LINC, a novel protein complex involved in the regulation of G2/M genes

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1 Introduction

1.1 Pocket proteins and E2F transcription factors in cell cycle progression

1.1.1 Retinoblastoma protein pRB and the pocket protein family

Regulated progression through the cell cycle is essential for ordered cell proliferation. Changes in the balance between cell cycle-driving proto-oncogene-dependent pathways and inhibiting signals from tumor suppressors are a common cause for cancer. One of the best characterized tumor suppressors is the retinoblastoma protein pRB, which was the first cloned tumor suppressor (Lee et al., 1987). This gene was first described as a susceptibility gene for retinoblastoma, an eye tumor in children, but is now also known to be mutated in many cancers (Sherr, 1996).

pRB belongs to the family of the pocket proteins together with p130 and p107. They share about 30-35 % sequence homology, especially in a region called the pocket domain. p130 and p107 are even closer related and share a homology of 50 % (Lipinski and Jacks, 1999). The pocket domain consists of two conserved regions A and B separated by a linker that is different in the three pocket proteins. Through the pocket region, pocket proteins bind to the E2F transcription factors as well as to cofactors carrying a conserved LXCXE motif. Some viral oncoproteins like adenoviral E1A, papilloma E7 or SV40 large T antigen have such a domain and are able to bind pRB, leading to a conformational change in pRB and thereby to mitogen-independent cell cycle progression (Felsani et al., 2006). Other proteins binding to the pocket proteins through their LXCXE domain include histone deacetylases (HDAC) and cyclins (Giacinti and Giordano, 2006).

pRB is regulated by cell cycle-dependent phosphorylation. In quiescent cells and in early G1, pRB is unphosphorylated and active. It binds to and inactivates E2F transcription factors which results in a cell cycle arrest (Chellappan et al., 1991; Goodrich et al., 1991). In late G1, cyclin D expression is induced by an active receptor tyrosine kinase pathway. Cyclin D binds to cdk4 and cdk6 and activates them. These active cyclin/cdk complexes then phosphorylate the pocket protein pRB. After phosphorylation by cyclin D/cdk4/6, the conformation of pRB changes and it

releases E2F. These transcription factors activate the expression of cyclin E, which in turn activates cdk2. The activated cyclin E/cdk2 complexes act as a positive feedback loop by also phosphorylating pRB. In addition to inducing cyclin E, E2Fs also activate other genes that are important for the further progression through the cell cycle (Bracken et al., 2004). Pocket proteins define the restriction point. Before this time point, mitogens are necessary for the cell cycle progression. Once pRB is phosphorylated by cyclin E/cdk2, no further mitogens are required for the entry into S-phase (Frolov and Dyson, 2004). The phosphorylation of pRB is inhibited by the cdk inhibitor families Cip1/Kip1 and INK4, leading to cell cycle arrest (Sherr and Roberts, 1999). The pocket proteins are redundant regarding cell cycle control. pRB knock-out cells can still arrest (Jacks et al., 1992; Lee et al., 1992), and a triple knock-out is required to completely abolish G1 arrest (Dannenberg et al., 2000; Sage et al., 2000).
In addition to its repressive function in cell cycle, pRB has also been described to positively act in development, differentiation (Lipinski and Jacks, 1999) and apoptosis (Chau and Wang, 2003).

1.1.2 E2F transcription factors

The family of human E2F transcription factors is composed of eight members.
E2F1, E2F2 and E2F3 are transcriptional activators that directly induce genes important for cell cycle progression. Some target genes with functions in cell cycle progression and DNA replication, for example cyclin E or cdc6, are expressed when the cell progresses from G1 to S phase. Others, e.g. cyclin A2 or cdc2, are expressed later in the cell cycle, namely in G2, and are responsible for later events in cell cycle progression. It is assumed that the expression of these delayed E2F target genes requires additional activators (Bracken et al., 2004).

E2F4 and E2F5 are pocket protein-dependent transcriptional repressors. In G0 and early G1, they are recruited to the nucleus by their interaction with pocket proteins. They carry nuclear export signals and therefore are inactivated in late G1 through their cytoplasmic localization (Gaubatz et al., 2001; Verona et al., 1997).

In addition to their opposing function, E2F1-5 also differ in their binding to pocket proteins. E2F1-3 preferentially bind to pRB, E2F4 can interact with all three pocket proteins and E2F5 only binds p130 and p107 (Cobrinik, 2005).

E2F6, E2F7 and E2F8 are pocket protein-independent repressors. E2F6 represses target genes by binding to polycomb group proteins, for example the oncoprotein Bmi1 (Gaubatz et al., 1998; Trimarchi et al., 1998; Trimarchi et al., 2001).

E2F1 to E2F6 dimerize with their interaction partners DP-1 or DP-2, which is necessary for their association with DNA. In contrast, E2F7 and E2F8 possess two DNA binding motifs and do not need DP dimerization (Christensen et al., 2005; de Bruin et al., 2003; Di Stefano et al., 2003; Logan et al., 2004; Logan et al., 2005; Maiti et al., 2005).

1.1.3 Mechanism of gene repression by pocket proteins

Unphosphorylated pRB binds to the transactivating motif of E2F1-E2F3 and thereby represses them in early G1. In addition, E2F4/pocket protein complexes and E2F5/p130 bind to the E2F-binding sites on their target gene promoters. This firstly prevents the association of activators and thereby gene activation. Secondly, repressive E2F/pocket protein complexes can also repress genes more directly. For example, they can recruit histone deacetylases (HDAC), leading to the local deacetylation of histone tails. This facilitates the nucleosome condensation and the genes are repressed due to lower accessibility for transcription factors (Frolov and Dyson, 2004).
Furthermore, pRB binds and recruits histone methyltransferases like SUV39H1 to promoters (Nielsen et al., 2001; Vandel et al., 2001). This leads to the methylation of Lysine 9 (K9) on histone H3 and subsequently to the recruitment of HP1 which stabilizes this modification (Jenuwein, 2001). Methylated H3K9 is linked to transcriptional repression (Kouzarides, 2007).

Besides leading to the covalent modification of histone tails, pocket proteins also recruit chromosome remodeling complexes. BRG1 and hBRM, homologues of yeast

SWI/SNF complex members, bind to pRB and this binding is essential for pRB-dependent G1 arrest (Dunaief et al., 1994; Zhang et al., 2000).
In summary, all these cofactors for pRB act on the chromatin structure or histone modifications and lead to gene repression and even propagated silencing in G0 and early G1 (Harbour and Dean, 2000; Trimarchi and Lees, 2002).

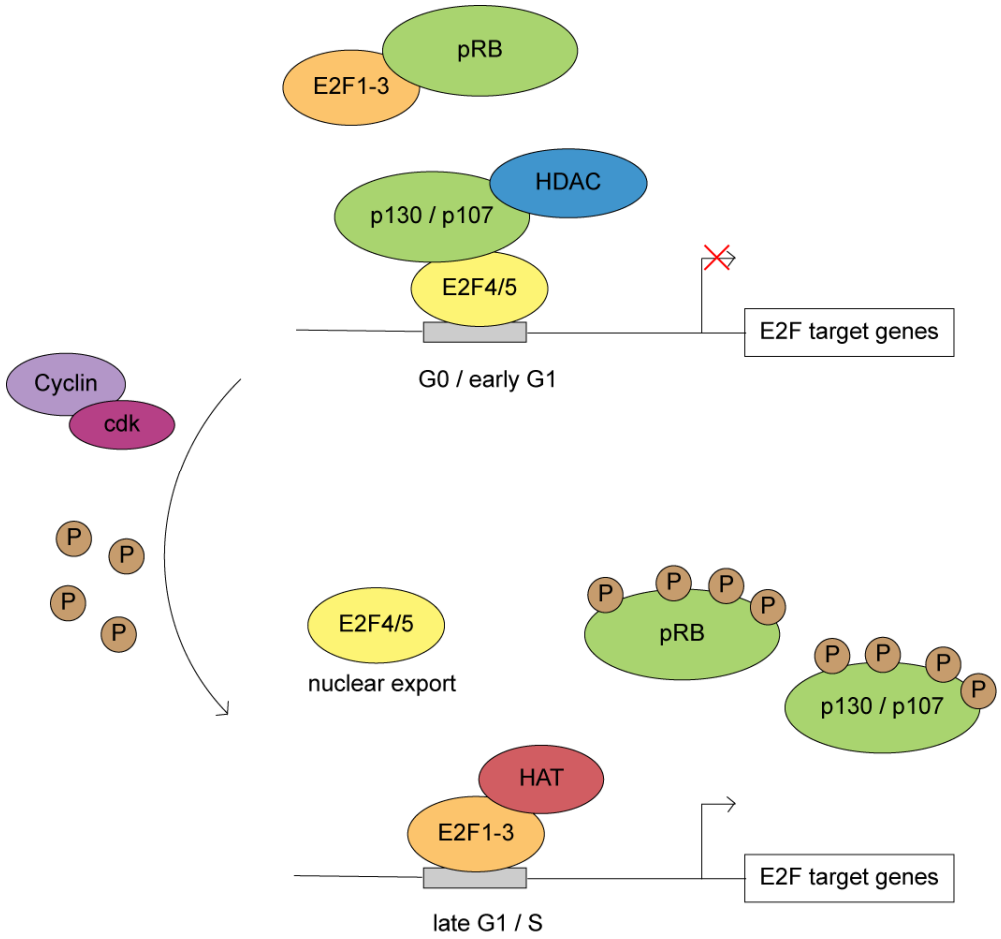


Fig. 1.1: Simplified schematic representation of E2F target gene regulation.
In G0 and early G1, activating E2Fs are bound and inactivated by unphosphorylated pRB. E2F4 and E2F5 bind to the promoters of E2F target genes together with p130 and p107. These pocket proteins recruit HDAC and other corepressors and the target genes are silenced. In late G1, pocket proteins are phosphorylated by cyclin/cdk complexes and E2Fs are released. The repressing E2Fs are exported from the nucleus and the activating E2Fs can bind to the promoters of their target genes, recruit coactivators and activate the expression of their target genes.

In late G1, when the pocket proteins are phosphorylated, the activating E2Fs are released and can bind to their target gene promoters. They recruit coactivators like the histone acetyltransferases (HAT) p300/CBP, P/CAF or Tip60 (Frolov and Dyson,

2004), which leads to the acetylation and activation of the genes. At the same time, the repressing E2Fs, which are only kept in the nucleus by their interaction with pocket proteins, are exported to the cytoplasm (Cobrinik, 2005). A simplified model of E2F target gene regulation by pocket proteins and E2F is shown in Fig. 1.1.

1.1.4 Regulation of the G2/M transition by E2F

Recently, genome-wide screens showed that E2F not only plays a role in the G1/S transition, but also regulates genes important for differentiation, development, apoptosis and G2/M transition (Ishida et al., 2001; Muller et al., 2001; Polager et al., 2002). Microarray experiments with cells overexpressing activating E2F transcription factors showed that for example cyclin B1, cyclin A2, cdc2 or polo-like kinase are activated by E2F1-3 (Ishida et al., 2001). ChIP-on-chip studies demonstrated that these genes are both regulated by E2F1 and E2F4. Target gene promoters are occupied by E2F4 in G0, which leads to gene repression, and by E2F1-3 at the G1/S transition, inducing the activation of the target gene (Ren et al., 2002). Target genes in this study comprise among others genes necessary for cell cycle progression (cyclin B1, cyclin A2, cdc2), chromosome segregation (securin, CENP-E), cytokinesis (PLK) and mitotic spindle checkpoint (Bub1, Mad2, CENP-E) (Ren et al., 2002; Takahashi et al., 2000). As there are two waves of E2F target gene expression, it was speculated that the expression of G2/M genes, in addition to E2F transcription factors, requires additional transcription factors. It was shown by Zhu and colleagues that the transcription factor B-MYB associates with the E2F G2/M target gene promoters when their expression is induced. This binding is dependent on a functional E2F site, and upon B-MYB depletion the target genes fail to be induced (Zhu et al., 2004). This strongly suggests that B-MYB, which is itself activated by E2Fs in G1, functions as a bridge between G1/S and G2/M E2F target genes.

1.2 Rb/E2F complexes in model organisms

Since the families of pocket proteins and E2F transcription factors have many members and often compensate for the loss of each other, functional studies in mammalian cells are very complicated. Therefore many studies were performed in

model organims, where the RB/E2F network is easier in comparison. In C. elegans, there is only one homologue of pRB and E2F each (LIN-35 and Efl-1) and the Drosophila melanogaster genome encodes for two pocket protein homologues (RBF1 and RBF2) and two E2F homologues, the activating dE2F1 and the repressive dE2F2.

1.2.1 The DRM complex in C. elegans is composed of synMuv class B proteins

In C. elegans, many genetic experiments have been performed. It was shown that LIN-35 and Efl-1 together with other proteins act in vulval development. They belong to the synMuv class B family of genes (Ceol and Horvitz, 2001). Mutation of one gene belonging to this class B together with one mutation in the synMuv class A or class C leads to a multivulva phenotype, suggesting that classes A and B function as two pathways repressing a vulval cell fate (Ferguson and Horvitz, 1989). Later it was shown that the synMuv gene products antagonize a Ras/MAPK pathway leading to the differentiation of the vulva (Beitel et al., 1990; Han et al., 1990).
In addition to pRB and E2F homologues and some genes with unknown or recently described homologues in mammalin cells (LIN-9, LIN-37, LIN-52, LIN-54), synMuv B genes comprise homologues of the nucleosome remodeling and histone deacetylation (NuRD) complex.
The synMuv B genes were identified in genetic studies where it was analyzed whether mutants of one gene give rise to a multivulva phenotype in a synMuv A mutant background (Beitel et al., 2000; Ceol et al., 2006; Davison et al., 2005; Ferguson and Horvitz, 1989). Due to the limitations of genetic screens and experiments however, it was not clear for a long time if all the synMuv class B proteins act together biochemically or if they belong to different complexes. It was speculated in analogy to other organisms that there is a NuRD complex. Solari and colleagues proposed a model, where the NuRD complex is recruited to promoter DNA by two redundant complexes of synMuvA and B proteins and represses developmental genes (Solari and Ahringer, 2000).

Some synMuv class B genes have recently been shown to interact in a biochemical complex called DRM (DP, Rb, MuvB). Members of this complex are: LIN-9, LIN-35

(pRB homologue), LIN-37, LIN-52, LIN-53 (RbAp48 homologue), LIN-54, DPL-1 and the E2F homologue EFL-1 (Harrison et al., 2006). This complex is indeed distinct from a NuRD-like complex containing the products of other synMuv B genes like HDA-1 (HDAC homologue), LET-418 (Mi2 homologue) and also LIN-53 (Harrison et al., 2006).

1.2.2 dREAM/MybMuvB complexes in Drosophila melanogaster

In 2004 two groups independently reported the identification and biochemical purification of complexes containing synMuv B homologues in Drososphila. These complexes, called dREAM (Korenjak et al., 2004) or Myb-MuvB (MMB) (Lewis et al., 2004), appear to function mainly as repressors of developmental genes.

dREAM contains dE2F2, the repressive E2F protein and either RBF1 or RBF2. Additional proteins in the complexes are dDP, the dimerization partner of E2F, p55/Caf1, the Drosophila homologue of mammalian RbAp48, the dMyb transcription factor and three previously identified Myb-interacting proteins mip120, mip130 and mip40 (homologues of LIN-54, LIN-9 and LIN-37 respectively) (Korenjak et al., 2004). The MMB complex is similar to the dREAM complex but contains an additional small protein, dLIN-52, and loosely associates with L(3)MBT and Rpd3, a homologue of HDAC (Lewis et al., 2004).

The dREAM complex binds to transcriptionally inactive sites on polytene chromosomes in-vivo and to deacetylated histone H4 in-vitro (Korenjak et al., 2004). Depletion of dREAM/MMB complex members leads to a derepression of genes known to act in differentiation (Dimova et al., 2003; Korenjak et al., 2004; Lewis et al., 2004), suggesting a role in the transcriptional repression of differentiation-specific genes for these complexes.
Interestingly, derepression of target genes did not occur upon dMyb depletion (Lewis et al., 2004), suggesting that dMyb is not involved in the transcriptional repression. Instead dMyb was shown to be essential for the amplification of the chorion gene cluster (Beall et al., 2002). Before the biochemical purification of dREAM and MMB, it was already shown that dMyb, p55/Caf1 and the dMyb-interacting proteins mip40, mip120, mip130 build a complex. This complex binds to the regulatory promoter

elements ACE3 and ori-β through dMyb and mip120, leading to the site-specific re-replication of the chorion gene cluster (Beall et al., 2002). The authors of these studies propose a model in which the dREAM/MMB complex has a dual function: It represses replication and transcription with a passive role of dMyb. Depending on the cellular context, dMyb can activate the complex which leads to gene amplification and target gene expression (Beall et al., 2004; Beall et al., 2002). This model is supported by a recent genome-wide study that used RNAi for every MMB/component and analyzed expression levels as well as localization of MMB on chromatin (Georlette et al., 2007). Georlette and colleagues could show that dREAM/MMB acts in the repression and in the activation of different target genes and that complex members preferentially colocalize near transcriptional start sites (Georlette et al., 2007).

Recently, a complex similar to dREAM/MMB, the testis-specific tMAC (Meiotic Arrest Complex), was purified from Drosophila (Beall et al., 2007). It contains the dREAM/MMB subunits mip40 and p55/Caf1 and the testis-specific proteins Always early (Aly), Tombola (Tomb) (homologues of LIN-9 and LIN-54 respectively), Cookie Monster (comr) and Matotopetli (Topi). Depletion of tMAC complex members by mutations in-vivo leads to reduced fertility or sterility through meiotic arrest in spermatogenesis (Beall et al., 2007; Jiang et al., 2007; Jiang and White-Cooper, 2003; Perezgasga et al., 2004).

Similarly to the situation in C. elegans, Drosophila also possesses a NuRD complex that is distinct from the dREAM/MMB complexes (Brehm et al., 2000; Kehle et al., 1998). Due to the association of proteins with known homologues in other organisms, the Drosophila NuRD complex has been linked to functions in transcription (Marhold et al., 2004; Stielow et al., 2008) and development (Kon et al., 2005).

The Drosophila and C. elegans complexes with their members are summarized in Fig. 1.2.

Drosophila			C. elegans
Lewis et al. 2004	Korenjak et al. 2004	Beall et al. 2007	Harrison et al. 2006
Myb/MuvB	**dREAM**	**tMAC**	**DRM**
mip130	mip130	Aly	Lin-9
mip40	mip40	mip40	Lin-37
dLin-52			Lin-52
mip120	mip120	Tomb	Lin-54
p55	p55	p55	Lin-53
dDP	dDP		dpl-1
dE2F2	dE2F2		Efl-1
RBF1/2	RBF1/2		Lin-35
dMyb	dMyb		
Rpd-3			
L(3)MBT			

Fig. 1.2: RB/E2F containing complexes in Drosophila and C. elegans.

1.3 A human synMuv-like complex?

In human cells, a NuRD complex was identified ten years ago (Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998). It was described as a complex with ATP-dependent nucleosome remodeling activity and a histone deacetylase activity, leading to transcriptional repression. It contains the ATPase MI-2, the histone deacetylase HDAC and RbAp48, which is present in many chromatin-associated complexes (Bowen et al., 2004).

This raises the question if there is also a mammalian RB/E2F complex similar to the situation in both Drosophila and C. elegans. Some human homologues of dREAM/MMB and DRM have already been characterized and are described in the following sections.

1.3.1 B-MYB

The transcription factor B-MYB is a classical cell cycle-regulated E2F target gene. It is repressed by E2F4/pocket protein complexes in G0. Its expression is induced in G1 by activating E2Fs and reaches its peak in S-phase. B-MYB then induces genes necessary for further progression through the cell cycle, e.g. cyclin A. In a positive feedback loop, B-MYB is phosphorylated by active cyclin A/cdk2 complexes and thereby gains its full activity (Ziebold et al., 1997). B-MYB sequence-specifically binds to the consensus sequence C/TAACNG on promoter DNA through the highly conserved N-terminal region (Biedenkapp et al., 1988) and it transcriptionally activates its target genes. B-MYB knock-out mice die in early embryonic development due to defects in inner cell mass formation (Tanaka et al., 1999).
In addition to the cell cycle-dependent expression, B-MYB is also regulated by its interaction with corepressors like N-Cor and SMRT (Li and McDonnell, 2002). It only displays full transactivating activity, when it is phosphorylated and binds to coactivators like p300 (Johnson et al., 2002), CREB-binding protein CBP (Bessa et al., 2001) or poly(ADP-ribose)polymerase PARP (Cervellera and Sala, 2000).
As described above, B-MYB activates target genes that are important for G2 and mitosis together with E2F transcription factors (Zhu et al., 2004).

1.3.2 The human LIN-9

1.3.2.1 LIN-9 in transformation

The human LIN-9 protein was first described as a chromatin associated protein that interacts with pRB. This binding was confined to the N-terminal part of LIN-9 and the pocket domain of pRB (Gagrica et al., 2004). The expression of LIN-9 cooperated with pRB in the induction of flat cells in the Saos-2 cell line. This effect resembels a senescent-like phenotype and flat cells are markers for differentiation in this system (Sellers et al., 1998). This indicates that LIN-9 acts in the pRB-pathway to promote differentiation (Gagrica et al., 2004). This finding was supported by the fact that LIN-9 together with pRB activated differentiation-specific genes. However, LIN-9 did not cooperate with pRB to induce G1 arrest in Saos-2 or Hela cells or in the repression or activation of E2F-dependent cell cycle genes.

In addition to the cooperation with pRB in differentiation, LIN-9 was also shown to inhibit transformation. LIN-9 overexpression rescued morphological changes induced by oncogenic RasV12 in NIH-3T3 cells and pRB-dependently inhibited growth of colonies in soft agar assays, pointing to a role for LIN-9 as a tumor suppressor (Gagrica et al., 2004). Additionally, in a primary cell system overexpressing RasV12 and SV40 small t and lacking p53, the additional depletion of LIN-9 lead to colonies in soft-agar. This shows that LIN-9 can substitute for the loss of pRB in oncogenic transformation. As the combined loss of LIN-9 and pRB did not lead to a cooperative effect, LIN-9 is a component of the pRB pathway in controlling transformation (Gagrica et al., 2004).

1.3.2.2 LIN-9 and its role in cell cycle progression

In primary human fibroblasts, LIN-9 is essential for cell cycle progression and proliferation. If LIN-9 is depleted, the cells are delayed before the entry into mitosis. This is, at least in part, due to the regulation of genes that are important for G2 and mitosis by LIN-9 (Osterloh et al., 2007). These target genes include genes essential for the entry into mitosis (cyclin A2, cyclin B1, cdc2), spindle assembly (PLK, Aurora-A), mitotic spindle checkpoint (Bub1, Mad2, CENP-E, Birc5), chromosome segregation (CENP-E) and exit from mitosis (Ubch10) (see 1.3.2.3 for details). The effect on the target genes is likely direct, as LIN-9 binds to their promoters in chromatin immunoprecipitations and activates them in S-phase together with B-MYB (Osterloh et al., 2007).

1.3.2.3 LIN-9 target genes

LIN-9 G2/M target genes are responsible for entry into and exit from mitosis as well as checkpoints during mitosis. Many of their promoters have similar binding sites known to be regulated by E2F transcription factors (Ren et al., 2002; Takahashi et al., 2000). They contain activating and repressive E2F binding sites. The repressive E2F-binding element is called CDE (cell cycle-dependent element) and is essential for transcriptional repression in G0 and early G1 (Tommasi and Pfeifer, 1995; Zhu et al., 2004). Often, a CHR domain (cell cycle genes homology region) is found near the CDE region. Although an intact CHR region is needed for transcriptional repression (Fajas et al., 2000; Lange-zu Dohna et al., 2000; Liu et al., 1998), and some factors

were described to bind to CHR (Kishore et al., 2002; Liu et al., 1998; Philips et al., 1999), no CHR-binding protein was clearly identified yet.
In addition to CDE and CHR elements, the LIN-9 target gene promoters contain several putative MYB binding sites and CAAT boxes where the transcription factor NF-Y can bind.

LIN-9 cell cycle target genes: cyclin A2, cyclin B1 and cdc2/cdk1

Cyclins have first been described as proteins that are expressed in a cell cycle-dependent manner (Evans et al., 1983). They share a homology in the cyclin-box, which is essential for the binding to cyclin-dependent kinases (Kobayashi et al., 1992).
Cyclin A2 is expressed from the beginning of the S-phase until its degradation by the anaphase-promoting complex (APC) in mitosis. Before the onset of S-phase, the cyclin A promoter is inhibited by binding of E2F4/p107 complexes to the repressive CDE element and interaction of an unknown protein to the CHR region (Liu et al., 1998; Philips et al., 1999; Zwicker et al., 1995). As cyclin A2 can interact with cdk2 and with cdc2 (Pagano et al., 1992), it has functions as well in S-phase as in mitosis. In S-phase, cyclin A2/cdk2 drive DNA synthesis (Resnitzky et al., 1995; Rosenberg et al., 1995; Yam et al., 2002). Later in the cell cycle, cyclin A2/cdc2 complexes accelerate the entry into mitosis (Furuno et al., 1999).
Cyclin B1 is expressed later than cyclin A2 and only associates with cdc2. In addition to its regulation by expression levels, cyclin B1 is also regulated through its localization. At the onset of mitosis, cyclin B1 accumulates in the nucleus (Porter and Donoghue, 2003). Cyclin B1/cdc2 complexes are directly involved in the entry into mitosis by phosphorylating lamin subunits, which leads to changes in microtubule structures and eventually to the breakdown of the nuclear envelope (Nigg, 1992).
Cdc2 is activated by binding to cyclin A and cyclin B. In addition to this, posttranslational modifications are necessary for cdc2 to gain full activity (Porter and Donoghue, 2003).

Spindle assembly and spindle checkpoint genes regulated by LIN-9

In metaphase, the chromatids have to be attached bipolarly to the mitotic spindle in order to distribute the genetic material equally upon the two daughter cells. The

organization of the spindles is ensured by Aurora-A and its activator polo-like kinase (PLK-1) (Barr and Gergely, 2007).
After replication in S-phase, the chromatids stay attached to each other. The chromosome segregation at the end of mitosis is triggered by the anaphase-promoting complex (APC), a ubiquitin ligase, that among other proteins contains the E2 ligase Ubch10.
The mitotic spindle assembly checkpoint prevents the chromosome segregation from occuring too early. Proteins like Bub1, BubR1, Mad2 and CENP-E bind to the free kinetochores and thereby inhibit the APC until all the chromatids are bound to the spindles (Lew and Burke, 2003; Peters, 2006; Smits and Medema, 2001).

LIN-9 target genes acting in the exit from mitosis and cytokinesis

Exit from mitosis is triggered by the anaphase-promoting complex. The APC ubiquitinates certain cell cycle proteins and thereby marks them for degradation, which leads to chromosome segregation. APC activity is enhanced by PLK-1 through an unknown mechanism (van Vugt and Medema, 2005).
Survivin/Birc5 together with other proteins correct misattachment of spindles to the kinetochores. These proteins are essential for the ordered cytokinesis and finally activate a pathway resulting in the formation of a cleavage furrow (Lens et al., 2006; Vader et al., 2006).

1.3.3 Other human homologues of the dREAM complex

In addition to the proteins described above, three other proteins belong to the DRM and dREAM/MMB complexes. These are LIN-37/mip40, LIN-52/dLIN-52 and LIN-54/mip120. The human homologue of LIN-52 has not been described yet. Human LIN-37 and LIN-54 have been described as pRB-interacting proteins in-vitro (Korenjak et al., 2004). Database searches showed expressed sequence tags (EST) for LIN-37, LIN-52 and LIN-54. As all the members of the Drosophila and C. elegans complexes have homologues in human cells, a synMuv like complex was very likely to exist in human cells.

1.4 Aim of this project

In this project, the following four questions were addressed:

First, it was analyzed whether a complex similar to dREAM/MMB also exists in human cells. For this, the necessary tools like expression plasmids and antibodies had to be produced.

A second important goal of this study was to characterize the composition and the function of the complex in the context of the cell cycle.

Third, because LIN-9 together with B-MYB functions in the regulation of G2/M genes, it was analyzed whether novel synMuv-like proteins also function in this process.

Finally, LIN-54, one member of the complex was characterized in more detail regarding its possible protein and DNA interaction ability.

2 Materials and methods

2.1 Materials

2.1.1 Chemical Stocks

Unless otherwise indicated, commonly used chemicals were purchased from Applichem, Roth, Invitrogen or Sigma with analysis quality.

