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„Regulation of Transcription by a Dual Modification Mark at Histone H3“

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**ABSTRACT**

The genomic DNA in eukaryotes organized as nucleoprotein complex, called chromatin. The basic component of chromatin, the nucleosome consists of 146bp DNA wrapped around an octameric protein complex made of two copies of each of the four core histones H2A, H2B, H3 and H4. The N-terminal tails of histones protrude from the histone core and can be posttranslationally modified. Different modifications can be recognized by specific proteins and changes in histone modification patterns frequently result in alterations in the chromatin structure and thereby in modulation of accessibility to specific genome regions.

The phosphorylation of serine10 on histone H3 is linked to chromosome condensation during mitosis, whereas it is associated with transcriptional activation of a number of genes in interphase. Although only a small portion of histones is subjected to phosphorylation, the number of genes known to be regulated by this process is increasing. Serine10 phosphorylation on histone H3 is accompanied by lysine14 acetylation and this dual modification, called phosphoacetylation, is also important for the regulation of transcriptional induction of some target genes like HDAC1 and p21.

The aim of the project was to investigate the role of histone H3 phosphoacetylation in transcriptional induction of target genes in Swiss 3T3 fibroblasts. The response and the different expression profiles of these genes upon activation of the MAP kinase pathway by stress were analyzed by quantitative Real-time PCR experiments and the conditions for a ChIP sequencing technique to examine the distribution of the phosphoacetylation mark on the entire mouse genome were optimized. Furthermore, it was verified that the signaling cascade that is triggered by stress leads to phosphorylation of specific serine residues but not threonine residues on histone H3.

Finally, a potential interaction between the H3S10ph detector 14-3-3 zeta and the elongation complex P-TEFb was analyzed to clarify the idea whether there is an interaction between 14-3-3 proteins and the transcription machinery.

## ZUSAMMENFASSUNG

Die genomische DNA eukaryotischer Zellen ist als Nukleoprotein-Komplex, der Chromatin heißt, organisiert. Dies erlaubt, mehrere Meter DNA in den Zellkern einzupacken. Der wesentliche Baustein des Chromatins, das Nukleosom, besteht aus einem oktamerischen Proteinkomplex, der aus je zwei Molekülen der 4 Core-Histone H2A, H2B, H3 und H4 aufgebaut ist und 146 Basenpaare DNA. Die N-terminalen Histoneschwänze ragen aus dem Nukleosom heraus und können posttranslational modifiziert werden. Verschiedene Modifikationen können von spezifischen Proteinen erkannt werden und Veränderungen in den Histonmodifikationsmustern führen häufig zu Änderungen in der Chromatinstruktur und dadurch zu einer veränderten Zugänglichkeit von definierten Genomregionen.

Die Phosphorylierung von Serin10 an Histon H3 korreliert mit Kondensation des Chromatins während der Mitose, während sie andererseits mit der transkriptionellen Aktivierung von einer Anzahl an Genen in der Interphase gekoppelt ist. Während der Interphase trägt nur ein sehr kleiner Anteil der H3 Histone die Phosphorylierung an Serine10, dennoch steigt die Anzahl an Genen, deren Expression mit dieser Modifikation korreliert, stetig. Die Serine10 Phosphorylierung an Histon H3 ist häufig der Azetylierung des benachbarten Lysin14 begleitet. Die resultierende duale Modifikation, genannt Phosphoazetylierung, hat eine wichtige Funktion für die Regulation der transkriptionellen Induktion von manchen Zielgenen wie HDAC1 und p21.

Ziel dieses Projekts war es, die Rolle der Phosphoazetylierung während der transkriptionellen Induktion von potentiellen Zielgenen, die von zwei voneinander unabhängigen Expression-Arrays stammen, zu untersuchen. Die Induktion und die verschiedenen Expressionsprofile dieser Zielgene durch Stress wurden mittels quantitativer Realtime-PCR analysiert und die Bedingungen für eine Tiefsequenzierung-Technik, um auf die Verteilung der Phosphoazetylieungs-Marker auf gesamten Genom zu schauen, wurden optimiert. Weiters wurde gezeigt, dass durch Stressinduziertedie Signaling-Kaskade spezifisch zur Phosphorylierung von Serinresten und nicht von Threoninresten auf Histon H3 führt.

Schließlich wurde die mögliche Interaktion zwischen 14-3-3 Proteinen und dem Elongationskomplex P-TEb analysiert, um die Idee, ob es eine Bindung zwischen 14-3-3 Proteinen und der Transkriptionsmaschinerie gibt, abzuklären.

# 1 INTRODUCTION

## 1.1 Eukaryotic Transcription

The transcription, one of the most important processes in the nucleus, is responsible for the transfer of genetic information carried by DNA into RNA and is followed by translation that mediates the synthesis of gene products based on the information lying on RNA sequence. Both transcription and translation are discussed under the term "gene expression" and is controlled stringently. The work presented in this thesis is based on the control of gene expression in transcriptional level.

Transcription is divided into three main steps called initiation, elongation and termination and mediated by RNA polymerases and basal transcription factors. The binding sites for basal transcription factors are provided by a TATA-box (O'Shea-Greenfield and Smale 1992) that is located 30bp upstream of transcription site on promoter regions of many genes (Wiley, Kraus and Mertz 1992). The basal transcription factors together with RNA polymerase II form the pre-initiation complex and give start to transcription. The transition from initiation to elongation is achieved by phosphorylation of amino acids on carboxy-terminal domain (CTD) of RNA polymerase II (RNAP II). During elongation, the mRNA processing takes place and the newly synthesized transcript is cleaved in the termination step.

