Aus dem Zentrum für Humangenetik
der Philipps-Universität Marburg
Director: Professor Dr. Karl-Heinz Grzeschik

Identification and functional characterization of protein domains in the transcription factor TWIST

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Dedicated

To my Loving and Adorable

Daughter

Parinita

Contents

Abbreviations

APS	Ammonium peroxodisulfate
ATP	Adenosintriphosphate
Bp	base pair
BSA	Bovine serum albumin
cDNA	complementary DNA
DAPI	4', 6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotidetriphosphate
dT	Deoxythymidinate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
FCS	Fetal calf serum
g	gravity
GFP	Green fluorescence protein
g	gram
HEPES	1-Piperazineethane sulfonic acid
HPLC	High performance liquid chromatograpy
IPTG	Isopropyl-ß-thiogalactopyranoside
kb	kilobase
kD	kilodalton
Mb	Mega base pair
mRNA	messenger Ribonucleic acid
NaAc	Sodium acetate
NCBI	National Center for Biotechnology Information
ng	nanogram
NLS	Nuclear localization signal
OD	Optical density

ORF	Open reading frame
Pa	Pascal
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pmol	picomol
PBS	Phosphate buffer saline
PBT	Phosphate buffer saline + Tween
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase-PCR
SDS	Sodium Dodecylsulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SV40	Simian Virus 40
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
TE	Tris-EDTA buffer
Temp	Temperature
TEMED	Tetramethylethylene diamine
Tris	Tri-hydroxymethyl aminomethane
U	Unit
UV	Ultra-violet
V	Voltage
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-ß-galactosidase
3-AT	3-amino-1,2,4-triazole

1 Introduction

1.1 Craniosynostosis

Craniosynostosis, the premature closure of one or more cranial sutures, is a congenital malformation with a frequency of ~1 in 2,500 infants (reviewed by Wilkie 1997). The abnormal skull growth may be associated with raised intracranial pressure, impaired cerebral prefusion, airway obstruction, impaired vision and hearing, learning difficulties and adverse psychological effects, as well as significant aesthetic deformity (Renier et al. 1982; David et al. 1996; Gosain et al. 1996). More than 100 different forms of isolated craniosynostosis and craniosynostosis syndromes are known, showing etiologic and pathogenetic heterogeneity (Winter and Baraitser 1994). In about half of the syndromes, a genetic cause has been confirmed or suggested. Most craniosynostosis syndromes with a genetic background are inherited as monogenic autosomal dominant traits (Cohen 1993).

1.2 Acrocephalosyndactyly

The acrocephalosyndactylies (ACS), a subtype of craniosynostosis syndromes form a heterogenous group of autosomal dominant disorders, with clinically similar symptoms etiology. Coronal or multiple synostosis, as well as partial syndactyly of fingers and/or toes are the main clinical features of these syndromes (Winter and Baraitser 1994). The shape of the skull is altered depending on which of the sutures are fused prematurely. This leads to brachycephaly or short-headness, trigonocephaly, anterior and posterior plagiocephaly, and dolichocephaly (Fig. 1).

On the basis of phenotypic characteristics the acrocephalosyndactylies were divided into a typical and an atypical form (Blank 1960). The typical form is Apert syndrome (MIM 101200), characterized by syndactyly of hands and feet of a special type (complete distal fusion with tendency for fusion also of the bony structures). The second group comprises a heterogeneous collection of disorders and includes Crouzon (MIM 123500), Jackson-Weiss (MIM 123150), Pfeiffer (MIM 101600), and Saethre-Chotzen syndrome (MIM 101400).

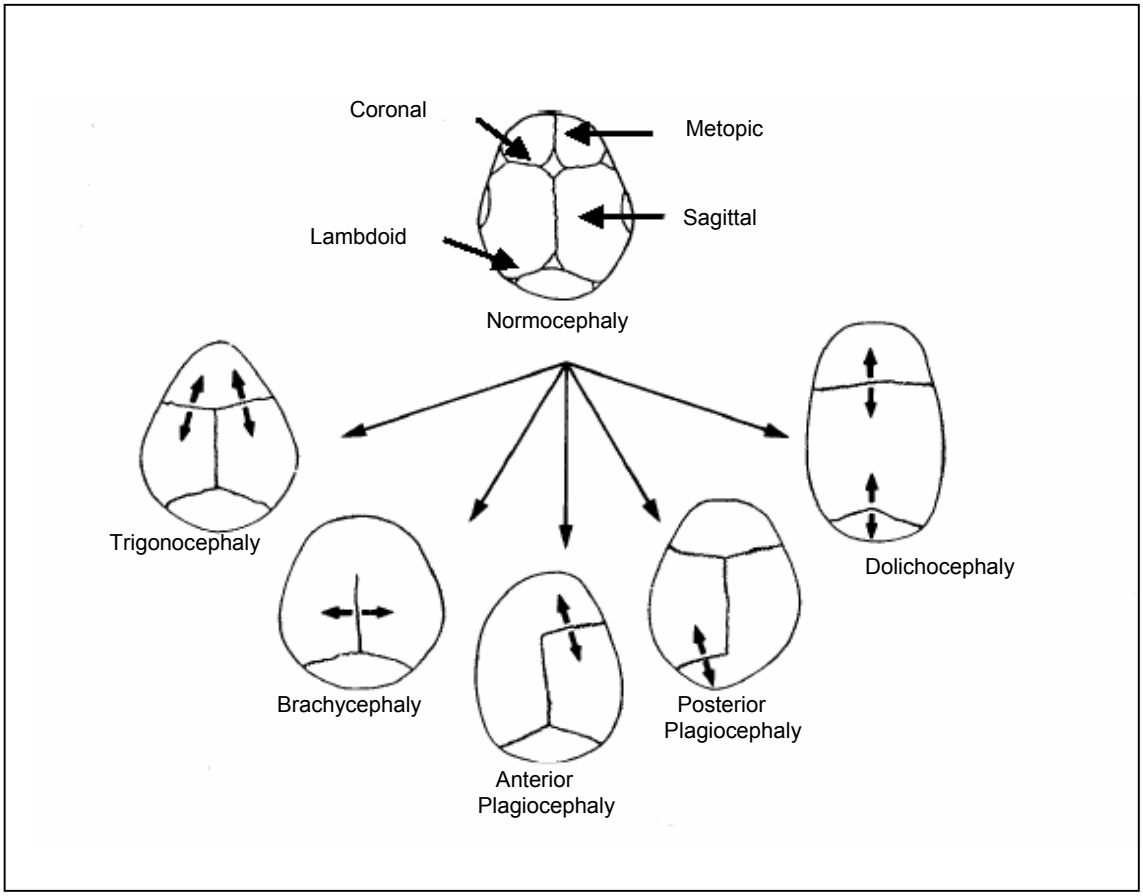


Fig. 1: Diagram of the sutures of the skull. Superior view of the normal infant skull. The anterior fontanelle is boardered by the metopic, coronal, and sagittal sutures, and the posterior fontanelle is bordered by the sagittal and the lamboid sutures. Premature fusion of the sutures leads to an abnormal head shape. Compensatory expansion (arrows) occurs particularly at neighbouring unfused sutures (modified from Cohen and Maclean 2000).

1.3 The Saethre-Chotzen syndrome (SCS)

The Saethre-Chotzen syndrome (Acrocephalosyndactyly type III [ACS3]; MIM 101400) was first described and named after the authors of its first reports (Saethre 1931; Chotzen 1932). The craniofacial features of this autosomal dominant disorder include eyelid anomalies, isolated coronal or multiple craniosynostosis, hypertelorism, facial asymmetry, low frontal hairline, ptosis, strabismus, deviated nasal septum, cleft palate, and small, posteriorly rotated ears with prominent crura. The limb abnormalities are often subtle and can include brachydactyly, mild cutaneous 2/3 syndactyly of the hands and/or feet,

clinodactyly, single-palmar crease, and broad or bifid halluces with valgus deviation (Reardon and Winter 1994). The variability of expression and occasional mild phenotype seen in Saethre-Chotzen syndrome patients makes this one of the more difficult craniosynostosis syndromes to diagnose clinically.

The syndrome was mapped to human chromosome 7p21 (Brueton et al. 1992; Lewanda et al. 1994; Rose et al. 1994) by linkage analysis in affected families and the determination of chromosomal breakpoints in sporadic cases. Howard et al. (1997) and El Ghouzzi et al. (1997*b*) showed first that the Saethre-Chotzen syndrome results from mutations in the *TWIST1* gene.

TWIST1 codes for a nuclear DNA-binding protein containing a basic helix-loop-helix (bHLH) motif suggesting it most likely functions as a transcription factor.

1.4 *TWIST1* gene structure

In *Drosophila* the *Twist* gene was originally identified as one of the zygotic genes required for dorso-ventral patterning during embryogenesis; its name is derived from the distorted, "twisted" appearance seen in the recessively lethal mutant (Simpson 1983; Nüsslein-Volhard et al. 1984). The human *TWIST1* gene consists of two exons, which are separated by an intron of 536 bp nucleotides from position +965 to +1500 downstream of stop codon. The first ATG (+315) is followed by an uninterrupted open reading frame of 609 bp that ends at +922 including with TAG codon and cover the first exon (GenBank Accession No. NM_000474) (Howard et al. 1997), coding therefore for a 202 amino acids protein. Two putative TATA boxes are upstream at the transcription start located -32 and/or -110 nucleotides. The 3'-end of the gene reveals two potential polyadenylation signals at positions +1565 and +1915 (Fig. 2).

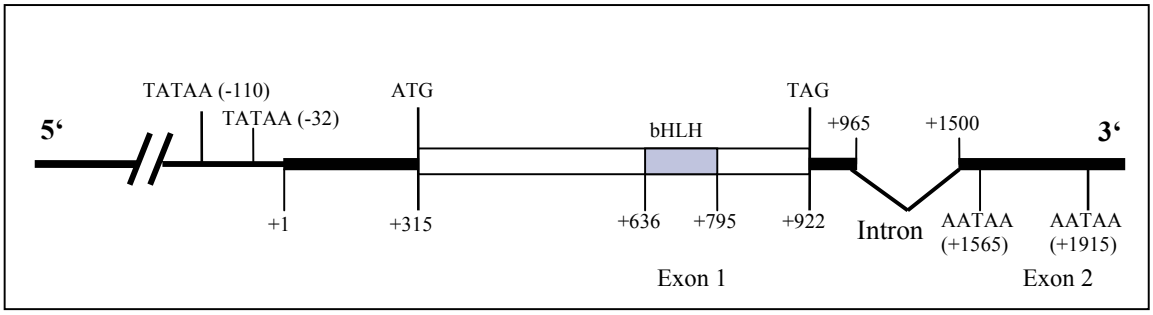


Fig. 2: Schematic representation of the human *TWIST1* gene structure. Nucleotide sequence of human *TWIST1* gene contains two exons separated by an intron. Two TATA boxes (-32, -110) present at upstream, and two polyadenylation sites (+1565, +1915) present at the downstream of transcription initiation (Genbank Acc.No. NM_000474).

1.5 The Saethre-Chotzen syndrome based on mutations in the human *TWIST1* gene

The Saethre-Chotzen syndrome is based on intragenic mutations, as well as specific genetic changes outside the coding region of the *TWIST1* gene. These intragenic mutations can include nonsense and missense alterations and small insertions, deletions, and duplications which resulting haploinsufficiency of the gene (Howard et al. 1997; El Ghouzzi et al. 1997*a*). All of the intragenic mutations like missense, deletions, and insertions as well as nonsense mutations are located within the coding region; particularly these mutations are frequently detected in the highly conserved basic helix-loop-helix domain. No splice site mutations, intronic mutations or changes within the second exon have been reported (Gripp et al. 2000). Currently, more then 70 intragenic mutations have been reported in unrelated SCS patients (Fig. 3) (Gripp et al. 2000; Muenke and Wilkie 2001).

Recently, two intragenic mutations have been reported in *TWIST1* in a child with mild SCS and her unaffected father and grandmother (Funato et al. 2005), located within glycine rich domain and putative nucleus localization signal of TWIST protein.

In addition to these intragenic mutations, several translocations and deletions have been reported which also caused the Saethre-Chotzen Syndrome (Lewanda et al. 1994; Rose et al. 1997; Johnson et al. 1998; Heer et al. 2004).

Deletions of 3.5-10.2 Mb in the *TWIST1* region were identified in SCS patients with mental retardation. Since the occurrence of such a mental retardation in SCS patients is atypical, this phenomenon cannot exclusively be attributed to the absence of the *TWIST1* gene. The hypothesis that this learning difficulties is caused by the absence of one or several genes, is

supported in three patients with megabase-sized deletion, which suggests that haploinsufficiency of genes neighboring *TWIST1* contribute to developmental delay. It is appropriate the assumption near that in this region further genes are located, for their deletion is responsible for this phenotype (Johnson et al. 1998). A 150 kb deletion of *TWIST1* gene was detected in a Dutch family (Heer et al. 2004), though these people were phenotypically normal.

Likewise, translocations at the 3'-end of *TWIST1* also caused the Saethre-Chotzen syndrome. A translocation breakpoint 5 kb 3' was mapped from *TWIST1* gene in a patient affected with Saethre-Chotzen syndrome (Krebs et al. 1997) and was also reported in 4 SCS patients at 3' which did not interrupt the coding sequence of the *TWIST1* gene and thus most likely were acting through a positional effect (Rose et al. 1997).

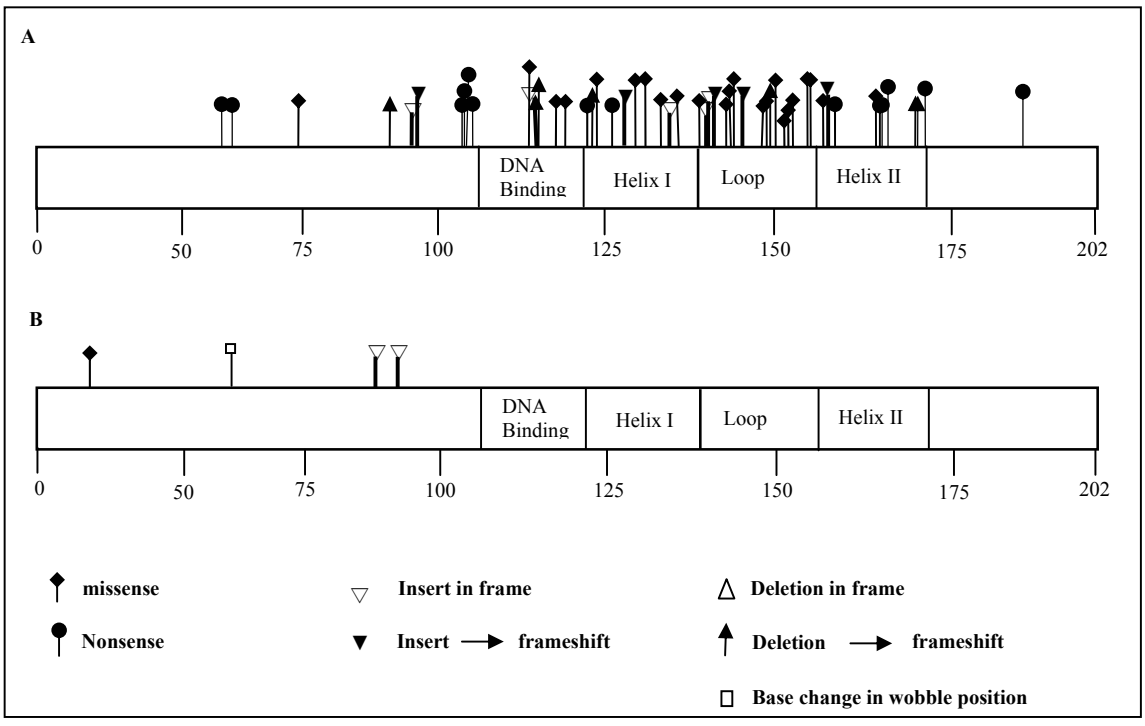


Fig. 3: Linear representation of types of mutations and polymorphisms, and their position relative to the *TWIST1* cDNA sequence. Numbers under the linear graph refer to the codon numbers. **A:** Mutations in the coding sequence. **B:** Polymorphisms in the coding sequence (Modified from Gripp et al. 2000).

In the Real time PCR gene dosage analysis assay of 55 patients with features of Saethre-Chotzen syndrome, it was found that 11% had deletions of the *TWIST1* gene, 2 patients had a translocation or inversion at least 260 kb at 3' of the *TWIST1* gene, suggesting the presence of position-effect mutations (Cai et al. 2003). The translocation breakpoint region is likely causes a position effect perhaps by separating *cis*-acting regulatory region from gene. The position effect phenomenon is likely to abolish or reduce the level of transcription of one copy of the gene resulting in haploinsufficiency or reduced protein levels at critical stages during development (Krebs et al. 1997, Rose et al. 1997).
In some patients, much smaller deletions at 3' of *TWIST1* lead to the confusion in the diagnosis of SCS with other craniosynostosis syndromes. These frequently arises invisible deletions, resulting the variability of expression and phenotypic overlap due to missing genotype-phenotype correlations, which make the Saethre-Chotzen syndrome is one of the more difficult clinically diagnosis syndrome.

1.6 TWIST protein

TWIST encodes a basic-helix-loop-helix (bHLH) transcription factor crucial for mesoderm development in *Drosophila* (Thisse et al. 1987). The HLH motif was first identified in the murine DNA-binding proteins E12 and E47 (Murre et al. 1989*b*). The two amphipathic helices are separated by a loop of varying length. The HLH region is necessary and responsible for protein dimerization. A basic amino acid rich region precedes the first helix and is required for DNA binding (reviewed by Kadesch 1993).
H-TWIST protein contains 202 amino acids (Howard et al. 1997). In an evolutionary sequence comparison of humans, mouse, rat, chicken, claw frog, zebrafish and fugu twist proteins based on the homology, different highly conserved domains were identified within the twist proteins (Spring et al. 2000; Baylies and Michelson 2001; Kosan 2002; Brand et al. 2003).
A novel domain with unknown function, named NSEEE-domain, which is characterized by the amino acid sequence NSEEE (position 19-23), is located at the N-terminus. Then two conserved motifs with the consensus of a nuclear localization signal (NLS) presents within the N-terminal region of the TWIST protein sequence (Godmann 2001). These NLS motifs are present at the amino acids positions 37-40 (RKRR; NLS1), and 73-77 (KRGKK;

NLS2). A glycine-rich and the basic domain are present at the amino acid positions 80 - 98 and 108 - 120, respectively. The bHLH domain represents the amino acids position 108-160, in which HLH domain is located at the amino acids position 121-161. At the very close of the C-terminal end of TWIST protein, there is a further evolutionary highly conserved WR domain named after two amino acids occurs (Fig. 4).

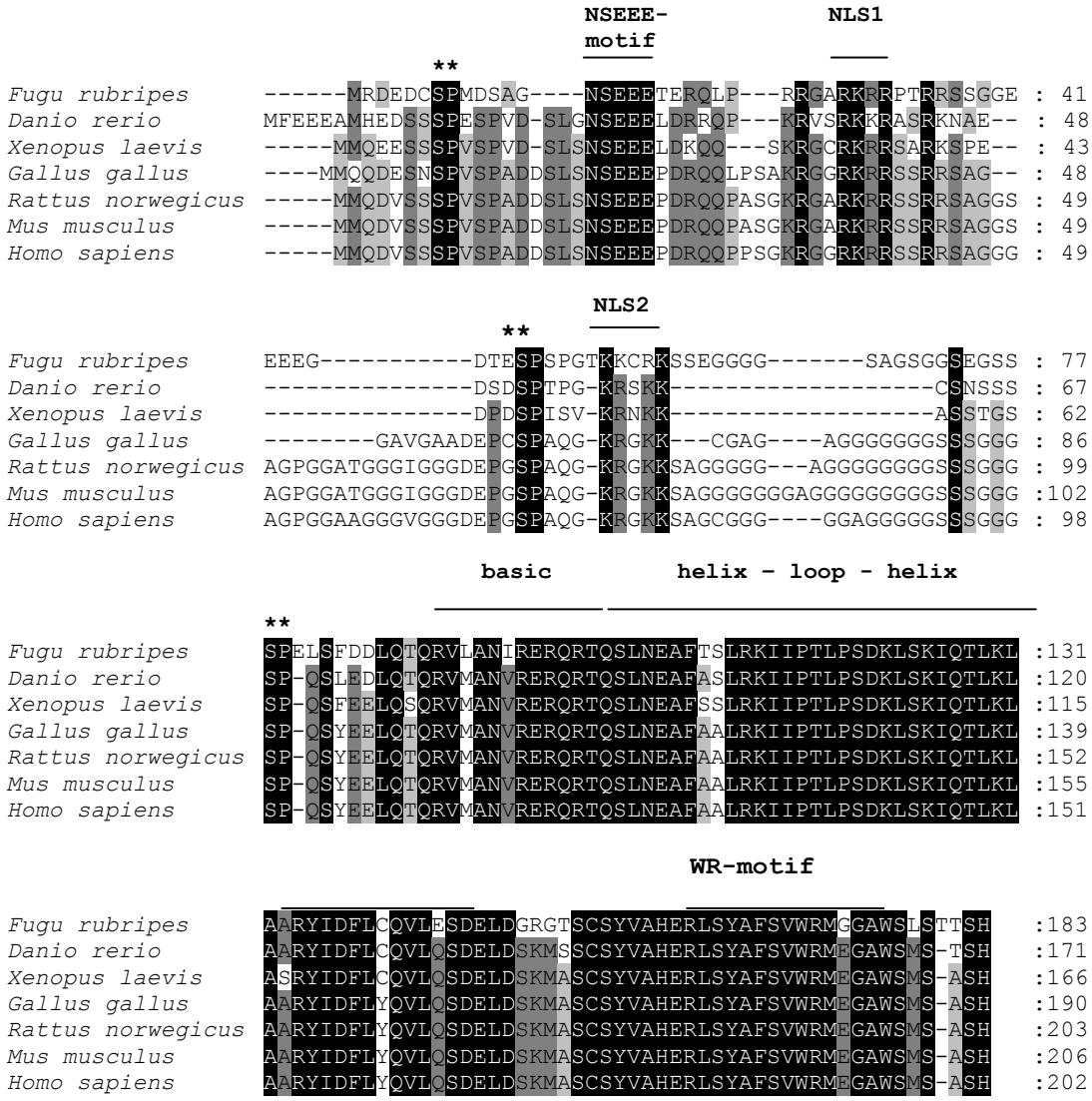


Fig. 4: Evolutionary alignment of vertebrate Twist proteins. Alignment of amino acid sequences encoded by the Twist gene from different species (*F. rubripes*, *D. rerio*, *X. laevis*, *G. gallus*, *R. norvegicus*, *M. musculus,* and *H. sapiens*) shows the well known basic Helix-Loop-Helix (bHLH) motif, and four additional protein regions in TWIST. The NSEEE-motif is a highly conserved sequence domain in all vertebrate species analysed at the N-terminal of the protein. The function is still unknown. NLS1 and NLS2 mark putative nuclear localization signals. WR-motif at the C-terminal of the protein shows remarkable conservation between all species. Gaps are indicated as hyphens. Black boxes indicate identical amino acids which are present in all species. Grey boxes are present in identical amino acid in six species and light grey boxes in five species, respectively. Asterisk indicates two amino acids (serine and proline) at three positions in the protein which showed high conservation in all species which could be of structural importance for the protein. Multiple sequences alignment was generated by ClustalW.