	Stock concentration
Ammonium Persulfate (APS)	10 % in H_2O
Ampicillin	100 mg/ml in H_2O
Blasticidin (Invivogen)	10 mg/ml in 10 mM Hepes pH 7.4
BrdU	32.5 mM in PBS
Bovine serum albumin (BSA)	20 mg/ml
[α^{32}-P]-dCTP (Hartmann Analytic)	50 µCi/µl
dNTP (Promega or Invitrogen)	2 mM dATP, dCTP, dGTP, dTTP each
4´-6-Diamidino-2-phenylindiole (DAPI)	1 mg/ml
DTT	1 M in H_2O
Ethidium bromide	10 mg/ml in H_2O
Isopropyl- β-D-1-thiogalactopyranoside (IPTG)	1M in H_2O
Luminol	250 mM in DMSO
p-coumaric acid	90 mM in DMSO
Phenylmethylsulphonyl fluoride (PMSF) (Roche)	10 mg/ml in isopropanol
Hexadimethrine bromide (Polybrene)	4 mg/ml in H_2O
Ponceau S solution	0.1 % Ponceau S in 5 % acetic acid
Propidium Iodide (PI)	1 mg/ml in H_20
Proteinase K	10 mg/ml in 50 mM Tris-HCl pH 8.0/1 mM $CaCl_2$
random primer	0.5 mg/ml in H_2O

RNase A	10 mg/ml in 10 mM Tris-HCl pH 7.4/150 mM NaCl
Sodium dodecyl sulfate (SDS)	10 % in H_2O
Salmon sperm ssDNA	10 mg/ml
[^{35}S]-methionine (Amersham)	50 µCi/µl

2.1.2 Buffers

2.1.2.1 General Buffers

5X DNA Loading Buffer	15 % Ficoll 0.05 % Bromphenol blue 0.05 % Xylene Cyanol 0.05 M EDTA in 1X TAE
2X HBS	280 mM NaCl 1.5 mM Na_2HPO_4 50 mM HEPES-KOH, pH 7.05
Luria Bertani (LB) Agar	40 g powder in 1 l H_20, autoclave
Luria Bertani (LB) Medium	25 g powder in 1 l H_20, autoclave
Miniprep-Solution S1	25 mM Tris-HCl pH 8.0 10 mM EDTA
Miniprep-Solution S2	200 mM NaOH 1 % SDS
Miniprep-Solution S3	29.44 g potassium acetate 11.5 ml acetic acid 28.5 ml H_2O

Phosphate buffered saline (PBS) (1x)	13.7 mM NaCl 0.3 mM KCl 0.64 mM Na_2HPO_4 0.15 mM KH_2PO_4 adjust pH to 7.4 with HCl
Protease inhibitors (PI) Stock-concentrations:	0.1 mg/ml Aprotinin 10 mg/ml AEBSF 0.5 mg/ml Bestatin 0.5 mg/ml E64 1 mg/ml Leupeptin 0.1 mg/ml Pepstatin
TAE buffer (1X)	40 mM Tris base 5 mM glacial acetic acid 10 mM EDTA, pH 8.0
TBS (1X)	50 mM Tris-HCl, pH 7.4 150 mM NaCl
TE	10 mM Tris-HCl, pH 7.5 1 mM EDTA
Trifast	Peqlab

2.1.2.2 Buffers for lysates and nuclear extracts

TNN buffer	50 mM Tris-HCl, pH 7.5
	120 mM NaCl
	5 mM EDTA
	0.5 % NP-40
	10 mM $Na_4P_2O_7$
	2 mM Na_3VO_4
	100 mM NaF
	ad 500 ml H_2O
	PI 1:100 (add freshly)
	PMSF 1:200 (add freshly)
Cell lysis buffer (Nuclear Extracts)	10 mM Hepes pH 7.4
	10 mM NaCl
	3 mM $MgCl_2$
	PI 1:200 (add freshly)
	PMSF 1:200 (add freshly)
Nuclear lysis buffer (Nuclear Extracts)	20 mM Hepes pH 7.4
	400 mM NaCl
	1.5 mM $MgCl_2$
	0.1 mM EDTA
	1 % NP-40
	15 % Glycerin
	0.5 mM DTT (add freshly)
	PI 1:100 (add freshly)
Bradford Solution	50 mg Coomassie Brilliant Blue G250
	23.75 ml Ethanol
	50 ml 85 % (v/v) ortho-phosphoric acid
	ad 500 ml H_2O
	filter twice

2.1.2.3 Buffers for immunoprecipitation and immunoblot

Coomassie blue stain	250 ml methanol 35 ml acetic acid 1 g Coomassie blue R-250 ad 500 ml H_2O
Coomassie destain	250 ml methanol 35 ml acetic acid ad 500 ml H_2O
Acrylamidbuffer for SDS-Gels	30 % (w/v) Acrylamide 0.8 % (w/v) N,N´-Methylenbisacrylamide
Blotting buffer (1x)	0.6 g Tris base 2.258 g Glycine 150 ml Methanol ad 1 l H_2O
Blocking solution	5 % (w/v) milk powder in TBST
3X Electrophoresis Sample Buffer (3X ESB)	300 mM Tris-HCl pH 6.8 15 mM EDTA 150 mM DTT 12 % (w/v) SDS 15 % (w/v) glycerol 0.03 % (w/v) bromphenol blue
TBS (1x)	50 mM Tris-HCl pH 7.4 150 mM NaCl
TBST	0.05 % Tween in 1x TBS

Substrate Solution	10 ml 100 mM Tris-HCl pH 8.5
	50 µl 250 mM Luminol
	22 µl 90 mM p-coumaric acid
	3 µl 30 % H_2O_2

2.1.2.4 Buffers for chromatin immunoprecipitation

Lysis buffer	5 mM PIPES pH 8.0
	85 mM KCl
	0.5 % Nonidet P-40
Nuclear lysis buffer	50 mM Tris-HCl pH 8.1
	10 mM EDTA
	1 % SDS
IP dilution buffer	0.01 % SDS
	1.1 % Triton X-100
	1.2 mM EDTA
	16.7 mM Tris-HCl pH 8.2
	167 mM NaCl
Elution buffer	50 mM Tris-HCl pH 8.0
	1 % SDS
	10 mM EDTA
LiCl wash buffer	0.25 M LiCl
	0.5 % Nonidet P-40
	0.5 % Sodium deoxycholat (DOC)
	1 mM EDTA
	10 mM Tris-HCl pH 8.0
Blocking buffer	3 ml IP dilution buffer
	150 µl BSA (20 mg/ml)
	30 µl ssDNA (10 mg/ml)

2.1.2.5 Buffers for gelshift

Bacterial lysis buffer	20 mM Hepes pH 7.5 120 mM NaCl 2 mM EDTA 10 % Glycerol PI 1:100 (add freshly)
Elution buffer	100 mM Hepes pH 8.0 100 mM NaCl 0.1 % Triton X-100 0.5 mM DTT (add freshly) 15 mg/ml glutathione (add freshly)
5x gelshift binding buffer	65 mM Hepes pH 7.9 65 % Glycerol 320 mM KCl 0.65 mM EDTA 2.5 mM $MgCl_2$

2.1.2.6 Buffers for GST pulldown

STE buffer	10 mM Tris pH 8.0 150 mM NaCl 1 mM EDTA 5 mM DTT (add freshly) PMSF 1:200 (add freshly)
Elution buffer	50 mM Tris pH 8.0 100 mM NaCl 0.1 % Triton X-100 0.5 mM DTT 15 mg/ml glutathione (add freshly)

IPH buffer	50 mM Tris pH 8.0 150 mM NaCl 5 mM EDTA 0.5 mM Nonidet P-40 PI 1:100 (add freshly)

2.1.3 Antibodies

2.1.3.1 Primary antibodies

Antibody against	Company	Origin	Application	Concentration	Internal number
LIN-9	Davids Biotech	Rabbit polyclonal serum (Osterloh et al., 2007)	IP	1:50	# 136
			WB	1:500	# 137
			ChIP	20 µg	# 81
LIN-37	Davids Biotech	Rabbit polyclonal serum This work	IP	1:50	# 131
			WB	1:500	
			ChIP	20 µg	
LIN-52	Davids Biotech	Rabbit polyclonal serum This work	IP	1:50	# 141
			WB	1:500	
LIN-54	Davids Biotech	Rabbit polyclonal serum This work	IP	1:50	#129
			WB	1:500	
			ChIP	20 µg	
B-MYB (N-19)	Santa Cruz Sc-724	Rabbit polyclonal (200 µg/ml)	IP	1:100	# 79
			WB	1:1000	
			ChIP	8 µg	
E2F4 (C-20)	Santa Cruz Sc-866	Rabbit polyclonal (200 µg/ml)	IP	1:100	# 6
			WB	1:1000	
			ChIP	4 µg	
p130	Santa Cruz Sc-317	Rabbit polyclonal (200 µg/ml)	WB	1:1000	# 33

p107	Santa Cruz Sc-318	Rabbit polyclonal (200 µg/ml)	IP	1:100	# 32
			WB	1:1000	
RbAp48	Abcam ab 488	Mouse monoclonal (1 mg/ml)	WB	1:2000	# 140
β-tubulin	Chemicon MAB3408	Mouse monoclonal (1 mg/ml)	WB	1:5000	# 102
IgG	Sigma I5006	Rabbit polyclonal (1 mg/ml)	IP	1:500	# 104
			ChIP	4 µg	
BrdU-FITC	Beckton Dickinson 347583	Mouse monoclonal	FACS	20 µl / 10^6 cells	
Phospho-H3	Upstate 06-570	Rabbit polyclonal (1 mg/ml)	IF	1:100	# 57
Flag-M2-Agarose	Sigma A-2220-5ml	Mouse monoclonal	IP	20 µl / IP	
Flag-M2	Sigma F-3165	Mouse monoclonal (5 mg/ml)	WB	1:5000	# 93
HA	Covance MMS-101P	Mouse monoclonal (1 mg/ml)	IP	1:200	# 92
			WB	1:2000	

2.1.3.2 Secondary antibodies

Antibody against	Company	Application	Concentration
Anti-mouse HRP-linked	Amersham	WB	1:5000
Anti-protein A HRP-linked	Amersham	WB	1:5000
Anti-rabbit rhodamin	Jackson Immuno Research (1.5 mg/ml)	IF	1:200

2.1.4 Plasmids

Unless indicated otherwise, all the plasmids encode for human sequences.

2.1.4.1 Plasmids for overexpression

Plasmid number	Plasmid name	Description
# 374	pCDNA3-flag	Empty vector control for overexpression constructs
# 375	pCDNA3-flag-LIN-9	Overexpression of flag-LIN-9 (Gagrica et al., 2004)
# 727	pCDNA3-HA-LIN-37	Overexpression of HA-LIN-37 (this work)
# 697	pCDNA3-flag-LIN-37	Overexpression of flag-LIN-37 (this work)
# 714	pCDNA3-HA-LIN-52	Overexpression of HA-LIN-52 (this work)
# 695	pCDNA3-flag-LIN-54	Overexpression of flag-LIN-54 (this work)
# 726	pCDNA3-HA-LIN-54	Overexpression of HA-LIN-54 (this work)
# 892	pCDNA3-flag-LIN-54-ΔN	Overexpression of the C-terminal part of LIN-54 (this work)
# 890	pCDNA3-flag-LIN-54-CXC	Overexpression of flag-tagged CXC domain of LIN-54 (this work)

# 893	pCDNA3-flag-LIN-54-HCH	Overexpression of flag-tagged HCH domain of LIN-54 (this work)
# 891	pCDNA3-flag-LIN-54-ΔCXC	Overexpression of flag-LIN-54 lacking the CXC domain (this work)
# 894	pCDNA3-flag-LIN-54-ΔHCH	Overexpression of flag-LIN-54 lacking the HCH domain (this work)
# 806	pCDNA3-HA-RbAp48	Overexpression of HA-RbAp48 (this work)
# 730	pCDNA3-flag-B-MYB	Overexpression of flag-B-MYB (mouse) (Johnson et al., 1999)
# 888	pCDNA3-HA-B-MYB	Overexpression of HA-B-MYB (mouse) (Johnson et al., 1999)
# 875	pCDNA3-HA-p130	Overexpression of HA-p130 (Hansen et al., 2001)
# 174	pEGFP-N1	Overexpression of eGFP for transfection efficiency control

2.1.4.2 Plasmids for recombinant proteins

Plasmid number	Plasmid name	Description
# 397	pGex-4T2	Recombinant expression of GST
# 401	pGex-4T2-LIN-9	Recombinant expression of GST-LIN-9 (Gagrica et al., 2004)

# 889	pGex-4T2-CXC	Recombinant expression of GST-CXC (this work, Sarah Cremer)
# 944	pGex-4T2-CXC-C525/527Y	Recombinant expression of GST-CXC with point mutations (this work)

2.1.4.3 Plasmids for knock-down

Plasmid number	Plasmid name	Description
# 652	pMSCV-puro	Empty vector control for knock-down constructs
# 766	pMSCV-shLIN-54#2	Retroviral expression vector for LIN-54-shRNA
# 767	pMSCV-shLIN-54#3	Retroviral expression vector for LIN-54-shRNA

2.1.4.4 Plasmids for gelshift

Plasmid number	Plasmid name	Description
# 789	pXP2-cdc2-wt	See appendix for sequence
# 805	pXP2-cdc2-CDEmut	TAGCGCgGT mutated to TAGCGCtGT
# 804	pXP2-cdc2-CHRmut	AGTttgaAAC mutated to AGTagctAAC
# 907	pXP2-cdc2-CHRup mut	ATttGAA mutated to ATccGAA

# 908	pXP2-cdc2-Myb1mut	GAActGTG mutated to GAAtcGTG
# 819	pXP2-cdc2-Myb4mut	AGAaacAGT mutated to AGAggaAGT
# 812	pXP2-cdc2-Myb5mut	CAGttgGCG mutated to CAGcctGCG

2.1.5 Primers

Unless indicated otherwise, all the primers were intended for human sequences.

2.1.5.1 Primers for cloning

Primer number	Sequence	Application	
SG 464	GCGGATCCGAGGTGGTGCCAGCTGAG	LIN-54	sense
SG 465	GCCTCGAGAATCAAGTGTCCCTGCACCT	LIN-54	antisense
SG 475	AAGGATCCATGTTCCCTGTGAAGGTGAAA	LIN-37	sense
SG 476	AAGAATTCGGGGATGTTTACTGGTGTGG	LIN-37	antisense
SG 477	AAGGATCCATGAGACTGGGCTGCGAGT	LIN-52	sense
SG 478	AAGAATTCTCCGAGGCTAAGAAGGTTCA	LIN-52	antisense
SG 464	GCGGATCCGAGGTGGTGCCAGCTGAG	LIN-54-ΔC	sense
SG 537	GCCTCGAGGCTTGCTGAAGAGGCTGAGT	LIN-54-ΔC	antisense
SG 908	GGCGGATCCAAGCCAGTGGTTGTTAATAC	LIN-54-ΔN	sense
SG 465	GCCTCGAGAATCAAGTGTCCCTGCACCT	LIN-54-ΔN	antisense
SG 464	GCGGATCCGAGGTGGTGCCAGCTGAG	LIN-54-ΔCXC	sense
SG 910	GCTCTAGAGAATGGAAGCCGTGCCTG	LIN-54-ΔCXC	antisense
SG 911	GCTCTAGATTGGCAGATGCAGCTGAAGTA	LIN-54-ΔCXC	sense
SG 465	GCCTCGAGAATCAAGTGTCCCTGCACCT	LIN-54-ΔCXC	antisense
SG 464	GCGGATCCGAGGTGGTGCCAGCTGAG	LIN-54-ΔHCH	sense
SG 912	GCCTCGAGTTAAACTTCGTCTTGGCTGC	LIN-54-ΔHCH	antisense
SG 860	ATGGATCCCCATCAGAGTCGGCCAGT	LIN-54-CXC	sense
SG 861	CGCTCGAGCATCTGCCAAATGCATCA	LIN-54-CXC	antisense
SG 909	GCGGATCCAGGGTACAGCAACAAACAGC	LIN-54-HCH	sense
SG 465	GCCTCGAGAATCAAGTGTCCCTGCACCT	LIN-54-HCH	antisense

2.1.5.2 Primers for point mutagenesis

Primer number	Sequence	Application	
SG 919	GGGGATCCATCCGAACTGTGCCA ATGCTGGGA	cdc2 CHRup mut	sense
SG 623	GTCAAGCTTCACTGTACCCGGCTT ATTATT	cdc2 CHRup mut	antisense
SG 920	GGGGATCAATTTGAATCGTGCCAA TGCTGGGAGAAAA	cdc2 Myb1 mut	sense
SG 623	GTCAAGCTTCACTGTACCCGGCTTA TTATT	cdc2 Myb1 mut	antisense
SG 932	AGTCGGCCCCGAAAGCCCTATAAT TATACAAAATCACTGTGTTTG	LIN-54-C525/527Y	sense
SG 933	CAAACACAGTGATTTTGTATAATTAT AGGGCTTTCGGGGCCGACT	LIN-54-C525/527Y	antisense

2.1.5.3 Primers for quantitative RT-PCR

Primer number	Sequence	Application	
SG 645	GCCCAATACGACCAAATCC	GAPDH	sense
SG 646	AGCCACATCGCTCAGACAC	GAPDH	antisense
SG 620	GGCAGACCGAGATGAATCCTCA	S14	sense
SG 621	CAGGTCCAGGGGTCTTGGTCC	S14	antisense
SG 787	GCCACATCAGCCAGTAGCTC	lin54	sense
SG 788	TAACAACCACTGGCTTTGCTT	lin54	antisense
SG 572	GGTACTGAAGTCCGGGAACC	cyclin A2	sense
SG 573	GAAGATCCTTAAGGGGTGCAA	cyclin A2	antisense
SG 574	CGCCTGAGCCTATTTTGGT	cyclin B1	sense
SG 575	GCACATCCAGATGTTTCCATT	cyclin B1	antisense
SG 576	TGGATCTGAAGAAATACTTGGATTCTA	cdc2	sense
SG 577	CAATCCCCTGTAGGATTTGG	cdc2	antisense
SG 590	TGCCGAGCTCTGGAAAAA	Ubch10	sense
SG 591	AAAAGACGACACAAGGACAGG	Ubch10	antisense
SG 568	GCCCAGTGTTTCTTCTGCTT	Birc5	sense
SG 569	CCGGACGAATGCTTTTTATG	Birc5	antisense

SG 680	AAGATCTGGAGGTGAAAATAGGG	PLK	sense
SG 681	AGGAGTCCCACACAGGGTCT	PLK	antisense
SG 731	CCTGTTCTCCTCGTGTAAAAGC	cdc6	sense
SG 732	GTGTTGCATAGGTTGTCATCG	cdc6	antisense
SG 733	GGCAAACTCACTAGTATGCACTTC	RR1	sense
SG 734	AAATAATACATCCCAGTCTTCAAACC	RR1	antisense

2.1.5.4 Primers for chromatin immunoprecipitation

Primer number	Sequence	Application	
SG 540	GGCAGCAAGAGTCACTCCA	GAPDH2	sense
SG 541	TGTCTCTTGAAGCACACAGGTT	GAPDH2	antisense
SG 538	AAGAAGAACGGAGCGAACAG	cdc2	sense
SG 539	CGGGAGAGTGTCGTCCTACT	cdc2	antisense
SG 552	GCCCTTTAATGGTTAGCGTTT	Ubch10	sense
SG 553	GCTGCCATTAACTAACGAATCC	Ubch10	antisense
SG 612	CCATTAACCGCCAGATTTGA	Birc5	sense
SG 613	GCGGTGGTCCTTGAGAAAG	Birc5	antisense
SG 586	AAGAAACGCGACTCTCAGGA	Bub1	sense
SG 587	TGGAGGTCTTTGAGACAGAAAAA	Bub1	antisense
SG 735	CTGTGGCCATTCGGATTT	cdc6	sense
SG 736	CCCCTGAACAAACTGCACA	cdc6	antisense
SG 737	GGAGAGGCGTAGTCTTCTGG	RR1	sense
SG 738	AGACTGACAGGCGACGTGTA	RR1	antisense
SG 781	CTGGCTGCTGCGCGA	PCNA	sense
SG 782	CACCACCCGCTTTGTGACT	PCNA	antisense

2.1.5.5 Primers for gelshift competitions

Primer number	Sequence	Application	
SG 622	GGGGATCCATTTGAACTGTGCCAATGC	Full length	sense
SG 623	GTCAAGCTTCACTGTACCCGGCTTATTATT	Full length	antisense
SG 750	GCGAGCAGTTTCAAACTCACC	PCR 9	antisense
SG 748	GCGCCAACTGAGTGCGA	PCR 10	antisense
SG 539	CGGGAGAGTGTCGTCCTACT	PCR 11	antisense
SG 925	TGTTCGCTCCGTTCTTCTTT	PCR 12	antisense

SG 938	GCTGGGAGAAAAAATTTAAAAGAAGAACG	PCR 14	sense
SG 538	AAGAAGAACGGAGCGAACAG	PCR 15	sense
SG 747	GGACGACACTCTCCCGACTG	PCR 16	sense
SG 749	TGGGCTCTGATTGGCTGCTTT	PCR 17	sense

2.1.6 shRNA sequences

Plasmid #767 pMSCV-sh-LIN-54#3 Target sequence:
GCAGTTACAGGACAGACAA
(bases 763 – 781 from transcriptional start)

Plasmid #766 pMSCV-sh-LIN-54#2 Target sequence:
GTATCAATAGCAAGCAACT
(bases 1459-1477 from transcriptional start)

2.1.7 Cell lines and cell culture media

DMEM (4.5 g Glucose/L-Glutamine)	Cambrex
Penicillin/Streptomycin (10 U/µl each)	Cambrex
Trypsin (EDTA) (200 mg/l)	Cambrex
Foetal Bovine Serum (FCS)	Invitrogen
Serum Supreme	Cambrex

HeLa	DMEM 10 % FCS/1 % PenStrep
293T	DMEM 10 % FCS/1 % PenStrep
BJ-ET	DMEM 10 % FCS/1 % PenStrep
T98G	DMEM 10 % FCS/1 % PenStrep
Phoenix	DMEM 10 % Serum Supreme/1 % PenStrep

2.1.8 Enzymes

Restriction endonucleases	New England Biolabs/Invitrogen/Fermentas
T4-DNA-ligase (400 U/µl)	New England Biolabs
M-MLV-RT (200 U/µl)	Promega
RiboLock (RNase-Inhibitor)	Fermentas
Phusion proofreading Taq (2 U/µl)	Finnzymes

2.1.9 Markers

1 Kb DNA Ladder	Fermentas
SDS Page Ruler Mix	Fermentas

2.1.10 Kits

Jetstar Gel Extraction Kit	Genomed
Jetstar Plasmid Purification Midi/Maxi Kit	Genomed
Plasmid Mini/Midi/Maxi Kit	Promega
QIAquick PCR Purification Kit	Qiagen
Absolute QPCR SYBR Green Mix	Thermo
T7 Quick coupled Translation System	Promega
Sephadex G-50 spin columns	Pharmacia

2.1.11 Beads

Protein A Sepharose (Pierce)
Protein G Sepharose (Pierce)
Glutathione Sepharose (Amersham)

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Passageing of cells

Eukaryotic cells were cultivated in a tissue culture incubator at 37 °C and with 5 % carbon dioxyde. For passageing, cells were washed once with PBS and incubated with Trypsin/EDTA for a few minutes at 37 °C. The detached cells were plated on new culture dishes.

2.2.1.2 Transient transfection

Hela, Phoenix and 293T cells were transfected using calcium-phosphate. 30-60 µg of DNA was mixed with 50 µl of 2.5 M $CaCl_2$ and with H_2O to a final amount of 500 µl.

500 µl of 2x HBS were continuously bubbled while DNA/$CaCl_2$ mixture was added dropwise. This solution was slowly added to the cells. After incubation of about 16 h, the cells were washed with PBS and fed with fresh medium. Whole cell lysates were prepared 48 h after transfection.
T98G cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions.

2.2.1.3 Infection of BJ-ET cells

For the production of ecotrophic virus supernatants, Phoenix cells carrying the ecotrophic receptor were transiently transfected with the plasmid of interest using calcium-phosphate (2.2.1.2). 48 h after transfection, the virus supernatants were harvested and used immediately or frozen in liquid nitrogen and stored at -80 °C.
BJ-ET cells were splitted 1:3 and infected the next day. For the infection, the viral supernatant was mixed with 8 µg/ml Polybrene, filtered (0.45 µm) and added to the cells. 16 h after infection, the cells were fed fresh medium and selection was started 48 h after infection.

2.2.1.4 Growth curve

$1x10^5$ of the infected and selected BJ-ET cells were plated on a 6-well-plate in triplicates. Every 4 days, they were counted and $1x10^5$ cells replated. Mean values of the cumulative cell numbers were plotted against the time.

2.2.1.5 Synchronization of T98G cells by serum starvation

For the cell cycle experiments, T98G cells were washed twice with PBS and fed with DMEM without FCS for 72 h. The cells were then arrested in G0 and could be released into the cell cycle by addition of 15 % FCS.

2.2.1.6 Determination of cell cycle phases: flow cytometry

For flow cytometry measurement, the cells were harvested by trypsinization, washed with PBS and fixed with 1 ml 80 % ethanol. After incubation at -20 °C over night, the cells were again washed with PBS and resuspended with 500 µl 38 mM NaCitrate and 25 µl RNase A (10 mg/ml) was added. The cells were incubated for 1-2 h at 37 C, stained with 30 µl propidium-iodide (1 mg/ml) and measured by flow cytometry.

In order to better discriminate cells in S-phase, the cells in culture were incubated with 10 µM BrdU for 1 h and then fixed with 80 % ethanol over night. The DNA was denaturated with 1 ml of 2 M HCl/0.5 % Triton-X-100 for 30 minutes at room temperature. The mixture was neutralized by adding 1 ml of 0.1 M $Na_2B_4O_7$. $1x10^6$ cells were resuspended in 50 µl PBS/0.5 % Tween 20/1 % BSA and 20 µl of an α-BrdU-FITC antibody was added. After an incubation of 1 h at room temperature in the dark, the cells were washed with 50 µl PBS/0.5 % Tween 20/1 % BSA and resuspended with 500 µl 38 mM NaCitrate/25 µl RNase A (10 mg/ml). The cells were incubated for 1-2 h at 37 °C, dyed with 30 µl propidium-iodide (1 mg/ml) and measured by flow cytometry.

To calculate the length of the S-phase, the results from a BrdU-pulse assay were evaluated with the following method (Begg et al., 1985).
First, the relative movement (RM) of BrdU positive cells compared to G1 and G2/M cells was calculated with this formula:

$$RM = (F_L - F_{G1})/(F_{G2/M} - F_{G1})$$

With the following values given by the software:
F_L: mean propidiumiodide-fluorescence value of BrdU positive cells
F_{G1}: mean propidiumiodide-fluorescence value of G1 cells
$F_{G2/M}$: mean propidiumiodide-fluorescence value of G2/M cells

The length of the S-phase is determined by this formula:

T_S = [0.5/(RM-0.5)] x time from BrdU-removal until harvesting [h]

2.2.1.7 Immunofluorescence

Infected and selected BJ-ET cells were plated and grown on coverslips for 24 h. After washing with PBS, they were fixed for 10 min with PBS/3 % paraformaldehyde/2 % sucrose, washed 3 times with PBS, permeabilized for 5 min with PBS/0.2 % Triton-X-100 and washed with PBS/0.1 % Triton-X-100 (PBST). Unspecific staining was minimized by blocking for more than 15 min with PBST/5 % goat serum. The cells were washed 3 times in PBS/5 mM $MgSO_4$ and incubated with the primary antibody

diluted in PBS/5 mM $MgSO_4$ for 1 h in a humid chamber. The coverslips were washed 3 times with PBST and incubated with the rhodamine-labeled secondary antibody in PBST for 15 min. After washing, the nuclei were stained with DAPI (1:500 in PBS), washed with PBS and mounted.

2.2.2 Expression analysis

2.2.2.1 RNA

Total RNA was isolated from cell culture cells with Trifast (Peqlab). After removing the medium, 0.5 - 1 ml Trifast was added and cells were scraped into a reaction tube. 100 – 200 µl chloroform was added and after vortexing the tubes thoroughly, they were spinned at 12000 g and 4 °C for 10 min. The aqueous phase was precipitated with 500 µl isopropanol, left at RT for 10 min and spinned for 10 min at 12000 g and 4 °C. The pellet was washed with 80 % ethanol and resuspended with 25 µl RNase-free water.

2.2.2.2 Reverse transcription

To transcribe RNA into cDNA 1 – 2.5 µg RNA was brought to 9.5 µl with water and mixed with 0.5 µl random primers (0.5 µg/µl). This mixture was incubated for 5 minutes at 70 °C and chilled on ice. The following mixture was added on ice:
5 µl M-MLV 5x reaction buffer
6.25 µl dNTPs (2 mM)
0.5 µl Ribolock RNase inhibitor (40 U/µl)
0.5 µl M-MLV-RT (100 U)
2.75 µl H_2O
The reactions were incubated for 60 min at 37 °C and inactivated for 15 min at 70 °C.