### 1.1.1 RNA Polymerase II

The RNA polymerase II in eukaryotes is the key machine to manage the transcription of the protein-coding genes (Dahmus 1995). The carboxy-terminal domain (CTD) of the largest subunit of the RNA polymerase II is subjected to posttranslational modifications and provides binding sites for the proteins like mRNA processing factors, transcription factors, chromatin remodeling complexes or chromatin modifiers (Hirose and Ohkuma 2007, Buratowski 2009). CTD consists of repeats of Tyr-Ser-Pro-Thr-Ser-Pro-Ser which appears 52 times on a CTD in eukaryotic cells (Dahmus 1995). The amino acids, serine and threonine (Zhang and Corden 1991) as well as tyrosine (Baskaran, Dahmus and Wang 1993) of these repeats are subjected to phosphorylation. The phosphorylation events on the CTD are associated with initiation and elongation steps of transcription (Dahmus 1996) and performed by different kinases.

The transcription factors can bind to the RNAP II and form the preinitiation complex, if CTD of RNAP II is not phosphorylated. TFIIH with the subunit CDK7 is the last factor of this complex and possesses CTD kinase activity (Svejstrup, Vichi and Egly 1996).

Besides TFIIH, the mediator complex consisting of CDK8 has also CTD kinase activity. Both TFIIH and the mediator complex phosphorylate the serine5 on CTD (Hirose and Ohkuma 2007), whereas serine2 of the CTD is phosphorylated by a different protein called P-TEFb.

### 1.1.2 P-TEFb

In 1992, P-TEF was discovered by Marshall and Price as a positive transcription elongation factor in *Drosophila* $K_c$ cell nuclear extracts. Although it was seen after the initiation of the transcription, its association with the preinitiation complex was not observed. The role of P-TEF was to produce long RNA transcripts (Marshall and Price 1992). Later in 1995, regarding experiments done in *Drosophila*, Marshall and Price reported that P-TEFb, out of three fractions, plays a role in the regulation of transcription (Marshall and Price 1995). One year later, it was proven that P-TEFb possesses a kinase activity to phosphorylate serine2 on CTD of RNAP II and, thereby, to allow the transition from initiation to elongation during transcription (Marshall, Peng, et al. 1996). Later, CDK9, a cyclin-dependent kinase, and cyclin T were identified as two subunits of P-TEFb where cyclin T was required to trigger the kinase activity of CDK9 (Peng, Marshall and Price 1998). In 1998, the human P-TEFb was also identified as a heterodimer of CDK9 and either cyclin T1 or T2 (Peng, Zhu, et al. 1998). The CDK9 is the only identified kinase to phosphorylate serine2 on CTD of RNAP II. While the coding regions of genes are associated with CTD phosphorylated at serine2 and this serine2 phosphorylation causes the shift from initiation into elongation during transcription, the serine5 phosphorylation of CTD was found at promoter-associated regions and had no effect on the transition between initiation and elongation of transcription. (Komarnitsky, Cho and Buratowski 2000).

The unphosphorylated state of CTD is achieved by phosphatases. The Fcp1, TFIIF-dependent CTD phosphatase, was first shown to dephosphorylate both serine2 and serine5 with the same efficiency regarding experiments done in HeLa cells (Lin, Dubois and Dahmus 2002). Three years later, another group reported that the yeast Fcp1 is responsible only for the dephosphorylation of serine5 (Kong, et al. 2005). Besides Fcp1, two additional phosphatases also perform the dephosphorylation of CTD; PP1, type 1 protein phosphotase, and SSU72 that dephosphorylates specifically the tyrosine residue of CTD (Palancade and Bensaude 2003) .

Besides the role to phosphorylate serine2 on CTD of RNAP II and to initiate the transition from transcriptional initiation to elongation, P-TEFb interacts with many proteins and fulfils different functions in the cell. One of the well-known interaction partners of P-TEFb is the Tat, a transactivator of HIV-1 transcription (Chen, Fong and Zhou 1999, O´Keeffe, et al. 2000). A histone methyltransferase of S. cerevisiae, Set2, was also reported to interact with P-TEFb and it was suggested that this interaction can facilitate the methylation of histone H3 by Set2 (Li, Moazed and Gygi 2002).
Brd4 is a nuclear protein that has two bromodomains and is another interaction partner of P-TEFb. Brd4 recognizes the acetylated histones by its bromodomains and bind to CycT1 subunit of the P-TEFb to bring the P-TEFb to the genes which are supposed to be transcribed and trigger the phosphorylation of serine2 on CTD of RNAP II (Zhou and Yik 2006, Brés, Yoh and Jones 2008).

## 1.2 Eukaryotic Chromatin

The genomic DNA carrying the genetic information possesses an enormous length. It is assembled into a nucleoprotein complex called chromatin. One of the roles of chromatin is to reduce the length of DNA and to make it fit into the nucleus. Another role of chromatin emerges importance during cellular processes such as transcription, replication and DNA repair. A various number of proteins including enzymes, activators and enhancers have to reach specific genomic regions to fulfill their functions. However, some DNA sequences are not accessible because they are hidden within the chromatin structure. This difficulty is solved by the chromatin structure itself. Dynamic chromatin modifications allow the break of binding between DNA and chromatin and this makes DNA sequences accessible. In this context, chromatin serves as an important regulator for processes required for the vital continuity of a cell.

