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Diploma Thesis:

Tumour Suppressor HIC1 – a novel inhibitor of Wnt Signalling

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I hereby declare that this thesis is my own work and effort. I have not plagiarised it from any source. I have referenced all literature sources and I have worked under supervision of Vladimír Kořínek, PhD.

Vendula Pospíchalová
May 15th, 2008

1. ABSTRACT

In all metazoan organisms Wnt ligands regulate various developmental and physiological processes during embryogenesis and also in adult tissues. Moreover, deregulation in Wnt-related signalling is associated with several human disorders including cancer. In the so-called canonical pathway, activation of the Wnt receptor complex Frizzled/LRP triggers a complex network of molecular events that ultimately lead to the stabilization of β-catenin and formation of TCF/β-catenin heterodimers in the nucleus. These protein complexes drive expression of a specific set of genes that control the cell fate decision. The Wnt pathway is tightly regulated by several mostly negative feedback loops involving scores of extracellular, cytoplasmic or nuclear proteins.

Recently, we have identified tumour suppressor Hypermethylated in cancer 1 (HIC1) as a negative modulator of canonical Wnt signalling. The HIC1 gene encodes a BTB/POZ-zinc finger transcriptional repressor. Interestingly, the HIC1-dependent repression of the TCF/β-catenin-dependent genes is not mediated by direct association of HIC1 with the regulatory regions of the Wnt-responsive genes but rather by a sequestration of TCF/β-catenin complexes to the nuclear speckle-like structures - the HIC bodies. These data indicate quite a pleiotropic role of HIC1 in the cells.

As the first aim this diploma thesis we prepared several laboratory tools to study the HIC1 gene and protein. These tools included antibodies recognizing HIC1; primer sets and total RNAs for expressional profiling of HIC1 mRNA, a Luciferase reporter containing HIC1-responsive promoter of the SIRT1 gene, and finally, we generated two constructs for gene-targeting experiments in the Hic1 locus in mouse. In the second part of the thesis we used these tools to show that (1) mouse uterus and primary human cells CCD841 have the highest levels of HIC1 mRNA; (2) HIC1 transcript is upregulated in stimulated T-cells; (3) using immunocytochemistry we observed endogenous HIC bodies in primary human fibroblasts WI38; (4) we showed that the repressive function of HIC1 depends on the intact Zn finger DNA binding and BTB/POZ oligomerization domains; (5) we prepared several clones of mouse embryonic stem cells with the conditionally disrupted Hic1 gene or with a citrine reporter gene introduced downstream of the Hic1 promoter. These cell clones will be used for blastocysts injections to generate Hic1 mutant mice. We hope that the mice will be a valuable resource to study the HIC1 function *in vivo*.

Key words: Wnt signalling, HIC1 (Hypermethylated in Cancer 1), tumour suppressor, transcriptional repression, HIC bodies, β-catenin, gene targeting, conditional knock-out mouse, qRT PCR.

2. LIST OF ABBREVIATIONS

aa amino acid
AEC 3-amino-9-ethyl carbazole
APC adenomatous polyposis coli
Arg, R arginine
BSA bovine serum albumin
C.e. *Caenorhabditis elegans*
CKI casein kinase I
CtBP C-terminal binding protein
Dkk Dickkopf
DMSO dimethylsulphoxide
Dsh Dishevelled
DTT dithiothreitol
EDTA ethylene diamine tetraacetic acid
EGTA ethylene glycol tetraacetic acid
EGF Epidermal Growth Factor
EGFP Enhanced Green Fluorescent protein
ESC embryonic stem cell
EtBr etidium bromide
EYFP Enhanced Yellow Fluorescent Protein
FBS Foetal Bovine Serum
FAP familial adenomatous polyposis
Fz Frizzled
GFP green fluorescent protein
GSK3 glycogen synthase kinase 3
H, His histidine
h, m, x human, mouse, gene /protein
hr hour
HDAC histone deacetylase
HIC 1 hypermethylated in cancer 1
ICC immunocytochemistry
IHC immunohistochemistry
IPTG isopropyl-beta-D-thiogalactopyranoside
KI knock-in
KO knock-out
LEF lymphocyte-enhancer factor
LIF leukaemia inhibitory factor
LOF/GOF loss/gain of function
LOH loss of heterozygosity
Lys, K lysine
MPEF Mouse Primary Embryonic Fibroblasts
NLS nuclear localization signal
O/N over night
Phe, F phenylalanine
PBS phosphate buffered saline (137mM NaCl, 10mM Phosphate, 2.7mM KCl, pH 7.4)
PCR Polymerase Chain Reaction
PEI polyethylenimine
PMSF phenylmethanesulphonylfluoride, a serine protease inhibitor
RT room temperature
S, Ser serine
SD Standard deviation, a statistical measure of the spread of values
SDS-PAGE sodium dodecylsulphate polyacrylamide gel electrophoresis
SFRP secreted Frizzled related protein
SLS Sodium lauryl sulphate
TCF T-cell factor
TE Tris/EDTA buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0)
W, Trp tryptophan
WB Western blot(ting)
Wg wingless
Wls wntless
WIF Wnt inhibitory factor
wt, fl wild type, full-length gene/protein
Y, Tyr tyrosine

3. INTRODUCTION

Our lab has been focused on studying the Wnt signalling pathway, one of the fundamental pathways involved in development and disease, at various levels. Recently, a novel nuclear inhibitor of Wnt signalling, tumour suppressor HIC1 (Hypermethylated in cancer 1), has been discovered (Valenta et al., 2006; Wales et al., 1995). A detailed characterization of the expression pattern and molecular functions of this newly described Wnt signalling modulator is a subject of my diploma work.

During my diploma work I have focused mainly on preparation of tools for studying the role of Hic1 *in vivo*. These tools, especially the knock-out and knock-in reporter mice, are intended to be used during my PhD study in which I'd like to continue with HIC1 project and further elucidate functions of this multi-functional protein *in vivo*.

Detailed aims of the study were:

(1) To prepare monoclonal antibodies recognizing HIC1 protein

(2) To study the level of expression of HIC1 in adult mouse tissues and human cell lines using qRT PCR approach

(3) To study the ability of full length and mutant HIC1 forms to repress luciferase reporter plasmids

(4) To generate conditional Hic1 knock-out mouse

(5) To generate reporter Hic1-citrine knock-in mouse

4. REVIEW OF THE LITERATURE

Wnt signalling is a common theme in animal development and the aberrant activation of the Wnt pathway is also implicated in the cellular transformation and cancer. The signalling cascade initiated by the secreted Wnt ligands is one of a relatively small number of the cell signalling pathways that relay information from the extracellular milieu via the cytosolic components up to the nucleus. In the current view, three different pathways are activated upon the association of Wnt proteins with the Frizzled/LRP receptor: the canonical Wnt cascade and the noncanonical planar cell polarity and Wnt/Ca^{++} signalling. The stabilization of cytosolic β-catenin is a central molecular mechanism of the canonical Wnt signalling. Stable β-catenin enters the cell nucleus where it associates with the TCF/LEF transcription factors to regulate expression of the specific Wnt-responsive genes. The pathway itself is tightly regulated at various levels. Activity of the Wnt ligands is stimulated or attenuated in the extracellular space by a diverse group of antagonists, cofactors and coreceptors. In the cytoplasm, the amount of β-catenin is constitutively reduced by a large destruction protein complex. In the nucleus, the transcriptional stimulation of the target genes is modulated by a complicated interplay between various activators, repressors and other auxiliary factors.

One of the numerous modulators is Hypermethylated In Cancer 1 (HIC1), a transcriptional repressor essential for mammalian development, that is epigenetically silenced in solid cancers and involved in a complex pathway regulating p53 tumour suppression activity.

4. 1. The Wnt signalling pathway

Wnt signalling pathway is an evolutionarily conserved signal transduction cascade (Rothbacher et al., 1995), which has been found virtually in all metazoan organisms from cnidarians to mammals (Frame and Cohen, 2001). Wnt proteins and their downstream cellular effectors were originally described mainly in *Drosophila,* nevertheless many of the signalling pathway mechanisms were also characterized in other animal models as mouse and *Xenopus* (Miller, 2002).

The pathway plays a crucial role during embryonic development (Wodarz and Nusse, 1998) and also in adult self-renewing tissues (Gregorieff and Clevers, 2005; Korinek et al., 1998; Reya and Clevers, 2005). Wnt proteins function as concentration-dependent long-range morphogenic signals regulating various cellular phenotypes as proliferation, differentiation, survival, cell polarity, movement and, according to the newest findings (Pereira et al., 2008), inflammation. Non-physiological activation of Wnt signalling is one of the key features of many cancers (Morin et al., 1997; Pinto and Clevers, 2005; Polakis, 2000). It is generally accepted that the tightly regulated self-renewal and differentiation of stem and progenitor cells is controlled by Wnt signalling. A failure of such control leads to the cell transformation, malignant proliferation and cancer.

Figure 1. The Wnt canonical pathway (Reya and Clevers, 2005)

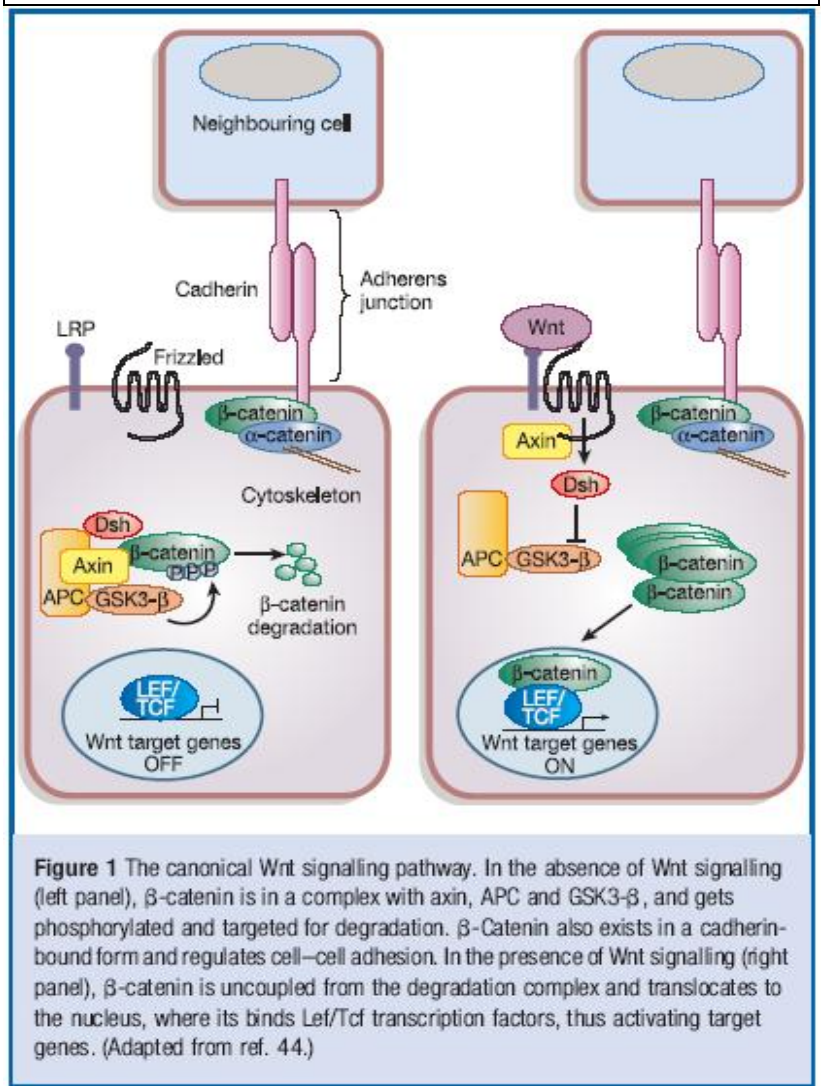


Figure 1 The canonical Wnt signalling pathway. In the absence of Wnt signalling (left panel), β-catenin is in a complex with axin, APC and GSK3-β, and gets phosphorylated and targeted for degradation. β-Catenin also exists in a cadherin-bound form and regulates cell–cell adhesion. In the presence of Wnt signalling (right panel), β-catenin is uncoupled from the degradation complex and translocates to the nucleus, where its binds Lef/Tcf transcription factors, thus activating target genes. (Adapted from ref. 44.)

The Wnt signals can be transmitted via at least three independent cellular pathways, one of which, so called canonical Wnt signalling, is β-catenin dependent (Fig. 1.). It includes binding of the Wnt proteins to the Frizzled receptors, activation of several intracellular proteins that leads to the inhibition of the β-catenin-degradation complex and the accumulation of β-catenin. Stabilized β-catenin translocates to the nucleus where it is engaged with the factors of the TCF/LEF family. The TCF(LEF)/β-catenin heterocomplexes drive transcription of a specific set of the Wnt signalling target genes.

4.1.1. The Wnts

The Wnt (*wingless* and *int*) proteins form a family of highly conserved cysteine rich and lipid-modified secreted glycoproteins. The first Wnt gene, *Wnt1,* was cloned from the mouse genome as a proto-oncogene in 1982 by Nusse and Varmus. *Wnt-1* was isolated during a search for genes that are insertionally activated by proviral DNA in mammary carcinomas induced by the mouse mammary tumour virus (MMTV) (Nusse and Varmus, 1982). Until 1991 *Wnt1* was called *Int-1* (for MMTV integration site). *Wingless* (*wg*) is the *Wnt1* homolog in *Drosophila* (Sharma and Chopra, 1976).

Membership in the Wnt family is defined by aminoacid sequence rather than functional properties (Miller, 2002). 19 Wnt genes were found in human and mouse, 16 in *Xenopus,* 11 in chicken, 12 in zebrafish, 7 in *Drosophila*, 5 in *C. elegans* and at least one in *Hydra* (Miller, 2002). Little is known about the mechanisms underlying Wnt secretion. Recently, Wntless (Wls/Evi/Srt) has been identified as a conserved multi-pass transmembrane protein whose function seems to be dedicated to promoting the release of Wnts (Banziger et al., 2006; Bartscherer et al., 2006). Wls

localization and levels critically depend on retromer, a conserved protein complex that mediates endosome-to-Golgi protein trafficking (Belenkaya et al., 2008; Eaton, 2008). Taken together, Wg, clathrin-mediated endocytosis and retromer sustain a Wls traffic loop from the Golgi to the plasma membrane and back to the Golgi, thereby enabling Wls to direct Wnt secretion (Port et al., 2008).

4.1.1.1. Wnt proteins in the extracellular milieu

The way how Wnt proteins move as morphogens over long distances is not known (Strigini and Cohen, 2000). Once released from the cell, Wnts can interact with a number of binding proteins (Kawano and Kypta, 2003). Some of them are thought to be positive regulators of Wnt activity, *e.g.* Heparan sulfate proteoglycans (HSPGs) (He, 2003), while the others, SFRPs (secreted Frizzled related proteins) (Lin et al., 1997), WIF (Wnt inhibitory factor) (Hsieh et al., 1999), the Dickkopf (Dkk) (Gonzalez-Sancho et al., 2005) and Kremen proteins (Mao et al., 2002), are inhibitors of the Wnt signalling.

4.1.2. The Wnt receptors

Members of the Frizzled family of seven-pass transmembrane proteins are the primary receptors for Wnts. Ten *Fz* genes are found in the human genome (He, 2003). These receptors possess a long amino-terminal extension (120 to 125 aa) called CRD (cysteine-rich domain), seven transmembrane domains and a short cytoplasmic tail with the PDZ domain binding motif (Dann et al., 2001). Wnt signalling requires not only a functional Fz, but also a presence of LRP (LDL receptor related protein), a long single-pass transmembrane molecule. The genome of vertebrates encodes two highly homologous genes, *LRP5* and *LRP6,* a single homolog in *Drosophila* is called *arrow* (Herz and Strickland, 2001). It has been proposed that the Wnt molecules can bind to LRP and form a trimeric complex with Fz receptors (Tamai et al., 2004).

4.1.3. Dishevelled (Dsh)

Once Wnts bind to Fz/LRP, the adapter protein Dishevelled is recruited to the membrane via interaction with Fz intracellular domain or/and phospholipids. There, it becomes phosphorylated and activated (Malbon and Wang, 2006). In parallel, Wnt-induced phosphorylation of the cytoplasmic tail of LRP allows docking of Axin to LRP (Tamai et al., 2004). Recruitment of Axin to the membrane is thought to disrupt the β-catenin destruction complex.

4.1.4. β-catenin destruction complex

In unstimulated cells newly synthesized cytoplasmic β-catenin is destabilized by a multiprotein complex containing two scaffolding proteins - Adematous polyposis coli (APC) (Hart et al., 1998) and Axin (or its homolog Conductin) (Ikeda et al., 1998). Casein kinase Iα (CKIα) (Amit et al., 2002) and Glycogen synthase kinase 3β (GSK3β) (Kishida et al., 1998) are kinases residing in a multiprotein complex that phosphorylate conserved Ser/Thr residues (S^{33}, S^{37}, $T^{41}$and S^{45}) in the N-terminal part of β-catenin (van Noort et al., 2002). Such phosphorylation earmarks β-catenin for ubiquitination by the ubiquitin E3 ligase (Latres et al., 1999) and further degradation in 26S proteasome (Aberle et al., 1997).

4.1.4.1. GSK3β (glycogen synthase kinase 3β)

Glycogen synthase kinase-3 was originally identified as a regulator of glycogen metabolism. Today, it is known that it may also play important roles in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics and cell motility (Frame and Cohen, 2001). There are two closely related isoforms, GSK3α and GSK3β, which are expressed ubiquitously in mammalian tissues (Frame and Cohen, 2001). The substrate specificity of GSK3 is quite unusual as its function requires the presence of another phosphorylated residue optimally located four amino acids downstream of the site of GSK3 phosphorylation. CKI serves as such priming kinase, for S^{45}, in the β-catenin degradation complex (Yanagawa et al., 2002).

4.1.4.2. Axin/Conductin scaffold protein

In addition to Wnt, several growth factors including EGF, insulin, and IGF1 inactivate GSK3. However, there is no direct evidence that these growth factors cause accumulation of β-catenin (Frame and Cohen, 2001). Therefore, Axin may act as a scaffold protein that selectively channels the signal from Wnt ligands to β-catenin. Recent data show that the number of Axin molecules in an individual cell is much lower (5000-fold) than other proteins in the complex. Therefore, Axin could be the limiting component of the Wnt signalling cascade that may promote the rapid assembly and disassembly of Wnt pathway components in order to regulate the β-catenin stability in the cell (Logan and Nusse, 2004).

There are alternative *Axin* splicing products, termed forms 1 and 2. Although Axin (Axin1) and Conductin (Axin2) share an overall identity in their amino acids sequence (45%) and they seem to have similar biochemical functions, they display quite different expression patterns (Kikuchi, 1999). Axin is ubiquitously expressed during embryonic development and in the adults, whereas Conductin appears to be specifically expressed in tissues with active Wnt signalling (Jho et al.,

2002). Moreover, Conductin (but not Axin) is strongly up-regulated in colon, liver, and ovarian tumours (Lustig et al., 2002). The promoter of the *conductin/axin2* gene contains functional TCF binding sites and therefore it is a direct target of the Wnt pathway (Leung et al., 2002). Interestingly, it provides a negative feedback loop for the signalling.

4.1.4.3. APC tumour suppressor

APC is a large protein with multiple functions and interactions within the cell, including Wnt signalling transduction, intercellular adhesion, cytoskeleton stabilization and presumably cell cycle regulation and apoptosis (Nathke, 2004).

APC stands for adenomatous polyposis coli. The *APC* gene is mutated in familial adenomatous polyposis (FAP), a hereditary form of colon cancer (Nakamura et al., 1991). Patients with such mutations develop thousands of colon tumours (called polyps), some of which inevitably progress to malignancy. Inactivating mutations in *APC* are also found in the large majority of sporadic colon cancers (Kinzler and Vogelstein, 1996). APC is therefore an important tumour suppressor in the colon (Korinek et al., 1997).

The APC protein consists of an oligomerization domain at its very N-terminus which is followed by the armadillo repeat region that binds β-catenin (Armadillo in *Drosophila*) (Huber et al., 1997). A set of 15- and 20-aa repeats binding Axin and CtBP is located in the central part of the protein. The C-terminal part contains the basic domain and the EB1 (end binding protein 1) and HDLG (human discs large) binding sites. The mutation cluster region (MCR) lies between codons 1286 an 1513. The name of this region corresponds to the fact that over 60% of all somatic mutations in *APC* "hit" this region (Fearnhead et al., 2001).

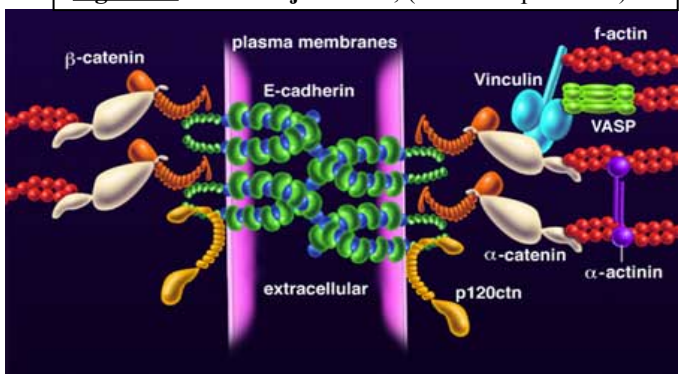
4.1.5. β-catenin – more than a key player in the Wnt canonical pathway

β-catenin is a dual-function protein encoded by a single gene in most animals; in humans, the gene is termed *CTNNB1*. The *Drosophila* homolog is called *Armadillo*. *C. elegans* has three β-catenin-like genes (Moon et al., 2002). β-catenin was originally reported to be involved in intercellular adhesion. In addition, it transduces the Wnt signals from the cytoplasm to the nucleus.

The adhesion function is based on a subcellular pool of β-catenin which is membrane-associated and stable (Gottardi and Gumbiner, 2001). On the other hand, the signalling function of β-catenin is triggered by a soluble cytoplasmic pool that is highly unstable in the absence of the Wnt signal. Such sharing of a critical component between two fundamental processes may reflect a need for coordinate control between them. Cell signalling is accompanied by disengagement of adhesion between epithelial cells during epithelial–mesenchymal transitions and also other developmental processes (Perez-Moreno et al., 2003). In addition, cancer progression and

malignancy typically depends on inappropriate cell signalling and loss of cadherin-mediated adhesion (Birchmeier et al., 1995). Although the two β-catenin pools are usually functionally separated from each other, experimental manipulations of one pool can affect the function of the other under some circumstances (Gottardi and Gumbiner, 2004). The switch between these pools and subsequently functions has been discovered recently (Lilien and Balsamo, 2005) and involves phosphorylation at crucial Y^{142}. Phosphorylation of Y^{142} determines this protein to bind with BCL9-2 and to be translocated into nucleus (Sampietro et al., 2006). Unphosphorylated Y^{142} binds with α-catenin and mediates intercellular adhesion (Bienz, 2005). All catenins (α-catenin, β-catenin and γ-catenin, also called plakoglobin) are associated with cadherins that mediate intercellular adhesion in the epithelia (Huber et al., 1996) (Fig. 2).

Figure 2. Adherens junctions; (www.humpath.com)



E-cadherin is a transmembrane protein the extracellular domains of which can homodimerize, keeping neighbouring cell together in zonula adherens junctions. Its cytoplasmic domain binds to β-catenin. β-catenin additionally binds α–catenin providing the link to the actin cytoskeleton (Kemler, 1993).

4.1.6. Wnt signalling inside the nucleus

The increased stability of β-catenin following Wnt signalling leads to its accumulation in the both cytoplasm and nucleus. Once in the nucleus, β-catenin binds members of the TCF/LEF family of transcription factors (Graham et al., 2000) and this complex stimulates expression of Wnt/β-catenin target genes. This is the final step of the Wnt canonical cascade.

4.1.6.1. β-catenin

The mechanism of β-catenin transport to the nucleus and regulation of this process remains unclear. For a long time it was believed that APC shuttles β-catenin in and out of the nucleus, as β-catenin contains no recognizable NLS (Bienz and Clevers, 2000). Recently, it has been proposed that in non-stimulated cells APC actively exports β-catenin from the nucleus, whereas in cancer and Wnt-stimulated cells the accumulated β-catenin can move between the nucleus and cytoplasm independently of APC (Henderson and Fagotto, 2002). It has been also demonstrated that β-catenin can enter the nucleus by binding directly to the nuclear pore machinery. The central armadillo repeats are required for such transport and they are structurally related to the importin β-HEAT

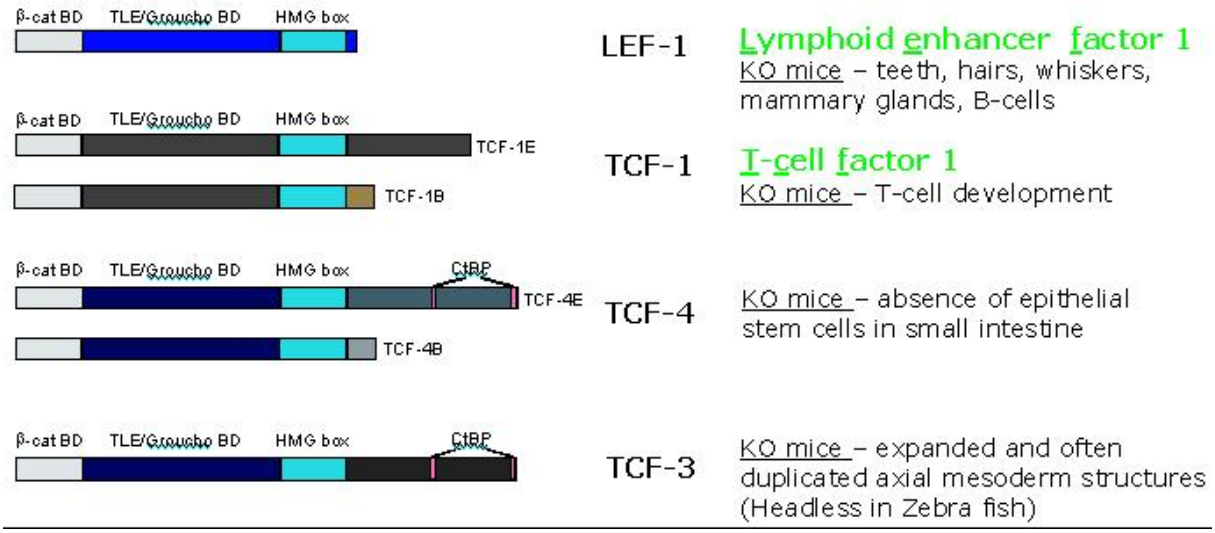
repeats which have been shown to associate with the nuclear pore complex (Henderson and Fagotto, 2002).

After translocation into the nucleus and binding to TCF/LEF factors, β-catenin activates transcription. Mutational analyses have identified two regions in β-catenin important for transactivation, one near the N-terminus and one at the C-terminus (Hecht et al., 1999).

4.1.6.2. TCF/LEF transcription factors

There are four TCF/LEF proteins in mammals: TCF-1 (T-cell factor 1), LEF-1 (lymphocyte-enhancer factor 1), TCF-3 and TCF-4 (Fig. 3).

Figure 3. TCF/LEF proteins and consequences of their knock outs (KO) in mice (Kořínek V.)



TCFs include an 80 aa residues HMG (high mobility group) box that binds to the minor groove of DNA. Its binding dramatically bends DNA and thus TCFs are thought to have an architectural function when organizing the spatial structure of enhancers (van de Wetering et al., 2002).

In the absence of the Wnt signal, TCFs act as repressors by forming a complex with TLE/GROUCHO corepressors (TLE stands for transducin-like enhancer of split). These corepressors bind both the hypoacetylated N-terminal tail of histone H3 and the human histone deacetylase 1 (HDAC1) (Brantjes et al., 2001). Their action is establishing the DNA conformation that prevents transcription. In contrast, β-catenin recruits a number of nuclear factors responsible for the transactivation of the TCF target genes. These factors are the histone acetylase CBP/p300 (cyclic AMP response element-binding protein) (Hecht et al., 2000), the SWI/SNF (switching-defective and sucrose nonfermenting) component BRG1 (Barker et al., 2001), Bcl9 (B-Cell Lymphoma; Legless in *Drosophila*) and Pygopus proteins (Kramps et al., 2002).

Recently, other proteins binding to β-catenin in the nucleus and modulating TCF driven transcription have been identified (Stadeli et al., 2006) and it is very likely that more β-catenin interaction partners will be discovered in future. Other nuclear proteins that affect β-catenin-driven transcription, like HIC1 or CtBP, will be discussed later.

4.1.7. The Wnt target genes

Up to date, more than 50 Wnt target genes have been described. Some of them are components of the pathway itself (*Fz, Axin2, Tcf1, Lef1* and others) triggering a feedback control loop (Logan and Nusse, 2004).

A large majority of the Wnt target genes is cell type-specific. Such specificity is common in developmental signalling pathways, and reflects the basic mechanism of gene control by extracellular signals: the cell rather than the signal determines the nature of the response. These genes include developmental regulatory genes such as *siamois* (Brannon and Kimelman, 1996), *twin* (Laurent et al., 1997), and *Xnr-3* (McKendry et al., 1997) in Xenopus, *ultrabiothorax* (Riese et al., 1997) in Drosophila. In addition, Wnt signalling also controls genes that are more widely induced. These target genes listed in the Tab. 1. and provide a clear link to invasion and metastasis of tumour cells.

A regularly updated list of Wnt target genes as well as much other information about Wnt signalling can be found at The Wnt Homepage: http://www.stanford.edu/~rnusse/wntwindow.html

The pathway itself offers ample targeting nodal points for cancer drug development (Paul and Dey, 2008). Few strategies have already been developed (Takahashi-Yanaga and Sasaguri, 2007) but a number of others are waiting to be discovered providing a great hope for people suffering from tumours induced by non-physiological activation of the Wnt signalling cascade.