2.2.2.3 Quantitative PCR

To determine the amount of a specific mRNA compared to a housekeeping gene, the following reaction was prepared:

12.5 µl Absolute QPCR SYBR Green Mix
10.5 µl H2O
1 µl fw/rv primer mix (10 pmol/µl each)
1 µl cDNA or precipitated chromatin

PCR program (40 cycles):
95 °C 15 min
95 °C 15 s
60 °C 1 min

The relative expression of a gene compared to a housekeeping gene was calculated with this formula: $2^{-\Delta\Delta Ct}$

with $\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (reference)
and ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

The standard deviation of $\Delta\Delta Ct$ was calculated with $s=\sqrt{(s_1^2+s_2^2)}$

with s_1 = standard deviation (gene of interest)
and s_2 = standard deviation (housekeeping gene)

The margin of error for $2^{-\Delta\Delta Ct}$ was determined by this formula: $2^{-\Delta\Delta Ct +/-s}$
and the error used for the error bars was calculated with: error = $2^{-\Delta\Delta Ct +s} - 2^{-\Delta\Delta Ct}$

Values in chromatin immunoprecipitations are shown as % of input:

% input = $2^{Ct (1\% Input) – Ct (IP)}$

Standard deviation, error margins and error were calculated as shown above.

2.2.3 Biochemical methods

2.2.3.1 Whole cell lysates

Hela and 293T cells were scraped with PBS, pelleted and resuspended with 10 times their amount of TNN + protease inhibitors (PI 1:100 + PMSF 1:200) by pipeting up and down 20 times and incubation on ice for 20 minutes. Lysates were spinned at 14000 rpm to remove the cell debris. The supernatant was immediately used for immunoprecipitation or boiled in 3x ESB for 5 minutes and frozen at -20 °C.

2.2.3.2 Nuclear extracts

T98G cells were scraped with PBS, pelleted and swollen with 1 ml cell lysis buffer for 20 minutes on ice. The cells were dounced (20 strokes, tight), nuclei were pelleted and lysed with 300 µl nuclei lysis buffer per dish for 20 minutes on ice. The nuclei

were spinned for 10 minutes at full speed and the supernatant was diluted 1:2 with 20 mM Hepes pH 7.4 + PI (1:100). Nuclear extracts were immediately used for immunoprecipitation or boiled in 3x ESB for 5 minutes and frozen at -20 °C.

2.2.3.3 Determination of protein concentration (Bradford)

The protein concentration was determined with the method described by Bradford (Bradford, 1976). 2 µl of whole cell lysate or nuclear extract were mixed with 1 ml of Bradford solution. Extinction at 595 nm was measured and compared to a standard BSA dilution series.

2.2.3.4 Immunoprecipitation

1 – 2 mg of whole cell lysate or diluted nuclear extract were incubated over night with the desired antibodies on a rotating wheel at 4 °C. 30 µl of protein A- (polyclonal antibodies) or protein G sepharose (monoclonal antibodies) were added and incubated for 1 h at 4 °C on a roating wheel. The beads were washed 5 times with TNN and spinned in between for 1 minute at 3000 rpm and 4 °C. After the last wash, the supernatant was removed completely, the beads were resuspended in 3x ESB and boiled for 5 minutes at 95 °C. Then the samples were ready for electrophoresis.

2.2.3.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed using the discontinuous method (Laemmli, 1970). An 8-14 % separating gel was prepared and after polymerization, the stacking gel was poured on the top. Electrophoresis was carried out in 1X SDS running buffer for about 1 h at 35 mA/gel. The gels were prepared as follows:

Separating gel (10 %)	Stacking gel
6.1 ml H_2O	6.9 ml H_2O
3.7 ml 1.5 M Tris pH 8.8	1.4 ml 0.5 M Tris pH 6.8
5 ml Acrylamid/Bisacrylamid	1.6 ml Acrylamid/Bisacrylamid
75 µl 20 % SDS	50 µl 20 % SDS
100 µl 10 % APS	50 µl 10 % APS
20 µl TEMED	20 µl TEMED

The gels were either used for immunoblotting or stained for 30 minutes with Coomassie blue to visualize the proteins.

2.2.3.6 Immunoblotting

The transfer of proteins onto PVDF membranes was done via electroblotting. The PVDF membrane was preincubated with 100 % methanol and rinsed with blotting buffer. The membrane was laid onto a layer of Whatman filter paper and the SDS-polyacrylamide gel was placed on the membrane, followed by a second layer of filter paper. This “sandwich” was clasped on both sides by sponges and placed in a cooled wet blotting tank (Biorad). The transfer occurred for 90 min at 300 mA in 1X Blotting Buffer. Successful and equal transfer of proteins was visualized by staining of the membrane with a Ponceau S solution and destaining with H_2O.

To detect specific proteins with their respective antibodies, the membranes were blocked with 3 % milk powder in TBST (Blocking solution) for 1 - 2 h and then incubated overnight at 4 °C with the primary antibody diluted in Blocking solution. Afterwards, the membrane was washed 3 times for 10 min in TBST and incubated with the secondary HRP-conjugated antibody for 1 h at RT. After 3 wash steps of 10 min in TBST, specific bands were detected using a Luminol-substrate-solution. The membrane was wrapped in plastic foil and exposed to an ECL-film

2.2.3.7 Affinity purification of polyclonal antisera

A protein-A-sepharose column was prepared by washing protein A sepharose beads with 20 ml 10 mM Tris pH 7.5. 1 ml of the antiserum was diluted in 10 ml 20 mM Tris pH 7.5 and incubated with the prepared protein A sepharose beads for 30 minutes on a rotating wheel. The serum was discarded and the beads washed with 20 ml 10 mM Tris pH 7.5, 20 ml 10 mM Tris pH 7.5/500 mM NaCl and again 20 ml 10 mM Tris pH 7.5. The antibodies bound to the protein A beads were eluted with 500 µl fractions of 100 mM Glycin pH 2.5 and mixed with 50 µl Tris pH 8.0. 20 µl of each eluate fraction was mixed with 100 µl Bradford solution. The peak fractions were mixed and concentration was determined. The affinity purified antibodies were used for chromatin immunoprecipitations.

2.2.3.8 Chromatin immunoprecipitation

The used protocol was based on the protocol in (Wells et al., 2000).

Proteins were crosslinked to DNA by adding 540 µl formaldehyde dropwise to the 20 ml medium. After 10 minutes, the reaction was stopped by addition of 2.5 ml 1 M Glycine and incubation of 5 minutes. Then the cells were washed twice in PBS and scraped with PBS + PMSF (1:200). The cell number per dish was determined by trypsinizing and counting cells from a spare dish. Crosslinked cells were aliquoted in $4x10^6$ cell portions, frozen in liquid nitrogen and stored at -80 °C or used immediately. $4x10^6$ cells were lysed in 1 ml cell lysis buffer for 10 minutes on ice. After spinning at 1500 rpm and 4 °C for 5 minutes, the supernatant was discarded, the nuclei lysed in 800 µl nuclei lysis buffer for 10 minutes on ice and sonified to fracture the chromatin. For T98G cells, the optimal conditions to get chromatin of 500 – 1000 basepairs was 12 pulses of 10 seconds at 25 % amplitude and 1 minute break in between the pulses. The chromatin was spinned at 14000 rpm for 10 minutes. 50 µl of the supernatant was removed to control chromatin size.

Therefore 2 µl 5 M NaCl was added to 50 µl chromatin to reverse the crosslink at 65 °C in a shaker over night. After 3 h of proteinase K treatment (2 µl 20 mg/ml) at 55 °C, the chromatin size was visualized on a 1.2 % agarose gel.

The remaining chromatin was diluted 1:10 with ChIP dilution buffer, precleared with 60 µl protein A sepharose for 15 minutes at 4 °C, and 4 immunoprecipitations were set up from the initial $4x10^6$ cells with the desired antibodies. 1 % precleared chromatin was removed for input and the IPs were incubated over night at 4 °C on a rotating wheel. The next day, 30 µl blocked protein A sepharose (500 µl packed beads were blocked over night with 3 ml blocking buffer and resuspended in 500 µl blocking buffer) was added for 1 h. The beads were washed 7 times with LiCl wash buffer, the buffer completely removed after the last wash and the Protein/DNA complexes were eluted twice with elution buffer, once with 120 µl, then with 150 µl. The input samples were brought to 250 µl with elution buffer and treated as the immunoprecipitated samples from this step on.

The crosslink was reversed for 250 µl of the eluate by adding 5 µl 5 M NaCl and incubating the samples over night at 65 °C in a shaker. The proteins were degraded by a proteinase K digest (2 µl 20 mg/ml) at 55 °C for 3 h.

To purify the chromatin, 230 µl phenol/chloroform 1:1 was added to 230 µl chromatin, vortexed thoroughly and spinned for 5 minutes at room temperature and full speed. The upper phase was purified with a PCR Purification Kit (Qiagen) according to the manufacturer´s instructions. 1 µl of purified chromatin was used for quantitative PCR analysis and precipitated samples were compared to input chromatin. The values were calculated as described in 2.2.2.3

2.2.3.9 Purification of GST-proteins from recombinant bacteria for antibody production and GST-pulldowns

Chemocompetent bacteria were transformed with the desired GST-construct with heat-shock. 0.1 µg plasmid was incubated with the bacteria for 10 minutes. Then the mixture was heated to 42 °C for 45 seconds and cooled on ice. 400 µl LB was added, the bacteria incubated at 37 °C for 0.5-1 h and plated on LB-agar plates containing a selection antibiotic.

50 ml over-night culture of one single bacterial colony was diluted 1:10 and grown for 1 h at 37 °C. Then the recombinant protein expression was induced for 4 h with 1 mM IPTG. For fusion protein recovery, bacterial cultures were pelleted by centrifugation at 6000 rpm for 10 minutes at 4 °C and resuspended in 10 ml of STE buffer where 1.5 % of sarcosyl was added. The bacteria were lysed on ice by sonication (2 times 10 seconds, 10 %), centrifuged at 6000 rpm for 10 minutes at 4 °C and after that 2 % Triton X-100 was added. The supernatant was shaked for 30 min at 4 °C with 250 µl of Glutathione-sepharose beads which were then washed four times with PBS. For analysis of bound fusion protein, the beads were boiled in 3X SDS sample buffer and loaded onto SDS-polyacrylamide gels. Proteins were visualized by Coomassie blue stain.

For antibody production, the protein was eluted from the beads with elution buffer and the concentration was determined with a Bradford assay. The protein was sent to Davids Biotechnology for rabbit injection.

2.2.3.10 In-vitro translation

0.5-1 µg of vector DNA was in-vitro transcribed and translated with a TNT-kit (T7 Quick Coupled Transcription/Translation System, Promega) according to the manufacturer's instructions. During the translation, proteins were labeled with [^{35}S]-methionine (Amersham). Proteins were prepared freshly for every experiment.

2.2.3.11 GST-pulldown

10 µl of the GST fusion proteins on beads were incubated with 1 ml IPH buffer and 5 µl of [^{35}S]-labeled in-vitro translated protein on a rotating wheel for 2 h at 4 °C. The beads were washed 5 times with 1 ml IPH buffer, pelleted at 3000 rpm for 1 min and boiled in 3X SDS sample buffer. Bound proteins were resolved by SDS-PAGE and subjected to autoradiography.

2.2.4 Molecular biology

2.2.4.1 Isolation of plasmid DNA from bacteria

Single colonies were picked from an LB-agar plate after transformation and incubated in 4 ml LB containing Ampicillin over night in a shaker at 37 °C. 1.5 ml bacterial solution was pelleted and resuspended in 200 µl S1. The bacteria were lysed by adding 200 µl S2 for 5 minutes. This reaction was neutralized with 200 µl S3. The bacterial debries were pelleted for 5 minutes at full speed and plasmid DNA in the supernatant was precipitated with 600 µl Isopropanol. After centrifugation for 10 min at room temperature, the pellet was washed with 1 ml 70 % Ethanol. The pellet was air-dried and resuspended in 30 µl TE. Correct bacterial clones were identified by restriction digest.

2.2.4.2 Isolation of plasmid DNA fragments from agarose gels

Plasmid DNA was digested with the desired enzymes and incubated at 37 °C for more than 1 h. The restriction was loaded on a 0.8 – 1.2 % agarose gel and fragments were seperated by electrophoresis at 100 – 130 V for 1 h. The desired bands were cut out and isolated with the JetStar Gel Extraction Kit (Genomed) according to the manufacturer´s instructions.

2.2.4.3 Site-directed mutagenesis

For site-directed mutagenesis, primers were designed carrying the desired mutation. These primers were used in a PCR reaction amplifying the whole plasmid.

10 µl 5x HF Phusion buffer	PCR program (12 cycles)
5 µl dNTP	98 °C 30 s
1 µl fw primer (10 pmol/µl)	98 °C 30 s
1 µl rv primer (10 pmol/µl)	55 °C 1 min
50 ng template	68 °C 6 min
1 µl Phusion	68 °C 10 min
ad 50 µl H_2O	10 °C hold

5 µl of the PCR reaction were loaded on a 1 % agarose gel to control the purity of the PCR product. To the remaining product, 1 µl DpnI was added and the mixture incubated at 37 °C for 1 h. During this digest, the template plasmid but not the newly generated product was digested. Supercompetent JM109 bacteria (Promega) were transformed with 5 µl of the digested PCR reaction and plated on LB-agar plates with ampicillin. Single clones were picked from the agar plate and grown in 4 ml LB over night. Plasmid minipreparation and control restriction digest was performed and the positive clones sequenced to confirm the insertion of the mutation.

2.2.5 Gelshift

2.2.5.1 Labeling of DNA fragments

As a probe, a cdc2 promoter fragment was prepared by PCR from the wild-type or mutant cdc2 promoter construct with the following reaction:

2 µl 10x PCR buffer
0.25 µl dNTP (Stock: 2 mM)
0.25 µl dATP/dTTP/dGTP (Stock: 2 mM each)
3.5 µl SG 622 (Stock: 10 pmol/µl)
3.5 µl SG 623 (Stock: 10 pmol/µl)
1 ng plasmid template
1 µl alpha-[^{32}P]-dCTP (Stock: 10 µCi/µl)
1 µl TaqC
7.5 µl H_2O

PCR program (40 cycles):
94 °C 2 min
94 °C 30 s
58 °C 1 min
72 °C 30 s
72 °C 4 min
10 °C hold

The PCR product was brought to 100 µl with TE and purified through Sephadex G-50 spin columns (ProbeQuant G-50, Pharmacia). The efficiency of the labeling was determined by measuring 1 µl labeled probe in a scintillation counter. 10000 counts were used in each gelshift reaction.

2.2.5.2 Purification of GST-proteins for gelshift analysis

For the production and purification of GST-fusion proteins from recombinant bacteria for gelshift experiments, the protocol described in 2.2.3.9 was used with a few changes. The bacteria were lysed in bacterial lysis buffer (20 mM Hepes pH 7.5, 120 mM NaCl, 2 mM EDTA, 10 % Glycerol, protease inhibitors) and the glutathione-sepharose was incubated over night at 4 °C. After 4 wash steps with PBS, the protein was eluted from the beads twice with 250 µl elution buffer for 15 minutes on a shaker. The protein concentration was measured in a Bradford assay (see 2.2.3.3).

2.2.5.3 Preparation of DNA fragments for competition

DNA fragments for competition were prepared by PCR using wild-type or mutated plasmid-DNA as template and primers resulting in different fragment sizes of the cdc2 promoter. The reactions were submitted to the PCR program described in 2.2.5.1.

10 µl 10x buffer
10 µl dNTP (Stock: 2 mM)
4 µl SG 622 (Stock: 10 pmol/µl)
4 µl SG 623 (Stock: 10 pmol/µl)
0.5 µl template (Stock: 1 µg/µl)
2 µl TaqC
69 µl H_2O

The PCR products were extracted from an agarose gel and purified with the Gel Extraction Kit (Genomed). The concentration was determined with the NanoDrop and 50 – 300 ng purified PCR fragment was used for competition.

2.2.5.4 Gelshift binding reaction

The binding reaction was prepared on ice.

5 µl	5x Gelshift binding buffer
0.05 µl	ssDNA (10 mg/ml)
1 µl	BSA (20 mg/ml)
0.3 µg	GST-CXC or GST-CXC-C525/527Y
50-300 ng	cold Oligo for competition
ad 24 µl	H_2O

After incubation on ice for 30 minutes, 10000 counts of the labeled probe were added and the mixture incubated for 30 more minutes at room temperature. Then the reactions were separated in a non-denaturating electrophoresis.

2.2.5.5 Non-denaturating gel electrophoresis

The non-denaturating gel was prepared with the following reaction between detergent-free glass plates.

Gel conditions (6 % acrylamid/bisacrylamid (75:1), 0.5x TAE):

10 ml	30 % acrylamid (w/v)
5 ml	0.8 % bisacrylamid (w/v)
500 µl	50x TAE
0.4 ml	10 % APS
34 µl	TEMED
ad 50 ml	H_2O

The gel was prerun in 0.5 % TAE for 1 h at 250 V in the cold room. The samples were loaded and run 30 minutes at 250 V and 1.5 h at 400 V. The gel was dried and exposed to an X-ray-film with a screen at -80 °C over night.

3 Results

3.1 Cloning of cDNAs encoding human LIN-37, LIN-52 and LIN-54

For the overexpression in mammalian cells, human LIN-37 and LIN-54 were amplified by PCR from plasmid clones containing the desired EST sequence from a cDNA library and cloned into pCDNA3-flag and pCDNA3-HA vectors. The cDNA clones IRATp970G126D6 (LIN-37) and DKFZp686bG04165Q (LIN-54) were used as templates in a PCR reaction and the fragments were cloned into pCDNA3-flag and pCDNA3-HA plasmids. Björn von Eyss cloned LIN-52 from the cDNA clone IRAKpS61G1214Q2. Phylogenetic trees comparing protein sequences of known homologues are shown in Fig. 3.1, for full sequence of the human cDNAs see Appendix (7.1).

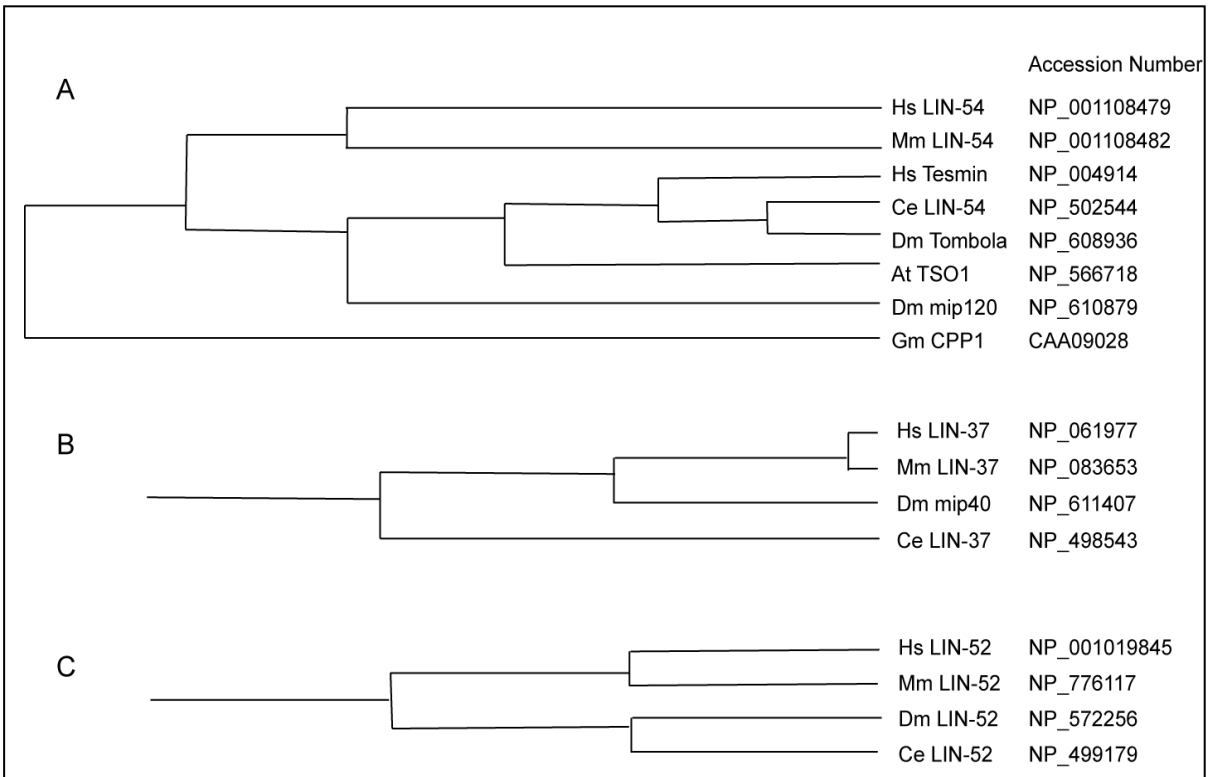


Fig. 3.1: Phylogenetic trees showing homologues of LIN-54 (A), LIN-37 (B) and LIN-52 (C).
Hs: Homo sapiens; Mm: Mus musculus; Ce: Caenorhabditis elegans; Dm: Drosophila melanogaster; At: Arabidopsis thaliana; Gm: Glycine max. Protein alignment was performed by ClustalW2 at http://www.ebi.ac.uk/Tools/clustalw2 (Larkin et al., 2007).

3.2 Binding studies with overexpressed proteins

To determine if the human proteins of a potential dREAM-like complex interact with each other, HeLa cells were transfected with tagged overexpression constructs and protein interactions were studied using immunoprecipitation experiments. Since human LIN-9 was already known and LIN-9 homologues are members of both the Drosophila and C. elegans complexes, flag-LIN-9 was first coexpressed with HA-LIN-37, HA-LIN-52, HA-LIN-54 or HA-RbAp48. Whole cell lysates were immunoprecipitated with an HA antibody and bound LIN-9 was detected by immunoblots with a flag antibody. As shown in Fig. 3.2.A, LIN-9 interacted with all the tested proteins but was not detected when no HA-tagged protein was coexpressed. Because LIN-54 overexpression was very weak and therefore the interaction not as clear as the other interactions, HA-LIN-54 and flag-LIN-9 were coexpressed and their interaction confirmed in an independent experiment (Fig. 3.2.B). Similarly, the interactions between flag-LIN-9 and HA-LIN-52 (Fig. 3.2.C) and flag-LIN-9 and HA-RbAp48 (Fig. 3.2.D) were confirmed in independent experiments. Furthermore, HA-LIN-54 interacted with flag-LIN-37 (Fig. 3.2.E) and flag-B-MYB (Fig. 3.2.F). Interactions between LIN-9 and B-MYB have been shown before (Osterloh et al., 2007). In summary, all the tested overexpressed proteins interact with each other. These data give first evidence for the existence of a human dREAM-like complex.

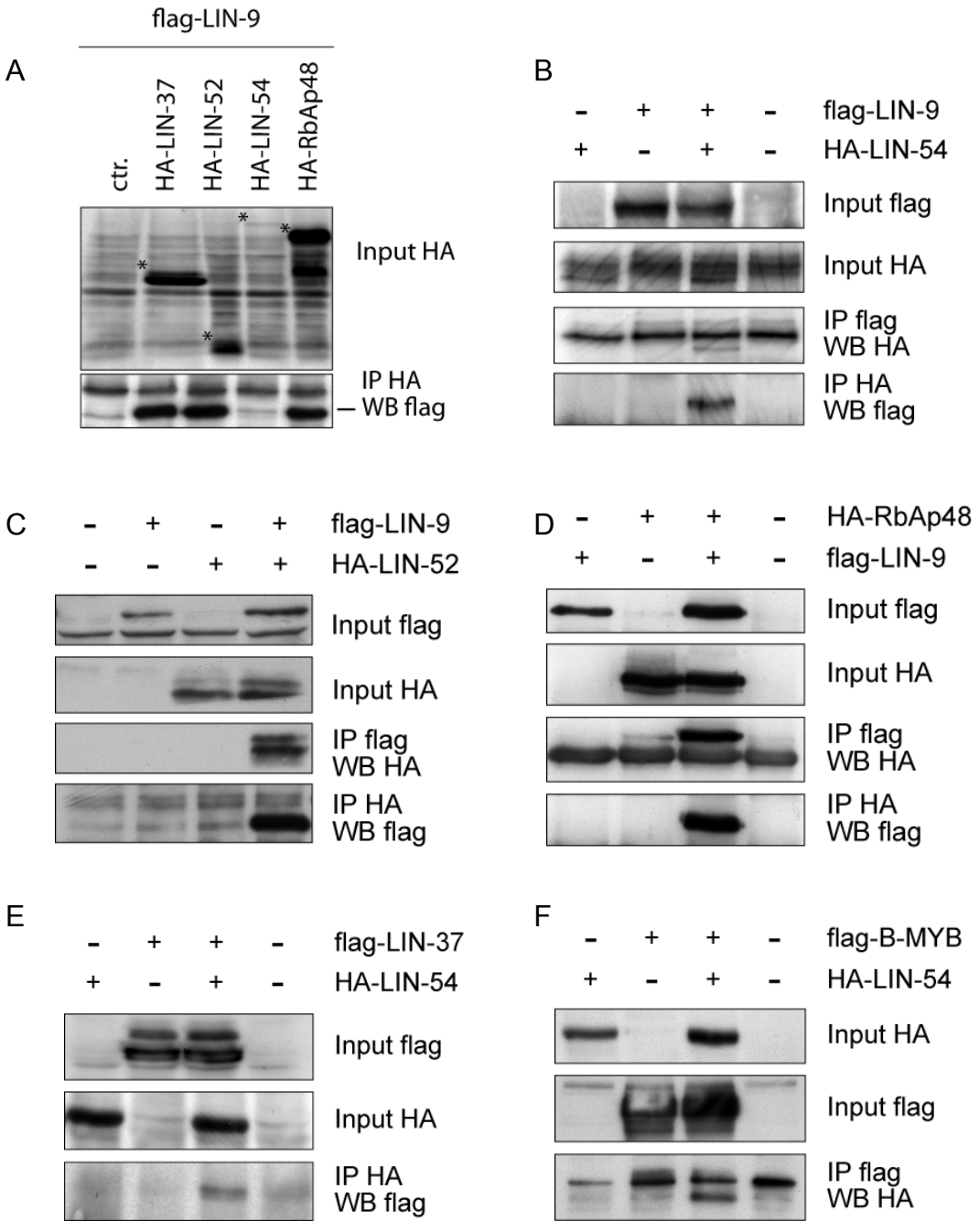


Fig. 3.2: Overexpressed human members of a putative dREAM-like complex interact with each other.
HeLa cells were transfected with the indicated overexpression constructs. Lysates were precipitated with α-HA or α-flag-antibodies as indicated and detected in immunoblots with the indicated antibodies. (A) Interaction of LIN-9 with LIN-37, LIN-52, LIN-54 and RbAp48. (B) Interaction of LIN-9 and LIN-54. (C) Interaction of LIN-9 and LIN-52. (D) Interaction of LIN-9 and RbAp48. (E) Interaction of LIN-37 and LIN-54. (F) Interaction of B-MYB and LIN-54. IP: Immunoprecipitation, WB: Western Blot.

3.3 Binding studies with endogenous proteins

3.3.1 Raising specific polyclonal antibodies

Since antibodies against LIN-37, LIN-52 or LIN-54 were not commercially available, recombinant GST-tagged LIN-37, LIN-52 and LIN-54ΔC (amino acids 1-389) were used to immunize rabbits. The immunesera and their corresponding preimmunesera were tested for specificity in immunoprecipitation/immunoblotting experiments. HeLa whole cell lysates were precipitated with immunesera against LIN-37 (Fig. 3.3.A), LIN-54 (Fig. 3.3.B) or LIN-52 (Fig. 3.3.C) or their corresponding preimmunesera and blotted with the same specific antisera. LIN-37 and LIN-54 antisera detected proteins of the expected size and thus can be used in immunoprecipitation and immunoblotting experiments. In contrast, signals obtained with the LIN-52 antiserum were very weak and many unspecific bands were detected. Therefore, in-vitro translated and [^{35}S]-methionine-labeled HA-LIN-52 was immunoprecipitated with the rabbit immunesera and HA and IgG as positive and negative controls and detected by autoradiography (Fig 3.3.D). This experiment showed that LIN-52 antisera can be effectively used for immunoprecipitation, but are not useful for immunoblotting. Antiserum against LIN-9 has been described before (Gagrica et al., 2004; Osterloh et al., 2007).