### 1.2.1 Chromatin Structure

The nucleosome is the basic and repeating component of chromatin. It is an octameric protein complex made of two copies of four histone molecules called H2A, H2B, H3 and H4 (Kornberg 1977). Histones H3 and H4 are highly conserved in evolution regarding the amino acid sequences (McGhee 1980) and build tetramers, whereas histones H2A and H2B form dimers and are responsible for the stabilization of the histone octamer (Kornberg 1977). The 146 base pairs long and negatively charged DNA, wrapped around this histone octamer, and basic histone molecules together define

the nucleosome core. The nucleosomes as spherical particles with 70Å diameter and are organized "beads on a string" configuration (Olins 1975).

They are cross-linked with each other to be organized into higher-order structures that are stabilized by binding of histone H1 to entry and exit site of the linker DNA between the nucleosome cores (McGhee 1980, Luger, et al. 1997, Thoma, Koller and Klug 1979). The folding of the "beads on a string" form of chromatin results in the formation of 30nm chromatin fiber (Woodcock, Frado and Rattner 1984) and the reduction of length of DNA.

### 1.2.2 Histone Modifications

The carboxy-terminal domain of the core histones is more structured than the amino-terminal tails and performs the stabilization of histone octamer by histone-histone and histone-DNA interactions (Lilley and Pardon 1979, Hansen, Tse and Wolffe 1998), whereas the highly basic N-termini are responsible for the binding to the nucleosomal DNA as well as for the condensation of chromatin. They protrude from histone octamer and provide recognition sites for the non-histone proteins such as chromatin remodeling complexes or chromatin modifying enzymes (Hansen, Tse and Wolffe 1998). Hence, they are subjected to posttranslational modifications (PTMs) such as acetylation, phosphorylation, methylation and ADP-ribosylation (see Fig. 1 for PTM of N-terminal tail of histone H3) that can be mediated by signal transduction pathways (McGhee 1980, van Holde 1988).


**Fig.1: The posttranslational modifications on the N-terminal tail of histone H3.** (Epigenetics: Allis, Jenuwein, Reinberg)

Regarding these modifications, the N-terminal tails of core histones present a "histone code" that can be read out by other proteins. The translation of the histone code by effector proteins results in the alteration of chromatin structure. In some cases, this change in chromatin structure makes DNA accessible to proteins like transcription factors, whose binding arise expression of genes. However, the alteration of chromatin folding can also result in the heterochromatin formation. Both of these outcomes depend

on the type of modification mark presented at the N-terminal tails of histones and which histone molecule is subjected to the modification. Therefore, the covalent modification marks on N-terminal tails of histones are important for the chromatin folding as well as for nuclear process like transcription (Cheung, Allis and Sassone-Corsi 2000, Strahl and Allis 2000)

### 1.2.3 Histone Acetylation

The histone acetylation is the first-discovered modification that occurs at the N-terminal tails of core histones. In 1960's it was observed that the histone acetylation plays a crucial role in transcriptional regulation. Regarding the investigations, it is a well-understood process nowadays.

Histones whose N-terminal tails are rich in lysine residues are one of the targets of the acetylation process. The histone acetylation is an amidation process causing a neutralization of charge of lysines (Garcia-Ramirez, Rocchini and Ausio 1995) which results in interruption of the interaction between DNA and chromatin (Allfrey, Faulkner and Mirsky 1964). The loss of DNA-chromatin interaction then makes DNA accessible by action of chromatin remodeling complexes that can perform removal or sliding of nucleosomes. The DNA sequences released from nucleosomes can be bound for example by RNA polymerases that trigger the transcription of target genes (Allfrey, Faulkner and Mirsky 1964, Clayton, Hazzalin and Mahadevan, Enhanced Histone Acetylation and Transcription: A Dynamic Perspective 2006).

The histone acetylation seems to be a general process that activates transcription, whereas the deacetylation of histones is referred to transcriptional repression according to the deacetylated lysines on histone H3 and H4 found in heterochromatin (Grösch, et al. 2005, Davie and Chadee 1998). The lysines that were found to be acetylated are lysines 9, 14, 23, 18 and 56 on histone H3, and lysines 5, 8, 12 and 16 on histone H4 (Thorne, et al. 1990, Grunstein 1997).

Histone acetylation is a reversible process and its steady-state is achieved by two enzymes; histone acetyltransferases (HATs) and histone deacetylases (HDACs). The HATs are responsible for the acetylation of lysines on histones, whereas the HDACs remove the acetyl groups from these lysine residues. There are two types of HATs, the B-type being cytoplasmic and the A-type being nuclear. The B-type HATs acetylate histones to transport them into the nucleus, whereas the A-type HATs generate acetylated histones to initiate the transcription. In general, the HATs are divided into

three protein families called GNAT, MYST and p300/CBP (Roth, Denu and Allis 2001) and each family contains a catalytic domain. Besides the chromodomain, a bromodomain is allocated to the nuclear HATs [ (Zeng and Zhou 2002, Jeanmougin, et al. 1997) and was found first in Drosophila in 1992. It had been believed to play a role in protein-protein interactions (Haynes, et al. 1992). Later in 2000, it was shown that the amino terminal tails of histone H3 and H4 are targets for the binding of bromodomains (Ornaghi, et al. 1999). Due to increasing number of the investigations, it is known nowadays that the bromodomain specifically binds to the acetylated histones. This was shown for the first time for the bromodomain of Gcn5p of S. cerevisiae that was bound to the acetylated lysine 16 on histone H4 (Zeng and Zhou 2002, Owen, et al. 2000).

