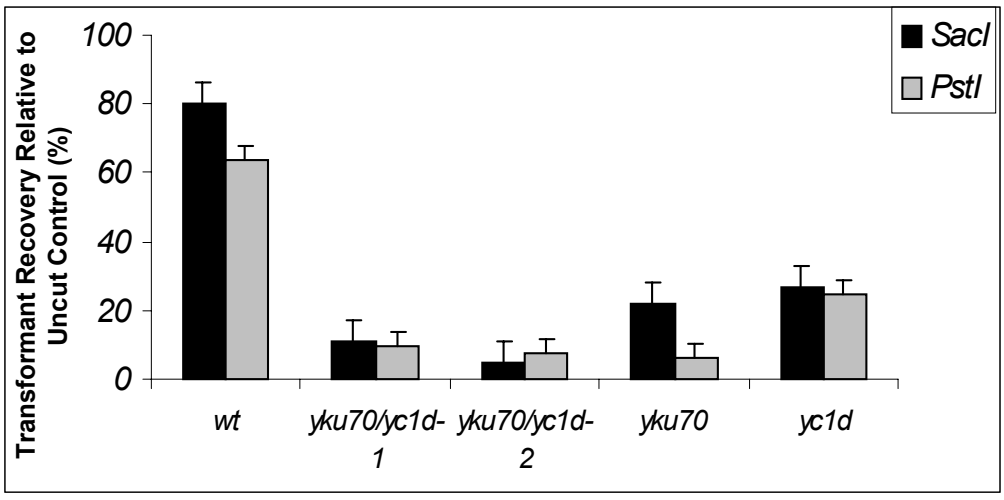


Figure 32: Yku70 and Yc1d are involved in the same pathway to rejoin 3'-overhanging ends

4.4.4. Analysis of *yc1d-yku70* Double Mutant for HR

Although Yku70 is mainly involved in NHEJ, we regularly observed a 1.5-fold increase in the efficiency of HR in the *yku70* mutant strain (Figure 26 and data not shown). This finding suggests that the Yku70 has a slight inhibitory effect on HR. Indeed, it was reported that in eukaryotes, Ku70 acts as a switch between NHEJ and HR repair pathways and induces NHEJ while repressing the other (Goedecke et al., 1999). Using the in vivo plasmid-based recombination assay described in section 4.3., it was demonstrated that the *yc1d/yku70* double mutants are also 1.5-fold more efficient in HR with respect to the wild type strains (Figure 33). As presented before and as seen in Figure 33, *yc1d* mutant strain is defective in HR. Although Yc1d is required for HR, obviously the repressive effect of *YKU70* is dominant to *YC1D*. This result is in accordance with the γ-irradiation sensitivity of double knockout mutant strains shown in Figure 33. Although the haploid *yc1d* mutant strain was slightly sensitive to γ-irradiation, the *yc1d/yku7*0 double knockout mutants, similar to the *yku70* mutants, exhibited resistance to this kind of damage. Therefore, it can be concluded that the functional effect of Yku70 in HR is dominant to the effect of Yc1d and that possibly these two proteins act on HR through different pathways.

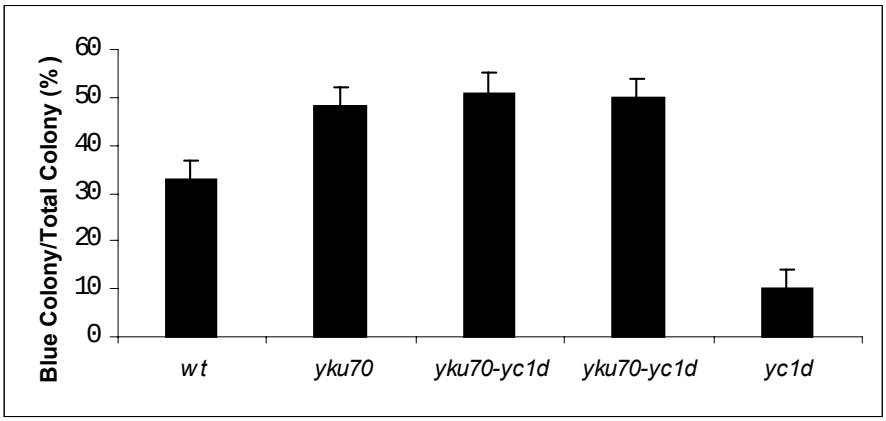


Figure 33: *yku70-yc1d* double mutant strain, similar to *yku70* mutant strain, exhibited 1.5 fold increase in HR

DISCUSSION

CHAPTER 5

IDENTIFICATION OF C1D INTERACTING PROTEINS

In human diploid nuclei, $6.6x10^9$ base pairs (bp) of DNA are compartmentalized into chromosomes. The resulting chromatin is further organized into looped domains by the dynamic binding of tethered bases to a network of intranuclear proteins, the so called nuclear matrix (NM) (Berezney et al., 1995). The 25 million nucleosomes in a mammalian nuclei are organized into about 60,000 chromatin loops by periodic attachments to NM at positions separated on average by 70 Kb (Gasser and Laemmli, 1986; Vogelstein et al., 1980). Dynamic association between DNA loops and NM allows stringent regulations of replication, differentiation, gene expression, recombination and repair in response to functional requirements of the cell (Nickerson, 2001) .

DNA sequences that exhibit high affinity binding to NM are called matrix/scaffold attachment regions (S/MARs) (Gasser and Laemmli, 1987). MARs are found in exons, introns, centromers, telomeres and also at gene break point cluster regions (Bode et al., 2000). Although MARs don't have clear consensus sequences, they consist of a specialized AT rich DNA content which is prone to base unpairing when subjected to negative superhelical strain (Benham et al., 1997). This unique sequence of MARs allow them to regulate DNA replication and transcription (Boulikas, 1995), DNA organization (Strick and Laemmli, 1995) and recombination (Sperry et al., 1989). Arising evidence indicates that MARs are recombinogenic elements or in other words recombination hot spot regions. For instance, they are the preferred targets for entry of retroelements and retrosposons (Baer et al., 2000; Mielke et al., 1996) and are sensitive to DNAses, topoisomerases and S1 nucleases (Boehm et al., 1989). In addition to these findings, it was shown that MARs

associate with proteins involved in recombination and repair, such as, TOPO II, PARP, HMG-1 and DNA-PK (Ku70/Ku86 and DNA-PK_{CS}) (Bode et al., 2000; Galande and Kohwi-Shigematsu, 2000), suggesting that MARs recruit DNA repair proteins to NM to perform their functions. MARs were also identified at breakpoint junctions of many chromosomal translocations such as 11q23, 9p22, 4q21, 11q23, 22q11, 8q24, 14q21 and 18q21 (Bode et al., 2000) .

C1D is the first non-histone NM protein to have been characterized at the sequence level. It was previously shown that protein and mRNA levels of NM protein C1D increase upon γ-irradiation. In addition, C1D interacts with DNA-PK and is an efficient substrate for this kinase. The interaction between C1D and DNA-PK is significant in the sense that C1D is the first substrate identified that is capable of activating DNA-PK in the absence of free DNA-ends (Yavuzer et al., 1998). Therefore, it is highly possible that upon activation by γ-irradiation, NM protein C1D through associating with chromatin loops, could recruit and activate DNA-PK to the site of MARs in response to DNA damage.

In an attempt to identify proteins that interact with C1D, we have applied yeast two hybrid system to screen a human B-lymphocyte library. As a result of this screen, Translin associating protein, TRAX was found to interact with C1D (Figure 5). Translin is a DNA binding protein that recognizes and binds specific consensus sequences found at the break point junctions in many chromosomal translocations including 1p32, 3q27, 5q31, 8q24, 9q34, 9q34.3, 10q24, 11p13, 11q13, 14q11, 14q32, 17q22, 18q21, 19p13, and 22q11 (Aoki et al., 1995; Kasai et al., 1997). Interestingly, Translin binding consensus sequences resemble the recognition signal (Chi-χ) of the RecBCD recombinase of *E.coli* (Smith, 1994). Similar to DNA structure of MARs, DNA region flanking the Translin binding consensus sequences exhibits non-B DNA structure that is sensitive to DNAaseI and TOPOII (Aoki et al., 1995). Of particular interest is the observation that many of the chromosomal translocations correlated with MARs including 22q11, 9q34, 14q21, 18q21, 8q24 (Meuth, 1989; Sperry et al., 1989) coincides with Translin associated chromosomal translocations.

Beside Translin's proposed role in recombination (Aoki et al., 1997), it also functions as a RNA binding protein that regulates mRNA transport (Wu and Hecht, 2000) and translation (Han et al., 1995).

To date, little is known about TRAX. Both human and mouse TRAX can form heterodimers with Translin/TB-RBP (Aoki et al., 1997; Taira et al., 1998; Wu et al., 1999). Although TRAX alone doesn't bind to DNA or RNA, TRAX/Translin heterodimer is capable of binding to DNA but not RNA (Chennathukuzhi et al., 2001). Therefore, TRAX enhances binding of Translin to DNA while decreasing its binding to RNA.

Recently, DNA-PK was found to associate with major breakpoint region (MBR) of BCL2 gene in which 70% of translocations occur (Ramakrishnan et al., 2000). In this region DNA-PK particularly shows binding affinity to unique octamer consensus sequence, which is termed as χ-sequence due to its high homology to prokaryotic recombination activator. Notably, this sequence was previously identified as Translin binding consensus sequence (Aoki et al., 1995).

In accordance to these findings, as being a nuclear matrix protein C1D could regulate the activity of Translin-TRAX complex and DNA-PK by organising their association with chromatin. Actually, our results give important clues about the molecular basis of NM associated regulation of DNA repair and recombination.

To define the functional relationship between TRAX and C1D, the interaction between them was verified *in vitro* and *in vivo* conditions. (Figures 7 and 8). The regions of TRAX responsible for TRAX-C1D and TRAX-Translin interactions were identified (Figures 12 and 13). Although, the putative LZ region of TRAX is important for interaction with both C1D and Translin, C1D and Translin show different requirements for interaction with TRAX outside the LZ region. The N-terminal region of TRAX that contains an intact LZ region is sufficient for its interaction with C1D (Figure 12), whereas Translin requires the full-length TRAX with an intact LZ region (Figure 13). Consistent with this, C1D and Translin bind to TRAX in a mutually exclusive manner and TRAX preferentially binds to C1D when three of them are found together (Figure 14). The most interesting finding is that, in mammalian cells C1D and TRAX interact in response to γ-irradiation (Figure 15).

Although the biological functions of these proteins are not yet clear, these findings suggest that both C1D and TRAX might play a role in the repair of DNA DSBs.

Previous data demonstrated that Translin is primarily cytoplasmic in many cell lineages, whereas it is found in nucleus in haematopoietic cell lines (Kasai et al., 1997). Interestingly, however, treatment of non-haematopoietic cells with mitomycin C and etoposide, agents that induce DNA-DSBs, was reported to cause translocation of Translin into the nucleus (Kasai et al., 1997). The subcellular localization of TB-RBP (mouse homolog of Translin) changes in meiotic and post-meiotic cells (Gu et al., 1998; Morales et al., 1998), but agents causing DNA DSBs did not affect localization of TB-RBP (Chennathukuzhi et al., 2001). In agreement with this latter study, and consistent with Translin containing a Rev-like nuclear export signal (Chennathukuzhi et al., 2001), Translin was located in the cytoplasm in all but around 6 % of cells where a nuclear/cytoplasmic expression pattern was observed (Figure 18Aa, B), and treatment of transfected COS7 cells with etoposide or γ-irradiation did not induce translocation of Translin into nucleus (Figure 18B). It is not clear at the moment why 6% of cells exhibit a different localization for Translin. These cells may represent those that have just undergone mitosis and Translin gained access to DNA as the nuclear envelope has been broken down.

In contrast to Translin and to a previous report indicating that mouse orthologue of human TRAX is predominantly a cytoplasmic protein (Chennathukuzhi et al., 2001), our results show TRAX is located in the nucleus in all cells examined (Figure 18Ab), consistent with TRAX containing a nuclear localization signal (Aoki et al., 1997). However, co-expression of TRAX and Translin resulted in a different pattern of localization of both proteins. When TRAX and Translin were co-expressed, an increase in the number of cells with a nuclear Translin and a significant increase in the cells expressing cytoplasmic TRAX was detected (Figure 19Ad,e; 19B). As TRAX and Translin interact with each other, co-expression of these proteins could effect their subcellular localizations. Therefore, the relative expression levels of TRAX and Translin in a cell could affect ability of these proteins to traffic between nucleus and cytoplasm. In fact, this explains why the reported subcellular localizations of exogenously expressed TRAX or Translin differ in different cell types and conditions.