1.7 Twist functions

In vertebrates, the basic helix-loop-helix (bHLH) transcription factor TWIST is involved in cell type determination and differentiation during myogenesis (Olson 1990), neurogenesis (Anderson et al. 1997), cardiogenesis (Thomes et al. 1998), hematopoieses (Lister and Baron 1998), and osteogenesis (Murray et al. 1992; Cserjesi et al. 1995). These bHLH transcription factors form either homo-or heterodimer with other bHLH proteins. The dimers bind to the E-box core sequence (CANNTG), on the promoter region of target gene through the basic region (Murre et al. 1989*b*).

The sutures are the primary sites of osteoblast differentiation and bone formation during development of the skull. TWIST down regulated expression suggest that its expression maintains cells in an osteoprogenitor or a preosteoblast like state, thus preventing premature or ectopic osteoblast differentiation (Lee et al. 1999). It has also been reported in mouse that TWIST protein is expressed early in the undifferentiated mesenchymal layer beneath the epidermis that develop into a distinct dermal layer at later stages (Füchtbauer 1995). Morriss-Kay et al. (2001) postulated that the balance between proliferation and differentiation is based on complicated molecular signal transduction cascades, in which the transcription factor TWIST as well as the fibroblast growth factors (FGFs) and their receptors (FGFR) play key roles.

It is well known that TWIST protein bind the myogenic factor MyoD and negatively regulates its activity (Spicer et al. 1996; Hamamori et al. 1997). TWIST mutations R120A, R122A, R124A disrupted the interaction between TWIST and MyoD and inhibit the MEF2 transcription factor, a cofactor of myogenic bHLH proteins (Hamamori et al. 1997).

Twist-null mice die at embryonic day 11.5 due to defects in the head mesenchyme, somites, limb buds, and failure of neural tube closure in the cranial region (Chen and Behringer 1995). *Twist*-heterozygous mice display skull defects resulting in poorly developed squamosal bones and of over-developed intra parietal bones and limb abnormalities. Heterozygote *Twist* mutants (+/-), however, exhibited mild phenotypes at the hind limbs and anomalies of head, which are comparable to those of the human Saethre-Chotzen syndrome (Bourgeois et al. 1998; Spring et al. 2000).

Firulli et al. (2005) reported that the heterodimers of *TWIST1* and *Hand2*, basic helix-loop-helix (bHLH) transcription factor proteins required for the limb development. The

dimarization partner choice can be modulated by altered threonine and serine, which are highly conserved in the helix I domain of TWIST proteins and necessary for the protein kinase A and protein phosphatase 2A-regulated phosporylation. Furthermore, they confirmed that altered threonine and serine leads to the development of SCS symptoms in mouse and chick models.

N-terminal domain of M-Twist directly binds 2 independent domains of histone acetyltransferases (HAT), p300 and p300/CBP-associated factor (PCAF) and regulates their HAT activities (Hamamori et al. 1999). Histone acetyltransferases (HATs) play a critical role in transcriptional control by relieving of repressive effects of the chromatin (Struhl 1998).

At the C-terminal of Twist protein a highly conserved WR motif was characterized in the evolutionary alignment (Kosan 2002). The anti osteogenic function of the Twist proteins was mediated by a Runx2-binding 'Twist box' (aa 183-202) characterized at the C-terminus. TWIST proteins transiently inhibit Runx2 function during skeletal development in mice. *Twist1* and *Twist2* were expressed in *Runx2*-expressing cells throughout the skeleton during early development, and osteoblast-specific gene expression occurred only after their expression decreased. Double heterozygotes for *Twist1* and *Runx2* showed none of the skull abnormalities observed in Runx2 (+/-) mice, a *Twist2* null background rescued the clavicle phenotype of Runx2 (+/-) mice, and *Twist1* or *Twist2* deficiency led to premature osteoblast differentiation (Bialek et al. 2004).

TWIST protein is reported as a negative regulator of NF-κB activation. NF-κB has been linked to numerous human diseases, especially cancer, because of the elevated expression of genes encoding antiapoptotic proteins, cytokines, chemokines, and cell adhesion molecules. *TWIST1* and *TWIST2* repress cytokine gene expression through interaction with RelA/p65. These factors bind DNA at kB sites and regulate expression of many genes with different biological functions. Twist mainly affects the transcriptional activity of nuclear-translocated NF-κB by competing with cofactors or directly preventing the tethering of histone acetyltransferases (HATs) by NF-κB subunit RelA/p65 (Chen 2004).

Mouse homozygous for a *Twist2* null allele or doubly heterozygous for *Twist1* and *Twist2* alleles showed elevated expression of proinflammatory cytokines, resulting in perinatal death from cachexia. There is an evolutionarily conserved signalling circuit in which

TWIST proteins regulate cytokine signalling by establishing a negative feedback loop that represses the NF-κB-dependent cytokine pathway (Fig. 5) (Sosic et al. 2003).

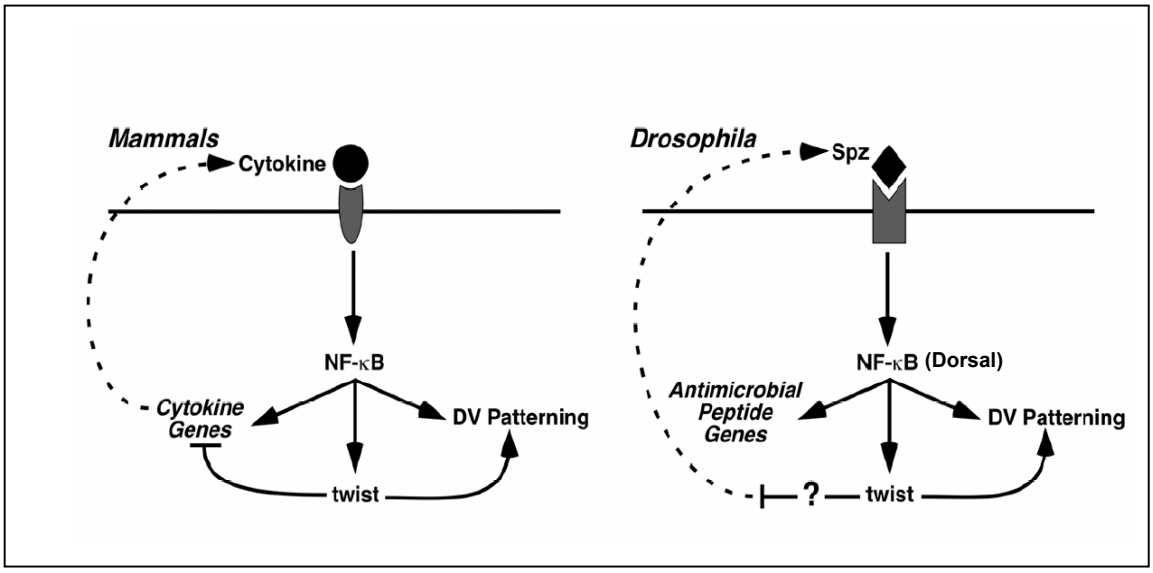


Fig. 5: Twist as a negative modulator of cytokine expression. Twist activation by NF-κB is part of an evolutionarily conserved pathway involved in mesoderm formation and dorsoventral (DV) patterning. While NF-kB plays a role in the immune response in both flies and mammals, twist has been adopted as a repressor of cytokine expression in mammals (Sosic et al. 2003)

Recently, it is repoted that TWIST plays an essential role in metastasis in a murine breast tumor model. Suppression of TWIST expression in highly metastatic mammary carcinoma cells specifically inhibited their ability to metastasize from the mammary gland to the lung. Ectopic expression of TWIST resulted in loss of E-cadherin mediated cell-cell adhesion, activation of mesenchymal markers, and induction of cell motility, suggesting that TWIST contributes in metastasis by promoting an epithelial-mesenchymal transition (EMT). In human breast cancers, high TWIST expressions were correlated to invasive lobular carcinoma, a highly infiltrating tumor type associated with loss of E-cadherin expression (Yang et al. 2004).

1.8 Aims of the study

Twist protein seems to be one of the key regulator of development and the process of epithelial-mesenchyme transition (EMT) during tumorgenesis. Two NLSs has been recognized at the N-terminal of Twist by sequence homology. Nuclear localization has lead to the concept that transport across the nuclear envelope is mediated by nuclear localization signals. Regarding the nucleus transport of TWIST, the possibility exist an important inspection point that a functional loss of the TWIST protein can be attributed to the inhibition of nuclear import and thus lead to an abnormal subcellular distribution. Furthermore, NLS-binding proteins allow the transport of NLS containing protein to the nucleus. From that point of view, functional domains particularly NLSs of the bHLH transcription factor TWIST should be analyzed.

It is evident from the literature about the involvement of cell type-specific bHLH proteins in the control of tissue-specific gene expression in vertebrates. For the better understanding of the involvement of TWIST in different regulatory pathways, the knowledge of the interacting partners during the regularization processes with TWIST is necessary. Possible interacting partners for TWIST and its motifs may be helpful to find out the TWIST role in various regulatory pathways. The objectives of the study can be devised as described below:

1. The first aim of thesis is to characterize the functionality of bHLH transcription factor TWIST NLSs using two criteria: that TWIST NLSs are sufficient to promote nuclear accumulation of an otherwise cytoplasmic protein when fused to it genetically or biochemically; in counterpart genetically altered NLSs leads to its cytoplasmic retention.
2. The second aim of the thesis is designed to find out the TWIST protein interactions using the protein-protein interaction methodology *i.e.* Yeast-Two-Hybrid assay. One of the most promising interacting partners was examined in the connection by further investigations for its functionality in the regulation of a target gene by TWIST using a combination of genetic and fluorescence microscopic approaches.

2 Materials and Methods

2.1 Materials

2.1.1 Equipments

ABI Prism 310 Genetic Analyzer	Applied Biosystems, Foster City, USA
ABI Prism 377 DNA-Sequencer	Applied Biosystems, Foster City, USA
Balance Model 2254	Sartorius, Göttingen
Balance PM 2000	Mettler, Switzerland
Cryostat	Julabo labortechnik GmbH, Seelbach
Centrifuge 5417 R	Eppendorf, Hamburg
Centrifuge 5415 C	Eppendorf, Hamburg
Megafuge 1.0	Heaeus, Osterode
Centrifuge Sorvall GLC-2B	Du Pont, Dreieich
Cooling centrifuge, Sorvall RT 6000	Du Pont, Dreieich
ECPS 500/400 Fluorometer TKO 100	Hoefer Scientific Instruments, San Francisco, USA
Electrophoresis Constant Power Supply	Pharmacia, Uppsala, Sweden
Hamilton pipette	Hemilton, Bonaduz, Switzerland
Horizontal Electrophoresis System	Owl Scientific, Inc., USA
Incubator Shaker, Model G25	New Brunswick Scientific co, Edison,USA
Incubator, CO_2-Auto-Zero	Heraeus; Hanau
Incubator for bacterial culture	Heraeus; Hanau
Laminar Air Flow	Heraeus; Hanau
Laminar Air Flow	Heraeus; Hanau

Sterilebank Microflow Intermed	Stolco, Düsseldorf
Sterilebank Lamin Air HA 2448 GS	Heraeus, Hanau
Mini Trans-Blot	Bio-Rad Laboratories GmbH, München
Microscope Axioplan	Zeiss, Jena
Microscope Axiophot Epifluoresnence	Zeiss, Jena
Mini-Protean 3 Cells	Bio-Rad Laboratories GmbH, München
pH meter CG 836	Schott, Hofheim
Gene Quant II, Photometer	Pharmacia Biotech, Sweden
Pharmacia Novospec II, Photometer	Pharmacia Biotech, Sweden
Spectrophotometer, Smartspec 3000	Bio-Rad Laboratories GmbH, München
Thermocycler PE 9600	Applied Biosystems, Foster City, USA
TRIO-Thermoblock TB1	Biometra, Göttingen
Gradient Cycler	Bio-Rad Laboratories GmbH, München
Water bath type 3042	Köttermann, Hänigsen
Water bath type 1083	GFL, Burgwedel

2.1.2 Chemicals

Alexa Flour 488 phallodin	Molecular-Probe Europe EV, Leiden, The Netherlands
Compelete Mini, Protease Inhibitor cocktail	Roche Molecular Biochemicals, Mannheim
Triton X-100	Serva, Heidelberg
Tween-20	Sigma, Deisenhofen
DMSO	Serva Reinbiochemica, Heidelberg
Long Ranger Gel solution	Bio Whittaker Molecular Application Rockland, USA
IGEPAL (NP 40)	Vysis Inc., Downers Grove, USA
TEMED	Serva Reinbiochemica, Heildelberg
Salmon sperms DNA	Sigma, Deisenhofen
SDS	Serva, Heidelberg
Vectashield Mounting Medium	Vectors laboratories Inc., Burlingame,USA
3-amino-1,2,4-triazole (3-AT)	Sigma, Deisenhofen

2.1.3 Buffers and solutions

Sterilization of solutions and equipments

All solutions that are not heat sensitive were sterilised at 121°C for 20 min in an autoclave (Webeco, Bad Schwartau). Heat sensitive solutions were filtered through disposable sterile filter (0.2 to 0.45 µm pore size). Plastic wares were autoclaved as above. Glass wares were sterilized overnight in an oven at 220°C.

6.6 µM Alex Fluor 488 Phalloidin	300 units Phalloidin (200 units/ml) was diluted in 1.5 ml methanol and store at –20° C
Comassie Blue Gel Stain	0.04% Coomassie blue R-250 (w/v) in methanol /acetic acid/aqua dest (40/10/40, v/v/v)
Comassie Blue Gel Destain	Methanol/Acetic acid/H_20 (10/10/80 v/v/v)
DAPI stock solution (250 ng/ml)	15 µl DAPI /50 ml 0.5% Tween-20/PBS

1M DTT	1.542 g DTT in 10 ml 0.01 M NaOAc pH 5.2 and store at –20°C
6 X Loading Buffer	50% glycerol (v/v) 0.1 M EDTA 0.1% SDS (w/v) 0.05% bromo-phenol blue (w/v) 0.05% xylene cyanol FF (w/v)
5 X TBE Buffer	0.5 M Tris 0.5 M Boric acid 10 mM EDTA
TE Buffer	10 mM Tris/ HCl 1 mM EDTA
10 X PBS (pH 7.4)	29.6 mM Na_2 $HPO_4.7H_20$ 10.6 mM KH_2PO_4 1.55 M NaCl
PBS-Tween 20	0.5% Tween-20 (v/v) in PBS
PMSF stock solution	0.25mM dilute in isopropanol and store at –20° C
Buffer A for Protein Extraction	10 mM HEPES 1.5 mM $MgCl_2$ 10 mM KCl adjust the pH 7.9 with KOH before use add 0.5 mM DTT and 0.2 mM PMSF

Buffer C for Protein Extraction	20 mM HEPES 1.5 mM $MgCl_2$ 420 mM NaCl 0.2 M EDTA adjust the pH 7.9 with KOH 25% Glycerine (v/v) before use add 0.5 mM DTT 0.2 mM PMSF
3 X SDS-PAGE Loading Buffer	0.5 M Tris/HCl (pH 6.8) 20% Glycerol 0.4% SDS 10% β-Mercaptoethanol
Transfer Buffer	39 mM Glycine 48 mM Tris 0.037% SDS (w/v) 20% Methanol
Tris- Glycin protein electrophoresis Buffer	250 mM Glycine 25 mM Tris 0.1% SDS (w/v)

2.1.4 Enzymes

All restriction enzymes were obtained from New England Biolabs, Schwalbach.

Calf Intestinal Alkaline phosphatase (CIAP)	Gibco BRL, Life Technologies, Karlsruhe
T4 DNA Ligase	New England Biolabs, Schwalbach
RNase H	Roche Molecular Biochemicals, Mannheim
Taq DNA Polymerase	Amersham Pharmacia Biotech, Piscataway, NJ, USA
Lyticase	Fa. Sigma, St. Louis, USA

2.1.5 DNA and protein size standards

100 bp DNA Ladder	Gibco BRL,Life Technologies, Karlsruhe
1 kb DNA Ladder	Gibco BRL, Life Technologies, Karlsruhe
Multicoloured protein markers wide range	New Life Science Products, Boston, USA

2.1.6 Oligonucleotides

The PCR primers were designed using the online program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi). All synthetic oligonucleotides were supplied by SIGMA. The optimal annealing temperature was also calculated using the primer 3 software (Table 1).

Table 1: Oligonucleotides used for amplification of different cDNA constructs

Gene	Genebank	Primer name	Sequence	Anneling Temp.	Product size
TWIST1	NM000474	Twist F	ccggaattccggctagtgggacgcggacat	60°C	630 bp
		Twist R	ccgcgatgatgcaggacgtgtcc		
E12	M31222	E12 F	ccggaattccggatggcgcctgtgggcaca	65°C	1900 bp
		E12 R	cgcggatccgccgcatgtgcccggcggggttgt		
TCF4	NM003199	Tcf4 F	ccggaattccggatgcatcaccaa	60°C	1966 bp
		Tcf4 R	cgcggatccgcgcatctgtcccat		
ETV5	NM004454	Etv5 F	ccggaattccggatggacgggttttatgatcag	60°C	1500 bp
		Etv5 R	cgcggatccgaggtaagcaaagccttcggcata		

Table 2: Oligonucleotide used in yeast-two-hybrid assays for TWIST cDNA and its domain: The oligonucleotide served as primers for the PCR, which are specified in this work.

Target	Primer name	Sequence	Anneal. temp.	Product size
TWIST	Y2hTW F Y2hTW R	CCGGAATTCCGGatgatgcaggacgtgtcc CGCGGATCCGCGcgtgggacgcggacatg	60°C	630 bp
TWIST WR domain	Y2hWR F Y2hWR R	CCGGAATTCCGGctggcggccaggtacatc CGCGGATCCGCGcgtgggacgcggacatg	60°C	250bp
TWIST NSE domain	Y2hNSE F Y2hNSE R	CCGGAATTCCGGatgatgcaggacgtgtcc CGCGGATCCGCGgcgcagacttcttgccgc	60°C	270 bp
Yeast mated clones	5 LD Inscr 3 LD Inscr	ctattcgatgatgaagataccccaccaaacccac aagtgaacttgcggggtttttcagtatctacg	57°C	variable

Capital latter represent nucleotide endonuclease linkers

Table 3: Oligonucleotide for the sequencing of cloned TWIST cDNA and TWIST domains in yeast vector used in yeast-two-hybrid assay

Target	Vector name	Primer name	Sequence	Annealing temp
DNA-BD seq-Primer	pGBKT7 (bait)	DNA-BD primer	taagagtcactttaaaatttgtat	56°C
Bait Forward	pGBKT7 (bait)	T7	taatacgactcactatagggcga	56°C
DNA-AD seq-Primer	pGADT7(prey)	3'AD Primer	agatggtgcacgatgcacag	55°C
5-LD seq-primer	PGADT7 (prey)	5-LD primer	cgatgatgaagataccccac	56°C

Table 4: Oligonucleotides used for site-directed mutagenesis to create the point mutations in TWIST cDNA

Mutation	Clone name	Primer name	Sequence	Annealing temp
K38R	NLS1 mut	NLS1 F NLS1 R	cgcgggggacgcaggcggcgcagcagc gctgctgcgccgcctgcgtcccccgcg	55°C
K73R	73NLS2 mut	73NLS2 F 73NLS2 R	cccagggcaggcgcggcaagaagt acttcttgccgcgcctgccctggg	55°C
K76R	76NLS2 mut	76NLS2 F 76NLS2 R	caagcgcggcaggaagtctgcgg ccgcagacttcctgccgcgcttg	55°C
K77R	77NLS2 mut	77NLS2 F 77NLS2 R	gcggcaagaggtctgcgggct agcccgcagacctcttgccgc	55°C

Table 5: Primers used for the proof of endogenous TWIST expression and cDNA synthesis

Primer	Sequence (5'-3')	Extension cycle	Product size (cDNA)
TWIST- f TWIST-r	cgc gga tcc gcg aga tga tgc agg acg tgt cc ccg gaa ttc cgg agt ggg acg cgg aca tgg acc a	30	635 bp
GAPDH-f GAPDH-r	cgt ctt cac cac cat gga ga cgg cca tca cgc cac act tt	30	300 bp

Table 6: Oligonucleotide used for verification of fusion of TWIST with c-myc

Primer	Sequence (5'-3')	Number of cycles	Product size
PCMV3C/twist f	gag cag aaa ctc atc tct gaa gag g	35	70 bp
PCMV3C/twist r	aca cgt cct gca tca ttc ttc ga		

2.1.7 Vectors

pBluescript -II-KS-Phagemid vector	Stratagene, Heidelberg
pCMV- Tag epitope tagging	Invitrogen, Groningen, Netherlands
eGFP, Mammalian expression vector	Invitrogen, Groningen, Netherlands
PCR 2.1 TOPO vector	Invitrogen, Groningen, Netherlands
PGBKT7 Bait vector	Fa. Clontech, Palo Alto, USA
pGADT7-Rec	Fa. Clontech, Palo Alto, USA

2.1.8 Kit Systems

DYEnamic ET Terminator Cycle Sequencing Kit	Amersham Pharmacia Biotech, Piscataway, USA
ECL Western blotting analysis system	Amersham Pharmacia Biotech, Piscataway, USA
ECL Western blotting detection reagents	Amersham Pharmacia Biotech, Piscataway, USA
ECL+Plus Western blot detection reagents	Amersham Pharmacia Biotech, Piscataway, USA
Immunoprecipitation Kit (Protein G)	Roche Molecular Biochemicals
QIAfilter Plasmid Maxi Kit	QIAGEN, Hilden
QIAprep Spin Miniprep Kit	QIAGEN, Hilden
QIAquick PCR Purification Kit	QIAGEN, Hilden
QIAquick Gel Extraction Kit	QIAGEN, Hilden
Ready-To-Go PCR Beads	Amersham Pharmacia Biotech, Piscataway, USA
Superscript First-Strand Synthesis System for RT-PCR	Gibco BRL, Life Technologies, Karlsruhe
TOPO TA Cloning Kit	Invitrogen, Groningen, Netherlands
QuikChange Site Directed Mutagenesis Kit	Stratagene, Cedar Creek, USA
Effectene Transfection Reagent	QIAGEN, Hilden
Matchmaker Yeast-2-Hybrid System	Clontech, Palo Alto, USA

2.1.9 Bacterial System

MAX Efficiency DH5α competent cells Gibco BRL, Life Technologies, Karlsruhe

Genotyp: *SupE*44 lacU169 (80lacZM15)

*hsd*R17 *rec*A1 *end*A1 *gyrA*69 *thi*-1

*relA*149 (Sambrook et al. 1989)

SUBCLONING efficiency	Gibco BRL, Life Technologies, Karlsruhe

DH5α competent cells

(Genotyp: F- _80d*lac*Z_M15 _(*lac*ZYA-*arg*F)

U169*deo*R *rec*A1 *end*A1 *hsd*R17 (rK-,mK+)

*pho*A *sup*E44 _- *thi*-1 *gyr*A96 *rel*A1.)

2.1.10 Antibiotic stock solutions

Ampicillin	100 mg/ml in H_2O
Kanamycin	20 mg/ml in H_2O
Chloramphenicol	25 mg/ml in EtOH 7.5µg/ml

All antibiotics stock solutions listed above were sterile filtered and stored at -20°C.

2.1.11 Nutrition medium and media plates

All bacterial medium were prepared with aquadest and autoclaved.