HATs can also be parts of protein complexes like chromatin remodeling complexes SWI/SNF complex (Roth, Denu and Allis 2001) and SAGA complex that is an important player in the transcriptional activation (Larschan and Winston 2001). Unlike HATs whose function is linked to the transcriptional activation, histone deacetylases are responsible for the transcriptional repression due to the formation of more condensed chromatin achieved by the removal of acetyl group from lysine residues on histones. There are 18 deacetylases identified in humans, which are divided into four groups. The class I histone deacetylases contain the HDAC1/2, HDAC3 and HDAC8, where the HDAC1 and HDAC2 enzymes show the most sequence similarity (Brunmeir, Lagger and Seiser 2009, Gregoretti, Lee and Goodson 2004). HDAC1 can regulate transcription on its own or as heterodimer with HDAC2 (Zupkovitz, et al. 2006). Although these enzymes differ very little in their function, they cannot compensate each other which was shown in case of HDAC1 KO embryonic mice. The lethality of these mice could not be rescued by the increased presence of HDAC2 (Lagger, et al. 2002).

HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10 are listed as class II histone deacetylases, whereas SIRT1 to SIRT7 are called class III histone deacetylases. The class IV of HDACs contains only the HDAC11 (Zupkovitz, et al. 2006). Like HATs, HDACs especially HDAC1 and HDAC2, were also found in the protein complexes such as Sin3, NuRD and CoREST, NODE, and SHIP1 (Brunmeir, Lagger and Seiser 2009). The recent investigations have shown that some of the HDACs can acetylate other proteins as well.

### 1.2.4 Histone Methylation

Histone methylation is the least understood modification compared to other covalent histone modifications. First, it was discovered for the lysine residues that can exist in mono-, di- and trimethylated form (Murray 1964, Sims III, Nishioka and Reinberg 2003).

Today, it is known that arginine residues of histones can also be methylated. Unlike acetylation and phosphorylation of histones, methylation does not affect the charge of lysines and arginines on histone molecules (Sims III, Nishioka and Reinberg 2003) Out of the four core histones, histone H3 is the most methylated histone molecule, especially on lysines 4, 9, 27 and 36. Compared to histone H3, histone H4 is methylated on lysine 20 (Strahl and Allis 2000, Strahl, Ohba, et al. 1999, Lachner and Jenuwein 2002). The histone methyltransferases (HTMs) are responsible for this modification mark on histones. For example, Su(var)3-9, one of the histone methyltransferases in *Drosophila*, and its human homologue Suv29H1 were identified as the first HTMs methylating specifically lysine 9 on histone H3 (Rea, et al. 2000). The histone molecules methylated by HTMs can be recognized by the proteins possessing a chromodomain. These chromodomain proteins allocated to chromosomal proteins (Eissenberg 2001) can regulate transcription either positively or negatively and play also an important role in the chromatin remodeling. Among chromodomain proteins, the heterochromatin protein 1 being first-discovered recognizes specifically the methylated lysine 9 on histone H3 that was created by HTM Su(var)3-9 (Aaslan and Steward 1995, Lachner, O´Carroll, et al. 2001)

Upon the recognition of methylated lysine 9 on histone H3, the HP1 binds and leads to formation of silent chromatin, whereas the methylation of lysine 4 on histone H3 is found in euchromatin and linked to transcriptional activation of target genes (Sims III, Nishioka and Reinberg 2003, Nakayama, et al. 2001).

Thereby, the methylation mark on histone H3 presents a dual role being either positively or negatively linked with transcription.

### 1.2.5 Histone Phosphorylation

Protein phosphorylation, in general, is the most studied modification which plays a crucial role in the regulation of the signal transduction pathways. The role of a signal transduction pathway is to transfer the signals gained from the outside of the cell into the nucleus and to activate the cellular and nuclear processes. One of the languages that

the components of this pathway use to communicate with each other is the phosphorylation. In many cases, there is a signaling cascade consisting of kinases that phosphorylate each other or other proteins to trigger their activation for which the MAP-kinase pathway can be given as an example. In addition to enzymes and other proteins, the chromatin is also a target of phosphorylation. For instance, histone phosphorylation can induce the transcription of some genes which was observed first for the immediate early genes associated with histone H3 (Mahadevan, Willis and Barratt 1991). The alteration of chromatin structure due to the phosphorylation of histones is explained by the nature of phosphate groups. Since the phosphate groups are negatively charged, their addition to amino acids on histones causes a break in interaction between negatively charged DNA and basic nucleosomes (Grant 2001, Wie, et al. 1999). The amino acids on the amino-terminal tail of histone H3 that undergo phosphorylation are serines on position 10 and 28 and threonines on position 3 and 11 (Polioudaki, et al. 2004). Recently, threonine45 on histone H3 has also been identified as target for phosphorylation in cells entering apoptosis and PKCδ kinase seems to be responsible for this modification (Hurd, et al. 2009).