Table 1. Wnt target genes (Behrens, J. 2005)

Table 1. *List of β-catenin/Tcf target genes tested functionally* in vitro *or* in vivo

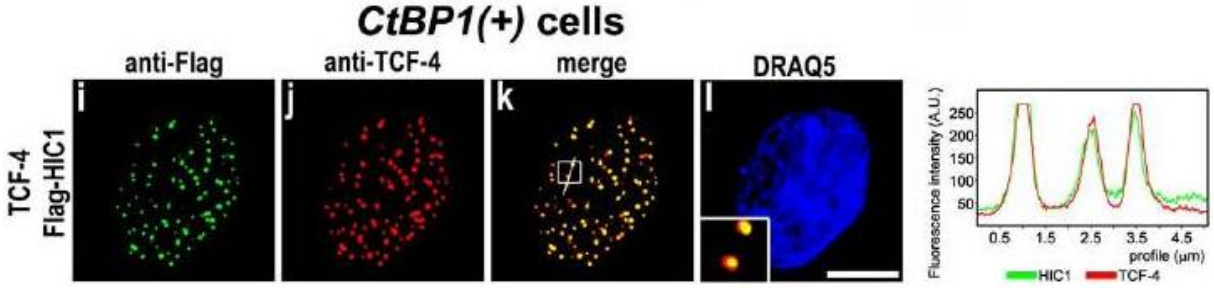
Gene	Type	LOF/GOF	References
c-Myc	-bHLH transcription factor	-knockdown blocks proliferation	He et al. 1998; van de Wetering et al. 2002
Cyclin D1	-cell cycle regulator	-cyclinD1$^{-/-}$/APC$^{min/+}$ show reduced polyp burden	Shtutman et al. 1999; Tetsu and McCormick 1999; Hulit et al. 2004
Id2	-inhibitor of bHLH transcription factors	-Id2$^{-/-}$ develop tumors and show impaired differentiation	Rockman et al. 2001; Russell et al. 2004
ITF-2	-bHLH transcription factor	-overexpression promotes neoplastic transformation	Kolligs et al. 2002
Tcfl	-Wnt signaling	-Tcfl$^{-/-}$/APC$^{min/+}$ show increased polyp burden	Roose et al. 1999
PPARδ	-ligand-activated transcription factors	-PPARδ$^{-/-}$/APC$^{min/+}$ show increased polyp burden -treatment with PPARδ agonist, GW501516, increases number and size of polyps in APC$^{min/+}$	He et al. 1999; Gupta et al. 2004; Harman et al. 2004; Reed et al. 2004
COX-2	-prostaglandin pathway	-COX-2$^{-/-}$/APC$^{\Delta 716/+}$ show reduced polyp burden	Oshima et al. 1996; Hsi et al. 1999; Araki et al. 2003
HDAC2	-histone deactylase	-treatment with HDAC2 inhibitor, valproic acid, reduces polyp number in APC$^{min/+}$ mice	Zhu et al. 2004
FGF18	-growth factor	-knockdown suppresses growth of CRC cells	Shimokawa et al. 2003
FGF20	-growth factor	-knockdown suppresses anchorage-independent growth	Chamorro et al. 2005
Endothelin	-growth factor	-rescues growth arrest and apoptosis resulting from blocking β-catenin	Kim et al. 2005
Gastrin	-gastrointestinal growth factor and hormone	-Gastrin$^{-/-}$/APC$^{min/+}$ show reduced polyp burden	Koh et al. 2000
BAMBI	-BMP and activin membrane-bound inhibitor	-overexpression blocks TGFβ-mediated growth inhibition	Sekiya et al. 2004
MMP7/Matrilysin	-ECM protease	-MMP7$^{-/-}$/APC$^{min/+}$ show reduced polyp burden	Wilson et al. 1997
Nr-CAM	-adhesion	-overexpression increases cellular motility	Conacci-Sorrell et al. 2002
Mdr1	-ABC transporter	-Mdr$^{-/-}$/APC$^{min/+}$ show reduced polyp burden	Yamada et al. 2000, 2003
ENC1	-BTB/Kelch protein family member	-overexpression increases growth rate in CRC cells	Fujita et al. 2001
APCDD1	-unknown	-knockdown inhibits cell/tumor growth	Takahashi et al. 2002

4.2. HIC1 tumour suppressor

HIC1 (Hypermethylated In Cancer 1) was isolated as a candidate tumour suppressor gene during the screening of chromosome 17p, that is frequently altered in human cancers (Hoff et al., 2000; Wales et al., 1995).

HIC1 interacts with at least two proteins involved in Wnt signalling, CtBP and TCF-4 (Valenta et al., 2003). Furthermore, HIC1 can oligomerize via the N-terminal BTB/POZ domain and form discrete nuclear structures called HIC1 bodies. Recently, we described that HIC1 recruits TCF-4/β-catenin complexes to the nuclear bodies and prevents transcription of the Wnt-responsive genes (Valenta et al., 2006) (Fig. 4.)

Figure 4. Simultaneous interaction between CtBP, TCF-4 and HIC1 is essential for the efficient nuclear sequestration of TCF-4 into the HIC1 bodies (Valenta et al., 2006). Confocal microscopy images of *CtBP1(+)* cells transfected with the indicated constructs (left) and stained with anti-Flag and anti-TCF-4 antibody. The right panel shows the overlap of fluorescence intensity peaks along profiles as indicated in the merged micrographs.



Overexpression of HIC-1 following transfection leads to decreased cell proliferation and induction of cell death/senescence (Wales et al., 1995). Recently, it has been also shown that HIC-1 plays a role in p53-dependent apoptosis (Chen et al., 2005) and both epigenetic and genetic loss of HIC-1 function accentuates the role of p53 in tumourigenesis (Chen et al., 2004).

4.2.1. *HIC1* gene

HIC1 gene is located at 17p13.3, locus SMG6. This region is hypermethylated or subject to allelic loss in many human cancers (Fujii et al., 1998; Chekmareva et al., 1997; Issa et al., 1997; Kanai et al., 1999; Li et al., 1998). It resides also within the 350-kbp critical region deleted in most patients with Miller-Dieker syndrome (MDS) (Yingling et al., 2003).

MDS is a contiguous gene deletion syndrome, all cases of which are found to be associated with haploinsufficiency on chromosome 17p13.3. The frequency in population is 1:50,000. Another syndrome connected with deletion in this region is Isolated lissencephaly sequence (ILS). Patients with MDS have a severe grade of lissencephaly, a heterogeneous human developmental brain disorder caused by defects in neuronal migration events that normally occur at 9–13 week of embryonic development (Yingling et al., 2003). The lissencephaly and mental retardation have been

clearly attributed to haploinsufficiency in the *LIS1* gene. Other anomalies, like craniofacial dysmorphology, defects of the limbs and digits, and omphalocele, are also observed in *Hic-1*$^{-/-}$ mouse embryos. Additionally, parts of the *HIC-1* expression territories as defined by *in situ* hybridization studies of mouse embryos overlap with regions that exhibit abnormalities in MDS patients (Carter et al., 2000).

The gene spans about 5 kb and the *HIC1* transcript has several alternatively spliced variants, which comprise a common 3-terminal exon 2 and a different 5-terminal exons, exon 1a, exon 1b, or exon 1c (Pinte et al., 2004), in different human and mouse tissues (Fig. 5) Recently, a novel transcript, which is not under direct transcriptional control of wild-type p53 has been identified in human cancer cells exposed to adverse growth conditions (Mondal et al., 2006).

Figure 5. HIC1 transcripts, adapted from (Mondal et al., 2006)

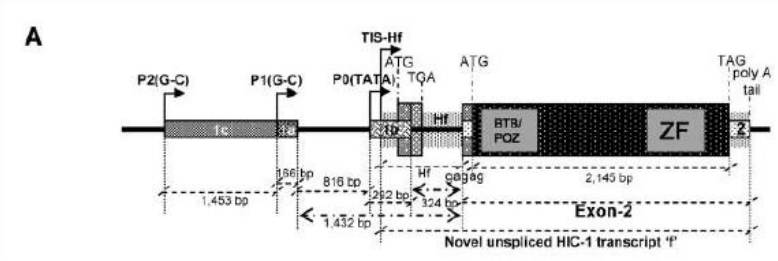


Figure 1. *A*, schematic structure of *HIC-1* gene locus with different promoters and exons for various transcripts. ; ➔, promoters P0, P1, and P2 and the transcription initiation site of the novel transcript (*TIS-Hf*). ▭ unspliced transcript **f** that is expressed from TIS-Hf. Exon 2 is common for all the transcripts. gagag, splicing acceptor site for all the spliced transcripts. Large boxes represent the coding regions from different transcripts. ▮ common region coding for the Bric-a-brac Tramtrack Broad complex/ Pox virus and Zinc finger (*BTB/POZ*) and zinc-finger (*ZF*) domains of HIC-1 protein. ATG start codon is same in both exon 1b and 5′ end of transcript **f** (Hf), but there is a premature TGA stop codon in Hf.

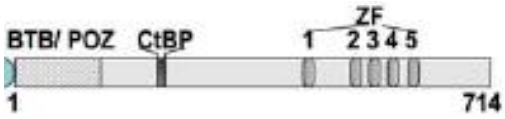
Various transcripts are present in many human tissues (Guerardel et al., 2001), but exon 1a transcript is prevalent, expressed as a "classical" 714-amino acid BTB/POZ protein.

HIC1 is extremely GC-rich; exon II presents CpG island itself and both promoters are encompassed within CpG islands (Rood et al., 2002). In addition, there are six *Not*I restriction sites (each with 2 CpG dinucleotides) in the 11 kb region flanking the *HIC1* gene (the average frequency in the genome is one *Not*I site every 150 kb). The density of CpG dinucleotides in the *HIC1* gene makes it an ideal target for inactivation through methylation (Fujii et al., 1998).

4.2.2. HIC1 protein

Human HIC1 is a polypeptide of 714 aa, that belongs to the BTB/POZ family (Broad-Complex, Tramtrack, Bric à brac/ Pox virus and Zinc finger) of transcription factors. Besides the N-terminal BTB/POZ domain, it possesses five Krüppel-like C_2H_2 (Cys_2-His_2) zinc finger motifs located at the C-terminus and a CtBP (C-terminal binding protein) interacting motif in the central part (Fig. 6).

Figure 6. HIC1 protein (Valenta et al., 2006)



The similar organization of zinc fingers is found in several transcription factors, for example BCL-6 and PLZF that are involved in human neoplasia (Dhordain et al., 1997).

HIC1 is evolutionary conserved, its avian homologue gammaFBP-B was isolated as a transcriptional repressor of the gammaF-Crystallin gene (Deltour et al., 1998) and HIC1 counterpart has been found in zebrafish as well (Bertrand et al., 2004; Guerardel et al., 1999).

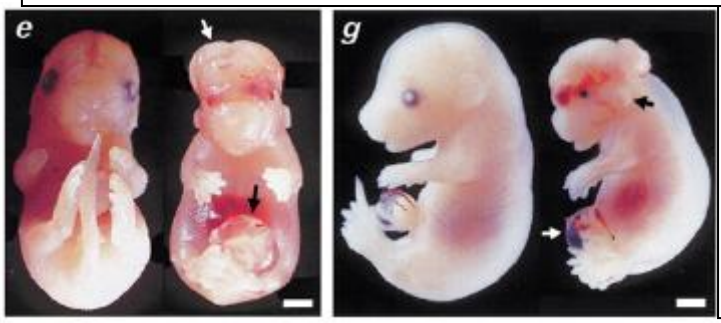
HIC2 (Hypermethylated In Cancer 2) or *HRG22 (HIC-related gene on chromosome 22)* is the closest to HIC1 sharing 80% of the sequence homology. Its precise function remains unknown, however, HIC2 colocalizes with HIC1 in nuclear dots and their BTB/POZ domains heterodimerize and repress transcription from a reporter plasmid (Deltour et al., 2001). Our results show that HIC2 overexpression fails to induce formation of the HIC1 bodies in the absence of HIC1.

HIC1 represses transcription via two autonomous repression domains, the N-terminal BTB/POZ and the central region, by a trichostatin A (inhibitor of histone deacetylase)-insensitive and trichostatin A -sensitive mechanisms, respectively. The HIC1 central region recruits the corepressor CtBP (C-terminal binding protein) through a conserved GLDLSKK motif (Deltour et al., 2002). It has been shown that HIC1 interacts with both CtBP1 and CtBP2 and that this interaction is stimulated by agents increasing NADH levels. Mutation of the central leucine residue (leucine 225 in HIC1) abolishes the interaction of HIC1 with CtBP1 or CtBP2. As expected from the corepressor activity of CtBP, this mutation also impairs the HIC1-mediated transcriptional repression (Stankovic-Valentin et al., 2006). Interestingly, CtBP appears to be a potential modulator of apoptosis and epithelial-to-mesenchymal transition (EMT), an important feature of embryonic development and tumourigenesis (Grooteclaes et al., 2003).

During embryonic development, mouse *Hic1* is expressed in mesenchyme of the sclerotomes, lateral body wall, limb, and craniofacial regions that embed the outgrowing peripheral nerves during their differentiation. *Hic1* is also expressed in mesenchyme opposed to the precartilaginous condensations, at many interfaces to budding epithelia of inner organs, and weakly in muscles (Grimm et al., 1999). In adulthood HIC1 is found in all tissues (Yingling et al., 2003). Constitutive expression of *HIC1* by stable transfection in various cancer cell lines results in a significant decrease in their survival, which suggests that HIC1 might suppress cell growth (Wales et al., 1995).

Mice KO approach revealed HIC1 as essential molecule for development. *Hic1* knockout results in embryonic and perinatal lethality and are much smaller than wt ones and exhibit a range of gross developmental defects such as acrania, exencephaly, cleft palate, limb abnormalities and omphalocele (Carter et al., 2000) (Fig. 7.).

Figure 7 . Developmental defects of Hic1$^{-/-}$ embryos (adapted from Carter *et al.* , 2000)



e) Frontal view of E16.5 grossly normal *Hic1*+/- (left) and *Hic1*-/- (right) embryo. The *Hic1*-/- animal is missing the right eye, has an underdeveloped left eye, acrania with protrusion of the brain (white arrow) and a large ventral wall defect with protruding intestinal loops and liver (black arrow).
g) Lateral view of the same two embryos shown in e).

Disruption of only one *Hic1* allele predisposes mice to a gender-dependent spectrum of malignant tumours with an onset after 70 weeks of life. 75% of the malignancies were epithelial cancers in males, whereas among the females 85% of the malignancies were lymphoid and mesenchymal cancers. *Hic1*$^{+/-}$ showed another distinct phenotype - over 25% of these mice develop severely pruritic ulcerative dermatitis around the facial area (Chen et al., 2003).

Knudson's hypothesis says that both alleles of a gene must be inactivated (either by methylation, mutation, or deletion) for a tumour suppressor gene to be inactivated. The complete loss of *Hic1* function in the *Hic1*$^{+/-}$ mice seems to involve dense methylation of the promoter of the remaining wild-type allele. Interestingly, in malignancies isolated from males, hypermethylation was observed mainly in the downstream promoter (1b) while in females it was mainly in the upstream (1a) promoter (Chen and Baylin, 2005).

4.2.3. Epigenetical silencing of HIC1

Genes can be inactivated by different ways. DNA methylation is one of the mechanisms that silence transcription. The addition of a methyl group at the 5-position of cytosine occurs predominantly at the CpG dinucleotides. In the vertebrate genome, CpG dinucleotides represent only 1% of it. In some regions, known as CpG islands, CpG residues can make 6% or more of all dinucleotides (Ariel, 2001). CpG islands are usually associated with the promoter regions of housekeeping genes and are unmethylated. Methylation of the CpG islands occurs at genes to be silenced or on the inactive X chromosome and in parentally imprinted genes. In addition, the CpG islands may become methylated upon oncogenic transformation. However, such DNA methylation is not always associated with gene silencing (Li, 2002).

DNA methylation regulates gene expression through several distinct mechanisms. It can directly block transcription regulatory factors from binding to their target sequences, but more often, DNA methylation represses gene expression through several methyl-CpG-binding proteins

(MECPs). For instance, MECP2 forms a complex with HDACs and a co-repressor protein, Sin3a, to repress transcription (Nan et al., 1998).

Silencing is present in tumour suppressor loci such as *p15, p16, Rb, VHL, e-cadherin, ER,* and *HIC1* or *APC* (Klarmann et al., 2008). *HIC1* has been reported methylated in a large number of adult solid tumours, including those of the colon (Goel et al., 2007; Lassmann et al., 2007; Maekawa et al., 2001), lungs (Fukasawa et al., 2006; Hayashi et al., 2001; Stephen et al., 2007; Zemliakova et al., 2003), breast (Muller et al., 2003; Nicoll et al., 2001; Parrella et al., 2004; Parrella et al., 2005), brain (Uhlmann et al., 2003), adrenals (Margetts et al., 2005), liver (Zhao et al., 2005), cervix (Dong et al., 2001; Gustafson et al., 2004; Narayan et al., 2003), ovary (Rathi et al., 2002; Strathdee et al., 2001; Tam et al., 2007; Teodoridis et al., 2005), prostate (Kekeeva et al., 2007), and in acute myeloid leukaemia (Aggerholm et al., 2006; Britschgi et al., 2008; Ekmekci et al., 2004; Melki et al., 1999; Melki et al., 1999), and diffuse large B-cell lymphoma (Stocklein et al., 2007). It has also been observed in various paediatric tumours, like medulloblastomas (the most common malignant brain tumour in children) (Lindsey et al., 2004; Rood et al., 2002), ependymomas (Waha et al., 2004), retinoblastomas, rhabdomyosarcomas (Rathi et al., 2003), germ cell (Koul et al., 2002; Koul et al., 2004; Lind et al., 2007; Xue et al., 2004) and neuroblastic tumours (Rathi et al., 2003).

Interestingly, the *HIC1* promoter is methylated also in some healthy human tissues (Eads et al., 2001). Normal breast ductal tissues and normal prostate epithelium includes approximately equal amount of densely methylated and completely unmethylated *HIC1* (Fujii et al., 1998). A baseline level of methylation was also found in normal brain tissues (Rood et al., 2002). However, the correlation between hypermethylation of *HIC1* and its reduced or lost expression does not reach statistical significance in all the tissues (Waha et al., 2004). There was not statistical significant correlation between methylation status and LOH in medulloblastomas. Methylation was neither correlated with clinical risk category and is therefore an independent prognostic factor. Furthermore, LOH status was not correlated with overall survival (Rood et al., 2002).

Epigenetic alteration of HIC1 expression along that of with other tumour suppressor genes has emerged as a potential marker of tumourigenesis. This is of particular importance, as new non-invasive methods of detection of such an aberrant cancer associated DNA methylation were developed (Muller et al., 2004). It is possible to assess the marker from the body fluids, as tumour-derived extracellular DNA is found in the blood and stool of cancer patients (Lenhard et al., 2005), and this approach can be useful in early detection of neoplasms (Rykova et al., 2004; Sabbioni et al., 2003; Skvortsova et al., 2006), as the hypermethylation of tumour suppressor genes is differential and occur early in the carcinogenesis.

4. 2. 4. HIC1 and p53

TP53 is a very potent tumour suppressor gene. Its product, p53, is a transcription factor that integrates a number of pathways mediating apoptosis in response to a wide range of cellular stresses, including DNA damage, hypoxia and nutrient deprivation, cell survival and proliferation (Feng et al., 2008). Thus, it couples stimuli promoting both cell proliferation and cell death.

TP53 resides at 17p13.1. Knowing that it is also location of *HIC1* gene, two tumour suppressor genes are located closely to each other on the same chromosome. Additionally, *HIC1* expression can be up-regulated by p53 as a functional p53 binding site was found in its promoter, located 500 bp upstream of TATA-box containing P0 promoter of HIC1 (Britschgi et al., 2006). Deletion of these regions on at least one chromosome is frequently observed in cancers. The intact copy of *p53* is then often mutated, and that of *Hic1* is frequently hypermethylated (Chen and Baylin, 2005).

In mice, both mentioned genes are localized closely on chromosome 11. A double KO approach was used to study a relationship between these tumour suppressors (Chen et al., 2004). $Hic1^{+/-}$, $p53^{+/-}$ mice were either *trans* (alleles deleted on separate chromosomes) or *cis* (on the same chromosome). Each of these cases yields distinct tumour phenotypes. *Trans* mice develop breast and ovarian carcinomas and metastatic osteosarcomas with epigenetic inactivation of the wild-type *Hic1* allele and interstitial deletion of the wt copy of *p53*, whereas *cis* germline deletion results in earlier, prevalent and more aggressive osteosarcomas with genetic deletion of both wt alleles. It underscores the selection pressure for rapid tumourigenesis as the deletion can simultaneously include the wild-type *p53* on the same chromosome, whereas promoter hypermethylation inactivates only *Hic1* (Chen and Baylin, 2005).

4. 2. 5. SIRT1, HIC1 target gene

So far, the only HIC1 target gene, SIRT1, a histone deacetylase that is regulated by direct binding of HIC1 to its promoter sequences, has been identified (Chen et al., 2005).

Histone deacetylases are divided into three categories based on their homology to yeast proteins Rpd3p (class I), Hda1p (class II), and Sir2p (class III). Unlike class I and II, the deacetylase activity of class III enzymes relies on the cofactor NAD^+ (de Ruijter et al., 2003). Yeast Sir2p has broad biological functions linking metabolism to transcriptional silencing, suppression of recombination, chromatin functions and longevity (Guarente, 2000). In mammals there are seven homologues of yeast Sir2p, termed sirtuins, with nuclear protein SIRT1 having the highest homology. SIRT1 levels increase upon calorie restriction or nutrient starvation. SIRT1 deacetylates nonhistone proteins and allows mammalian cell survival under oxidative stress and DNA damage through several mechanisms (North and Verdin, 2004). One of them is deacetylation of p53 and

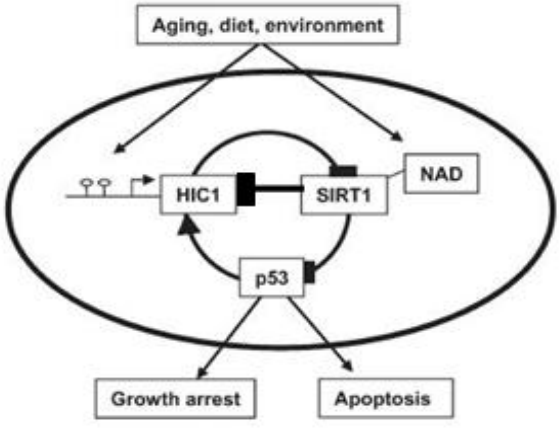
attenuation of its ability to *trans*-activate its downstream target genes, such as p21 for cell-cycle arrest and Bax for apoptosis (Luo et al., 2001). SIRT1 involvement in transcriptional regulation is through deacetylation of histones and non-histone proteins that interact with SIRT1. The recruitment of SIRT1 also induces deacetylation of linker histone H1 and promotes further formation of facultative heterochromatin (Vaquero et al., 2004).

HIC1 forms a transcriptional repression complex with SIRT1 deacetylase (via the BTB/POZ domain), and this complex directly binds the *SIRT1* promoter and represses its transcription (Chen et al., 2005). Inactivation of HIC1 results in upregulated *SIRT1* expression in normal and cancer cells. This deacetylates and inactivates p53 and allows cells to bypass apoptosis and survive DNA damage. Inhibition of SIRT1 function in cells without HIC1 abolishes the resistance to apoptosis (Chen et al., 2005).

It has been shown, that SIRT1 activity can be regulated at the transcriptional level through the redox-sensing ability of the transcriptional corepressor, CtBP, HIC1 interacting protein (Zhang et al., 2007). The redox changes that can be induced *e.g.* by the glycolytic inhibitor 2-deoxyglucose (2-DG), decrease CtBP interaction with HIC1. The consequent reduction of CtBP recruitment decreases transcriptional repression of HIC1 and induces *SIRT1* expression. This mechanism allows the specific regulation of SIRT1 in response to nutrient deprivation. Recently, SIRT1 has been proposed as a marker of tumourigenesis (Lim, 2007).

Endogenous HIC1 has been shown to be SUMOylated in vivo on a phylogenetically conserved lysine, K314, located in the central region (Stankovic-Valentin et al., 2007). K314R mutation does not influence HIC1 subnuclear localization but significantly reduces its transcriptional repression potential. Furthermore, HIC1 is acetylated in vitro by P300/CBP. The fact that the K314R mutant is less acetylated than wild-type HIC1, suggests this lysine is a target for both SUMOylation and acetylation. HIC1 transcriptional repression activity is positively controlled by two types of deacetylases, SIRT1 and HDAC4, which increase the deacetylation and SUMOylation, respectively, of K314. Finding that HIC1 is a target for SIRT1 identifies a new posttranslational modification step in the P53-HIC1-SIRT1 regulatory loop (Fig. 8.)

Figure 8. P53-HIC1-SIRT1 regulatory loop
(adapted from *Chen, WY et al.; 2005*).



Other posttranslational modification of HIC1 protein includes O-linked N-acetylglucosamine (O-GlcNAc) occurring preferentially in the DNA-binding domain. Such O-GlcNAc modification of HIC1 does not affect its specific DNA-binding activity and is highly

sensitive to conformational effects, namely to its dimerization through the BTB/POZ domain (Lefebvre et al., 2004).

Very recently, Hic1 has been identified as a direct transcriptional repressor of *Atonal Homolog 1* (*Atoh1*), a proneural transcription factor essential for cerebellar development. Atoh1 is also a putative target of the Hedgehog signalling. In addition, based on the fact that chromosome 17p deletion is the most frequent genetic lesion in medulloblastoma (Ferretti et al., 2005), it was suggested that *Hic1* and *Ptch1* tumour suppressors cooperate in silencing *Atoh1* expression during development of medulloblastoma, most common malignant CNS tumour in children (Briggs et al., 2008).

5. MATERIALS AND METHODS

5.1. Bacterial strains, Cell lines and Media

5.1.1. *Escherichia coli* strains

- **TOP-10** (*Invitrogen*): F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
- **BL-21** (*Invitrogen*): F-, *omp*T, *hsd*S(r-B, m-B), *gal*, *dcm*, *lac*I, *lac*UV5-T7 gene 1, *ind*1, *sam*7, *nin*5
- **SURE®** (*Stratagene*): *e14– (McrA–) D(mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5* (Kan^r) *uvrC* [F' *proAB lacI*^{q}*ZDM15 Tn10* (Tet^r)]

5.1.2. Eukaryotic cell lines

The cell lines used during this study are listed in Tab. 2.

Table 2. Cell lines and their specifications

Name of cell line	Source organism	Type of cells, tissue of origin	Source of the cell line
COS-7	monkey	SV40 transformed fibroblasts, kidney	Utrecht University, Netherlands
HEK 293	human	transformed epithelial, embryonic kidney	ECACC, Sigma
HEK 293 FT	human	HEK 293 cells containing SV40 large T antigen	ECACC, Sigma
HeLa	human	epithelial, cervical adenocarcinoma	Prof. Hořejší, IMG, ASCR
WI-38	human	fibroblasts, embryonic lungs	ATCC, USA
LEP 19	human	fibroblasts, embryonic lungs	Dr. Šloncová, IMG, ASCR
CCD 841	human	transformed epithelial, normal colon	ATCC, USA
HIEC	human	untransformed epithelial, normal intestine	Dr. Jean-Francois Beaulieu, Canada
FHC	human	untransformed epithelial, normal colon	ATCC, USA
HT 29	human	secretory epithelial, colon adenocarcinoma	ATCC, USA
Colo 320	human	secretory epithelial, colon adenocarcinoma	ATCC, USA
SW 480	human	secretory epithelial, colon adenocarcinoma	ATCC, USA
LS 174T	human	secretory epithelial, colon adenocarcinoma	ATCC, USA

DLD-1	human	epithelial, colon cancer	ATCC, USA
DLD-1/HIC1	human	epithelial, colon cancer	produced using ARGENT Regulated Transcription Retrovirus Kit (*ARIAD*)
MPEF	mouse	primary embryonic fibroblasts	StemCell Technologies, Canada
NeoR MPEF	mouse	Neomycin-resistant primary embryonic fibroblasts	StemCell Technologies, Canada
v6.5	mouse	ESC, blastocyst	Dr. Divoký, LF UPOL

5.1.3. Culture media

5.1.3.1. Culture medium for bacteria - LB (Luria-Bertani) medium

The liquid medium was prepared by dissolving 20 g of LB concentrate *(Amresco)* in 1 liter distilled water and sterilized by autoclaving at 1 Bar and 120°C for 20 minutes (*Astell*).

Antibiotics (ampicilin or kanamycin) were added just prior to culture at final concentration 100 μg/ml. Agar plates with LB medium were prepared by adding agar for bacterial culture 18g/l LB medium (Sigma Aldrich,). 7g agar per liter of LB medium was added to prepare top agar used for plating lambda phage. The suspension was cooled down to approximately 50°C and selection antibiotics (ampicilin or kanamycin) (if required) were added at final concentration 100 μg/ml. 20ml of sterile suspension were sterilely poured on polystyrene plates and let solidify.

5.1.3.2. Culture media for eukaryotic cell lines

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS (*Hyclone*) and antibiotics (50 000 IU/l penicillin; 0.1 g/l streptomycin; 0.08g/l gentamycin; all *Sigma*) was used to culture COS-7, HEK 293, HEK 293 FT, DLD-1, HT 29, Colo 320 cell lines.

For growing HeLa cell line we utilize DMEM supplemented with 5 % calf serum (*Zvos Hustopeče*) and antibiotics.

SW 480 were maintained in RPMI supplemented with 10 % FBS (*Hyclone*) and antibiotics.

WI38 and LEP19 primary cells and LS 174 T cell line were cultured in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10 % FBS (*Hyclone*), non-essential amino acids (*Gibco*) and antibiotics (50 000 IU/l penicillin; 0.1 g/l streptomycin; 0.08g/l gentamycin; all *Sigma*)

HIEC cells were grown in OPTI-MEM® I (*Gibco*) supplemented with 5 % foetal bovine serum GOLD (*PAA*), 10 mM Hepes, 5ng/ml EGF and 2mM L-glutamine.