According to our experiments, γ-irradiation didn't have any significant effect in the subcellular localizations of TRAX, C1D or Translin. Therefore the finding that the SDS-resistant homodimers of C1D and TRAX interact only after γ-irradiation (Figure 16) may indicate that a post-translational modification is required for the interaction. After DNA damage, damage sensor proteins such as DNA-PK and ATM, are activated and transmit the signal by phosphorylating the downstream effector molecules to activate cell signalling mechanisms for cell cycle arrest, repair, or apoptosis (Khanna and Jackson, 2001). Considering the previous report that shows phosphorylation of C1D by DNA-PK (Yavuzer et al., 1998), it is possible that γ-irradiation activates DNA-PK and activated DNA-PK phosphorylates C1D, which in turn interacts with TRAX. However, this doesn't exclude the possibility that TRAX could be phosphorylated by DNA-PK or a related kinase in response to DNA-DSBs. In fact, mouse TRAX has been found to be phosphorylated in testis germ cell nuclear extracts where many DNA DSBs are found due to ongoing recombination events (Chennathukuzhi et al., 2001).

TRAX enhances Translin DNA binding while decreasing its RNA binding capacity (Chennathukuzhi et al., 2001). Therefore, TRAX could regulate Translin function by adjusting its DNA-RNA binding ability. One biological consequence of TRAX-C1D interaction could be to regulate the Translin-TRAX interaction. Because interaction with either C1D or Translin appears to require the putative LZ motif of TRAX (Figures 12 and 13), the increased expression of C1D that occurs in response to γ-irradiation (Yavuzer et al., 1998) (a signal that also triggers the stable interaction between TRAX and C1D) could result in C1D masking the Translin interaction site of TRAX and thereby preventing the Translin/TRAX complex formation and binding to DNA. In fact, this possibility supports the proposed function of C1D in maintenance of genomic integrity, because increased C1D/TRAX interaction upon γ-irradiation might represent a mechanism to inhibit any unwanted Translin-mediated recombination events before the damaged DNA is repaired. Indeed, in our *in vitro* competition experiments, we showed that binding of C1D and Translin to TRAX is mutually exclusive. Importantly, however, if TRAX binds to Translin first, then it is no longer available to bind to C1D. Therefore, C1D may act

as a regulator of TRAX-Translin complex formation, rather than regulate association of any preformed TRAX-Translin complex.

Our studies using flourescently tagged proteins also failed to identify any dramatic changes in subcellular localization of TRAX, Translin or C1D when expressed in any combination in response to γ-irradiation. This is in agreement with others (Chennathukuzhi et al., 2001) who also found no difference in the subcellular localization of Translin in response to DNA damaging agents. We suspect that the results may reflect the relative timing of expression of the proteins in transfected cells. Thus, in the absence of γ-irradiation, C1D will not interact stably with TRAX and no significant effect of C1D on the subcellular localization of TRAX and Translin would be expected. On γ-irradiation C1D would interact with TRAX, but this association will only inhibit the formation new TRAX-Translin complexes.

Against the high background of preformed TRAX-Translin complexes it would be difficult to detect any change in overall localization of the florescent proteins before the overexpression of C1D eventually results in apoptosis. Nevertheless, we suspect that the interaction between C1D and TRAX will represent an important mechanism regulating the associations of TRAX and Translin and leading to alterations in the relative amounts of TRAX/Translin targeted to nucleus.

C1D could also regulate the activity of Translin and DNA-PK in a cell cycle dependent fashion because it was reported that Ku complex (DNA targeting subunit of DNA-PK) and Translin bound to the same consensus sequences, found at the Bcl2 breakpoint region, in a cell cycle dependent manner. In G1 and early S phases, this sequence is occupied by the Ku complex, however in G2-M phase it is replaced by Translin (DiCroce and Krontiris, 1995). In eukaryotes, DNA-DSB repair mediated by HR occurs mainly during late S and G2 phases of the cell cycle, when sister chromatids are present and other repair pathway, NHEJ is predominant in G1-early S-phase (Hendrickson, 1997). Therefore by regulating Translin and DNA-PK activities, C1D could assist DNA-DSBs repair pathways in a cell cycle dependent manner.

Cancer susceptibility genes have been classified into two groups; gatekeepers and caretakers (Kinzler and Vogelstein, 1997). Gatekeepers are genes that control cell proliferation and death, whereas caretakers are DNA repair genes whose

inactivation leads to genetic instability. Abrogation of both caretaker and gatekeeper's function markedly increases cancer susceptibility. Repair genes are caretakers of the genome when they fail to repair DNA damage, this leads to genetic instability, gross chromosomal rearrangements and accumulation of mutations that trigger cell cycle checkpoints resulting in permanent growth arrest or death of affected cells. If this system is inactivated this leads to tumorigenesis. It has been previously shown that DNA-PK complex has a caretaker role in maintaining genomic stability. Considering previous reports and our results both C1D and Translin-TRAX complex could be caretakers. In this context, it is important to identify roles of these proteins in epigenesis of cancer. We believe that analysis of the function of C1D, TRAX or Translin by mouse knockout models or analysis of expression pattern of these proteins in cancer cells will provide important clues about the role of nuclear matrix in tumorigenesis.

CHAPTER 6

IDENTIFICATION OF *S.CEREVISIAE* HOMOLOG OF C1D

There is no doubt that repair of the genotoxic lesions is critically important for prevention of genomic instability. Our published work (Chapter 3) and previous reports suggest that the nuclear matrix protein C1D could be a care-taker protein that is involved in maintenance of genomic integrity by regulating activity of proteins involved in DSB repair. However, these results do not reveal the precise molecular mechanisms that C1D employs when performing its function. Obviously, transgenic animal models that over express C1D or animals knocked out for C1D would be very useful to demonstrate the role of this nuclear matrix protein in important cellular processes. These types of experiments require well-established animal house facilities and heavily depend on financial support. Therefore, we decided to use a simpler organism, *S.cerevisiae*, to demonstrate the direct consequences of C1D deletion. Since, yeast carries out all essential processes such as replication, cell cycle control, DNA repair and protein trafficking using proteins conserved among all eukaryotes including humans, it is accepted as a very useful *in vivo* test tube to analyse functions of genes from other organisms.

The yeast C1D homolog, *YC1D*, exhibits 27% identity to human C1D. Therefore, the mutant strains deleted for *YC1D* were analysed to decipher the function of this gene. In agreement with the proposed role for human C1D, the *yc1d* mutant strain is sensitive for growth at 39^0C (Figure 21), which is a common phenotype of mutant strains defective in preservation of genomic integrity (Boulton and Jackson, 1996; Hryciw et al., 2002; Peggie et al., 2002). Moreover, the *yc1d* mutant strains were found to be slightly sensitive to γ-irradiation but not to other DNA damaging agents such as MMS or u.v., supporting the results obtained for human C1D. In support of our findings on *yc1d* mutant strain, recently, it has been

reported that some *S.cerevisiae* genes are only slightly sensitive to γ-irradiation, whereas exhibit resistance to all other DNA damaging agents (Bennett et al., 2001). In this study, a genome-wide screen of diploid mutants homozygous to deletions of 3,670 nonessential genes revealed 107 new loci that have an effect on γ-sensitivity. Interestingly, about 90% of these were sensitive also to other DNA damaging agents. However, a group of diploid mutants containing 14 newly identified genes, amongst which *YC1D (YHR081W)* was also found, exhibited resistance to all DNA damaging agents but not to γ-irradiation. Therefore, it can be concluded that the activities of both the human and yeast C1D are regulated by the presence of double-strand DNA breaks and that these two proteins could be functionally homologous. One direct way of demonstrating that *hC1D* and *YC1D* are orthologs, would be to show that the human C1D complements the phenotypic defects in a *yc1d* mutant strain. This approach was tried by expressing the hC1D under the control of a galactose inducible promoter in *yc1d* mutant strains. However, because the promoter used for expression of hC1D is a heterologous one and does not resemble the native endogenous *YC1D* promoter, the over expressed protein was toxic to the cells and results were not informative (data not shown). Therefore, this experiment should be repeated by integrating the hC1D coding sequences downstream of the native *YC1D* promoter. This approach will enable us to detect the effects of hC1D in yeast and therefore will be a direct way of showing that the function of *C1D* gene is evolutionarily conserved.

Nevertheless, because the phenotype of *yc1d* mutant strain suggested a role for *YC1D* in DSB repair, the functional analyses were performed. Since DSBs can be repaired both by NHEJ and HR, the effects of deletion of *YC1D* was analysed for both pathways. The *yku70* and *rad52* mutant strains were analysed along with *yc1d* mutants since they are the best-studied examples of NHEJ and HR in *S.cerevisiae*.

Using the plasmid -based DNA repair assay, it was demonstrated that the *yc1d* mutant strains are defective in DNA-end joining to a similar extent as the *yku70* mutant strain (Figure 23). The only difference between the *yc1d* and *yku70* mutant strains was in the efficiency of repair of the 5'-overhanging ends. Although still defective with respect to the wild type strains, the *yc1d* mutant strain was slightly more efficient than *yku70* in repairing the 5'-overhanging DNA ends, suggesting that

the role of Yc1d in NHEJ is mainly in the 3'-end processing, an essential step in appropriate joining of DNA ends. Moreover, our work has demonstrated that in the absence of Yc1d, around 50 % of 3'-overhanging ends are repaired inaccurately (Figure 24), indicating that another role of Yc1d in NHEJ is to repress the error-prone double-strand break repair pathways. This finding is yet another supporting evidence to the proposed role for C1D in maintenance of genomic integrity. When NHEJ is impaired in *S.cerevisiae*, cohesive ends are repaired by error-prone pathways, which is highly mutagenic (Boulton and Jackson, 1996). Thus, by inhibiting this pathway, Yc1d may prevent formation of mutations on the DNA.

In order to identify the function of Yc1d in HR, a novel *in vivo* recombination assay was developed. This assay is easy to apply and quicker with respect to the other conventional assays used to measure the HR efficiency. Indeed, using this novel assay, a *rad52* mutant strain was found to be defective in HR, whereas a *yku70* mutant strain was found to behave like a wild type strain, both of which are in agreement with the previous findings. After confirming that the results obtained using this assay is reliable, a *yc1d* mutant strain was analysed for efficiency of HR. The *yc1d* mutant strain exhibited a twofold reduction in HR efficiency with respect to the wild type strain. Although this is not a dramatic response as observed in a *rad52* mutant strain, nevertheless it suggests that Yc1d has a role in HR pathway (Figure 26, 27). More importantly, these results were also confirmed using a conventional HO endonuclease assay, which measures the HR efficiency in yeast cells. The *yc1d* mutant strain again exhibited a twofold reduction in HR and when a plasmid expressing wild type C1D was introduced, the deficiency was restored back to the wild type levels. In conclusion, the results obtained on *YC1D* so far demonstrate that *yc1d* mutant strains show sensitivity to increased temperature and γ-irradiation and Yc1d functions in both HR and NHEJ.

Since both Yc1d and Yku70 act on NHEJ, the effects of disrupting both genes were also tested to see whether *YKU70* and *YC1D* genes work together. If that was the case, we would expect to have a more profound effect in the efficiency of NHEJ. Similar to the *yc1d* and *yku70* mutant strains, *yc1d-yku70* double mutant strain displayed temperature sensitivity for growth at 39^0C (Figure 30). The response of this double mutant strain to various DNA-damaging agents was rather unexpected.

Consistent with a previous report (Boulton and Jackson, 1996), the *yku70* mutant strain exhibited resistance to DNA damaging agents such as u.v. and γ-irradiation, however, showed slight sensitivity to MMS (Figure 31B). On the contrary, *yc1d* mutants were resistant to MMS and u.v. but slightly sensitive to γ-irradiation (Figure 22). It was seen that the *yc1d-yku70* double mutant strains behaved similar to the *yku70* mutant strain (Figure 31A,B and C), thus, exhibiting slight sensitivity to MMS but resistance to u.v. and γ-irradiation. In other words, slight γ-irradiation sensitivity of *yc1d* mutant strain was complemented in double-mutant strains. At the moment, it is not easy to understand how Yku70 deficiency is dominant over Yc1d deficiency. We believe that analysis of Yc1d role in other damage response processes such as cell cycle, DNA end processing and telomere maintenance will provide important clues about the particular response of *yc1d-yku70* mutant strain to DNA damaging agents.

When the *yc1d-yku70* mutant strains were analysed for NHEJ, it was demonstrated that, the double knockouts were even less efficient in DNA end-joing with respect to the *yc1d* or *yku70* mutant strains (Figure 32). This result indicates that as long as NHEJ is concerned, *YC1D* and *YKU70* genes are in the same epistasis group. Although we cannot exclude the possibility that this collaboration may be through direct interaction of Yku70 and Yc1d proteins, studies performed in mammalian systems demonstrated that the human C1D and Ku70 do not interact directly (Yavuzer et al., 1998). Therefore, collaborative work of Yc1d and Yku70 proteins in NHEJ in yeast, could involve different protein complexes. Alternatively, it is also possible that *YC1D* could have a general role in RNA and protein stability, thus, it may effect the action of Yku70 by regulating the stability of Yku70. Indeed, a recent study, in which 1739 gene products in *S.cerevisiae* were analysed according to protein complexes they form, revealed that *YC1D* interacts with proteins involved in RNA processing (Gavin et al., 2002).