Bacterial medium:

LB medium (Sambrook et al. 1989)	10 g/l Bacto-Tryptone
	5 g/l Bacto-Yeast Extract
	5 g/l NaCl
	1000 ml H_2O
LB-Agar	LB-medium with 15 g/l Bacto agar
SOB-Medium	20 g/l Bacto tryptone
	5.0 g/l Bacto yeast extract
	0.5 g/l NaCl
	10 ml 250 mM KCl

SOC Medium	20 g/l Bacto tryptone
	5.0 g/l Bacto yeast extract
	0.5 g/l NaCl
	10 ml 0.25M KCl
	5 ml 2M $MgCl_2$
	20 ml 1M Glucose
	pH 7.0 with NaOH
NZY^+-Medium	10 g/l NZ-Amine (Caseinhydrolysate)
	5 g/l Yeast extract
	5 g/l NaCl
	2g/l $MgSO_4.7H_2O$
	adjust pH 7.5 with NaOH

Yeast medium:

YPD-medium	20 g/l Bacto peptone
	10 g/l yeast extract
	10 g/l glucose
	autoclave at 121°C for 15 min

YPDA-medium: For the production of YPDA medium, 15 ml sterile filtered 0.2 % adeninehemisulfate was added into the YPD medium.

Dropout medium

Synthetic Drop-out (SD-)	Medium 425 ml SD-Bases
	50 ml 10x Dropout Supplement
	adjust pH 5.8 with NaOH
	autoclave at 121°C for 15 min
	25 ml 40% (w/v) glucose (sterile filtered)

10x-Dropout Supplement: 10x-Dropout supplement solution consist of individual amino acids as well as adenine and uracil, by which those components, on whose auxotrophy genes is to be selected, are omitted in each case ("dropped out"). A solution with all amino acids consists of:

L-Adenine hemisulfate	200 mg/l
L-Arginine HCl	200 mg/l
L-Histidine HCl monohydrate	200 mg/l
L-Isoleucine	300 mg/l
L-Leucine	1000 mg/l
L-Lysine HCl	300 mg/l
L-Methionine	200 mg/l
L-Phenylalanine	500 mg/l
L-Threonine	2000 mg/l
L-Tryptophan	200 mg/l
L-Tyrosine	300 mg/l
L-Uracil	200 mg/l
L-Valine	1500 mg/l

It omitted for:

SD-Trp: Tryptophan (for AH109 *S. cerevisiae*)

SD-Leu: Leucine (forY187 *S. cerevisiae)*

DDO (-Double Dropout, two-fold selective medium): Leucine and Tryptophan

four-fold selectively (-Quadruple Dropout-, four-fold selective medium): Leucine, Tryptophan, adeninhemisulfate and histidine HCl mono hydrate

The dropout solutions were autoclaved at 121°C for 15 min and stored at 4°C.

2.1.12 Yeast strains

Table 7: Yeast strains used in this study for yeast-two-hybrid screening

S. N.	Genotype	Source
1	AH109 competent *S. cerevisiae* MATα *ura3-52 leu2-3, 112 trp1-901 his3-200 ade2-101 gal4Δ met⁻ gal80Δ* *URA3: GAL1uas-GAL!tata-lacZ MEL1*	Clontech (Heidelberg) Harper et al. 1993
2	Y187 pretransformed *S. cerevisiae* MAT**a** *ura3-52 leu2-3, 112 trp1-901 his-200 gal4Δ gal80Δ* *LYS2: GAL1uas-GAL1tata-HIS3* *GAL2uas-GAL2tata-ADE2* *URA3: MEL1uas-MEL1tata-lacZ MEL1*	Clontech (Heidelberg) James et al. 1996

2.1.13 Human cell-line

U2-OS Osteosarcoma cells (ATCC HTB-96)

(Origin: *Homo sapiens)*. The U2-OS-cell line obtained from American Type Culture Collection.

2.1.14 Cell culture: medium, salts, and transfections reagents

DMEM with Glutamax I,	Gibco, LifeTechnologies**,** Karlsruhe (4500 mg/L D-Glucose, without Sodium pyruvate)
Penicillin/Streptomycin	Gibco, LifeTechnologies**,** Karlsruhe
Fetal Calf serum	Roche molecular biochemicals, Mahnheim
10% DMEM	440 ml DMEM 50 ml fetal Calf serum 5 ml nonessential aminoacids 5 ml Penicillin/Streptomycin
Effectene Transfection Reagent	QIAGEN GmbH, Hilden
PBS-EDTA	0.02 % EDTA in PBS

0.05 % Trypsin per 1 Liter	0.5 g Trypsin
	2.69 g tri-sodiumcitrate-dihydrate
	6.0 g NaCl, adjust the pH 7.8

Culture bottles, bowls, six-hole culture plates (six well plates), tubes, culture chamber slides (lab Tek II Chamber Slide) were obtained by the companies Greiner, Frickenhausen, Nalge Nunc international, Naperville the USA and Becton Dickinson, USA.

2.1.15 Antibodies

Table 8: Antibodies for detection of TWIST-cmyc constructs

Name of antibody	Organism	Company
Monoclonal anti-c-myc, mouse IgG1	Mouse	Molecular probes, Eugene, Oregon, USA
Texas-Red anti mouse IgG	Goat	Molecular probes, Eugene, Oregon, USA
anti-Mouse-IgG, Texas-Red conjugated	Mouse	Kaninchen Sigma, Saint-Louis, Missouri USA
Anti-mouse-IgG, Horseradish-Peroxidase linked antibody	Sheep	Amersham Biosciences, Piscataway, USA
Monoclonal Anti-human-c-myc (clone 9E10)	Rabbit	AG Dr. Frank Seeber, FB Biology, Philipps-Universität, Marburg

2.1.16 Fluorochrome used for fluorescence detection

Table 9: Reagents used for fluorescence microscopic detection of different cell compartments

Target	Fluorochrome	Absorption	Emission	Dye
Cell nucleus	DAPI	358 nm	461 nm	Blue
F-Actin	Alexa Fluor 488	495 nm	519 nm	Green
TWIST-c-myc-fusion protein	Texas Red	596 nm	620 nm	Red

2.1.17 Databases and software used for sequences analysis

For the analysis and the characterisation of the nucleotides and aminoacids sequences following internet sources were used.

Table 10: Web links and programmes used for the biometrics analysis

Appliance	**Databases/ programme**	**Resources/links**
Literature search	PubMed	*NCBI www.ncbi.nlm.nih.gov/entrez
Comparisons Amino acid sequences Nucleotide sequences	Blast Blast p Blast n	*NCBI www.ncbi.nlm.nih.gov/blast
Inquiries Protein function Protein domains Protein sequences	SWISS-PROT	**SIB SWISS-PROT www.expasy.ch/sprot
Informations Protein families Protein domains and their functions	PROSITE	**SIB www.expasy.ch/prosite
Homology Amino acids sequences	MultAlin	www.protein.toulouse.inra.fr/ multalin/multalin.html
Genome data bank	UCSC	http://genome.ucsc.edu
Primer design software	Primer3	primer/primer3_www.cgi

*National Center of Biotechnology Information (NCBI)

**Schweizerisches Institutes of Bioinformatics (SIB)

2.2 Methods

2.2.1 Isolation of plasmid DNA (Sambrook et al. 1989, Sambrook et al. 2000)

2.2.1.1 Small-scale isolation of plasmid DNA (adapted from Birnboim and Doly 1979)

A single *E.coli* colony was inoculated in 5 ml of LB medium with the appropriate antibiotic and incubated in a shaker for 16 hrs at 37°C. One militre of saturated culture was used for making glycerol stock and rest of the culture was sedimented by centrifugation for 15 min at 13000 x g. The pellet was resuspended in 250 µl of solution P1. The bacterial cells were lysed with 250 µl of P2 solution and than neutralised with 350 µl of solution P3. The precipitated solution was incubated on ice for 15 min, and centrifuged at 13000 x g at 4°C. The supernatant was transferred into a new tube, and 1 ml of 100% isopropanol was added to precipitate the DNA. It was then incubated on ice for 15 min, centrifuged for 20 min, and the pellet was washed with 70% ethanol. After air-drying dissolved in 30 µl of TE buffer or dH_2O.

P1:	50 mM Tris/HCl (pH 8.0)
	10 mM EDTA
	100 µg/ ml RNase A
P2:	200 mM NaOH
	1% SDS
P3:	3.0M Potassium acetate, pH 5.5

2.2.1.2 Large-scale preparation of plasmid DNA (Qiagen Maxi- Kit)

A single clone was inoculated in 2 ml LB medium with appropriate antibiotic as a pre-culture for 8 hrs in 37°C shaker. This pre-culture was added to 100 ml LB medium with appropriate antibiotic at a dilution of 1/100 fold and incubated overnight at 37°C with shaking. The saturated culture was centrifuged for 15 min, pellet was resuspended in 10 ml of solution P1 and cells lysed with P2 and neutralised with P3 as described above. The precipitated solution was centrifuged at 10,000 rpm for 30 min at 4°C. Meanwhile, the column (Qiagen-tip) that was provided with the Maxi preparation kit was equilibrated with

10 ml of QBT solution. After centrifugation the lysate was poured into this equilibrated column thus allowing the DNA to bind with the resin present in the bed of the column. The column was then washed twice with 30 ml of solution QC. Finally, the DNA was eluted with 15 ml of QF solution. To precipitate the DNA, 10.5 ml of isopropanol was added and mixed thoroughly and centrifuged at 14,000 x g for 30 min at 4°C. The DNA pellet was washed with 70% ethanol and dissolved in 200 µl of TE or DNA grade H_2O.

QBT:	750 mM Sodium chloride
	50 mM MOPS (pH 7.0)
	15 % Ethanol
	0.5 % Triton X-100
QC:	1 mM Sodium chloride
	50 mM MOPS (pH 7.0)
	15 % Ethanol
QF:	1.25 M Sodium chloride
	50 mM Tris/HCl (pH 8.5)

2.2.2 Gel electrophoresis

Gel electrophoresis is the technique by which mixture of charged macromolecules, especially nucleic acids and proteins are separated in an electrical field according to their mobility which is directly proportional to macromolecule's charge to mass ratio.

2.2.2.1 Agarose gel electrophoresis of DNA

Agarose gels are used to electrophoresis nucleic acid molecules from as small as 50 bases to more than 50 kilobases, depending on the concentration of the agarose and the precise nature of the applied electrical field (constant or pulse). Usually, 1 g of agarose was added in 100 ml 1.0 x of TBE buffer, and boiled in the microwave to dissolve the agarose, then cooled down to about 60°C before adding ethidium bromide (10 mg/ml). This 1% agarose gel was poured into a horizontal gel chamber.

2.2.3 Isolation of DNA fragments after agarose gel electrophoresis

2.2.3.1 QIA quick gel extraction method

This method is designed to extract and purify DNA of 70 bp to 10 kilobase pair (kb) in length from agarose gels. Up to 400 mg agarose can be processed per spin column. The principle of this method depends on selective binding of DNA to uniquely designed silica-gel membrane. To the excised DNA fragment from agarose, gel slice was weighed and three volumes of QG buffer was added and incubated at 50°C for 10-15 min. After the gel slice was dissolved completely, equal amount of isopropanol was added and mixed well and then it was applied over a QIAquick column and centrifuged for 1 min. The flow through was discarded and the column was washed with 750 µl of PE buffer. After drying the column it was placed into a fresh microcentrifuge tube. To elute DNA, 35-50µl of EB buffer was applied to the centre of the QIAquick membrane and centrifuged for 1 min.

2.2.4 Restriction enzyme digestion of DNA

Restriction enzyme digestions were performed by incubating double-stranded DNA with an appropriate amount of restriction enzyme in its respective buffer as recommended by the supplier, and at the optimal temperature for that specific enzyme. Standard digestion included 2-10 U of enzyme per microgram of DNA. These reactions were usually incubated for 1-3 hrs to ensure complete digestion at the optimal temperature for enzyme activity, which was typically 37°C. However for genomic DNA digestion the reaction solution was incubated overnight at 37°C.

2.2.5 Ligation of DNA fragment

The ligation of an insert DNA into a vector (digested with appropriate restriction enzyme) carried out in the following reaction mixture:

30 ng	vector DNA (digested)
50-100 ng	DNA (1:3, vector: insert ratio)
1µl	ligation buffer (10x)
1µl	T4 DNA ligase (5 U/µl)
	make a total volume of 10 µl

Blunt-end ligations were carried out at 16°C overnight, whereas sticky-end ligations were carried out at room temperature for 2-4 hrs.

2.2.6 *E. coli* transformation of bacteria

In order to obtain plasmid DNA for purposes of transfection, sequencing and generating probes, the ligation was transformed into a clone of DH5α ™ bacterial cells. The competent cells were removed from a –70°C freezer and thawed on ice. Two microlitre of β-mercaptoethanol and 2 µl of the ligation reaction were added into 100 µl cells aliquot. The tube was gently tapped to mix and then incubated on ice for 30 min. To heat shock the cells after the incubation, the sample was placed in a 37°C water bath for 45-60 sec, and then placed on ice for two minutes. After that 450 µl of LB medium was added, and incubate for 1 hour at 37°C while shaking at 225 rpm. A 1:10 dilution of the sample was made and 100 µl of the dilution was plated out on LB agar growth plates containing appropriate antibiotics. The plate was incubated in a bacterial cell incubator at 37°C overnight. Transformed bacteria form a single colony growth on the appropriate antibiotic selective medium. The growth plates were stored at 4°C.

2.2.7 Polymerase chain reaction (PCR)

PCR is a very sensitive and powerful technique (Saiki et al. 1988) that is widely used for the exponential amplification of specific DNA sequences *in vitro* by using sequence specific synthetic oligonucleotides (primers). The general principle of PCR starts from a pair of oligonucleotide primers that are designed so that a sense primer directs the synthesis of DNA towards an antisense primer, and vice versa. During the PCR, the *Taq* DNA polymerase (a heat stable polymerase) (Chien et al. 1976) catalyses the synthesis of a new DNA strand that is complementary to a template DNA from the 5' to 3' direction by a primer extension reaction, resulting in the production of the DNA region flanked by the two primers. It allows the rapid and unlimited amplification of specific nucleic acid sequences that may be present at very low concentrations in very complex mixtures. The amplification cycles were performed in an automatic thermocycler. The PCR reaction contains in general, the following substances:

10 ng	DNA
1µl	forward primer (10 pmol/µl)
1µl	reverse primer (10 pmol/µl)
1µl	10 mM dNTPs
5µl	10x PCR buffer
1.5µl	50 mM $MgCl_2$
0.5µl	*Taq* DNA Polymerase (5U/µl)
Up to 50 µl	H_2O

The reaction mixture was placed in a 200 µl reaction tube and placed in thermocycler. A standard PCR program is shown here:

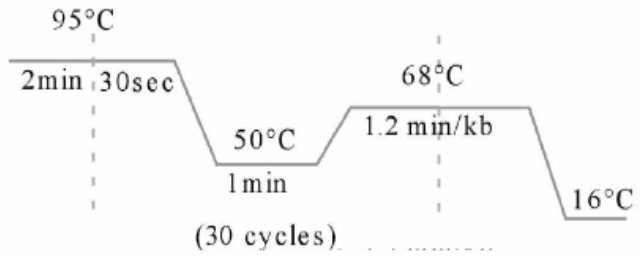
Initial denaturation	95°C for 5 min
Elongation	95°C for 30 sec (denaturation)
30-35 cycles	58°C for 45 sec (annealing)
	72°C for 1-2 min (extension)
Final extension	72°C for 10 min

2.2.8 DNA sequencing (Sanger et al. 1977)

In order to verify clones for the presence of any point mutations, appropriate DNA preparations were sequenced. Clean plamids were prepared using the Nucleospin plasmid prep kit or QIA plasmid prep kit. The purity and concentrations were analyzed spectroscopically using DNS method. For a sequencing PCR reaction, plasmid concentrations 100 ng/kb were used in a reaction mix of 10 µl which contained 10 pmol primer and 2 µl of termination mix (ABI). A standardized PCR reaction program was used with an initial denaturation at 95°C for 60 sec, 30 cycles of denaturation at 95°C for 10 sec, primer annealing at Tm-2°C for 5 sec, extension at 60°C for 1 min, and terminated with 60°C for 5 min to facilitate the completion of extension reaction. After PCR completion, the products were purified by precipitating with and 75% isopropanol, then the pellet was washed with 70% ethanol. The sample was denatured at 90°C for 2 min and resuspended in 15 µl High Dye formamide before subjecting to analysis by the ABI 310 sequence analyzer.

2.2.9 Site-directed mutagenesis

In vitro site-directed mutagenesis is a valuable technique for studying protein structure-function relationships. This procedure utilizes a vector carrying the gene to be modified and two complementary primers carrying the desired point mutation. These two complementary primers were designed such that the mutation region is located at the centre: (15-18 bp)-(mutation region)-(15-18 bp). The primers each complementary to opposite strands of the vector, are extended in the PCR reaction with a *turbo pfu* polymerase. The PCR reaction was carried out in a reaction volume of 50 µl containing 1 µl of the plasmid (from standard plasmid prep).



The PCR reaction allows for the incorporation of the primers which results in a mutated plasmid with staggered nicks. In order to remove the parental vector the reaction was digested with *Dpn*I for 2-3 hours. *Dpn*I endonuclease is specific for methylated and hemimethylated DNA which is the case only for the parental vectors. The product was then purified through the column and used for transformation in *E.coli*. The presence of mutations was confirmed by sequencing the plasmids after their isolation from the transformants.

To mutate TWIST^{NLS1} and TWIST^{NLS2} the Quick change^{M} site directed mutagenesis kit (Stratagene) was used. Therefore, about 30 bp long primers were designed (Table 4), which contained the desired mutation and annealed at the same sequence on the opposite strands of the plasmid. The reactions were prepared as followed.

5 µl	10 x Pfu Ultra reaction buffer
50 ng	template DNA
10 pmol	each primer
1 µl	dNTP mix (25 mM each dNTP)
2.5 U	*Pfu* Ultra
5.0 µl	DMSO
dd H_2O to final volume of	50 µl

The reaction was cycled using the following parameters.

1. Denaturing:	95 °C for 30 sec
2. Denaturing:	95 °C for 30 sec
3. Annealing:	55 °C for 1min
4. Polymerization:	68 °C for 1 min per kb of plasmid length

Step 2 to 4 was cycled 12 times. The PCR was treated with 10U of *DpnI* restriction enzyme for digestion of the PCR product to remove the methylated parental DNA.

2.3 Cell biology methods

2.3.1 Culture of human cells

Handling and propagation of all cell lines were performed in a cell and tissue culture laminar-air flow under aseptic conditions. All solutions were stored at 4°C and warmed up to 37°C in a water bath before using. All solutions were only opened under aseptic conditions in a laminar flow. The adherent U2-OS (Osteosarcoma) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % FCS, 5% non essential amino acids and 5% penicillin/streptomycin in the incubator at 37° C, 5% CO2 and 88% moisture.

2.3.2 Freezing cultured human cells

To freeze cells for long term storage, cells were harvested (at least one T75 flask of 80 % confluent cells) and centrifuged at 1,000 x g for 5 min. The media was then aspirated and cells were resuspended in 1 ml of cell culture freezing medium. The resuspended cell solution was transferred to cryo-vials and kept at –70° C in an isopropanol-containing cell

freezing container, which guarantees a slow freezing process. After 24 hrs the tube can be stored at –70°C or in liquid nitrogen in a tube.

2.3.3 Splitting the human cells

All cell lines were grown in a 37°C incubator and split in certain ratios depending on the stage of confluence and the proliferation rate of each cell line. The amount of medium added to the flask was dependent on its size. After that 10 ml were added into medium sized flasks (T75). The medium was changed every second day. 1/10 of the amount of culture medium was used as amount of trypsin/EDTA supplied to a culture flask in order to detach the cells. To split cells the medium was completely aspirated from the flask and the cells were washed briefly with 1 ml of trypsin to remove traces of antitrypsin originating from the serum in the media. The cells detached after about 5 min upon occasional gentle tapping. Trypsin was then immediately inactivated by adding 3 ml of medium to each flask. This suspension was transferred into a 15 ml tube and centrifuged at 1,000 x g for 5 min. The supernatant was aspirated and the cell pellet was resuspended in medium and split into 3 to 5 new flasks. For each experiment, each individual cell line was pooled during the splitting process to assure equal amounts of cells plated into each flask.

2.3.4 Maintenance of human cell lines

The cell line aliquots were stored in 1 ml freezing medium at -80°C. Cell lines were grown by quickly thawing the frozen cells in a 37° C water-bath then transferring them into a 15 ml tube to be mixed with 10 ml of medium. The suspended cells were centrifuged at 1,000 x g for 5 min. in order to isolate a cell pellet free of DMSO residues from the freezing medium. After removing the supernatant the pellet was resuspended in 10 ml medium and transferred into a cell culture flask. The cells were incubated at 37°C with 5% CO_2.

2.3.5 Transient transfection of mammalian adherent cells

In this study the QIAGEN effectene transfections reagent was used for transferring foreign DNA into mammalian cells. Effectene Transfection Reagent is an innovative non-liposomal lipid formulation used in conjunction with a special DNA-condensing enhancer and optimized buffer to achieve high transfection efficiencies. The enhancer first condenses the

DNA molecules and effectene reagent subsequently coats them with cationic lipids providing a particularly efficient way of transferring DNA into eukaryotic cells. Effectene Reagent is used in conjunction with the enhancer and the DNA-condensation buffer (Buffer EC) to achieve high transfection efficiencies. In the first step of effectene–DNA complex formation, the DNA is condensed by interaction with the enhancer in a defined buffer system. Effectene reagent is then added to the condensed DNA to produce condensed effectene–DNA complexes. The Effectene–DNA complexes are mixed with medium and directly added to the cells. Effectene transfection reagent spontaneously forms micelle structures that show no size or batch variation, as found with preformulated liposome reagents. This unique feature ensures excellent reproducibility of transfection complex formation. The process of highly condensing DNA molecules and then coating them with effectene reagent is a particularly effective way to transfer DNA into eukaryotic cells.

2.3.6 Cell density at the time of complex addition

Table 11 lists the recommended number of adherent cells to seed per culture plate/dish the day before transfection, and the recommended number of suspension cells to seed the volume of medium to use the day of transfection. For adherent cells, the optimal confluency at the time of transfection complex addition is normally 40–80%.

Table 11: Recommended number of cells per culture vessel for transfection assay

Assay	Sowing cells numbers	Amount of DNA	Enhancer	EC Buffer	Effectene	Medium
RT-PCR	$8x10^5$ cells per 56 cm^2	1–2 µg	10 µl	250 µl	12 µl	2 ml
Immuno-fluorescence	$1.5x10^5$ cells per 10 cm^2	400 ng	3.2 µl	100 µl	4 µl	600 µl
SDS-PAGE, Western blot	$1x10^6$ cells per 74 cm^2	2–3µg	14 µl	400 µl	16 µl	4 ml

2.3.7 Isolation of total RNA from the human cells

Total RNA from cells was isolated by following the manufacturer's protocol describing the application of the Qiagen RNeasy mini kit. The concentration and purity of RNA was determined by measuring the absorbance at 260 nm (A260) in an UV/VIS spectrophotometer and by visual inspection of the preparation on an Rnase free agarose gel to exclude degradation. Absorbance 1 at A260 corresponds to a RNA concentration of 40µg/ml such that the RNA concentration is given by:

$$[RNA] = a \cdot 40 \frac{\mu g}{ml}$$

Where [*RNA*] is the RNA concentration in µg/ml and *a,* the absorbance measured at 260 nm.