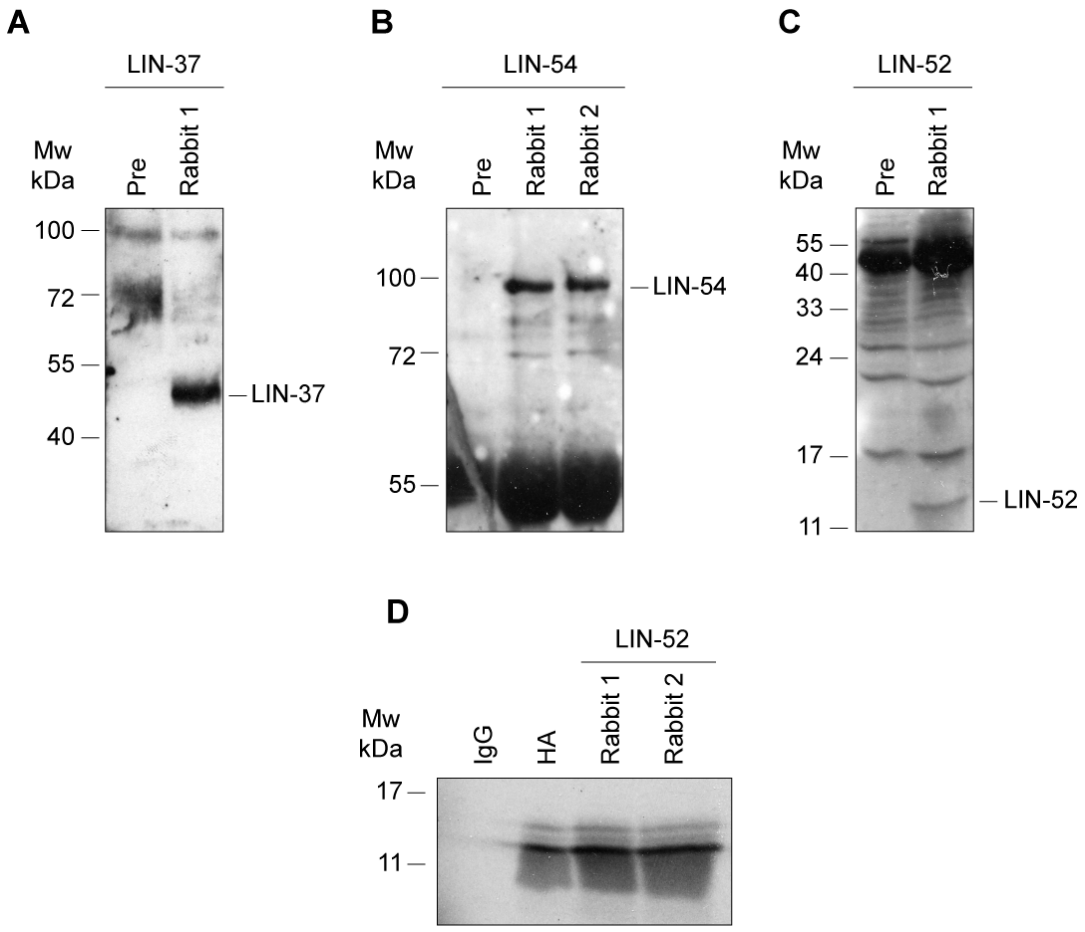


Fig. 3.3: Specific polyclonal antisera were raised against LIN-37, LIN-54 and LIN-52.
HeLa cell lysates were immunoprecipitated with antisera against LIN-37 (A), LIN-54 (B) or LIN-52 (C) and with their corresponding preimmunesera and detected by immunoblotting with the same immunesera. (D): In-vitro translated [^{35}S]-methionine-labeled HA-LIN-52 was precipitated with unspecific IgG, HA antibody and LIN-52 antisera. Labeled LIN-52 was detected by autoradiography. Mw: molecular weight, kDa: kiloDalton.

3.3.2 Immunoprecipitation of endogenous proteins

Since overexpressed proteins showed multiple interactions (see Fig. 3.2), the next important question was whether the endogenous proteins also interact. To address this, cell lysates from untransfected HeLa cells were immunoprecipitated with antisera against LIN-9, LIN-37, LIN-52 or LIN-54 or with their respective preimmunesera or with B-MYB antibody or IgG as a control. Bound LIN-9, LIN-37, LIN-54, B-MYB and RbAp48 were detected by immunoblotting. These interactions were not detected with the preimmunesera, indicating that all of these endogenous proteins specifically interact with each other (Fig. 3.4).

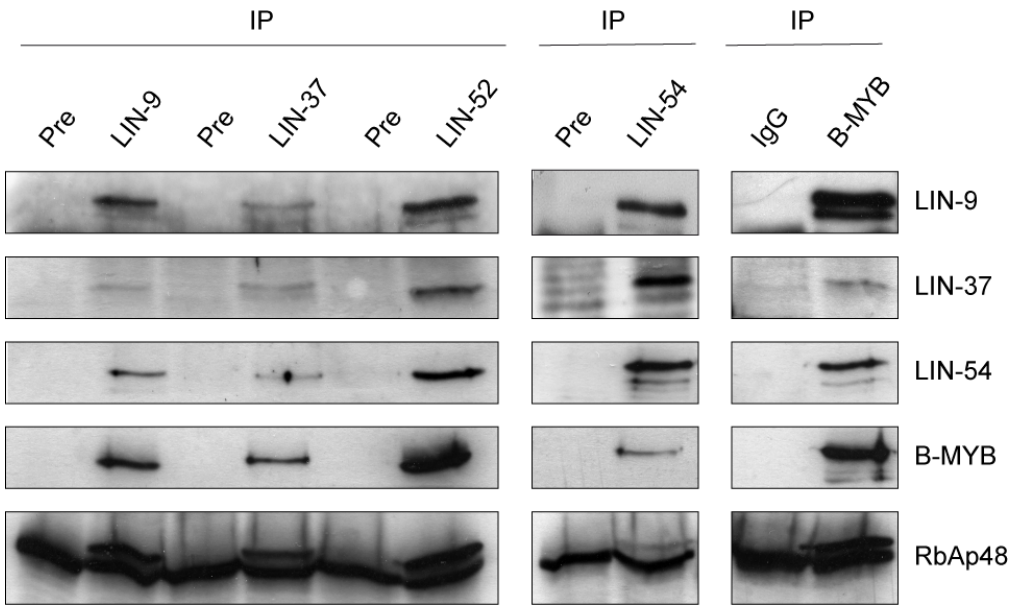


Fig. 3.4: Endogenous human members of a putative dREAM-like complex interact with each other.
HeLa cells were lysed and precipitated with specific immunesera or the corresponding preimmunesera as indicated on top. Interaction partners were detected by immunoblotting with the antibodies indicated on the right.

3.3.3 Direct protein-protein interactions

3.3.3.1 Yeast-two-hybrid

Interactions shown by immunoprecipitation experiments can be direct or mediated by a third protein. Yeast-two-hybrid experiments performed by Claudia Franke and Frank Hänel (Jena) should give information about which of the interactions are direct. They could show that LIN-9 directly interacts with LIN-52 and RbAp48. In yeast, LIN-54 and LIN-37 did not bind to any other assayed proteins directly (Fig. 3.5).

	AD-Fusion				
BD-Fusion	RbAp48	LIN-37	LIN-52	LIN-54	B-MYB
LIN-9 (pGBT9)					
RbAp48					
LIN-37					
LIN-52					
LIN-54					
B-MYB					

Fig. 3.5: Summary of yeast-two-hybrid experiments.
Yeast cells were transformed with the indicated AD (activating domain) and BD (binding domain) fusion constructs. Interaction of two proteins results in β-galactosidase expression, which was detected by filter assay. Yellow: no interaction. Grey: self-activation of the BD-fusion alone. Blue: Yeast-two-hybrid interaction. Experiments performed by Claudia Franke and Frank Hänel (Jena).

3.3.3.2 Verification of direct interactions by GST-pulldowns

To verify the yeast-two-hybrid data, GST-pulldown experiments were performed to see whether LIN-9 directly interacts with LIN-52 and RbAp48 in-vitro. GST-tagged LIN-9 and GST as a control were expressed in recombinant bacteria and bound to glutathione-sepharose beads. In-vitro translated (IVT) and [^{35}S]-methionine-labeled LIN-52 or RbAp48 were incubated with GST- and GST-LIN-9 beads, the samples loaded on an SDS gel and labeled IVT-protein was detected by autoradiography (Fig. 3.6). The input lanes served as a positive control for the in-vitro translation. LIN-52

and RbAp48 proteins bound to GST-LIN-9 but not to GST alone, showing that these two proteins directly interact with LIN-9 (Fig 3.6).

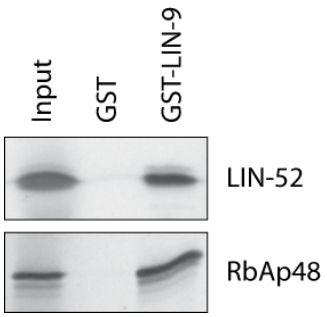


Fig. 3.6: LIN-9 directly interacts with LIN-52 and RbAp48.
Recombinant GST-LIN-9 or GST were incubated with in-vitro translated [^{35}S]-met-labeled LIN-52 or RbAp48. Bound labeled protein was detected by autoradiography.

3.3.3.3 The LIN complex LINC

All the experiments shown and described above suggest that there is a human dREAM-like complex containing LIN-9, LIN-37, LIN-52, LIN-54, RbAp48 and B-MYB. As there are many LIN proteins involved, we named this human core complex LINC (for LIN complex). In parallel to this work, our cooperation partners Michael Korenjak and Alexander Brehm biochemically purified the LIN complex from cycling MOLT-4 cells and identified LIN-9, LIN-37, LIN-54, B-MYB and p107 by mass spectrometry (data not shown, (Schmit et al., 2007)).

3.4 LINC is differentially composed during the cell cycle

Immunoblotting of the final fractions after biochemical purification of LINC showed that LIN-9, LIN-37, LIN-54, RbAp48 and B-MYB perfectly co-eluted, suggesting that they are part of a complex larger than 669 kDa. In contrast, p107 only partially co-eluted in the same fractions (Michael Korenjak and Alexander Brehm, data not shown, (Schmit et al., 2007)), indicating that p107 might only be loosely or context-dependently associated with LINC. Therefore experiments with synchronized cells

were performed as pocket proteins play essential roles in the regulation of the cell cycle and are expressed in a cell cycle-dependent manner.

3.4.1 Cell cycle-dependent interaction of LINC with pocket proteins and transcription factors

To determine the composition of the LIN complex during cell cycle, the T98G glioblastoma cell line was used as it can be synchronized by serum starvation (Stein, 1979). Cells were washed twice with PBS to remove the remaining serum and fed with DMEM without serum for 72 h. Most of the cells were then arrested in G0. The cells could be released from cell cycle block by addition of 15 % serum. After 20 h, many cells were in S-phase (Fig. 3.7).

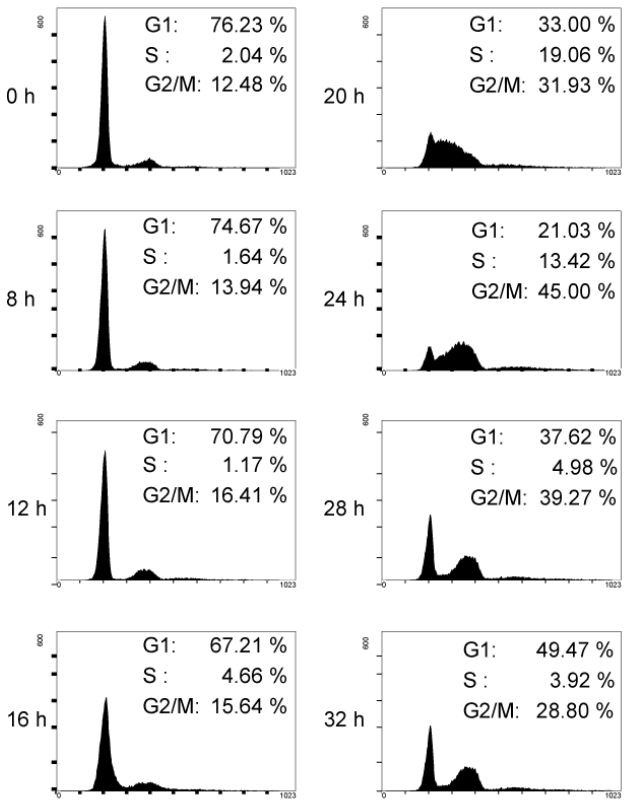


Fig. 3.7: Synchronization of T98G cells.
T98G cells were synchronized in G0 by serum starvation for 72 h and released by addition of 15 % FCS for the indicated times. DNA content was determined by intensity of intercalated PI.

To test LINC composition, T98G cells were harvested in G0 and in S-phase, nuclear extracts were prepared and immunoprecipitated with E2F4, LIN-9, LIN-37 and LIN-54 antibodies. Bound p130, p107, E2F4 and B-MYB were detected by immunoblotting.

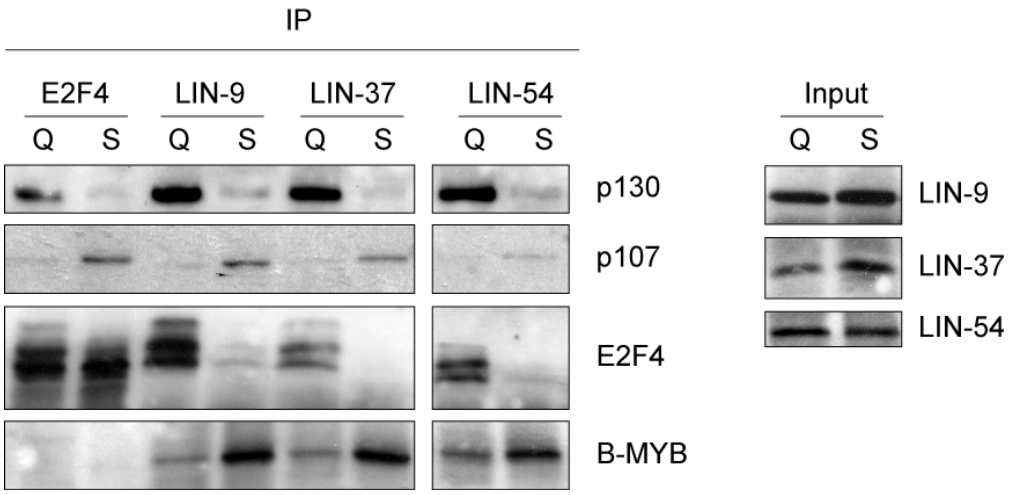


Fig. 3.8: LINC composition is cell cycle-dependent.
T98G cells were synchronized in G0 (Q: quiescent) or in S-phase. Nuclear extracts were immunoprecipitated with the antibodies on top. Bound proteins were detected with the antibodies on the right.

LIN-9 associated with p130 and E2F4 in G0. This binding was lost in S-phase, when LIN-9 bound to p107 and B-MYB (Fig. 3.8, left panel, lanes 3 and 4). Interestingly, LIN-37 and LIN-54 showed the same binding pattern to the tested pocket proteins and transcription factors (Fig. 3.8, left panel, lanes 5 to 8). Input controls showed that LIN-9, LIN-37 and LIN-54 protein levels were comparable in G0 and S-phase (Fig. 3.8, right panel). This experiment proved that the LINC composition is cell cycle-dependent.

3.4.2 B-MYB and p107 interact in S-phase

Since p107 is mainly known as a transcriptional repressor and B-MYB as an activator, it was surprising that both proteins bind to LINC at the same time during the cell cycle. To determine if they belong to the same complex, unsynchronized T98G cell lysates were precipitated with LIN-9, B-MYB and p107 antibodies. Although both p107 and B-MYB strongly interacted with LIN-9, no interaction between p107 and B-MYB could be detected in cycling cells (Fig. 3.9.A). In S-phase synchronized cells however, a weak interaction between p107 and B-MYB could be detected (Fig. 3.9.B), suggesting that there is a short period of time during the cell cycle, where both proteins associate to the LIN complex.

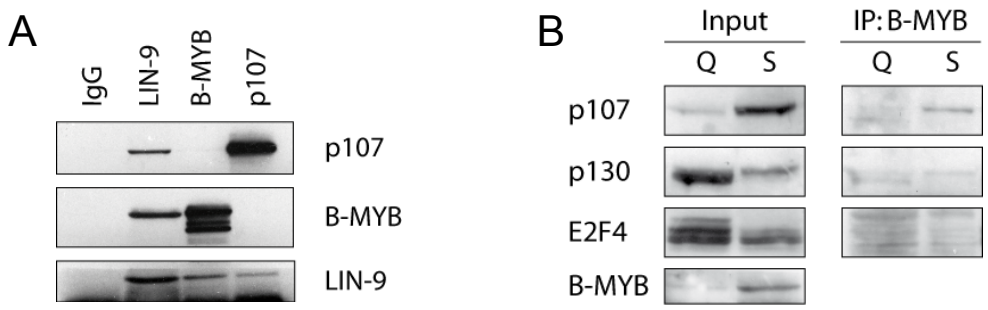


Fig. 3.9: B-MYB and p107 bind in S-phase.
A: Nuclear extracts of (A) unsynchronized and (B) synchronized T98G cells were immunoprecipitated with the antibodies as indicated on top and bound proteins were detected with the antibodies on the right or left. Q: quiescent, S: S-phase

3.4.3 p107 is recruited to the complex earlier than B-MYB

To determine the time frame of p107 and B-MYB association to LINC, T98G cells were harvested at different time points after release from a G0 block. As shown in Fig. 3.7, the cells started entering S-phase after 16 h. After 20 h, most cells were in S-phase, after 24 h, they were in G2/M phase and after 28 h they entered the G1 phase of the next cell cycle. Fig. 3.10 shows that LIN-9 was expressed during the whole cell cycle. p107 was expressed at low levels in G0 and its expression was strongly induced when the cells entered S phase. B-MYB was not expressed at all in G0 and was induced at the same time than p107. As soon as it was expressed, B-MYB associated to LIN-9. In contrast, p107 already associated to LIN-9 a few hours earlier, before its expression was strongly induced (Fig. 3.10).

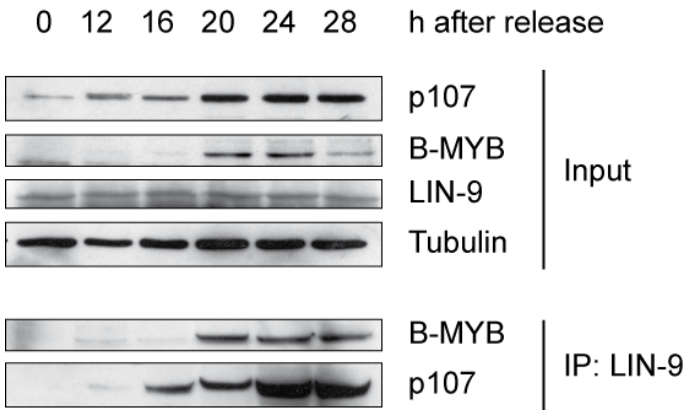


Fig. 3.10: p107 is recruited to LINC earlier than B-MYB.
T98G cells were synchronized in G0 and released from cell cycle block for different time points. Input of nuclear extracts and immunoprecipitated extracts were blotted with the indicated antibodies. IP: Immunoprecipitation.

3.4.4 Stable LINC core complex

To determine whether there is a stable LINC core complex, several rounds of immunodepletions were performed from S-phase T98G cells with a LIN-9 antibody or an IgG antibody as a control. The remaining lysate was tested for protein amounts that were left after LIN-9- or control depletion. After LIN-9 depletion, LIN-37 and LIN-54 were also completely removed from the remaining lysate, indicating that these proteins strongly associate with each other. B-MYB too was nearly completely codepleted after three rounds of LIN-9 depletion, but not after IgG depletion, suggesting that a large percentage of the B-MYB protein in the cell associates to LINC. In contrast, p107 levels were not diminished after LIN-9 depletion, indicating that only a small fraction of p107 proteins bind to LINC and that p107 also binds to other complexes or exists as a free protein in the cell (Fig. 3.11).

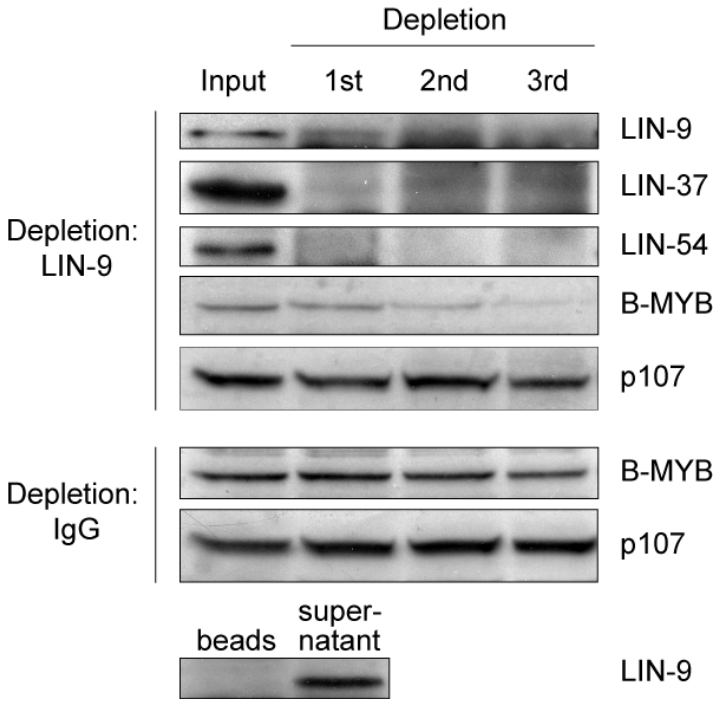


Fig. 3.11: LIN-proteins form a stable core complex.
Nuclear extracts from S-phase T98G cells were immunoprecipitated several times with a specific LIN-9 antiserum or IgG as indicated on the left. Remaining lysate was blotted with the antibodies on the right.

3.4.5 Overexpression of B-MYB in G0 is not sufficient to switch LINC

One interesting mechanistic question is how LINC switches from p130/E2F4 binding to p107/B-MYB. The most obvious explanation is that B-MYB is not expressed early in the cell cycle, and that upon its expression, B-MYB replaces p130 and E2F4 on LINC. To test this possibility, T98G cells were transfected with an overexpression construct for B-MYB, serum starved for 72 h, and tested for the composition of LINC with immunoprecipitation/immunoblotting experiments. Overexpressed B-MYB alone in the context of a G0 cell was not able to switch LINC, since LIN-9 still bound to p130 and E2F4 (Fig. 3.12). This suggests that other events in addition to B-MYB overexpression are responsible for the switch of LINC.

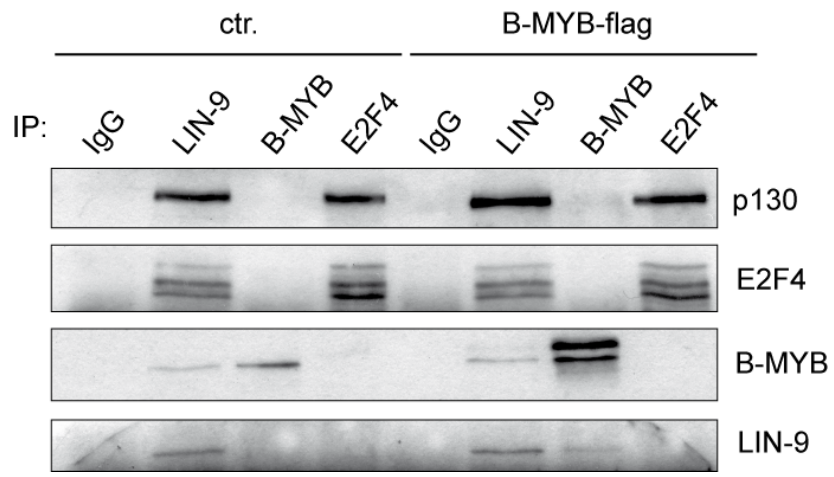


Fig. 3.12: Overexpression of B-MYB in G0 cells is not sufficient to switch LINC. Control cells and B-MYB overexpressing cells were synchronized in G0. Nuclear extracts were immunoprecipitated with the antibodies indicated on the top and bound proteins were detected with the antibodies as indicated on the right.

3.5 LIN-54 is involved in G2/M transition

3.5.1 LIN-54 depletion in primary fibroblasts leads to growth defects

LIN-9 depletion from primary human fibroblasts was already known to induce cell cycle defects (Osterloh et al., 2007). To address whether this defects depend on LINC or whether this is an isolated function of LIN-9, the possible function in proliferation of LIN-54, another LINC core complex member, was examined. Therefore, immortalized BJ cells containing the ecotrophic receptor (BJ-ET) were infected with a murine virus encoding for an shRNA against LIN-54 or an unspecific control sequence. After selection, the efficiency of the knock-down was tested on mRNA (Fig. 3.13.A) and protein levels (Fig. 3.13.B). After LIN-54 depletion, LIN-54 mRNA was reduced by 70 % (Fig. 3.13.A), and a significant reduction of the protein level was detected in immunoblots (Fig. 3.13.B). To analyze the role of LIN-54, the proliferation of LIN-54 depleted cells compared to control cells was quantified with a cumulative proliferation curve. Therefore, infected and selected cells were monitored for 12 days for their proliferation behavior. $1x10^5$ cells were plated in triplicates, counted every 4 days and $1x10^5$ cells were replated. Total cell numbers were calculated and plotted against time (Fig. 3.13.C). These experiments showed that LIN-54 depleted cells grow much slower than control cells, suggesting a strongly reduced cell proliferation (Fig. 3.13.C).

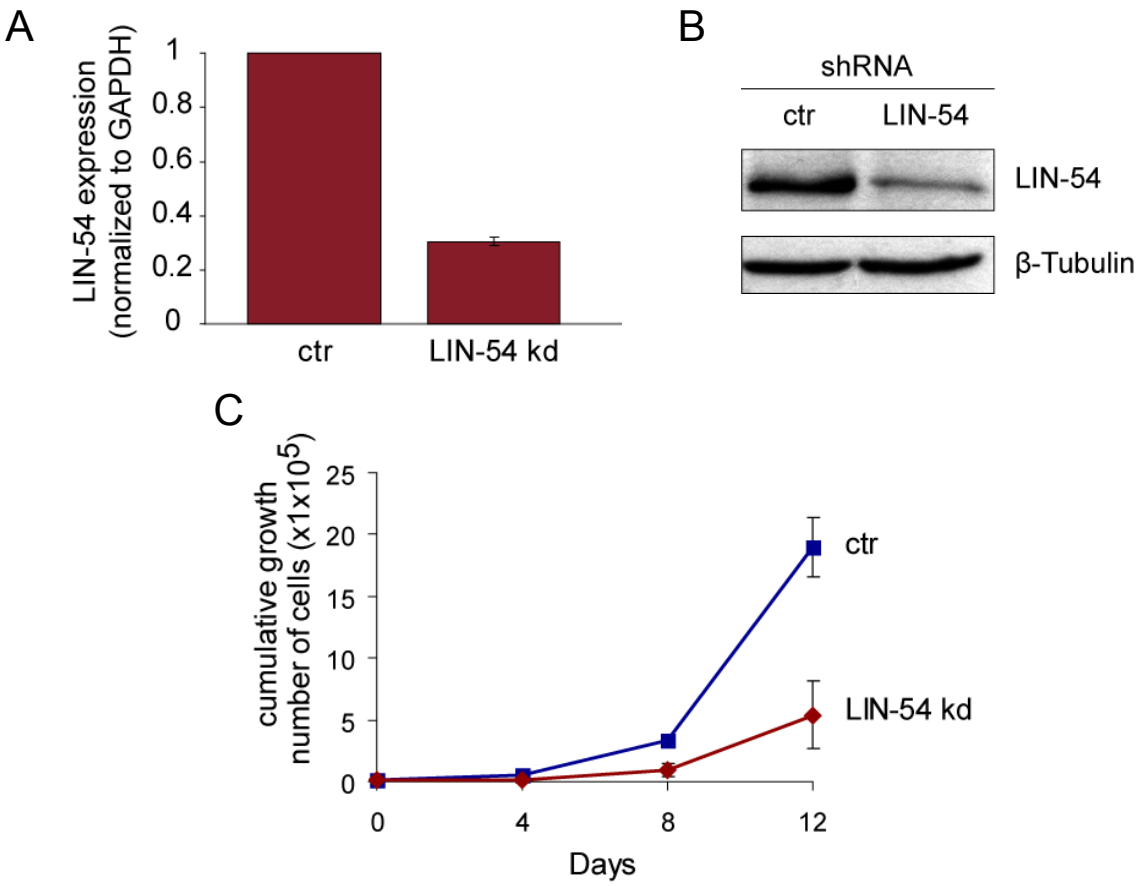


Fig. 3.13: LIN-54 depletion leads to reduced proliferation of BJ-ET cells. BJ-ET cells were infected with control or shRNA virus against LIN-54. Knock-down (kd) was detected on RNA (A) and protein (B) levels. (C) Cumulative growth curve of control cells and LIN-54 depleted cells (Mean values of three independent experiments). ctr: control.