It was reported that in eukaryotes, Ku70 acts as a switch between NHEJ and HR repair pathways and induces NHEJ while repressing the other (Goedecke et al., 1999). In accordance with this report we observed a 1.5 fold increase in the efficiency of HR in the *yku70* mutant strain (Figure 33). Interestingly, the *yc1d-yku70* double mutant strain was also 1.5-fold more efficient in HR with respect to the

wild type strain (Figure 33). Although, *yc1d* mutant strain shows a defect in HR, clearly it is not an essential gene in HR and the *RAD52* epistasis group of proteins plays the major role in homologous recombination. Therefore, relieving the inhibition of Yku70 on this major pathway will lead to an increase on the efficiency of HR performed by Rad52 group of proteins. Under these circumstances, it will not be easy to detect the effect of deleting a redundant gene like *YC1D*. This hypothesis could also be used to explain the complementation of the γ-irradiation sensitivity of Yc1d deletion in a *yku70* mutant background. According to the findings on *YC1D*, a hypothetical diagram is shown below to summarize how *YC1D* may affect the NHEJ and HR (Figure 34).

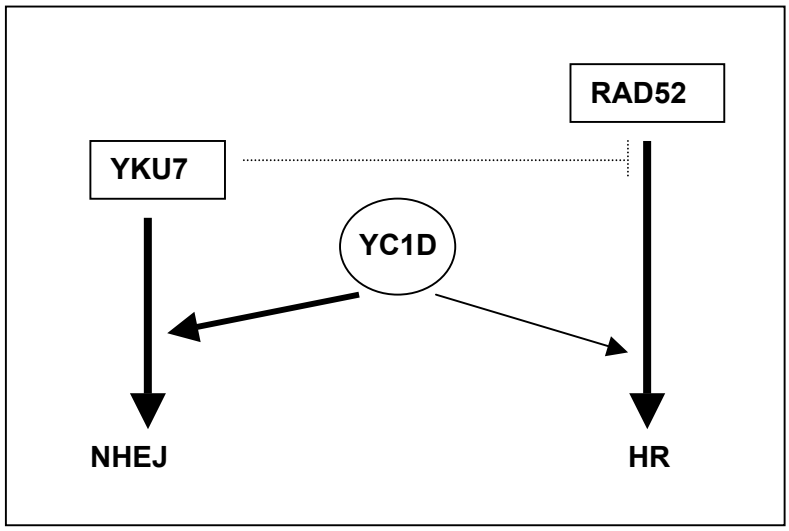


Figure 34: Effect of Yc1d on NHEJ and HR

Two main repair pathways in eukaryotes, HR and NHEJ, begin in the same way; the ends of the DSB are resected by 5' to 3' exonucleases or by a helicase coupled to an endonuclease to produce 3'-overhanging DNA tails (Haber, 2000b). Therefore, processing of broken DNA ends is critical for repair of DNA-DSBs and absence of proteins involved in this process can affect the efficiency of DNA-DSB repair pathways. As previously shown in humans and yeast, Rad50, Mre11, and Xrs2 (RMX) complex is involved in processing of broken DNA ends (Haber, 2000a;

Haber, 2000b). Interestingly, genetic studies have implicated the role of RMX gene products in the same NHEJ pathway as Yku70 and Yku80 (Boulton and Jackson, 1998; Critchlow and Jackson, 1998). In addition to play a major role in NHEJ, RMX complex is also involved in HR, telomere maintenance, mating type switching and meiotic recombination (Haber, 2000a). More recently this complex has also been implicated in the suppression of chromosomal rearrengements (Chen and Kolodner, 1999) and in cell cycle checkpoint signalling (D'Amours and Jackson, 2001; Grenon et al., 2001; Usui and Schiebel, 2001). These findings suggest that the biological role of RMX complex is in maintenance of genomic integrity. Similar to RMX complex, Yc1d has dual function in both HR and NHEJ repair pathways, which raises the possibility that Yc1d could also have a role(s) in other RMX complex related functions. Indeed, this suggestion coincides with our previous results that imply a role for human C1D in preservation of genomic integrity by regulating factors involved in DNA repair and recombination (Chapter 5).

6.1. FUTURE PERSPECTIVES

As indicated in the beginning of this section, studies on *YC1D* provided rather useful information on the function of this newly identified gene. More importantly, the data obtained so far strongly suggests that the human and yeast C1D genes are functionally homologs. Therefore, future experiments will be directed towards deciphering the other molecules *YC1D* works with and directly demonstrating that the human C1D and *YC1D* genes are orthologs. These experiments are summarized below:

Expression of hC1D protein under the control of native YC1D promoter: As described in the relevant section, by replacing the *YC1D* coding sequences with the human C1D, we expect to detect complementation of phenotypic and functional deficiencies of *yc1d* mutant strains. This experiment will directly indicate that human and yeast C1D genes are evolutionarily conserved.

Demonstrating if YC1D is involved in RMX complex-regulated events: Targeted disruption of *YC1D* in *mre11, rad50* and *xrs2* mutant strains and analysing of double mutants for cell cycle arrest, telomere maintenance and 5'-3' exonuclease activity are critically important strategies, to understand whether Yc1d functions in the same pathway with RMX complex. Besides these genetic assays, we also plan to perform biochemical interaction studies between RMX complex and Yc1d. Using homologous recombination it is possible to tag the endogenous genes with various epitopes, such as GST, HA, Myc or GFP. By this approach, we will be able to tag *YC1D* and any of the genes in RMX complex and perform immunoprecipitations using antibodies raised against various epitopes. Tagging the endogenous *YC1D* gene with an epitope will also be useful in analyzing the expression pattern of the endogenous Yc1d protein. In *S.cerevisiae*, mating type-switching status influences NHEJ and HR. Haploid yeast strains perform NHEJ about 10-fold more efficiently than diploid strains (Lee et al., 1999). Previous studies suggest that some genes are repressed by the diploid specific a/α factor (Kegel et al., 2001). Therefore, analysis of the expression pattern of Yc1d in haploid and diploid strains could provide important clues about the role of Yc1d in DNA-DSB repair.

Analysis of S.pombe C1D: The fission yeast *S.pombe* provides a good model system to study DNA-DSB repair pathways in humans since DNA-DSB repair pathways between human and *S.pombe* are highly conserved. For instance, similar to humans, in *S.pombe*, the major DNA-DSBs are repaired by NHEJ (Wilson et al., 1999). Studying the *S.pombe* C1D will also be useful, since both TRAX and Translin homologs are found in *S.pombe*. In chapter 3, we showed that C1D interacts with TRAX both *in vivo* and *in vitro* and proposed a model for C1D-TRAX interaction. According to this model, by regulating TRAX and Translin interaction, C1D assists recombination and repair, which are nuclear processes critical for maintenance of genomic stability. Therefore, in order to analyse the biological significance of TRAX-C1D/ TRAX-Translin interaction, the functions of *S.pombe* homologs of human C1D, TRAX or Translin could be analysed.

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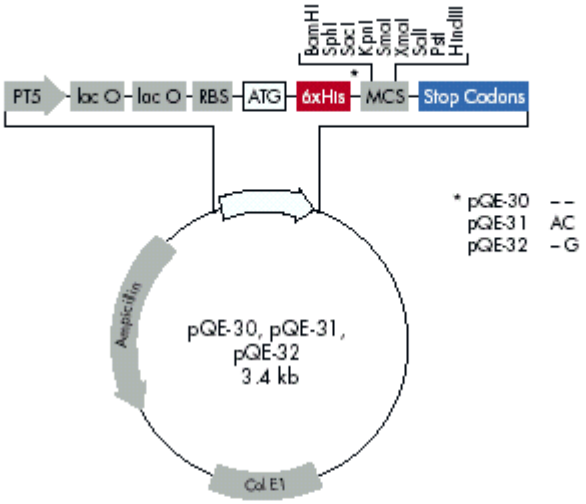
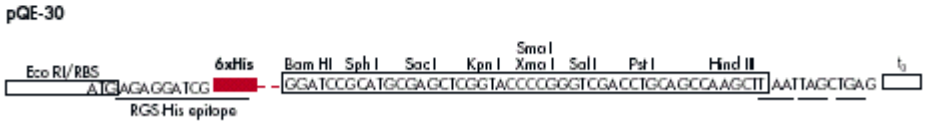
APPENDICES