Out of these phosphorylated residues of histone H3, the serine10 phosphorylation is the most studied one that occurs either in interphase or during mitosis and can be triggered by various signals.

**Phosphorylation of histone H3 during mitosis**

Serine10 phosphorylation on histone H3 during mitosis has different roles compared to histone H3 phosphorylation during interphase. While serine10 phosphorylation during interphase is targeted only to a minute fraction (Barratt, Hazzalin and Mahadevan 1994), mitotic histone phosphorylation is distributed on the entire chromosome and linked to the chromosome condensation (Thomson, Mahadevan and Clayton 1999). In *Tetrahymena,* it has been observed that it also mediates the chromosome segregation (Wie, et al. 1999). In S. cerevisiae and C. elegans, AIR2-Ipl1p of aurora kinase family seems to be responsible for serine10 phosphorylation of histone H3 during mitosis [ (Hsu, et al. 2000)].The mitotic phosphorylation of histone H3 starts at G2 phase (Hendzel, et al. 1997) and proceeds in prophase, metaphase, and anaphase. The dephosphorylation of histone H3 occurs at the exit of the anaphase (Gurley, et al. 1978). Like serine10, the serine28 on histone H3 has also been found to be phosphorylated during mitosis and it is also linked to the chromosome condensation. However, the

phosphorylation levels of the serine10 and serine28 show differences where Ser10 seems to be more phosphorylated than serine28 (Goto, et al. 1999).

Besides phosphorylation of serine residues on histone H3, threonine residues on histone H3 are also phosphorylated during mitosis. Although mitotic phosphorylation of threonine3 on histone H3, mediated by haspin (Dai, et al. 2005), correlates with the phosphorylation of threonine11 on same histone, it differs from phosphorylated serine10 (Polioudaki, et al. 2004).

Compared to the localization of phosphorylated serine10 of histone H3, phosphorylation of threonine11 on histone H3 is restricted to centromeric regions and starts in prophase and lasts until early anaphase and is mediated by Dlk (Death-associated protein (DAP)-like kinase). Interestingly, the Dlk and Aurora B can selectively target different amino acids on histone H3, although both are located in centromeric regions (Preuss, Landsberg and Scheidtmann 2003).

**Phosphorylation of histone H3 in interphase**

In interphase, induction of immediate early genes c-fos and c-Jun as a response to extracellular signals such as growth factors was found to be accompanied by histone H3 phosphorylation (Mahadevan, Willis and Barratt 1991). Depending on the signal, whether it is an epidermal growth factor (EGF), stress or UV radiation (Hazzalin, et al. 1996), distinct MAP kinase pathways are activated whose downstream kinases phosphorylate serine10 on histone H3 (Thomson, Clayton and Hazzalin, et al. 1999). Out of three MAP kinase pathways (Thomson, Mahadevan and Clayton 1999), the ERK and p38 pathway have been defined as inducers for the transcription of IE genes, whereas no effect on the histone H3 phosphorylation has been observed in case of the JNK/SAPK pathway that can be activated by anisomycin or UV radiation like the p38 pathway (Thomson, Mahadevan and Clayton 1999, Thomson, Clayton and Hazzalin, et al. 1999).

The activation of the ERK and p38 pathways upon the stimuli leads to a “nucleosomal response” (Thomson, Clayton and Hazzalin, et al. 1999) that appears as serine10 phosphorylation on histone H3 mediated by the downstream kinase, Msk1/2 (Cheung, Allis and Sassone-Corsi 2000). Compared to ERK and p38 pathways, Msk1/2 cannot be activated via JNK/SAPK kinase pathway (Thomson, Clayton and Hazzalin, et al. 1999). Besides serine10 phosphorylation on histone H3, *in vitro* experiments revealed that the Msk1 is also able to phosphorylate serine28 on histone H3 (Dyson, et al. 2005).

Phosphatases also exist to dephosphorylate proteins. The published phosphatase for serine10 on histone H3 is PP2A that was identified in *Drosophila* to be responsible for the transcriptional repression of the non-heat shock genes (Nowak, Pai and Corces 2003).

### 1.2.6 Histone Phosphoacetylation

Besides single modifications, histones can undergo combinatorial and multiple modifications. The best-studied combinatorial modification of histones is the phosphoacetylation (Jenuwein and Allis 2001) which was first observed in 1994 by Barratt and his colleagues. They showed that the histone H3 phosphorylation at serine10 was a minute fraction upon EGF stimulation and this small fraction was also subjected to the histone H3 acetylation at lysine14 which results in the transcriptional activation of the immediate-early genes (Barratt, Hazzalin and Mahadevan 1994). Further investigations have focused on the role of this dual modification on the gene activation and on the underlying mechanisms.

Two models have been proposed to elucidate the mechanism of simultaneous phosphorylation and acetylation of H3 tail (Clayton and Mahadevan 2003). One model argued that the phosphorylation and acetylation of histone H3 are "synergistically coupled" (Cheung, Tanner, et al. 2000). In this model serine10 phosphorylation on histone H3 occurs before lysine14 acetylation. This is based on the observation that in *in vitro* experiments serine10 phosphorylation facilitates subsequent lysine14 acetylation, whereas prior acetylation has no effect on the subsequent phosphorylation of histone H3 (Lo, et al. 2000). This phenomenon depends on the higher affinity of the lysine14 histone acetyltransferase Gcn5 for the phosphorylated histone H3 (Cheung, Tanner, et al. 2000, Lo, et al. 2000, Kuo, et al. 1996).