Medium for CCD841 cells consists of Ham's F12 medium and DMEM (ratio 1:1), 10% FBS *(Hyclone),* 10 mM Hepes, 0.01 mg/ml insulin *(Sigma),* 0.01 mg/ml transferrin *(Sigma)*, 25 nM sodium selenite, 50 nM hydrocortisone, 0.5% (w/v) bovine serum albumin *(Sigma)* , 100 pM

triiodothyronine, 1 ng/ml EGF, 0.01 mM ethanolamine, 0.01 mM phosphorylethanolamine, 0.5 mM sodium pyruvate, 2mM L-glutamine, and 1.2 g/l sodium bicarbonate.

FHC cells were maintained in Ham's F12 medium and DMEM (ratio 1:1), with 10% FBS *(Hyclone),* 25 mM Hepes, 10 ng/ml cholera toxin, 5 ng/ml insulin, 5 ng/ml transferrin and 100 ng/ml hydrocortisone.

DLD-1/HIC1 cells were maintained in selection media to ensure maintenance of expression plasmids, *i.e.* DMEM with 10 % FBS (*Hyclone*) and standard antibiotics supplemented with 200 μg/ml hygromycin B (*Sigma)* and 700 μg/ml Geneticin (G-418) (*Sigma*). The expression of HIC1-EGFP protein was induced by adding the dimerizer AP21967 (*ARIAD*) to final concentration of 30nM.

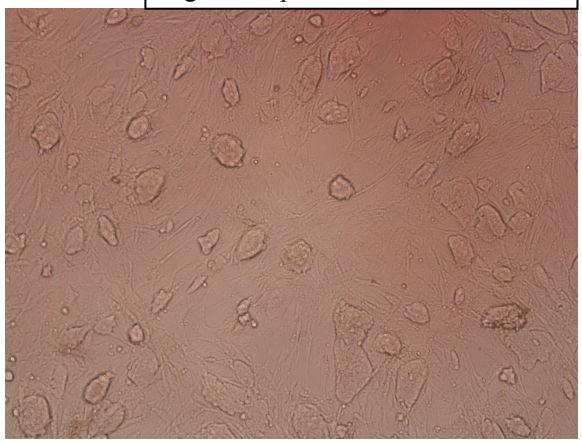
All primary cells and cell lines were grown in culture incubators (*Sanyo*) at 37°C, 5% CO_2 with the only exception being v6.5 cells that were grown in 37°C, 10% CO_2.

All work was done using sterile equipment and standard tissue culture techniques. Cell cultures were regularly checked for the presence of mycoplasma.

5.2. Mouse Embryonic Stem Cells manipulation

5.2.1. Embryonic Stem Cells culture

Figure 9. Optimal mESC culture



Murine embryonic stem cells are derived from the inner cell mass of the 3.5 days- old mouse embryo. The manipulation of embryonic stem cells to generate targeted mutations via homologous recombination has proved an invaluable resource for researchers.

ES cell cultures to be used in the genetic engineering of mice should be subconfluent (~70%), well established, and contain colonies that maintain well-defined edges and have not yet begun to differentiate (Fig. 9.). Embryonic stem cells generally require feeder layer of cells to grow on. These feeder cells provide mechanical support and release factors maintaining undifferentiated and pluripotent status of ESC. Leukaemia inhibitory factor (LIF) can substitute for feeder cells. We combined both approaches (feeder cells and LIF) to ensure growth and maintenance of the unique properties of the ESC.

v6.5 mESC cell line was cultured in DMEM Glutamax (*Gibco*), supplemented with 15% FBS *(Hyclone, lot #ARK27856,* this lot was previously tested not to cause differentiation of the mESC), 2 mM L-glutamine, 1 mM sodium pyruvate, 1x non-essential amino acids, 100 UI Penicillin/Streptomycin, 0.1 mM β-mercaptoethanol (all *Gibco*) and 1.25 ml LIF conditioned

media/500 ml media (*Dr.Divoký*). This mESC medium was replaced daily. ESCs were split at 1:8 every other day (corresponding to 60% confluency) onto a new feeder plate.

MPEF and NeoR MPEF were used as feeder cells. These were allowed to reach high confluency and then used for mitomycin C treatment. MPEF medium was prepared using DMEM Glutamax (*Gibco*), 15% FBS *(Hyclone),* 100 UI Penicillin/Streptomycin and 0.1 mM β-mercaptoethanol (all *Gibco*). MEPF were mitotically inactivated by 2 hrs incubation with 10 μg/ml Mitomycin C (*Sigma)* in MPEF media. After washing the cells three times with PBS, these were trypsinized (0.05% Trypsin- 0.53 mM EDTA; *Gibco*) and frozen down in MPEF media with FCS (ratio 1:1) and 8% DMSO.

Inactivated feeders were thawed and plated 24hours before being used for ESC culture.

5.2.2. Electroporation of mESC

This procedure is highly efficient for the introduction of foreign DNA into mammalian cells. I utilized it to introduce my two gene-targeting constructs in mESCs.

Plasmid DNA was linearized using *Cla*I restriction endonuclease, extracted using phenol/chloroform and resuspended in TE to give a final concentration of ~ 1 μg/μl. Four 10-cm plates of ES cells at optimal density were washed twice with PBS and a single cell suspension was generated using 0.05% Trypsin- 0.53 mM EDTA *(Gibco*). Cells were pooled, counted and spined down. Two different setups were performed:

(1) Pellet of 10^7 cells was resuspended in PBS to a total volume of 0.8 ml and transferred to a 0.2mm electroporation cuvette (*Bio-Rad*) containing 20 μg of linearized construct (either pEF-mHic1 conditional KO or pEF-mHic1-citrine KI). The cuvette content was mixed carefully, incubated for 20 min at RT and electroporated by one 230 V pulse, 500 μF using a Gene Pulser II (*Bio-Rad).* pEF-mHic1 conditional KO zap yielded time constant of 8.3 ms, while pEF-mHic1-citrine KI zap time constant of 10 ms.

(2) $5x10^6$ cells were resuspended in mESC medium to give a total volume of 0.8 ml and were transferred to an electroporation cuvette containing 10 μg of DNA (either pEF-mHic1 conditional KO or pEF-mHic1-citrine KI). The content of cuvette was mixed gently and immediately electroporated by a 340 V pulse, 250 μF at RT. For pEF-mHic1 conditional KO I recorded time constant of 3.6 ms, while pEF-mHic1-citrine KI time constant was 3.7 ms.

After electroporation the cells from each zap were split in ratio 1: 3: 6 onto three 10-cm plates with fresh feeder layer (NeoR MPEF) and mESC medium and placed in incubator.

The plates were labelled “KO” or “KI” according to targeting vector, “1” or “2” regarding to the zaps and “10%” or “30%” or “60%” according to the amount of cells plated. Afterwards we

determined that there was no significant difference between the number of colonies selected after the two types of zap and we further didn't distinguish between "1" and "2" plates.

5.2.3. Selection of targeted clones

Selection medium contains antibiotic in doses lethal to all ESCs except those that are expressing the resistance gene included in the targeting vector. Random insertion of the targeting vector into the ESC genome was selected against with gancyclovir as the vectors contain a viral thymidine kinase cassette at the end of one of the homology arms. Such addition of this negative selection to routine G-418 positive selection decreased the number of colonies to be screened by approximately 15-fold as was determined by growing one of the plate of mESCs (the one containing 10% of electroporated mESCs) under positive selection only.

After allowing the electroporated cells to grow for 24 hours in mESC medium, I began selection. G-418 (*Gibco*) at concentration 350 μg/ml and 2.5 μM gancyclovir (*Sigma*) were used as selection agents. Selection of the cells took 14 days. Very little cell death was observed until day 2–3 and after 6 days of selection small round colonies became visible.

5.2.4. Picking colonies

Colonies were picked from eight (four "KO" and four "KI") 10-cm plates containing 30% and 60% of electroporated mESCs subjected to both positive and negative selection. Altogether 119 "KO" colonies and 97 "KI" colonies smaller than a pipette tip were picked between day 7 and 11.

The procedure was done as follows. Optimal colonies were identified using a microscope and marked with pen on the bottom of the dish. The dish was washed once with PBS and 15 ml PBS was added to the dish. Under a microscope, placed in a laminar flow hood, we nudged the colony loose from the bottom of the dish with the tip of a pipette and aspirated the colony into the tip in one motion. The colony was placed into individual well of a clean (no feeders) 96-well dish containing 50 μl trypsin/EDTA *(Gibco)*. Following picking of 15-20 colonies (taking approximately 10 minutes) the trypsinization was stopped by adding 150 μl ES cell medium to each well of the dish. Pipetting up and down mechanically dissociated colonies and all 200 μl of single cell suspension was transferred into the 48-well dish containing fresh feeders (NeoR MPEF). Afterwards, the cells were fed daily with mESC medium.

5.2.5. Expansion and cryopreservation of colonies

Once the 48-well dish was approximately 70% confluent, the culture was split in half. Half of the culture was seeded onto a new 6-well dish of feeders (NeoR MPEF) to be cryopreserved,

while the other half of the culture was seeded onto a new 6-well dish coated with 0.1% gelatine to be lysed for DNA extraction. To subculture the ESCs, these were washed twice with 300 µl PBS, 50µl trypsin/EDTA was added and incubated for 2 minutes at 37°C.

After reaching approximately 60% confluency all 216 clones were cryopreserved separately each in single cryotube in freezing media containing mESC media and FCS (*Hyclone*, *lot #ARK27856*) (ratio 1:1) and 8% DMSO.

The cells in remaining 6-well were grown to high density before lysing for DNA extraction. This is necessary because although all the colonies picked survived selection, homologous recombination of the construct is rare and must be ascertained via Southern analysis of extracted DNA.

Once correctly targeted clones have been identified from Southern analysis, individual colonies were thawed and plated onto a 6-well dish for expansion. We checked their appearance and repeated Southern blot analysis, to exclude misnumbering of the clones. Finally, the clones were sent to Transgenic Core Facility, Max Planck Institute, Dresden, to be microinjected into blastocysts.

5.3. Transformations and Transfections

5.3.1. Bacterial transformation

Transformation is an introduction of "naked" DNA into bacteria or yeast. Bacteria are treated so they will take the DNA up into their cells. These are called competent cells and are made so by treatment with calcium chloride in the early log phase of growth.

Such competent cells can be prepared ready for use and stored for long term in -70°C. TOP10 *E.coli* strain was used for multiplying plasmids and BL21 (DE3) strain was used for production of recombinant proteins.

Heat shock transformation

The 50µl aliquot of competent cells was thawed on ice. 1µg of plasmid DNA or a ligation mixture was added to the suspension and mixed by pipetting. Following 20 minute incubation on ice, the cells were heat-shocked in a 42°C water bath for 90 seconds and returned directly to ice. 1 ml of LB media was added and the cells were transferred to 37°C to help them recover from the heat shock and to take time for plasmid genes to be expressed. After 45 min, the cells were briefly centrifuged, resuspended in 200 µl of fresh LB media, seeded onto agar plates with the selective antibiotics and incubated at 37°C overnight.

5.3.2. Transfection of eukaryotic cells

5.3.2.1. Lipofectamine

Transfections of DLD-1 cell line were performed using the Lipofectamine™ 2000 reagent *(Invitrogen)* as described by the manufacturer. In brief, 24h before transfection cells were seeded so that they were 90-95% confluent at the time of transfection. Plasmid DNA (0.8 μg/ well/ 12-well plate) was diluted in OPTI-MEM® I (*Gibco*) and mixed with Lipofectamine 2000 diluted in OPTI-MEM® I (ratio 1:2 μg DNA/μl Lipofectanime). The mixture was incubated for 30 minutes at room temperature and then added dropwise onto cells.

5.3.2.2. FuGENE

Transfections of HeLa cell line were performed using the FuGENE® 6 transfection reagent *(Roche)* according to the manufacturer's instructions. In short, FuGENE (3:2 ratio μl Fugene/ μg DNA) was incubated with OPTI-MEM® I medium for 5 minutes at RT before adding the undiluted DNA into the mixture. The Fugene/DNA mixture was incubated for further 15-30 minutes at RT before dropwise pipetting onto exponentially growing cells in OPTI-MEM® I.

5.3.2.3. Polyethylenimine (PEI)

HEK 293, HEK 293 FT and COS-7 cell lines were transfected using PEI. One day before transfection the cells were split to reach 60-70 % density at the time of transfection. Following procedure is designed for transfection of one well (=9 cm^2) of 6-well plate; it can be adjusted according to transfected area. 10x PEI = 100mM monomeric solution of PEI (25 kDa), pH 7.4.

Two mixtures were prepared: mixture A = 5 μg of vector DNA + 125 μl of sterile 150mM NaCl, mixture B = 15 μl of 1x PEI + 110 μl of sterile 150mM NaCl. Both mixtures were incubated (separately) for 15 min at RT, then combined and incubated for further 10 minutes at RT. Meanwhile, culture medium from the cells was replaced with medium without serum and without antibiotics. A+B mixture was added dropwise to the cells. COS-7 cells were centrifuged (5 min, 200 rcf, 25ºC) afterwards while HEK293 cells were not.

After 3 hours, the medium was changed for medium supplemented with 10% FBS and antibiotics.

5. 4. DNA manipulation

The basic manipulation with DNA (restriction digest, ligation, precipitation and separation in agarose gel) was performed according to standard molecular protocols (Ausubel et al., 2003) chapters 1.53-1.72.

Following chemicals were used:

- Restriction endonucleases *Aat*II, *Age*I, *Apa*I, *Asc*I (*Sgs*I), *Ase*I, *BamH*I, *Bgl*I, *Bgl*II, *BssH*I, *BssH*II, *BstE*II, *Cla*I (*Bsu*15I), *Dpn*I, *EcoR*I, *EcoR*V, *Eco47*III, *Hae*III, *Hind*III, *Kpn*I, *Mlu*I, *Msc*I, *Nco*I, *Nar*I, *Nde*I, *Nhe*I, *Not*I, *Pme*I (*Mss*I), *Pst*I, *Pvu*I, *Sac*I, *Sal*I, *Sca*I, *Sfi*I, *Sma*I, *Spe*I, *Ssp*I, *Stu*I, *Xba*I, *Xho*I, *Xma*III (*Eco52*I) and *Xmn*I (*Fermentas*)
- Alkaline phosphatase CIAP or SAP (*Fermentas*) to hydrolyse 5´-phosphate groups from vector DNA
- *ZymoClean Gel DNA Recovery Kit* (*Zymo Research*) for the purification and concentration of DNA from agarose gel slices
- T4-DNA-ligase (*Fermentas*) to ligate DNA fragments
- T4-DNA-polymerase (*Fermentas)* to blunt the cohesive ends of DNA fragments

All procedures were performed according to manufacturers' instructions.

Experiments were designed *in silico* using Lasergene® software (DNAStar).

5.4.1. PCR

Polymerase Chain Reaction was used to amplify specific DNA fragment.

5.4.1.1. Oligonucleotides

Primers used for PCR are listed in Tab. 3. and Tab. 4. Restriction sites (underlined) are often included in primers to be used for cloning the fragment into vector. Primers were designed using Primer3 Tool available at http://frodo.wi.mit.edu

All oligonucleotides were purchased from *Sigma-Genosys*.

Table 3. PCR primers

Name	5'->3' sequence
FhSIRT1#promoter#XhoI	AGGCTCGAGTATGTCAACCACTAGGAG
RhSIRT1#promoter#HindIII	CCCAAGCTTCCTCTGCTCCCGCTCGAC
FmHic1-insertion-Bam#Sal	AGCATCTGGGGCTGGATCCGTCGACAGGCTAACCTGGCCTA
RmHic1-insertion-Bam#Sal	TAGGCCAGGTTAGCCTGTCGACGGATCCAGCCCCAGATGCT
FmHic1#78390#XhoI	CACACTCGAGGATTCAAGGAAGTAATGTGT
RmHic1#78854#HindIII	ATTAAGCTTGTCTGCGTCCTCTGCTCTCTG
FmHic1#92211#HindIII	AGTACTAAGCTTGCCTATAAAAGC
RmHic1#92672#XhoI	ATCTAGGCTCGAGTCACCGCTCTG
FFLPrec#PstI	CGGCTGCAGCCCAAGCTTCCACCAT
RmHic1#2883#SspI	GCTAATATTAAAAATATAAATCTCCCTTCC
FFlirt#40#BamHI	ACGGATCCATGAAGTTCCTATACTTTCTAG
Fcitrine#Hind#KasI#start	TTTAAGCTTGAGGCGCCTATGGTGAGCAAGGGCGAGGAG
Rcitrine#EcoRV	GGAGATATCACTTGTACAGCTCGTCCA
FFlirt#40#BamHI	ACGGATCCATGAAGTTCCTATACTTTCTAG

mHic1 rec out F	CAAGCTGAAAGGAACCTTCGTC
mHic1 rec out R	GTCCACTTGTCCCTCACTAGTAAAA
mHic1 rec in F	GACTGTTGGTGGTCCTTACCTGTC
mHic1 rec in R	CTCTGTGAGGCTGGTAAGGGATATT

Table 4. Sequencing primers

Name	5'->3' sequence	Name	5'->3' sequence
FpBS seq	GTAAAACGACGGCCAGT	RpBS seq	GGAAACAGCTATGACCATGA
FpEGFP#4635	TTACGGTTCCTGGCCTTTTG	RpKmyc#1397	CCCGTCAAGCTCTAAATCGG
T7 primer	GTAATACGACTCACTATAGGGCG	T3 primer	CCCTTTAGTGAGGGTTAATT
FmHic1#3B#3224	GCATTCTGCACCACGATGAT	FmHic1#3B#2076	TGGGGAAGCCTTTGCCACATG
RmHic1#3B#3990	CTCCTGCCCCGATATAACGC	seq pEF #8997 F	ATTAATGCAGCTGGCACGAC
RmHic1#3B#2460	AGACCCGTTTCGAGGAAGCG	seq pEF #4576 R	CGACATTGGGTGGAAACATT
seq mHic1 long arm #24#5833 F	TGAGGAACTTCACATTATCAGTCC	seq mHic1 short arm NotI R	CCCCCAGTTCCTCCTCTG
seq pEF #4296 F	GTGGGCTCTATGGCTTCTGA	FFlirt#416	CTCTGGCCAGCCTAGGACTTC

Table 5 . Other oligonucleotides

Name	5'->3' sequence
FloxP#BamHI#SspI#Eco	GATCCAATATTATAACTTCGTATAGCATACATTATACGAAGTTATGA
RloxP#BamHI#SspI#Eco	ATCATAACTTCGTATAATGTATGCTATACGAAGTTATAATATTG

5.4.1.2. PCR reaction

All PCR reactions (except for screening for 5'arm recombination in gene targeting) were performed using Phusion™ Hot Start DNA polymerase (*Finnzymes*). I followed the manufacturer's guidelines described at http://www.biocat.de/bc/pdf/Phusion%20Hot%20Start%20DNA%20Polymerase%20Manual.pdf

The reaction mixtures were set up as follows. I worked with GC rich templates so betaine and DMSO were included.

0.01 – 0.05 µg	DNA template
10 µl	5x Phusion GC Buffer
1 µl	10 mM dNTPs
3.2 pmol	primer 1 (5´)
3.2 pmol	primer 2 (3´)
0.5 µl	Phusion DNA Polymerase (1 U)
20 µl	2.5M betaine
1.5 µl	DMSO
to final volume 50 µl	dH_2O

5.4.2. Recombinant plasmids

During my diploma thesis I cloned several constructs. The application is described for each construct separately. All the cloning was verified by restriction analysis and further by sequencing.

5.4.2.1. pET42b-HIC1 for immunization

The protein coding sequence was gained by restriction of pET28b-HIC1 for immunization construct with *BamH*I and *Hind*III enzymes. The fragment was cloned into pET42b vector keeping the appropriate reading frame. GST fused to the N-terminal of HIC1 was used for affinity purification of the recombinant protein (chapter 5.6.2.).

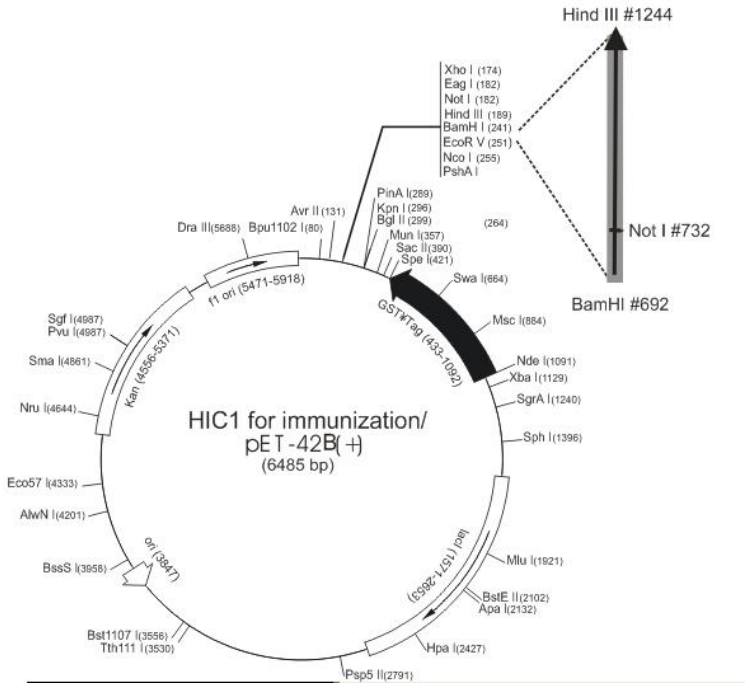


Figure 10. Map of pET42b-HIC1 for immunization construct
Nucleotide positions are derived from the sequence gi:5729870. Internal *Not*I site was used for restriction analysis.

5.4.2.2. pGL3 basic-Sirt1 promoter

Sirt1 is so far the only identified gene regulated by HIC1 binding to its promoter sequence. I used this construct for luciferase reporter assay (chapter 5.11.).

The promoter sequence was obtained by PCR using FhSIRT1#promoter#XhoI and RhSIRT1#promoter#HindIII primers and genomic DNA as a template. The fragment was extracted with phenol/chloroform and subjected to double digestion with *Hind*III and *Xho*I and ligated into *Hind*III and *Xho*I sites of dephosphorylated pGL3 basic vector.

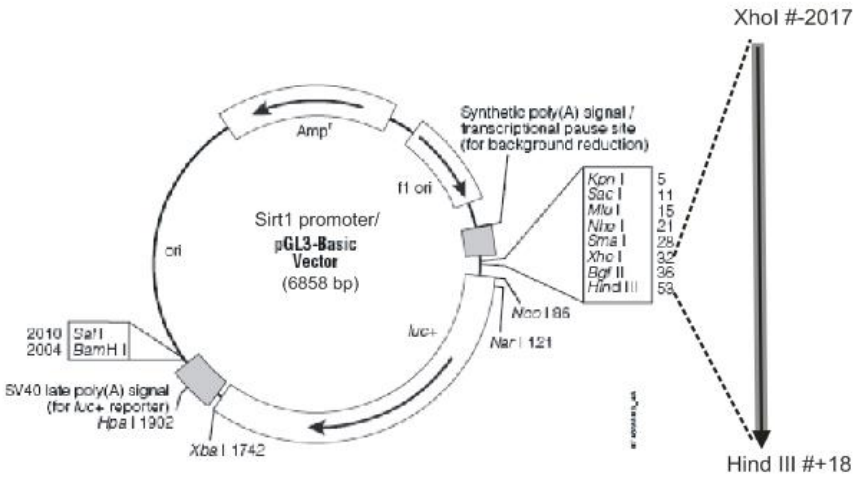


Figure 11. Map of pGL3 basic-Sirt1 promoter construct
Nucleotide positions are relative to the transcriptional

5.4.2.3. pEF-mHic1 conditional KO

To prepare gene targeting constructs I emanated from two bacteriophage clones 3B and 4A. I subcloned all parts of the targeting construct into a homemade vector pKB (kanamycin Bluescript). We chose pKB vector for its different resistance compared to Lambda DASH® II and pEF (pEasyFlirt) vectors carrying ampicilin resistance cassette. This resistance switch has proved crucial in cloning long fragments of DNA.

Fig. 12. shows the cloning scheme and the maps of vector used.

(1) The isolated lambda DNA (chapter 5.4.3.3.) was digested with *Not*I and resolved in agarose gel. Isolated *Not*I fragments were cloned into pKB vectors. Midipreps were done for all fragments cloned in both orientations.

(2) Two constructs representing 4A-9.5kb NotI fragment cloned in pKB in both orientations were digested with *BamH*I and fragments containing pKB vector sequence were religated.

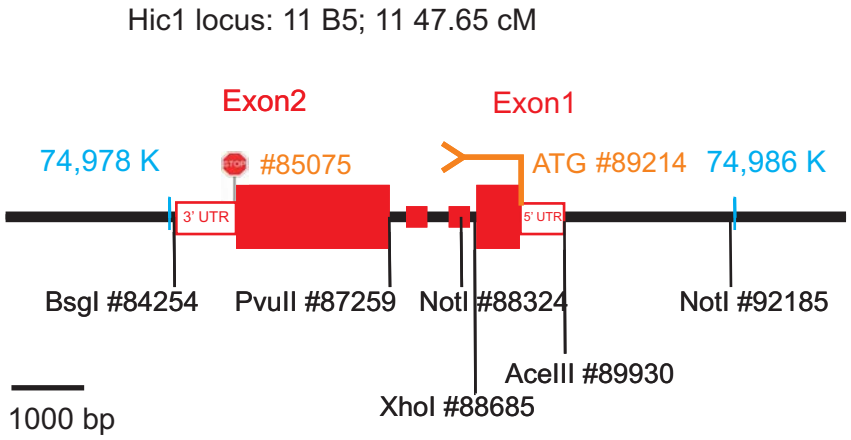
(3) pKB-4A-2.1kb fragment was used as a template for PCR mutagenesis reaction primed with FmHic1-insertion-Bam#Sal and RmHic1-insertion-Bam#Sal primers. After the PCR, the template DNA was digested by adding 5UI of *Dpn*I restriction enzyme. This enzyme cleaves the methylated DNA while it preserves the DNA synthesised during PCR (non-methylated) uncleaved. TOP10 *E.coli* cells were transformed with the reaction mixture and four colonies were obtained on kanamycin selection. One of the colonies contained the desired vector with *Sal*I and *BamH*I inserted sites that were used for further cloning steps.

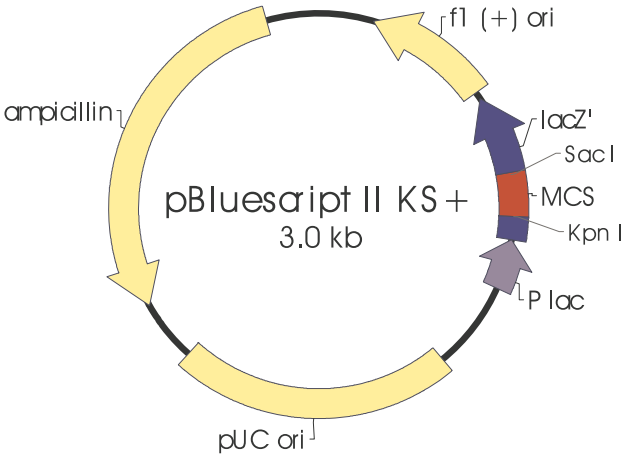
(4) FloxP#BamHI#SspI#EcoRV and RloxP#BamHI#SspI#EcoRV oligos were annealed (oligos at equimolar concentration were incubated at 95ºC for 5 min and then let to cool down to room temperature on the workbench) and ligated into *BamH*I and *EcoR*V sites of pBluecript II KS vector (*Fermentas*)

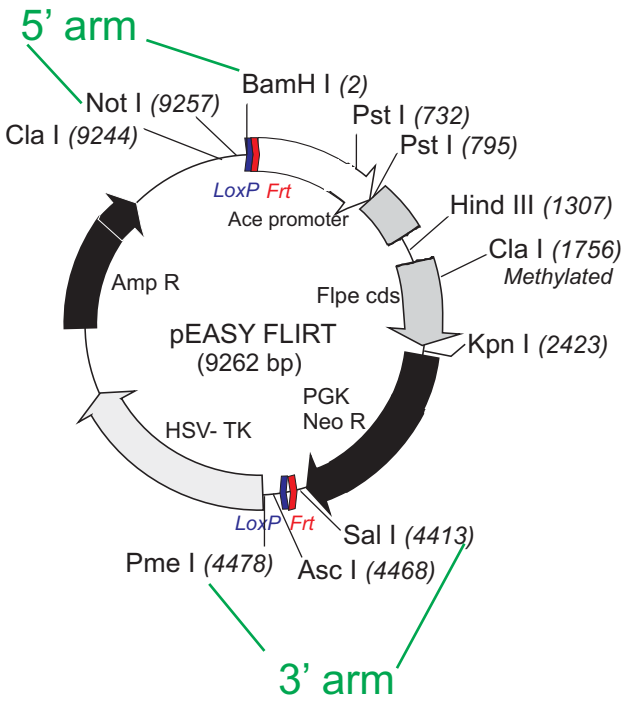
(5) Three-point ligation was performed to acquire pBS/mHic1 loxP construct. The first fragment (1323 bp) was obtained from PCR reaction with RmHic1#3B#2460 and RmHic1#2883#SspI primers and pKB-4A-9.5 kb NotI fragment construct as a template (PCR product 1494 bp in length) followed by digestion with *BamH*I and *Ssp*I. The second fragment was the loxP site with cohesive *Kpn*I and *Ssp*I ends from pBS-loxP construct. These two fragments were ligated into the rest of pBS-loxP (from which the loxP site was cut with *Kpn*I and *Ssp*I enzymes) into *BamH*I and *Kpn*I sites.

(6)1.3 kb BamHI fragment from pKB-4A-mutated 2.1kb fragment construct was cloned into pBS-mHic1 loxP. The right orientation was verified by *BamH*I, *Sal*I restriction digest.