APPENDIX 1

MAPS OF PLASMIDS

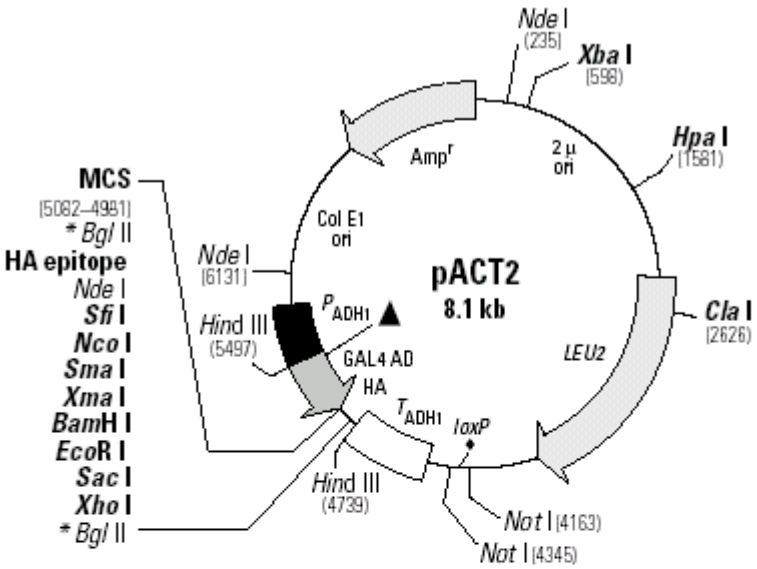
A1: Bacterial Expression Plasmids

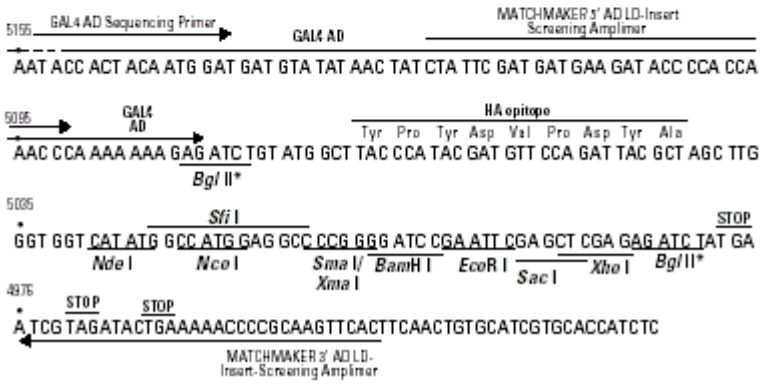




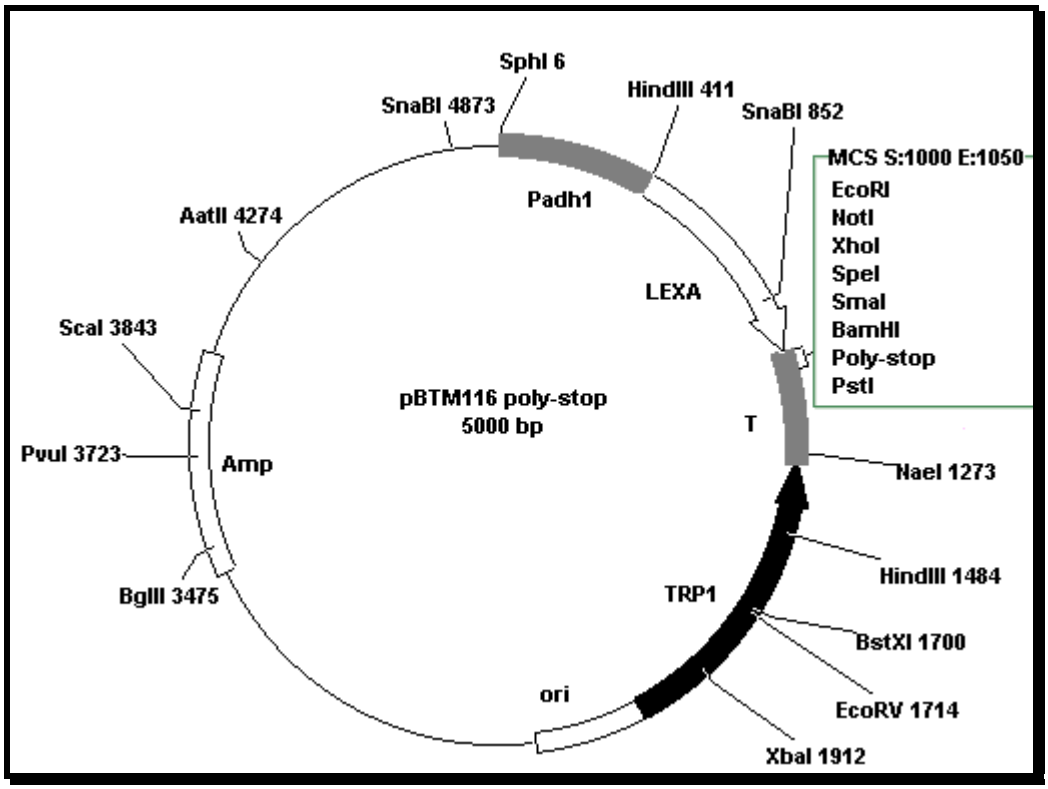
pQE-30 vector and its MCS

A2: Yeast Plasmids



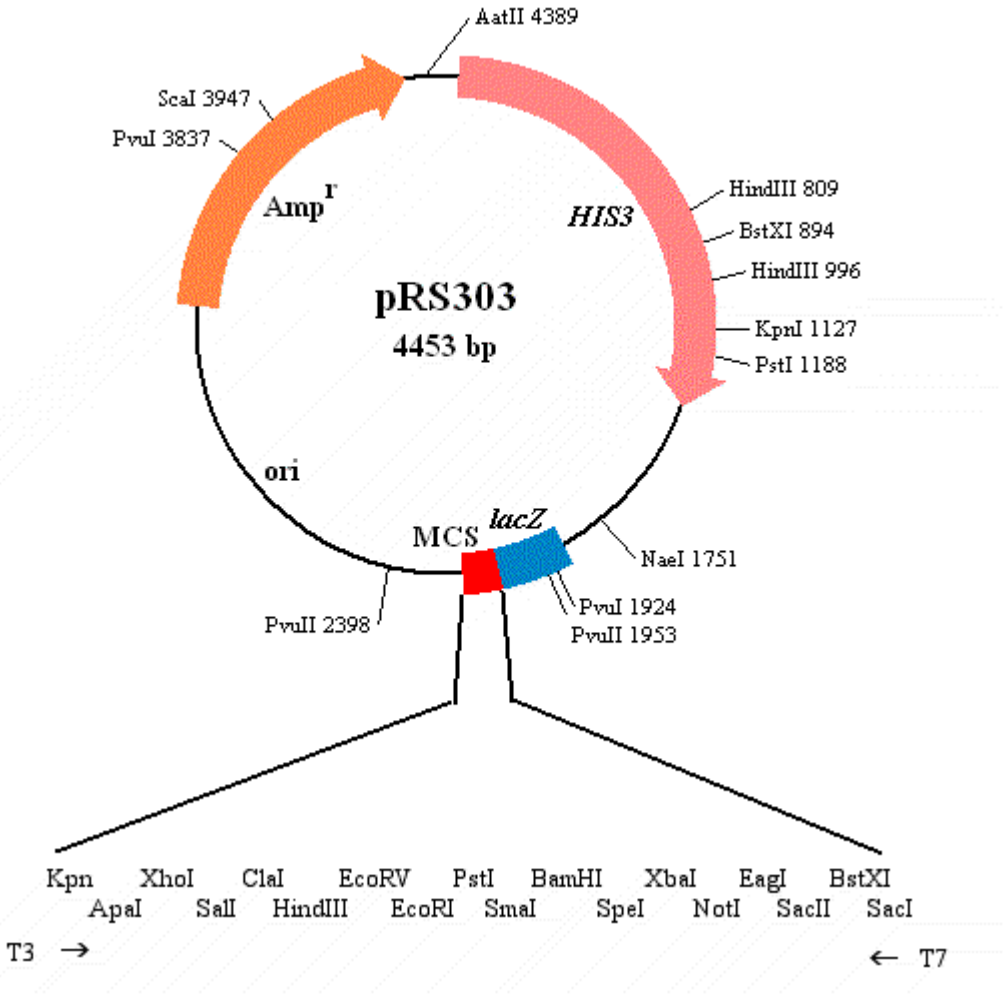


pACT2 and its MCS

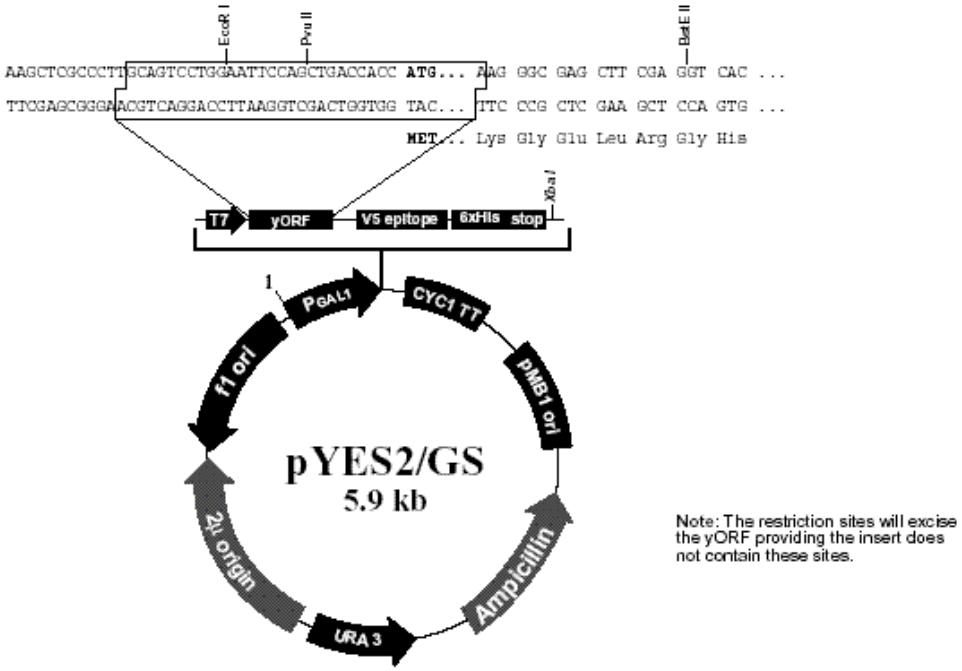


CCATG GCC GGA TCC GAA TTC CTC GAG ATC GAT TAG ACTAGTCTAAGA AATTC
BamHI EcoRII XhoI ClaI SpeI XbaI

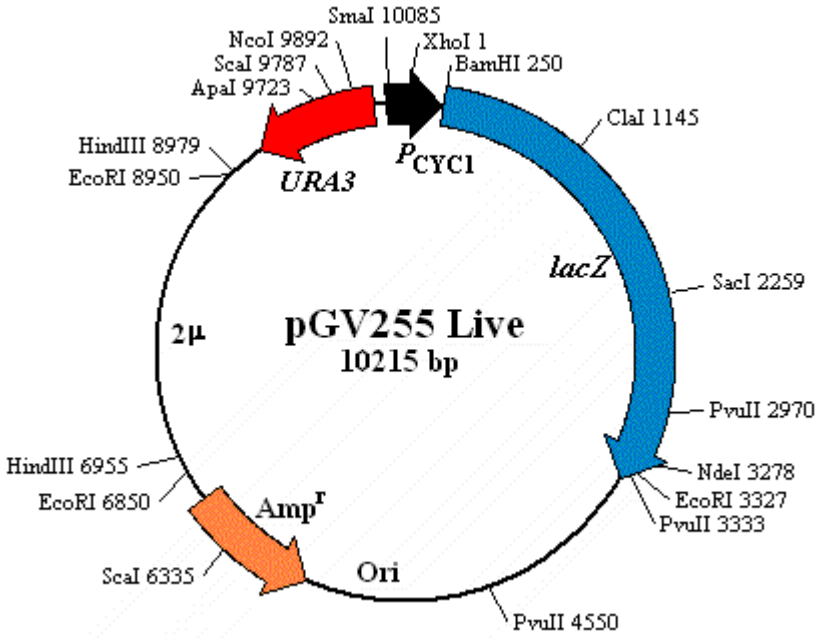
pBTM116 and its MCS



pRS303 used for *in vivo* plasmid end joining assay



pYES2/GS: used to expresses Yc1d

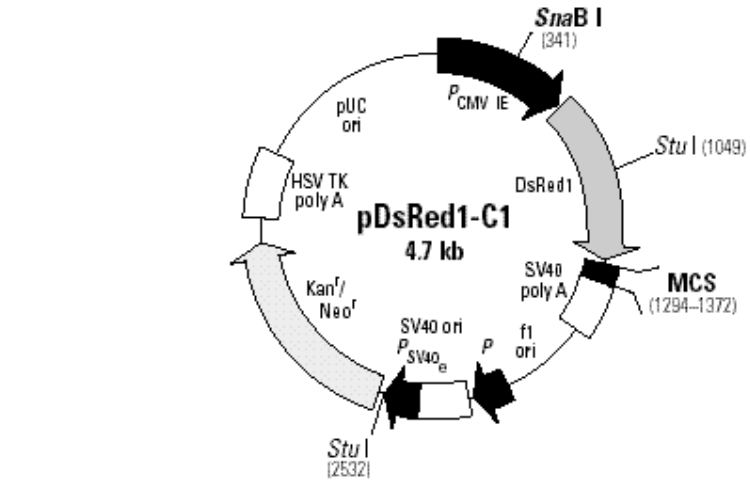


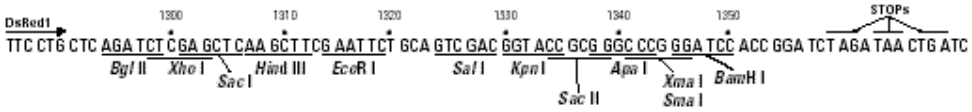
pGV255: used for accuracy test and *in vivo* recombination assay

A3: Mammalian Expression Plasmids

pDsRed1-C1 Vector Information PT3425-5

Catalog #6924-1



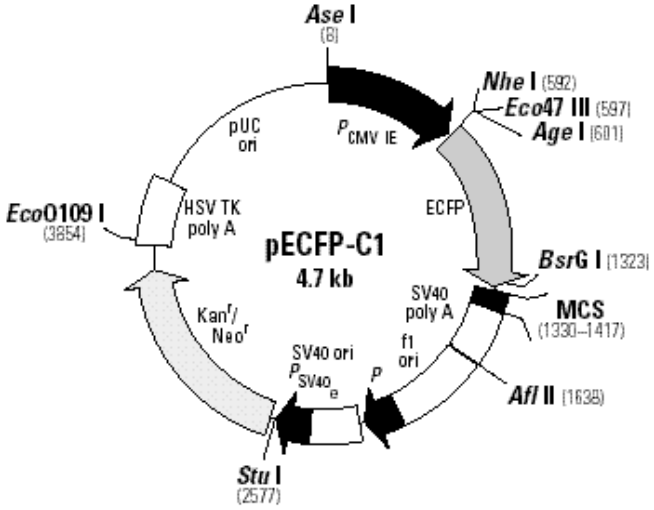


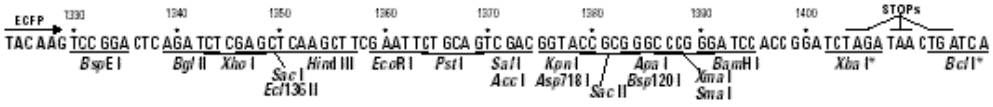
Restriction Map and Multiple Cloning Site (MCS) of pDsRed1-C1. Unique restriction sites are in bold.

pECFP-C1 Vector Information

PT3259-5

Catalog #6076-1



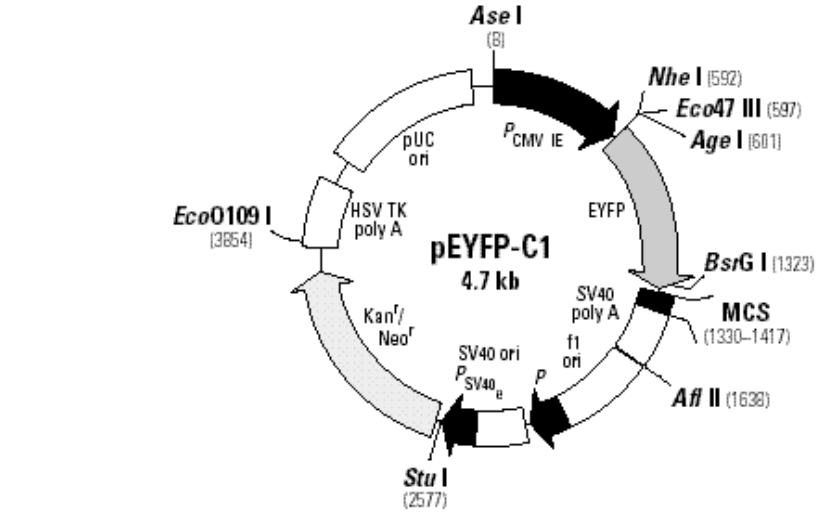


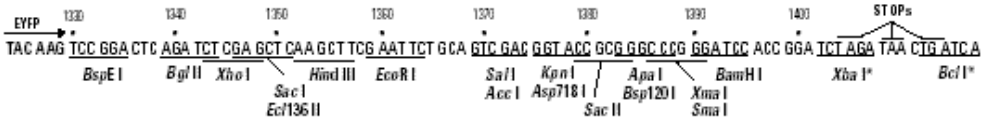
Restriction map and multiple cloning site (MCS) of pECFP-C1. Unique restriction sites are in bold. The *Xba* I and *Bcl* I sites (*) are methylated in the DNA provided by CLONTECH. If you wish to digest the vectors with these enzymes, you will need to transform the vector into a *dam*$^-$ host and make fresh DNA.