3.5.2 LIN-54 depleted cells accumulate in G2/M

To monitor where the cell cycle defect takes place, flow cytometry profiles of control cells and LIN-54 depleted BJ-ET cells were compared. The infected and selected cells were treated with BrdU for 1 h, harvested and fixed for cytometry measurement. BrdU was incorporated into the DNA in S-phase, which allowed a clear distinction between S-phase cells and cells in G1 or G2/M. After fixation, the cells were stained with an anti-BrdU-antibody and additionally with propidium-iodide, which allowed the discrimination between G1 and G2/M. LIN-54 depleted cells show an accumulation in G2/M and lower amounts of S-phase cells (Fig. 3.14).

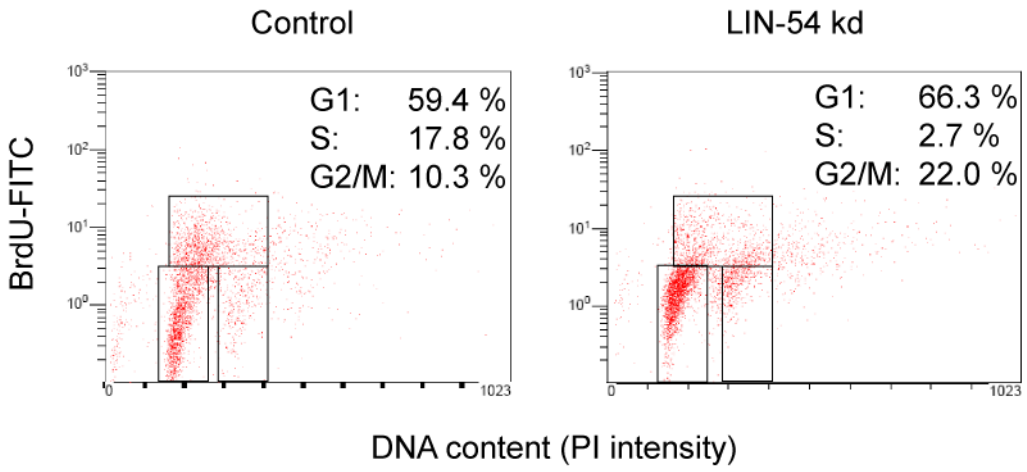


Fig. 3.14: LIN-54 kd cells accumulate in G2/M.
BJ-ET cells were infected with control or shRNA virus against LIN-54 and labeled with BrdU after selection. 1 h after labeling, cells were harvested and fixed for FACS analysis. Cell cycle phases are distinguished by measurement of BrdU-incorporation (S-phase) and propidium-iodide staining. kd: knock-down.

3.5.3 LIN-54 depleted cells arrest before the entry into mitosis

As cells in G2 and mitosis have equal DNA amounts, the flow cytometer can not distinguish between these two phases. Therefore LIN-54 depleted cells and control cells were plated on cover slips and stained with an antibody against phosphorylated serine 10 on histone H3 (PH3). This modification is present in late G2 and early mitosis and H3S10 is dephosphorylated in anaphase. If cells arrest in G2, lower amounts of cells with PH3 staining are expected, whereas cells with PH3 staining accumulate if they arrest in mitosis. When LIN-54 was depleted, the amount of phospho-H3 positive cells decreased from 14.8 % to 5.8 %, indicating that the cell cycle defects occur before the entry into mitosis (Fig. 3.15).

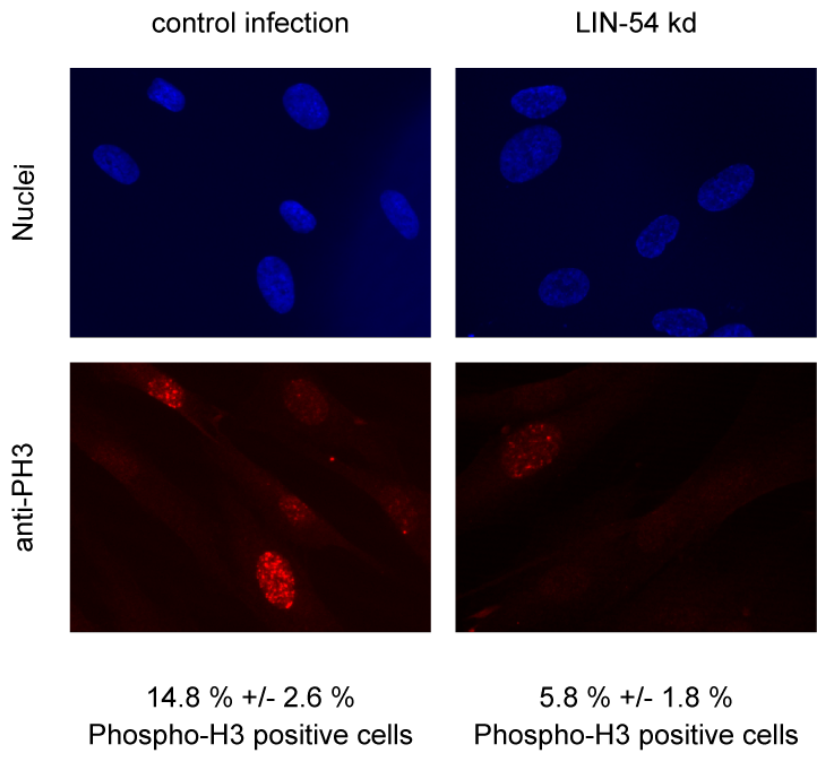


Fig. 3.15: LIN-54 depleted cells arrest before mitosis.
BJ-ET cells were transfected with control virus or LIN-54 kd virus. After selection they were plated on cover slips and stained with an anti-phospho-H3 antibody, a rhodamine-labeled secondary antibody and DAPI. Each experiment was done in triplicates and 300 cells of every triplicate were counted. The numbers show mean values of three independent experiments. kd: knock-down.

3.5.4 LIN-54 depleted cells are delayed in G2

To address whether the cells block completely or are strongly delayed, the kinetics of the cell cycle progression was analyzed. Therefore infected cells were labeled with BrdU for 1 h. Then, the BrdU was removed by washing the cells twice with PBS. 6 h later the cells were harvested, fixed, stained with an anti-BrdU-antibody and with propidium-iodide and measured by flow cytometry. 11.6 % of the control cells that were in S-phase during labeling were in the next G1 phase at 6 h after labeling (see Box in Fig. 3.16). In contrast, only 4.5 % of the labeled LIN-54 depleted cells had reentered the next G1 phase at 6 h after labeling (Fig 3.16). This shows that the cells were strongly delayed in cell cycle progression but did not block completely after LIN-54 depletion.

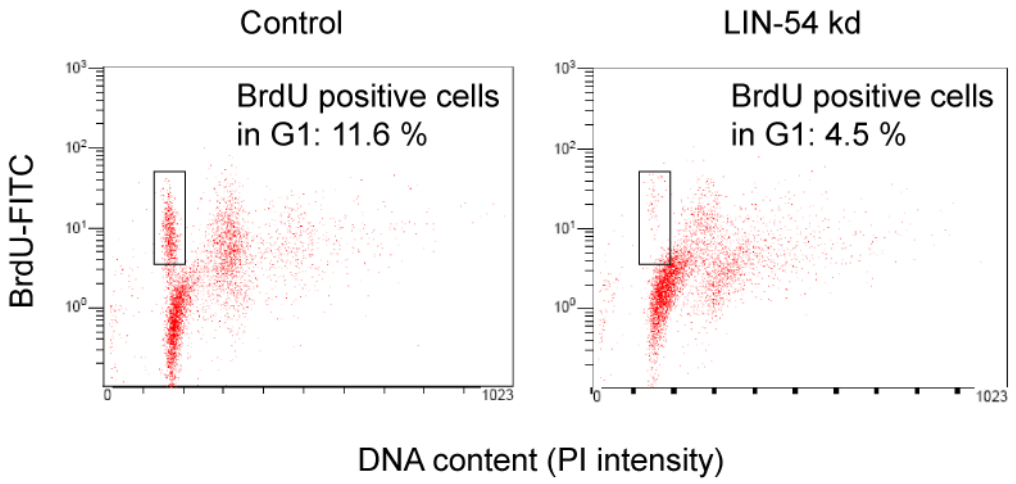


Fig. 3.16: LIN-54 kd cells reenter the next cell cycle.
BJ-ET cells were infected with control or shRNA virus against LIN-54. After selection, cells were labeled with BrdU for 1 h, held in culture for 6 h and harvested and fixed for FACS analysis. Cell cycle phases are distinguished by measurement of BrdU-incorporation (S-phase) and propidium-iodide staining. kd: knock-down.

To determine if the cells needed more time for their progression through S-phase or if they were delayed in G2, the S-phase length was calculated from the raw data of three independent BrdU-pulse experiments like the experiment shown in Fig. 3.16 (see methods for formulas).

The S-phase length was not significantly altered when LIN-54 was depleted (Fig. 3.17).

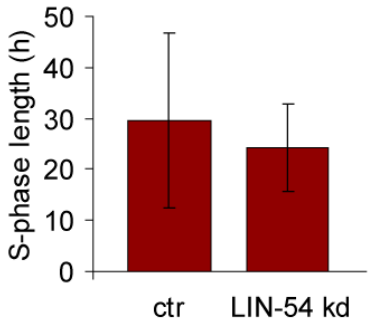


Fig. 3.17: LIN-54 kd cells progress normally through S-phase.
The length of the S-phase was calculated from three independent BrdU-pulse experiments. See text for details.

In summary, these data show that LIN-54 depleted cells are strongly delayed in the G2 phase, suggesting that LIN-54 plays an important role for the entry into mitosis.

3.6 LINC regulates G2/M genes

LIN-9 depletion is known to downregulate several genes important in G2 and mitosis. To determine if this is an isolated function of LIN-9 or if LIN-54 also plays a role in the regulation of these genes, LIN-9 target genes were analyzed after LIN-54 depletion.

3.6.1 LIN-54 depletion downregulates G2/M target genes

BJ-ET cells were depleted of LIN-54 by infection with a knock-down virus. After selection, RNA was prepared and quantitative RT-PCR performed. After LIN-54 depletion, the target G2/M genes were downregulated, but the expression of control G1/S genes was not significantly altered (Fig. 3.18).

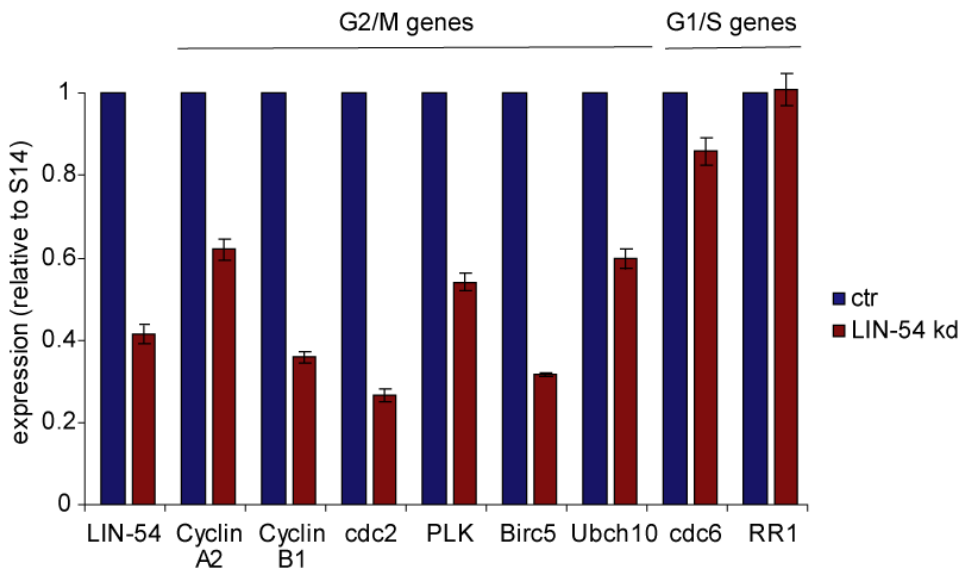


Fig. 3.18: Lower expression of G2/M genes in LIN-54-depleted cells.
BJ-ET cells were infected with a control virus or a LIN-54-kd virus. After selection, RNA was isolated and quantitative RT-PCRs performed.

3.6.2 The regulation of target genes is a direct effect

To determine if the regulation of these target genes could be a direct effect, chromatin immunoprecipitation experiments were performed from synchronized T98G cells. These experiments allow to detect interactions of proteins with the promoters of target genes.

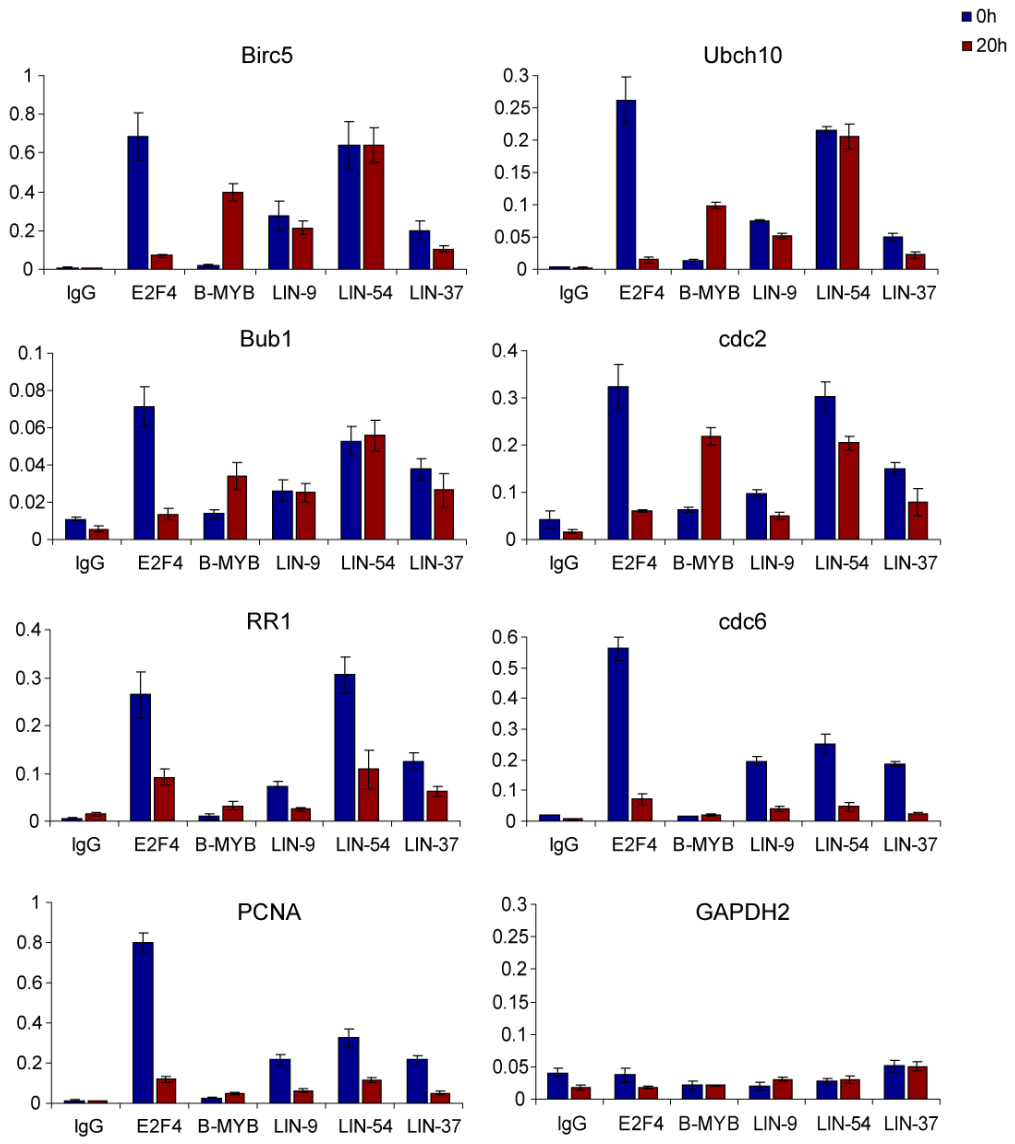


Fig. 3.19: LINC occupies the promoters of its target genes in G0 and S-phase. T98G cells were synchronized in G0 and S-phase. Chromatin was immunoprecipitated with the antibodies indicated below the graphs and quantitative real-time PCRs were performed with primers annealing in the promoter regions of G2/M genes (Birc5, Ubch10, Bub1, cdc2), G1/S genes (RR1, cdc6, PCNA) or an unregulated control gene (GAPDH2).

As known before, it was shown that E2F4 occupied the promoters of the assayed G1/S (RR1, cdc6, PCNA) and G2/M (Birc5, Ubch10, Bub1, cdc2) genes in G0 and left them in S-phase. B-MYB regulated the G2/M genes and bound to their promoters in S-phase. Interestingly, the LINC core complex proteins occupied the promoters of G1/S and G2/M genes together with E2F4 in G0. In S-phase, the LIN-proteins only occupied the promoters of the G2/M genes together with B-MYB and left the G1/S genes (Fig. 3.19). This indicates that LINC together with E2F4 and B-MYB can regulate the target genes directly as it occupies their promoters.

3.7 Characterization of LIN-54 – a protein with evolutionary conserved domains

How LINC is recruited to the promoters of its target genes is not clear. As many of the target genes have known or putative E2F- and B-MYB binding sites, the binding could be mediated by E2F4 in G0 and by B-MYB in S phase. However, LIN-54, a protein that has been conserved through evolution (Fig. 3.1), also carries a potential DNA binding domain, the cysteine-rich region (CXC). In addition, LIN-54 has a helix-coil-helix motif (HCH) that potentially is responsible for protein-protein interactions. To address the functions of these domains in human LIN-54 in detail, a set of LIN-54 deletion mutants were generated (Fig. 3.20).

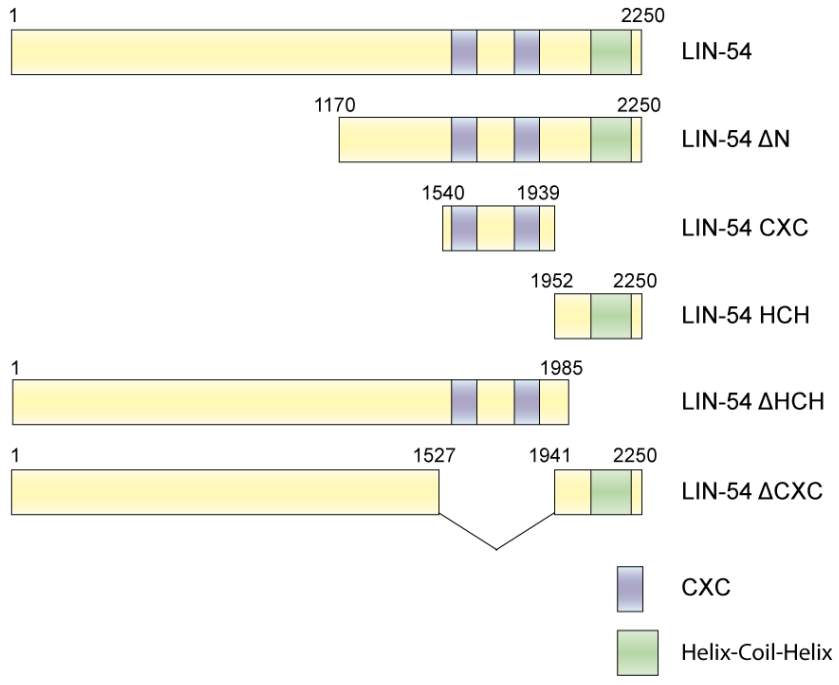


Fig. 3.20: Scheme of LIN-54 deletion mutants.

3.7.1 The HCH domain of LIN-54 is responsible for protein interactions

To test which part of LIN-54 mediates protein interactions, 293T cells were cotransfected with overexpression constructs for HA-p130 and flag-tagged LIN-54 deletion mutants. Whole cell lysates were immunoprecipitated with an anti-flag antibody and bound p130 was detected with an anti-HA antibody. p130 binding could be detected to full length LIN-54 and the ΔN, ΔCXC and HCH mutants, all of which carry an intact HCH region. In contrast, the ΔHCH and CXC mutants that lack the HCH region did not bind to p130 (Fig. 3.21 A). Similarly, binding of B-MYB to LIN-54 could be confined to the HCH region (Fig. 3.21 B), proving that HCH is responsible for protein interactions.

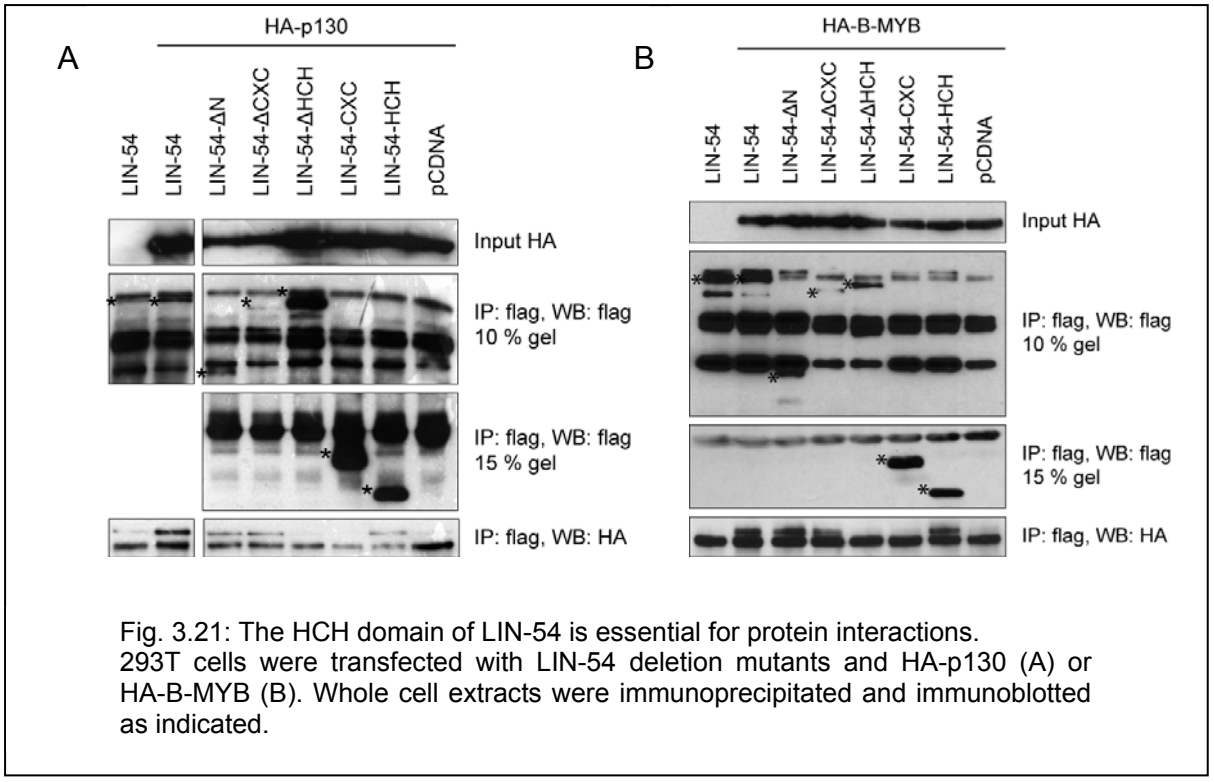


Fig. 3.21: The HCH domain of LIN-54 is essential for protein interactions.
293T cells were transfected with LIN-54 deletion mutants and HA-p130 (A) or HA-B-MYB (B). Whole cell extracts were immunoprecipitated and immunoblotted as indicated.

3.7.2 The CXC domain of LIN-54 interacts with DNA

3.7.2.1 The conserved cysteines in the CXC domain of LIN-54 are required for DNA-binding

To test whether the CXC domain of LIN-54 (see Fig. 3.20 and sequence in the Appendix 7.1.1) directly binds to the promoter of cdc2, one of its target genes, gel shift experiments were performed. First, GST-tagged CXC was purified from recombinant bacteria by affinity purification (Fig. 3.22.A). A 400 basepair fragment of the cdc2 promoter was amplified by PCR spiked with 10 µCi [^{32}P]-dCTP (see Fig. 3.23, wt, for full sequence see Appendix 7.1.4). This labeled probe was incubated with purified GST-CXC protein and the mixture run on a non-denaturating polyacrylamide gel (Fig. 3.22.B). Lane 1 showed the running properties of the free probe. The presence of GST-CXC in lane 2 resulted in a shifted band compared to lane 1, which shows that GST-CXC can bind to the cdc2 promoter fragment. This band was competed when 250 ng unlabeled cdc2 promoter fragment was added to the mixture, indicating that this is a specific band (Fig. 3.22.B lane 3).

To test whether the conserved cysteines in the CXC domain are required for DNA interaction, a mutated GST-construct, where two cysteines (amino acids 525 and 527 counted from the beginning of the LIN-54 full length sequence) were mutated to tyrosine, was expressed and purified from recombinant bacteria (For sequence information see Appendix Fig. 7.1.1). This mutated GST-CXC-C525/527Y protein was not able to bind to the cdc2 promoter in gelshift experiments (Fig. 3.22.B, lane 4), indicating that the cysteines are essential for DNA binding.

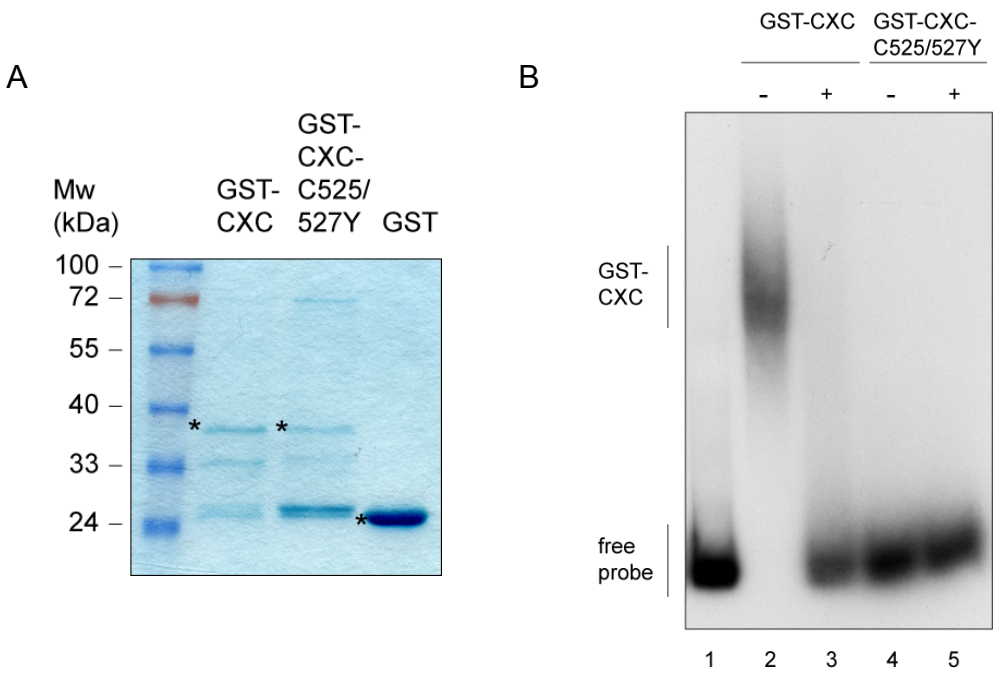
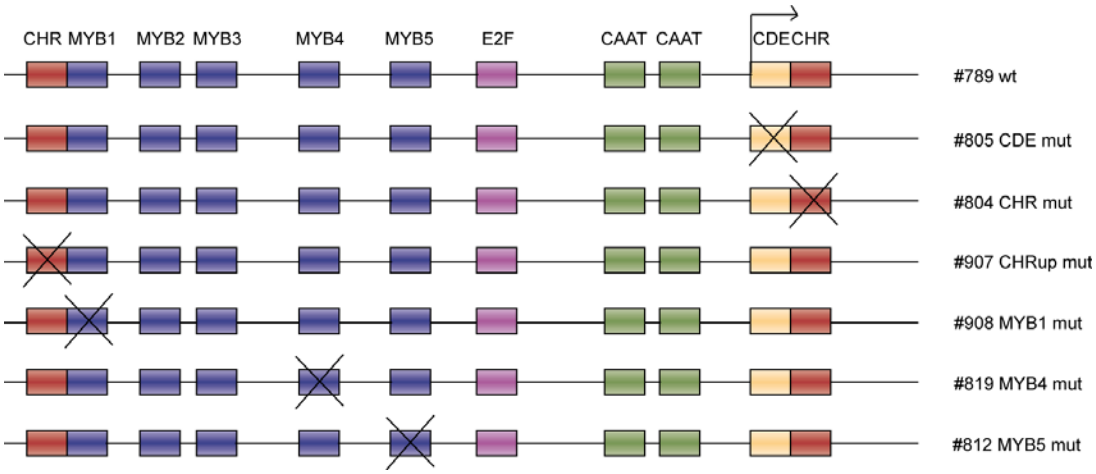


Fig. 3.22: The CXC domain of LIN-54 binds to the cdc2 promoter.
(A) Coomassie stained gel of recombinant GST-CXC, GST-CXC-C525/527Y and GST.
(B) Recombinant GST-tagged CXC and CXC-C525/527Y were incubated with a [^{32}P]-labeled fragment of the cdc2 promoter and separated on a non-denaturating acrylamide-gel. 250 ng non-labeled cdc2 promoter fragment was used to compete for the binding of the GST-protein to the labeled probe (+). Labeled DNA fragment was detected by autoradiography. kDa: kiloDalton.