Besides Gcn5, the PCAF and p300, human homologues of the Gcn5, have been also shown to acetylate lysine14 of histone H3 (Shiltz, et al. 1999).

Opposite to first model, the second model assumed that these two histone modifications are independent and dynamic events. There is a steady state of histone acetylation in quiescent cells achieved by HATs and HDACs, whereas an external signal is required to trigger the histone phosphorylation via MAP kinases (Thomson, Clayton and Mahadevan 2001, Hauser, et al. 2002, Nowak and Corces 2004).

Therefore, both histone modifications are regulated in different ways, although they can activate together same subset of genes. Compared to the first model, the acetylation

does not require any previous phosphorylation which was observed for example for c-jun gene (Thomson, Clayton and Mahadevan 2001).

After it was shown that the transcriptional activation of IE genes is triggered upon the phosphoacetylation of histone H3 (Winter, Simboeck and Seiser 2007), the distribution of this dual modification mark was investigated and it was found preferentially at the promoters of the immediate-early genes c-fos (Cheung, Tanner, et al. 2000, Thomson, Clayton and Mahadevan 2001) and c-jun (Clayton, Rose, et al. 2000). However, this transcriptional activation by histone phosphoacetylation is not restricted to the IE genes and has also been found at the promoters of other genes like HDAC1 gene. Compared to the IE genes whose transcription is a rapid event and where both modifications occur independently, a synergy between phosphorylation and acetylation mark on histone H3 as well as a prolonged phosphoacetylation on histone H3 is required for the transcriptional activation of HDAC1 gene (Hauser, et al. 2002). In general, the HDAC1 gene is regulated by a negative feedback loop that leads to the transcriptional repression by HDAC1 itself. The treatment of cells with trichostatin A, an HDAC inhibitor, blocks the local HDAC1 predominance on one hand and causes a hyperacetylation of promoter-associated histone H3 on the other hand. If trichostatin A (TSA) treatment is accompanied by the treatment with the MAP kinase inducers, the phosphoacetylation on histone H3 at HDAC1 promoter is prolonged. Hence, the transcription of HDAC1 gene is induced (Hauser, et al. 2002, Schuettengruber, et al. 2003). Besides IE genes and HDAC1 gene, further investigations revealed that the p21 gene is also subjected to the phosphoacetylation of histone H3 at its promoter region. Importantly in the absence of MAP kinase signaling TSA treatment is not sufficient for the activation of the p21 gene. In contrast, cells simultaneously treated with anisomycin, an inducer for the p38 stress pathway, and TSA showed transcriptional activation as well as prolonged transcription of the p21 gene. Therefore, the p21 gene is believed to be a new target gene of histone H3 phosphoacetylation [Simboeck, E., unpublished data].

### 1.2.7 Histone Code

The posttranslational modifications on N-terminal tails of histone molecules define the "histone code". It can be read out by proteins such as bromo- or chromodomains that are components of chromatin remodeling complexes or chromatin modifiers (Strahl and Allis 2000) and these different readouts are associated with distinct downstream events (Strahl and Allis 2000).

Translation of the histone code by effector proteins results in alteration of chromatin structure that is associated either with transcriptionally active or repressive state of chromatin. The interplay between acetylation and phosphorylation mark at histone H3 amino tail can be given as an example for the transcriptionally active state because it was identified to induce the immediate early gene expression (Barratt, Hazzalin and Mahadevan 1994).

Phosphoacetylation is not only combinatorial mark existing at histone H3 tail. Our group could verify the existence of a triple modification mark at that histone H3 tail by mass spectrometry analysis. This triple modification bearing a repressive and two active marks at same time was used as a hypothesis to explain the transcriptional regulation of the HDAC1 gene by 14-3-3 proteins (Winter, Simboeck and Fischle, et al. 2008). This hypothesis and the potential role of this multiple histone modification will be discussed further in the chapter 1.3.1.

## 1.3 14-3-3 Proteins

Members of 14-3-3 protein family have been found in various tissues and play an important role in the signal transduction (Aitken 1995). They are highly acidic and are found abundantly expressed in the brain tissue. They consist of seven isoforms with a molecular weight of 27- 30kDA. All isoforms show sequence similarity, but none of them is identical with other one (Ichimura, et al. 1988). They exist in the cell as homo- or heterodimers that is formed by their N-terminal domains (Jones, Ley and Aitken 1995). 14-3-3 proteins are “phosphoserine/-threonine binding” proteins and two binding motifs have been identified: RSXpSXP (Muslin, et al. 1996) and RXXXpSXP, where p indicates the phosphorylated serine residue (Yaffe, Rittinger, et al. 1997). The proteins that 14-3-3s interact with are important players in a variety of cellular processes like cell cycle regulation, transcription, protein synthesis etc (Yaffe and Smerdon 2001). Due to their homo- or hetero-dimerization, they are also able to mediate the scaffolding within the protein complexes (Tzivion, Shen and Zhu 2001). The isoforms of the 14-3-3 proteins show specificity for target proteins. The binding of non-phosphorylated proteins which have been also revealed as interacting partners of 14-3-3s is also isoform-specific (Aitken 2002, Aitken, Baxter, et al. 2002)