(7) To insert a loxP site into the 3'UTR of Hic1 gene pBS-loxP + BamHI fragment was digested with *EcoR*V and *Sal*I. Fragment of 2655 bp was isolated and swapped with *EcoR*V, *Sal*I







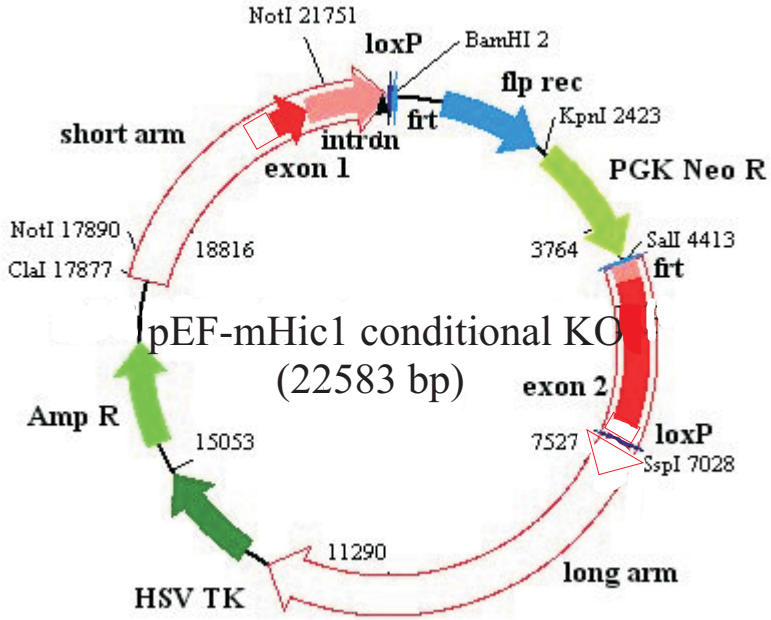
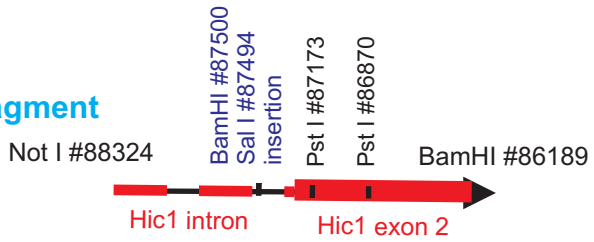


Figure 12A:Hic1 locus and vectors used for cloning of the gene-tergeting constructs and the final pEF-mHic1conditional KO construct

(2) **pKB-4A- 9.5 kb Not I fragment (+)**
pKB-4A- 9.5 kb Not I fragment (-) → BamHI digest → religation of fragments containing pKB sequence → **pKB-4A-2.1kb fragment** **pKB-4A-9kb fragment**

(3) **pKB-4A-2.1kb fragment** → PCR mutagenesis + DpnI digest → **pKB/4A- mutated 2.1kb fragment**



(4) Lox P site oligos annealing → ligation into BamHI and EcoRV sites of pBS → **pBS-LoxP**

(5) Hic1 3'UTR as a PCR product **pBS-LoxP** → SspI, KpnI, BamHI digests → Three-point ligation → **pBS/mHic1 loxP**

(6) **pKB/4A- mutated 2.1kb fragment** → BamHI digest 1.3 kb fragment isolation → ligation into BamHI site of **pBS/mHic1 loxP** → **pBS/mHic1 loxP + BamHI fragment**

(7) **pBS/mHic1 loxP + BamHI fragment** → EcoRV, SalI digest 2.7 kb fragment isolation → ligation into EcoRV, SalI sites of **pKB-4A- 9.5 kb Not I fragment (+)** → **pKB-mHic1 3' arm**

(8) **pKB/4A- mutated 2.1kb fragment** → BamHI, Not I digest 0.8 kb fragment isolation → ligation into BamHI, SalI sites of pEF → **NotI-BamHI fragment/pEF**

(9) **pKB-4A- 3.8 kb Not I fragment (+)** → Not I digest 3.8 kb fragment isolation → ligation into NotI site of **NotI-BamHI fragment/pEF** → **pEF/mHic1 5' arm**

(10) **pKB-mHic1 3' arm** → Not I digest, blunting with T4 polymerase, SalI digest, 8.7 kb fragment isolation → ligation into PmeI, SalI sites of **pEF/mHic1 5' arm** → **pEF/mHic1 conditional KO**

Figure 12C. Cloning scheme of pEF-mHic1 conditional KO construct, part II

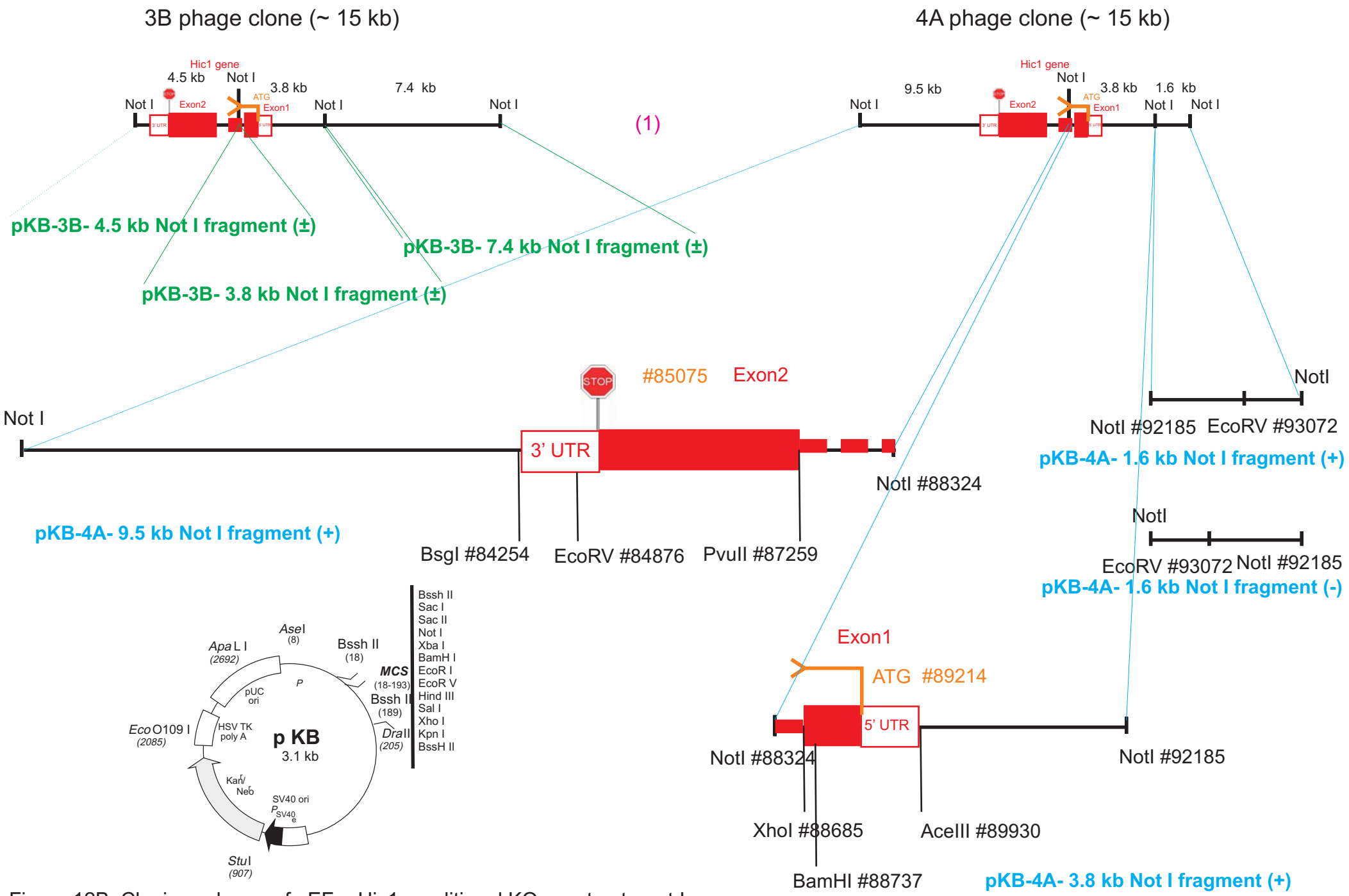


Figure 12B. Cloning scheme of pEF-mHic1 conditional KO construct, part I

3509 bp fragment of pKB-4A- 9.5 kb Not I fragment. The resulting construct was named pKB-mHic1 3' arm.

(8) pKB-4A-mutated 2.1kb fragment was digested with BamHI and NotI endonucleases and fragment 824 bp in length was cloned into pEF.

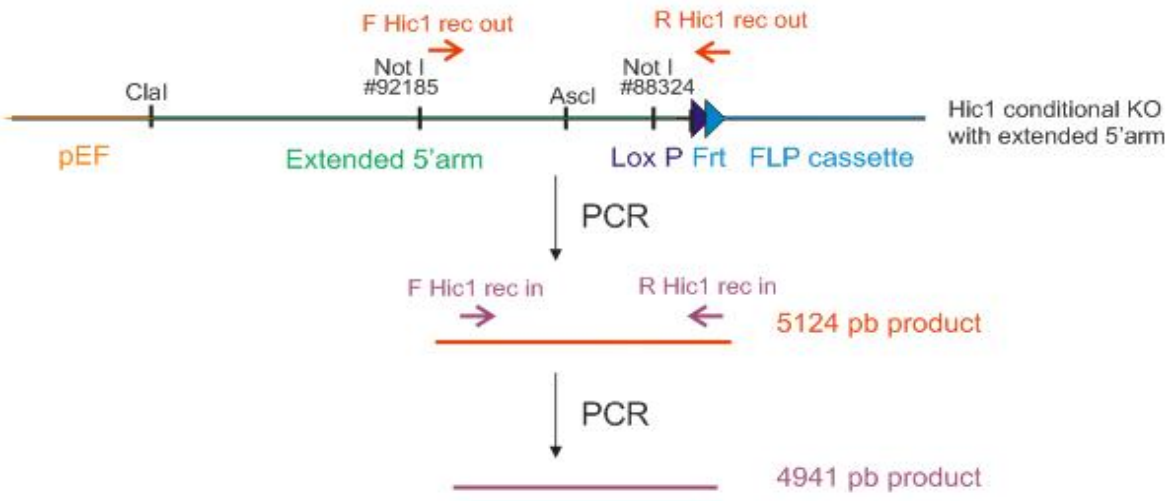
(9) 3.8 kb *Not*I fragment from pKB-4A- 3.8 kb NotI fragment was cloned into pEF-NotI-BamHI fragment and the orientation of the fragment was verified. The resulting construct was named pEF-mHic1 5' arm.

(10) Finally the mHic1 3' arm was cloned into pEF already containing the mHic1 5'arm. pKB-mHic1 3' arm was digested with NotI and the cohesive ends were blunted with T4-DNA polymerase (dNTPs mix was added to final concentration of 0.1 mM and incubated at 11°C for 20 min followed by 10 min incubation at 70°C to inactivate the enzyme). Following the phenol/chloroform extraction, the construct was digested with SalI. The isolated fragment (8,698 bp) was ligated into *Pme*I (*Mss*I) and *Sal*I sites of pEF-mHic1 5' arm

5.4.2.4. pEF-mHic1 conditional KO with extended 5'arm

PCR approach can be used for verifying the homologous recombination of targeting vector. We designed our PCR screening strategy with one primer annealing to a region of the gene that is NOT included in the construct, and the other annealing to the FLP cassette. This means, that the desired product will be amplified only after undergoing the desired recombination. I designed two set of primers to conduct the nested PCR to lower the detection limit of the reaction. To test the primers, mHic1 conditional KO with extended 5'arm/pEF construct was prepared.

Figure 13. PCR screening strategy for 5'arm recombination



(1) HindIII fragment was digested from pKB-3B-7.4 NotI fragment and the construct was religated.

(2) 3.8 kb Not I fragment from 3B was inserted into construct from step (1) resulting in pKB-extended 5'arm construct.

(3) The extended 5'arm was cloned into pEF using *Cla*I and *Asc*I(*Sgs*I) restriction endonucleases.

PCR reactions were performed using Long PCR Enzyme Mix (*Fermentas*) according to manufacturer's protocol

http://www.fermentas.com/profiles/pcr/pdf/coa_k0181.pdf

5.4.2.5. pEF-mHic1-citrine KI

For cloning the second construct for gene targeting I emanated from intermediate constructs gained during preparation of pEF-mHic1 conditional KO.

(1) The citrine fluorescent protein coding sequence was obtained by PCR from Citrine GT46 vector using Fcitrine#Hind#KasI#start and Rcitrine#EcoRV primers and ligated into *Hind*III and *EcoR*V restriction sites of pBS II KS.

(2) mHic1-citrine fused protein was generated by cloning the citrine coding sequence to internal *Nar*I site of a second exon of the Hic1 gene. This resulted in almost complete replacement of exon 2 with citrine. NarI/SalI citrine fragment from pBS-citrine was ligated into *Nar*I and *Sal*I sites of pKB-4A-mutated 2.1kb fragment giving the pKB-mHic1-citrine construct.

(3) pKB-mHic1-citrine 3'arm construct was acquired similarly to mHic1 3' arm/pKB by swapping EcoRV, SalI fragment of pKB-4A- 9.5 kb NotI fragment with EcoRV, SalI fragment of pKB-mHic1-citrine construct.

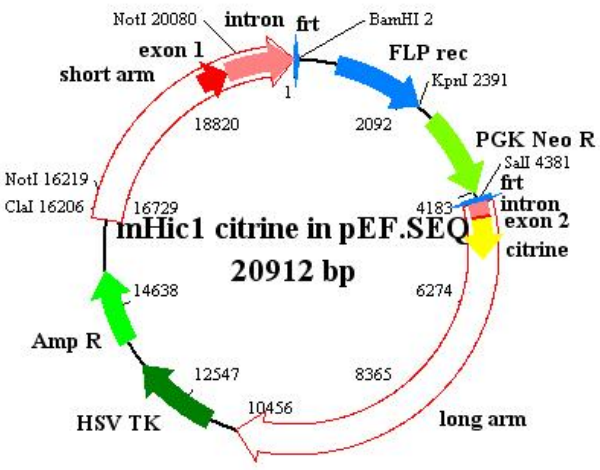
(4) BamHI, KpnI 2422 bp fragment of pEF containing the loxP and Frt sites was cloned to pKB.

(5) To delete the proximal loxP site from pEF PCR using FFlirt#40#BamHI FFlirt#416 primers on pEF as template was conducted. The PCR product was subjected to digestion with *BamH*I and *Age*I enzymes and ligated to *BamH*I and *Age*I sites of pKB-pEF construct from step (4)

to obtain pKB-pEF(-)loxP.

(6) The proximal loxP site in pEF-NotI-BamHI fragment was deleted by

Figure 14 . pEF-mHic1-citrine KI construct



swapping NdeI/BamHI fragment (2128 bp) of this construct with NdeI/BamHI fragment (2096 bp) from pKB-pEF(-)loxP.

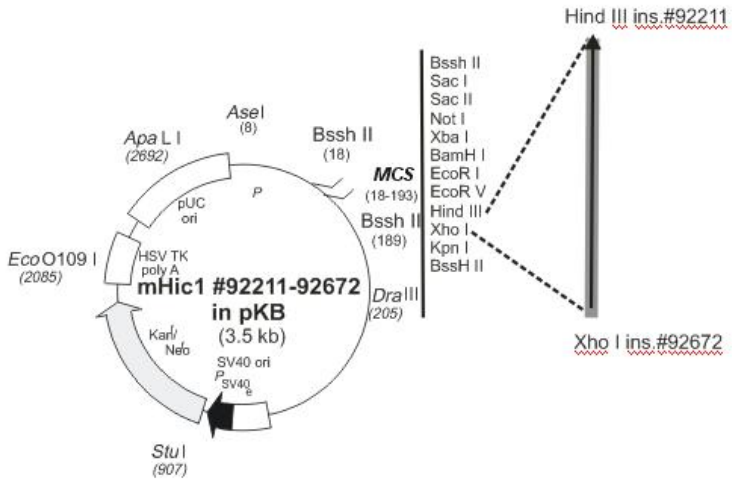
(7) (9) 3.8 kb *Not*I fragment from pKB-4A- 3.8 kb Not I fragment was cloned into (-)loxP pEF-NotI-BamHI fragment and the orientation of the fragment was verified. The resulting construct was named (-)lox pEF mHic1 5' arm.

(8) mHic1-citrine 3'arm was cloned to the final construct (Fig. 14.) in the same way as in pEF-mHic1 conditional KO, described in step (10).

5.4.2.6. pKB-Southern blot probe for 5' arm

The probe sequence was derived from the genomic DNA using PCR primed with FmHic1#92211#XhoI and RmHic1#92672#HindIII oligonucleotides. The PCR product was digested with *Xho*I and *Hind*III and ligated into the pKB vector.

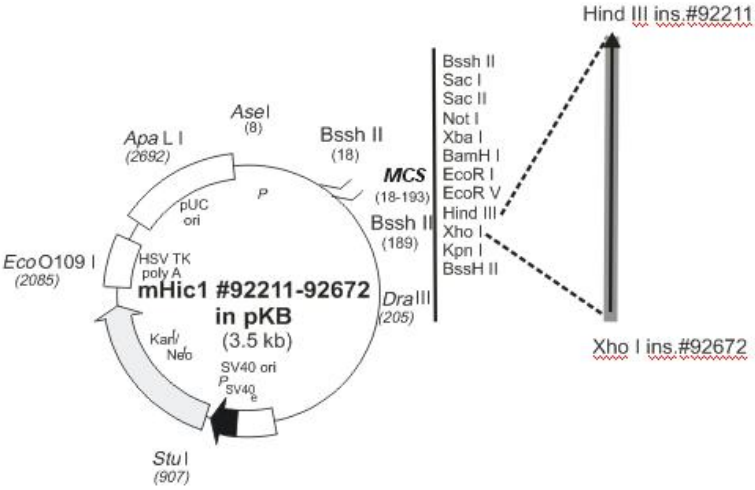
Figure 15. pKB-Southern blot probe for 5' arm map



5.4.2.7. pKB-Southern blot probe for 3' arm

The second probe for Southern blot analysis was derived similarly to the first one. Primers used were FmHic1#78390#HindIII and RmHic1#78854#XhoI. Nucleotide positions are derived from mouse DNA sequence RP23-143A14 located on chromosome 11.

Figure 16. pKB-Southern blot probe for 3' arm map



5.4.3. DNA isolation

5.4.3.1. Plasmid DNA minipreparation

Zyppy™ Plasmid Miniprep Kit II (*Zymo Research*) was used for isolation of plasmid DNA from small scale bacterial cultures (~ 1.5 ml). This is particularly useful for restriction analysis or sequencing. I followed the manufacturer's instructions available at: http://www.zymoresearch.com/products/dna/zyppy_plasmid_miniprep_kit.asp

5.4.3.2. Large scale plasmid DNA preparation

To obtain larger amounts of highly pure DNA (up to 100 µg) for transfection, electroporation and radioactive labelling I utilized JETSTAR Plasmid Purification Kit (*Genomed).* Detailed protocol can be found on: http://www.genomed-dna.com/pdf/Star-PDFs/JETSTAR-Protocol.PDF

5.4.3.3. Isolation of Lambda DNA

Two clones 3B and 4A of bacteriophage library were obtained from Dominique Leprince. These clones contain mHIC genomic fragments of sv129 mouse strain in Lambda DASH® II vectors (*Stratagene*). Both clones were propagated to obtain genomic DNA, a starting material for cloning in the target vectors.

Amplifying Lambda library clones

E.coli SURE® strain culture was grown to an OD_{600} of 0.6 in LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$ to induce production of λ receptor (lamB protein). The aliquot of library suspension was mixed with 100 µl of host cells and incubated 15 min at 37°C, at which time the phage infects the bacteria. 6.5 ml of cooled top agar (~ 48°C) was mixed with an aliquot of infected bacteria and spread evenly onto freshly made 10-cm bottom agar plate. The plates were incubated at 37°C for 8h to prevent the plaques from getting larger than 1-2 mm. The plates were overlaid with 10 ml of SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatine) and stored at 4°C with gentle rocking overnight to allow the phage to diffuse into the SM buffer.

Preparing Lambda DNA from phage lysates

15 µl (1U/µl) of DNase and 25 µl of 10mg/ml RNase were added to 20 ml of SM buffer with the phage and incubated at 37°C for 1h to degrade the bacterial DNA and RNA. The suspension was ultracentrifuged (100,000 x g, 2h, 4°C), supernatant discarded and the phage pellet resuspended in 800 µl of 50 mM Tris-HCl pH 7.0. The DNA was extracted with phenol and

chloroform. I performed two extractions with phenol pH 8.0 (1:1) and two with chloroform (1:1). 1/10 volume of 3M sodium acetate pH 4.8 was added to the aqueous layer and DNA was precipitated in 1 volume of isopropanol. I washed the pellet by adding 1ml of 70% ethanol and spun it for 5 min, dried it and resuspended it in 150 µl of TE pH 8.0. Finally, the DNA was precipitated with polyethylene glycol (PEG) to ensure its purity. 1 volume of 1.6 M NaCl containing 13% (w/v) PEG 8000 was mixed well with the DNA solution. DNA was recovered by centrifugation (16,000 x g, 5 min, and 4°C) and the pellet dissolved in 360 µl TE pH 8.0 and 36 µl 3M sodium acetate pH 4.8 for further precipitation with 2 volumes of ethanol. I again washed the pellet by adding 1ml of 70% ethanol, spun it for 5 min, dried it and resuspended it in 150 µl of TE pH 8.0 containing 10 µg/ml RNase.

5.4.3.4. Genomic DNA isolation

For verification of homologous recombination of the targeting vector in mESCs, it is necessary to isolate genomic DNA and perform Southern blot analysis.

The cells in 6-well plates were cultured to the point of the medium turning bright yellow. Wells were rinsed twice with 1ml PBS. The cells were lysed with 500 µl lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% SLS with 1.0 mg/ml Proteinase K (*Fermentas*) for 10 minutes at 37°C. The content of wells was transferred to 1.5 ml eppendorf tubes and incubated at 60°C overnight. The next morning any moisture from lid was spined down. The DNA was precipitated by adding 1ml of absolute ethanol (*Merck*) and 50 µl of 3M sodium acetate, pH 5.2 and inverting the tube. The DNA precipitates centrifuged (16,000 x g for 5 minutes at RT). The pellet was washed twice with 0.8ml of 70% ethanol and air dried for 20 minutes or longer. Then it was resuspended in 150 µl of TE, pH 8.0 with RNase A.

5.4.4. Spectrophotometric evaluation of nucleic acids

The concentration and purity of DNA and RNA was measured by Eppendorf BioPhotometer. Spectrophotometer measures the absorbance at 260 nm and 280 nm. A_{260} determines the concentration of nucleic acid (A_{260} = 1 equals 50 µg/ml dsDNA and 40 µg/ml RNA), A_{280} determines protein contamination. The purity of nucleic acids is expressed as ratio A_{260}/A_{280}, highly pure samples have this ratio between 1.8 – 2.0.

5.4.5. DNA sequencing

ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kit (*Applied Biosystems*) was utilized to sequence DNA samples. I followed the manufacturer's manual found at http://docs.appliedbiosystems.com/pebiodocs/04337035.pdf

In brief, each PCR reaction consisted of ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kit, specific primer and 2μg of template DNA. Sequencing itself was provided by Sequencing centre located at Microbiological Institute AS CR.

5.4.6. Southern blot

Southern blotting was named after Edward M. Southern who developed this procedure in the 1970s. DNA molecules are transferred from an agarose gel onto a membrane. Southern blotting is designed to locate a particular sequence of DNA within a complex mixture with a specific probe. Under optimal conditions, one can expect to detect 0.1 pg of the DNA.

5.4.6.1. Restriction digest and electrophoresis of genomic DNA

Genomic DNA isolated from mESC (chapter 5. 4. 3. 1) was digested overnight with the restriction endonuclease. After heat inactivation of the enzyme (65°C, 20 min) the DNA was subjected to electrophoresis in 0.9% agarose gel with EtBr.

The gel was photographed under UV light with a fluorescent ruler so the band positions can be later identified on the membrane.

5.4.6.2. Southern blotting onto a nylon membrane with an alkaline buffer

Alkaline transfer to positively charged nylon membrane worked better in my hands than the traditional blotting onto nitrocellulose membrane with high salt buffer.

The gel was rinsed in distilled water and treated in ~ 10 gel volumes of 0.25 M HCl while shaking for 30 min at RT. This step led to partial depurination of the DNA fragments and in turn to strand cleavage necessary for efficient transfer of DNA molecules longer than 4 kb. After rinse with distilled water, the DNA was denatured in ~ 10 gel volumes of denaturing solution (0.4 M NaOH, 1M NaCl). Downward capillary transfer was set up according to the standard protocol (Ausubel et al., 2003) chapter 2.9.7.; with the 0.4 M NaOH, 1M NaCl as the transferring solution to blot the DNA fragments to positively charged nylon membrane (*Ambion*).

After blotting, the membrane was rinsed in 2xSSC (300 mM NaCl, 30 mM sodium citrate), placed on a sheet of Whatman 3MM paper and air-dried.

5.4.6.3. Radioactive labelling of DNA probe

Genomic DNA fragments (~ 460 bp) to be used as specific probes were cloned into pKB plasmids (chapters 5.4.2.5. and 5.4.2.6.). The fragments were obtained by digesting the constructs with appropriate restriction enzymes, separating on agarose gel and isolating them using

ZymoClean Gel DNA Recovery Kit (*Zymo Research*). This purified DNA served as a template for the labelling reactions.

Labelling itself was conducted using Amersham Ready-To-Go DNA Labelling Beads (-dCTP) (*GE Healthcare*) according to manufacturer's instruction. In brief, denatured DNA (25–50 ng), 5 µl [α-^{32}P]dCTP (3 000 Ci/mmol) (=50 µCi) and distilled water in total 50 µl were added to the tube containing the Reaction Mix bead and incubated at 37°C for 30 min. Unincorporated nucleotides were removed by gel filtration on Sephadex™ G-25 column and the labelled DNA was denatured by heating at 95–100°C for 2 minutes and cooled immediately on ice.

5.4.6.4. Hybridization analysis of DNA blots

The membrane carrying the immobilized DNA was wetted in distilled water and placed DNA-side-up in a hybridization tube. 10 ml of hybridization solution was added. The tube was incubated in the hybridization oven 3h with rotation at 42°C to block non-specific DNA binding sites on the membrane. The hybridization solution was replaced with 10 ml of fresh hybridization solution including the denatured DNA labelled probe and incubated overnight at 42°C, rotating. The next morning the probe in hybridization solution was poured out and stored for next hybridization (to be done within a week) or disposed appropriately to a radioactive waste. The membrane was washed twice with 20 ml of 2x SSC, 0.1% SDS for 5 min at RT. Then it was washed three times with 20ml of 0.2xSSC, 0.1% SDS for 30 min each at 68°C with rotation.

Finally, the membrane was rinsed in 2x SSC at RT and checked with the Geiger counter. If too much radioactivity was left, wash with 0.2xSSC, 0.1% SDS at 68°C was repeated. The membrane was wrapped in plastic wrap and an autoradiograph was set. The radioactivity was recorded on Imaging Plate (*Fuji*) and visualized using BAS 5000 Phospho Imager (*Fuji*).

Buffers and solutions used for Southern blotting:

Hybridization solution
- 5x SSPE
- 5x Dernhardt solution
- 0.1% SDS
- 100 µg/ml salmon sperm DNA
- 50% formamide
- 10% dextran sulphate

20x SSPE, pH 7.0
- 3M NaCl
- 0.2M NaH2PO4
- 20 mM EDTA

50x Dernhardt solution
- 1% BSA
- 1% Ficoll 400
- 1% Polyvinyl pyrrolin

5. 5. Production of monoclonal antibody against HIC1

Monoclonal antibodies are of exceptional purity and specificity and therefore have various applications. Monoclonal antibodies to HIC1 are commercially available from Abnova, but these are suitable only for Western blotting or ELISA.

The central part of hHIC1 protein (aa 223-408) was chosen as an immunogenic peptide as it shares the lowest level of homology with the mostly related protein HIC2. DNA encoding this fragment was cloned into an expression vector pET28b to obtain His-tag for purification of the recombinant protein using TALON® resin. The denatured protein was subsequently used to immunize a mouse.

5.5.1. Production of recombinant proteins using pET vectors

Target genes are cloned in pET plasmids (*Novagen*) under control of strong bacteriophage T7 transcription signal. Expression is induced in host cells (*E.coli* BL21) containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control, and expression is induced by the addition of IPTG, a non-metabolizable analogue of galactose.

1 ml of an overnight culture of *E. coli* BL21 (DE3) strain transformed with HIC1 for immunization/pET28b expression plasmid was inoculated to 1 liter of LB medium with kanamycin and incubated while shaking at 37°C for another 3–4 hr, until the culture had an absorbance of ~0.8 OD_{600}. 1 ml sample of the culture was removed, centrifuged at 13,000 x g for 1 min at 4°C, and the cell pellet stored at –20°C for electrophoretic analysis.

Expression of HIC1 protein was induced by adding 1mM IPTG (*Alexie Corporation*) and incubation continued for another 2.5 hr at 25°C, shaking. 1 ml sample of the culture after induction was removed, centrifuged at 13,000 x g for 1 min at 4°C, and the cell pellet stored at –20°C for electrophoretic analysis.

The bacterial culture was centrifuged at 4,000 x g for 15 min at 4°C and the cell pellet was resuspended in 35ml of denaturing lysis buffer (8M urea, 20mM Hepes pH 7.9, 300mM NaCl, 0.1% Tween 20, 5% glycerol) supplemented with 1mM PMSF to inhibit proteases and sonicated using Branson Sonifier® Cell Disrupter 18x 10 s pulses. Finally, the lysate was centrifuged (7,000 x g at 4°C for 15 min) and the supernatant was applied to TALON® Resin.