3.7.2.2 The CHR element is necessary for CXC binding to the cdc2 promoter

To narrow down the CXC-binding site in the cdc2 promoter, mutated cdc2 promoter fragments were used for competition in gelshift experiments (Fig. 3.23). If a mutated fragment used for competition leads to the disappearance of the specific band, this shows that GST-CXC can bind to the competitor. This indicates that the mutated part of the cdc2 promoter is not essential for binding. If the specific band is not competed by addition of the mutated cdc2 promoter fragment, this mutation affects the binding of CXC to it and the mutated region is essential for DNA-protein interaction.

A



B

	Wildtype sequence	Mutated sequence
# 805 CDE mut	TAGCGCgGT	TAGCGCtGT
# 804 CHR mut	AGTttgaAAC	AGTagctAAC
# 907 CHRup mut	ATttGAA	ATccGAA
# 908 MYB1 mut	GAActGTG	GAAtcGTG
# 819 MYB4 mut	AGAaacAGT	AGAggaAGT
# 812 MYB5 mut	CAGttgGCG	CAGcctGCG

Fig. 3.23: Scheme (A) and sequence changes (B) of cdc2 promoter mutants used for competition in gelshift.
For full sequence of the wt promoter see Appendix 7.1.4.

When GST-CXC was incubated with labeled cdc2 promoter, a specific band showed protein-DNA interaction (Fig. 3.24, lane 2), confirming the data shown in Fig. 3.22. As expected, this band was competed with 100 ng of the wildtype DNA fragment (Fig. 3.24, lane 3). Fragments in which the CDE region, the upstream CHR region or MYB binding regions 1, 4 or 5 are mutated (Fig. 3.24, lanes 4 and 6-9) also specifically competed for binding, indicating that GST-CXC requires none of these regions for binding. In contrast, 100 ng of a cdc2 promoter fragment with mutated CHR region (Fig. 3.23) did not efficiently compete (Fig. 3.24, lane 5), suggesting that the CHR region is important for the binding of GST-CXC to the cdc2 promoter.

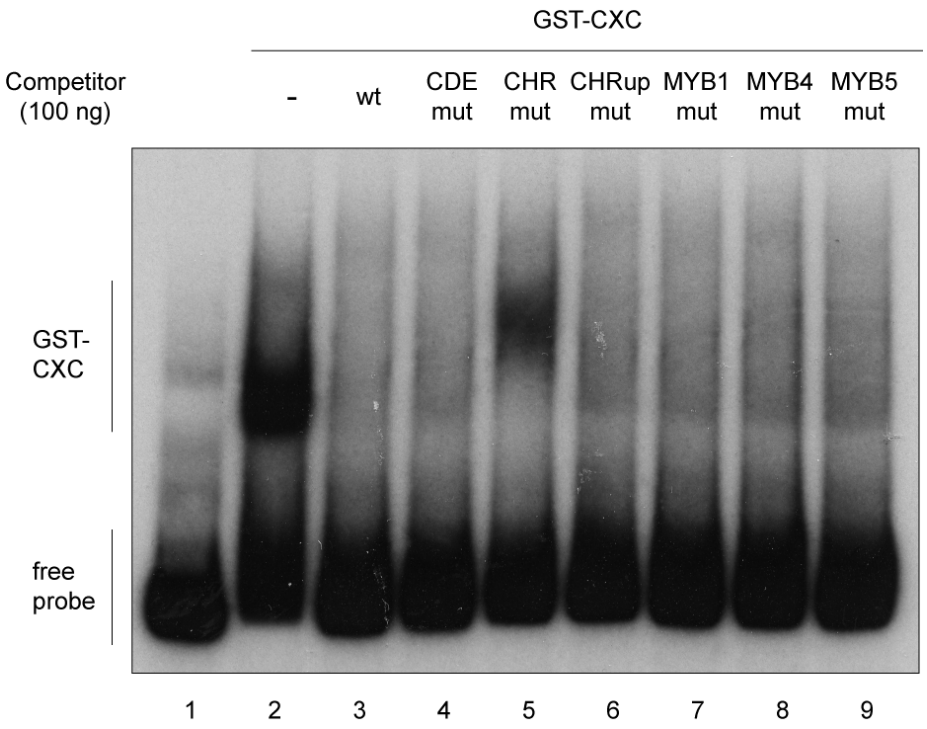


Fig. 3.24: The CHR region is essential for binding of GST-CXC to the cdc2 promoter.
GST-CXC protein was incubated with a [^{32}P]-labeled fragment of the cdc2 promoter (lanes 2-9). Bound labeled probe was competed with 100 ng of non-labeled wildtype cdc2 promoter (lane 3) or different promoter mutants (lanes 4-9).

3.7.2.3 The CHR element is not the only binding site for GST-CXC

Although the cdc2-CHRmut fragment did not compete for binding of GST-CXC to the cdc2 promoter when low amounts were used (Fig. 3.24 and Fig. 3.25, lane 6), a partial competition was observed with higher amounts of competitor (Fig. 3.25, lanes 7-8). This indicates that the binding affinity is reduced but not completely abolished when the CHR element is mutated, suggesting that it is not the only site important for CXC binding.

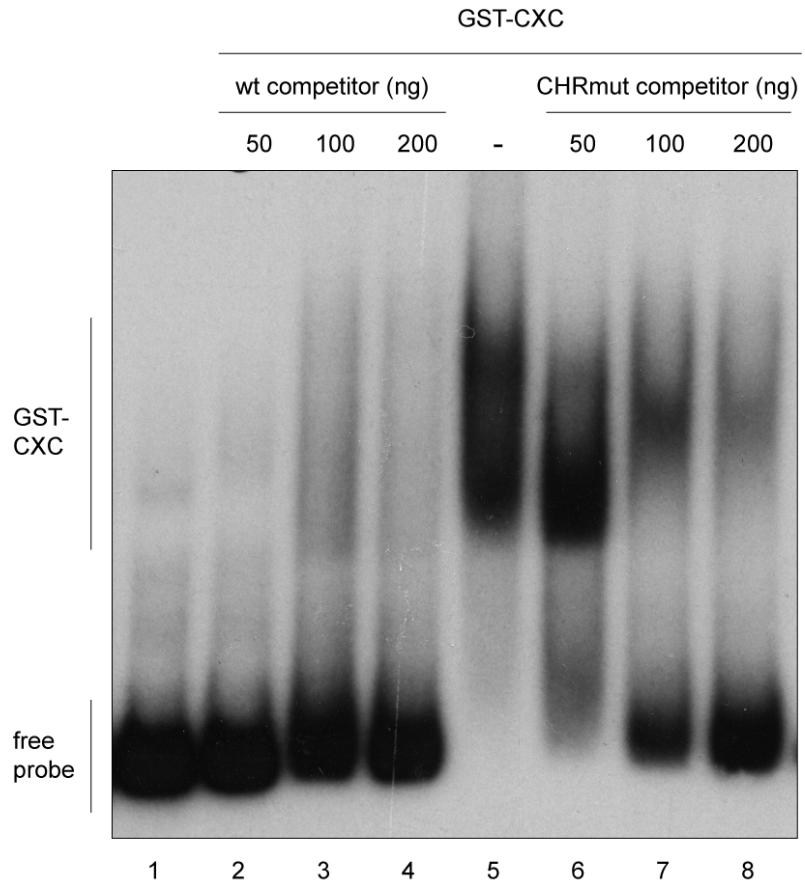


Fig. 3.25: The CHR region is not the only essential region for CXC binding to the cdc2 promoter.
GST-CXC protein was incubated with a [^{32}P]-labeled fragment of the cdc2 promoter (lanes 2-8). Bound labeled probe was competed with increasing amounts of wildtype cdc2 promoter (lanes 2-4) or increasing amounts of the CHR mutant promoter fragment (lanes 6-8).

The assumption that CXC not only associates to the CHR region of the cdc2 promoter is reinforced by the finding that the cdc2-CHRmut construct was shifted by the GST-CXC fusion protein, indicating that the CXC domain also binds to the cdc2 promoter when CHR is mutated. This binding is specific as the band was competed by non-labeled wildtype and CHRmut fragments (Fig. 3.26).

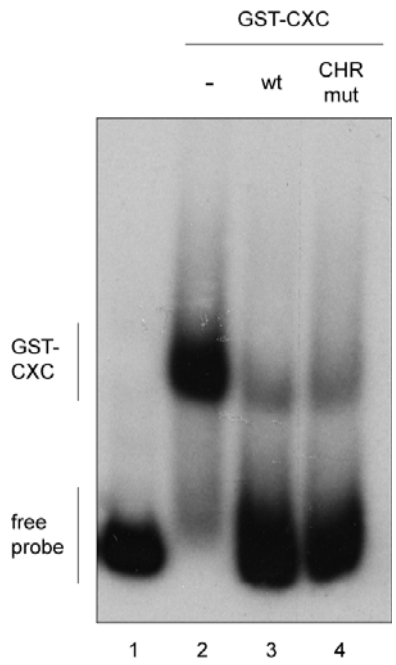


Fig. 3.26: CXC binds to the cdc2-CHRmut promoter fragment.
GST-CXC protein was incubated with a [^{32}P]-labeled fragment of the cdc2 promoter carrying mutations in the CHR region. Bound labeled probe was competed with 100 ng of wildtype cdc2 promoter or CHR mutant promoter fragment.

3.7.2.4 A region near the transcriptional start site is necessary for CXC binding

To find out which region of the cdc2 promoter is important for CXC-interaction, further gelshift experiments were performed with PCR fragments of different length (Fig. 3.27).

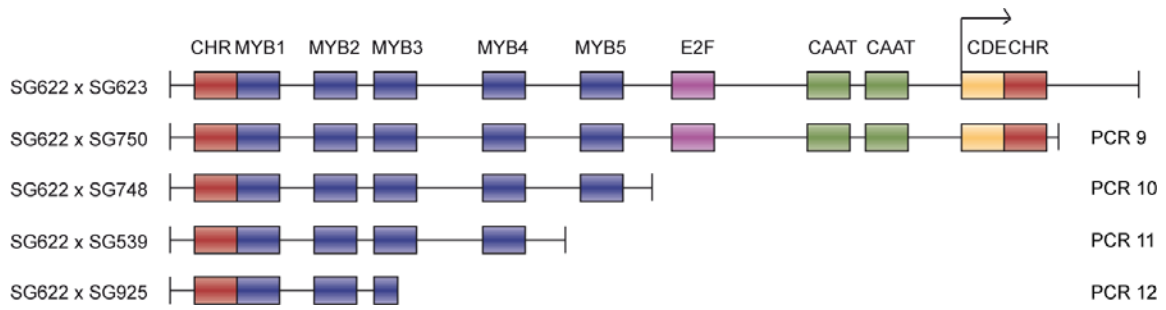


Fig. 3.27: Scheme of the PCR fragments of the cdc2 promoter used for competition in gelshifts.

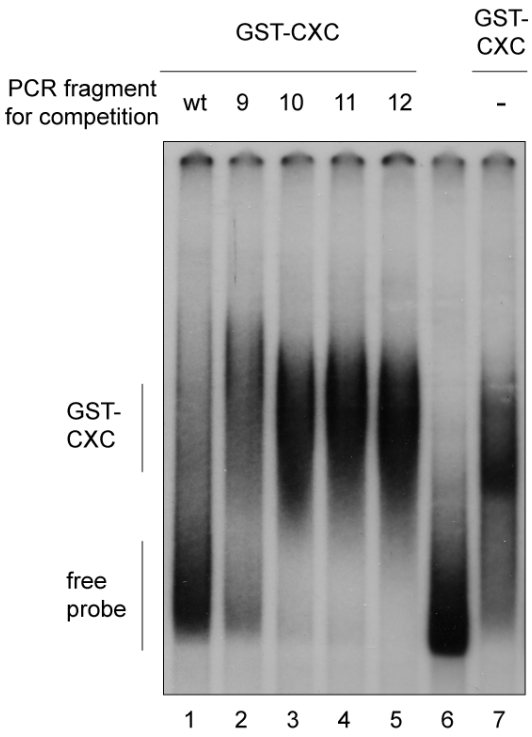


Fig. 3.28: PCR fragments 10-12 do not compete for the binding of GST-CXC to the cdc2 promoter.
GST-CXC protein was incubated with a [^{32}P]-labeled fragment of the cdc2 promoter. Bound labeled probe was competed with 150 ng of the indicated PCR fragments.

The small fragments 10, 11 and 12 (see Fig. 3.27) did not compete for the binding of CXC to the cdc2 promoter, indicating that the CXC domain does not bind to these small fragments (Fig. 3.28 lanes 3-5, compare to lane 7 (without competitor) and to lane 1 (wildtype competitor)). The PCR fragment 9, that lacks 99 basepairs from the wildtype sequence, showed partial competition, indicating that the binding affinity is diminished when a small part near the CHR region is missing (Fig. 3.28, lane 2).

To test if the binding affinity is reduced if PCR fragment 9 is used for competition, the CXC-cdc2 binding was competed with increasing amounts of different competitors. Wildtype cdc2 promoter (Fig. 3.29, lane 3 in each panel) strongly competed already at the lowest concentration. PCR 10 (Fig. 3.29, lane 5 in each panel) did not compete regardless of the amount used for competition. PCR 9 did not compete at the lowest concentration, but the more competitor was used, the more efficiently the specific CXC-cdc2 band was competed (Fig. 3.29, lane 4 in each panel), indicating that the binding affinity of the CXC domain to PCR 9 is reduced compared to the wildtype.

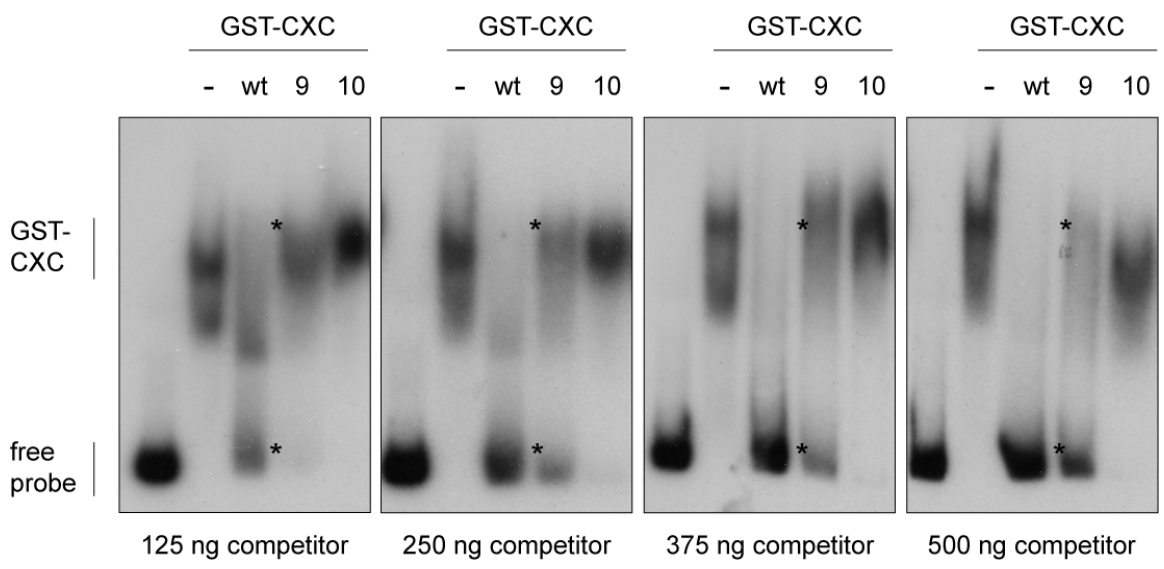


Fig. 3.29: A region downstream of the CHR region is required for the efficient binding of GST-CXC to the cdc2 promoter.
GST-CXC protein was incubated with a [^{32}P]-labeled fragment of the cdc2 promoter. Bound labeled probe was competed with different PCR fragments at the indicated concentrations.

3.7.2.5 An upstream region in the cdc2 promoter is necessary for CXC binding

The experiments shown above indicate that the binding affinity of GST-CXC to the cdc2 promoter is diminished if the CHR region is mutated or if a small part downstream of this region is missing. To determine if there are other binding sites in the cdc2 promoter, competition experiments with cdc2 promoter fragments missing upstream parts were performed (Fig. 3.30).

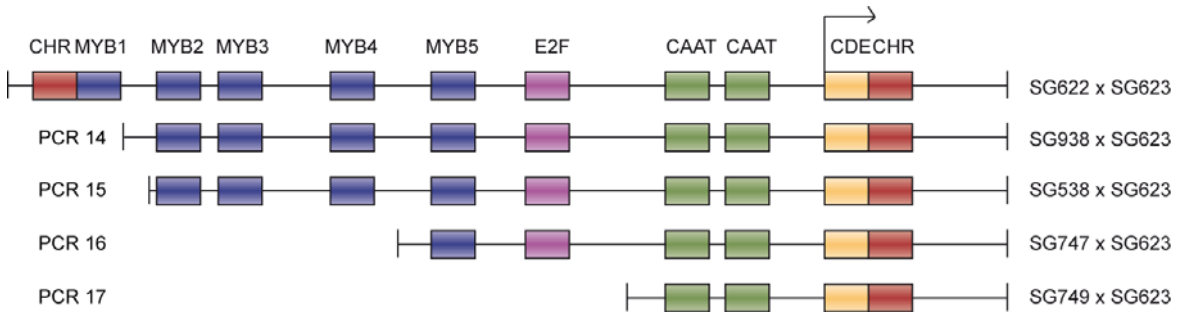


Fig. 3.30: Scheme of the PCR fragments of the cdc2 promoter used for competition in gelshifts.

The three smallest fragments, PCRs 15, 16 and 17, did not compete for binding, indicating that the CXC domain does not bind to these promoter regions, although they contain an intact CHR region (Fig. 3.31, lanes 5-7, compare with lane 2). PCR fragement 14 partially competed for the binding of CXC to the cdc2 promoter (Fig. 3.31 lane 4). PCR 14 only lacks a small part carrying the upstream CHR und MYB1 regions, both of which alone are not necessary for CXC binding (see Fig. 3.24). Taken together these experiments suggest that CXC requires an upstream part of the cdc2 promoter for efficient binding.

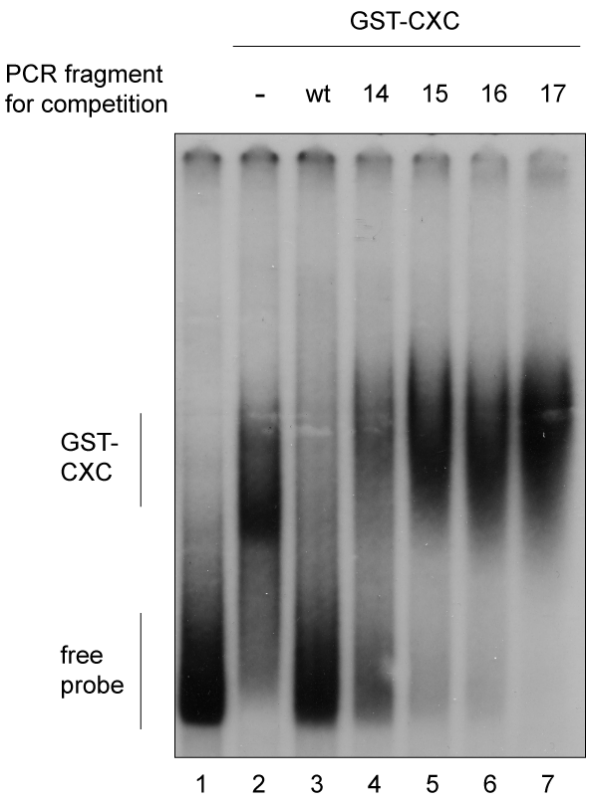


Fig. 3.31: Binding of GST-CXC to the cdc2-promoter is reduced if a small part upstream in the cdc2 promoter is missing.
GST-CXC protein was incubated with a [^{32}P]-labeled fragment of the cdc2 promoter. Bound labeled probe was competed with 200 ng of the indicated PCR fragments.

3.7.2.6 CXC binding to the cdc2 promoter needs upstream and downstream promoter regions

The above results suggest that CXC needs two different parts of the cdc2 promoter for effective binding: the CHR element and one upstream element missing in the PCR fragment 14. To prove this assumption, gelshift experiments were performed comparing the competition behavior of PCR fragment 14 with the PCR fragment 14mut, that was prepared from the CHR mutated template (Fig. 3.32). As shown before, the PCR fragment 14 competed for the binding of GST-CXC to the cdc2 promoter (Fig. 3.33.A, lanes 1-7). Similarly, the binding of GST-CXC to cdc2-CHRmut was competed by PCR14. Importantly, this fragment contains an intact CHR element (Fig. 3.33.A, lanes 8-14). In contrast, the PCR fragment 14mut, which neither contains the upstream part of the cdc2 promoter nor an intact CHR region, was unable to compete the binding of CXC to the cdc2 promoter (Fig. 3.33.B, lanes 1-7) or to the cdc2-CHRmut promoter (Fig. 3.33.B lanes 8-14). Taken together, these data strongly suggest that GST-CXC can interact with the cdc2 promoter at two different binding sites, and that efficient binding needs both binding sites.

Fig. 3.32: Scheme of PCR products 14 and 14mut used for competition

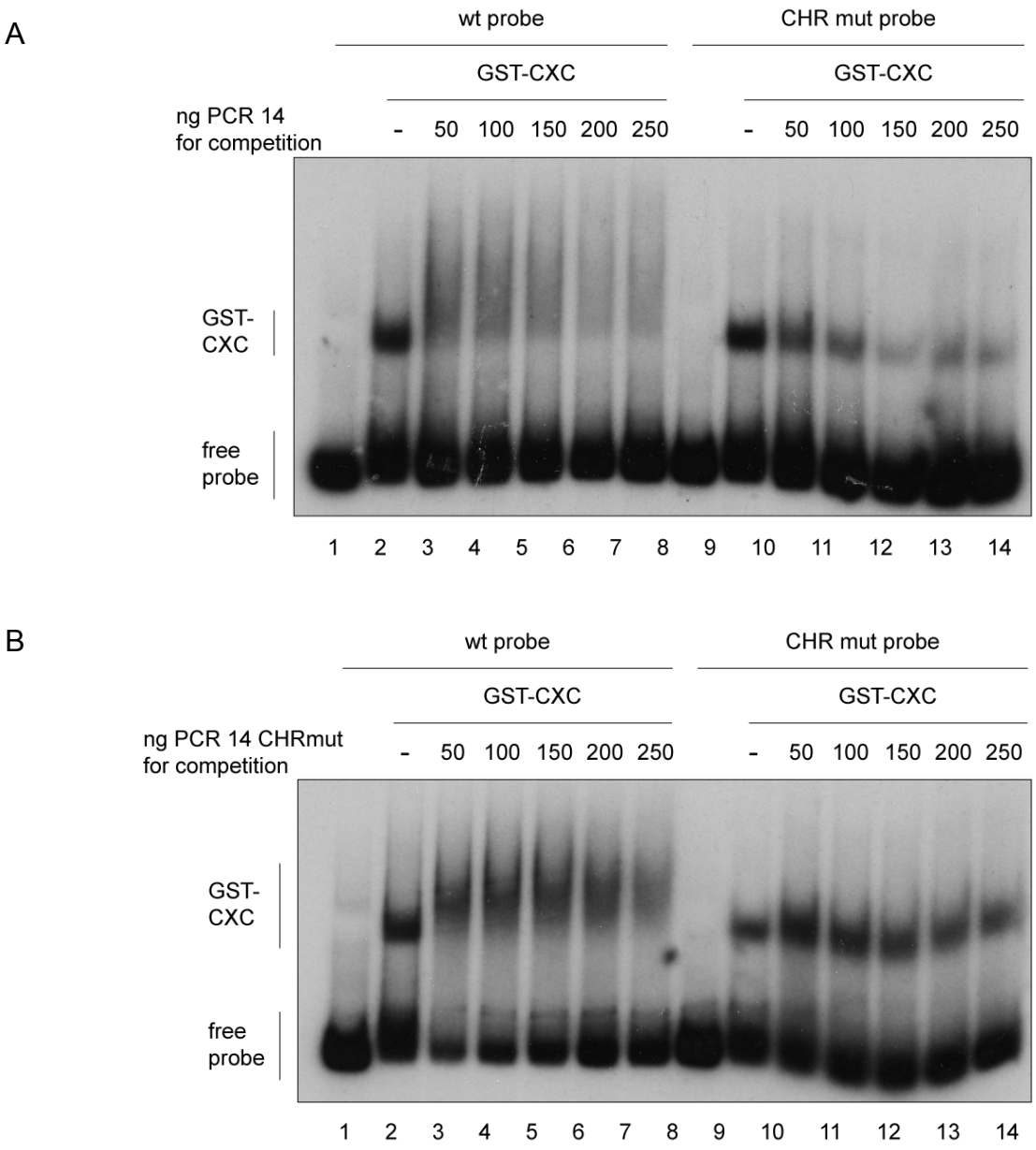


Fig. 3.33: Binding of GST-CXC to the cdc2 promoter depends on an upstream region and on the downstream CHR region.
GST-CXC protein was incubated with a [^{32}P]-labeled fragment of the cdc2 wildtype promoter (A+B lanes 1-7) or CHRmut cdc2 promoter (A+B lanes 8-14). Competition experiments were performed with different amounts of PCR14 (A) or PCR14 carrying a mutation in the CHR region (B)

4 Discussion

pRB and E2F transcription factors play an important role in cell cycle regulation. As the network of pocket proteins and E2Fs is relatively simple in model organisms compared to human cells, complexes containing pRB and E2F homologues have been identified in C. elegans (DRM) (Harrison et al., 2006) and Drosophila (dREAM, MMB) (Korenjak et al., 2004; Lewis et al., 2004) (see 1.2.2). This work addressed the question if a similar complex also exists in human cells and which function it assures.

4.1 Existence of the human LINC complex

All the members of the C. elegans and Drosophila complexes have homologues in humans. Human homologues of dREAM complex members comprise the transcription factors B-MYB and E2F, pocket proteins, the RB-interacting protein RbAp48, which is a member of many chromatin associated complexes and LIN-9, which was first described in our lab. Two other proteins, human LIN-37 and LIN-54, had been described as pRB-interacting proteins in-vitro (Korenjak et al., 2004). dLIN-52, which is present only in the MMB and DRM complexes but not in dREAM, has not been described in human cells yet.

To address whether these proteins are part of a complex in human cells, cDNAs of the human LIN-37, LIN-52 and LIN-54 proteins were cloned and antibodies generated. Since complexes were known from other organisms, the first question addressed was whether there is also a human synMuv-like complex. For this, coimmunoprecipitations from cells overexpressing different combinations of the human homologues were performed, showing that all the tested proteins interact with each other. In addition, association of the endogenous proteins was demonstrated, giving important evidence for the existence of a human complex.
In parallel to this work, our cooperation partners Michael Korenjak and Alexander Brehm (Munich, Marburg) used a stable MOLT-4 suspension cell line expressing flag-LIN-37 to identify LIN-37 binding proteins. They found stoichiometrical amounts of proteins identified as LIN-9, LIN-37, LIN-54, B-MYB and p107 by mass spectrometry. LIN-52, whose homologues are present in the DRM and MMB complexes, is a 16 kDa protein that was too small to be detected in the purification.

Moreover, Korenjak and Brehm biochemically purified nuclear cell extracts and found that LIN-9, LIN-37, LIN-54, B-MYB and RbAp48 cofractionated over six chromatography columns. The final eluates showed a perfect overlap of these proteins in the same fractions, strongly suggesting that they interact. In contrast, p107 partly coeluted but also eluted in different fractions, suggesting that p107 additionally to binding to the LIN proteins also associates with other protein complexes.
Taken together, these data show that a complex similar to the complexes in C. elegans and Drosophila exists in human cells. As many LIN proteins are involved, the stable human core complex was named LINC (for LIN complex).