pEYFP-C1 Vector Information

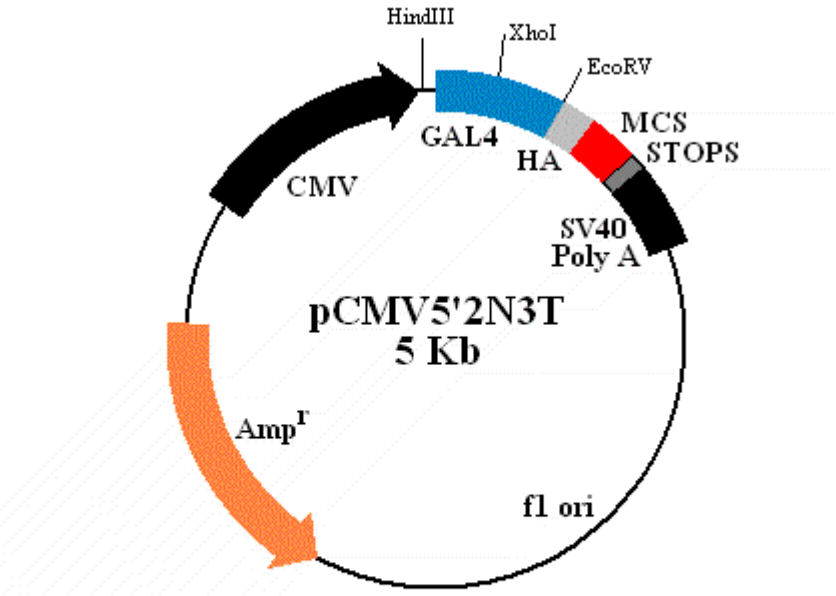
PT3176-5

Catalog #6005-1





Restriction map and multiple cloning site (MCS) of pEYFP-C1. Unique restriction sites are in bold. The *Xba* I and *Bcl* I sites (*) are methylated in the DNA provided by CLONTECH. If you wish to digest the vectors with these enzymes, you will need to transform the vector into a *dam*$^-$ host and make fresh DNA.



MCS:

BamHI	SalI	KpnI	SmaI	EcoRI	SacI	XbaI
						xxx
G GAT CCC	CGT CGA	CGG TAC	CCC GGG	GAA TTC	GAG CTC	TAG A

DNA damage-dependent interaction of the nuclear matrix protein C1D with translin-associated factor X (TRAX)

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Summary

The nuclear matrix protein C1D is an activator of the DNA-dependent protein kinase (DNA-PK), which is essential for the repair of DNA double-strand breaks (DSBs) and V(D)J recombination. C1D is phosphorylated very efficiently by DNA-PK, and its mRNA and protein levels are induced upon γ-irradiation, suggesting that C1D may play a role in repair of DSBs in vivo. In an attempt to identify the biological function of C1D, we have employed the yeast two-hybrid system and found that C1D interacts specifically with Translin-associated factor X, TRAX. Although the biological function of TRAX remains unknown, its bipartite nuclear targeting sequences suggest a role for TRAX in the movement of associated proteins, including Translin, into the nucleus. We show that C1D and TRAX interact specifically in both yeast and mammalian cells. Interestingly, however, interaction of these two proteins in mammalian cells only occur following γ-irradiation, raising the possibility of involvement of TRAX in DNA double-strand break repair and providing evidence for biological functions of the nuclear matrix protein C1D and TRAX. Moreover, we show, using fluorescently tagged proteins, that the relative expression levels of TRAX and Translin affect their subcellular localization. These results suggest that one role for C1D may be to regulate TRAX/Translin complex formation.

Key words: C1D gene, Nuclear matrix, Translin, TRAX, DNA-PK

Introduction

Higher order organization of genomic DNA is one of the important mechanisms involved in control of cell-type-specific gene expression. Polypeptides involved in higher order chromatin folding are non-histone proteins and are tightly bound to DNA, even after treatment with harsh denaturants (Neuer et al., 1983). Although the biological functions for these proteins are still largely obscure, some of them have been reported to be associated with highly repetitive DNA sequences and to be involved in targeting a subset of genomic DNA to the nuclear matrix (Neuer and Werner, 1985; Neuer-Nitsche et al., 1988; Pfutz et al., 1992; Werner and Neuer-Nitsche, 1989). The nuclear matrix protein C1D is the first of such non-histone proteins to have been characterized at the sequence level (Nehls et al., 1998). Independently, C1D was found to be associated with the transcriptional repressor RevErb and the nuclear coreceptors N-Cor and SMRT, indicating that it could act as a component of the complex involved in transcriptional repression (Zamir et al., 1997). C1D is also an activator of DNA-dependent protein kinase (DNA-PK) (Yavuzer et al., 1998), which plays an important role in DNA double-strand break (DSB) repair and V(D)J recombination, a process specific to lymphocytes that is required for development of the immune system (Smith and Jackson, 1999). Interestingly, C1D is induced upon γ-irradiation and proposed to play a role in targeting DNA-PK to specific nuclear regions in response to DNA damage. Indeed, the *xrs5* cells deficient in one of the subunits of DNA-PK, Ku-80, exhibit irregularly shaped nuclear envelope and altered nuclear matrix compared with their wild-type controls (Korte and Yasui, 1993; Yasui et al., 1991). In connection with its role to regulate DNA-PK, C1D has also been shown to induce apoptosis in a p53-dependent manner (Rothbarth et al., 1999). Although the physiological function for C1D is not yet clear, accumulating evidence indicates that C1D is a multifunctional protein and, possibly by influencing chromatin structure, is capable of regulating different cellular events, such as DNA-repair, transcription and apoptosis.

In an attempt to identify the possible biological functions of C1D and the nuclear matrix, we have applied the yeast two-hybrid system (Fields and Song, 1989) to screen for polypeptides that interact with C1D. This approach has led us to identify a specific interaction between C1D and a recently identified protein, Translin-associated factor X (TRAX). TRAX has been shown to interact with Translin through yeast two-hybrid screening (Aoki et al., 1997) and exhibits extensive amino acid homology to Translin. Translin was first identified as a single-strand DNA binding protein that recognizes the consensus sequences, ATGCAG and GCCC(A/T)-(G/C)(G/C)(A/T), found at the breakpoint junctions of various chromosomal translocations seen in many lymphoid and solid tumours (Aoki et al., 1995; Chalk et al., 1997). Interestingly, homologues of TRAX and Translin are found in various other

organisms including mouse, fission yeast, frogs, insects and plants (Devon et al., 2000), suggesting that these molecules have important biological functions**.** Indeed, numerous functions have been proposed for Translin, ranging from mRNA transport and translational regulation to DNA recombination and repair.

In this paper we report that C1D and TRAX are capable of interacting specifically in yeast and in mammalian cells. However, in mammalian cells their interaction could be detected only after γ-irradiation and interestingly, only the dimeric but not the monomeric form of C1D can interact with TRAX. In addition, C1D and Translin interact with TRAX in a mutually exclusive fashion, and co-expression of TRAX and Translin results in their altered subcellular localization. The potential implications of these results in regard to the biological roles of C1D and TRAX in DNA repair and recombination are discussed.

Materials and Methods

Cloning, mutagenesis and sequencing

Wild-type TRAX, Translin and C1D open reading frames (ORFs) were isolated by PCR with primers placing suitable restriction sites to the 5′ and 3′ ends and cloned into various yeast and mammalian expression plasmids. The yeast expression plasmid is pACT. The mammalian expression constructs include a HA-epitope (a peptide derived from influenza haemagglutinin protein)-tagged expression vector pCMV 5′2N3T (kindly provided by T. Kouzarides), and plasmids expressing various chromophores of the *Aequorea victoria* green fluorescent protein (GFP) obtained from Clontech: pEGFP (enhanced green fluorescent protein), pEYFP (enhanced yellow fluorescent protein), pECFP (enhanced cyan fluorescent protein) and pDsRed1 (red fluorescent protein). All other C1D constructs have been described previously (Yavuzer et al., 1998). MutLZ-Trax (the mutated LZ motive of TRAX) was generated in two steps. First, the region between amino acids 1-194 was isolated by PCR with mismatch primers placing *Sal*I/*Bam*HI sites at the 5′ end and a *Bgl*II site at the 3′ end. The region between amino acids 194-272 was then amplified via PCR, using primers that place a *Bgl*II site at the 5′ end and *Sal*I/*Bam*HI sites at the 3′ end. The two PCR fragments were digested with *Sal*I/*Bgl*II and cloned in a triple ligation into the *Xho*I site of pACT. This construct produced a mutant TRAX where a *Bgl*II site is inserted, which changes the 194th amino acid leucine to arginine and the preceding lysine to serine. The whole fragment was then cut out with *Bam*HI and cloned into T7-Myc/plink; it was transcribed and translated in vitro using TnT Coupled Reticulocyte Lysate system (Promega) to confirm that it gives a similar size product to wild-type TRAX. Sequencing of PCR fragments, point mutations and cDNA inserts from positive clones of the two-hybrid screen was performed with an automatic sequencer (ABI 377, version 2.1.1). Sequence comparisons were conducted using GenBank database searches.

Yeast two-hybrid screen

The yeast two-hybrid screen was performed as described previously using a B-lymphocyte cDNA library in pACT (Harper et al., 1993) and full-length C1D in fusion with the DNA-binding domain of bacterial LexA protein (DBD-C1D) (Yavuzer et al., 1998). The yeast strains and the control plasmids (DBD-Lamin and DBD-Daughterless) were kindly provided by S. Hollenberg. *Saccharomyces cerevisiae* L40 (*MAT**a** trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) was grown at 30°C in YPD medium (1% yeast extract, 2% polypepton and 2% glucose) and was transformed with DBD-C1D. Human B-lymphocyte cDNA library was then transformed into this strain using the lithium acetate method. Double transformants were plated on selective medium lacking Trp, Leu, His, Lys and uracil and were grown at 30°C for 3 days. Colonies were transferred on Whatman 40 filters to test for β-galactosidase activity using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Positive clones were rescued and tested for specificity by mating analysis or retransforming into L40 together with the control plasmids mentioned above. For the mating assay, DNA from positive clones was extracted using glass beads and then transformed by electroporation into the bacterial strain HB101, which has a defect in the leuB gene that can be complemented by LEU2 from yeast, and therefore provides selection of the library plasmids. The transformants were plated onto M9 medium containing ampicillin and proline and incubated at 37°C for 24 hours. DNAs of five colonies from each plate were then isolated by the alkaline lysis method, transformed into the yeast strain AMR70 (*MATα his3 lys2 trp1 leu2 URA3::(lexAop)$_8$-lacZ*) and were mated with the L40 strain carrying the bait plasmid. For liquid β-galactosidase assays using ONPG (o-nitrophenyl β-D-galactopyronoside) as a substrate, a yeast colony was grown at 30°C to mid-log phase (OD_{600}) and the cells were resuspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) and lysed by freeze-thaw cycles. Seven hundred μl of Z buffer and 160 μl of ONPG (4 mg/ml) were added to 100 μl of lysate and incubated at 30°C until a yellow colour appeared. The reaction was stopped by the addition of 400 μl 1 M Na_2CO_3 and OD_{420} of the samples were read. β-Galactosidase units were calculated according to the formula: $1000\times OD_{420}/(t\times V\times OD_{600})$ where t is elapsed time (in minutes) of incubation and V is 0.1 ml×concentration factor.

Ni-NTA pull-down assays

His-tagged C1D was expressed in bacteria and bound to Ni-NTA column as described previously (Yavuzer et al., 1998). His-tagged hepatitis-C virus core protein, p16 (kindly provided by Mehmet Ozturk), was purified under denaturing conditions and immobilized on Ni-NTA beads. TRAX was transcribed and translated in vitro using TnT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. Ten μg of radiolabelled translation product and the immobilized fusion proteins were incubated in 200 μl binding buffer (20% glycerol, 100 mM NaCl, 0.1×TE pH 8.0, 0.1% NP-40, 1 mM DTT, 30 μg BSA) at room temperature for 20 minutes followed by four washes using wash buffer (20% glycerol, 100 mM NaCl, 0.1×TE pH 8.0 and 0.5% NP-40). The beads were then resuspended in 20 μl of 2×SDS sample buffer, boiled, centrifuged at 10,000 ***g*** for 5 minutes and loaded on 10% SDS-PAGE. The gel was soaked in Amplify solution (Amersham) for 30 minutes, dried and visualized by autoradiography.