More than hundred interaction partners have been identified for 14-3-3 proteins. For example, p53, a tumor-suppressor protein, was identified to be an interacting partner of 14-3-3s (Waterman, et al. 1998). The deacetylases HDAC4 and HDAC5 have been also

revealed as interaction partners. The interaction between HDAC4/ HDAC5 and 14-3-3 proteins inhibits the translocation of these proteins to the nucleus due to the sequestration. Hence, 14-3-3 proteins are supposed to be negative regulators of the HDAC4 and HDAC5 activity (Grozinger and Schreiber 2000, Wang, et al. 2000, Bertos, Wang and Yang 2001).

### 1.3.1 The Role of 14-3-3 Proteins in The Gene Expression

Similar to bromodomain proteins, which recognize the acetylated histones (Zeng and Zhou 2002) and chromodomain proteins, which recognize the methylated histones (Lachner, O´Carroll, et al. 2001), 14-3-3 proteins are the detectors for the phosphorylation mark on histones what makes the histones interaction partners of 14-3-3 proteins. First hint regarding the interaction between histones and 14-3-3 proteins was gained after cross-linking experiments that showed that the 14-3-3 binding inhibits the dephosphorylation of histones (Chen and Wagner 1994). Later, the group of Mahadevan showed the binding of 14-3-3 isoforms ε, γ and ζ to phosphorylated serine10 on histone H3 (Macdonald, et al. 2005). Importantly, recruitment of the 14-3-3 proteins to the nucleosomes associated with the IE genes coincides with serine10 phosphorylation on histone H3 during the transient activation of transcription of these genes. Besides the binding of 14-3-3 isoforms to histone H3, Mahadevan and his colleagues also showed that the acetylation of lysine 9 and 14 on histone H3 does not inhibit the binding of the 14-3-3 (Macdonald, et al. 2005). Later, it was observed by Winter et al. that the acetylation mark on lysine14 of histone H3 even increases the binding of 14-3-3 proteins to phosphorylated histone H3 and two 14-3-3 isoforms ε and ζ specifically recognize the phosphoserine on histone H3. In addition to the proof of 14-3-3 binding to phosphoacetylated histone H3, the RNAi experiments performed by Winter et al. suggest that 14-3-3 proteins are required for the transcriptional induction of the HDAC1 gene. These results assume the fact that the 14-3-3 proteins can be mediators of the signal transduction pathways leading to phosphoacetylation and subsequent nucleosomal response (Winter, et al. 2008).

Experiments performed in yeast also verified the interaction of 14-3-3 proteins with phosphoacetylated histone H3 and the recruitment of Bmh1, a yeast homologue of 14-3-3 proteins, to the promoter of Gal1 gene. Like HDAC1 gene, the Gal1 gene also required the binding of 14-3-3 proteins to its promoter, which is associated with phosphoacetylated histones, to be transcriptionally activated (Walter, et al. 2008). The

results from these two groups indicate that the dual modification mark on histone H3 is recognized by 14-3-3 proteins and this interaction is necessary to activate specific target genes.

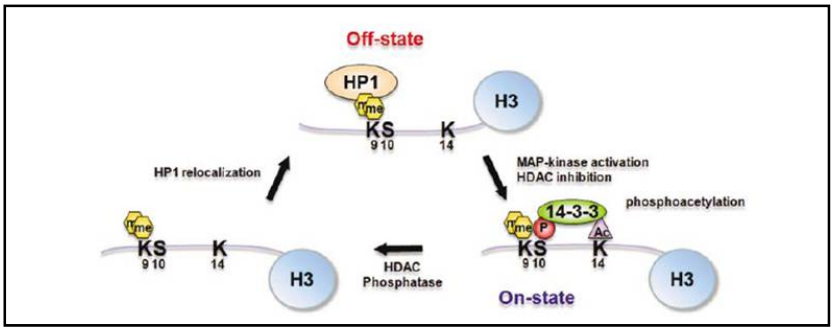



**Fig. 2: The model for the role of 14-3-3 proteins in the regulation of expression of HDAC1 gene.** In the off-state, promoter-associated histone H3 is bound by HP1 on the dimethylated lysine9. In the on-state, the binding of 14-3-3 to the phosphoacetylated histone H3, which is mediated by activation of MAP-kinase and inhibition of HDACs causes to dissociation of HP1 (Winter, Simboeck and Fischle, et al. 2008).

Based on the results obtained from the study on the transcriptional activation of the HDAC1 gene (Winter, Fischle and Seiser 2008) the following model has been proposed (Fig. 2).