The loose association of p107 to LINC raised the possibility that the composition of LINC is context-dependent. As p107 is cell cycle-regulated, binding assays were performed in synchronized cells. It could be shown that LINC associates in G0 to E2F4 and p130. This binding was lost in S-phase, where LINC switched to B-MYB and p107. This situation is different from the complexes in the model organisms. In Drosophila, the binding to RBF and dMyb does not seem to be context-dependent although depending on the cellular context dMyb is only a silent member of dREAM/MMB (Beall et al., 2002; Korenjak et al., 2004; Lewis et al., 2004). dMYB and dE2F2 were shown to recruit MMB to the promoters of different gene classes, but there is no evidence for distinct complexes containing either dMyb or dE2F2. In contrast they always coimmunoprecipitated, indicating that they are members of the same complex at the same time (Georlette et al., 2007).
In addition to the dREAM and MMB complexes, which are widely expressed in different tissues in Drosophila, one tissue-specific complex tMAC (testis Meiotic Arrest Complex) has been purified from Drosophila testes (Beall et al., 2007), suggesting that in Drosophila context-dependent complexes may also exist.

In parallel to this work, another group also described a similar complex in human cells called DREAM (DP, RB-like, E2F and MuvB) (Litovchick et al., 2007). This complex like LINC contains LIN-9, LIN-37, LIN-52, LIN-54 and RbAp48 as core proteins. DREAM binds to E2F4 and p130 in G0 and to B-MYB in S-phase (Litovchick et al., 2007). A context-dependent composition of a human dREAM-like

complex has also been confirmed by Pilkinton and colleagues although they found an association of p107 to the complex in G0 (Pilkinton et al., 2007).
A summary of the complexes with their members is shown in Fig. 4.1.

Drosophila melanogaster			**C. elegans**	**Homo sapiens**	
Lewis et al. 2004	Korenjak et al. 2004	Beall et al. 2007	Harrison et al. 2006	Schmit et al. (LINC) Litovchick et al. (DREAM) 2007	
Myb/MuvB	**dREAM**	**tMAC**	**DRM**	**G0**	**S**
mip130	mip130	Aly	Lin-9	LIN-9	LIN-9
mip40	mip40	mip40	Lin-37	LIN-37	LIN-37
dLin-52			Lin-52	LIN-52	LIN-52
mip120	mip120	Tomb	Lin-54	LIN-54	LIN-54
p55	p55	p55	Lin-53	RbAp48	RbAp48
dDP	dDP		dpl-1	DP	DP
dE2F2	dE2F2		Efl-1	E2F4	
RBF1/2	RBF1/2		Lin-35	p130	(p107)
dMyb	dMyb				B-MYB
Rpd-3					
L(3)MBT					

Fig. 4.1: Summary of E2F/pocket protein complexes in different species.
p107 is present in the LIN complex but not in the DREAM complex.

Although the components of LINC are known, its three-dimensional structure is unclear. It would be interesting to known if there is one central protein that keeps the complex together and if E2F4/p130 and B-MYB/p107 interact with the same core complex member. To address these questions, yeast-two-hybrid experiments were performed by Claudia Franke and Frank Hänel (Jena). These assays showed that LIN-9 directly interacts with LIN-52 and with RbAp48 (Claudia Franke, Frank Hänel, data not shown (Schmit et al., 2007)). These interactions were confirmed by GST-pulldown assays. LIN-37 and LIN-54 however did not bind directly to any other LINC member in yeast. Possibly these proteins need a modification for interactions that cannot take place in yeast. Possible posttranslational modifications of the LINC core complex members could be analyzed using mass spectrometry. Furthermore, interaction studies with synchronized knock-down cells could show if one of the LINC

members is essential for the formation of LINC or for the association with pocket proteins and transcription factors.

The molecular mechanism of the LINC switch during the cell cycle remains unclear. The most obvious explanation is that B-MYB is not expressed in G0 (Lam et al., 1995; Lam et al., 1992). Upon B-MYB expression, it might compete for a binding site with p130 or E2F4 and displace them. This is possible, as p130 and B-MYB both bind to the HCH region of LIN-54. To test if the mere expression of B-MYB leads to the switch of LINC, B-MYB was overexpressed in T98G cells and the cells were synchronized in G0. Although B-MYB was strongly overexpressed, LIN-9 still bound to p130 and E2F4 and not to B-MYB. This showed that the mere overexpression of B-MYB in the context of a G0 cell was not sufficient to switch LINC, possibly because B-MYB has to be phosphorylated to reach its full activity (Ziebold et al., 1997), and this might not be possible in a G0 cell. Another possible explanation for the LINC switch is that upon cell cycle entry, p130 is phosphorylated and releases E2F4, which is then exported from the nucleus (Gaubatz et al., 2001; Verona et al., 1997). Possibly at that time, p130 also leaves LINC, which could then result in a free binding site for phosphorylated B-MYB. In addition to this, LINC core proteins might also be modified upon cell cycle entry, leading to a conformational change and finally to LINC switch. This hypothesis could also explain why some LIN proteins did not show any interactions in yeast.

It was surprising that B-MYB, a transcriptional activator, and p107, which has been described mostly as a repressor, are present in the same complex. Although the interaction of B-MYB and p107 was not detectable in cycling T98G cells, the interaction was shown in S-phase cells. Also, binding of B-MYB to p107 was already shown before (Joaquin et al., 2002). Furthermore, there are studies describing p107 as a transcriptional activator (Batsche et al., 2005; Liu et al., 2000), suggesting that p107 and B-MYB might have similar functions in the LIN complex.

To learn more about the composition of LINC in S-phase, immunodepletion experiments were performed. These experiments showed a codepletion of LIN-37, LIN-54 and B-MYB with LIN-9, but not with an unspecific IgG antibody. This suggests that these proteins build a stable complex and that they mostly occur as a part of

LINC. In contrast, p107 was not efficiently codepleted with LIN-9, suggesting that a large percentage of p107 present in the cell is a free protein or bound to other complexes than LINC. This result matches the gelfiltration and fractionation experiments that showed only a partial overlap between p107 and other LINC members (Michael Korenjak and Alexander Brehm, data not shown, (Schmit et al., 2007)).

4.2 LINC function

From previous work in our lab it was known that the knock-down of LIN-9 in primary fibroblasts leads to a delay in the G2-phase of the cell cycle (Osterloh et al., 2007). To see whether this is an isolated function of LIN-9 or if LINC is involved in cell cycle regulation, LIN-54, another core component of LINC was depleted. These experiments showed that the depletion of LIN-54 results in the accumulation of cells in G2. This effect was not due to a longer S-phase but to a delayed entry into mitosis.

Microarray experiments with LIN-9 knock-down cells revealed target genes involved in G2 and mitosis (for details see 1.3.2.3 and (Osterloh et al., 2007)). The regulation of these genes by LIN-9 explained the LIN-9 knock-down phenotype. To analyze if the regulation of these G2/M genes is dependent only on LIN-9 or on the LIN complex, target gene expression was analyzed after LIN-54 knock-down. These experiments showed that LIN-54, like LIN-9, is involved in the regulation of the G2/M target genes. In addition, the depletion of LIN-52 caused G2/M gene downregulation (Mirijam Mannefeld, (Schmit et al., 2007)), suggesting that the LIN complex, and not an isolated protein, regulates the expression of G2/M target genes. Thereby LINC is essential for cell cycle progression.

Chromatin immunoprecipitation experiments showed that the LINC components bind to the promoters of their target genes in S-phase, when the expression of these genes is induced, suggesting that their regulation is a direct effect of LINC. Surprisingly, LINC core proteins also associated to the G2/M target genes and to G1/S E2F target genes in G0. This raised the question whether LINC might have a dual function by repressing the genes in G0 and activating them in S-phase. This question was addressed by synchronizing T98G cells in G0 and subsequently

depleting LINC members. Then the cells were released into the cell cycle and at different time points the expression of LINC members and their target genes was analyzed (performed by Mirijam Mannefeld, Stefanie Hauser and Stefan Gaubatz (Osterloh et al., 2007; Schmit et al., 2007)). These experiments confirmed the role of LINC in the activation of G2/M target genes. When LIN-9, LIN-52, LIN-54 or B-MYB were depleted, the G2/M target genes were not induced as strongly as in control cells. Similarly, the knock-down of p107 had the same effect, indicating that p107, which is a member of LINC in S-phase, is also involved in the activation of G2/M genes (Mirijam Mannefeld, data not shown). In G0 however, no difference between control cells and knock-down cells was observed, suggesting that LINC does not play a role in the repression of G2/M genes in this experimental setup. Litovchick and colleagues also performed expression analyses after knock-down of LINC components. They used a different protocol where they first depleted the LINC proteins and then synchronized the cells. In this experimental setup, they observed a derepression of the target genes after LINC protein depletion (Litovchick et al., 2007). Taken together, these experiments suggest that LINC may play a role in establishing the repressive state but not for its maintenance.

Early studies of the Drosophila complexes only described a function for replication (Beall et al., 2004; Beall et al., 2002) and transcriptional repression (Dimova et al., 2003; Korenjak et al., 2004; Lewis et al., 2004), but not for transcriptional activation. One recent paper however analyzed the function and DNA-binding properties of MMB in a genome-wide screen (Georlette et al., 2007). They could show that MMB indeed represses many developmental genes. In addition to this function they described MMB as an activator of G2/M genes (Georlette et al., 2007). This activating function in Drosophila confirms our results in human cells.

4.3 Human LIN-54 and its homologues

To better understand the molecular function of LINC, it is helpful to analyze the LINC members in more detail. Since database searches showed that LIN-54 has been conserved through evolution and possesses predicted homologous regions, the human LIN-54 protein was further characterized. Sequence alignments show that LIN-54 has homologues in many species. There are two characterized homologues

in Drosophila (mip120 and Tombola) and one known homologue in C. elegans (LIN-54), humans (Tesmin), Arabidopsis (TSO1) and Soybean (CPP1) (Fig. 3.1 and 4.2). These proteins show sequence homologies, especially in the C-terminal CXC regions (Fig. 4.2 and 4.3). Tombola lacks the first CXC region, and the first CXC domain of Tesmin is truncated. An additional C-terminal region, although not as strongly conserved as the CXC domains, is predicted to form Helix-coil-Helix structures (Jiang et al., 2007) (Fig. 4.2 and 4.4).

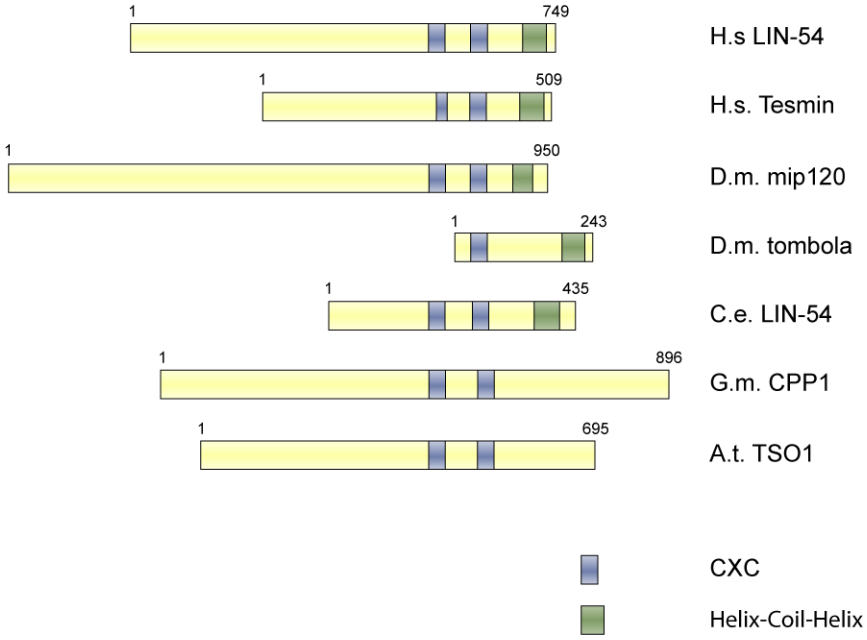


Fig. 4.2: Schematic alignment of conserved LIN-54 homologues.

The CXC region of the soybean (Glycine max) CPP1 protein was shown by gelshift analysis to bind to the promoter DNA of the leghemoglobin c3 gene (Cvitanich et al., 2000). Other LIN-54 homologues also directly bind to DNA. The C. elegans LIN-54 was recently found in a yeast-one-hybrid screen to bind to different promoter DNA fragments (Deplancke et al., 2006). Additionally, recombinant Drosophila mip120 was shown to sequence-specifically bind to ACE3 (Amplification Control Element for the 3rd chromosome) and Ori-β regions in the chorion gene locus by DNAseI footprining assays (Beall et al., 2002). Although the DNA-binding region was not confined to a certain domain for C. elegans LIN-54 and Drosophila mip120, it is possible that, similar to CPP1, the CXC region is essential for the DNA binding due to its conservation during evolution.

Most of the characterized LIN-54 homologues play an essential role in transcriptional regulation.

The C. elegans LIN-54 belongs to the synMuv class B genes, which inhibit a Ras-pathway leading to the development of the vulva (Fay and Han, 2000). It was proposed that the synMuv proteins repress the transcription of vulval differentiation-specific genes, although it is not clear at which step the synMuv and Ras pathways interfere (Lu and Horvitz, 1998). A recent biochemical study suggested that the DRM complex with LIN-54 as a member is recruited to the promoter DNA and acts together with a NuRD-like complex in the transcriptional repression (Harrison et al., 2006).

The soybean CPP1 protein is essential for the transcriptional repression of the leghemoglobin c3 gene although it was not shown if this effect is due to the DNA binding of CPP1 (Cvitanich et al., 2000).

Similarly, mip120 represses many developmental genes and activates other genes that are responsible for G2-phase and mitosis (Georlette et al., 2007). In addition, mip120 plays an important role in chorion gene amplification. In this system it was shown that the DNA binding of mip120 is essential for this effect, since mutations in the mip120 binding sites on the DNA result in a severely reduced amplification (Beall et al., 2002). In addition, mip120 mutants completely lack chorion gene amplification (Beall et al., 2007).

The Arabidopsis TSO1 protein is required for the development of organs in floral tissues (Liu et al., 1997) and for the differentiation of male and female reproductive tissues (Andersen et al., 2007). The molecular mechanism of these effects remains unclear. Possibly, TSO1 transcriptionally regulates target genes important for development and differentiation.

The human Tesmin is expressed in early development of male germ cells (Sugihara et al., 1999) and in embryonic ovaries (Olesen et al., 2004). This allows the speculation that Tesmin might have a function in development, although it has only been linked to heavy metal metabolism (Matsuura et al., 2002; Sugihara et al., 1999). Interestingly, Tesmin is localized mostly in the cytoplasm but it translocates to the nucleus at the G2/M transition in meiosis (Matsuura et al., 2002). This finding links Tesmin to the cell cycle and possibly Tesmin, like LIN-54, transcriptionally regulates cell cycle genes.

Tombola has also been linked to the cell cycle. It is a meiotic-arrest gene as tomb mutants arrest at the G2/M border in meiosis (Jiang et al., 2007).

In this work, it was first shown that the human LIN-54 protein, like some of its homologues, directly binds to DNA. This binding was confined to the CXC region. Recombinant CXC protein binds to the promoter of cdc2, a LINC target gene. The binding was abolished when two conserved cysteine residues were mutated to tyrosine, indicating that the cysteines are essential for the binding of CXC to the cdc2 promoter. The gelshift experiments performed in this study show that CXC needs two elements on the cdc2 promoter for efficient binding. One of them is the CHR region, which is a known repressive element on the cdc2 promoter and on the promoters of other cell cycle-regulated genes (Haugwitz et al., 2002; Lange-zu Dohna et al., 2000; Wasner et al., 2003; Zwicker et al., 1995). The other region essential for the binding of CXC is upstream in the used promoter construct and distinct from an upstream CHR domain.

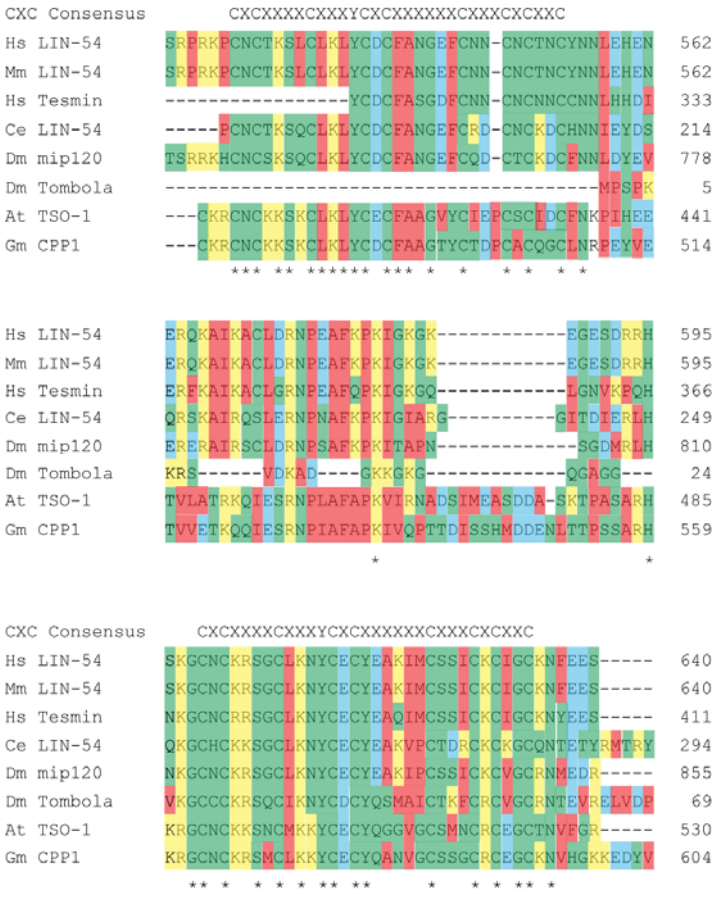


Fig. 4.3: Multiple sequence alignment of the CXC domains of different LIN-54 homologues.
CXC consensus sequence is indicated. Hs: Homo sapiens; Mm: Mus musculus; Ce: Caenorhabditis elegans; Dm: Drosophila melanogaster; At: Arabidopsis thaliana; Gm: Glycine max. *: same amino acid in all the displayed sequences. Red: small and hydrophobic amino acids; blue: acidic amino acids; yellow: basic amino acids; green: hydroxyl, amine and basic amino acids.
Accession Numbers: Hs LIN-54 NP_001108479, Mm LIN-54 NP_001108482, Hs Tesmin NP_004914, Ce LIN-54 NP_502544, Dm mip120 NP_610879, Dm Tombola NP_608936, At TSO1 NP_566718, Gm CPP1 CAA09028.
Alignment was performed by ClustalW2 at http://www.ebi.ac.uk/Tools/clustalw2 (Larkin et al., 2007).

Since the CXC domain of CPP1 and human LIN-54 binds to DNA ((Cvitanich et al., 2000) and this study), and other homologues also bind to DNA, it is possible that all the proteins carrying a CXC domain bind to DNA. This allows the speculation that the DNA binding of CXC-proteins is essential for the transcriptional regulation. This hypothesis has to be proved experimentally. For example, the phenotype of a cell line expressing a LIN-54 construct with a mutation that is unable to bind to DNA could be compared to a cell line expressing the wild-type LIN-54.

A cysteine-rich region similar to CXC is also found in polycomb group proteins (PcG), e.g. in Drosophila enhancer of zeste (E(z)) (Jones and Gelbart, 1993) and its homologues Curly Leaf in Arabidopsis (Goodrich et al., 1997) and EZH1 in human cells (Abel et al., 1996). Polycomb complexes are essential for maintaining developmental genes silenced by modifying histones and forming heterochromatin-like structures (Schuettengruber et al., 2007). E(z) only has one CXC region which has been linked to chromosome binding (Carrington and Jones, 1996), probably by its interaction with the DNA-binding protein PHO (Wang et al., 2004). In addition, the CXC-domain of E(z) increases the histone methyltransferase activity of its SET domain (Ketel et al., 2005).

The Helix-Coil-Helix structure was first described in the Drosophila testis-specific tombola (tomb). This protein was identified in a yeast-two-hybrid assay being a binding protein to aly, the testis-specific Drosophila homologue of LIN-9. The binding region was confined to the Helic-Coil-Helix (HCH) region in tomb (Jiang et al., 2007).

This study shows that the HCH domain of human LIN-54 is also responsible for protein-protein interactions.

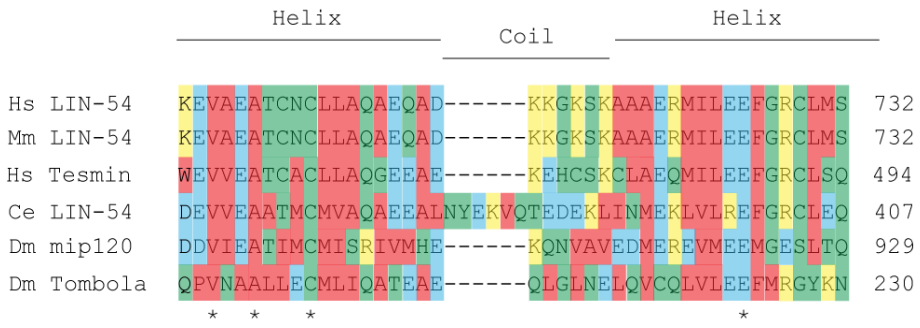


Fig. 4.4: Multiple sequence alignment of the HCH domains of different LIN-54 homologues.
Hs: Homo sapiens; Mm: Mus musculus; Ce: Caenorhabditis elegans; Dm: Drosophila melanogaster. *: same amino acid in all the displayed sequences. Red: small and hydrophobic amino acids; blue: acidic amino acids; yellow: basic amino acids; green: hydroxyl, amine and basic amino acids.
Accession Numbers: Hs LIN-54 NP_001108479, Mm LIN-54 NP_001108482, Hs Tesmin NP_004914, Ce LIN-54 NP_502544, Dm mip120 NP_610879, Dm Tombola NP_608936.
Alignment was performed by ClustalW2 at http://www.ebi.ac.uk/Tools/clustalw2 (Larkin et al., 2007).

4.4 Mechanism for LINC DNA binding

As shown and discussed above, LINC regulates G2/M target genes and thereby is important for the entry into mitosis. The effect on the G2/M genes is direct as chromatin immunoprecipitations demonstrated that members of LINC bind to their target gene promoters.

The binding of LINC to DNA could be mediated by at least three DNA binding proteins or a combination of these. LINC associates cell cycle-dependently with E2F4 or B-MYB, both of which are known DNA-binding transcription factors (Biedenkapp et al., 1988; Takahashi et al., 2000; Trimarchi and Lees, 2002). In addition, it was shown in this work that LIN-54 can directly bind to DNA and that it requires two binding sites on the cdc2 promoter. If the situation is the same in-vivo, this might result in a loop structure of the DNA.

As LINC binds to E2F4 or B-MYB during the cell cycle, it is also possible that these two proteins are involved in the binding of LINC to DNA. The promoters of the LINC target genes carry putative E2F and B-MYB binding sites. Although Drosophila dMyb is not necessary for the recruitment of MMB to promoter DNA (Georlette et al., 2007), it cannot be excluded that the situation may be different in human cells. It is possible that the LIN-54 binding to DNA is stabilized by a simultaneous binding of E2F4 or B-MYB to adjacent DNA binding sites. It is known that in G0 E2F4 binds to the CDE region and that for this an intact CHR element is necessary (Zwicker et al., 1995). Possibly at this time LIN-54 binds to the CHR element. In S-phase, LIN-54 might bind to an upstream region of the cdc2 promoter and this binding is possibly stabilized by the binding of B-MYB at a neighbouring B-MYB binding motif. Naturally this model has to be verified experimentally. Chromatin immunoprecipitation experiments from LIN-54, E2F4 or B-MYB knock-down cells could show if the binding affinity of LINC to DNA is reduced compared to control cells. To analyze to which binding motif on the cdc2 promoter LINC binds at different phases of the cell cycle, gelshift analyses with mutant competitors and nuclear extracts of synchronized cells would be useful. Additionally, this question could be addressed by reporter ChIPs. Therefore reporter plasmids carrying the wild-type cdc2 promoter or mutant promoter fragments are transfected into cells and the interaction of LINC members to the transfected promoter fragments is analyzed.

A model in which LIN-54 and another transcription factor bind to adjacent binding sites on promoter DNA is sterically possible. It has been shown that E2F4 and Smad bind to adjacent motifs on the c-myc promoter, leading to the repression of this gene (Chen et al., 2002). Such a model is also supported by experiments performed by Sarah Cremer. Gelshift experiments from synchronized cell extracts showed that in G0 there is a complex binding to the CDE-CHR element of the cdc2 promoter. This complex contains E2F4 and LIN-54 (Sarah Cremer, unpublished). In S-phase extracts such a complex could not be detected on this promoter fragment, suggesting that LINC binds to another motif in S-phase. Whether LINC directly contacts a different binding site on the promoter DNA or whether it leaves the promoter and is recruited again before S-phase remains unknown.

The binding of LIN-54 to DNA has only been analyzed on the cdc2 promoter so far. The promoters of some other LINC target genes also carry E2F- and B-MYB binding sites as well as CDE and CHR elements. The arrangement of these elements is however different in every promoter. Therefore the molecular regulation might differ between the target genes.

4.5 Mechanism for LINC function

As LINC does not contain any enzymes, the exact molecular mechanism for LINC function is not clear. It probably needs to recruit cofactors to ensure different functions in G0 and S-phase. Three important findings of this work have to be taken into account for a possible mechanistic model. First, the composition of LINC is cell cycle-dependent. Second, LINC contains different proteins with DNA-binding ability that most likely interact with different promoter regions. Last, LINC has different functions in the cell cycle, namely the activation of G2/M genes in S-phase and either a silent or a repressive role in G0. All this evidence suggests that the association of LINC to its target gene promoters and the chromatin structure may change in different phases of the cell cycle.

The repression of the LINC target genes by E2Fs and pocket proteins has been extensively studied. It is known that E2F and pocket proteins can mediate histone tail modifications. The E2F/pRB pathway was linked to the mediation and the maintenance of certain histone methylations that are associated with chromatin condensation through polycomb repressor complexes (Blais and Dynlacht, 2007). It would be interesting to analyze if LINC is involved or affected by these modifications.
Other markers for silenced chromatin are deacetylated histone residues. Interestingly, pocket proteins have been shown to recruit histone deacetylases (HDAC) (Macaluso et al., 2006). Although the Drosophila homologue of HDAC Hda-1 associates with MMB and HDAC is involved in the repression of some E2F target genes (Luo et al., 1998), HDACs do not seem to be essential to repress dE2F2 target genes (Dimova et al., 2003; Luo et al., 1998). In contrast p55/dCaf-1, the Drosophila homologue of RbAp48, is essential for the repression of dE2F2 target genes (Taylor-Harding et al., 2004). RbAp48 is a member of LINC as well as of the NuRD complex.
The NuRD complex acts in chromatin remodeling and deacetylation and has been linked to transcriptionally silenced chromatin. In C. elegans, members of the NuRD and DRM complexes belong to the synMuv class B, but it has been shown biochemically that the DRM complex is distict from the NuRD complex (Harrison et al., 2006). The authors of this study have postulated that the DRM and NuRD complexes act together to inhibit vulval differentiation genes. As the Drosophila dREAM complex associates with deacetylated histone tails (Korenjak et al., 2004),

the NuRD complex may be essential to deacetylate histone tails before DRM can bind. A similar model, where the LINC and NuRD complexes bind together or sequentially to repress E2F target genes is also possible in human cells.
In a recent RNAi screen from Drosophila, two other proteins were linked to the repression of E2F target genes (Lu et al., 2007), Domino, a SWI/SNF ATPase (Ruhf et al., 2001), and L3mbt, a polycomb group protein (Koga et al., 1999; Wismar et al., 1995) that associates with MMB (Lewis et al., 2004).
It would be interesting to analyze whether LINC is involved in or dependent on the regulation of E2F target genes by the discussed proteins by interaction studies and by ChIP and expression experiments from knock-down cells. Another question that remains to be investigated is whether LINC binds to the target gene promoters to actively repress the expression, to recruit corepressors or chromatin remodelers or whether LINC plays a more passive role by binding to E2F4/p130 and preparing for its function in S-phase.

The mechanism of E2F target gene activation is not well understood. It is known that in late G1 and S-phase, E2F1-3 bind to their target gene promoters and that at this time, the chromatin is acetylated (Takahashi et al., 2000). Recently, new evidence showed that these two events are linked and that E2F promotes the acetylation of histone tails (Taubert et al., 2004). E2F binds to p300/CBP, PCAF (Martinez-Balbas et al., 2000; Marzio et al., 2000) and Tip60 (Taubert et al., 2004), which enhances E2F-dependent gene activation.
Trimethylation of histone 3 on lysine 4 is also linked to transcriptionally active chromatin. This modification is established by MLL (mixed lineage leukaemia) and SET families of histone methyltransferases mediated by E2F and HCF-1 proteins (Tyagi et al., 2007).
Even less is known about the delayed activation of the G2/M E2F target genes. Data in this study provide an explanation for this delayed expression. B-MYB itself is a G1/S E2F target gene. Upon its expression in S-phase, B-MYB binds to LINC. This S-phase LIN complex with B-MYB is essential for the activation of the G2/M genes.