Cell culture, transient transfections and co-immunoprecipitations

COS 7 cells were maintained in DMEM supplemented with 10% fetal calf serum and grown at 37°C with 5% CO_2 as monolayers. Twenty four hours before transfections, cells were plated into 35 mm 6-well plates at a density of 3×10^4. Transfections were performed using FuGENE 6 reagent (Roche) with 0.5 μg of DNA according to the manufacturer's instructions. Cells were harvested 24 hours after transfection and lysed in lysis buffer (PBS containing 0.5% Triton-X, 5 mM EDTA and protease inhibitor cocktail tablets; Roche, 1873580). Cells that were subjected to 20 Gy of gamma (Cs^{137} source) or 50 j/m^2 of UV irradiation (Stratagene) were incubated for 1 hour at 37°C before harvesting. Thirty μl of Protein G agarose beads per sample was used and washed twice in PBS. The lysates were incubated with either an anti-GFP (Clontech) or anti-HA monoclonal antibody (Roche) for 2 hours at 4°C and then with protein G agarose beads for another 2 hours. The beads were washed five times with PBS followed by five washes with the lysis buffer. Precipitates were then separated

by 10% SDS-PAGE and blotted onto nitrocellulose. Western blotting was performed with anti-GFP antibody or a polyclonal antiserum raised against the full-length C1D and immunoreactive bands were visualized using ECL (Amersham) according to the manufacturer's instructions.

Microscopy and microphotography

Vectors expressing C1D, TRAX and Translin in fusion with GFP and various GFP variants were transiently transfected into COS 7 cells as described above. Twenty four hours after transfections, expression of the fusion proteins were examined in the control and γ-irradiated living cells for 24 hours. Fluorescence microscopy was performed on a Zeiss Axiovert 135TV microscope equipped with a CCD camera (Hitachi Denshi, Ltd) and appropriate filter sets, and the images were processed by using OpenLab software (Improvision).

Results

Identification of proteins interacting with the nuclear matrix protein C1D

A yeast two-hybrid screen was employed in an attempt to identify proteins interacting with C1D. To this end, we generated the yeast expression plasmid DBD-C1D, which directs the synthesis of full-length open reading frame of C1D, fused C-terminal to the bacterial LexA DNA binding protein. DBD-C1D was then introduced into the yeast reporter strain L40 (Hollenberg et al., 1995) that contains both the *HIS3* and *lacZ* genes under the control of LexA operators. Strain L40 containing DBD-C1D is His^- and does not express detectable β-galactosidase activity, indicating that C1D is transcriptionally inert when fused to LexA.

Strain L40 containing DBD-C1D was then transformed with a human B-lymphocyte cDNA library in the vector pACT. A total of ~1×10^8 transformants were obtained, of which ~3×10^3 were capable of growing on medium lacking histidine. Of these, 280 were found to produce elevated β-galactosidase activity. False positives were eliminated by using a mating assay and 120 transformants were identified as containing cDNAs that conferred a His^+, $LacZ^+$ phenotype in a fashion that was specific to L40 cells expressing the DBD-C1D fusion protein. The cDNA expression vectors from 80 of these transformants were then rescued, and sequencing revealed five cDNA groups, one of which comprised C1D. This finding served as a positive control for the screening because C1D homodimerisation has been demonstrated previously (U.Y., unpublished). A search of the GenBank database revealed that a second group of overlapping sequences that derived from the same cDNA encodes a recently identified protein termed TRAX (GenBank accession no. X95073) (Aoki et al., 1997).

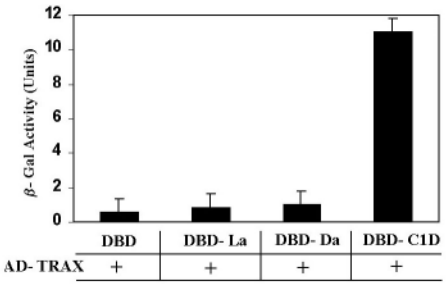


Fig. 1. C1D and TRAX interact specifically. TRAX fused to the Gal4 activation domain (AD-TRAX) was transformed into the reporter yeast strain together with DBD alone, DBD-lamin (La), DBD-Daughterless (Da) or DBD-C1D and interactions were measured by β-galactosidase activity using ONPG.

C1D interacts with TRAX specifically

To establish the specificity of the two-hybrid interaction between C1D and TRAX, the full-length open reading frame of TRAX was expressed in yeast as an activation domain tagged fusion protein (this fusion is referred to as AD-TRAX). AD-TRAX was transformed into L40 together with baits comprising the LexA DNA-binding domain (DBD) alone, DBD-Lamin, DBD-Daughterless or DBD-C1D; the level of activity from the integrated *LacZ* reporter was determined in each case using the ONPG assay. TRAX interacts specifically with DBD-C1D given that no interaction is detected with DBD alone, DBD-Lamin or DBD-Daughterless (Fig. 1). In reciprocal two-hybrid experiments where C1D was fused to the activation domain and TRAX to the C-terminal DNA-binding region of LexA and introduced into the strain L40, a similar level of β-galactosidase activity was also determined (data not shown). Therefore, we concluded that C1D and TRAX interact specifically.

C1D and TRAX interact in vitro

Next, we determined whether C1D is able to bind TRAX in vitro. For this, C1D was expressed in *E. coli* as a His-tagged fusion protein and was purified by use of Ni-NTA beads. Hepatitis C core protein, which has a similar size to C1D, was also expressed and purified under the same conditions as C1D and was used as control. The immobilized fusion proteins were then incubated with the in vitro transcribed and translated TRAX and pull-down assays were performed. As shown in Fig. 2, although no interaction is observed between TRAX and pQE alone or hepatitis C core protein, a substantial proportion of the input TRAX (one-tenth) is bound by C1D, indicating that C1D and TRAX also interact specifically in vitro.

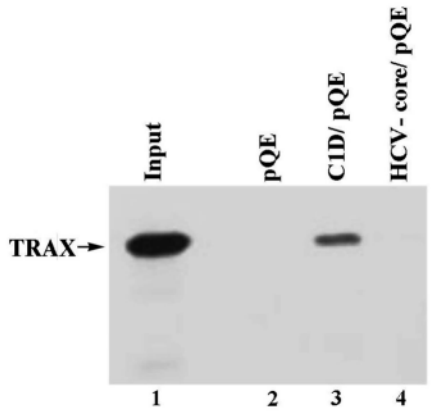


Fig. 2. C1D and TRAX interact in vitro. C1D and hepatitis C virus core proteins (HCV-core/pQE) were expressed in bacteria using the plasmid pQE30 (Qiagen) and purified by Ni-NTA column under denaturing conditions. One μg of each of the immobilized fusion proteins and pQE alone were incubated with In vitro Transcribed and Translated (IVTT) TRAX product and pull-down assay was performed. One tenth of the input TRAX was bound by C1D.

The putative leucine zipper region of TRAX is involved in interaction with C1D

TRAX contains a putative leucine zipper (LZ) (Aoki et al., 1997) motif between amino acids 73-208. Because this motif may be involved in protein-protein interactions, we tested whether this region has any role in its interaction with C1D. We introduced mutations into the putative LZ motif of TRAX within an otherwise intact full-length protein (this mutant is termed mutLZ-TRAX). We then subcloned mutLZ-TRAX to a yeast expression plasmid as a fusion to the Gal4 activation domain (AD-mutLZ-TRAX) and transformed into strain L40 together with the plasmid expressing DBD-C1D and determined the β-galactosidase activity. As expected, a significant activity was observed with AD-TRAX, whereas a low level of β-galactosidase activity was detected when mutLZ-TRAX-AD was tested for a two-hybrid interaction with DBD-C1D (Fig. 3A). To confirm the requirement of the putative LZ region of TRAX in C1D interaction, we used the N-terminal region of TRAX (between amino acids 1-210) and expressed this truncated protein as a fusion with the Gal4 activation domain (AD-N-TRAX) and determined the β-galactosidase activity. As seen in Fig. 3A lane 2, the truncated version of TRAX protein, which contains the N-terminal region including the putative LZ, interacted with C1D as strongly as the wild-type TRAX protein.

Because TRAX can interact with both Translin and C1D, and the putative LZ region is important in its interaction with C1D, it was important to determine whether the same region also plays a role in Translin/TRAX interaction. The yeast two-hybrid assay employed for this purpose showed that the putative LZ region of TRAX is also important for TRAX/Translin interaction, as the mutLZ-TRAX did not interact with Translin (Fig. 3B). However, unlike the C1D/TRAX interaction, which is mainly governed through the N-terminal region of TRAX containing the LZ motif, TRAX/Translin interaction is likely to require the full-length TRAX protein with an intact LZ region, as neither the N-terminal nor the C-terminal regions of TRAX alone interacted with Translin (Fig. 3B, lanes 3,4). Because the mutLZ-TRAX did not interact with either C1D or Translin, it was important to check whether it is expressed in yeast. As an HA-epitope was tagged to the N-terminal region of mutLZ-TRAX, we were able to detect the protein levels by western blotting in total yeast extracts, and we showed that the mutLZ-TRAX was expressed in both of the strains carrying DBD-C1D and DBD-Translin (data not shown).

C1D and translin bind to TRAX in a mutually exclusive manner

According to the yeast two-hybrid results, TRAX binds to both C1D and Translin. Therefore, we wanted to determine the relative interaction between TRAX and C1D or Translin when three molecules are found together. His-tagged C1D was immobilized on Ni-NTA beads, and in vitro transcribed and translated TRAX and Translin were incubated with the immobilized fusion protein either alone (Fig. 4, lanes 3,4) or together (lanes 5, 6 and 7). Similar to the previous result (Fig. 2), around 10% of input TRAX was bound to C1D (lane 3), whereas binding of Translin to C1D was barely detectable (lane 4). When TRAX and Translin were incubated simultaneously with the immobilized C1D, the binding of TRAX to C1D was similar to the levels obtained when TRAX was incubated with C1D alone (compare lanes 3 and 5). By contrast, when TRAX and Translin were pre-incubated for 1 hour and then added to the immobilized C1D, only a small fraction of TRAX was able to bind to C1D (lane 6). When TRAX was incubated with C1D for 1 hour prior to the addition of Translin (lane 7), binding of TRAX to C1D was increased almost twofold with respect to its binding without pre-incubation in the absence of Translin (compare lanes 3 and 7). Taken together with the fact that both C1D and Translin require the putative LZ region of TRAX to bind (Fig. 3), these results would suggest that C1D and Translin bind TRAX in a mutually exclusive fashion.

C1D and TRAX interaction is stimulated by γ-irradiation in mammalian cells

Because C1D mRNA and protein levels have been shown to

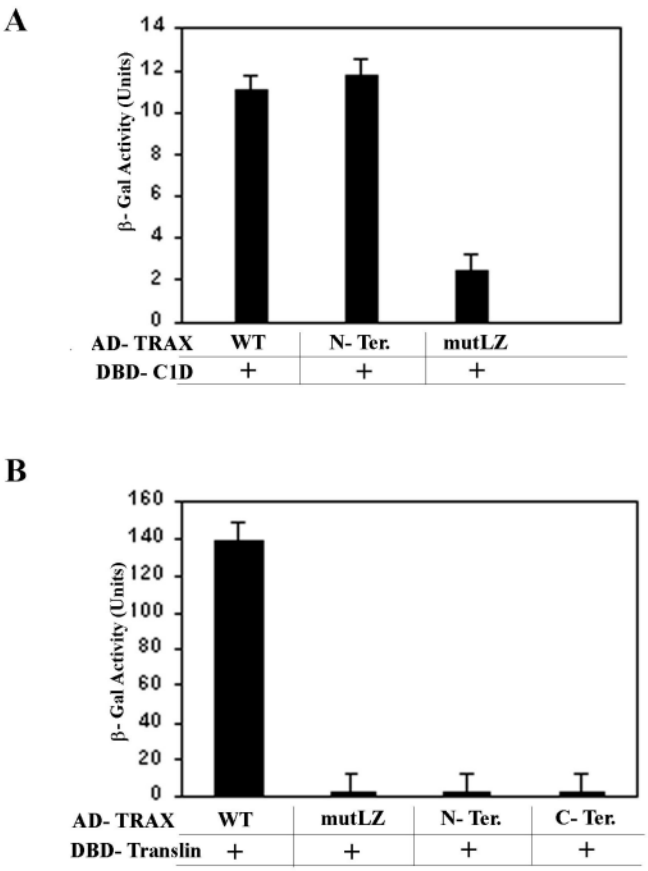


Fig. 3. The putative LZ region of TRAX is essential for interactions with C1D and Translin. (A) Yeast two-hybrid assay was performed using DBD-C1D and an activation domain (AD) tagged forms of TRAX; wild-type (WT), N-terminal region containing the LZ motif (N-Ter) or mutLZ where the LZ region of TRAX has been mutated within an otherwise intact protein. The interactions were measured by β-galactosidase activity using ONPG. (B) Constructs as in (A) were used to detect interaction with the Translin protein expressed as a fusion to the bacterial LexA protein (DBD-Translin). Mutations that disrupt the LZ region of TRAX abolish its interaction with Translin and neither the N-terminal region of TRAX carrying an intact LZ nor the C-terminal region alone (C-Ter) are sufficient for interaction with Translin.

be induced upon γ-irradiation (Yavuzer et al., 1998), we wished to determine whether γ-irradiation would affect TRAX/C1D interaction in mammalian cells in vivo. C1D protein is insoluble and remains tightly bound to DNA during whole-cell or nuclear extract preparations and therefore cannot be detected by western blotting of soluble cell extracts (Yavuzer et al., 1998). Therefore, COS 7 cells were transiently transfected with vectors expressing HA-epitope-tagged version of C1D (HA-C1D) and YFP-tagged TRAX (YFP-TRAX). Cells expressing YFP only and untransfected cells were also included as controls. Transfection of cells with C1D-expression vectors enables the production of sufficient soluble C1D and thus allows co-immunoprecipitation assays to be performed (Yavuzer et al., 1998). Twenty four hours following transfection, 20 Gy of γ-irradiation was applied to a set of transiently transfected cells and after incubating for an additional hour, lysates were prepared from the irradiated and unirradiated cells. Immunoprecipitations were performed with anti-GFP, which also recognizes YFP, or anti-HA antibodies, followed by immunoblotting with an antibody against C1D. In both unirradiated and irradiated cells, C1D monomers (16 kDa) and SDS-resistant dimers (32 kDa) (Nehls et al., 1998) were detected in immunoprecipitations using anti-HA antibody followed by immunoblotting with anti-C1D (Fig. 5A, lanes 1,4). As expected, C1D was detected in lysates from HA-C1D-transfected cells but not in lysates from untransfected or YFP-expressing cells (lanes 6 and 5, respectively). When HA-C1D and YFP-TRAX were cotransfected and immunoprecipitated with anti-GFP antibody and probed with anti-C1D antibody, we were not able to detect C1D interaction with TRAX from unirradiated cells (lane 2). However, the 32 kDa SDS-resistant C1D homodimer, but not the 16 kDa monomer, was readily detectable upon γ-irradiation (lane 3), indicating that γ-irradiation induces TRAX/C1D interaction.