2.3.8 Reverse transcription PCR (RT-PCR)

RT-PCR generates cDNA fragments from RNA templates. In an autoclaved tube the total RNA was mixed with 1 µl random hexamer primer (concentration) and 1 µl dNTPs (concentration) in a total volume of 10 µl. To avoid the possible secondary structure of the RNA, which might interfere with the synthesis, the mixture was heated 65°C for 5 min, and then quickly chilled on ice. After a brief centrifugation, 9 µl of master mix prepared from the following reagents was added.

2 µl	10X RT Buffer
4 µl	25 mM $MgCl_2$
2 µl	0.1 mM DTT
1 µl	RNase inhibitor

The content of the tube was mixed gently and incubated at 25°C for 2 min.One microlitre of reverse transcriptase enzyme (Superscript II) was then added, and further incubated at 25°C for 10 min, then 42°C for 50 min and 70°C for 15 min for the first strand cDNA synthesis. Afterthat, 1 µl of *Rnase*I added and again incubated for 20 min. The synthesized cDNA was store at –20°C for further use. One microlitre of the first strand reaction was used for the PCR reaction (as described above).

2.3.9 Immunofluorescence and microscopy

2.3.9.1 Paraformaldehyde fixation

Cells grown in slide chamber were washed twice in 1X PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After rinsing the slides once in PBS, the cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min at room temperature. The slides were again rinsed twice in PBS and then incubated in PBS for 5 min. The cells were blocked in 10% BSA in PBS for 30 min at RT or over night at 4°C.

2.3.9.2 Staining and microscopy

Fixed cells were incubated with a primary antibody, diluted in 1% BSA/PBS-Tween 20 from 1:200 cover over the slide and incubate for 1 h at 37°C. The slides were washed three times for 5 min in 0.5% Tween-20 in PBS and stained with a secondary antibody conjugated with Texas red in a 1:1000 dilution in 1% BSA in PBS for 1 h at 37 °C. The slides were washed again three times for 5 min in 0.5% Tween-20 in PBS. To stain the nucleus, 1 µg DAPI was added to the 50 ml PBS. After 3 min incubation the slides in DAPI, were again washed three times in 0.5% Tween-20/PBS and mounted using mounting media (Vectorshield). The slides were sealed with the mounting medium and the cells were analyzed using an Axiovert 135 microscope (Zeiss) with 40X objective (Zeiss) with a numeric aperture of 1.3. The pictures were taken with a CCD-camera and saved with metaview software. Further preparation of the pictures was performed with Adobe Photoshop.

2.4 Techniques related to protein

2.4.1 Preparation of protein extract

For the preparation of protein extracts from cell lysates, cell density was monitored spectroscopically at 600 nm (OD_{600}nm), culture volumes corresponding to OD_{600}nm values of 5 or 10 were harvested, and cells were isolated by centrifugation at 4°C. This procedure ensured that comparable amounts of cells were withdrawn for protein extract preparation even when cells of different growth stages had to be compared. The isolated cell pellets were lysed by lysozyme treatment. For cell lysis by lysozyme treatment, the cell pellet was

resuspended in a lysis buffer (50 mM EDTA, 0.1 M NaCl, pH 7.5) containing 50 µg/ml of lysozyme and incubated for 10-15 min at 37°C until the dense solution started clearing. Independent of the cell lysis method applied, the cell lysates were centrifuged to remove the cell debris. An aliquot of each lysate was stored at -20°C for future use and 100 µl of the lysates were boiled with the denaturing protein loading buffer and equal volumes of these samples were loaded on a SDS gel for inspection of whether the protein contents were of comparable concentration.

2.4.2 Separation of proteins by SDS-polyacrylamide gel electrophoresis (Laemmli 1970)

The protein sample to be resolved was denatured by heating at 95°C for 2 min in the presence of SDS and β-mercaptoethanol (see protein loading buffer composition below). While β-mercaptoethanol reduces disulfide bonds, SDS denatures and anneals to the amino acid chains of the proteins giving each protein a negative net charge proportional to the polypeptide chain length. As a consequence, the proteins are separated essentially based on their molecular mass (Laemmli, 1970). The sieving effect of the gel matrix is achieved by adjusting an appropriate ratio of acrylamide to N, N' methylene bisacrylamide (37.5/1).The polymerization of acrylamide is catalyzed by adding 0.1% APS (w/v) and 0.05% TEMED just before pouring into the casket. The migration of the proteins was carried out in running buffer under a constant current of 25 mA for 2 h.

Loading buffer:

100 mM	Tris/HCl, pH 6.8
10%	(v/v) glycerol
2.0%	(w/v) SDS
3.0%	(v/v) β-mercaptoethanol
0.1%	(w/v) bromophenol blue

Running buffer:

25 mM	Tris/HCl, pH 8.3
250 mM	Glycine
0.1%	(w/v) SDS

2.4.3 Protein staining with coomassie blue

After electrophoresis, the proteins in the gel were fixed and stained in staining solution with gentle agitation for 1-2 hours. In order to remove non-specific dye from the protein gels, the gel was destained in the destaining solution.

Staining solution:

0.125%	(w/v) Coomassie blue
10%	(v/v) acetic acid
25%	(v/v) ethanol

Destaining solution:

10%	(v/v) acetic acid
20%	(v/v) ethanol

2.4.4 Determination of protein concentration (Bradford 1976)

To determine the protein concentration, Bio-Rad protein assay based on the differential colour change of a dye in response to various concentrations of protein was used. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomasie Blue G-250 shifts from 494 to 595 nm when the binding to protein occurs. The BSA stock solution of 1 mg/ml was diluted in order to obtain standard dilutions in range of 10 µg/ml-100 µg/ml. The Bio-Rad's colour reagent was diluted 1:5 with H_2O, and filtered through 0.45 µm filters. In a 96 well microtiter plate, 20 µl of each standard dilution and the samples to be measured were pipetted with 280 µl of the color reagent. The absorption of the colour reaction was measured at 595 nm in spectrophotometer (Bio-Rad).

2.4.5 Western blotting

For the detection of specific proteins on protein gels, a technique termed western blot was applied in which the protein bands were first transfered to a polyvinylidene fluoride (PVDF) 0.45 µm microporous membrane (Immobilon-P, Millipore). An air bubble-free sandwich was formed from Whatman 3MM filter papers embedding the membrane and the gel. All components were pre-soaked in transfer buffer and the electro transfer was carried out in a semi dry transfer system (Bio-rad) for 90 minutes under a constant current calculated by the area of the gel (in cm) multiplied by 0.8 mA. After transfer, the proteins

were visualized by staining the membrane with amido black solution for 1-2 minutes and destaining with dH_2O.

Transfer buffer:

48 mM	Tris base
39 mM	glycine
1.3 mM	SDS
20%	methanol

2.4.6 Immunodetection

After transferring the proteins to a PVDF membrane, the non-specific sites were blocked by incubating the membrane in blocking buffer for 30 min at RT. The membrane was then incubated in blocking buffer with a defined dilution of the primary antibodies (see below) overnight at 4°C or for 1 h at 37°C. The membrane was washed to eliminate the unbound antibodies, once for 10 minutes and twice for 5 min with PBS-T at RT. The membrane was then incubated in blocking buffer with the secondary antibody coupled to horseradish peroxidase (1:10000 dilutions of antirabbit or 1:5000 of anti-mouse) for 1 h at 37°C. The membrane was washed again as described earlier to eliminate the unbound secondary antibodies. The proteins recognized by the primary antibodies were detected using ECL (enhanced chemiluminescence).

PBS-T:

80 mM Na_2HPO_4
20 mM NaH_2PO_4
100 mM NaCl, pH 7.5
0.2% (v/v) Tween-20

2.5 Yeast-two-hybrid system

Yeast-two-Hybrid System is a complete GAL4-based two-hybrid system providing a transcriptional assay for detecting specific protein-protein interactions in yeast. The MATCHMAKER yeast-two-hybrid systems can be used either to screen a library for a gene encoding a novel protein that interacts with a known bait protein or to test two previously cloned proteins for interaction.

The yeast two-hybrid assay is based on the fact that many eukaryotic trans-acting transcription factors are composed of physically separable, functionally independent domains. Such regulators often contain a DNA-binding domain (DNA-BD) that binds to a specific enhancer-like sequence, which in yeast is referred to as an upstream activation site (UAS; Heslot and Gaillardin 1992). One or more activation domains (AD) direct the RNA polymerase II complex to transcribe the gene downstream of the UAS (Keegan et al. 1986; Hope and Struhl 1986; Ma and Ptashne 1987). Both the DNA-BD and the AD are required to activate a gene and normally, as in the case of the native yeast GAL4 protein, the two domains are part of the same protein. If physically separated by recombinant DNA technology and expressed in the same host cell, the DNA-BD and AD peptides do not directly interact with each other and thus cannot activate the responsive genes (Brent and Ptashne 1985; Ma and Ptashne 1988). However, if the DNA-BD and AD can be brought into close physical proximity in the promoter region, the transcriptional activation function will be restored. In principle, any AD can be paired with any DNA-BD to activate transcription, with the DNA- BD providing the promoter specificity (Brent and Ptashne 1985).

2.5.1 Vectors used in yeast-two-hybrid assay

In the MATCHMAKER Two-Hybrid system, the DNA-BD and the AD are both derived from the yeast GAL4 protein (aminoacids 1–147 and 768–881, respectively). Two different cloning vectors are used to generate fusions of these domains to genes encoding proteins that potentially interact with each other. The recombinant hybrid proteins are coexpressed in yeast and are targeted to the yeast nucleus (Chien et al. 1991). An interaction between a bait protein (fused to the DNA-BD) and a library encoded protein (fused to the AD) creates a novel transcriptional activator with binding affinity for a GAL4-responsive UAS (Fig. 6). This factor then activates reporter genes having upstream GAL4- responsive elements in their promoter and this makes the protein-protein interaction phenotypically detectable.

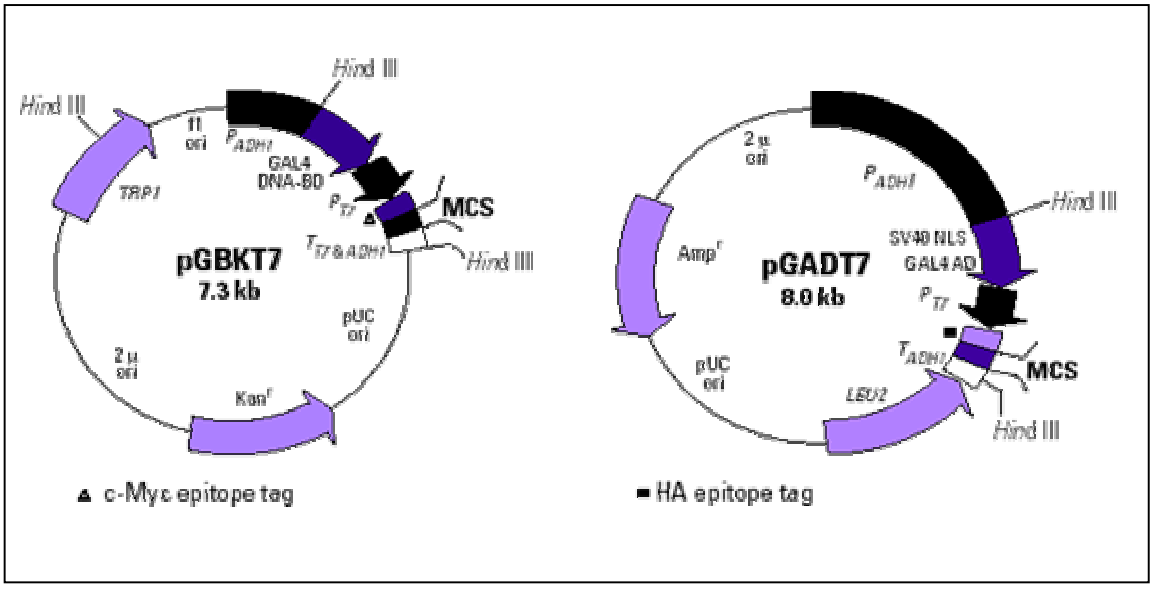


Fig. 6: The yeast two hybrid bait pGBKT7 and library pGADT7 cloning vectors. The entire TWIST, NSEEE, and WR were cloned into the MCS of pGBKT7 vector and pGADT7 are expressed as fusion to the GAL4 binding domain (DNA-BD) and a activation domain (AD9, respectively). Interaction of both proteins allows activation of a down stream reporter.

2.5.2 Transformation of bait plasmid in yeast *S. cerevisiae* AH109

In this study we used yeast *S. cerevisiae* AH109 strain. The bait plasmids were transformed in the commercially available AH109 chemical competetent cells. One vial of frozen cells from –70°C was used and thawed at RT. In a sterile 1.5 ml prechilled tube, 400 ng bait plasmid and 200 µl competent yeast cells were combined and gently mixed by pipetting. 1.2 ml PEG/LiAC solution was then added and the mixture vortexed for 3-5 sec. The tube was incubated at 30°C for 45 min, with vortexing every 10 min and then at 42°C in a water bath for 15 min. After 15 min the yeast cell was centrifuged at 700 x g for 5 min, the supernatant discarded and the cells resuspended in 0.25 ml recovery medium. The cells then incubated again 30°C for 90 min. The yeast cells were centrifuged and the supernatant discarded, the pellets were resuspended in 0.5 ml TE and plated out on to the QDO–Trp plates and then incubated at 30°C for one week.

2.5.3 Yeast mating

Yeast mating is a convenient method of introducing two different plasmids into the same host cells (Harper et al. 1993; Finley and Brent 1994). One colony of each type picked to

use in the mating. Only large (2–3 mm), fresh (<2-months old) colony from the working stock plates were used, and each colony placed in a 1.5 ml microcentrifuge tube containing 0.5 ml of YPD medium. The tubes were vortexed to completely resuspend the cells. A concentrated overnight culture of the bait strain was prepared (*i.e.* AH109 [bait]) as follows:

One large (2–3 mm), fresh (<2 months old) colony of AH109 [bait] was inoculated into 50 ml of SD/–Trp, and incubated overnight (16–24 hrs) at 30°C with shaking at 250–270 rpm. The entire AH109 [bait] culture and the 1 ml library culture was combined in a 2 L sterile flask, and 45 ml of 2X YPDA/Kanamycin added and gently swirled. Two aliquots of 1 ml of 2X YPDA/Kanamycin was used to rinse cells from the library tube followed by incubation at 30°C overnight (20–24 hrs) with gentle swirling (30–50 rpm). The mating mixture was then transfered into a sterile centrifuge bottle and the cells spun down by centrifuging at 1,000 x g for 10 min. Meanwhile, the mating flask was rinsed twice with 2X YPDA/Kanamycin (50 ml each rinse), the rinses combined and used to resuspend the first pellet. The cells again spun down at 1,000 x g for 10 min, the cell pellet resuspended in 10 ml of 0.5X YPDA/Kanamycin, and the total volume of cells measured in YPDA medium. The library mating mixtures were plated as follows: 100 µl of a 1:10,000, 1:1,000, 1:100, and 1:10 dilution of the mating mixture was spread on SD/–Leu, SD/–Trp, and SD/–Leu/–Trp plates for mating efficiency controls (90 mm plates). The remaining mating mixture was spread on ~50 large (150 mm) plates, at 200 µl per plate, equivalent to ~ 2 x 10^4–2 x 10^5 cells per plate.

Important Notes:

• The flask size must be at least 2 L to permit sufficient aeration of the mating culture at low-speed swirling.

• Low-speed swirling is necessary to keep cells from settling to the bottom of the flask. Shaking the culture at speeds >50 rpm will significantly reduce mating efficiency.

2.5.4 Plasmid isolation from yeast

A large 2–4 mm, from 2-4 days old yeast colony was used to inoculate a 0.5 ml of the appropriate SD liquid medium. The tube was vortexed vigorously to completely resuspend the cells. 10 ml of lyticase solution was then added to each tube, and the cells resuspended

thoroughly by vortexing or repeatedly pipetting up and down, followed by incubation at 37°C for 30–60 min with shaking at 200-250 rpm. After that 10 ml of 20% SDS was added to each tube and the tubes vortexed vigorously for 1 min to mix. The samples were subjected through one freeze/thaw cycle (at –20°C) and vortexed again to ensure complete cell lysis. The volume of the sample was brought up to 200 ml in TE buffer (pH 7.0), and 200 ml of phenol: chloroform: isoamyl alcohol (25:24:1) added, followed by vortexing at maximum speed for 1 min. After centrifugation at 14,000 rpm for 10 min, the aqueous (upper) phase was transferred to a fresh tube, and then 8 µl of 10M ammonium acetate and 500 µl of 95–100% ethanol were added. The suspension was incubated at –70°C or in a dry-ice/ethanol bath for 1 h, and then centrifuged at 14,000 rpm for 10 min. The supernatant was discarded and the pellet dried prior to pellet resuspension in 20 µl of H_2O

2.5.5 PCR amplification from yeast

The amplification cycles were performed in an automatic thermocycler. The PCR reaction contains in general, the following substances:

1 µl	Yeast plasmid
1 µl	5 LD Inscr primer (10 pmol)
1 µl	3 LD Inscr primer (10pmol)
2.5 µl	10x Buffer
0.125 µl	dNTPSs
1 µl	$MgCl_2$
0.125 µl	Taq polymerase (units/ml)
up to 25 µl	H_2O

The reaction mixture was prepared in a 200 µl reaction tube and reaction was carried out in a thermocycler. A standard PCR program is shown here:

Initial denaturation	95° C for 2 min
Elongation	95°C for 30 sec (denaturation)
35 cycles	58°C for 30 sec (annealing)
	72°C for 4 min (extension)
Final extension	72°C for 4 min
Store	4°C

3 Results

TWIST nuclear import mechanism was studied to observe misdistribution of bHLH transcriptional factor TWIST in the human cells, particulary when nucleus localization signals (NLSs) of TWIST were altered. In order to examine the functionality of N-terminal part of bHLH transcriptional factor TWIST, we comapred different Twist proteins sequences using the PROSITE program and predicted that two highly conserved nucleus localization signals (NLS1 and NLS2) present at amino acid positions 37-40 and 73–77, respectively in the N-terminal part of the TWIST protein. NLSs are generally rich in the basic amino acids lysine or arginine, and responsible for the ability of proteins to translocate from the cytoplasmic compartment of the cell to the nucleus.

3.1 Selection of human cell line

The importance of bHLH transcription factor TWIST is well established in osteogenesis and suggests that its expression preventing premature or ectopic osteoblast differentiation. Therefore, it is important to select a human cell line originated from the mesoderm and in which TWIST is expressed endogenously. Furthermore, to select a human cell line several factors were considered such as constant growth conditions, proliferate steadily and easy to transfect. Considering all these factors alltogether, several cell lines were tested like OHS, CCL-136, COS7, and U2-OS. Finally, U2-OS (Osteosarcoma) cell line was selected for further experiments. The U2-OS cell is originated from the mesoderm and in which TWIST was expressed endogenously, which is important for the nucleus import mechanism.

3.2 Confirmation of endogenous expression of TWIST in U2-OS cells by RT-PCR

RT-PCR was performed to examine the endogenous expression of TWIST in U2-OS cells. The cDNA was synthesized using the Oligo (dT) primers from total RNA isolated from U2-OS cells and TWIST expression was detected in cDNA using the TWIST specific oligonucleotides TWIST f/r.

To exclude the possible contamination of genomic DNA in cDNA synthesis, *GAPDH* gene was used as a positive control, which is ubiquitously expressed for the glyceraldehyde-3-phosphate dehydrogenase. The GAPDH-f and GAPDH-r primers were selected in such a way that it spans an intron of the *GAPDH* gene. Due to different product sizes, it could be

differentiated between genomic DNA and cDNA amplification. All blank PCRs were done with the help of (-) RT control for genomic impurities examination (Fig. 7).

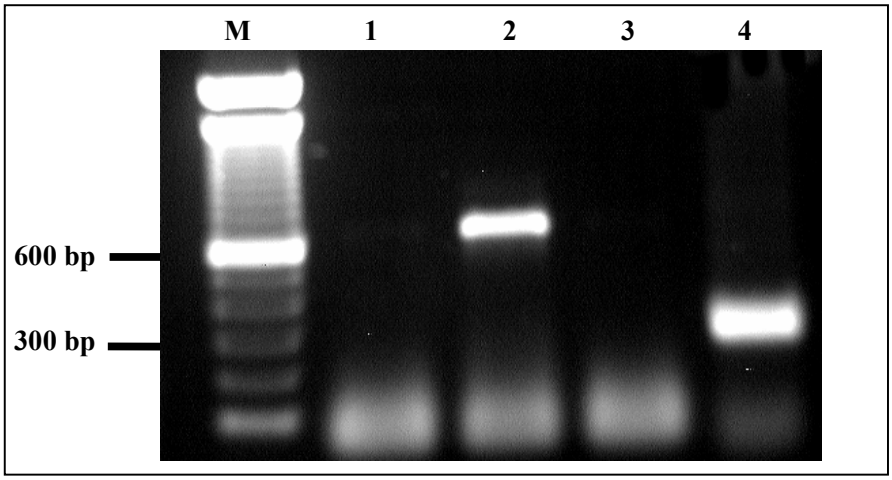


Fig. 7: The endogenous expression of TWIST in the U2-OS cell line. Detection of coding region of TWIST in the U2-OS cells using Twist-f and Twist-r primers: (M) 100 bp size standard DNA ladder; (1) negative control without reverse transcriptase to exclude the contamination by genomic DNA; (2) (+) 630 bp fragments containing coding region of TWIST (3) (-) negative control for RT PCR GAPDH; (4) (+) RT PCR GAPDH used as positive control.

3.3 Generation of TWIST fusion constructs

3.3.1 Generation of TWIST fusion constructs with c-myc epitope

TWIST fusion construct with c-myc epitope was prepared to analyze the functionality and subcellular localization using indirect immunofluorescence assay. TWIST cDNA was cleaved by *XhoI* and *EcoRI* from the pBluescript II KS Phagemid vector. The cleaved cDNA was cloned into PCMV vector at the 5'-over-hanging end. The pCMV-tag expression vector is derived from the pCMV-script vector which contains sequences for c-myc epitope at either N or C- terminal. The c-myc epitope tag is small, highly immunoreactive, not likely to interfere with the function of the target protein, and allow easy purification and detection of the fusion protein by using anti c-myc antibodies. The c-myc epitope is derived from the human c-myc gene and contain 10 amino acid residues (EQKLISEEDL). In addition to the epitope tag sequences, the pCMV-tag vector contains features for expression of the fusion proteins in eukaryotic cells.

A 6 to 8 bp long oligonucleotide was created, which contains *BamHI* restriction endonuclease site. A complementary strand of DNA was synthesized using the Klenow using one simply mixed single-stranded template, primers and the enzyme with an appropriate buffer for the creation of *BamHI* restriction sites at both ends. After ligation, TWIST was fused with c-myc epitope at N-terminal site (Fig. 8).