All the effects and models discussed above are dependent on the recruitment of cofactors, chromatin modifiers or enzymes. In G0, LINC with the associated E2F4 and p130 might recruit factors that modify histone tails and condense chromatin,

which can lead to the inhibition of gene expression. In S-phase, when p130 and E2F4 have dissociated from LINC, histones may be acetylated by recruited acetyltransferases and the chromatin may be decondensed. This could lead to transcriptional initiation and elongation (see model in Fig. 4.5.A).

Alternatively, as LINC does not contain any enzyme and is differentially composed during the cell cycle, LINC may sterically block the binding of activating proteins in G0, leading to a passive block of gene expression. Another possibility that does not require recruitment of any protein is that LINC may on its own induce a different chromatin structure in G0 and S phase, allowing the expression of target genes in S-phase but not in G0. Such a model has been described for the CD4 gene in developing thymocytes. Runx1 and the transcription factor P-TEFb bind and bring a silencer element on the CD4 promoter in close proximity of an enhancer element. In this case, P-TEFb can not activate the RNA-polymerase, which stays at the transcriptional start site. When Runx1 is downregulated, the chromatin conformation changes and the enhancer element gets closer to the transcriptional start site. There the RNA polymerase is activated by P-TEFb and the CD4 gene is derepressed (Jiang and Peterlin, 2008). Similarly, the association of LINC with E2F4 and p130 in G0 could lead to a different chromosomal structure than the association with B-MYB in S-phase, resulting in active target genes in S-phase and silent target genes in G0 (see model in Fig. 4.5.B).

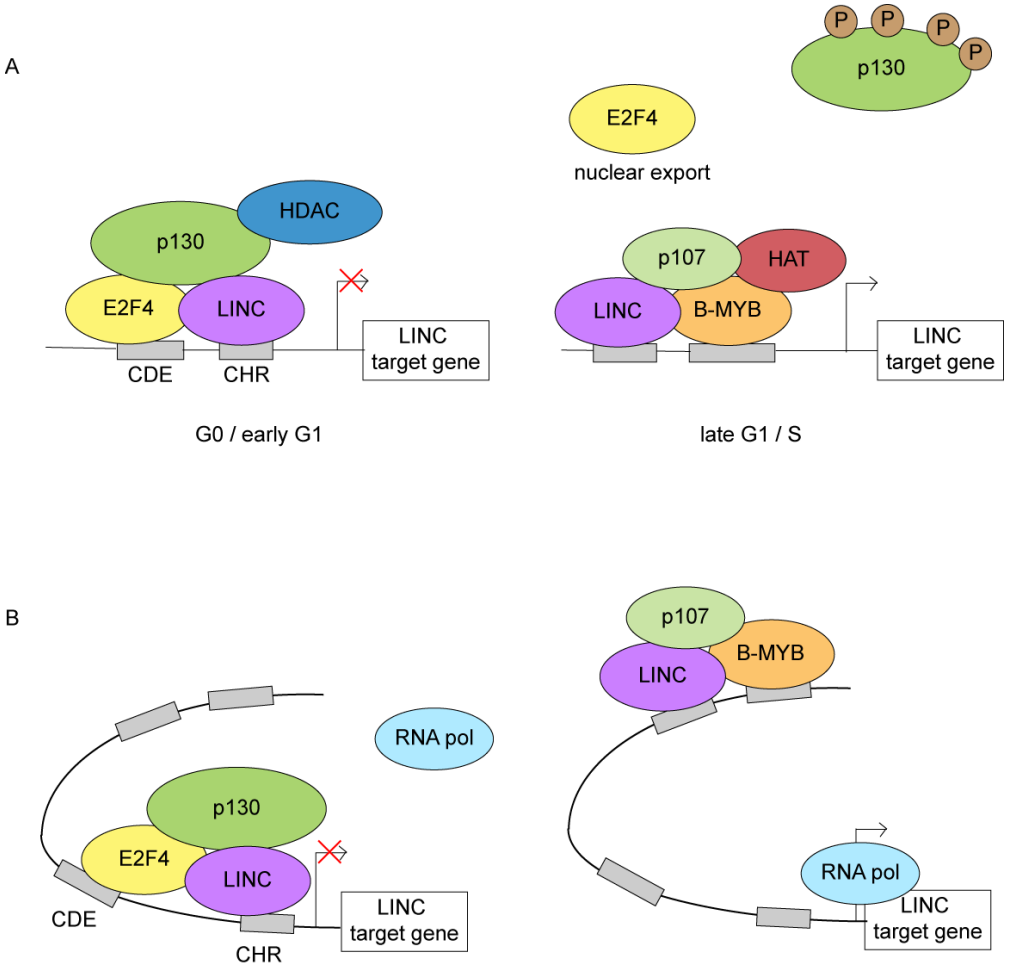


Fig. 4.5: Possible models for LINC function.
A: In G0, LINC is bound to E2F4 and p130. They recruit histone deacetylases, which leads to chromatin condensation and gene silencing. In S-phase, LINC binds to B-MYB and p107, which recruit histone acetyltransferases. The chromatin is decondensed and the target gene is expressed.
B: In G0, LINC with E2F4 and p130 binds to the CDE/CHR element, which sterically blocks the RNA polymerase from initiating or elongating transcription. In S-phase, LINC binds to more distant promoter elements. This allows the RNA polymerase to bind to the transcriptional start site and target gene expression is activated.

5 Summary

Regulated progression through the cell cycle is essential for ordered cell proliferation. One of the best characterized tumor suppressors is the retinoblastoma protein pRB, which together with the E2F transcription factors regulates cell cycle progression. In the model organisms Drosophila melanogaster and Caenorhabditis elegans, RB/E2F containing multiprotein complexes have been described as transcriptional regulators of gene expression.

This work first describes a homologous complex in human cells named LINC (for LIN complex). It consists of a stable core complex containing LIN-9, LIN-37, LIN-52, LIN-54 and RbAp48. This core complex interacts cell cycle-dependently with different pocket proteins and transcription factors. In quiescent cells, LINC associates with p130 and E2F4. In S-phase cells these interactions are lost and LINC binds to B-MYB and p107.

The transient knock-down of LIN-54 in primary fibroblasts, as the depletion of LIN-9, leads to cell cycle defects. The cells are delayed before the entry into mitosis. This effect is due to the fact that the knock-down of LINC components leads to the downregulation of cell cycle genes responsible for the entry into and exit from mitosis as well as for checkpoints during mitosis. These LINC target genes are known E2F G2/M target genes, which are expressed later than the classical G1/S E2F target genes.

The transcriptional regulation by LINC is a direct effect as LINC binds to the promoters of its target genes throughout the cell cycle. LINC contains three DNA-binding proteins. E2F4 and B-MYB, which cell cycle-dependently bind to LINC, are known DNA-binding transcription factors. Additionally, it is show here that the LINC core complex member LIN-54 also directly binds to the promoter of a LINC target gene.

Although the exact molecular mechanism of LINC function needs to be analyzed further, data in this work provide a model for the delayed activation of G2/M target genes. B-MYB, a G1/S E2F target gene, binds to LINC upon its expression in S-phase. Then only LINC is a transcriptional activator that induces the expression of the G2/M genes. This provides an explanation for the delayed expression of these E2F G2/M target genes.

Key words: LINC, LIN-54, cell cycle, G2/M transition, transcription

6 Zusammenfassung

Die Regulation des Zellzyklus ist unerlässlich für die fehlerfreie Zellteilung. Einer der am Besten charakterisierten Tumorsuppressoren ist das Retinoblastom-Protein pRB, welches zusammen mit den E2F Transkriptionsfaktoren den Zellzyklus reguliert. In den Modellorganismen Drosophila melanogaster und Caenorhabditis elegans wurden Multiproteinkomplexe beschrieben, die pRB und E2F Homologe enthalten und transkriptionell die Expression von Zielgenen regulieren.

Diese Arbeit beschreibt erstmals LINC, einen homologen Komplex in humanen Zellen. Der LIN-Kernkomplex besteht aus LIN-9, LIN-37, LIN-52, LIN-54 and RbAp48 und assoziiert zellzyklus-abhängig mit Pocket Proteinen und Transkriptionsfaktoren. In ruhenden Zellen (G0) assoziiert LINC mit p130 und E2F4. In der S-Phase verlassen p130 und E2F4 den Komplex und B-MYB und p107 interagieren mit LINC.

Die transiente Depletion von LIN-54, ebenso wie die Depletion von LIN-9, führt zu Defekten im Zellzyklus. Die „knock-down"-Zellen treten verzögert in die Mitose ein. Dies konnte darauf zurückgeführt werden, dass die Depletion von LINC Mitgliedern Gene herunterreguliert, die für den Eintritt in und den Austritt aus der Mitose, sowie für Regulationsprozesse während der Mitose verantwortlich sind. Diese LINC Zielgene wurden bisher als G2/M E2F Zielgene beschrieben, welche verglichen mit klassischen E2F Zielgenen verzögert exprimiert werden.

Die transkriptionelle Regulation durch LINC ist ein direkter Effekt, da LINC in G0 und in der S-Phase an die Promotoren seiner Zielgene bindet. LINC enthält drei DNA-bindende Proteine. Die zellzyklus-abhängigen Komponenten von LINC E2F4 und B-MYB sind bekannte DNA-bindende Transkriptionsfaktoren. Zusätzlich konnte in dieser Arbeit gezeigt werden, dass das LINC Kernprotein LIN-54 direkt an den Promoter eines LINC Zielgens, cdc2, bindet.

Obwohl der genaue molekulare Mechanismus für die Funktion von LINC noch genauer untersucht werden muss, liefern Daten in dieser Arbeit ein Modell für die verzögerte Expression von G2/M Genen. B-MYB ist selbst ein E2F Zielgen und bindet an LINC sobald es exprimiert wird. Erst die Assoziation von B-MYB an LINC in der S-Phase macht LINC zu einem transkriptionellen Aktivator G2/M-spezifischer Gene. Dies erklärt die verzögerte Expression dieser E2F G2/M Zielgene.

Schlüsselwörter: LINC, LIN-54, Zellzyklus, G2/M Übergang, Transkription

7 Appendix

7.1 Sequences

7.1.1 LIN-54 cDNA

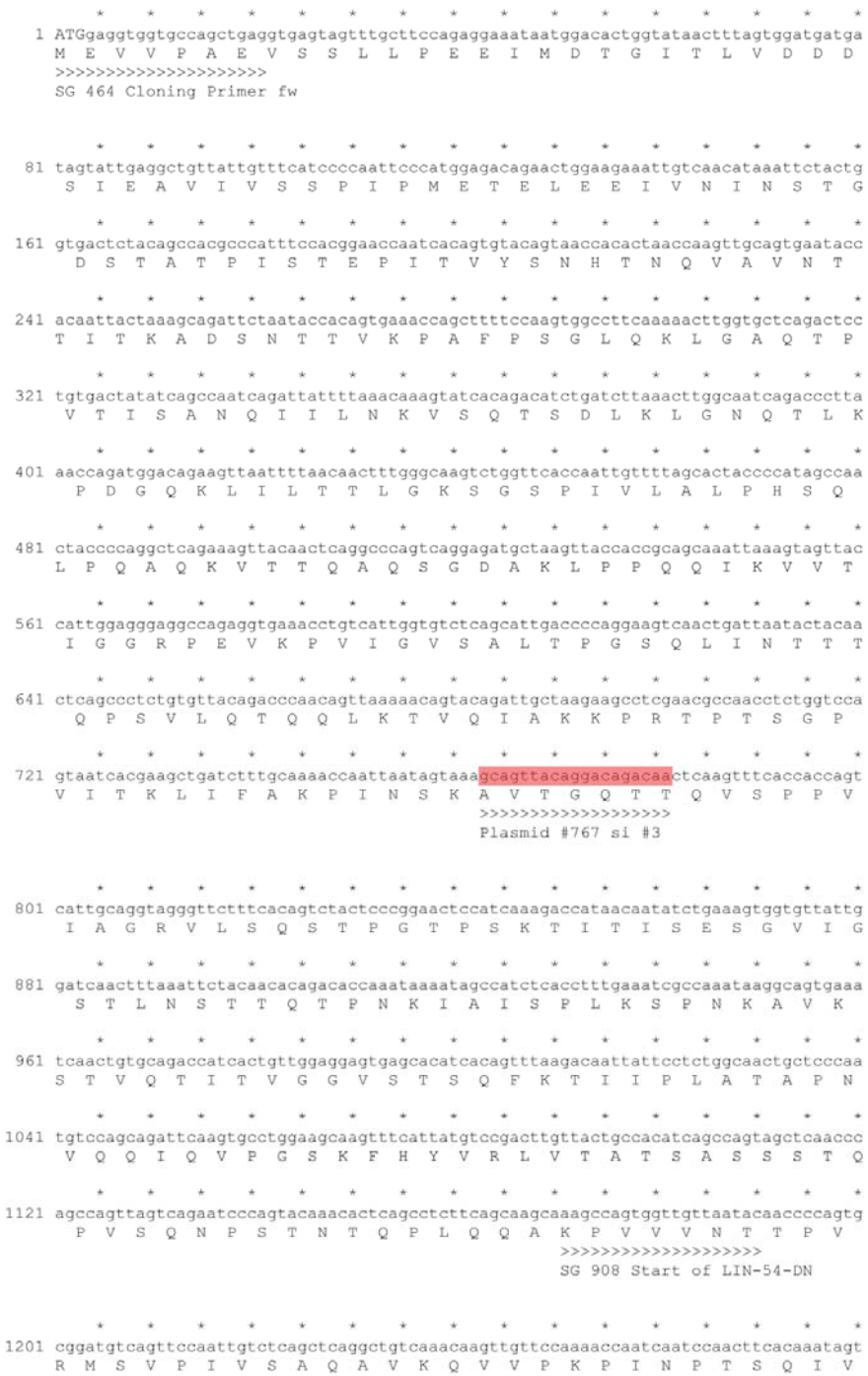


Fig. 7.1: LIN-54 cDNA (Accession number: NM_194282) and protein sequence (Part1).
red: shRNA target sequence

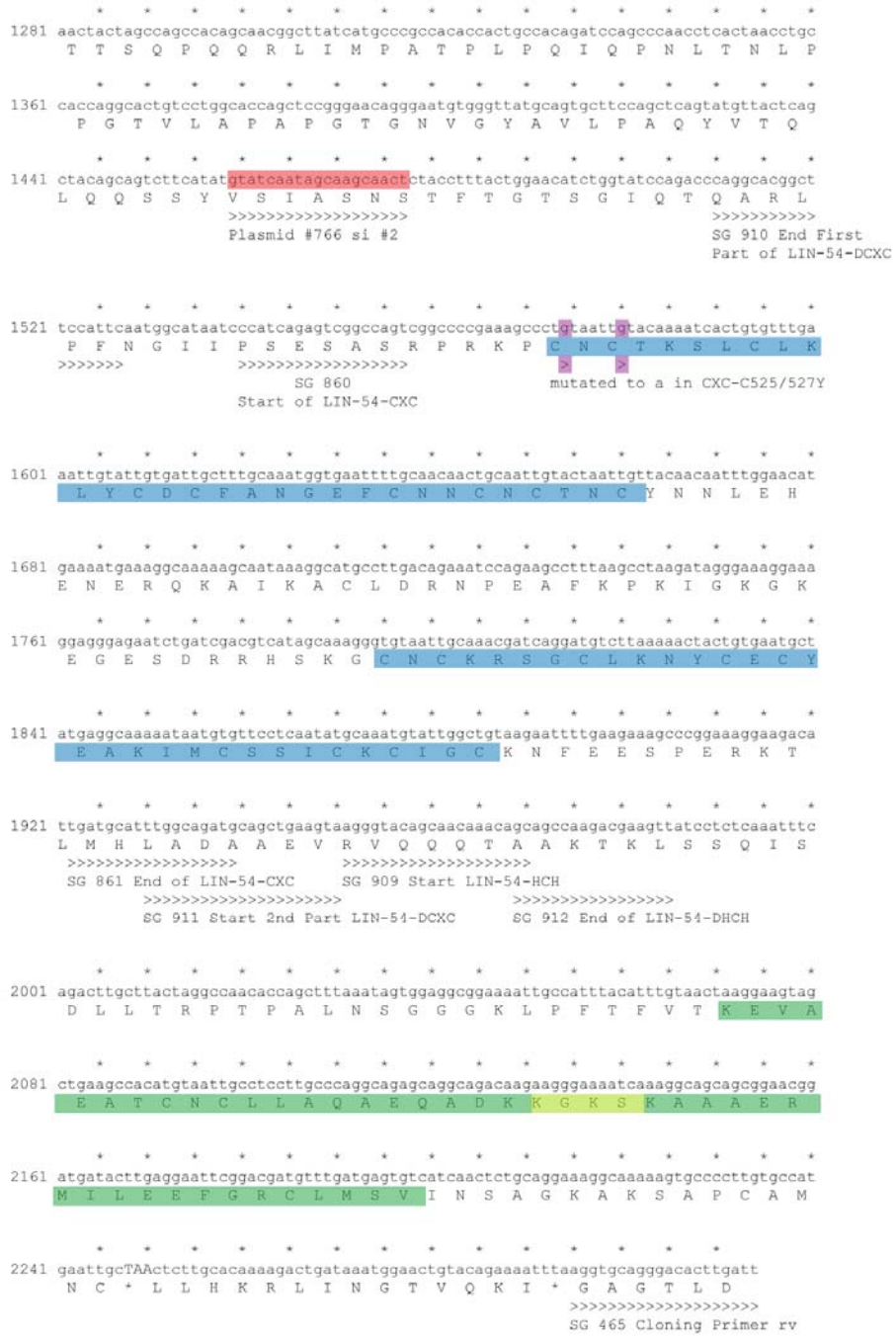


Fig. 7.1: LIN-54 cDNA (Accession number: NM_194282) and potein sequence (Part2).
blue: CXC regions, green: Helix-coil-Helix region, red: shRNA target sequence

7.1.2 LIN-37 cDNA

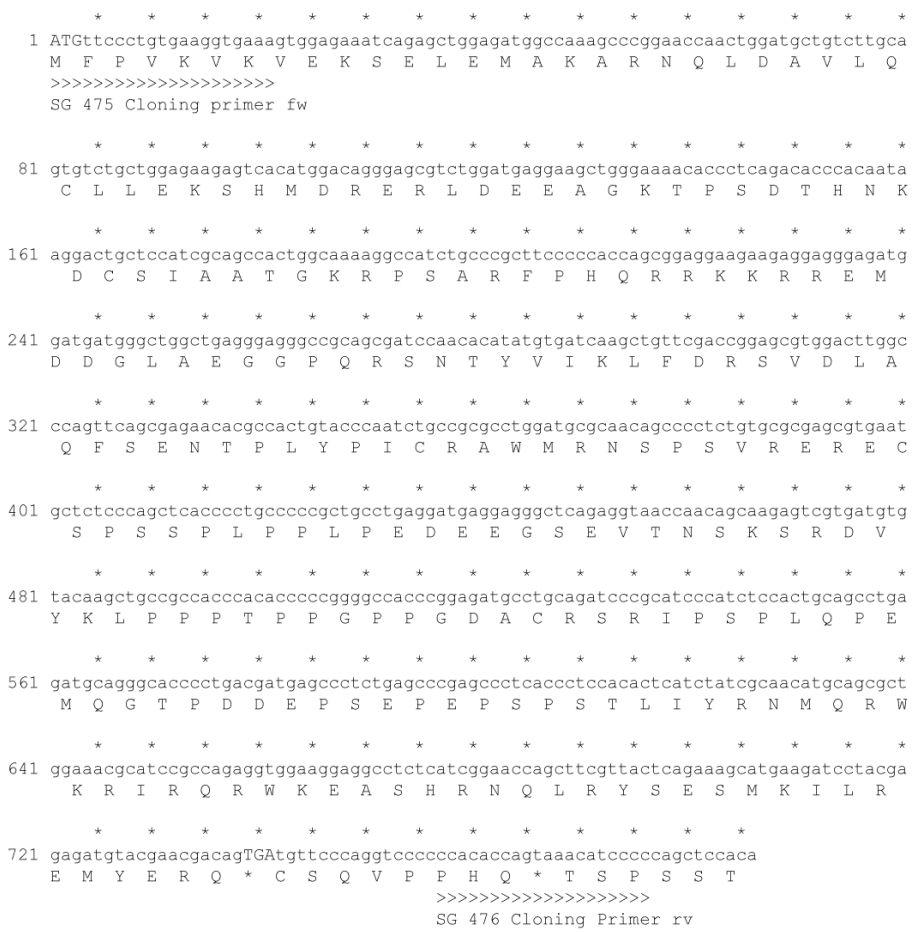


Fig. 7.2: LIN-37 cDNA (Accession number: NM_019104.1) and protein sequence.

7.1.3 LIN-52 cDNA

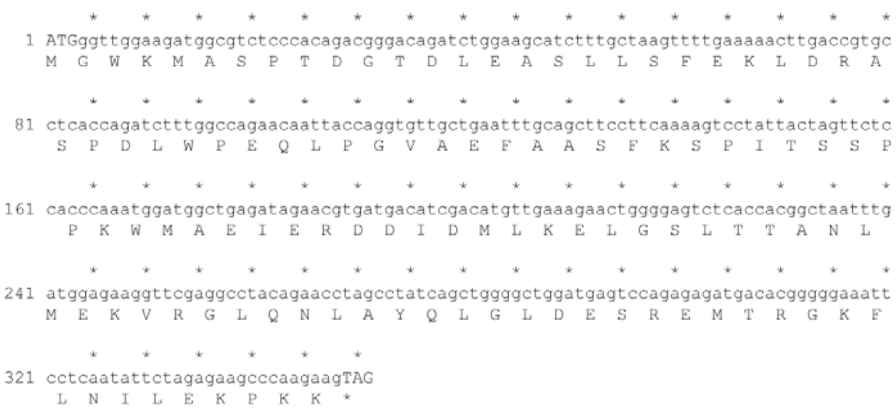


Fig. 7.3: LIN-52 cDNA (Accession number: NM_001024674.1) and protein sequence.

7.1.4 cdc2 promoter

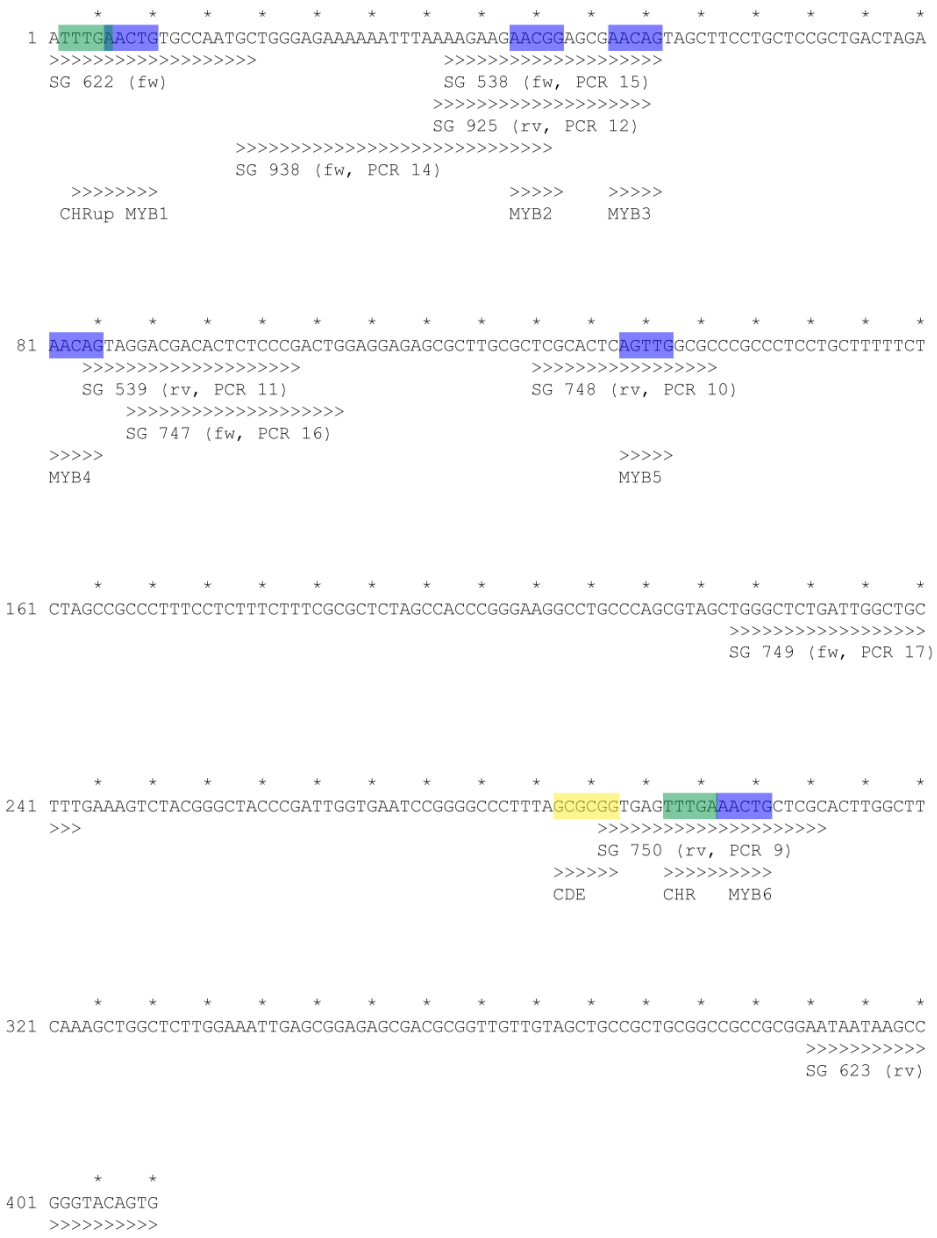


Fig. 7.4: cdc2 promoter sequence.
Blue: MYB binding site, green: CHR, yellow: CDE. Primer used for probes and competitors are indicated.

7.2 List of Figures

7.3 Abbreviations

APC	Anaphase promoting complex
APS	Ammonium persulfate
BrdU	Bromodeoxyuridin
BSA	Bovine serum albumine
DTT	Dithiothreitol
CDE	Cell cycle-dependent element
Cdk	Cyclin-dependent kinase
Ci	Curie
ChIP	Chromatin immunoprecipitation
CHR	Cell cycle genes homology region
DMSO	Dimethylsulfoxyde
dREAM	Drosophila RBF E2F and Myb complex
DREAM	DP, RB-like, E2F and MuvB complex
DRM	DP, RB and MuvB complex
ECL	Enhanced chemiluminescence
ESB	Electrophoresis sample buffer
FACS	Fluorescence-associated cell sorting
FCS	Fetal calf serum
Fig.	Figure
G0, G1, G2	Gap phases
GST	Glutathione S-transferase
HAT	Histone acetylase
HDAC	Histone deacetylase
HRP	Horseradish peroxydase
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
kd	Knock down
kDa	kiloDalton
mip	Myb-interacting protein
M-phase	Mitosis

MMB	Myb-MuvB complex
Mw	Molecular weight
NuRD	Nucleosome remodeling and deacetylase complex
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulphonyl fluoride
pRB	Retinoblastoma protein
qPCR	Quantitative polymerase chain reaction
rpm	Revolutions per minute
RT	Reverse transcriptase
	Room temperature
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
S-phase	Synthesis phase
synMuv	Synthetic multivulva
tMAC	Testis Meiotic Arrest Complex
WB	Western blot

7.4 References

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7.6 Own publications

Schmit F, Korenjak M, Mannefeld M, Schmitt K, Franke C, von Eyss B, Gagrica S, Hänel F, Brehm A and Gaubatz S.
LINC, a human complex that is related to pRB-containing complexes in invertebrates regulates the expression of G2/M genes.
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Osterloh L, von Eyss B, Schmit F, Rein L, Hübner D, Samans B, Hauser S and Gaubatz S.
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EMBO J. 2007, **26**: 144-157

Conference contributions:
Schmit F, Hanel F, Gagrica S, Gaubatz S. Characterization of novel human synMuv-like proteins. Poster. Mechanisms and Models of Cancer, August 16 – August 20, 2006, Cold Spring Harbor.

7.7 Lebenslauf

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Titel: LINC, a novel protein complex involved in the regulation of G2/M genes

7.8 Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbständig verfasst habe und dabei keine anderen, als die von mir angegebenen Hilfsmittel und Quellen benutzt habe. Zitate sind als solche gekennzeichnet.

Ich erkläre außerdem, dass die vorliegende Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ich habe früher, außer den mit dem Zulassungsantrag urkundlich vorgelegten Graden, keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 9. Juli 2008

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Fabienne Schmit