5.5.2. Purification of recombinant protein using TALON® Resin

TALON™ Superflow™ Metal Affinity Resin (*Clontech*) is cobalt-based IMAC resin designed to purify recombinant polyhistidine-tagged proteins. Under conditions of physiological pH, histidine binds by sharing electron density of the imidazole nitrogen with the electron-deficient

orbitals of transition metals. Elution occurs when the imidazole nitrogen (pKa of 5.97) is protonated (in low pH) generating a positively charged ammonium ion, which is repelled by the positively charged metal atom. Alternatively, the bound polyhistidine-tagged protein can be competitively eluted by adding imidazole to the elution buffer, because imidazole is identical to the histidine side chain.

We processed the lysate according to manufacturer's instructions: http://www.clontech.com/clontech/techinfo/manuals/pdf/pt3856-1.pdf.

HIC1 fusion protein was eluted by 150 mM imidazole in 8 fractions that equalled bed volume of the resin (200 µl). These fractions were subjected to SDS PAGE analysis and those containing majority of protein were dialysed against PBS with 6M urea to lower the molarity of urea and to remove imidazole. Finally the protein was concentrated to ~ 2 mg/ml using Microcon Centrifugal Devise (*Amicon*).

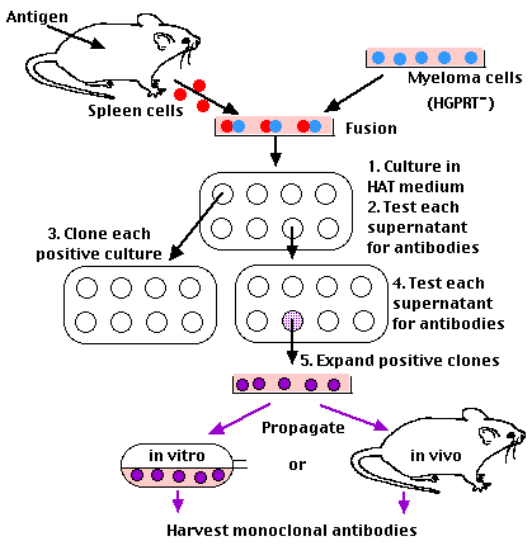
5.5.3. Immunization with recombinant HIC1 protein

Immunization of mouse was conducted according to standard intrasplenic immunization protocol.

5.5.4. Monoclonal antibodies production

Hybridomas producing HIC1 specific antibodies were obtained by Simona Benešová according to standard protocol. The procedure is briefly reviewed in Fig. 17.

Figure. 17. Monoclonal antibodies production
http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/M/Monoclonals.html



5.5.5. Testing of hybridomas

Each of approximately 1500 hybridomas was tested on ELISA (Enzyme Linked Immunosorbent Assay).

384-well plates (*Corning*) were coated with 100 µl of the immunogen or EGFP protein (3µg/ml in 0.1M carbonate buffer pH 9.6) at 4°C O/N. The EGFP protein was produced by expression of EGFP/pET28 vector used as a negative control to sort out non-specific antibodies reacting with the His-Tag of the immunogen. hHIC1 protein was produced by expression of hHIC1 from the immuno/pET28 vector. Next morning, the immunogen was discarded and the plates were blocked with 0.2% gelatine, 1 % glycine and 0.1% NaN_3 in PBS at 37°C for 1h. The wells were washed five times with PBS and 100 µl of hybridoma supernatant was added to well containing either hHIC1 or EGFP. After 1h incubation at RT the wells were washed five times with PBS. The secondary antibody, GAM-HRP (*Bio-rad*), diluted 1000 x in DMEM was applied for 45min at RT. Finally I added 100 µl of a substrate solution. Substrate solution consisted of 1/10 volume of 50mg ABTS (Azino-bis/3-ethylbenzthiazoline-6-sulphonic acid)/5ml H_2O, 9/10 volume of Mc Ilvains buffer (4.68 ml 0.2M Na_2HPO_4, 5.32 ml 0.1 M citric acid, pH 4.6) and 15 µl H_2O_2 /ml. Dark green colour developed in few minutes. The clones positive for hHIC1 and negative for EGFP were further subcloned.

Subcloned hybridomas were tested again on ELISA to verify their monoclonality. The most promising clones were than tested by immunocytochemistry (chapter 5.8.4.) and Western blots (chapter 5.8.2.).

5.5.6. Monoclonal antibodies purification

500 ml of supernatant produced by hybridomas was concentrated using 100 kDa cut-off membrane (*Vivascience*) to 50ml. IgG isotopes of antibodies (clones 1C5 and 4F8) were subjected to protein A isolation while IgM isotope (2D7 clone) was purified using differential precipitation or Mep HyperCel™.

5.5.6.1. IgG isolation using ProSep® Ultra Protein A column

Protein A, a surface protein *Staphylococcus aureus,* binds the Fc region of immunoglobulins through interaction with the heavy chain.

The concentrated supernatant was equilibrated with 1/10 volume of 10x binding buffer (0.5M Tris pH 8.9, 1M glycine, 1M NaCl) and supplied with NaCl powder to final concentration 2M NaCl. After spinning (3,200 x g, 15 min, RT) the supernatant was filtered through 0.45 µm filter. This mixture was applied to column (0.5 ml/min) packed with 5 ml ProSep® Ultra Protein A

(*Millipore*) that was equilibrated with three column volumes of 1x binding buffer with 2M NaCl loaded at 2ml/min using a peristaltic pump.

The unbound IgG was washed out with five column volumes of PBS with 2M NaCl. The antibody was eluted with 0.1M citrate pH 4.0 directly into 1/5 volume of 1M carbonate buffer pH 9.4. If the antibody was not completely eluted (assessed by measurement at A_{280nm}), second wash with 0.1M glycine pH 2.5 was employed. After dialysation against PBS (2 changes in 24h) the antibody concentration was assessed.

5.5.6.2. IgM isolation using Mep HyperCel™

MEP HyperCel® Hydrophobic Charge Induction Chromatography Sorbent (*Pall Life Sciences*) was used to purify IgM monoclonal HIC1 antibodies. This purification method is designed to purify IgG antibodies, but it works for IgM as well. I followed the manufacturer's instructions that are available at http://www.pall.com/variants/pdf/pdf/35677.pdf

Antibodies bind to this resin under physiological pH (in PBS) and were eluted step wise with 0.1M citrate pH 6, 0.1M citrate pH 5, 0.1M citrate pH 4 and finally 0.1M citrate pH 3 directly to ½ volume 2M Tris pH 8.8. The fractions were combined and dialysed against PBS for 24h at 4°C.

5.5.6.3. IgM purification using differential precipitation

The concentrated supernatant from 2D7 clone was dialysed against distilled water (two changes in 24h) and centrifuged (16,000 x g, 4°C, and 15 min). The pellet was washed with distilled water and then let freely resolve in PBS. The concentration of dissolved antibodies was assessed by spectrophotometry and further verified on SDS-PAGE.

Alternatively, the concentrated supernatant was directly used for immunostaining.

5.5.7. Monoclonal antibodies testing

Purified monoclonal antibodies from clones 1C5, 2D7 and 4F8 were subjected to tests to detect over-expressed and endogenous HIC1 protein using immunocytochemistry, immunofluorescence, immunoprecipitation and Western blotting.

5.6. Purification of polyclonal antibodies

Several purification methods were employed to isolate HIC1 antibodies from rabbit serum. The immunization of the rabbit was done by hHIC1 peptide (aa 223-408) equivalent to the immunogen I used for HIC1 monoclonal antibodies production.

5.6.1. Purification on protein A

Four millilitres of Protein A Sepharose™ CL-4B (*Amresham Bioscience*) in column were washed with 10ml of binding buffer (5M NaCl, 0.1M glycine, pH 8.9). 5 ml of rabbit sera diluted in 10ml of binding buffer was poured onto the column. Following washing with 20ml of binding buffer antibodies were eluted step wise with 1.5 ml of 0.1M citrate pH 6, 0.1M citrate pH 5, 0.1M citrate pH 4 and finally 0.1M citrate pH 3 to 1.5 ml 2M Tris pH 8.8. The fractions were combined and dialysed against PBS for 24h at 4°C.

5.6.2. Purification of antibodies on immobilized antigen

To purify specific antibodies from rabbit serum we adopted protocol by Bar-Pelet and Raikhel (Bar-Peled and Raikhel, 1996)

Purification of recombinant protein using Glutathione Sepharose™ 4B

The glutathione *S*-transferase (GST) catalyses the conjugation of reduced glutathione to electrophilic centres on a wide variety of substrates. This activity is useful in the detoxification of endogenous compounds. In molecular biology "GST gene fusion system" is used to purify and detect proteins of interest. In our lab we use pET42 vectors to create GST fusion proteins, where the GST part binds its substrate glutathione immobilized on Glutathione Sepharose™ 4B *(GE Health care)*.

HIC1-GST fusion protein was produced in BL21 (DE3) bacterial strain from HIC1 for immunization/pET42b along with GST protein from empty pET42b vector as described in chapter 5. 6. 1. 50ml of lysate (in 20mM Hepes pH 7.9, 300mM NaCl, 0.1% Tween 20, and 5% glycerol) of 1L of LB culture was applied to 1 ml Glutathione Sepharose™ 4B prewashed with lysis buffer. Sepharose beads were then washed five times with the lysis buffer to remove contaminating bacterial proteins. Such beads were utilized for antibody purification according to Bar-Pelet and Raikhel.

In short, GST and GST-HIC1 proteins were covalently crosslinked to the Sepharose beads with DMP solution (15.5 mg dimethyl pimelimidate-HCl in 2 ml of 0.2M triethanolamine pH 8.3). The crosslinking reaction was terminated by 0.2M ethanolamine pH 8.2. Crude rabbit serum was precleared on Sepharose beads with GST protein (4h incubation with rotation, 4°C) and then applied to GST-HIC1 protein crosslinked to Sepharose resin (O/N incubation with rotation, 4°C). Unbound antibodies were washed away using TBS and specific antibodies were eluted step wise with 0.1 M glycine pH 2.5, 0.1 M glycine pH 11.5 and 0.1 M glycine pH 12.5, 0.02% CHAPS and fraction were immediately adjusted to pH ~7 with 2M Tris. Finally, the eluted fractions were dialyzed for 24h against PBS at 4°C, and then stored in 50% glycerol in PBS, 0.02% sodium azide at -70°C.

5. 6. 3. Purification of antibodies using Melon™ Gel

ImmunoPure® Melon™ Gel IgG Purification Kit (*Pierce*) was used according to the manufacturer's instructions at http://www.technochemical.com/instruction/1512as4.pdf
In this system, the resin binds non-antibody serum proteins using a physiological pH allowing the antibody to flow through in a mild buffer suitable for storage and further applications.

5. 7. Quantitative Real time PCR

Real-time PCR is a powerful tool for gene expression studies. In qRT PCR (quantitative Real Time/Reverse Transcription PCR) techniques, signals (generally fluorescent) are monitored as they are generated and are tracked after they rise above background but before the reaction reaches a plateau. The time point when the fluorescent signal reaches a threshold level during amplification corresponds to the amount of original target sequence, thereby enabling quantification. In addition, the final product can be further characterized by subjecting it to increasing temperatures to determine when the double-stranded product "melts." This melting point is a unique feature dependent on product length and nucleotide composition and is used to verify specificity of the product (Kubista et al., 2006).

The most popular dye for this mode of qRT PCR is SYBR® Green. The SYBR® Green dye is added during the PCR, and binds to the minor groove of double-stranded DNA. The signal increases as the amount of double-stranded DNA elevates.

Several variables need to be controlled in gene-expression analyses, such as amount of starting material or overall differences in overall transcriptional activity. Internal controls, often referred to as housekeeping genes, are commonly used to normalize the mRNA fraction. These housekeeping genes should not vary in the cells or tissues under investigation, or in response to experimental treatment. However, the literature shows that the housekeeping gene expression can vary considerably and that ideal and universal housekeeping genes do not exist (Vandesompele et al., 2002). To overcome this drawback, I included several housekeeping genes (listed in Tab. 6.) in each experiment and normalized the data to all of them.

Table 6. Housekeeping genes for qRT PCR

Symbol	Name	Function
β-actin	Beta actin	Cytoskeletal structural protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis
SDHA	Succinate dehydrogenase complex, subunit A	Electron transporter in TCA cycle and respiratory chain
β2m	Beta-2-microglobulin	Beta-chain of MHC class I molecules
UBC	Ubiquitin C	Protein degradation
HPRT	Hypoxantinephosphoribosyl transferase I	Purine synthesis in salvage pathway

5.7.1. RNA isolation

For RNA isolation from both cell cultures and whole organs I utilized Trizol® reagent (*Invitrogen*) or very similar RNA blue reagent (*Top-Bio*). The reagents, mono-phasic solutions of phenol and guanidine isothiocyanate, improve the single-step RNA isolation method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). During sample homogenization or lysis, these reagents maintain the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol.

Mouse organs were homogenized using Ultra-Turrax T8 tissue homogenizator *(IKA Labortechnik)*.

I followed the manufacturer's instructions for TRIZOL:
http://www.invitrogen.com/content/sfs/productnotes/F_Trizol%20and%20Trizol%20LS-041018-RD-TL-HL0506021.pdf

or for RNA blue: http://www.top-bio.cz/htm/RNA_Blue-TI.asp

The isolated RNA was further purified using Mini RNA Isolation II™ (*Zymo Research*) according to the protocol provided by manufacturer at
http://www.zymoresearch.com/protocols/rna/mini_rna_2_kit.asp.

5.7.2. Reverse transcription

1 μg of pure total RNA (concentration determined spectrophotometrically) was used for reverse transcription to derivate cDNA. I employed SuperScript™ II RNase H – Reverse Transcriptase (*Invitrogen*) according to the manufacturer's instruction published at http://www.invitrogen.com/content/sfs/manuals/superscriptII_pps.pdf.

Alternatively I used RevertAid™ H Minus First Strand cDNA Synthesis Kit (*Fermentas*) as described by manufacturer at the website:

http://www.fermentas.com/profiles/kits/pdf/revertaidhminus1631.pdf

The reactions were primed using random hexamers (250 ng; *Invitrogen*). Each sample was processed in duplicate, one of which contained the reverse transcriptase. The other sample lacking the enzyme served as a negative control for genomic DNA contamination.

5.7.3. qRT PCR reaction

PCR reactions were performed using The LightCycler ® 480 Real-Time PCR System (*Roche*), that comprises reagents and disposables, instrument and software features.

PCR reaction mix	5 µl total volume
LightCycler® 480 SYBR Green I Master (2x concentrated)	2.5 µl
F and R primer mix (5 mM each)	0.5 µl
cDNA (10x diluted after RT)	1 µl
d H_20	1 µl

Table. 7. Primers for qRT PCR

Forward primer	5'->3' sequence	Reverse primer	5'->3' sequence
Primers for mouse cDNA			
FmBetaActin#332#real1	GATCTGGCACCACACCTTCT	RmBetaActin#469#real1	GGGGTGTTGAAGGTCTCAAA
FmGAPDH#539#real1	AACTTTGGCATTGTGGAAGG	RmGAPDH#607#real1	ATCCACAGTCTTCTGGGTGG
FmSDHA#1484#real1	AAGGCAAATGCTGGAGAAGA	RmSDHA#1596#real1	TGGTTCTGCATCGACTTCTG
FmUbb#857#real1	ATGTGAAGGCCAAGATCCAG	RmUbb#1016#real1	TAATAGCCACCCCTCAGACG
FmHIC1#1779#real2	CAACCTGTACGTGTGCATCC	RmHIC1#1844#real2	ACGTGTGCATTCAGCTGTTC
FmOct4#440 real1	GAGGAGTCCCAGGACATG	RmOct4#593 real1	AGATGGTGGTCTGGCTGAA
FmSox2#1903 real1	AAGGGTTCTTGCTGGGTTT	RmSox2#2052 real1	AGACCACGAAAACGGTCTT
FmNanog#570 real1	AAGTACCTCAGCCTCCAG	RmNanog#732 real1	GTGCTGAGCCCTTCTGAAT
Primers for human cDNA			
FhBetaActin#260#real1	GGCATCCTCACCCTGAAGTA	RhBetaActin#341#real1	AGGTGTGGTGCCAGATTTTC
FhGAPDH#1198#real	CACCACACTGAATCTCCCCT	RhGAPDH#1252#real	CCCCTCTTCAAGGGGTCTAC
FhSDHA#2146#real	AGATTGGCACCTAGTGGC	RhSDHA#2214#real	ACAAAGGTAAGTGCCACG
FhUbb#54#real1	GCTTTGTTGGGTGAGCTTG	RhUbb#154#real1	TCACGAAGATCTGCATTTT
FhCtBP2#2168#real	TTGCCTGTGGTAGACACCT	RhCtBP2#2250#real	CAGTTCAGATGTGAGAGGC
FhAxin-1#2966#real	CCTGTGGTCTACCCGTGTCT	RhAxin-1#3068#real	GCTATGAGGAGTGGTCCAGG
FhHPRT1#888#real	AAATGTCAGTTGCTGCATTCC	RhHPRT1#974#real	GTTAAACAACAATCCGCCCA
FhHIC1#real1	CGACGACTACAAGAGCAGCA	RhHIC1#real1	TGCACACGTACAGGTTGTCA
FhHIC1#est295 real2	TACCACCAGGTCTCCCACTC	RhHIC1#est398 real2	CTAAAGAGACCGCGTTCCAG
FhIL2Ralpha#608#real1	ATCAGTGCGTCCAGGGATAC	RhIL2Ralpha#804#real1	GACGAGGCAGGAAGTCTCAC
FhIL2Ralpha#1837#real2	TGGACACACAAGGTGCAAAT	RhIL2Ralpha#1975#real2	TGTGACCTCCATCCCTTCTC
FhSIRT1#1946#real1	GCAGATTAGTAGGCGGCTTG	RhSIRT1#2018#real1	AGCGCCATGGAAAATGTAAC
FhSIRT1#1006#real2	CTGGACAATTCCAGCCATCT	RhSIRT1#1247#real2	GCACCTAGGACATCGAGGAA

All samples were run in triplicates and a negative controls for the genomic DNA and primer contamination („-RT"samples) were run in duplicates. 384 samples in multiwell plates can be analysed in each run using LightCycler ® 480.

qRT PCR reaction

1. programme: Initial denaturation	95°C		5 min	
2. programme: Denaturation		95°C	20 s	
Primer annealing	61°C		20s	45 cycles
Elongation	72°C		30s	
3. programme: Melting curve		55-95°C		

The data were processed using The LightCycler® 480 software. Abs Quant/2nd derivate max formula was used to calculate Cp value. Meeting curve was called for all samples to check the specificity of the PCR product. Finally the basic statistics was calculated using Microsoft Excel.

5.8. Protein detection

5.8.1. SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) is used to separate proteins according to their electrophoretic mobility that is a function of molecular weight as well as higher order protein folding, posttranslational modifications and other factors.

Samples were prepared in 2x reducing Laemmli buffer [2R; (50 mM Tris.HCl pH 6.8, 20 % glycerol, 4 % SDS, 0.02% bromophenol blue, 0.5 % DTT)] and boiled for 5 min at 95°C just prior to loading onto polyacrylamide gel.

SDS PAGE was run according to a standard protocol (Sambrook et al., 1989), chapters 18.47-18.59).

Chemicals and equipment used:

- Standards (*Bio-Rad Laboratories, Cell Signalling, Fermentas*)
- To prepare resolving and stacking gels: 30% acrylamide (0.8% bisacrylamide); 1.5M Tris.HCl pH 8.8; 0.5M Tris.HCl, pH 6.8; 10% SDS; 10% APS; TEMED; n-butanol in aqueous solution
- Glass plates, combs, spacers
- SDS-PAGE *Mighty Small* (*Hoefer*)
- SDS-electrode buffer (250 mM glycine, 10 mM Tris.HCl, 0.1% SDS, pH 8.3)

After the electrophoresis the gel was subjected to either staining or to Western blotting (chapter 5. 9. 2.) .

0.05% solution of CBB-R250 (Coomassie Brilliant Blue - R250) was employed for polyacrylamide gels staining. These were incubated in CBB for at least 30 min. Subsequently the gel was destained in destaining solution (25% ethanol, 10% acetic acid) until only blue protein bands corresponding were left. Such a gel was then incubated in drying solution (25% ethanol, 2% glycerol) for at least 2h before it was vacuum-dried and stored.

5. 8. 2. Western blot

Western blot is a technique used to identify and locate proteins based on their ability to bind specific antibodies.

We followed a standard protocol for Western Blot (Sambrook et al., 1989), chapters 18.60-18.75 and utilized the following:

- Transblot (*Bio-rad)* (1.95mA, 15V, 30 min)
- Transferring buffer (48 mM Tris.HCl, 39 mM glycine, 20% methanol)
- Nitrocellulose membrane *PROTRAN* (*Schleicher&Schuell*)

After blotting the membrane was blocked with a generic protein (5% dried milk in PBS + 0.05% Tween 20) to bind to any remaining sticky places on the nitrocellulose. Then it was incubated with a primary antibody diluted with blocking buffer (1 □g/ml in 1% dried milk, PBS + 0.05% Tween 20) or a hybridoma supernatant. After washing unbound antibodies with PBS + 0.05% Tween 20 the secondary antibody was added (5,000 x diluted GAM-HRP (Goat-anti-mouse conjugated with horseradish peroxidase) or GAR-HRP (Goat-anti-rabbit conjugated with horseradish peroxidase) (*Bio-Rad*) in 1% dried milk, PBS + 0.05% Tween 20). Following washing the unbound secondary antibody with PBS + 0.05% Tween 20 the membrane was dried and incubated for 1 min in ECL (Enhanced Chemiluminiscence) solution freshly prepared by mixing ECL1 [2,5mM luminol (5-amino-2,3-dihydro-1,4-ftalazindion) in 100 mM Tris.HCl, pH 8.8] a ECL2 (5.4 mM H_2O_2 in 100mM Tris.HCl, pH 8.8) in ratio 1:1. Chemiluminiscence was visualized by using X-OMAT AS film (*Kodak*).

5.8.3. Protein lysates

Several approaches were employed to obtain whole cell or nuclear lysates of eukaryotic cells.

5.8.3.1. Whole cell lysates

Cell lysis using Laemmli buffer

This type of lysis was applied to all bacterial samples unless specified elsewhere. Twice concentrated reducing Laemmli buffer [2R; (50 mM Tris.HCl pH 6.8, 20 % glycerol, 4 % SDS, 0,02% bromophenol blue, 0.5 % DTT)] was added to bacterial pellet which was then homogenized by sonication (3 x 15 s, 70 V) and boiled at 95°C for 5 min.

Eukaryotic cell plates were placed on ice, washed with PBS and scraped with a cell scraper to a 15ml Falcon tube and after washing with PBS centrifugation (500 x g, 5 min, and 4°C) the cells were transferred to 1.5 ml Beckman ultracentrifugation tube and lysed in appropriate volume of 2R buffer and boiled 5min at 95°C. Genomic DNA was removed by ultracentrifugation (120,000 x g, 1h, and 15°C) and supernatant containing proteins was stored until further use.

High salt lysis

Hypertonic buffer was used in order to coisolate nuclear proteins as they easily detach from nucleus in high salt environment.

Cells were pelleted in PBS (500 x g, 5 min, and 4°C). About 6 volumes of lysis buffer (20 mM Tris pH 7.5, 200 mM NaCl, 0.5% Triton X-100, and 5 mM EDTA) were added and the suspension was incubated 30min on ice with occasional pipetting up and down. Membranes and DNA were removed by centrifugation (16,000 x g, 5 min, and 4 °C) and the supernatant was stored for further use.

5.8.3.2. Nuclear lysates

Nuclear Laemmli buffer lysate

The cytoplasm of scraped cells was dissolved using Cytoplasm lysis buffer (20 mM Tris pH 7.5, 80 mM NaCl, 1.5% Triton X-100, 5 mM EDTA) while rotated at 4°C for 30 min. Pelleted nuclei (5,000 x g, 2 min, 2 °C) were lysed in appropriate volume of 2R buffer and boiled 5min at 95°C.

STKM lysate

Cells were washed with PBS, scraped and spun down (500 x g, 5 min, and 4°C). Pellet (up to 100 µl) was resuspended in 500 µl STKM A buffer (40 mM Tris HCl pH 7.5, 37 mM KCl, 12 mM $MgCl_2$, 30% sucrose, 0.5 mM DTT and 1 mM PMSF). Then 500 µl of STKM buffer B (40 mM Tris HCl pH 7.5, 37 mM KCl, 12 mM MgCl2, 30% sucrose, 0.8% Triton X-100, 0.5 mM DTT and 1 mM PMSF) was added and put cells on ice. The nuclei were spun down (5,000 x g, 2 min, and 2 °C) and washed twice with STKM buffer A. Subsequently the nuclei were resuspended in 2.5 pellet volume of extraction buffer (10 mM Hepes/KOH pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EGTA, 20% glycerol, 0.5 mM DTT and 1 mM PMSF) and incubated 30min on ice with occasional pipetting up and down. Finally the suspension was spun at maximum speed (16,000 x g, 5 min, 5 °C) and the supernatant (= nuclear extract) was transferred into new tubes and stored at -70°C.

Dignam lysate

This is a modified version of a protocol originally written by *Lee et al., 1988.*

Cells in culture plates were placed on ice, washed with ice-cold PBS, scraped in ice-cold PBS to 15ml Falcon tubes, pelleted (500 x g, 5 min, 2 °C) and resuspended in ~3 volumes of Dignam A buffer [10 mM HEPES, pH 8.2, 1.5 mM $MgCl_2$, 10 mM KCl and protease inhibitor Complete™ (*Roche*)]. Following 15 min incubation on ice, cells were lysed by plunging 8 times through a 25xG needle. The nuclei were pelleted (5,000 x g, 2 min, 2 °C), resuspended in ~1,5 volume of Dignam C buffer (20 mM HEPES, pH 8.2, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA pH 8.0, 25% glycerol and protease inhibitor Complete™ (*Roche*)] and rotated at 4 °C for 30 min. The mixture was centrifuged to pellet membranes and chromatin residues (16,000 x g, 5 min, 5 °C) and the supernatant was transferred into new tubes and stored at -70°C.

5.8.4. Determining protein concentration

Several methods are commonly used for determination of protein concentration. Measurement of the UV absorbance at 280 nm is most useful for pure protein solutions while Bradford assay methods is routinely used during protein purification and protein lysate preparation.

Measurement of OD at A_{280}

Determination of protein concentration by ultraviolet absorption at 280 nm depends on the presence of aromatic amino acids in proteins. When the sequence of protein is known, extinction coefficient E_{280nm} can be calculated. I use Protparam Tool found at http://www.expasy.org/tools/protparam.html.

Protein concentration is then calculated using equation:

$$[\text{Protein}]\ (\text{mg/ml}) = A_{280nm} / [E_{280nm} \times (\text{cuvette path length in cm})]$$

Bradford assay

The Coomassie Brilliant Blue G-250 dye binds selectively to positively charged residues (Lys, Arg, His) and aromatic residues (Phe, Tyr, Trp) and this binding is accompanied by a shift in absorbance maximum from 465nm to 595nm.

The Bradford reagent (?) was diluted 1:4 with water and added to samples. The A_{595nm} of sample was compared to the A_{595nm} of standards to assess the protein concentration.

5.8.5. Immunocytochemistry

This assay was employed to test HIC1 monoclonal antibodies of capability to detect over-expressed HIC1 protein.

COS-7 cells in 96-well plate were PEI-transfected with hHIC1/pEGFP-N3 (chapter 5. 3. 2. 3.). Transfection efficiency was checked using fluorescent microscope (EGFP fused protein). The wells were washed twice with PBS and fixed with ice-cold methanol for 20 min. The fixation agent was washed away with PBS and the hybridoma supernatant or diluted antibody was applied for 1h at RT. Subsequently, the wells were washed with PBS and then incubated for 45 min with GAM-HRP (or GAR-HRP for rabbit polyclonal antibodies that were used as positive controls) diluted 1,000 x in DMEM. The secondary antibody was visualized by AEC (3-amino-9-ethyl-carbazol). AEC solution was prepared by dissolving 6 mg AEC in 2 ml DMF (dimethyl foramide), 5mM NaAc buffer pH 5.0 was added to final volume of 10 ml. This solution was filtered using 0.22 μm filter and 100 μl supplied with 5 μl 3% H_2O_2 was added to each well. Brown colour developed in 5-10 min in dark and the reaction was stopped by replacing the AEC solution with distilled water. Wells positive for HIC1 staining were visualized under a microscope.

5.8.6. Immunofluorescence

The capability of the monoclonal antibodies to detect endogenous protein was verified using immunofluorescence microscopy.

HIC1 transfected HeLa cells (chapter 5. 3. 2. 2.) or WI38 primary cells cultured on coverslips in 12-well plates were washed with PBS and fixed. I used two types of fixation. (1) Formaldehyde fixation: Cells were fixed with 4% formaldehyde in PBS for 10 min at RT and permeabilized with 0.1% Triton X-100 in PBS for 20 min at 4°C.

(2) Methanol/Acetone fixation: Alternatively the cells were fixed with ice-cold methanol for 10 min at -20°C and then immersed for a few seconds in ice-cold acetone.

After washing with PBS, the cells were blocked for 1h with 1% BSA in PBS. Primary antibody diluted in 1% BSA in PBS was applied to coverslip for 1h at RT or overnight at 4°C. The unbound antibody was washed away with PBS and secondary antibody conjugated to a fluorochrome diluted 500x in 1% BSA in PBS was added. The incubation took 45 min and was performed in dark and at RT. The solution was decanted and DAPI solution (4,6-diamidino-2-phenylindole, 8,000 x diluted in PBS) was applied for 1min. Coverslips were washed four times with PBS and mounted with inverted 10 µl of Mowiol (*Calbiochem*) dropped on a slide. Alternatively to DAPI staining, the nuclei were counterstained by DRAQ5™ (*Alexis Biochemicals*) dissolved in Mowiol used for mounting. The edges of each coverslip were sealed with transparent nail polish and allowed to dry. The coverslips were then viewed under a confocal laser scanning microscope SP5 (*Leica*). All images were scanned separately in the "sequential scanning mode" for the green, red and blue channels using a 100x/1.40 oil-immersion objective. The ratio of colocalization was quantified by measuring the overlap in the fluorescence intensities of corresponding channels along selected profiles using Leica confocal software. Image files were processed with CorelDRAW Graphics suite.