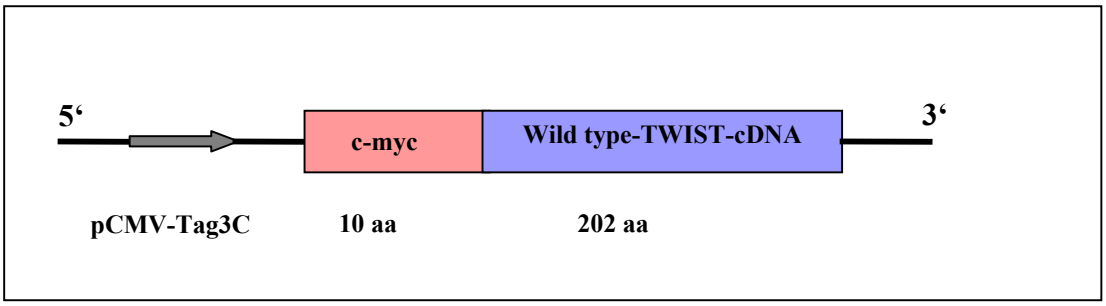


Fig. 8: Schematic representation the construction of fusions protein TWIST cDNA~c-myc^{N}. This figure represents the fusion of 202 amino acid wide TWIST and 10 amino acid long c-myc epitopes. In this construct the TWIST cDNA is cloned in pCMV mammalian expression vector which consists of c-myc epitope on N-terminal.

3.3.2 Cloning of E12 ORF into the pEGFP-N1 vector

Class B bHLH proteins to which H-TWIST belongs are known to form stable heterodimers with members of class-A bHLH transcription factors including E12 and E2-2 (Murre et al. 1989*b*; Henthorn et al. 1990). The E12 cDNA was cloned into pSP65 vector which was received from Christian Kosan. The E12 open reading frame was amplified by the PCR and cloned into Topo TA cloning vector. The E12 ORF was then released from the Topo vector by *EcoRI* digestion. After purification, the insert was ligated into the *EcoRI* sites of the multiple cloning site of the mammalian expression vector pEGFP-N1 (Fig 9).

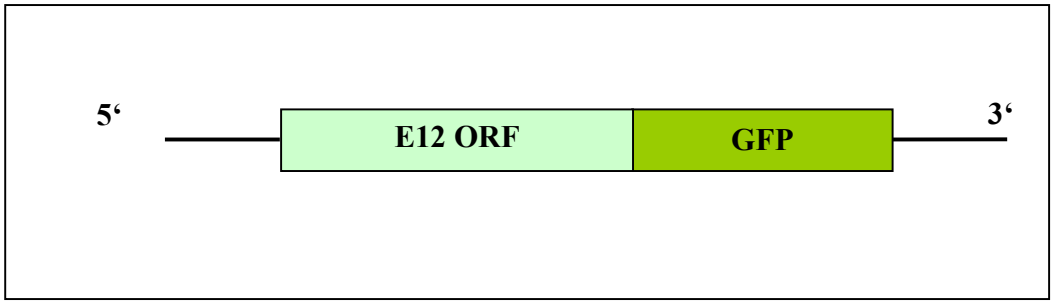


Fig. 9: Schematic representation of E12-pEGFPN1 fusion construct. This figure represents the fusion of 648 amino acids wide E12 ORF tagged with GFP. In this construct the E12 is cloned in eGFPN1 mammalian expression vector which consists of GFP on C-terminal.

3.3.3 Generation of mutations in TWIST by site directed mutagenesis

The active transport of proteins in both directions across the nuclear envelope requires the presence of specific targeting sequences within the protein (Görlich and Mattaj 1996). To determine the key role of the NLS in the TWIST protein transport, the basic amino acid residues of the two putative nucleus localization signals were substituted at the position 37-40 (RKRR, NLS1) and 73-77 (KRGKK, NLS2) using site directed mutagenesis (Table 12). Specifically, to test the functionality of these two motifs, each of these basic amino acid residue were systematically altered individually or in combination with others in the first NLS cluster K38R and the effect of these mutations on nucleus localization of TWIST protein in transiently transfected U2-OS cells were assayed.

Table 12: Wild type and mutated amino acid sequences of TWIST nuclear localization signals

Clone Name	NLS1	NLS2
$NLS1^{wt}$	RKRR	KRGKK
K38R	R**R**RR	KRGKK
K73R	RKRR	**R**RGKK
K77R	RKRR	KRGK**R**
K76R	RKRR	KRG**R**K
K38R / K73R	R**R**RR	**R**RGKK
K38R / K76R	R**R**RR	KRG**R**K
K38R / K77R	R**R**RR	KRGK**R**

The mutated amino acids are bold and underlined.

3.4 Expression control of the fusion constructs in transient transfected U2-OS cells

The expression of pCMV-TWIST fusion construct in U2-OS cells was analysed using RT-PCR and Western blot analysis. The primers were designed to amplify the target sequence in such a way that PCMV3C/twist f is located in the c-myc coding sequence and PCMV3C/twist r hybridize with a part of a TWIST-cDNA fragment (Fig. 10). A 70 bp target cDNA was amplified using the insert specific primers which allowed the distinct identification of the fusion constructs (Fig. 11). To avoid the contamination of genomic DNA a negative RT(–)PCR was used as a negative control.

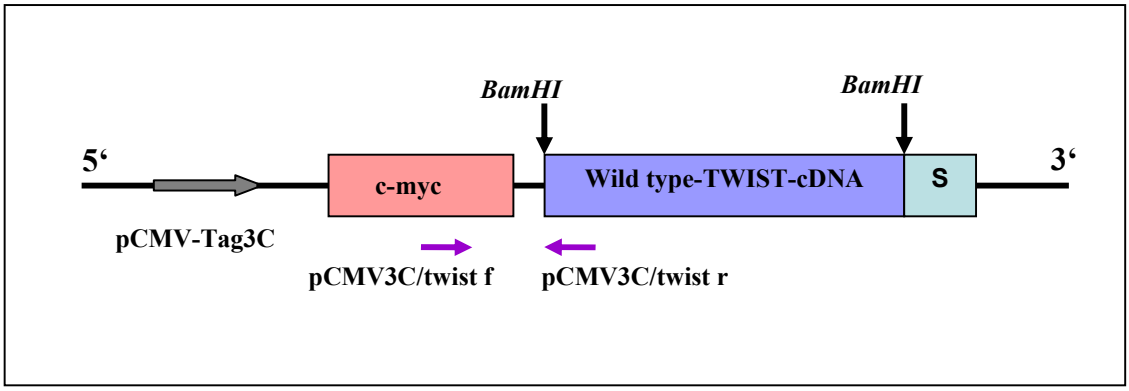


Fig. 10: The position of the used oligonucleotide in RT-PCR. The primer pairs used in RT-PCR for expression of fusion construct were created in such a way that one of the primer is located in the c-myc coding sequence and other primer hybridize with a part of a Twist-cDNA fragment: The Twist cDNA in pCMV Tag3C vector TWISTcDNA~c-myc^{N} and the position of the primer pCMV3C Twist forward and reverse is shown.

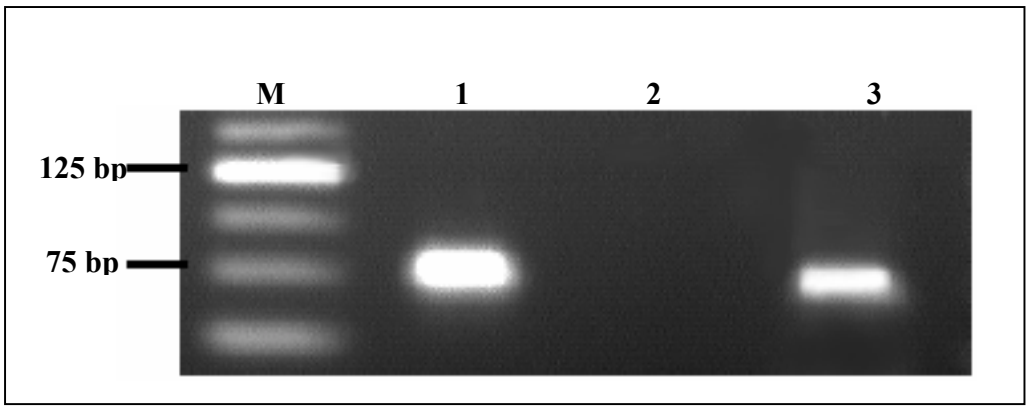


Fig. 11: Expression of TWISTcDNA~*c-myc*^{N} in transiently transfected U2-OS cells by using RT-PCR. M represents the 25 bp ladder; Line 1 and 3 represents represents the amplified TWISTcDNA~c-myc^{N} (70bp) pCMVtag 3C vector; Line 2 used as a negative control (-RT) to exclude the genomic contamination.

3.5 Subcellular localization of TWIST protein in an immunofluorescence assay

The subcellular localization of TWIST constructs into U2-OS cells was determined by an indirect immunofluorescence assay. The indirect immune detection of the TWIST c-myc fusion protein is undertaken by incubation with a monoclonal antihuman c-myc antibody (Molecular probes, Göttingen, Germany) as well as with a Texas Red-coupled anti mouse IgG secondary antibody (Molecular Probes). The sub-cellular distribution of the fusion

proteins could be determined accurately by using the cyto skeleton f-actins labelled with Alexa fluorine Phalloidine (Molecular Probes) as reference and by staining the cell nucleus with DAPI. The different structures were represented both individually and in an overall view fluorescence-microscopically, which was possible due to the use of different fluorochrome (Materials and Methods 2.1.16: Table 9).

3.5.1 Localization of TWIST cDNA fusion protein into human U2-OS cells

In order to test the hypothesis that the transcription factor TWIST is actively imported into the nucleus, the sub-cellular localization of the TWIST-cDNA~c-myc^{N} fusions proteins was determined by an indirect immunfluoresence assay into human U2-OS cells. The fusion construct TWISTcDNA~c myc^{N} was introduced into U2-OS cells using various liposomal and non-liposomal transfections reagents such as calcium phosphate, Lipogen^{TM}, and Effectene. Cell mortality and transfection efficiency were monitored by microscopy. The non-liposomal transfection reagent Effectene (Qiagen, Germany) was selected due to a lower cell mortality and satisfactory transfection efficiency.

The experimental procedures were optimized in this manner so that the immune colouring techniques used to detect the various TWIST c-myc fusion proteins, did not negatively affect adherent U2-OS cell morphology. In order to minimize non-specific signals during immune detection, different concentrations of the primary as well as the secondary anti-bodies were optimized and best signals were observed with 1:700 and 1:10,000 dilutions for anti- human c-myc antibody (primary antibody) and for anti- mouse IgG~TR antibody (secondary antibody), respectively.

3.5.2 Sub-cellular immune localization of TWIST cDNA~cmyc and galactosidase c-myc fusion constructs in U2-OS cells

To investigate the 25 kDa human TWIST subcellular localization, TWIST cDNA was fused in pCMVc-myc tag vector. Plasmids encoding c-myc-TWIST were transfected into U2-OS cells and protein verified using Western blotting. A 28 kDa protein was recognized by an anti-c-myc antibody suggesting that it was a fusion of c-myc (3 kDa) with TWIST (Fig. 12).

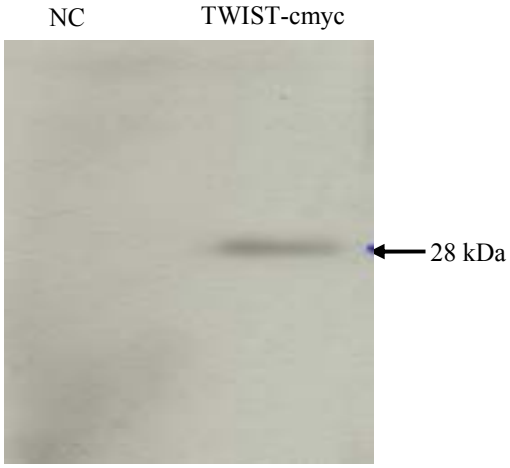


Fig. 12: Expression of Twist-cmyc fusion construct by western blot analysis using anti-c-myc antibody. Line 1 used for a negative control which contains the empty vector pCMV, and line 2 represents a 28 kDa fused TWIST-c-myc protein.

No back ground signals were detected in the subcellular localization of c-myc and the TWIST-cDNA-c-myc constructs in U2-OS cells using fluorescence microscopy, which showed that c-myc itself does not play any role in the U2-OS cells (Fig. 13 Ia-d). The fusion protein from β-galactosidase and c-myc epitope should only locate into the cytoplasm of eukaryotic cells, due to the high molecular weight of the bacterial metabolic enzyme (540 kDa), and missing topogene nucleus localization signals a free passage through the nuclear pore complex and an active nuclear import is not possible. The fusion protein could be detected, as expected, only in the cytoplasm of human U2-OS cells (Fig. 13 IIa-d). TWIST-cDNA-c-myc construct was predominantly localized into the nucleus (Fig 13 IIIa-d). Position of nucleus was determined using DAPI staining (Fig. 13 III b). Merged images summarize the results (Fig. 13 IIIa-d).

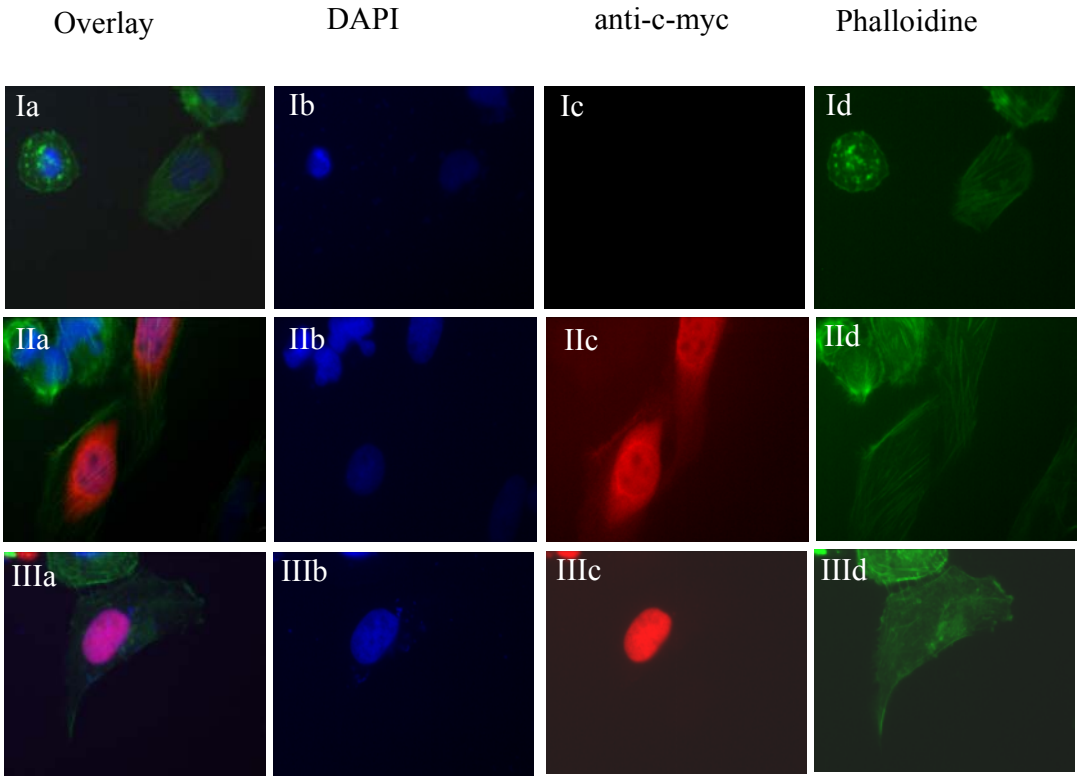


Fig. 13: Subcellular localization of tagged protein. TWIST is fused with c-myc; subcellular localization of the fusion construct is monitored following transient transfection in U2-OS cells. (Ia-d) represent the negative control in transient transfection (without TWIST); (II a-d) subcellular localization of c-myc~β-galactosidase fusion construct distributed in U2-OS cell cytoplasm: (III a-d) show U2-OS cells transiently transfected with c-myc~TWIST. The distribution of TWIST fusion protein is limited to the nucleus. (a-d): subcellular immune localization of under (a) the Merged picture of the objects are specified, (b) show DAPI Staining for cell nucleus; (c) with anti-human the C-myc and the anti- mouse IgG anti-body detected, Texas Red colored TWIST C myc Fusions proteine, (d) represents the picture with Alexa fluorine 488 Phalloidin marked cyto skeleton f-actins (Oil immersion 100X magnification).

The sub cellular localization of the TWIST~c myc fusion construct and distribution of the TWIST mutants had been observed in U2-OS cells with the help of fluorescence-microscopic analysis. The proteins were either imported completely into the nucleus or held back in the cytoplasm completely.

3.6 Subcellular localization of TWIST NLSs mutants

By the precise localization of the TWIST cDNA~c-myc fusion protein in U2-OS cells, a strategy for further analysis was formulated to identify and characterize potential TWIST protein nucleus localization signals. The sequence of the core import essential motifs could be determined based on the subcellular localization of NLS1 and NLS2 mutants. We substituted the lysine to arginine by using site directed mutagenesis as the basic amino acid residues form two clusters in H-TWIST (Table 12). One basic amino acid residue was mutated into the $TWIST^{NLS1}$ at the position 38 of this cluster (K38R), and the effect of mutated $TWIST^{NLS1}$ protein for the nucleus localization was evaluated into the transiently transfected U2-OS cells. The expression of the altered $TWIST^{NLS1}$ at the amino acid position 38 was observed in the cytoplasm of the U2-OS cells after the immunofluorescence assay for which the potential topogene nucleus import signals were systematically removed (Fig. 14 Ia-d).

To understand the role of the second cluster in the nucleus localization of $TWIST^{NLS2}$, the amino acids were substituted at the position 73, 76 and 77, respectively. The subcellular distribution of mutant 76 $TWIST^{NLS2}$ (Fig. 14 IIIa-d) was observed into the nucleus, while the subcellular distribution of the mutants 73 and 77 was accumulated in the cytoplasm (Fig. 14 IIa-d, and IVa-d, respectively). This result indicates that the amino acid at position 76 in NLS2 motif does not play an essential role in the nuclear localization of TWIST.

Although K76R mutant cannot inhibit nuclear localization by itself, it does play a synergistic role with the K38R mutant to further reduce nuclear localization which is described next.

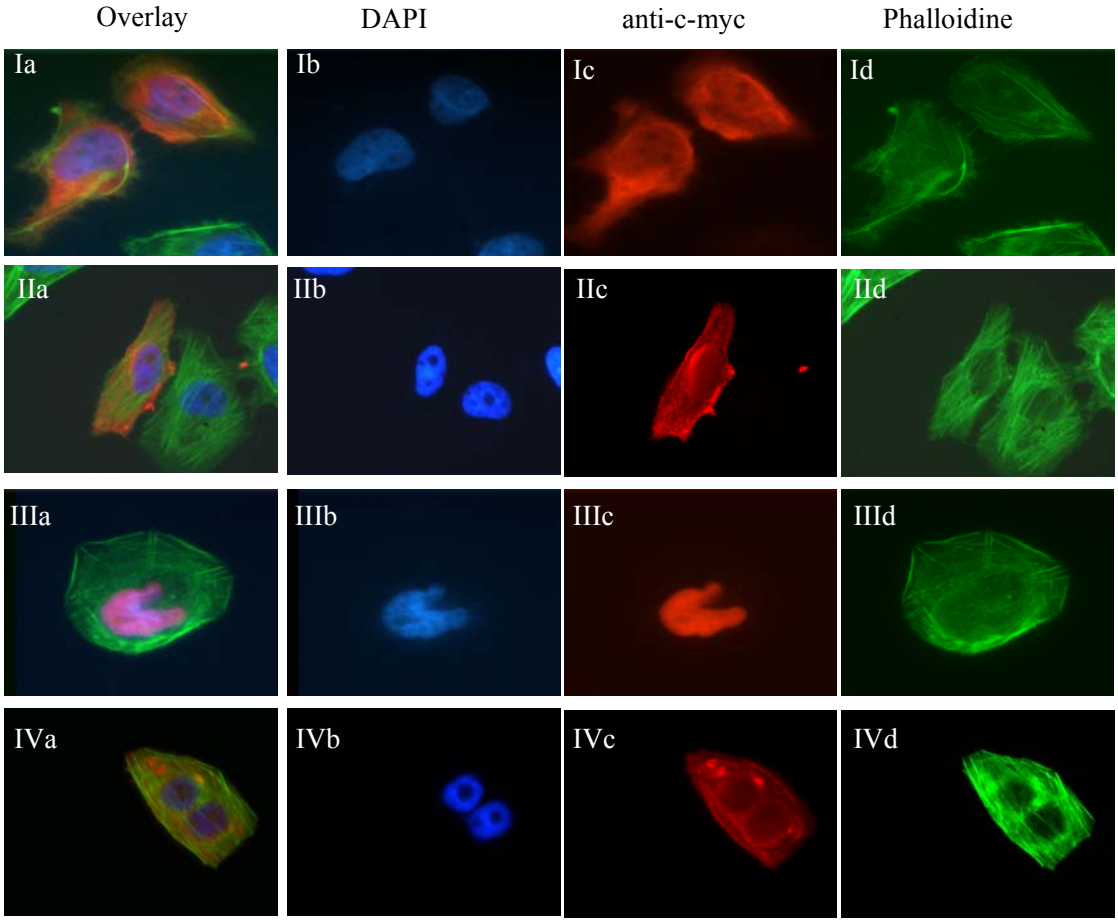


Fig. 14: Subcellular localization of tagged protein. Subcellular localization of TWIST NLSs mutants is monitored following transient transfection in U2-OS cells. The subcellular localization of the mutated constructs K37R (NLS1) (I a-d) was in the cytoplasm of U2-OS cell, K73R NLS2 (IIa-d) was monitered in the cytoplasm of U2-OS cells. The subcellular localization of the mutated constructs K76R (NLS2) (IIIa-d) was limited in the nucleus of U2-OS cell, K77R NLS2 (IVa-d) was monitered in the cytoplasm of U2-OS cells, as observed by immunostaining. The (a) pictures show composite exposures of the objects, (b) pictures show DAPI-stained nuclei, (c) pictures show the TWIST~c-myc fusion proteins detected by mouse anti-c-myc and Texas-Red-conjugated anti-mouse antibodies, (d) pictures represent exposures of the cytoskeletal F-actin marked with Alexa Fluor 488 phalloidine.

3.6.1 Subcellular localization of TWIST NLS2 mutants in combination with NLS1 mutant

To investigate potential synergistic or antagonistic regulation of nuclear localization of basic amino acid residues in both clusters, we altered the each NLS2 basic amino acid residue in combination with NLS1 clusters. TWIST having mutations at the positions K38R

(NLS1) and K76R (NLS2) inhibited the nuclear localization, suggesting that the two substituted residues in the first and the second cluster (K38R/K76R) strongly inhibit nuclear accumulation of TWIST protein. Similarly, the nuclear accumulation of (K38R/K77R) was also inhibited (Fig. 15, Table 13). These results confirm that both clusters work together in regulating nuclear localization of TWIST protein.