Because γ-irradiation results in DNA double-strand breaks, we next determined whether the interaction between C1D and TRAX was specific to DNA double-strand breaks or whether it could also be induced by other types of DNA damage. To test this, we used UV irradiation (50 j/m^2), which is known to activate mainly the nuclear excision repair (NER) pathways. COS 7 cells were transiently transfected with vectors expressing GFP-TRAX and HA-C1D and immunoprecipitations were performed using antibodies raised against GFP peptide or HA-epitope. As for controls, lysates from untransfected and HA-C1D- or GFP-TRAX-transfected cells were also immunoprecipitated using the same antibodies

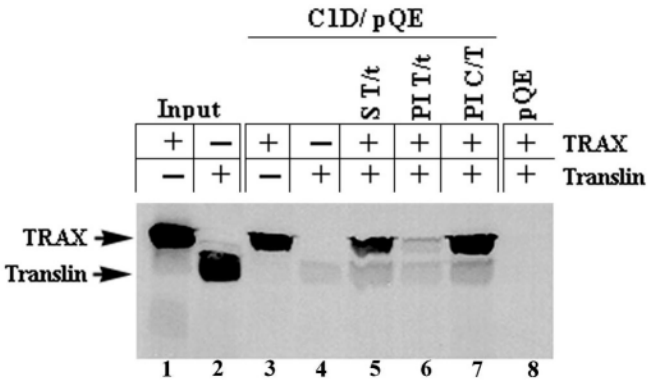


Fig. 4. Binding of C1D and Translin to TRAX is mutually exclusive. C1D was expressed in bacteria and immobilized on Ni-NTA column as explained in the legend to Fig. 2. IVTT TRAX and Translin products were incubated with the immobilized C1D either alone (lanes 3 and 4) or together. TRAX (T) and Translin (t) IVTT products were incubated simultaneously (S) with the immobilized C1D for 2 hours before washing (lane 5), TRAX and Translin IVTT products were pre-incubated (PI) for 1 hour before adding to immobilized C1D and incubated for another hour (lane 6), IVTT product of TRAX was incubated with the immobilized C1D (C) for 1 hour and then Translin was added and incubated for an additional hour (lane 7). The plasmid expressing His only (pQE) was expressed in bacteria, purified and immobilized under the same conditions as C1D and was used in lane 8 as a negative control.

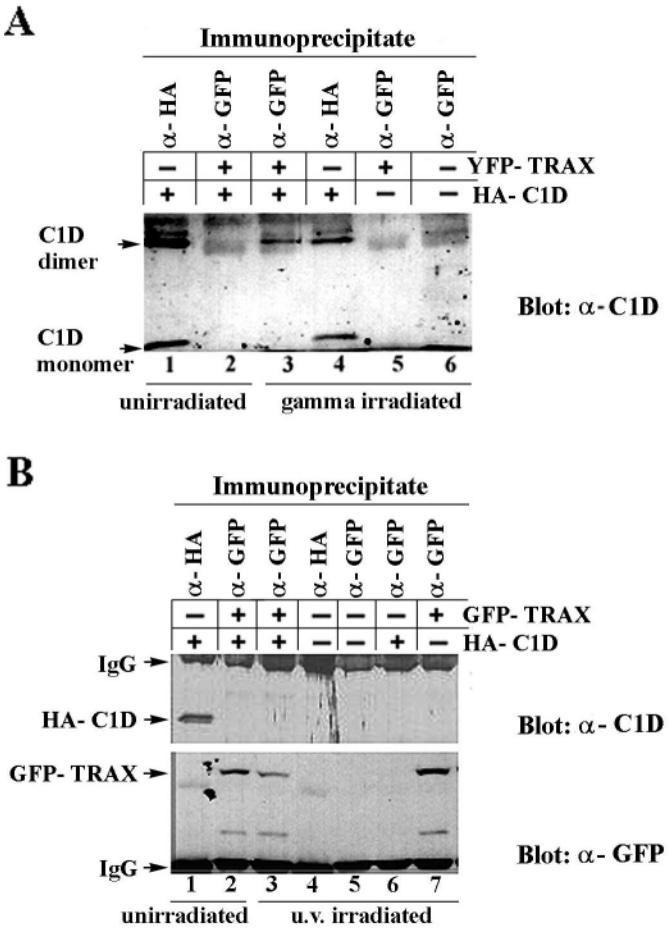
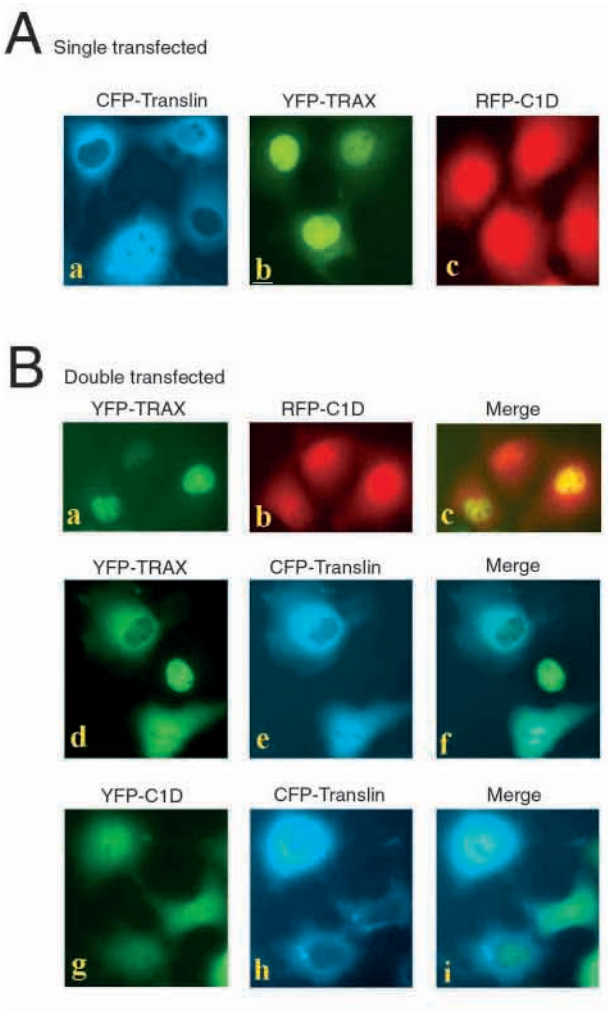


Fig. 5. SDS-resistant C1D homodimers interact with TRAX upon γ-irradiation. (A) Transiently transfected COS 7 cells were immunoprecipitated using the antibodies indicated. For lanes 3-6 the cells were treated with 20 Gy of ionizing irradiation before lysate preparation. Western blotting was performed by an anti-C1D antibody. The monomer and dimer forms of C1D are indicated. (B) Transiently transfected COS 7 cells were immunoprecipitated using the indicated antibodies. For lane 3-7, the cells were treated with 50 j/m^2 UV irradiation. The top panel shows the western blotting using an anti-C1D antibody, and in the bottom panel, the blot was stripped and reprobed with an anti-GFP antibody to demonstrate the presence of GFP-TRAX. For each lane, immunoprecipitations were performed using the cell lysates obtained from 3×10^5 cells.



D

Transfection	% Nuclear cytoplasmic: Translin pre	Translin post	TRAX pre	TRAX post	C1D pre	C1D post	% nuclear: TRAX pre	TRAX post	Irradiation
Single	6	4.5	—	—	100	100	100	100	
Double	10	11	—	—	—	—	—	—	
	34	43	58	61	—	—	—	—	
	19	15	—	—	100	100	—	—	
	—	—	—	—	100	100	100	100	
Triple	30	46	44	65	100	100	—	—	

(Fig. 5B, lanes 4-7). The immunoprecipitates were then blotted with an anti-C1D antibody (Fig. 5B, top panel). We were able to detect the 32 kDa dimeric form of C1D only in cell lysates from HA-C1D-transfected cells (lane 1). However, C1D was not detectable when cell lysates from GFP-TRAX- and HA-C1D-transfected cells were immunoprecipitated with an anti-GFP antibody (lane 2). In contrast to γ-irradiation, treatment of these double-transfected cells with UV irradiation did not induce interaction of TRAX with C1D (lane 3). To confirm that the lack of interaction between C1D and TRAX upon UV irradiation is not due to the absence of TRAX expression, we stripped the blot and reprobed with an antibody raised against GFP-peptide (Fig. 5B, bottom panel). As expected, a protein of ~60 kDa, which is the expected size for GFP-TRAX protein, was detected in all the cell lysates from GFP-TRAX-transfected cells (lanes 2, 3 and 7) but not in lysates from untransfected or HA-C1D-transfected cells (lanes 1, 4, 5 and 6). We conclude therefore that C1D and TRAX interact in mammalian cells in response to agents inducing DNA-double strand breaks but not in response to UV irradiation.

Subcellular localization of C1D, TRAX and Translin

To determine the subcellular localization of Translin, TRAX and C1D, we expressed these proteins in COS 7 cells as fusions with different GFP variants either alone or in combination, and determined the expression patterns in the living cells. The fluorescence results are shown in Fig. 6A-C with a summary shown in Fig. 6D. In agreement with previous results (Kasai et al., 1997; Chennathukuzhi et al., 2001), CFP-Translin when expressed alone was found to be cytoplasmic in over 90% of cells examined (Fig. 6Aa). In around 6% of cells however, CFP-Translin exhibited both nuclear and cytoplasmic localization. γ-Irradiation or other DNA damaging agents that induce DNA double-strand breaks did not cause any alteration in its subcellular localization in agreement with previous observations (Chennathukuzhi et al., 2001). By contrast, YFP-TRAX was localized to the nucleus in all cells examined,

Fig. 6. Subcellular localizations of TRAX, Translin and C1D. (A) Plasmids expressing CFP-Translin, YFP-TRAX and RFP-C1D were transfected into COS 7 cells and subcellular localizations were determined in the living cells. Translin was mainly cytoplasmic; however, 6% of cells exhibited nuclear/cytoplasmic staining (a). TRAX was nuclear (b) and C1D was mainly nuclear, but a cytoplasmic staining was also evident (c). (B) COS 7 cells were double transfected with the indicated plasmids. (a-c) TRAX/C1D double-transfected cells showing that TRAX is solely nuclear (a) whereas C1D is showing a nuclear/cytoplasmic staining pattern (b), and colocalizes with TRAX in the nucleus (c). In TRAX/Translin double-transfected cells (d-f), TRAX and Translin colocalize in the cytoplasm and in the nucleus. The middle cell in d and f expresses TRAX only. In C1D/Translin double-transfected cells (g-i), C1D is mainly nuclear (g) and Translin is cytoplasmic (h). (C) COS 7 cells triple transfected with TRAX-, Translin- and C1D-expressing plasmids. (a-c) A single cell in which a diffuse nuclear/cytoplasmic staining pattern is observed. In d-f, another field is shown where two of the cells were triple transfected and in which the upper cell TRAX and C1D were expressed in the nucleus (d and f) when Translin was mainly cytoplasmic (e); the lower cell shows a diffuse nuclear-cytoplasmic staining pattern as in a-c. (D) Summary of the results demonstrating the percentage of nuclear/cytoplasmic and solely nuclear localizations of Translin, TRAX and C1D.