5.8.7. Immunoprecipitation and co-immunoprecipitation

Immunoprecipitation and co-immunoprecipitation experiments were performed with nuclear Dignam lysates (chapter 5. 8. 3. 2.) of DLD-1/HIC1 cells and WI38.

The nuclear lysates were diluted in 2 volumes of Dignam A up to at least 350 µl for one IP (or Co-IP) reaction. Sepharose beads with bound antibody for each reaction were prepared by incubating 15 µl of prewashed (mixed Dignam A+C, ratio 2:1) Protein A/G PLUS-Agarose (*Santa Cruz*) with 10 µg of antibody for 2h in mixed Dignam A+C (ratio 2:1) at 4°C with rotation. The unbound antibody was washed away with mixed Dignam A+C (ratio 2:1), the sepharose beads were always centrifuged at 2 °C, 700 x g, for 3 min. The diluted nuclear lysates were added to the washed beads and rotated on rotation wheel at 4 °C overnight. The next day the mixtures were centrifuged and 20 µl of the supernatants were boiled in 20 µl of 2R SDS-PAGE sample buffer and

used as flow-through control. The beads with bound protein complexes were resuspended in 500 µl of mixed Dignam A+C (ratio 2:1), centrifuged and supernatants discarded. This washing step was repeated three times. 40 µl of 2R SDS-PAGE sample buffer was added to the washed beads and incubated in a shaker for 15 min at 37 °C and then 5 min at 70 °C. The mixture was filtered through a column (a tube cap cut with a scalpel and pipetman tip with a filter pitched into a hole) using centrifugation (16,000 x g, 1 min). Flow-through (IP or Co-IP) was transferred into new tubes, boiled at 95°C and used for SDS-PAGE analysis.

5. 9. Luciferase assays

Gene expression in transfected eukaryotic cells is generally studied by linking a promoter sequence to an easily detectable "reporter" gene such as that encoding firefly luciferase. The activity of reporter enzyme is dependent on its amount which in turn depends on the transcriptional activity of the promoter. The enzymatic reaction is then started by adding the luciferase assay reagent which contains all the components required for starting the chemiluminescence reaction. Photon emission is quantified using a luminometer.

Luciferase reporter tests were done to assess the ability of full-length and mutant forms of hHIC1 to repress the transcription activity of HIC1-responsive promoters. Two luciferase reporters were used.

(1) 5x HIRE SV40-Luc reporter is a gift from Dominique Leprince and is fully referenced in (Pinte et al., 2004). This luciferase reporter contains five HIC1 responsive element sites in the pGL3-promoter vector (*Promega*) upstream of the luciferase gene driven by an SV40 promoter.

(2) pGL3 basic-Sirt1 promoter construct (see chapter 5. 4. 2. 2.) represents an endogenous situation, as Sirt1 is a target gene for HIC1.

SV40-Rl construct (*Promega*) that codes for *Renilla* luciferase was used in all transfection experiments as internal control. Normalizing the activity of the experimental reporter to the activity of this internal control minimizes experimental variability relevant to differences in cell viability or transfection efficiency caused by different cell type, cell cycle phase and the quality of particular plasmid.

SV40-R1 vector as well as substrates for firefly and *Renilla* luciferase and lysis buffer is included in the Dual-Luciferase® Reporter Assay System (*Promega*).

For the experiment itself, cells were transfected with 1.6 µg DNA/well in 12well plate. Each transfection mixture contained 1.3 µg of HIC1, 0.25 µg of reporter and 0.05 µg of SV40-R1 and was repeated in triplicate. After 24h cells were lysed in 100 µl Passive lysis buffer and 10 µl of the lysate was applied to luminometer (*Berthold Detection Systems*) where it was automatically

combined with 50 µl of the substrate. The light emission was recorded by Sirius F12 software (*Berthold Detection Systems*).

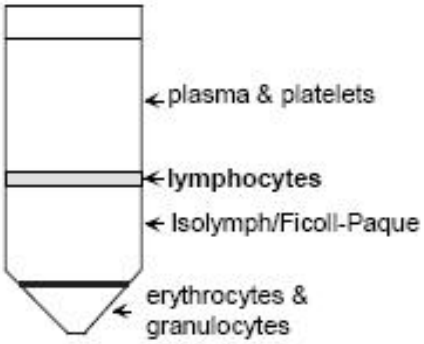
5. 10. Preparation and activation of primary human T lymphocytes

T- lymphocytes were prepared from buffy coats (leukopacks, 30 ml leukocytes enriched from 450 ml whole blood). We obtain pathogen-negative leukopacks from the blood bank located at The Institute of Haematology and Blood Transfusion (Prague).

5. 10. 1. Isolation of PBL using Ficoll gradient

The procedure was performed under sterile conditions. Content of leukopack was transferred to two 50 ml conical tubes (~15 ml/tube) and tepid PBS was added to adjust volume to 35 ml/tube. A separate set of two 50 ml conical tubes with 15 ml Ficoll-Paque PLUS (*BD Biosciences*) per tube was prepared. Diluted blood was poured gently on a top of the Ficoll-Paque to avoid layer mixing. The tubes were centrifuged at 900 x g for 30 min at 20°C with acceleration and deceleration set up very slow. Lymphocyte layer (see Fig. 18.) was transferred to another 50 ml conical tube (~5 ml/ tube was collected). PBS was added up to 45 ml and centrifuged at 650 x g for 10 min at RT with a very slow acceleration and deceleration.

Figure 18. PBL isolated in Ficoll gradient



Red blood cells were lysed with 10 ml RBC lysis buffer (155mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA). Pellet was gently resuspended in RBC lysis buffer, incubated 10 min at 37°C with occasional gentle mixing. Subsequently, 30 ml PBS was added and the mixture centrifuged at 800 x g for 5 min at RT. If RBCs were still visible, lysis step was repeated. Pellet was resuspended with 40 ml of PBS and centrifuged at 400 x g for 5 min at RT. Both pellets were combined in total volume of 45 ml PBS and very carefully laid onto 5ml of FBS in 50ml falcon tube. This step eliminates contamination with thrombocytes. The tube was centrifuged at 200 x g for 15 min at RT with a very slow acceleration and deceleration. The pellet was washed with 50ml of PBS and centrifuged at 200 x g for 10 min at RT. Cells were resuspended in 40ml complete RPMI medium (10% FCS, antibiotics)/ buffy coat (~ 4 x 10^6 cells/ml) and plated ~20 ml/ 150-mm plate. Then, the cells were incubated for 4 hr. at 37°C and 5% CO_2. During this step mononuclear cells adhered to plate bottom. Non-adherent cells, corresponding to PBL consisting mainly of T lymphocytes, were removed and transferred to a fresh T-flask and placed into incubator.

5. 10. 2. Activation of T lymphocytes

Activation of T cells was performed in two different ways, (1) 24h activation with CD3 antibody and (2) 3-day activation with CD3 and CD28 antibody.

(1) Culture plates were pre-coated with CD3 antibody (MEM-57 immobilized IgG2a antibody, *Exbio*) or irrelevant anti-FLAG antibody (…) at 10μg/ml in PBS for 1h at 37°C. (2) culture plates were pre-coated the same way with CD3 antibody (MEM-57, *Exbio*). Subsequently, the antibody was discarded and plates washed with PBS. PBL isolated the previous day using ficoll gradient were plated on the coated plates at concentration ~ 2 x 10^6 cells/ml and soluble anti CD28 antibody (*Exbio*) was added to the media at final concentration 2μg/ml.

Following 24h incubation with CD3 or irrelevant anti-FLAG antibody, the cells were lysed with Trizol reagent to perform qRT PCR analysis or with STKM lysis buffer to perform protein analysis using Western blotting. The remaining fractions of the cells were processed in the same way after 48h culture.

6. RESULTS

6. 1. Monoclonal antibodies to HIC1

The central part of hHIC1 was chosen for immunizing mouse to obtain HIC1 specific B-cells to be used for hybridoma production.

The recombinant protein was produced in BL21 *E.coli* strain by expression of a sequence for HIC1 cloned into the pET28b vector. Bacteria were lysed in a lysis buffer containing 8M urea and the protein was isolated by use of TALON™ resin. Protein was eluted in several fractions with 150mM imidazole (Fig. 19.). Fractions 2-5 were pooled together, dialysed against 6M urea in PBS and used for immunization.

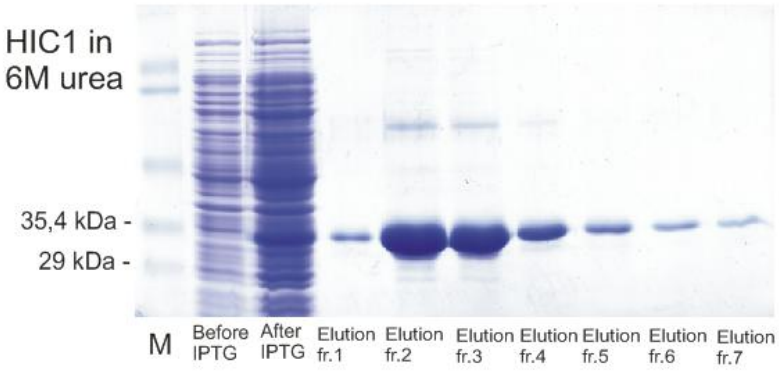


Figure 19. Isolation of the hHIC1fragment
10% PAAG stained with CCB shows 1/30 of the fractions of recombinant hHIC1 peptide eluted from TALON™ resin.
Enrichment of the hHIC1 peptide in the bacterial lysate is clearly visible after induction with IPTG.

More than 1500 hybridoma clones were screened by ELISA and thirteen of them (Tab. 8.) were subjected to further tests. ELISA with IgG and IgM class specific GAM-HRP was also performed to address the Ig class of the clone.

α HIC1 hybridomas					
Hybridoma	*ELISA*	*Subcloning*	*Ig class*	*ICC*	*WB*
1C5	**++**	**Yes**	**IgG**	**+-**	**+++**
1E 9	+	Yes	IgG	+	++
1E 12	+	Yes	IgM	+-	-
2C3	++	Yes	IgM	++	+-
2D7	**++**	**Yes**	**IgM**	**++**	**+**
2G10	++	Yes	IgM	+	NT
2H5	++	No	IgM	+	-
3B7	++	Yes	IgM	-	-
3C9	+	No	IgG	+-	-
3G3	+	Yes	IgM	+	-
4F8	**++**	**Yes**	**IgG**	**+-**	**+++**
6G1	++	No	IgM	-	-
4D11	+-	No	IgM	+	NT

Table 8. Anti HIC1 hybridoma clones
ELISA test identified thirteen positive clones that were further evaluated using immunocytochemistry (ICC) and Western blotting (WB). The evaluation ranges from +++ for very strong to – for negative, NT means not tested.
All IgG and some of IgM clones were subcloned prior to the tests.

The capability of these supernatants to detect HIC1 protein on Western blots was assessed. Laemmli buffer nuclear lysates were prepared with Laemmli buffer from HEK293 cells transfected with HIC1-EGFP, HIC-EYFP, HIC1-FLAG and EYFP-NLS constructs using PEI, and non-transfected cells. Fluorescent tag was employed for an easy evaluation of the transfection efficiency. Nuclear lysates obtained in the same way were prepared from DLD-1/HIC1 cells

activated with the dimerizer AP21967 and from WI38 primary cells. SDS-PAGE was done with 20 µl of the lysates loaded per well. After Western blotting the membranes were incubated with hybridoma supernatants. GAM-HRP was used as a secondary antibody. Rabbit polyclonal antibody purified on protein A served as a positive control and was visualised with GAR-HRP secondary antibody. Fig. 20. shows representative results from several experiments, all clones shown below are capable of detecting over-expressed HIC1 protein on WB. None of these clones cross-reacts with EYFP-NLS (Enhanced Yellow Fluorescent protein with nuclear localization signal) nor with other proteins from HEK 293 cells lysate. Unfortunately, none of these clones is capable of detecting endogenous HIC1 protein in WI38 primary fibroblasts.

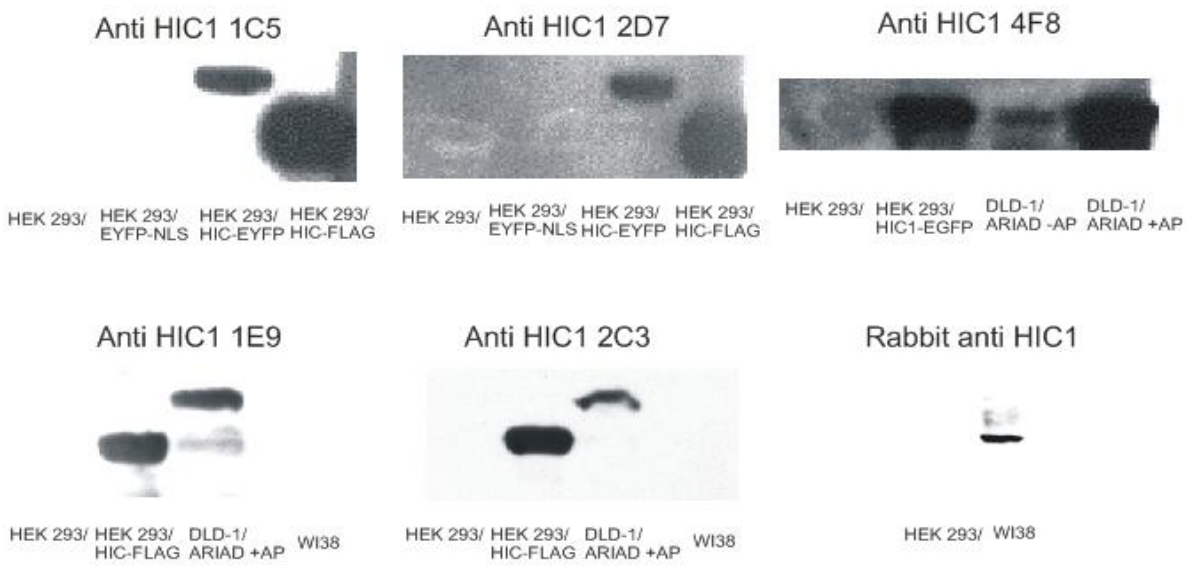


Figure 20. HIC1 hybridomas detect overexpressed HIC1 protein by Western blotting
Five hybridoma clones are capable of recognizing over-expressed HIC1 protein, but not the endogenous one. Rabbit polyclonal antibody that served as a positive control detects both over-expressed and endogenous levels of HIC1 protein.

The hybridoma supernatants were tested using immunocytochemistry on COS-7 cells transfected with HIC1-EGFP, HIC1-FLAG and EYFP-NLS constructs. Five out of eleven tested clones proved positive (summarised in Tab. 8.)

Three clones (1C5, 2D7 and 4F8) were cultured *in vitro* to obtain 1l of their supernatant. IgG antibodies (clones 1C5 and 4F8) were isolated using ProSep® Ultra Protein A column while several methods were chosen for isolation of the obtained IgM antibodies (Mep HyperCel™, differential precipitation or just concentrating the supernatant).

WB and ICC tests (Fig. 21.) were repeated with the purified antibodies with the same results acquired with hybridoma supernatants regardless on the method of their purification. Three clones were capable of detection of over-expressed protein but not the endogenous one.

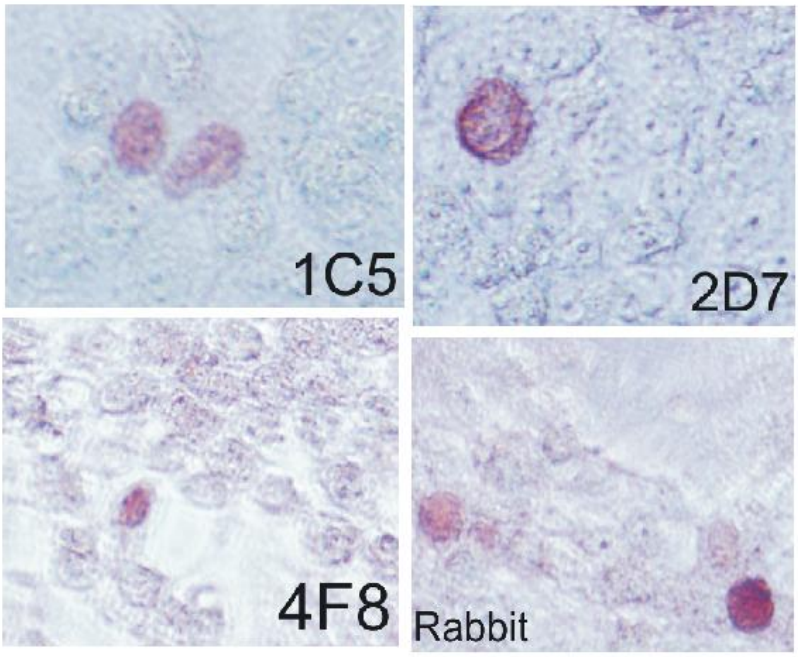
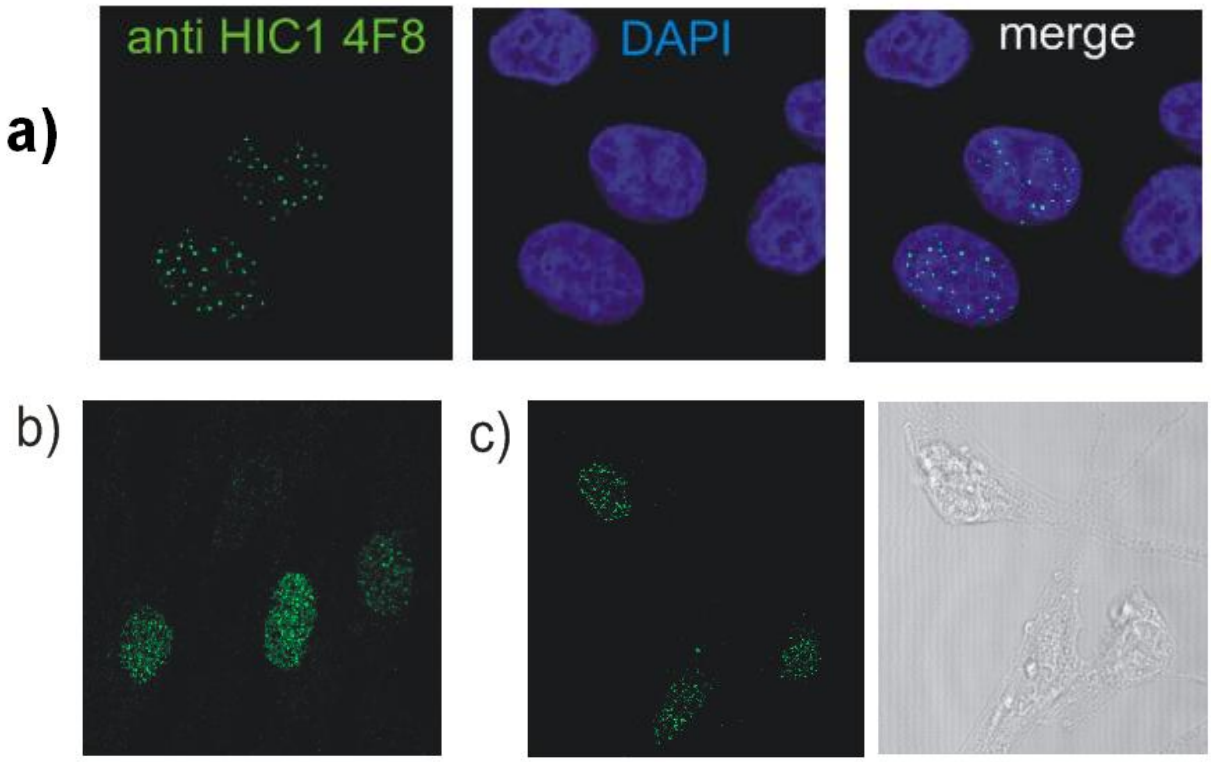


Figure 21. Nuclear staining pattern of HIC1 hybridomas in immunocytochemistry assay.
Monoclonal antibodies purified on protein A (1C5 and 4F8 500x diluted) and concentrated supernatant of clone 2D7 (20x diluted) detect over-expressed HIC1 protein in transfected COS-7 cells. Rabbit polyclonal antibody purified on the antigen (1000x diluted) served as a positive control. 1000x diluted GAM-HRP (or GAR-HRP) reacted with AEC. Magnification 100x.

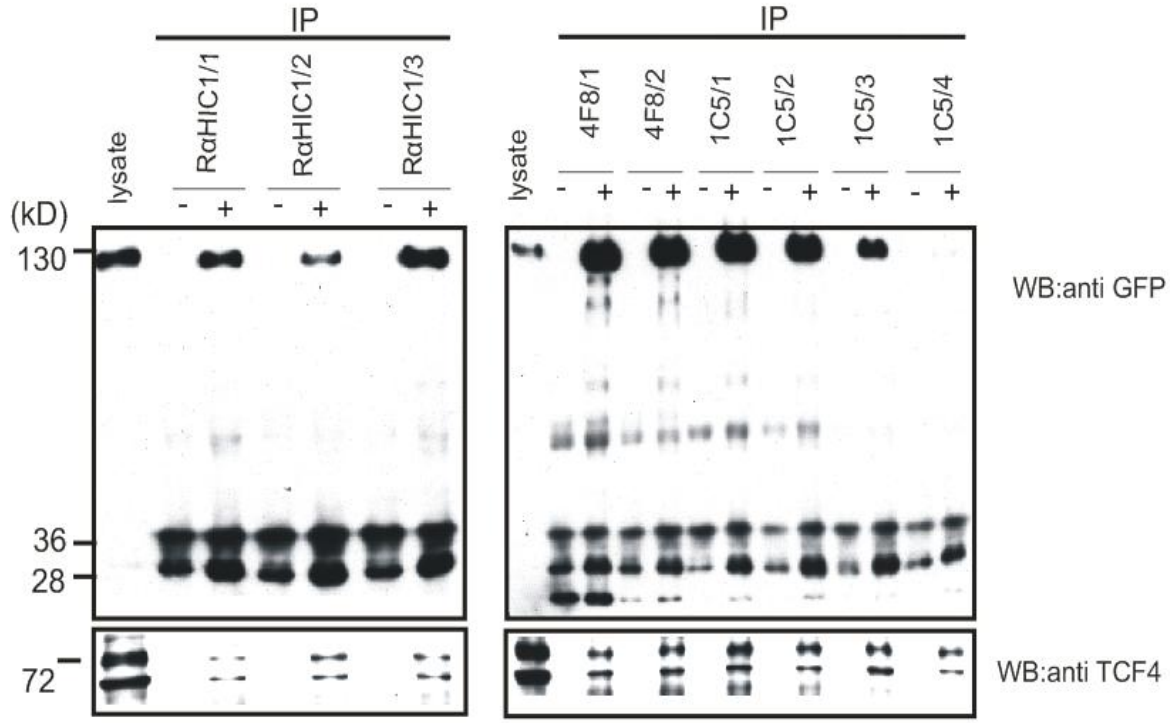
Isolated antibodies were subjected to immunofluorescent tests on the over-expressed and endogenous proteins (Fig. 22.). And again: unlike rabbit polyclonal antibodies, the antibodies from isolated clones were able to detect just an overexpressed protein, not endogenous one.

Figure 22. HIC1 bodies depicted by immunofluorescence
a) HeLa cells transfected with HIC1 were fixed using methanol/acetone fixation and stained with purified monoclonal antibodies. Staining of HIC bodies with 4F8 clone is shown, other clones looked very similarly. Secondary antibody was GAM-Alexa 488. Nuclei were counterstained with DAPI.
b) WI38 cells stained with rabbit anti HIC1polyclonal antibody purified on humam antigen.
c) WI38 cells stained with rabbit HIC1 polyclonal antibody purified on Melon™ Gel Bright field photo is also shown.



Finally, the capacity of the antibodies to immunoprecipitate HIC1 protein was tested. All three clones are capable to immunoprecipitace overexpressed HIC1 protein. Fig. 23. shows an co-immunoprecipitation experiment on DLD-1/HIC1 cells.

Figure 23. HIC1 antibodies co-immunoprecipitate TCF4 in DLD-1/HIC1 cells
Western blots stained with anti GFP antibody show an immunoprecipitation reaction with rabbit polyclonal and mouse monoclonal anti HIC1 antibodies. Rabbit serum was affinately purified on the human antigen. RαHIC/1 is an antibody fraction eluted with 0.1M glycin pH 2.5, RαHIC/2 and RαHIC/3 are antibody fractions of another purification eluted with 0.1M glycin, pH 2.5 or 0.1M glycin, pH 11.5 respectively. Monoclonal anti HIC1 antibodies (hybridoma clones 4F8 and 1C5) were purified on protein A. Fractions of three separate purifications were eluted with 0.1M citrate, pH 4.0 (4F8/1, 1C5/1, 1C5/3) and with 0.1M citrate pH 4.0 (4F8/2, 1C5/2, 1C5/4).
All the antibodies, except for 1C5/4, are capable to precipitate overexpressed HIC1 protein as well as to co-immunoprecitate TCF4, protein interacting with HIC1.



6. 2. HIC1 expression profiling

qRT PCR approach was employed to identify mouse tissues with the highest Hic1 expression as well as to determine HIC1 expression in various cell lines and primary cells of human origin.

Selected organs were isolated from four different mice. In the first experiment, male and female mice of Balb/c strain of three months age were used. To overcome possible strain variability, mice of different strains were chosen for the second experiment. These mice were eight-week-old female and male of the STOCK Tg(Fos-lacZ)34Efu/J strain -/- (*The Jackson Laboratory*).

A representative piece of a tissue (cca 100 mg if possible) was homogenized in Trizol® reagent (RNA Blue reagent in the second experiment), RNA was isolated and 1 μg of total RNA was subjected to reverse transcription using SuperScript™ II Rnase H – Reverse Transcriptase (RevertAid™ H Minus First Strand cDNA Synthesis Kit in the second experiment).

qRT PCR was performed in triplicates for each cDNA and primer set (duplicate in "–RT samples") using The LightCycler ® 480 Real-Time PCR System.

Fig. 24. demonstrates Ct values for Hic1 transcript (primer set FmHIC1#1779#real2 and RmHIC1#1844#real2) normalized to three housekeeping genes (beta actin $_{Ct=18}$, GAPDH $_{Ct= 21}$ and ubiquitin C $_{Ct=19}$). Ct values for normalization were determined by calculating the mean of all Ct values gained for each housekeeping gene. Normalization to several housekeeping genes is the most representative way of analysing the qRT PCR data, as no true housekeeping genes exist and the more housekeeping genes involved the more accurate normalization. The data shown represent mean values of two experiments performed in two mouse strains in which all samples were analyzed in triplicates, SD did not exceed 0.1 in any triplicate. In parallel with the "+RT" samples that represent the result, "-RT" samples were run in duplicates to exclude the contamination of primer sets and/or the contamination of cDNA with the genomic DNA. Typical Ct values for "-RT" samples were above 35 among housekeeping genes and above 40 (meaning undetectable) for Hic1 gene. It is not unusual, that the "-RT" samples products are unspecific as verified by the melting curves. The melting curves were analysed for all "+RT" samples as well, to ensure the specific PCR product. Presenting the data as a mean from two different mouse strains suggests there is no significant strain variability and indeed, we didn't find it.

Among thirty tissues isolated from adult mice, uterus revealed to be a tissue with the most abundant Hic1 transcript (Ct value ~27 after normalization). Other tissues presented with Ct values above 28 and most of tissue with values between 31 and 34. These Ct values represent a very low level of transcription when compared to Ct values of the housekeeping genes reaching about 20. This finding of low but ubiquitous Hic1 expression is consistent with the literature (http://symatlas.gnf.org/SymAtlas).