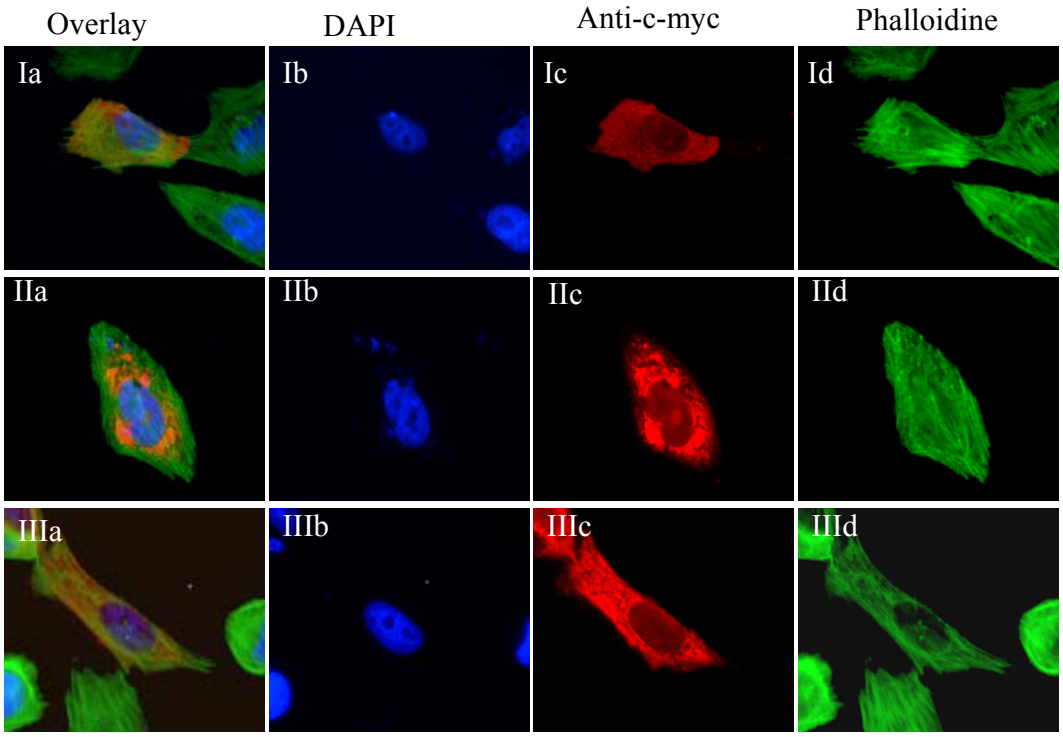


Fig 15: Subcellular localization of mutant construct. NLS2 mutants K73R, K76R and K77R in the combination with NLS1 mutant K38R showing the cytoplasmic subcellular localization in the U2-OS cells in an indirect immunofluoresence microscopy: (a) merged images of the objects are specified, (b) DAPI stained cell nucleus, (c) anti-mouse c-myc and anti-mouse IgG antibodies coupled with Texas red coloured TWIST c-myc fusion protein, (d) f-Actins cytoskeleton marked with Alexa fluorine 488 Phalloidine.

Table 13: Nuclear localization of wild type and mutant versions of TWIST

Clone Name	NLS1	NLS2	Localization	
			Nucleus	Cytoplasm
NLS1^{wt}	RKRR	KRGKK	+	
K38R	R**R**RR	KRGKK		+
K73R	RKRR	**R**RGKK		+
K77R	RKRR	KRGK**R**		+
K76R	RKRR	KRG**R**K	+	
K38R / K73R	R**R**RR	**R**RGKK		+
K38R / K76R	R**R**RR	KRG**R**K		+
K38R / K77R	R**R**RR	KRGK**R**		+

The mutated amino acids are in bold and underlined

3.7 *In vivo* interaction and co-localization of TWIST with E12

The bHLH transcriptional factor protein requires either homo/heterodimer for DNA binding. It is reported earlier that E12 forms a heterodimer with other bHLH protein. To confirm that TWIST NLS are functionally significant for nuclear localization and that the interaction with E12 is required for translocation to the nucleus, E12 cDNA was fused into the eGFP-tag vector. In order to investigate the direct interaction between TWIST and E12, transient co-transfections of TWIST^{WT} with E12, and TWIST mutants with E12 in U2-OS cells were performed. Co-localization was observed into the nucleus due to the heterodimer formation. TWIST^{WT} with E12 was present in the nucleus (Fig. 16 Ia-d), simultaneous expression of E12 led to preferential nuclear localization of mutant TWIST K38R in all transfected U2-OS cells (Fig. 16 IIa-d).

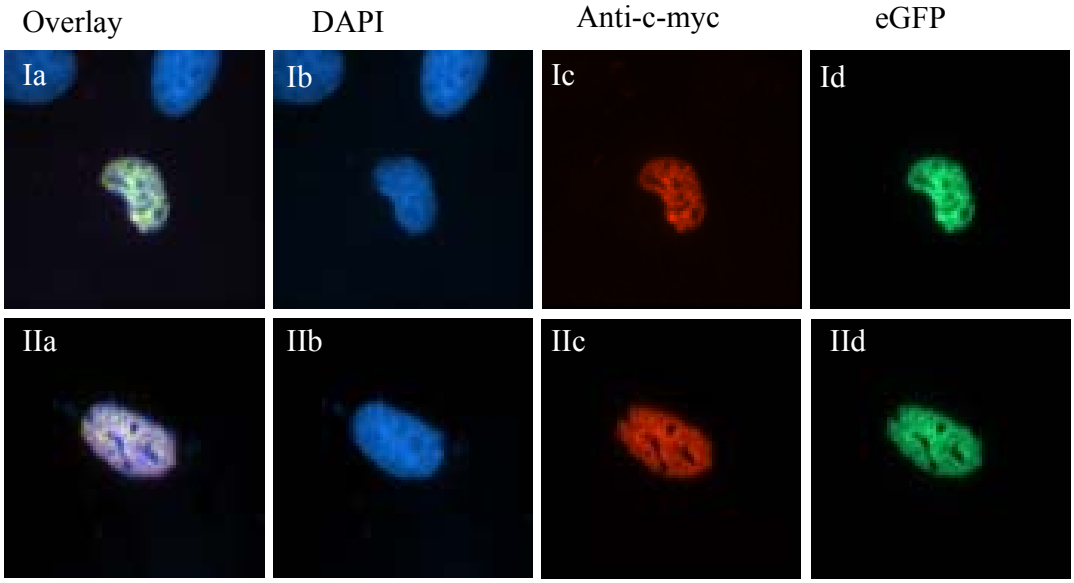


Fig. 16: Co-transfection of E12 with NLS1, and NLS2. TWIST is fused with c-myc, and E12 with eGFP; both proteins co-localized in the nucleus of U2-OS cells in transient transfection. The results indicate the formation of a heterodimer of E12 with Twist^{wt} (I a-d), and E12 with NLS1 mutated at K38R (II a-d) which is localized in the nucleus. The (a) pictures show composite exposure of the objects, (b) pictures show DAPI-stained nuclei, (c) pictures show the TWIST~c-myc fusion proteins detected by mouse anti-c-myc and Texas-Red-conjugated anti-mouse antibodies, (d) pictures represent exposure of E12 fused with eGFP.

3. 8 Identification of functional domains in TWIST by using yeast-two-hybrid assay

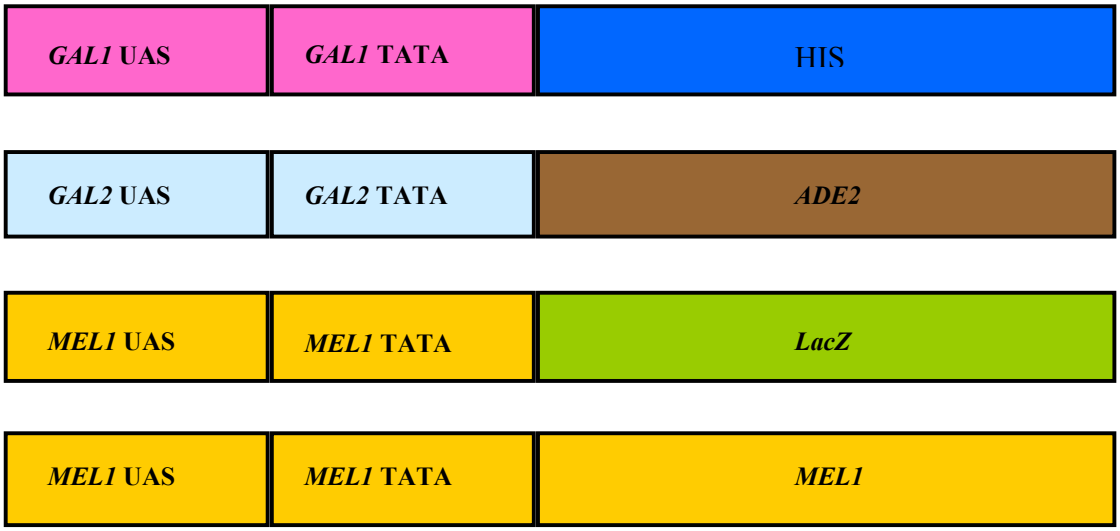
Yeast two-hybrid assay is a technique for identifying and analyzing protein–protein interactions. As we described early in the introduction that one aim of this work is the analysis of protein interactions of TWIST and its (NSEEE and WR) motifs with other proteins.

3.8.1 Principle of the two-hybrid assay: a protein-protein interaction assay

Two-hybrid assay is used to identify novel protein-protein interactions, confirm suspected interactions, and define interacting domains. In a BD Matchmaker Two-Hybrid assay, a bait gene is expressed as a fusion to the GAL4 DNA-binding domain (DNA-BD), while another gene or cDNA is expressed as a fusion to the GAL4 activation domain (AD) (Fields and Song 1989; Chien et al. 1991). When bait and library fusion proteins interact in a yeast reporter strain such as AH109 or mated AH109 and Y187 strains (Y187 alone has

just *MEL1* and *LacZ*; while AH109 alone has *HIS3*, *ADE2*, *MEL1* and *LacZ* (Fig 17), the DNA-BD and AD are brought into proximity and activate the transcription of the reporter genes: *ADE2*, *HIS3*, *LacZ*, and *MEL1* (Fig. 18).

AH109 (Mating Partner) reporter gene constructs



Y187 (library host strain) reporter gene constructs

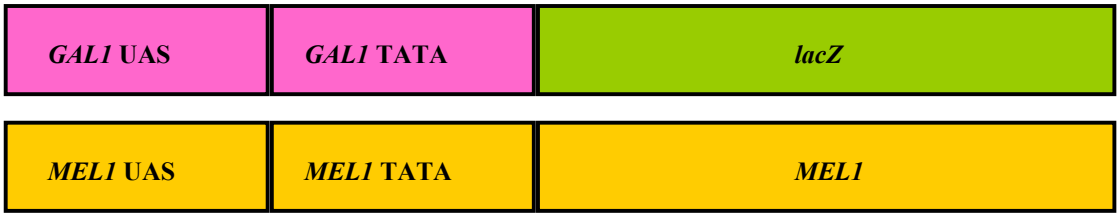


Fig. 17: These figure shows the different reporter gene constructs in AH109 and Y187 strains. While Y187 only contains the sequences for *LacZ* and *MEL1* reporter genes, the AH109 contains the sequences for *HIS3* and *ADE2* reporter genes. *MEL1* encodes β-galactosidase and is an endogenous *GAL4*-responsive gene. In AH109, the *HIS3*, *ADE2,* and *MEL1/lacZ* reporter genes are under control of three completly heterologous *GAL4*-responsive UAS and promotor elements – *GAL1*, *GAL2* and *MEL1*, respectively.

DNA-BD and AD fusion proteins are created by cloning target cDNAs into the yeast expression vectors pGBKT7 or pGADT7. The pGBKT7 expressed proteins are fused to the GAL4 DNA-BD (DNA Binding Domain), while pGADT7 expressed proteins are fused to the GAL4 AD (Activation Domain)

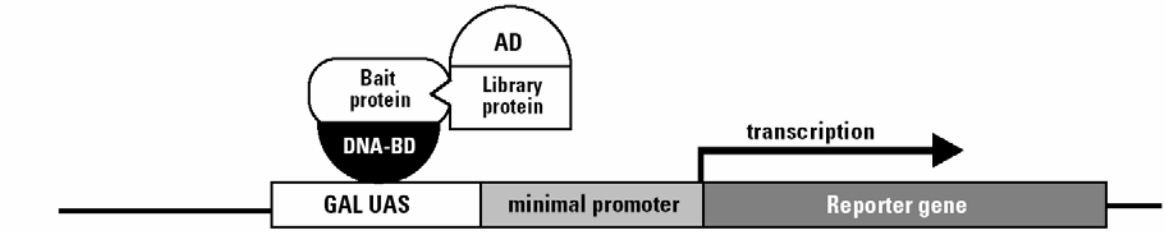


Fig. 18: The two-hybrid principle. GAL4 protein is a yeast transcription factor that normally controls genes responsible for galactose metabolism. Each GAL4-responsive gene contains a target site called an Upstream Activating Sequence (UAS). When GAL4 binds the UAS, transcription is activated from a downstream promoter (Clontech).

3.9 Cloning of different inserts into pGBKT7 bait vector

To find out the possible binding partners using a yeast-two-hybrid assay, as a bait protein we used

(1) TWIST ORF (NM_000474)

(2) N-terminal region with NSEEE domain

(3) C-terminal region with WR domain.

The TWIST-ORF, N-terminal region containing NSEEE domain and C-terminal region containing WR domain were amplified without stop codons. For this purpose, the primers were designed as described in materials and methods (2.1.6: Table 2), which contained adapters *EcoRI* and *BamHI*. The amplified PCR products were double digested with both enzymes (in *EcoRI* buffer) and isolated by the gel extraction, and the insert was ligated with pGBKT7-vector, which was also digested with these restriction enzymes (Fig. 19).

After transformation of these ligations in *E. coli,* several clones were to be seen on the agar plates, out of them 8 clones were picked for isolation of DNA from each plate and inoculated into a LB medium with Kanamycin (50 mg/ml). The isolated plasmids were digested with *BamHI* and *EcoRI* to check for successful ligation. The cloning products pGBKT7-TWIST, pGBKT7-NSEEE and pGBKT7-WR were transformed into *E.coli*. The final plasmids were sequenced to confirm the right frame and the absence of PCR artifacts.

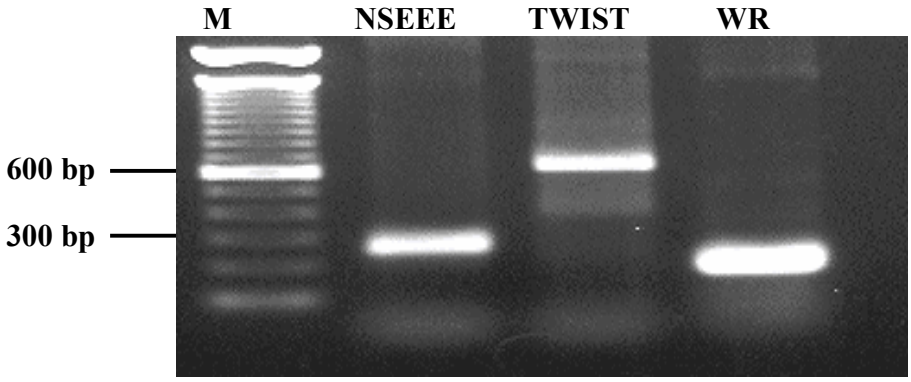


Fig. 19: Agarose gel electrophoresis of the PCR products of TWIST and its motifs NSEEE and WR. Line 1 shows a 100 bp DNA ladder; Line 2 an insert of 270 bp size NSEEE clone; line 3 an insert of 619 bp TWIST clone; and line 4 an insert of 250 bp WR clone.

3.10 Transformation of the bait plasmids into AH109 cells

In order to examine the expression of the bait protein, the generated bait plasmids were transformed into commercially available chemically competent *Saccharomyces cerevisiae* AH109 yeast cells as described in materials and methods (2.5.2). DNA isolated from the yeast clones and the transformed plasmid were detected by PCR using the insert specific primers and the correct size inserts confirmed in all the three appropriate clones pGBKT7-TWIST, pGBKT7-NSEEE and pGBKT7-WR.

3.11 Testing the DNA-BD/bait protein for transcriptional activation

First the self-activation of bait clones was verified. Self activation test is necessary to determine if fusion proteins (DNA-BD/bait protein) intrinsically activate unspecific transcription of reporter genes. In case of pGBKT7-TWIST, pGBKT7-NSEEE, and pGBKT7-WR in yeast strain AH109 no unspecific activation of reporter genes (for syntesis of adenine and histidine) was observed. The self-activation of reporter genes (without AD/prey) was checked on selective media plates lacking the three amino acids tryptophan, histidine, and adenine.

i. SD/–Trp (Positive control)
ii. SD/–Trp /–Ade/-His

AH109 with pGBKT7-NSEEE vector (expressing DNA-BD/bait protein NSEEE) was transformed. Transformed AH109 clones grew only on the –Trp plates, because these plates select plasmid pGBKT7 carrying cells. The colonies did not grow on SD/–Ade/–His/–Trp. In contrast, if the colonies would appear on the SD/–Ade/–His/–Trp plates that would be unspecific transcription activation. However, this was not the case with our transformed clones, so that the constructs for the execution of a two hybrid screen were applicable (Fig. 20).

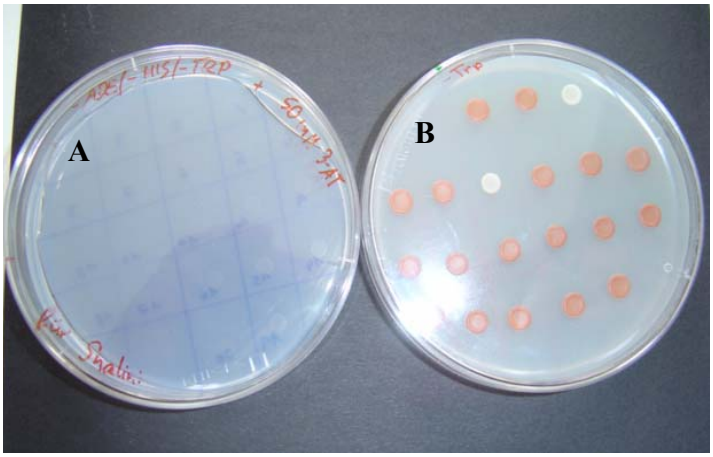


Fig. 20: Representing the testing of pGBKT7 with NSEEE bait Protein for transcriptional activation. **(A)** SD/–Ade/–His/–Trp plate showing no growth represents that there is no unspecific transcription activation, **(B)** –Trp plate showing the growth of AH109 containing pGBKT7–NSEEE vector.

3.12 Testing the DNA-BD/bait protein for toxicity effects

Many interactions could not be detectable due to increased cell mortality due to increasing toxicity, which is problematic for the execution of a two hybrid screen. To exclude this possibility, the toxicity of yeast cells growth was checked under control of the *ADH1*-promotors. On the basis of the yeast cells growth incubated at 30°C, the density of the cell suspensions was determined after every two hours at 600 nm for both the yeast strain AH109 carrying unmodified cloning vector (pGBKT7) and of AH109 carrying the bait vector (pGBKT7-TWIST, pGBKT7-NSEEE, pGBKT7-WR). The toxicity of the bait protein was checked on the host strain to compare the growth rate in liquid culture of cells transformed with the empty DNA-BD vector and cells transformed with the DNA-BD/bait plasmid (pGBKT7-TWIST, pGBKT7-NSEEE, pGBKT7-WR). If the bait strain would

grow noticeably slower, the DNA-BD/bait protein would be toxic; thus, this strain may need to grow on agar plates in preparation for mating with the library. We could not detect any toxicity in our plasmids, because growth was as fast as AH109 carrying the unmodified plasmid.

3.13 Mating of the bait vector with a pre-transformed human placenta cDNA library

Three transformed yeast clones one from each TWIST, NSEEE, and WR were randomly selected from the –Trp plates. The mating of these randomly selected yeast clones named Tw-5, NSEEE-2, and WR-8 was accomplished as described in materials and methods (2.5.3). The paired yeasts were resuspended in SD minimal media and plated out directly on quadruple selective plates (QDO–lacking the amino acids Ade, His, Leu, Trp), so that only positive mated cells survived, in which fusion proteins interact. Additionally for negative control, the unmated pGBKT7-TWIST, pGBKT7-NSEEE, pGBKT7-WR as well as an aliquot of the placental library placed on QDO plates, which showed no growth. Twenty four clones for TWIST-ORF, 1200 clones for NSEEE domain, and 100 clones for WR domain were obtained. These clones were grown up approximately after three weeks of incubation at 30°C and given the numbers. Glycerine stocks were prepared from the clones grown on these plates. The plates were incubated further for one more week at 30°C and prepared DNA from the regenerated clones. TWIST and WR clones were again recultured on replica plates without 3-AT for futher experiments. On the other hand, NSEEE clones were recultured on replica plates which contains 50 mM of 3-AT to select out the stronger interacting candidates. Six hundred clones were grown on the replica plate containing 50mM 3-AT and these strong interacting clones were used for further experiments.

3.14 Segregation of yeast clones in the bacteria

The plasmids were isolated from the 24 TWIST, 600 NSEEE, and 100 WR yeast clones. The isolated plasmids were transformed in *E. coli* DH5α cells and selected on LB agar plates containing ampicillin (100 mg/ml). This step helps to get rid of the yeast genomic contamination as well as the selection of single clone.

3.15 DNA preparation from bacteria and analysis of positive clones by PCR

DNA was prepared as described in materials and methods. The inserts were amplified from the library clones using the 1 µl template DNA and insert screening amplimer 5'-LD and 3'-LD primers. PCR product was visualized on 1% agarose gel.

3.16 Analysis of the entire TWIST positive clones by sequencing

PCR products were cleaned by gel extraction using Qiagen column, prior to sequencing using an ABI PRISM 377 DNA Sequencer. The obtained sequences were screened with the help of sequencher 4.1 program and the sequences containing inserts were compared in the NCBI blast search.

Table 14: List of the interacting proteins with entire TWIST

Clone	Result	Remark	Gene Bank Acc.No.
TW1-2	mRNA Splicing factor 2	Nuclear protein	L04636
TW1-8	SEF2-1A Transcription factor	HLH protein	M74718
TW1-10	pre mRNA splicing factor 2	Nuclear protein	L04636
TW1-11	pre mRNA splicing factor 2	Nuclear protein	L04636
TW1-15	KIAA1376-protein	unknown	XM_033042
TW1.-16	KIAA1376-protein	unknown	XM_033042
TW1-18	Clusterin	Sulfated-glycoprotein	BC010514

Out of 24 screened clones, 7 proteins were identified by BLAST searches. 4 of which (SF2p32, KIAA1376, SEF2, JAB1/CSN5) are candidates for an interaction with TWIST (Table 14). From the interacting candidates, SEF2 was selected for further studies because

SEF2 is also a HLH transcription factor gene, and it is hypothesized that TWIST makes heterodimer with other HLH/bHLH protein

3.17 Retest the direct interaction of TWIST with SEF2 protein in yeast cloning

It is well known that in two-hybrid screens relatively false positive interactions were found frequently. In order to verify that the found clones were actually carriers of positive interactions that TWIST and SEF2 protein were real, we cloned the SEF2-ORF into pGADT7 prey vector. The pGADT7-SEF2 plasmids were transformed into chemically competent Y187 yeast cells as described in materials and methods. Transformants were spreaded on –Leu plate and incubated at 30°C for one week. The pGBKT7-TWIST plasmid was allowed to grow on –Trp plate for one week at 30°C.

3.17.1 Yeast mating to verify the direct interaction of SEF2 protein with TWIST protein

After the growth of clones on both plates one clone from each plate was selected and streaked out AH109 (pGBK T7-TWIST) with Y187 (pGADT7–SEF2) on YPD plate and allowed to grow at 30°C for 24 h (Fig. 21). Upon growth on the YPD plate a retro-plate was prepared with the help of velvet onto –Leu/–Trp plates to select only mated cells (irrespective growth if the fusion-proteins interact or not). The retro plates were again incubated atleast for next 2 or 3 days at 30°C, at which time growth was observed on the –Leu/–Trp plates (Fig. 21).