whereas C1D was observed in a diffuse nuclear (predominantly)/cytoplasmic staining pattern as observed using anti-C1D antibody (U.Y., unpublished) (Fig. 6Ab and Fig. 6Ac, respectively). As with Translin, the localization of TRAX and C1D did not change following γ-irradiation (data not shown). Because Translin exhibited nuclear/cytoplasmic localization in 6% of the cells when expressed alone, we wanted to see whether an alteration would occur in its nuclear localization when expressed together with C1D or TRAX. To constitute a basal level for the percentage of cells exhibiting nuclear Translin in double transfections, Translin was co-expressed with a plasmid expressing YFP only and was found to show nuclear/cytoplasmic localization in around 10% of the cells (Fig. 6D). A different picture emerged with TRAX or C1D double transfections: co-expression of Translin with TRAX resulted in an almost 3.5-fold increase in the proportion of cells in which Translin was observed to be nuclear/cytoplasmic (34%) colocalizing with TRAX, and a striking alteration in the location of TRAX, with around 58% of cells expressing TRAX both in cytoplasm and nucleus (compared to 100% nuclear when expressed alone). In 42% of the cells TRAX was solely cytoplasmic and colocalized with Translin. Importantly, in these cells where TRAX was cytoplasmic, Translin was never found to be nuclear. In Fig. 6Bd-f, a representative area consisting of three cells is shown; two of the cells contain both TRAX and Translin. In one of the cells, TRAX and Translin are co-localized to cytoplasm, whereas in the other, a diffuse nuclear/cytoplasmic staining pattern is observed. The third cell expresses TRAX only and, as expected, when Translin is not co-expressed, TRAX is solely nuclear. This single TRAX-expressing cell in this particular area also acts as an internal control demonstrating that the filters used to detect YFP or CFP are highly specific. These alterations in the compartmentalization of TRAX and Translin presumably reflect interactions between these two proteins and indicates that the relative expression levels of these proteins is important in determining their subcellular localization. γ-Irradiation did not cause a significant alteration in the subcellular localizations of TRAX and Translin. On the contrary, when TRAX was expressed with C1D, neither C1D nor TRAX changed their localizations either before or after γ-irradiation and TRAX stayed in the nucleus (Fig. 6Ba) whereas C1D showed its usual nuclear/cytoplasmic staining pattern (Fig. 6Bb), colocalizing with TRAX in the nucleus (Fig. 6Bc). Co-expression of Translin and C1D (Fig. 6Bg-i) resulted in a slight increase in the proportion of the cells containing nuclear Translin (19%); however, once again γ-irradiation did not have any effect on the subcellular localizations of either Translin or C1D. These results demonstrate that γ-irradiation does not affect the localization of TRAX, Translin or C1D, but the otherwise nuclear TRAX changes its localization when Translin is co-expressed and stays in the nucleus when C1D is co-expressed.

In cells triple transfected with YFP-TRAX, CFP-Translin and RFP-C1D expression vectors, we were not able to detect any significant alteration in the subcellular localizations of TRAX or Translin compared to TRAX/Translin double-transfected cells (Fig. 6D). In other words, C1D over expression did not cause an overall alteration in the localization of TRAX or Translin. In Fig. 6Cd-f, a group of cells, of which two were triple transfected, is shown. C1D again exhibits a nuclear/cytoplasmic staining (Fig. 6Cf), whereas TRAX is solely nuclear in one of these cells (Fig. 6Cd, upper cell) and nuclear/cytoplasmic in the lower cell. Translin, however, is also nuclear/cytoplasmic in this lower cell, but is mainly cytoplasmic in the upper cell and therefore does not colocalize with TRAX. In the bottom, left-hand side, a third cell is seen (Fig. 6Cd,e) which only expresses nuclear/cytoplasmic TRAX and Translin, and a fourth cell (Fig. 6Ce) expressing Translin only, again acting as internal controls; this shows that the different filters used for GFP, YFP or DsRed are specific. Although in a few cells expression of C1D appears to maintain TRAX in the nucleus, whereas Translin was mainly cytoplasmic, the majority of cells showed a nuclear/cytoplasmic staining (Fig. 6Ca-c), making it difficult to determine whether TRAX was complexed with Translin or C1D. However, we noted a slight increase in the percentage of cell expressing nuclear TRAX or Translin after irradiation in the presence of C1D, compared to no increase in the double-transfected cells (Fig. 6D).

Discussion

The protein and mRNA levels of the nuclear matrix protein C1D increase upon γ-irradiation (Yavuzer et al., 1998). In addition, C1D interacts with DNA-PK and is an efficient substrate for this kinase, which plays an essential role in the repair of DNA double-strand breaks. The interaction between C1D and DNA-PK is significant in the sense that C1D is the first substrate identified that is capable of activating DNA-PK in the absence of free DNA-ends (Yavuzer et al., 1998). These findings raised the possibility that C1D might play a role in DSB repair. Given the role of the nuclear matrix in the regulation of important cellular events, such as DNA replication, transcription, RNA splicing, topoisomerase activity, nucleotide excision and DSB repair (Berezney, 1984; Cockerill and Garrard, 1986; Jackson, 1991; Johnston and Bryant, 1994; Kaufman and Shaper, 1991; Koehler and Hanawalt, 1996; Korte and Yasui, 1993; Nelson et al., 1986; Verheijen et al., 1988; Yasui et al., 1994; Yasui et al., 1991), it is clearly important to reveal the role of C1D in any of these events.

Using the yeast two-hybrid system to screen a human B-lymphocyte library we showed that C1D can interact specifically with the Translin-interacting protein, TRAX. Although the putative LZ region of TRAX is important not only in interaction with C1D but also in its interaction with Translin, C1D and Translin show different requirements for interaction with TRAX outside the LZ region. The N-terminal region of TRAX that contains an intact LZ region is sufficient for its interaction with C1D, whereas Translin requires full-length TRAX with an intact LZ region for interaction. However, consistent with Translin and C1D sharing an interaction motif within TRAX, we were able to show that binding of Translin and C1D to TRAX appears to be mutually exclusive and that stable interaction between C1D and TRAX in mammalian cells occurs upon γ-irradiation. Although the biological functions of these proteins are not yet clear, these findings suggest that both C1D and TRAX might play a role in the repair of DNA DSBs.

Cells use various mechanisms to protect their genome against genotoxic insults. One obvious mechanism is to detect

the damage and activate DNA repair machinery to repair the damage before cell division takes place. When the damage is beyond repair, cells can activate their suicide programs, and via apoptosis the damaged cell destroys itself. C1D may play a role in both types of mechanism as overexpression of C1D is capable of inducing apoptosis in tumour cell lines (Rothbarth et al., 1999) and C1D interacts with DNA-PK, which has an established role in double-strand DNA break repair.

An important feature of tumour cells is that they carry specific chromosomal abnormalities, including translocations, that result in the altered expression or structure of cellular gene products giving rise to functional activation which contributes to the initiation or progression of cancer (Rabbitts, 1994). Chromosome translocations in malignant tumours may arise by several mechanisms. The translocations seen in lymphomas and leukaemias involve the immunoglobulin or T-cell receptor genes and the Ig/TCR recombinase signal sequences are present on both affected chromosomes. In some other lymphoid malignancies, the breakpoint junctions are characterized by another consensus sequence, which is recognized by the Translin protein (Aoki et al., 1995). Translin recognition site sequences have also been identified in solid tumours (Chalk et al., 1997) indicating that Translin may be important in the genesis of chromosomal translocations in some types of neoplasms. As well as being a sequence-specific DNA-binding protein, Translin is also capable of binding to RNA, and the mouse homologue of human Translin, the testis brain RNA-binding protein (TB-RBP), was identified as a RNA-binding protein capable of recognizing conserved elements in the 3′ untranslated regions of specific mRNAs in brain and testis (Han et al., 1995; Wu et al., 1997; Wu and Hecht, 2000).

Although the role of TRAX in any of these events is not yet clear, both human and mouse TRAX can form heterodimers with Translin/TB-RBP (Aoki et al., 1997; Taira et al., 1998; Wu et al., 1999) and the TRAX/Translin heterodimer is capable of binding to both single-stranded DNA and RNA, raising the possibility that TRAX/Translin heterodimer is multifunctional. However, little is known about the biological functions of Translin and TRAX.

Previous data demonstrated that Translin is primarily cytoplasmic in many cell lineages but it is found in the nucleus in haematopoietic cell lines. Interestingly, however, treatment of HeLa cells with mitomycin C or etoposide, agents that induce DNA double-strand breaks, was reported to cause translocation of Translin into the nucleus (Kasai et al., 1997). The subcellular localization of TB-RBP changes in meiotic and post-meiotic cells (Gu et al., 1998; Morales et al., 1998), but agents causing DNA DSBs did not affect localization of TB-RBP (Chennathukuzhi et al., 2001). In agreement with this latter study, and consistent with Translin containing a REV-like nuclear export signal (Chennathukuzhi et al., 2001), Translin was located in the cytoplasm in all but around 6% of cells where a nuclear/cytoplasmic expression pattern was observed, and treatment of transfected COS 7 cells with etoposide or γ-irradiation did not induce translocation of Translin into the nucleus. Why around 6% of cells exhibit a different localization for Translin is unclear, but these cells may represent those that have recently undergone mitosis, a point in the cell cycle when Translin would have access to DNA by virtue of nuclear envelope breakdown. Following the completion of mitosis, the nuclear envelope would reform, causing the Translin that was retained in the nucleus either being exported to the cytoplasm or degraded.

In contrast to Translin, and to a previous report indicating that TRAX is a predominantly cytoplasmic protein (Chennathukuzhi et al., 2001), our results show TRAX located in the nucleus in all cells examined (COS7, MCF7 or 3T3 cell lines), consistent with TRAX containing a nuclear localization signal (Aoki et al., 1997). However, co-expression of TRAX and Translin resulted in a different pattern of localization of both proteins – an increase in the number of cells with a nuclear Translin and an increase in cells expressing cytoplasmic TRAX. The changes in localization occurring when TRAX and Translin are co-expressed are consistent with an interaction between the two proteins affecting their ability to traffic between the nucleus and cytoplasm, and they also indicate that the relative expression levels of TRAX and Translin are likely to be important in determining their subcellular localization. Indeed, differences in the levels of endogenous or ectopically expressed TRAX and Translin in different cell types may explain why the reported subcellular localization of ectopically expressed Translin or TRAX may differ.

Because, in our experimental sytems, γ-irradiation did not have a significant effect in the subcellular localizations of TRAX, Translin or C1D, the finding that the SDS-resistant homodimers of C1D and TRAX interact only after γ-irradiation may indicate that a post-translational modification is required for the interaction. Considering that C1D is phosphorylated by DNA-PK, it is possible that upon γ-irradiation DNA-PK is activated and phosphorylates C1D, which in turn interacts with TRAX. DNA-PK was not found to immunoprecipitate with TRAX from mammalian cells, nor was it able to phosphorylate recombinant TRAX in vitro (data not shown). However, this does not exclude the possibility that TRAX could be phosphorylated by DNA-PK or a related kinase in vivo in response to DNA damage. Indeed, mouse TRAX has been found to be phosphorylated in testis germ cell nuclear extracts where many DNA DSBs are found due to ongoing recombination events (Chennathukuzhi et al., 2001).

One biological consequence of C1D/TRAX interaction could be to regulate the Translin/TRAX interaction. In this regard, we note that TRAX has been shown to enhance the DNA binding capacity of TB-RBP (Translin), while decreasing its RNA-binding ability (Chennathukuzhi et al., 2001). Because interaction with either C1D or Translin appears to require the putative LZ motif of TRAX, the increased expression of C1D that occurs in response to γ-irradiation (Yavuzer et al., 1998) (a signal that also triggers the stable interaction between C1D and TRAX in mammalian cells) could result in C1D masking the Translin docking site on TRAX and thereby preventing the Translin/TRAX complex formation and binding to DNA. Thus, increasing the C1D/TRAX interaction on γ-irradiation might represent a mechanism to inhibit any unwanted Translin-mediated recombination events before the damaged DNA is repaired. Indeed, in our in vitro competition experiments, we showed that binding of C1D and Translin to TRAX is mutually exclusive. Importantly, however, if TRAX binds to Translin first, then it is no longer available to bind to C1D. Therefore, C1D may act as a regulator of TRAX/Translin complex formation, rather than regulate the association of any

preformed TRAX-Translin complex. This is an important point. Our studies using fluorescently tagged proteins also failed to identify any dramatic change in subcellular localization of TRAX, Translin or C1D when expressed in any combination in response to γ-irradiation. This is in agreement with others (Chennathukuzhi et al., 2001) who also found no difference in the subcellular localization of Translin in response to DNA-damaging agents. We suspect that the results may reflect the relative timing of expression of the proteins in transfected cells. Thus, in the absence of γ-irradiation, C1D will not interact stably with TRAX and so no significant effect of C1D on the subcellular localization of TRAX and Translin would be expected. On γ-irradiation C1D would interact with TRAX, but this association would only inhibit the formation of new TRAX-Translin complexes. Against the high background of preformed TRAX-Translin complexes it would be difficult to detect any change in overall localization of the fluorescent proteins before the overexpression of C1D eventually results in apoptosis. Nevertheless, we suspect that the interaction between C1D and TRAX will represent an important mechanism regulating the association of TRAX and Translin and leading to alterations in the relative amounts of TRAX/Translin targeted to the nucleus. Further studies dissecting the interaction between C1D, TRAX and Translin and their requirements for DNA binding should help to address some of these issues.

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