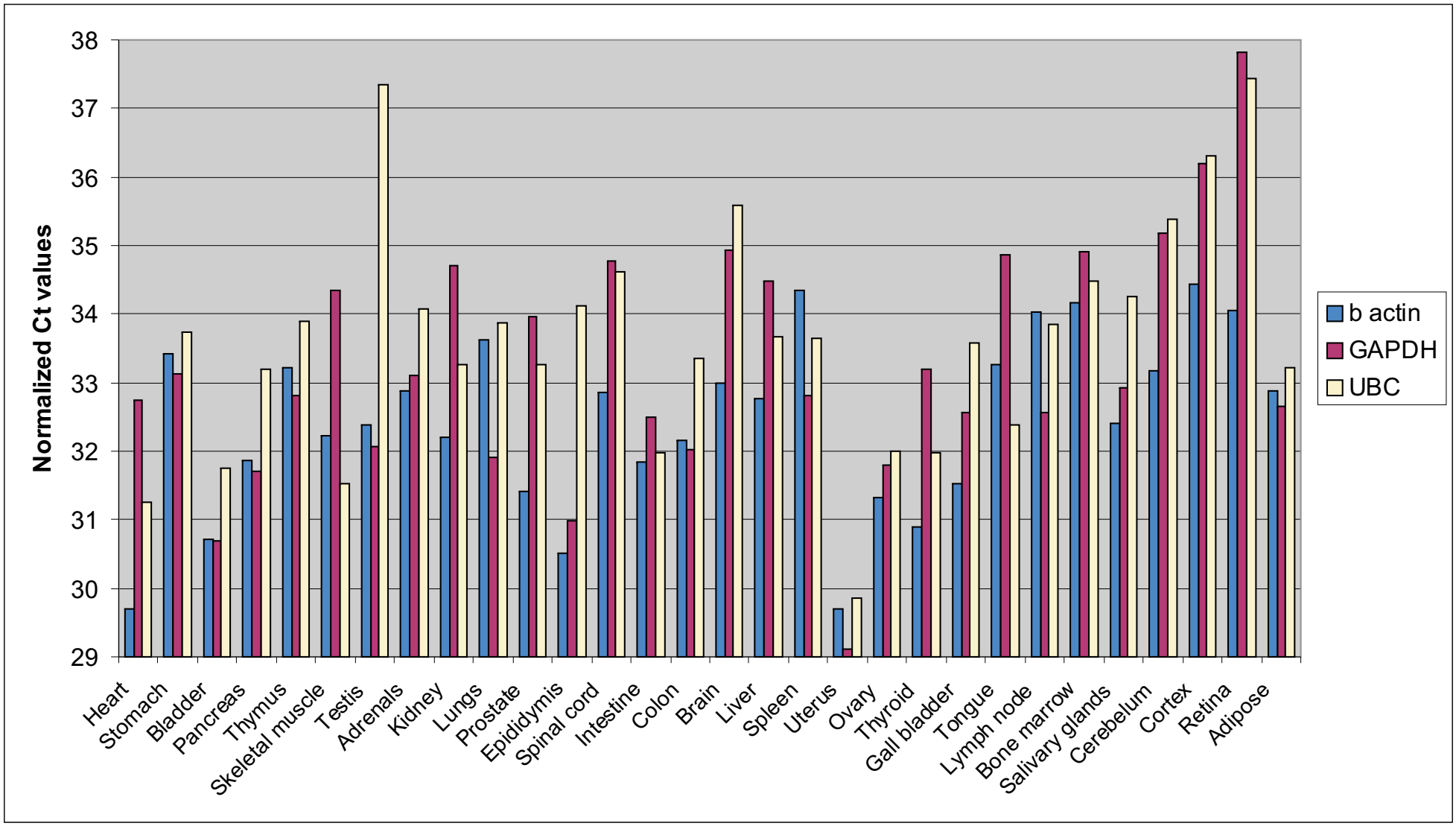


Figure 24. Hic1 expression in tissues of adult mouse
The results are means of normalized Ct values from qRT PCR analysis done in triplicate. Ct values were normalized to three housekeeping genes (b actin, GAPDH and UBC). The Ct value is reversely proportional to the level of expression. Uterus was identified as a tissue with highest Hic1 expression.

Besides uterus, also heart, bladder, epididymis, ovary and thyroid gland exhibited higher levels of mRNA.

HIC1 expression in several human cell lines and primary cells was determined.

qRT PCR was run on two primary fibroblast cell types (WI38 and LEP 19), two types of gut epithelial cells (HIEC and CCD 841), five colon cancer cell lines (DLD-1, Colo 320, SW620, LS 174T and HT29) and two non-colon cancer cell lines (HEK 293 and HeLa). The cell line with inducible HIC1 expression created in our laboratory (DLD-1/HIC1) was included to the screen as a positive control.

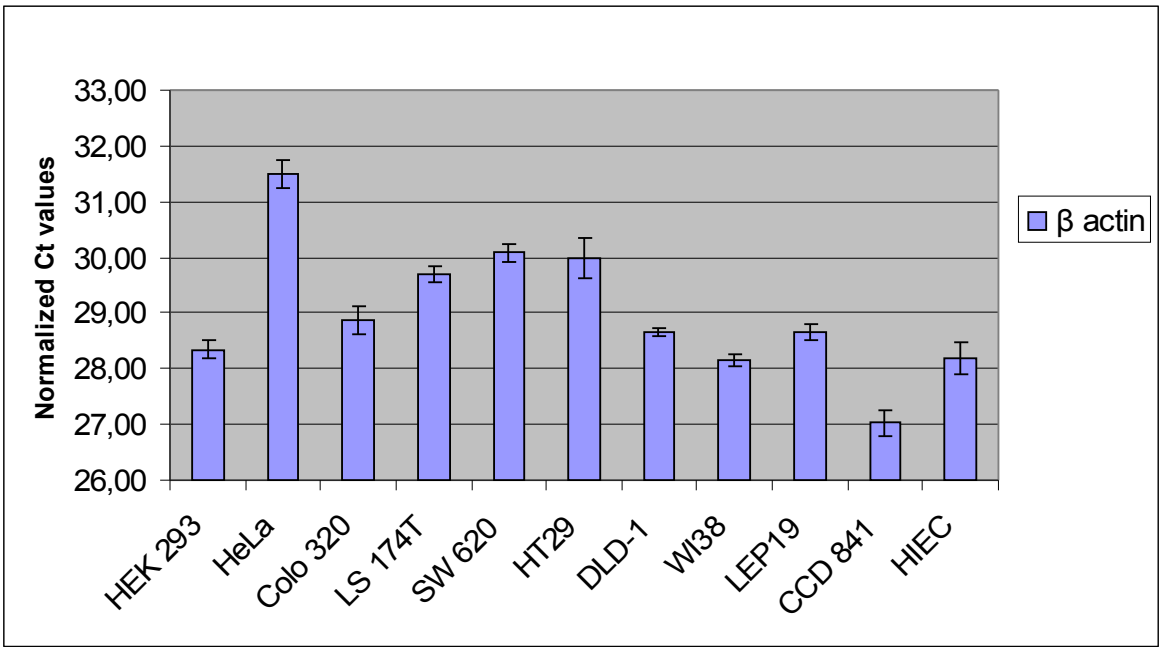


Figure 25. HIC1 expression is higher in primary cells than in cancer cells
The chart shows Ct values of HIC1 normalized to housekeeping gene beta actin (Ct=16.00), bars represent mean ±SD of technical triplicates. Ct values are lower (meaning higher level of expression) in non-cancer cells (WI38, LEP19, HIEC, and CCD841) compared to cancer lines.

Beta actin was chosen among four housekeeping genes for normalization as it showed the most stable expression among all human cells tested. Cp values normalized to beta actin are shown in Fig. 25.

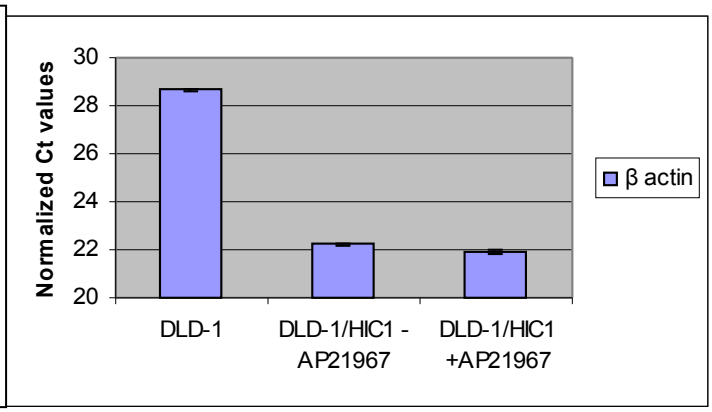
In the primary fibroblasts (WI38 and LEP 19), normalized Ct values were 28.14 and 28.66, respectively. These values are lower than the normalized Ct values recorded for all cell lines of cancer origin except for HEK 293 cell line (Ct value 28.35)

HIEC, WI38 and CCD841 cells were identified as cells with the highest expression of HIC1 mRNA (Ct 27.03 and 28.19) among the cells tested. Owing to high requirements of the cells derived from gut during culture I preferred to use WI38 cell line a source of HIC1 endogenous protein.

These qRT PCR data confirm our expectation that as a tumour suppressor, HIC1 would be silenced in cancer cells and higher expression levels would be detectable in the primary cells.

DLD-1/HIC1 cells, a system for inducible HIC1 expression was produced by my labmate Tomáš Valenta using ARGENT Regulated Transcription Retrovirus Kit (*ARIAD*). We intended to use this system for studying the HIC1 interacting partners and for evaluation of the tools I prepared (*e.g.* monoclonal antibodies). In this particular case I included this system as a positive control for HIC1 expression. It was clearly appointed that the system is very leaky in our hands, despite what the manufacturer states. Expression of HIC1 from retroviral constructs without induction with the dimerizing agent AP21967 is almost the same as the expression level assessed after the induction (Fig. 26.). Although it should rather resemble the expression level of the parental cell line DLD-1.

Figure 26. ARIAD system for inducible HIC1 expression is leaky
The chart shows Ct values for HIC1 normalized to housekeeping gene beta actin (Ct=16.00), bars represent mean ±SD done in triplicates.
The system is extremely leaky, opposed to the manufacturer's claim. Leakiness is demonstrated by a low Ct value in DLD-1/HIC1 cells where no AP21967 was added. The expression of HIC1 reaches almost the same level as in the presence of the dimerizer. Ct value of the parental DLD-1 cell line is also shown

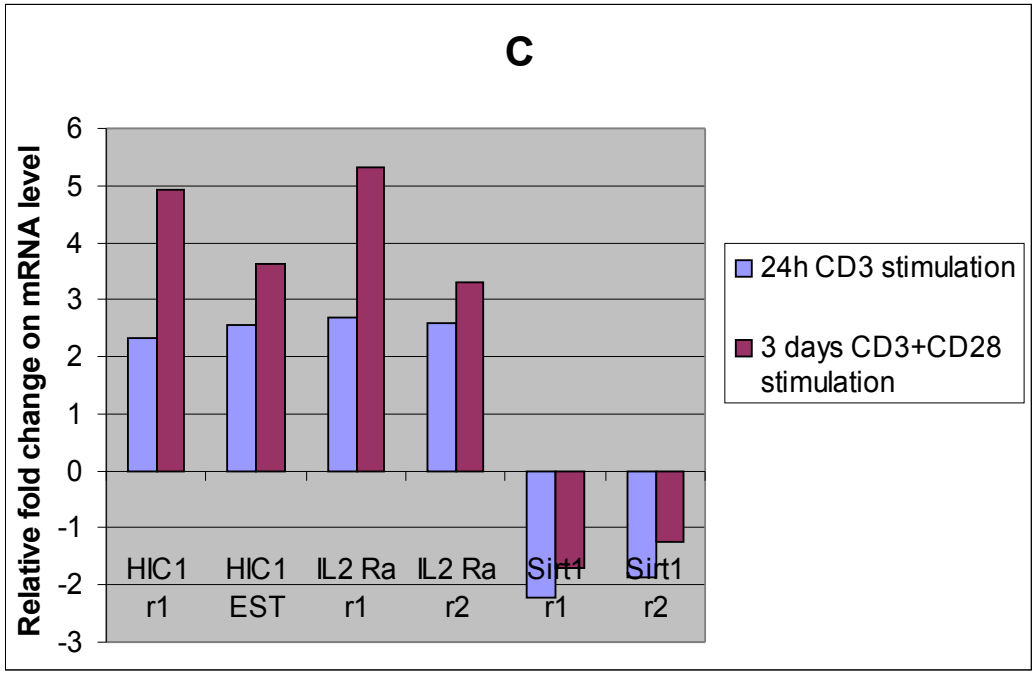


6. 3. HIC1 up-regulation upon T lymphocytes stimulation

During expression profiling studies conducted in our laboratory we found that HIC1 may be up-regulated upon stimulation of T lymphocytes. To confirm or exclude this hypothesis we isolated PBL (peripheral blood leucocytes) from buffy coats using ficoll gradient method. T lymphocytes were activated using CD3 or CD3 and CD28 antibodies and the expression of HIC1 mRNA was quantified using qRT PCR (Fig. 27.). The protein level was verified by WB using anti HIC1 rabbit polyclonal antibodies. The observed expression levels were compared to the levels of HIC1 in unstimulated T lymphocytes.

Figure 27 . HIC1 up-regulation upon T lymphocytes stimulation
The table shows normalized Ct values (means of triplicates). Primers in black represent housekeeping genes and genes not expected to change in the system (CtBP, Axin1). HIC1 primers are shown in green, marker of the T lymphocyte stimulation in red and HIC1 target gene Sirt1 in blue.
(A) 24h stimulation of T lymphocytes with CD3 or irrelevant anti FLAG antibody - Ct values of indicated genes and set of primers normalized to beta2 microglobulin (Ct=19) are shown.
(B) 3 day stimulation of T lymphocytes with CD3 and CD28 antibodies in comparison with unstimulated T lymphocytes - Ct values of indicated genes and set of primers normalized to beta actin (Ct=17) are shown.
(C) Fold change on mRNA level of HIC1, IL2-Rα (CD25) and Sirt1 genes - HIC1 as well as IL2-Rα (marker of T lymphocytes activation) are highly up-regulated upon T-lymphocytes stimulation. Sirt1, a negatively regulated target gene of HIC1, is down-regulated.

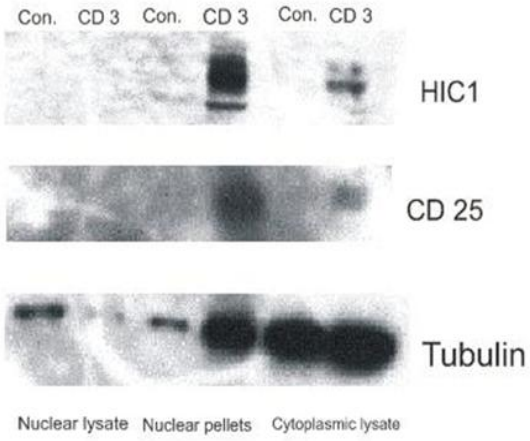
A				B		
Normalization	b2 microglobulin	Ct= 19.00		Normalization	b actin	Ct= 17.00
Ct values	***FLAG***	***CD3***		*Ct values*	***(-)***	***CD3+CD28***
b actin	17,78	17,67		**b actin**	17,00	17,00
GAPDH	24,91	23,76		**GAPDH**	26,43	26,15
SDHA	25,54	26,07		**SDHA**	26,00	25,84
b2 microglobulin	19,00	19,00		**b2 microglobulin**	18,99	19,49
CtBP2	26,24	27,02		**CtBP2**	25,44	26,80
Axin1	28,95	29,43		**Axin1**	28,31	29,14
HIC1 r1	29,35	28,13		**HIC1 r1**	28,62	26,32
HIC1 EST	33,69	32,34		**HIC1 EST**	33,90	32,04
IL2 Ra r1	23,92	22,50		**IL2 Ra r1**	25,45	23,04
IL2 Ra r2	24,27	22,89		**IL2 Ra r2**	25,65	23,93
Sirt1 r1	25,05	26,19		**Sirt1 r1**	25,18	25,94
Sirt1 r2	24,08	24,97		**Sirt1 r2**	24,23	24,54



HIC1 expression was compared in the T lymphocytes after short term and long term activation. For the long term activation (3 days), CD28 antibody was appended to the system. CD28 costimulation is well known to prevent cell death and anergy during T cell activation. Four housekeeping genes (beta actin, GAPDH, SDHA and beta2 microglobulin) and two additional genes expected not to change upon T lymphocytes activation (CtBP2 and Axin1) used as reference genes for qRT PCR analysis. As shown in Figs 27A and 27B, none of these worked perfectly. I chose beta2 microglobulin gene for normalizing the data from the short term T lymphocytes activation experiment and beta actin for normalizing results of the long term activation. Interleukin 2 receptor alpha chain (IL2-Rα, CD25) is a marker of T lymphocytes that displayed more than two fold increase in short term and about four fold increase in long term T lymphocytes activation at the mRNA production (Fig. 27C). Similar trend was observed in HIC1 mRNA. Sirt1 is a HIC1 target gene regulated negatively by binding of HIC1 to its promoter sequence. In consonance with the ratio of the experiment, Sirt1 expression level was down-regulated to about one half after T

lymphocytes stimulation. Two primer sets were employed for HIC1, CD25 and Sirt1 gene and the recorded results confirmed a well known fact that different primer sets exhibit different primer annealing efficiency and that's why the 100% doubling of PCR products is just a theoretical estimation. The data on mRNA level were confirmed on the protein level (Fig. 28).

Figure 28. HIC1 up-regulation upon T lymphocytes stimulation on WB
This Western blot shows HIC1 upregulation upon T lymphocytes stimulation on the protein level. Dignam lysates of T lymphocytes stimulated with CD3 antibody and control (Con.) antibody were employed. HIC1 protein is tightly bound to DNA and it is hard to release it from these complexes. That's why most of the protein remains in the nuclear pellets. CD25 is a marker of T lymphocytes stimulation and alpha-tubulin was used as a load control. .



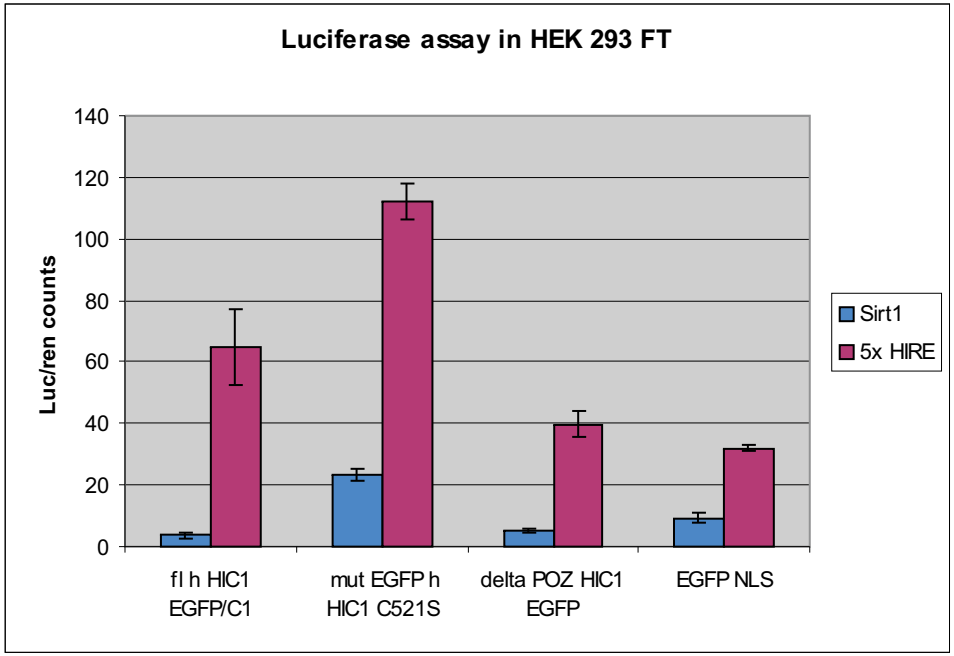
6. 4. Luciferase assays using HIC1 reporters

The repressive properties of HIC1 protein were evaluated using luciferase reporter system. Constructs encoding human full length form and two mutant forms of human HIC1 proteins together with two luciferase reporters were used. All the constructs were cloned into pEGFP vectors so sufficient transfection could have been verified under UV light prior to luciferase measurement. The first mutant form of HIC1 contains C521S substitution of the second cysteine in zinc finger 3 and abolishes the binding of HIC1 to DNA. The second mutant form of HIC1 protein lacks the BTB/POZ domain which mediates cooperative binding of HIC1 proteins to multiple sites in the promoter. 5x HIRE SV40-Luc reporter represents an artificial HIC1 responsive promoter, while pGL3 basic-Sirt1 promoter reporter represents an endogenous one.

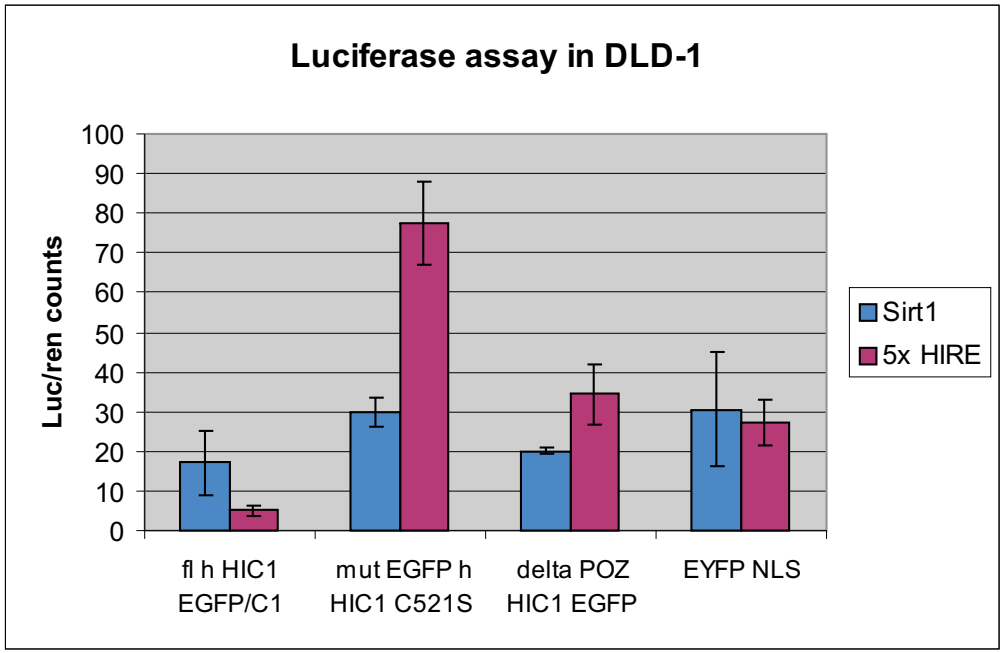
Cells in 12well-plate were transfected in triplicates using Lipofectamine™ with 1,6 ug DNA/well (1.3 μg of HIC1 construct, 0.25 μg of reporter and 0.05 μg of RT-TK) and firefly and *Renilla* luciferase were measured 24h later (Fig. 29.).

The luciferase system behaves differently depending on the cell line used but in general, full length form of HIC1 is capable of efficient repression of the HIC1 responsive promoter compared to the control cells transfected with EGFP-NLS protein. Mutant form of HIC1 that cannot bind DNA may virtually act as a transcriptional activator. This fact can be explained by pulling away other transcriptional reppressors containg oligomerization BTB/POZ domain. Mutant form of HIC1 protein lacking the BTB/POZ domain exhibits reduced repression properties and this drop is more pronounced on 5xHIRE SV40-Luc reporter containing five HIC1 binding sites.

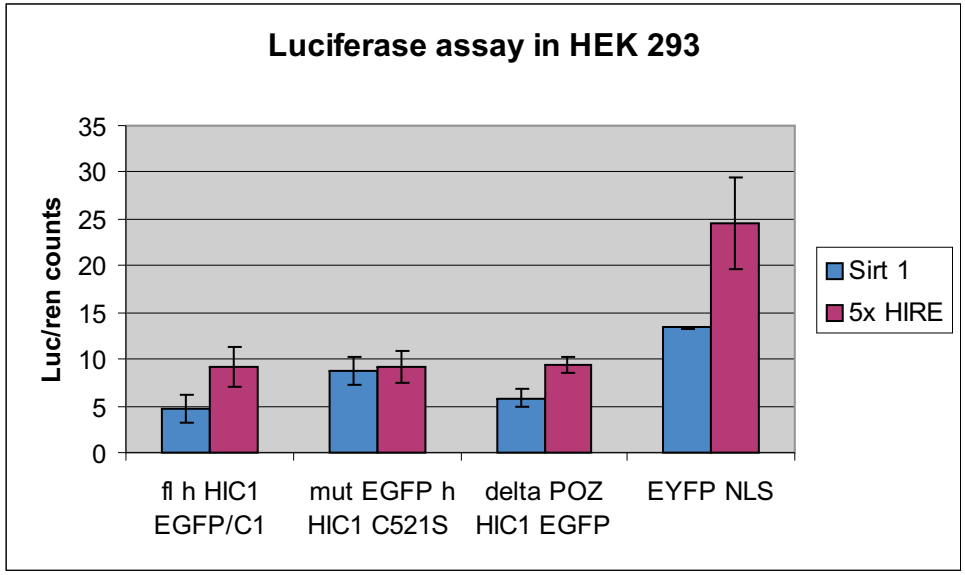
a)



b)



c)



d)

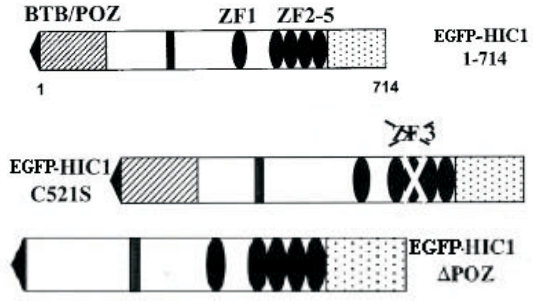


Figure 29 . Luciferase reporter assays
Charts represent the mean values +S.D. from one independent transfection in triplicate. Firefly luciferase counts are normalized to *Renilla* EG/YFP-NLS is a control to assess the basal luciferase activity.
a)HEK 293 FT cells **b)**DLD-1 cells **c)** HEK 293 cells
d) Schemes of the constructs
Full length of HIC1 protein is more potent repressor than the mutant forms.

6. 5. Producing Hic1 conditional KO and Hic1-citrine KI mice

mESC cell line v6.5 was targeted with pEF-mHic1 conditional KO or pEF-mHic1 citrine KI construct. DNA isolated from 220 clones that survived both positive and negative selections was isolated and subjected to verification of undergone homologous recombination.

Expression of pluripotency markers of mESC was verified by qRTPCR. All three master genes regulating pluripotency of ESC, Oct4, Sox2 and Nanog, showed very high expression levels when compared to MPEF that were used as feeder cells (Fig. 30.).

Cp values	mESCs	MPEF
β actin	18,89	18,81
GAPDH	22,02	23,68
UBB	20,35	20,55
Sox2	26,83	>40,00
Oct4	23,47	34,56
Nanog	24,33	32,90
HIC1 r2	34,76	31,85

Figure 30. Expression of *Oct4*, *Sox2* and *Nanog* in mESC
Mean of Cp values of technical triplicates are shown. Housekeeping genes are highlighted in red, markers of pluripotent ESC in blue and Hic1 in green. Great differences are seen in Sox2, Oct4 and Nanog expression between mESC and MPEF. Difference of 8 cycles (Nanog) on qRTPCR correspond to about 2^8=256 increase of abundance of mRNA. Difference of 9 cycles (2^9=512) was recorded for Oct4 and the Sox2 transcript was undetectable in MPEF. Lower expression of HIC1 in mESC compared to MPEF is consistent with evidence that Wnt signalling pathway is active in mESC.

PCR strategy was designed to screen for 5'arm recombination. As the strategy is designed in the way that only in the presence of recombination the reaction gains a product, primers were tested on a mHic1 conditional KO with extended 5'arm/pEF (Fig. 31).

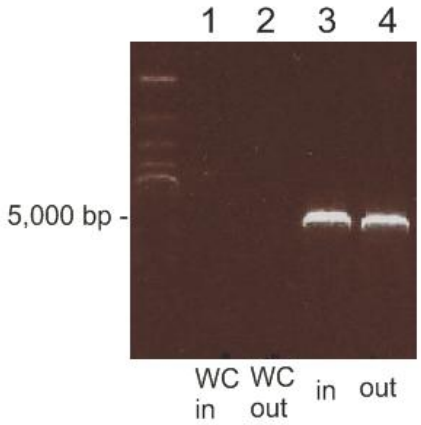


Figure 31. Optimizing PCR strategy to screen for the homologous recombination
pEF-mHic1 conditional KO with extended 5'arm construct was utilized to optimize PCR for screening the 5'arm recombination. Products gained with mHic1 rec in primer set [line(3), 4941 bp] and mHic1 rec out primer set [line(4), 5124 bp] are shown. Lines (1) and (2) are negative water controls. 1ng of template, 1M betain and Long PCR mix were employed.