Mated clones were then dissolved in water from the –Leu/–Trp, and dropped out onto the QDO plates and the –Leu/–Trp filter plates. After one week incubation the growth on QDO plate and on the filter of the –Leu/–Trp filter plate was observed.

To check the real interaction we used positive and negative controls onto QDO and –Leu/–Trp filter plates. As a positive control we used p53/pGBKT7 clone transformed into AH109 mated with Y187 strain containing T7-antigene in pGADT7-vector and as a negative control used p53/pGBK T7 in AH109 mated with LamC/pGADT7 which was transformed into Y187. These both mated strains grew on –Leu/–Trp plate at 30°C after 24 h of incubation. The p53 and LamC were unable to interact, and adenine and histidine genes not

transcribed. Therefore, mated cells with carrying non-interactive bait and prey could not grow on QDO plates and they are also negative for transcription of β-galactosidase (the filter-LacZ-test cells did not turn green-blue).

3.17.2 Growth test for mated pGBKT7-TWIST and pGADT7-SEF-2 yeast clones

To check the activation of reporter genes encoding histidine and adenine, strains carrying pGBKT7-TWIST (bait) and pGADT7-SEF-2 (prey) were mated. When bait and prey interact a transcription factor results, which then activates the transcription of the reporter genes histidine and adenine (and of β-galactosidase also). We found that the mated bait pGBKT7-TWIST and prey pGADT7-SEF-2 grew well on QDO plates (Fig. 21). Therefore, transcription of the reporter genes initiated because of interaction of bait pGBKT7-TWIST with prey pGADT7-SEF-2 proteins which is shown in Fig. 21.

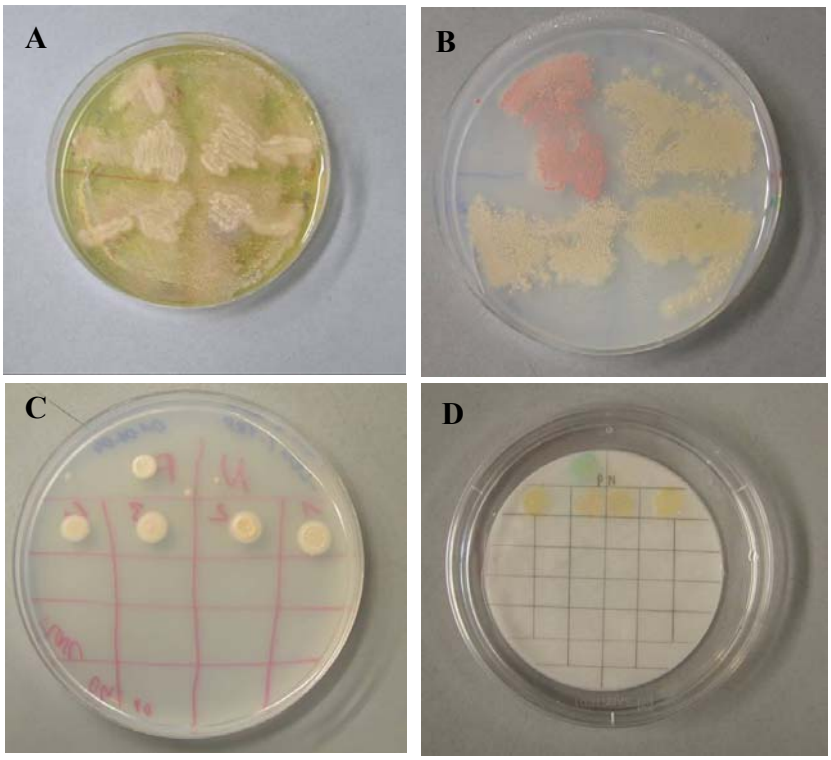


Fig. 21: Yeast mating to verify protein interactions. (A) AH109 TWIST and Y187 SEF2 clones were streaked together on the YPD plate; **(B)** growth on –Leu/–Trp shows the successful cell mating (irrespective if fusion proteins interact); **(C)** TWIST and SEF2 proteins interaction was confirmed on QDO plate and **(D)** in Filter-LacZ test activation assay on filter/–Leu/–Trp plates.

3.17.3 Filter-LacZ-test activation of the reporter gene ß-galactosidase

Filter-LacZ-assay was performed after 3 to 4 days cell growth on the filter/–Leu/–Trp plates. The filter was picked from the filter/–Leu/–Trp plates with sticking colonies and cells cracked in liquid nitrogen. After that, the filter containing cracked cells was placed on the new filter soaked in the freshly prepared Z buffer containing β-mercaptoethanol and X-Gal (colonies at upper side).

In this assay, p53/pGBKT7 clone transformed into AH109 mated with Y187 strain containing T7-antigene in pGADT7-vectors was used as a positive control and p53/pGBKT7 in AH109 mated with LamC/pGADT7 which was transformed into Y187 used as a negative control. Both the positive control as well as the positive interacting partners (pGBKT7-Twist and pGADT7-SEF-2) also turned green. The time to obtain a green signal varied from a few minutes to several hours later -the excact time being dependent on the strength of the interaction. In case of green positive clones (pGBKT7-TWIST and pGADT7-SEF-2), the transcription factor (interaction DNA-BD fusion protein and GAL4-AD fusion protein) activates β-galactosidase transcription resulting in X-Gal cleavage resulting in a green-blue colour which confirms the strong interaction of TWIST with SEF2 (Fig. 21).

3.17.4 Confirmation of protein interactions in mammalian cells

In order to examine, whether proteins possessing same subcellular address can colocalize with each other, they have to be viewed simultaneously in U2-OS cells. To accomplish this, the TWIST protein under investigation was tagged to c-myc and combined with strains expressing its potential interaction partner fused to eGFP. For this SEF2 open reading frame was subcloned into eGFP-N1 vector by using *EcoRI*. To verify the interaction of the SEF2 with TWIST protein *in vivo*, NLS rescue assay was performed. In this assay a NLS-mutated TWIST, which is unable to be transported into the nucleus, was cotransfected with SEF2 to check whether the lack of a functional NLS could be compensated by heterodimerization and subsequent co-import of the two proteins by fluorescence microscopy. While no nuclear localization of TWIST could be observed in the cells co-transfected only with 38NLS mutated TWIST, co-localization of both proteins in the

nucleus was detected in the cells co-transfected with both TWIST NLS^{mut} and SEF2 expression vector (Fig. 22).

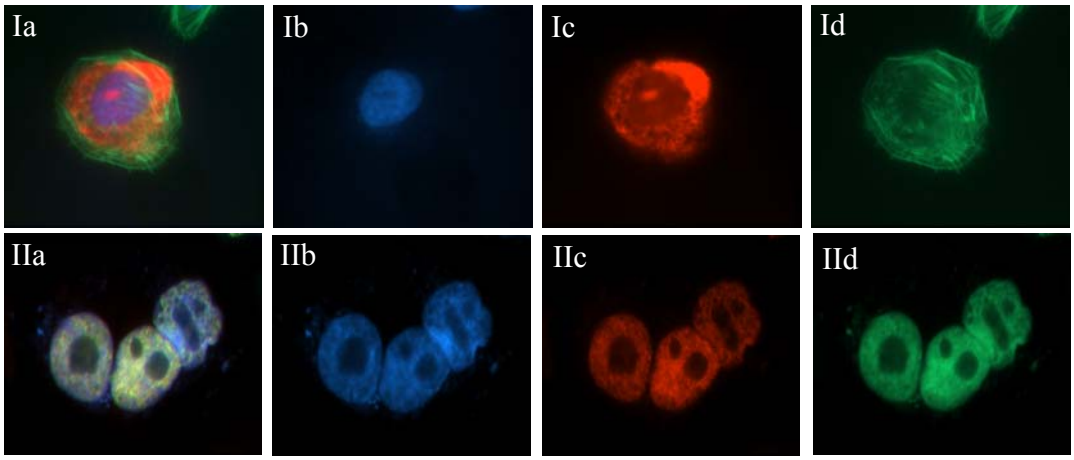


Fig. 22: *In vivo* NLS rescue assay to study the ability of SEF2 to assist in the nuclear localization of TWIST NLS^{mut}. Ia-d Show the subcellular localization of the mutated construct TWIST K38NLSR fused with C-myc due to the mutated NLS the protein mislocalized into the cytoplasm; IIa-d NLS rescue by interaction of SEF2 and mutated TWIST K38NLSR. Because of the interaction between these two proteins the functional NLS of TWIST with SEF2 is sufficient to get them both imported into the nucleus due to formation of heterodimer. The picture Ia-IIa show composite exposures of the objects, Ib-IIb represents the DAPI stained nuclei, Ic-IIc show the TWIST-cmyc fusion proteins detected by mouse anti-c-myc and Texas red conjugated anti mouse secondary antibody. In picture Id F-actin fluor 488 phalloidine showing the cytoskeleton, IId represents an exposure of SEF2 fused with eGFP.

3.18 Evaluation of NSEEE clones from the yeast two hybrid

In the first screening, more than 1000 yeast clones were obtained, In order to investigate the strongest interaction we used 3-amino-1,2,4-triazole (3-AT). This is a competitive inhibitor of the yeast HIS3 protein (His3p). 3-AT is used to inhibit the low levels of His3p leaky expression to better suppress background growth on SD medium lacking His (Fields 1993; Durfee et al. 1993).

Transformants derived from AH109 showed slightly elevated HIS3 expression because of intrinsic DNA-binding properties of the bait protein. A small amount of 3-AT is generally sufficient to suppress background growth on SD/–His. However, if the both HIS3 and

ADE2 are expressing, it is generally not necessary to suppress HIS3 leakiness in the initial library screen.

To optimize the 3-AT concentration, AH109 transformed with NSEEE bait plasmid on the SD/–Trp/–His plates containing 3-AT ranging from 0 to 50 mM (0, 2.5, 5, 7.5, 10, 12.5, 15 mM…..50mM). It is optimized that in case of NSEEE clones the strong interactions were obtained with 50 mM 3-AT concentration. Six hundred strongly interacted clones were obtained in this assay. DNA was extracted from these clones and amplified by using the vector specific primer in a PCR reaction. The nucleotide sequences of amplified products were sequenced and BLAST searched for the interesting interacted candidates. Several interesting clones found in this screening given below (Table 15).

Table 15: List of important screened NSEEE clones from yeast-two-hybrid assay

Clone	**Result**	**Remark**	**Gene Bank Acc.No.**
NSEEE(1-A1)	galactose mutarotase	(aldose 1-epimerase),	BC014916
NSEEE(1-A3)	Homo sapiens secreted protein	osteonectin	BC072457
NSEEE(1-A4)	Homo sapiens chorionic somatomammotropin hormone 1	Placental lactogen	BC062775
NSEEE(1-A5)	Homo sapiens ets variant gene 5	ets-related molecule	NM_004454
NSEEE(1-A8)	full-length cDNA clone CS0DI051YC24 of Placenta	Placental protein	CR626712
NSEEE(1-A11)	Homo sapiens KIAA0892 (KIAA0892), mRNA	Placental protein	NM_015329
NSEEE(1-B1)	Homo sapiens chorionic somatomammotropin hormone 1),	placental lactogen	BC002717
NSEEE(1-B2)	Homo sapiens pregnancy specific beta-1-glycoprotein 3	Placental protein	BC005924
NSEEE(1-B3)	Homo sapiens chromosome 17	unspecific	AC040958
NSEEE(1-B5)	Homo sapiens	clone CTB-25P15	AC011399

	chromosome 5		
NSEEE(1-B7)	Homo sapiens tyrosine 3-monooxygenase	participation in the protein kinase C signaling pathway	BC001359
NSEEE(1-B9)	Homo sapiens surfeit 4 (SURF4),	integral membrane protein	NM033161
NSEEE(1-B11)	Spast gene	SPG4-linked hereditary spastic paraplegia.	AJ246003
NSEEE(1-C4)	unspecific	-	-
NSEEE(1-C6)	Homo sapiens chorionic somatomammotropin hormone 1	(placental lactogen)	NM_022642
NSEEE(1-C8)	Homo sapiens chorionic somatomammotropin hormone 1	placental lactogen	NM_022642
NSEEE(1-C12)	Homo sapiens chorionic somatomammotropin hormone 1	placental lactogen	NM_022642
NSEEE(1-D1)	unspecific	-	-
NSEEE(1-E2)	Homo sapiens pregnancy specific beta-1-glycoprotein 4	Placental protein	BC063127
NSEEE(1-E7)	False positive		
NSEEE(1-E9)	False positive		
NSEEE(1-E12)	RP1-134O19 on chromosome 1p36.11-36.33	Contains the 3' end of the gene for SMART/HDAC1 associated repressor protein (SHARP), the 3' end of the ZNF151 genefor zinc finger protein 151 (pHZ-67) and a CpG island, complete sequence	AL034555
NSEEE(1-F6)	unspecific	-	-
NSEEE(1- F8)	Homo sapiens chorionic somatomammotropin hormone 2 (CSH2),	human placental lactogen genes	NM_022646

	transcript variant 4		
NSEEE(1-G1)	False positive		
NSEEE(1-G4.1)	Homo sapiens splicing factor, arginine/serine-rich 3	Placenta, choriocarcinoma	BC000914
NSEEE(1-G4.2)	False positive		
NSEEE(1-G5)	Homo sapiens PC2 (positive cofactor 2, multiprotein complex)	glutamine/Q-rich-associated protein	BC005027
NSEEE(1-G6)	False positive		
NSEEE(1-G8)	Homo sapiens chorionic somatomammotropin hormone 1	(placental lactogen)	NM_022642
NSEEE(3-A8)	unspecific	-	-
NSEEE(3 -B3)	Unknown genomic sequence on chromosome 10	unknown	AL359878
NSEEE(3 -B4)	Homo sapiens 3 BAC RP11-26P16 (Roswell Park Cancer Institute HumanBAC Library) complete sequence		AC117416
NSEEE(3-B5)	Homo sapiens chorionic somatomammotropin hormone 1	(placental lactogen)	NM_022642
NSEEE(3-B8)	cDNA clone of Neuroblastoma	Neuroblastoma protein	CR607127
NSEEE(4-B9)	prostaglandin D2 synthase gene	prostaglandin D2 synthase	M98538
NSEEE(4-C6)	Homo sapiens FK506 binding protein 9,		NM_007270
NSEEE(5-A62)	Homo sapiens asparagine synthetase (ASNS),		NM_001673
NSEEE(5-A7)	Homo sapiens ALR-like protein mRNA	involved in leukemia and brain development	AF264750
NSEEE(5-B4)	Homo sapiens chorionic somatomammotropin	(placental lactogen)	NM022641

	hormone 1 transcript variant 3		
NSEEE(5-B6)	tissue inhibitor of metalloproteinase 2		NM_003255
NSEEE(5-C6)	Synaptosomal associateprotein		BC000148
NSEEE(5-G7)	Homo sapiens cDNA FLJ38210 fis	highly similar to Mus musculus neuronal cell adhesion molecule (Punc) mRNA	AK095529
NSEEE(5-G9)	RAS p21 protein activator	GTPase activating protein	BC020761

Among them 6 candiates are recognized most promising candidates. These are ETV5, *Homo sapiens* SURF4, Spastin, *Homo sapiens* FK506 binding protein9, *Homo sapiens* tissue inhibitor of metalloproteinase 2, and ALR proteins.

4 Discussion

In eukaryotic cells, evolution of the nuclear envelope to define cytoplasmic and nuclear compartments led to a physical separation of transcription and translation. Nuclear proteins (structural polypeptides or regulatory factors), which are implicit in this compartmentalization and are synthesized in the cytoplasm, have to be imported into the nucleus to exert their activities for cellular growth and differentiation.

The active transport of karyophillic proteins allows another way to exert gene regulation by modulating localization in the nucleus (Vandromme et al. 1996) in contrast to other small molecules that can freely diffuse over the nuclear pore complex. Transport across the pore complex is a two-step process involving binding to the pore complex and translocation across the lumen of the pore complex (Dinwall and Laskey 1991; Nigg et al. 1991; Boulikas 1994, Görlich and Mattaj 1996). Various studies on the nuclear localization signal have led to the concept that transport across the nuclear envelope is an active process mediated by one or more nuclear localization signal sequences present within either the protein or an associated cofactors (Reviewed by Garcia-Bustos et al. 1991; Nigg et al. 1991). Specialized transporter protein known as NLS-binding proteins can recognize one or several nuclear-localization-signal (NLS) sequences in the primary structure of the target protein and transports these proteins to the nucleus (Boulikas 1994).

NLSs have been characterized in a growing number of nuclear proteins using two criteria: NLS is sufficient to promote nuclear accumulation of an otherwise cytoplasmic protein when fused to it genetically or biochemically and, deletion of an NLS(s) from a nuclear protein leads to it cytoplasmic retention. Although a strict consensus does not yet exist, it appears that most NLSs are in short and linear sequences (from 5 to 12 amino acids) generally containing several basic residues (arginine and lysine) (Boulikas 1994). Moreover, presence of a NLS on a protein is not always sufficient to direct its nuclear import, as the NLS might be modified or masked so that it is no longer recognized by the nuclear transport machinery. Masking the NLS might be achieved by post-translational modifications, such as phosphorylation and/or by intra- or inter-molecular interactions. The protein is then sequestered in the cytoplasm until the NLS is unmasked. Furthermore, an NLS-containing protein might be anchored in the cytoplasm by binding to another protein, as a result of a specific amino acid sequence whose effect is dominant (Vandromme et al. 1996).

NLS motifs are not limited to only nuclear protein contains but can be exhibited by some cytoplasmic proteins (Smith et al. 1985). However, these proteins are not available for a nuclear import, since they are held back due to their respective function by association with diaphragm proteins or cytoplasmic structures in the cytoplasm (Boulikas 1994).

Mutations in sequences relevant for nucleus transport, which exhibit resident proteins, shuttle molecules or any interaction partners in the cell nucleus, can lead to a false distribution of these proteins in the cell leading to loss of functionality. The expression of the target genes can then be impaired due a lack of nuclear import by the transcription factor.

Regulation of *TWIST1* gene expression was analyzed to characterize the poorly understood amino terminal region of the bHLH transcription factor TWIST.

In this study we provide evidence that nuclear import of the bHLH TWIST protein is mediated by two functional nucleus localization signals sequences present in the N-terminal region of the TWIST protein and that the heterodimerization of TWIST with class A bHLH proteins is prerequisite for nuclear translocation of TWIST protein. NLSs of TWIST protein substitutions were analyzed for the functional consequences.

4.1 Nuclear import directed around the functionality of two putative nucleus localization signals of TWIST

According to Boulikas (1994) postulate, the basic region of transcription factors often contains potential nucleus localization signals, although in the case of the bHLH transcription factors TWIST (amino acid position 108-120) no nucleus localization signals are observed in the basic domain. The identification of the TWIST NLSs therefore represents an important step in the understanding the regulation of TWIST localization. Two classical nucleus localization signals sequences were detected from data base searches at the N-terminal of the TWIST protein, which are found to be rich in basic amino acids lysine and arginine. The NLSs characterized in TWIST fulfil the requirements for nucleus localization signals as postulated by Boulikas criteria. The first cluster (NLS1) consists of 37-RKRR-40 and the second cluster (NLS2) consists of 73-KRGKK-77 respectively (Fig 23).

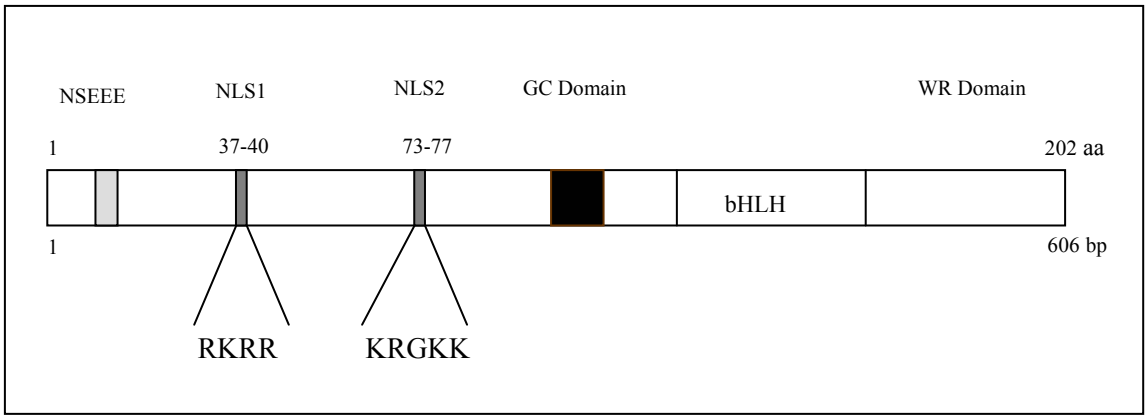


Fig. 23: Potential topogene core localization signals within the Twist protein sequence. The grey coloured boxes amino acids represent the potential NLS1 and NLS2 of TWIST, at the positions of the amino acids 37-40 (RKRR) as well as 73-77 (KRGKK), respectively. The glycine-rich as well as the basic domain are present at the amino acid positions 80-98, and/or 108-120. The Helix Loop Helix domain (aa position 121-160) is responsible for the homo and/or heterodimerization of the transcription factor TWIST.

NLS was first reported by Prendergast et al. (1991), who characterized NLS1 in Myn (murine homolog of Max) protein and reported that it forms a DNA-binding complex with c-myc oncoprotein through a helix-loop-helix/Leucin Zipper. Consistent with this, Blackwood and Eisenmann (1991) reported that the neither max nor myc alone exhibit appreciable DNA binding.

The second putative NLS sequence (NLS2) was reported to contain arginine and lysine separated by a glycine amino acid (KRGKK). This motif for nuclear import was reported as functional transportation signal in the oestrogen receptor (Green et al. 1986). Different types of mutations in TWIST protein such as missense or nonsense are responsible for the clinical features of the Saethre-Chotzen syndrome. However, the mutation in conserved region of the Twist proteins affects their nuclear localization and inability to bind the DNA (Takebayshi et al. 1996). As the recognition of NLS motifs can involve tertiary structure recognition as well as amino acid sequence, (Boulikas 1994; Vandromme et al. 1996), it is important to carry out mutational studies without compromising protein conformation in the N-terminal range of the TWIST protein to avoid masking of potential NLS motifs which can be analyzed by the c-myc-epitope. For this reason the nucleotide sequences coding for the c-myc-epitope was fused at the 5'-end of TWIST cDNA fragment and subcellular localization was examined in U2-OS cells by the indirect immune fluorescence microscopy.

The localization of TWIST protein predominantly in the U2-OS cell nucleus is consistent with previous reports of TWIST protein localization in COS7 cell nucleus (El Ghouzzi et al. 2000), suggesting that nuclear transport is an active process. Although the small molecular weight of TWIST protein (approximately 25 kDa), could allow it to localize easily to the core pore complex by free diffusion, it is assumed that Twist is imported actively into the nucleus during embryonic development of vertebrates and invertebrate, as it's transcriptional activity is critical during these developmental stages. Intracellular communication between nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs) present in the nuclear envelope that allow passive diffusion of molecules smaller than 40 to 60 kDa (up to 9 nm in diameter) (Görlich and Kutay 1999; Rout and Aitchison 2000). However, larger macromolecules, and even some proteins or RNAs smaller than 20 to 30 kDa, such as histones and tRNA, might be transported actively in a multi-step manner through NPCs, usually involving proteins encoding one NLS and using NLS-mediated import machinery (Lee and Aitchison 1999).