Despite the primers worked well on the plasmid DNA, we were not able to get the desired product using the DNA isolated from selected clones as the template. We tried several DNA polymerases from different suppliers but were unable to optimize the reaction sufficiently. We were only able to gain a non-specific smear of DNA products. We obtained this smear in all three primer sets tested (1) mHic1 rec out, (2) mHic1 rec in, (3) nested PCR with first 23 cycles using mHic1 rec out primer set and additional 23 cycles using mHic1 rec in primer set (1/25 of the first reaction volume served as a template for the second reaction) . We subjected these gels to Southern blotting,but we were not able to detect the specific band suggesting non-optimal reaction conditions

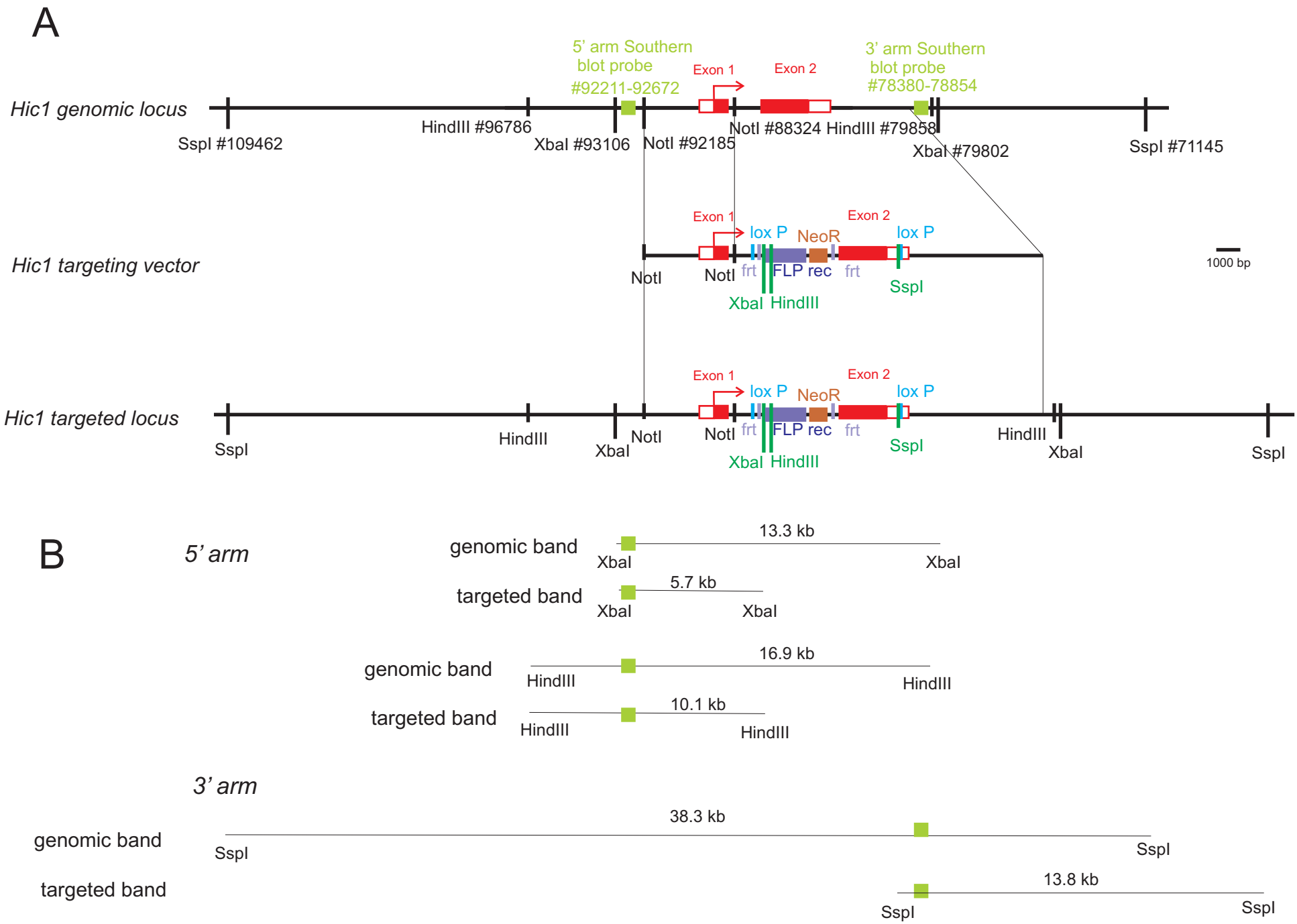


Figure 33. Southern blot strategy for verification of the homologous recombination of pEF-mHic1 conditional KO

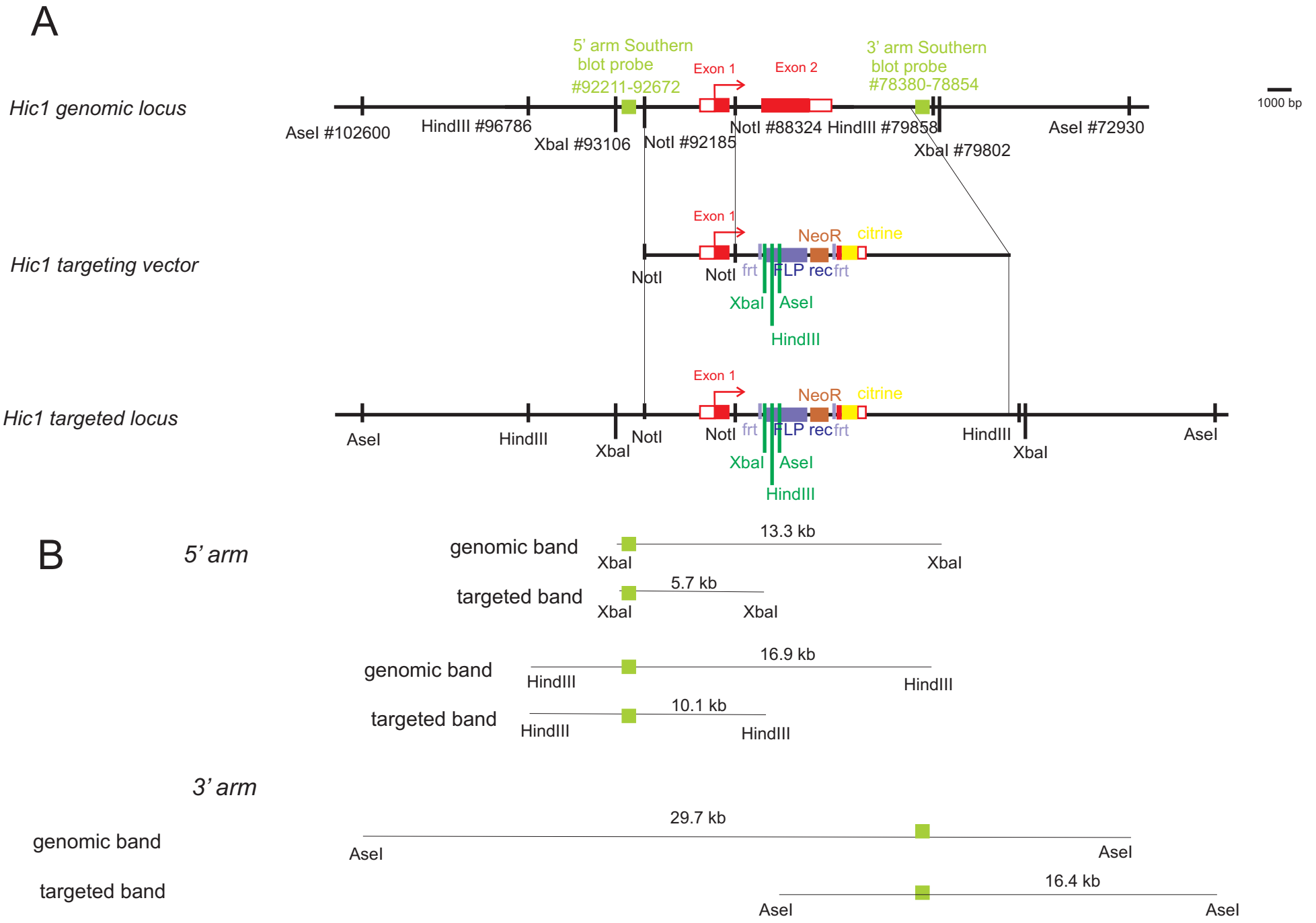


Figure 34. Southern blot strategy for verification of the homologous recombination of pEF-mHic1 citrine KI

rather than low amount of the PCR product that would be not visible on the EtBr stained agarose gel.

In the light of this experience, we employed the Southern blot approach (see Fig. 32. and Fig. 33. for the design of the strategy). The genomic DNA was digested with a restriction enzyme that cut around the Hic1 locus. And the idea is that with the construct we inserted a new restriction site that result in a shortening of the fragment. The fragment is then detected by radioactively labelled probe. We optimized our Southern blotting protocol using the genomic DNA from v6.5 cell line (Fig. 34.).

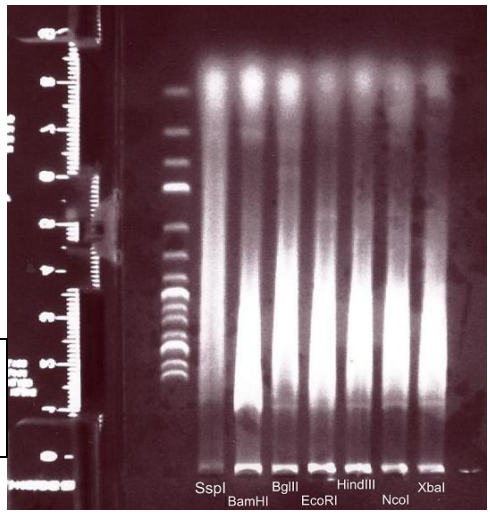


Figure 34. Restriction digest of genomic DNA
Agarose gel shows genomic DNA isolated from v6.5 cell line digested with several enzymes. Microsatelites are visible as thickened bands.

*Xba*I and *Hind*III enzymes were chosen for screen of the 5'arm recombination (Fig. 35.).

Clones positive for 5' arm recombination were digested with *Ssp*I (KO construct) or *Ase*I (KI construct) and the recombination of 3'arm was verified (Fig. 36.).

We identified thirteen positive clones for conditional KO and at least five for citrine KI.

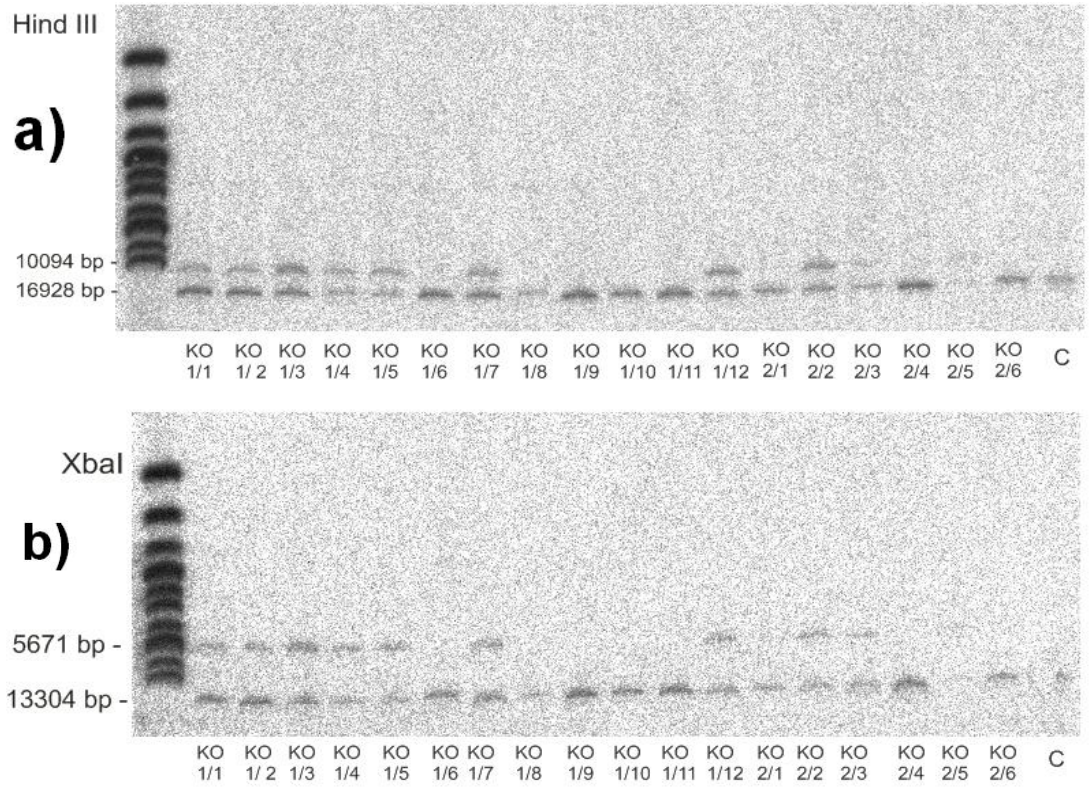


Figure 35. Southern blot analysis of 5'arm recombination
a) The genomic DNA isolated from the clones targeted with mHic1 conditional KO construct was digested overnight with *Hind*III, fragments were resolved in 0.9% agarose gel and blotted onto N+ nylon membrane. The fragments were visualized using[α-^{32}P]dCTP labelled probe for 5' arm. The 16,928 bp fragment represents the genomic band while the target band has length of 10,094 bp.C represents control DNA isolated from non-targeted cells.
b) The same DNA as in a) digested with *Xba*I. The genomic band is 13,304 bp long and the target one is 5,671 bp in length. Nine positive clones are identified on these blots.

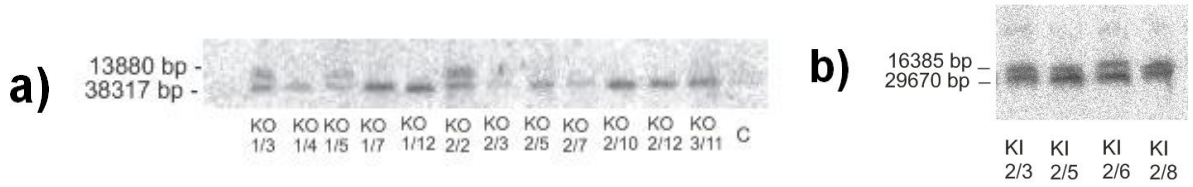


Figure 36. Southern blot analysis of 3'arm recombination
a) Clones positive for 5'arm recombination of mHic1 conditional KO vector were digested overnight with *Ssp*I, fragments were resolved in 0.9% agarose gel and blotted onto N+ nylon membrane. The fragments were visualized using[α-^{32}P]dCTP labelled probe for 3' arm. The 38,317 bp fragment represents the genomic band while the target band has length of 13,880 bp. Three positive clones (KO1/3, KO1/5 and KO2/2) were identified.
b) *Ase*I digest was employed to verify recombination of mHic1 citrine KI construct. The 29,670 bp fragment represents the genomic band while the target band has length of 16,385 bp. Four positive clones were identified.

We decided to use the citrine fluorescent protein instead of GFP for several reasons. (1) citrine is stable across wider range of pH and is resistant to commonly used fixatives. (2) citrine shows higher brightness and photostability, (3) citrine is of yellow colour, so the channel for Alexa 488 is left for a different primary antibody detection, (4) mCitrine is a monomeric protein (Shaner et al., 2005).
Prior to electroporation we tested the citrine fluorescent protein characteristics (Fig. 37.).

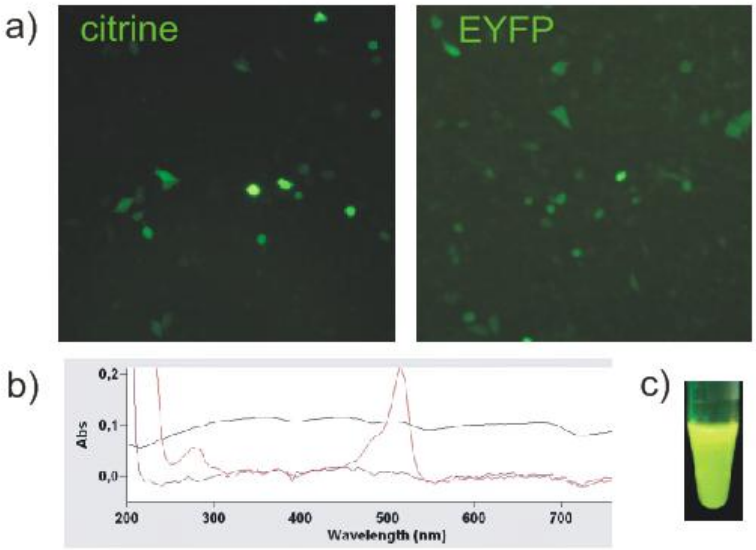


Figure 37. mCitrine fluorescent protein
a) Comparison of DLD-1 cells transfected with pK-myc C3-citrine or pEYFP N3. **b)** evaluation of the absorbance spectrum of citrine. Emission =529 nm **c)** citrine protein under UV light.

The three conditional KO clones (KO1/3, KO1/5, KO2/2) were thawed to be expanded, their phenotype checked and to be frozen down in more aliquots. Southern blot analysis was repeated to ensure these clones are "the ones" and two clones (KO1/5 and KO2/2) were sent to Dresden to be microinjected into blastocysts.
mESC targeted with pEF-Hic1 citrine KI construct will be microinjected in upcoming months.

6. DISCUSSION

The Wnt signalling pathway is essential in many biological processes and numerous studies of this pathway over the last years have lead to the identification of several novel components. The main focus of this diploma thesis is a recently discovered modulator of the canonical Wnt signalling pathway, the HIC1 protein.

HIC1 was originally identified as a potential tumour suppressor that shows reduced expression in various tumour cells. HIC1 is known to participate in p53-dependent apoptosis and both epigenetic and genetic loss of HIC1 function accentuates the role of p53 in tumorigenesis (Chen et al., 2004). Mouse knock-out approach revealed Hic1 essentiality for mammalian development and also recent studies in humans illustrate a clear role for HIC1 during neural development (Briggs et al., 2008) and granulocytic differentiation (Britschgi et al., 2008). Nevertheless, detailed information describing a biological function of HIC1 (including its involvement in the Wnt pathway) is still missing. As the final outcome of the molecular and cell biology research is strongly dependent on various laboratory tools and techniques available to the researchers we dedicated most of this diploma thesis to the development and preparation of such tools. We hope that with the techniques currently available in our laboratory we will be able to elucidate the *in vivo* role of tumour suppressor HIC1.

High-quality monoclonal antibodies to HIC1 protein are not commercially available so far. There is only one company selling HIC1 antibodies that can be applied in ELISA and Western blotting. To raise specific antibodies we immunized mice with the central part of hHIC1 (aa 223-408) produced as recombinant protein in *E. coli*. This region shares the lowest level of homology with the mostly related protein HIC2, Hybridomas from the splenocytes were generated according to the standard protocol and over 1,500 clones were subjected to ELISA test. Several clones positive in ELISA test were further tested for applications including Western blotting, immunocytochemistry, immunofluorescence and immunoprecipitation. Three most promising clones, two of IgG class (1C5 and 4F8) and one of IgM class (2D7) were cultured and antibodies were isolated in large quantities. Unfortunately, none of these clones met our expectations and all of them failed to detect HIC1 protein at endogenous level, which is quite low, in all the applications tested. However, all three clones work well on overexpressed HIC1 protein. Clone 4F8 seems to be the best for detection of HIC1 protein upon transient transfection as well as in DLD-1/HIC1 cells, a cell line with inducible HIC1 expression generated in our lab. The failure to produce monoclonal antibodies with sufficient affinity for applications where only the endogenous level of protein is reached can be attributed at least partially to underestimated immunization of the mice. Both mice were immunized with three dozes of the immunogen in three-week intervals. The excessive amount of IgM class immunoglobulins obtained suggests one more immunization would have been

desirable. This experience also confirms a well known fact that production of high-quality monoclonal antibodies to some particular peptides may be a challenging task.

Polyclonal anti HIC1 antibodies isolated from rabbit serum were purified in several ways to be used as controls in the experiments done with the monoclonal antibodies. These polyclonal antibodies are capable of detection of both overexpressed and endogenous HIC1 protein. Affinity purification on immobilized antigen and purification using Melon Gel revealed to be more suitable for immunofluorescent application giving a lower background than the antibodies purified on protein A. However, on Western blotting, antiserum purified on protein A worked better than the others.

qRT PCR method for screening the expression levels of HIC1 was chosen as a quick, reliable and high-throughput approach. However, it has its pitfalls mainly in the accurate normalization of the data. We tried to overcome this by employing several housekeeping genes. Three housekeeping genes (β-actin, GAPDH and UBC) were finally used to normalize the Ct values of Hic1 transcript. In some tissues, even the fourth housekeeping gene SDHA was used. It gained very similar values as the three previously mentioned housekeeping genes and as we are lacking the data from the complete panel of tissues, we decided not to report it.

During analysis of thirty tissues isolated from adult mice done in biological duplicates together with technical triplicates, uterus revealed to be a tissue with the most abundant Hic1 transcript (Ct value ~27 after normalization). Other tissues displayed Ct values above 28 and represent a very low level of transcription when compared to Ct values of the housekeeping genes reaching about 20. Our finding of low but ubiquitous Hic1 expression in adult mouse is consistent with the GNF SymAtlas database (http://symatlas.gnf.org/SymAtlas) that gathers the information mainly from microarrays. Besides uterus, also heart, bladder, epididymis, ovary and thyroid gland exhibited higher levels of mRNA than the rest of the tissues. Uterus is the organ of choice for further tests of antibodies using immunohistochemistry. We would like to optimize the staining protocols to be able to evaluate HIC1 expression in human tumours with the focus on cervical smears. HIC1 expression levels have been found low in the carcinoma of cervix uteri due to promoter methylation (Dong et al., 2001). Moreover, the methylation pattern correlates with some clinical characteristics, it is more dense in the more advance stage of the disease and HIC1 promoter methylation is often associated with microsatellite instability (Narayan et al., 2003).

HIC1 expression in several human cell lines and primary cells was also determined. Beta actin served as a housekeeping gene for normalization as it showed the most stable expression among all human cells tested. In the primary fibroblasts (WI38 and LEP 19), normalized Ct values were ~28, while Ct values recorded for all cell lines of cancer origin except for HEK 293 cells were

higher. This finding is in agreement with the fact that in the most of the solid tumours the HIC1 gene is silenced or down regulated.

Although human primary HIEC and CCD841 cells showed relatively high levels of HIC1 expression we encounter some technical difficulties to expand these cells in tissue cultures. Therefore, WI38 primary fibroblasts derived from human lung were used for immunocytochemistry and Western blotting analyses.

We also found that HIC1 expression is substantially upregulated upon activation of T lymphocytes. A stimulation of T-cells by crosslinking CD3 and CD28 with specific monoclonal antibodies is an artificial but commonly used laboratory approach. So-called buffy coats were used as cheap and convenient source of human blood cells. Centrifugation in Ficoll gradient was utilized in a standard protocol to separate T lymphocytes from the other cell types present in the peripheral blood. HIC1 expression was upregulated after 24h stimulation with the corresponding antibodies and the expression was even higher on the day three when compared to unstimulated T lymphocytes. The expression of SIRT1, which is negatively regulated by HIC1, was also assessed and it showed a marked decrease upon stimulation of T lymphocytes. In case of SIRT1, the downregulation was more pronounced on day one than on day three after stimulation.

Very recently, HIC1 mRNA levels have been found significantly induced during granulocytic differentiation (Britschgi et al., 2008). The authors of the article report 100-fold HIC1 mRNA induction in HL-60 and U-937 cells upon ATRA (*all-trans* retinoic acid) induced differentiation and about 5-fold induction of HIC1 expression in $CD34^+$ progenitor cells after granulocyte colony-stimulating factor-induced differentiation. To evaluate, whether HIC1 induction after T lymphocytes stimulation seen in our experiments can be truly attributed to T lymphocytes, we intend to perform the very same experiment on CD4- and CD8-sorted T lymphocytes. We also intend to use the model of stimulated T lymphocytes for studying the interacting partners and target genes of HIC1.

pGL3 basic-Sirt1 promoter reporter plasmid was prepared to study the repressive characteristics of HIC1 in the luciferase reporter assay. DNA constructs encoding full length and mutant forms of human HIC1 were transiently co-transfected with Luciferase reporters to three different cell lines. First we transfected HEK 293 FT cells with the synthetic HIC1-dependent reporter 5xHIRE. We observed that HIC1 proteins lacking the BTB/POZ domain or containing mutant Zinc finger domain (this form of HIC1 is unable to bind DNA) displayed compromised repressive function when compared to the full length protein. As all constructs encoded EGFP-tagged variants of HIC1 a plasmid producing EGFP-NLS was used as a negative control in the parallel transfections. In HEK 293 cells transfected with EGFP-NLS the luciferase activity was unexpectedly low. Since HEK 293 FT cells support the replication of plasmids containing the SV40

origin (SV40 ori) we explained this observation by the fact that some replication-competent plasmids can dominate the second co-transfected construct. Therefore, we decided to repeat the experiments in different cells which do not enable plasmid replication. Parental, i.e. SV40 large T antigen free, HEK 293 cells are easily transfectable cells and were therefore system of choice. The repression capacities of mutant HIC1 proteins were in this case comparable to the ones of the full length protein. DLD-1 cells were the third system tested. In DLD-1 cells the mutant form of HIC1 that cannot bind DNA virtually acted as a transcriptional activator. We explain the fact by HIC1 pulling away other transcriptional repressors containing oligomerization BTB/POZ domain, a phenomenon already observed by others (Pinte et al., 2004). Mutant form of HIC1 protein lacking the BTB/POZ domain, which promotes cooperative binding of HIC1 molecules to multiple binding sites, exhibited reduced repression properties and this drop was more pronounced on 5xHIRE SV40-Luc reporter containing five HIC1 binding sites. The results obtained with pGL3 basic-Sirt1 promoter reporter plasmid nicely correlate with the ones obtained with 5xHIRE SV40-Luc reporter (Pinte et al., 2004). Unlike 5xHIRE SV40-Luc reporter, Sirt1 promoter reporter represents the endogenous situation and therefore it brings more valuable information.

Generating knock-out mice is a method that is unfortunately not widely used in the Czech Republic so far. Establishing the method in our lab was a challenging task for us and required a lot of optimization during each step of the procedure. With my supervisor's previous experience with generating knock-out mice and ES culture know-how from Doc. Vladimír Divoký's lab we succeeded. We prepared two constructs and targeted the mESC with them. Two hundred and twenty G418-resistant colonies were picked, expanded, frozen down and genomic DNA isolated from them was subjected to Southern blot analysis to verify correct targeting the Hic1 locus. We obtained at least eighteen clones (thirteen for conditional inactivation and at least five for the citrine knock-in experiment) with the correctly recombined Hic1 gene. The frequency of homologous recombination was 11% for the pEF-mHic1 conditional KO and 5% at minimum for the pEF-mHic1 citrine KI construct. We attribute such frequency to the length of the "arms" for homologous recombination (9 kb "long arm", 7 kb in the citrine construct respectively, and 6 kb "short arm") and to the use of negative selection agent gancyclovir. Three clones for conditional inactivation and three clones for the citrine knock-in were expanded in tissue culture, re-tested by Southern blotting and sent to a collaboration laboratory in Dresden for the blastocysts injections.

Hopefully, in the near future we are going to use the tools prepared during my diploma thesis, especially the conditional knock-out and knock-in (reporter) mice to clarify the role of HIC1 *in vivo*.

7. SUMMARY

The Wnt signalling pathway plays a crucial role in the development of multicellular organisms and, moreover, abnormal Wnt signalling has been associated with various human disorders ranging from cancer to degenerative diseases. The signalling process itself is triggered by binding of extracellular Wnt ligands to the membrane Frizzled/LRP heterocomplex and subsequently proceeds via a series of biochemical events involving many cytoplasmic and nuclear proteins. A key molecule of the so-called canonical Wnt signalling is β-catenin. The activated Wnt pathway inhibits continuous degradation of β-catenin molecules and as the result the protein accumulates in cytoplasm and also enters the cell nucleus. Nuclear β-catenin forms complexes with the transcription factors of the Tcf/Lef family. Interestingly, β-catenin contains a strong transactivation domain at its C-terminus, thus, the TCF/β-catenin heterodimers activate transcription of a specific set of genes. Many of these Wnt-dependent target genes are involved in the control of the cell division (c-Myc, Cyclin D1 etc.). The Wnt pathway is tightly regulated and more than one hundred proteins located in the extracellular space, cytoplasm or the cell nucleus participates in a very complex regulatory network that modulates the final outcome of this signalling.

One of the recently described nuclear Wnt pathway modulators is Hypermethylated in cancer 1 (HIC1). HIC1 was originally described as a tumour suppressor gene inactivated by a chromosomal deletion or promoter hypermethylation in many types of human tumours. The tumour suppressor role of Hic1 was recently confirmed by gene-targeting experiments in mouse. Although Hic1-deficient mice die perinatally and exhibit combinations of gross developmental defects throughout later stages of the prenatal development, the $Hic1^{+/-}$ animals are viable and fertile. However, in about eighteen to twenty months of age $Hic1^{+/-}$ mice develop many different spontaneous tumours – mostly lymphomas or osteosarcomas - that are gender dependent and rapidly progress to the malignant phenotype. The genetic analysis of the dissected tumours showed the inactivation (symptomatically by chromosomal deletion or promoter methylation) of the second “healthy” Hic1 allele in the heterozygote animals confirming the Hic1 tumour suppressor function.

Although the role of the HIC1 gene in tumorigenesis was well established the biological function of HIC1 protein remains elusive. The HIC1 gene encodes a BTB/POZ-zinc finger transcriptional repressor which binds a specific sequence motif in chromosomal DNA. Nevertheless, for now only Sirt1 and Atoh1 have been defined as genes directly repressed by HIC1. In our previous study, we described an inhibitory effect of HIC1 on the expression of several Wnt signalling target genes. Surprisingly, the HIC1-dependent repression is not mediated by direct binding of HIC1 to the promoter regions of the selected genes but by a sequestration of TCF-4 (a member of the Tcf/Lef family) and β-catenin to the HIC1-enriched nuclear dot-like structures called

the HIC bodies. All these data indicate that further research is definitely needed to clarify a possible complex behaviour of HIC1 in the cell.

During my diploma work I prepared several tools to study the function of the HIC1 gene and protein in various experimental setups. These tools include the preparation of monoclonal and polyclonal antibodies recognizing the HIC1 antigen, the expressional profiling of HIC1 mRNA in mouse tissues and human cell lines; immunocytochemical detection of endogenous HIC1 protein in primary human cells; a construction of the Luciferase reporter plasmid carrying the endogenous HIC1-regulated promoter; and finally, a generation two independent constructs for the conditional inactivation of the mouse Hic1 gene and for the so-called knock-in experiments of the citrine reporter gene into the Hic1 chromosomal locus in mouse. Subsequently, I used these tools to demonstrate that:

(1) Uterus is the tissue with the highest expression of Hic1 mRNA in adult mouse; primary human cells were proven to have higher levels of HIC1 mRNA in comparison to the cells derived from different human cancers. Interestingly, we observed a robust HIC1 mRNA up-regulation in primary human T lymphocytes activated by crosslinking their CD3 membrane molecules.

(2) Using antigen- or affinity-purified rabbit anti-HIC1 polyclonal antibodies we readily detected endogenous HIC1 protein in primary human fibroblasts WI38. As expected, in the nuclei HIC1 forms hundreds of distinct speckle-like structures - the HIC bodies.

(3) Luciferase reporter assays conducted on both artificial and endogenous HIC1- responsive promoters revealed the necessity of Zn finger DNA binding domain and the BTB/POZ oligomerization region for the sufficient repression of the gene transcription by HIC1.

(4) We introduced the Hic1-targeting constructs into the mouse embryonic stem cells (mESs) and using specific DNA probes for Southern blot analysis (at both “short” and “long” arms of the constructs) we selected the mESs clones with correct homologous recombination of exogenous DNA in the Hic1 locus. From originally two hundred twenty five G418-resistant colonies of mES cells which were selected we obtained at least eighteen clones (thirteen for conditional inactivation and five for the citrine knock-in experiment) with the correctly recombined Hic1 gene. Several primary clones (three for conditional inactivation and four for the citrine knock-in) were expanded in tissue culture, re-tested by Southern blotting and sent to a collaboration laboratory for the blastocysts injections. We hope that the mice generated by the gene-targeting will be a valuable resource to study the Hic1 function *in vivo*. Last but not least these mice are intended to be used during my PhD study.

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