Substituted NLSs were studied in order to know that if the mutations in NLSs motifs altered the TWIST function through different mechanism. First a series of amino acid point mutations were constructed, resulting in lysine to arginine substitutions in the NLS of a TWISTcDNA~cmyc^{N} fusions construct. The subcellular localization of the NLS mutated constructs were analyzed by an immune fluorescence microscopy in U2-OS cells, so that both the nucleus import mechanism as well as nucleus localization signals functionality could be assayed. From these studies it was determined that alteration of the both NLS sequences resulted in a great loss of TWIST nuclear import. It is also observed that the either NLSs individually is required but not sufficient to promote nuclear translocation of the TWIST protein *i.e.* they synergistically play a role in the TWIST protein import.

Funato et al. (2005) reported a novel mutation at 115C>G, encoding A39G, located within the putative NLS1, in a SCS patient, where both nuclear and cytoplasmic expression was observed in COS7 cells. TWIST protein carrying NLS1 (K38R) was found to be retained in the U2-OS cell cytoplasm in this study (Fig. 13), consistent with the observations of Funato and co-workers, and confirm that the N-terminal NLS 1 is functional in nuclear import. Comparable observations were made for murine Dermo-1, a bHLH protein encoded by the *TWIST2* gene, in which engineered replacement of the

equivalent NLS (also containing the RKRR sequence) by four alanine residues impaired nuclear import (Gong and Li 2002).

Similarly, the functionality of NLS2 was checked in the U2-OS cells to substitute the amino acids at the positions K73R, K76R, and K77R. It was observed that NLS2, like NLS1, also played an essential role in the nuclear import of TWIST protein. From these findings, it is concluded that altered NLS variants carrying substitutions at all the above mentioned positions, except the substituted, lead to cytoplasmic retention. By contrast, NLS2 with substitutions at position K76R was found to be localized predominantly in the nucleus, suggesting that the in NLS2 lysine at position K76R alone does not play essential role in the nucleus import. By contrast, NLS2 K76R in combination with mutated NLS1 was found sufficient to promote the nuclear import of TWIST protein.

4.2 Dimerization and nuclear entry of TWIST protein in U2-OS cells

In class B bHLH proteins to which TWIST belongs, the helix-loop-helix are known to form heterodimers with members of class A bHLH transcription factors including E12 and E47 (collectively termed as E2A), which are alternatively spliced products of the *TCF3* gene (Murre et al. 1989*a*; Murre et al. 1989*b*), and E2-2, E2-5 (Henthron et al. 1990). In some proteins like MyoD the NLS are often present in the bHLH region, but this is not the case with TWIST protein. Missense mutations of the bHLH region of TWIST either lose the ability to recruit partner bHLH molecules (such as E2A) or abolish DNA binding, and hence fail to repress E2A-dependent activation of p21 expression (El Ghouzzi et al. 2000, 2001; Funato et al. 2001), resulting in craniosynostosis. TWIST/E2A heterodimers suppress osteoblast differentiation by down regulating the expression of CDKN1A, which encodes p21/WAF1/ CIP1 (referred as p21), an inhibitor of cyclin-dependent kinases (Harper et al. 1993; Funato et al. 2001). In a co-transfection assay, the findings in this study suggest that E12 strongly interacted with TWIST in U2-OS cells. Heterodimerization of HLH proteins have been previously reported to take place in the nucleus due to nuclear import (El Ghouzzi, 2000). This study confirmed that the expected nuclear localization of the wild-type TWIST protein appears to be a direct consequence of the *in vivo* interaction with E12.

It is also well known that a missense mutation in the helical domain lead to complete TWIST hetrodimerization loss with E12 and resulting in mislocalization (El Ghouzzi et al. 2000). Consequently, the nucleoplasmic location of TWIST proteins carrying single

amino acid change in the NLS1 as well as in NLS2 with E12 result from the dimer formation. This study concludes that in cotransfection experiments, E12 can functionally compensate non-functional NLS in TWIST.

TWIST protein loses its function as negative modulator during the differentiation of different mesodermal layers *e.g*. in the myogenesis, osteogenesis or neurogenesis (Hebrok et al. 1994; Stoetzel et al. 1995; Spicer et al. 1996; Hamamori et al. 1997). By contrast Vandromme and coworkers (1995) reported that a deletion in bHLH factors in MyoD is responsible to loss the dimerization ability with E12 and concluded that these proteins can be transported also as monomers into the cell core.

Twist can interact with other proteins through its N-terminal and C-terminal domains. In addition, individual homodimer and heterodimer bHLH combinations can confer differences in DNA binding affinity, target preference site and biological activities, emphasizing that partner choice is an additional key regulation point for bHLH proteins (Jones 1990; Kadesch, 1993). Besides the two putative nucleus localization signals and bHLH, two additional motifs are also present (NSEEE and WR) in Twist proteins. The function of these motifs in Twist is not yet fully understood. In order to investigate the characterization of these two domains in TWIST protein a yeast –two-hybrid assay was performed.

4.3 Yeast-two-hybrid systems to interact the protein using TWIST and its domains

Yeast-two-hybrid assay is used to analyse the target protein's ability to dimerize with bait protein. There are several advantages to using the two-hybrid system to isolate novel cell type specific partners for TWIST and its motifs. It is a functional assay, and based only on the ability of proteins to dimerize. The assay is also highly sensitive and specific. Additionally, there is an evidence that the bHLH motif interacts with co-regulators to induce muscle specific transcription (Brennan et al. 1991; Davis et al. 1990).

There is considerable evidence for cell type-specific bHLH proteins involvement in the control of tissue-specific gene expression in vertebrates. (i) Twist and several related E-proteins that are widely expressed, dimerize preferentially with cell type-specific HLH proteins such as MyoD and myogenin (Chakraborty et al. 1991; Murre et al. 1989*a*, 1989*b*). (ii) Id, a negative regulator of HLH protein DNA binding, is expressed in many undifferentiated cell types and is down regulated upon activation of differentiation, which has been proposed to release E-proteins to dimerize with cell type-specific

partners (Benezra et al. 1990). (iii) many tissue specific genes contain E-boxes in their control regions that are essential for cell type specific transcription (Murre et al. 1989a; Olson, 1990; Sartorelli et al. 1992), and (iv) the involvement of numerous cell type-specific bHLH proteins in cell fate specification in *Drosophila* suggests that these proteins constitute a conserved mechanism for regulation of cell type specific transcription (Alonso and Cabrera 1988; Thisse et al. 1988).

It is evident that '*twist*' gene can be isolated from species ranging jellyfish to man, it is less apparent what the actual functions of Twist and its domains are in these different organism. Twist protein was shown to contain an amino terminal histone-acetyl-transferase (HAT) binding domain (Hamamori et al. 1999), for which the NSEEE domain could be possibly responsible. The N-terminal region of D-Twist (amino acid 1-330) has been implicated in transcription activation (Shirokawa and Courey 1997) and in amino acids 141–330 of D-Twist have been shown to interact with TAF110, a subunit of the TFIID complex, to mediate target gene activation (Pham et al. 1999).

The WR motif is highly conserved from jellyfish to vertebrates located at the C-terminal end of Twist (Spring et al. 2000), but the function of this domain was uncharacterized at the beginning of this study. However, a nonsense mutation in the *TWIST1* gene just before the WR motif also causes a mild appearance of the Saethre–Chotzen syndrome, suggesting that C-terminal domain is required either for TWIST activity, for the stability of its mRNA or for normal protein folding (Gripp et al. 2000). The M-Twist C-terminal region point mutations protein prevented its interaction with the transcription factor MEF2 (Spicer et al. 1996).

Furthermore, Bialek et al. (2004) reported that WR domain is the binding domain for RunX2 protein, the master regulator of osteoblast differtiation. The interaction of TWIST (Twist box) and the Runt domain of RunX2; prevents DNA binding and gene activation by RunX2. A point mutation in the Twist box of *Twist-1* leads to accelerated bone formation in both heterozygous and homozygous mice. Bialek et al. (2004) conclude that *Twist-1* (previously called *Twist*), and Twist-2 (previously called *Dermo-1*) regulate the development action of RunX2 in bone formation through the direct interaction of these proteins. WR domain could be functional due to the interaction with transcription factors such as RunX2 or other unknown transcription factors. In this study, TWIST WR domain was evaluated to find out the interacting partners using yeast-two hybrid assay. One hundred twenty clones were obtained, but after screening

of these 120 seems positive clone by blast search analysis, the real interaction could not be detected. It is well known that in two hybrid screen relatively false positively interactions were found frequently. It might be the possibility that the interaction of this domain with other proteins needs the bHLH motif.

4.3.1 Potential interacting partners of TWIST

Several interesting candidates are found interacted with entire TWIST and NSEEE domain by using a human placental cDNA library in this study, are discussed below.

4.3.1.1 SEF2 Protein

As discussed above, TWIST heterodimerizes with other bHLH transcriptional factor proteins. Hamamori et al. (1997) reported a connection of Twist and MyoD shown to prevent the specific activation of target genes for osteogenic differentiation-a substantial mechanism of the molecular pathogenesis of the Saethre–Chotzen syndrome. In an attempt to further explore this interconnection of cross-talk between different types of transcription factors, a potential candidate of bHLH transcription factor encoded E2-2 protein also known as immunoglobulin transcription factor 2, (ITF2 or SEF2-1B or SEF2 or TCF4) referred here as SEF2 was identified, which is located on chromosome 18q 21.1 (Breschel et al. 1997).

The strong expression of SEF2 in the brain tissue (Liu et al. 1998); its binding and regulatory activity with some neuron-and neuroendocrine-specific promoters, including the tyrosine hydroxylase enhancer and the somatostatin receptor II promoter (Yoon and Chikaraishi 1994; Pscherer et al. 1996), along with SEF2 upregulation in osteoblast differentiation (Beck et al 2001) makes it an interesting candidates. Based on these previous reports and the interaction of SEF 2 with TWIST reported in this study along with their possible hetero dimerization confirmed by *in vitro* and NLS rescue assay, it might be possible that the heterodimer of TWIST with SEF2 involved in the osteogenesis and brain development and makes an interesting topic for further investigation.

Furthermore TWIST and SEF2 expression was confirmed in the U2-OS cells. The hypothesis was based on the observation that if the cotransfection with SEF2 NLS deficient TWIST restores into the nucleus then it could be responsible for a real interaction. As NLS deficient TWIST is unable to localize into the nucleus, this study illustrates that TWIST can form a functional complex together with SEF2-like with

E12. It is concluded that since heterodimerization of both proteins has to occur in the cytoplasm prior to nuclear import, the expected nuclear location of the wild type TWIST protein appears to be a direct reflection of the *in vivo* interaction with SEF2.
Hence, SEF2 appears as a putative partner *in vivo* for regulating the TWIST transcriptional activity. Interestingly, TWIST and E2A transcripts have been detected in mouse osteoblastic cells (Murray et al. 1992), and the inhibitory effect of TWIST in osteogenic differentiation (Bialek et al. 2004), the TWIST-SEF2 hetrodimer might be a negative regulator of transcription in the human osteoblastic cells.

4.3.2 Potential interaction partner of NSEEE domain

Using selection plates many different potentially positive clones were isolated, and some of these clones possibly could be very interesting candidate proteins which are described below.

4.3.2.1 *Homo sapiens* ETS variant gene

The most promising interacting candidate was the NSEEE clones (1-A5) *i.e. Homo sapiens* ETS variant mRNA (Etv5) which corresponds, gene bank AccNo NM_004454. The ETS family transcription factors have in common the ETS-domain, an 85 amino-acid domain organized in a winged helix-turn-helix (HTH) structure and responsible for DNA-binding to the specific core sequence GGA (A/T) (Sharrocks 2001). These factors can be sub-classified in 13 groups according to the amino-acid conservation not only in the ETS-domain but also in other domains, such as transactivation or protein interaction domains. The PEA3 group is made up of three members: Pea3 (E1af, Etv4), Er81 (Etv1) and Erm (Etv5). These three factors are more than 95% identical in the ETS-domain, and highly conserved in the N-terminal acidic and C-terminal transactivation domains (de Launoit et al. 2000; Kurpios et al. 2003). These transcription factors are involved in developmental processes and oncogenesis (de Launoit et al. 2000). For example, in the mammary gland, their overexpression is observed in certain human breast cancers as well as in oncogene-induced mammary tumors (Baert et al. 1997, Shepherd and Hassell 2001). They are also expressed upon different stages of normal mammary gland development, from embryonic emergence to postnatal postnatal evolution (Chotteau-Lelievre et al. 2001; Chotteau-Lelievre et al. 2003), with high levels of expression during extensive ductal outgrowth and branching. As mentioned above Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis (Yang et al

2004), offering the possibility that TWIST NSEEE domain is able to interact with Etv5 to play a role in human breast cancer thereby making this interaction a interesting topic for further investigation.

4.3.2.2 *Homo sapiens* surfeit locus

The other important interacting protein was NSEEE clone (1-B9*)* that contains integral membrane *Homo sapiens* surfeit 4 (SURF4) which corresponds, gene bank Acc. No. NM_033161.

The mouse Surfeit locus contains at least six housekeeping genes (Surf-1 to -6) 2 in the tightest mammalian gene cluster (Garson et al. 1995; Huxley and Fried 1990), with no DNA sequence or amino acid homology among the six surfeit genes. The locus contains a number of novel features including overlapping genes (Williams and Fried, 1986) and a bidirectional transcriptional promoter (Lennard and Fried 1991). The 5' end of each of the genes is associated with a CpG-rich island (Huxley and Fried 1990). The Surf-3 gene encodes the ribosomal protein L7a (Giallongo et al. 1989), the Surf-4 gene encodes an integral membrane protein associated with the ER (Reeves and Fried, 1995, the two proteins encoded by Surf-5 as a result of differential splicing are cytoplasmic (Garson et al. 1995, 1996), the Surf-6 protein is located in the nucleolus (Magoulas and Fried, 1996), and a yeast homologue of the Surf-1 gene encodes a mitochondrial membrane protein involved in respiration (Mashkevich et al. 1997). The human Surfeit locus maps to 9q34.2 (Yon et al. 1993), and the mouse Surfeit locus maps to a syntenic region within the proximal portion of chromosome 2 (Stubbs et al. 1990). The *Homo sapiens* Surfeit 4 protein is not yet well characterized.

4.3.2.3 Spastin protein

The other interacting candidate was the NSEEE clones (1-B11) *i.e.* Spastin protein contains SPG4-linked hereditary spastic paraplegia corresponds, gene bank Acc. No. AJ246003.

This protein is involved in an autosomal dominant hereditary spastic paraplegia (AD-HSP), a genetically heterogeneous neurodegenerative disorder characterized by progressive spasticity of the lower limbs. The hereditary spastic paraplegias comprise a group of inherited neurological disorders in which the primary manifestation is spastic weakness of the lower extremities. Troyer syndrome is an autosomal recessive form of spastic paraplegia caused by a frameshift mutation in the spartin (*SPG20*) gene. The

SPG4 locus at chromosome 2p21–p22 has been shown to account for 40–50% of all AD-HSP families. Sequence analysis of this gene in seven *SPG4*-linked pedigrees revealed several DNA modifications, including missense, nonsense and splice-site mutations. Both *SPG4* and its mouse orthologue were shown to be expressed early and ubiquitously in fetal and adult tissues. The sequence homologies and putative subcellular localization of spastin suggest that this ATPase is involved in the assembly or function of nuclear protein complexes (Hazen et al. 1999). Futhermore, Bakowska et al. (2005) suggested that spartin might be involved in endocytosis, vesicle trafficking, or mitogenic activities and that impairment in one of these processes may underlie the long axonopathy in patients with Troyer syndrome. The interaction of TWIST NSEEE with spastin protein could be possible to play an important role in the development of the lower limbs and might be an interesting topic for further investigation.

4.3.2.4 *Homo sapiens* FK506 binding protein 9

The other interacting candidate NSEEE (4-C6), was *Homo sapiens* FK506 binding protein 9 corresponds, gene bank Acc. No. NM_007270.

The FK506-binding proteins (FKBPs) are peptidyl–prolyl cis/trans isomerases (PPIases) that bind the immunosuppressive drug FK506. It is localized to the endoplasmic reticulumand associates with tropoelastin in the secretory pathway. Unlike other FKBP characterized so far, FKBP65 is developmentally regulated and may be intimately involved in organogenesis (Patterson et al. 2002).

4.3.2.5 *Homo sapiens* tissue inhibitor of metalloproteinase 2

The other important interacting candidate was the NSEEE clones (5-B6) *i.e.* tissue inhibitor of metalloproteinase 2 protein corresponds, gene bank Acc. No. NM_003255.

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play a role in the processes of extracellular matrix degradation. Changes in their expression levels have been observed in various tumor types, including lung carcinoma and breast cancer (Gouyer et al. 2005). However, their clinical significance and their prognostic importance in the progression of non small cell lung carcinoma (NSCLC) remain to be characterized.

4.3.2.6 ALR protein

The other important candidates was NSEEE clones (5-A7) *i.e.* protein ALR protein corresponds, gene bank Acc. No. AF264750.

A novel human gene (designated HALR for "homologous to ALR" and given the symbol *MLL3* by the HUGO Gene Nomenclature Committee) encodes a predicted large hydrophilic nuclear protein. HALR amino acid sequence is present in SUVAR3-9, an enhancer of zeste, trithorax (SET) domain, three plant homeodomain (PHD)-type zinc fingers, a high motility group (HMG)-1 box, a leucine-zipper-like pattern, two potential transactivating domains, several nuclear localization signals, and multiple nuclear receptor interaction signature motifs. Especially within the SET domain, PHD fingers and several other regions, the HALR protein exhibits significant similarity to ALR (acute lymphoblastic leukemia (ALL)-1 related), ALL-1/myeloid/lymphoid or mixed-lineage leukemia (ALL-1/MLL), and trithorax, evolutionarily conserved proteins that influence differentiation and development. (Tan and Chow 2001) isolated the partial cDNA of the murine homologue of HALR, which displays high homology to its human counterpart. Taking into consideration its notable protein motifs, ubiquitous expression, evolutionary conservation and chromosomal position, HALR is likely to play a housekeeping role in transcriptional regulation, and may be involved in leukemogenesis and developmental disorders.

The identification of several promising interacting partners in this work strongly suggests that transcriptional regulation is mainly executed by these interacting and potentially other interacting proteins. The fact that TWIST may potentially play critical roles in other unknown regulatory pathways leaves open the possibility of additional regulatory factors or novel mechanisms being discovered in the future. In order to fully elucidate this transcriptional mechanism, further studies are needed to fill gaps of knowledge.

5 Summary

Saethre-Chotzen syndrome is an autosomal dominant inherited disorder with premature fusion of cranial sutures. It is caused by nucleotide sequence changes within or in proximity of the *TWIST1* gene. This gene encodes for a bHLH transcription factor, which inhibits osteogenic differentiation by transcriptional control of various target genes. The aim of my work is to characterize functional domains in TWIST protein, and to determine the interacting partner for TWIST and its motifs particularly NSEEE and WR. The present study was thus undertaken to determine how *TWIST1* gene mutations affect protein function. Evolutionary alignment of Twist proteins from different species, indicate TWIST contain 4 additional conserved regions such as NSEEE, NLS1, NLS2, and WR-domain besides the bHLH motif. The bHLH domain is thought to be responsible for heterodimerization with other bHLH proteins such as E12 protein or SEF2 protein. The functions of NSEEE, NLS1, NLS2, and conserved WR motifs are poorly understood at present.

First, I focused on functional characterization of the NLS1 and NLS2 domains as potential nuclear localization signals in TWIST., Specifically the effects of various NLS substitutions in TWIST on cellular localization was assayed by immunoflourescence assay. In particular, TWIST NLS1 altered at amino acid position K38R was found to be retained in the cytoplasm of transiently transfected U2-OS cells, suggesting that NLS1 is functional and essential for the nuclear transport of TWIST. Additionally, to understand the role of the TWIST NLS2 in nuclear localization, amino acid at positions 73, 76 and 77 were substituted in this motif. These results demonstrated that substitution at NLS2 position 76 does not play an essential role in the nuclear localization of TWIST, in contrast to the K73R and K77R that inhibit nuclear accumulation. Although K76R mutants cannot inhibit nuclear localization by itself, we demonstrate it plays a synergistic role with the NLS1 K38R mutation to further reduce nuclear localization. This synergistic effect is consistent with the observation that combined K38R (NLS1) and K76R (NLS2) mutants dramatically reduced nuclear localization, further suggesting that both NLS1 and NLS2 work together in regulating nuclear localization of TWIST protein.

TWIST belongs to class B bHLH proteins which are known to form stable heterodimers with members of class A bHLH transcription factors including gene products of E12 and E47, respectively. Accordingly, the subcellular localization of NLS1 and NLS2 in TWIST protein was investigated in U2-OS cells following co-transfection with E12. The

cotransfections with heterodimerization partner E12 and NLS1-mutated TWIST led to a compensation of the mislocalization.

The second aim of my work was to identify the interacting proteins that could influence the functionality of TWIST using yeast-two-hybrid assay. I wanted to determine if the TWIST protein or its conserved motifs interact with other regulatory proteins to help to regulate the TWIST transcriptional activity. Using the entire coding sequence of *TWIST1* gene, an interesting candidate was found belonging to the class A bHLH transcription factors that includes the *SEF2* gene product. The direct interaction of SEF2 with TWIST was verified in a yeast mating assay and then confirmed in an *in vivo* NLS-rescue assay using U2-OS cells, showing that SEF2 forms a heterodimer with TWIST protein and co-localized into the nucleus.

Furthermore, two more highly conserved TWIST motifs NSEEE and WR were analyzed individually to find out their interacting proteins and their role in regulating the *TWIST1* transcriptional activity. I found more than 1000 yeast clones for NSEEE motif and 120 clones for WR motif. The second screening of the yeast clones suggested some promising candidates protein as interacting partners with the NSEEE motif such as ETV5, SURF4, Spastin, Metalloproteinase 2, and ALR-like protein mRNA. By contrast I could not detect any interesting interacting partners with the WR domain. A possible explanation may be the requirement of bHLH to mediate WR interaction with other proteins.

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Posters

1. Singh S, Rauschendorf M, Kunz J (2005) A bipartite nucleus localisation signal is responsible for the nuclear transport of the bHLH transcription factor TWIST. Poster presented in 6th Annual Meeting of the German Society of Human Genetics in Halle/Germany

2. Singh S, Uebe S, Kunz J (2004) TCF4 interact with TWIST and contributes to import the NLS. Poster presented in European conference of Human Genetics, Munich 13 June-17 June, 2004.

3. Singh S, Godmann M, Kosan C, Kunz J (2003) Functional characterisation of the nuclear localisation signal in the bHLH transcription factor gene TWIST. 14 Jahrestagung der Deutschen Gesellschaft für Humangenetik gemeinsam mit der österreichischen Gesellschaft für Humangenetik und der Schweizerischen Gesellschaft für Medizinische Genetik, Marburg, Germany. Medizinische Genetik (3), 348,

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Shalini Singh

Declaration

I hereby declare that the present piece of work entitled **"Identification and Functional Characterization of Protein Domains in the Transcription Factor TWIST"** is the result of my own investigation and as such it is being presented for the award of Dr. Rer. Physiol. Philipps University Marburg, Germany.

The thesis has not been submitted in the current or a similar form to any other University.

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