Dimethylated histone H3 is constitutively associated with the HDAC1 promoter. In the absence of stimulating signals, HP1 recognizes the dimethylation mark of lysine9 on histone H3 and mediates repression of the HDAC1.Upon stimulation of the MAP kinase pathway or p38 stress pathways the serine10 phosphorylation of histone H3 takes place and leads to dissociation of HP1. Alternatively, phosphoacetylation is required for HP1 dissociation (Mateescu, et al. 2004). Treatment with HDAC inhibitors results in stable H3 phosphoacetylation and thereby in creation of a triple modification mark H3K9me2S10phK14ac and stable 14-3-3 recruitment. Importantly, in this setting 14-3-3 association can occur in the presence of K9 dimethylation. Thus, the change between the proteins HP1 and 14-3-3 does not impact the K9me2 mark on histone H3 (Winter, Simboeck and Fischle, et al. 2008). In accordance with this model, mass spectrometry analysis has demonstrated the existence of the triple modification of histone H3 in stimulated cells. The binding of the 14-3-3 proteins to the phosphoacetylated histone H3 is required for the transcriptional activation of the HDAC1 gene. Therefore, it can be assumed that the 14-3-3 proteins are able to turn the repressive state into an active state which causes a transcriptional activation (Winter, Simboeck and Fischle, et al. 2008).

## 1.4 Aim of Work

The aim of the work presented here was to clarify the role of regulation of transcription by a dual modification mark at histone H3. It has been shown by various groups that the transcriptional activation of a small number of genes in response to stress or growth factors is associated with S10/K14 phosphoacetylation at the histone H3 tail. First, we asked, which potential target residues within the histone H3 tail are subject to stress-induced phosphorylation in mouse fibroblasts. Furthermore, we asked whether there is a more general role of H3 phosphoacetylation in the regulation of stress-inducible genes. To this end, data obtained from expression arrays done to identify potential target genes had to be validated. Potential target genes were tested for their response to activation by stress inducer anisomycin, the HDAC inhibitor TSA or combination of both stimuli. Finally, the conditions for a genome-wide mapping (ChIP-Seq) of the H3-S10phK14ac phosphoacetylation mark on entire mouse genome should be established.

The second aim of the work was to establish a system for the identification of potential interaction partners of 14-3-3 proteins that act as detectors for histone H3 phosphorylated at serine10 and mediate the transcriptional induction of some target genes. The set-up of cell lines producing TAP-tagged 14-3-3ζ protein could be used for screens for interaction partners and for a genome-wide analysis of the distribution of 14-3-3 in stress induced cells.

# 2 MATERIALS AND METHODS

## 2.1 Tissue Culture

### 2.1.1 Cell Lines

The cell lines used in this study were mouse Swiss 3T3 fibroblasts, the human embryonic kidney cell line 293 and the human cervix carcinoma cell line- HeLa.

### 2.1.2 Media and Solutions

DMEM (Dulbecco's Modified Eagle Medium) was supplemented with antibiotics (AB) and 10% fetal calf serum.

| **10x PBS** | |
|---|---|
| NaCl | 80g/l |
| KCl | 2g/l |
| $Na_2HPO_4$ | 16g/l |
| $KH_2PO4$ | 2g/l |
| pH7.4 | |

| **100x AB** | |
|---|---|
| Penicillin G potassium salt | 6g/l |
| Streptomycin sulfate | 10g/l |

| **TE** | |
|---|---|
| Trypsin 2.2 U/mg | 700mg |
| 10x PBS | 25ml |
| 1% Na-EDTA pH7.4 | 5ml |
| adjust to 250ml | |

### 2.1.3 Thawing of Cells

10ml medium was put in a 10cm Petri dish and saturated with 7.5% $CO_2$ by incubating at 37°C for 20 minutes. The cells stored in liquid nitrogen were thawed in a water bath (37°C) for several minutes and transferred into with 7.5% $CO_2$ into the medium.

### 2.1.4 Culture and Splitting of Cells

The cells were cultured in 15cm Petri dish supplied with 20ml DMEM medium under at 37°C in an atmosphere of 7,5% $CO_2$. When the cultured cells reached a proper density, they were split. For the splitting, they were washed twice with 1x PBS and incubated with a proper amount of TE at 37°C for several minutes. The cells in TE were resuspended with medium and split into new Petri dishes containing fresh DMEM medium.

### 2.1.5 Transfection of Cells with TAP- tagged 14-3-3ζ Construct

The transfection was performed with the Lipofectamine 2000 according to the manufacture's protocol. The cells were incubated with the vector for 2-3 hours and the medium was changed to transfection medium, DMEM, 10% FCS, AB, neomycin (50mg/ml).

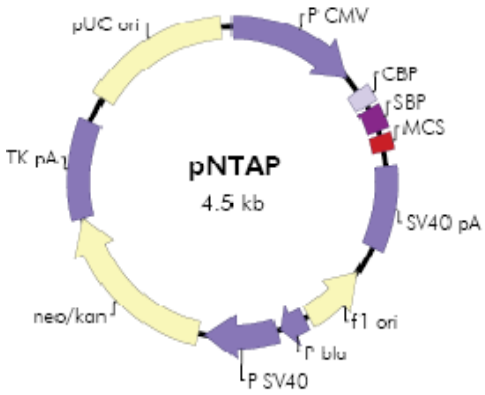



**Fig. 3: Map of expression vector** (InterPlay Mammalian TAP System, Instruction Manual, Ref 117)

### 2.1.6 Freezing of Cells

The cells were washed with 1x PBS twice and were incubated with some drops of TE at 37°C. After the trypsination, they were resuspended with 10ml medium and centrifuged at 1200rpm for 5 minutes. The freezing medium was prepared (90% FCS, 10% DMSO). The pellet was resuspended in freezing medium and the suspension was distributed into vials (cryo-tube). The cells were incubated on ice for 30 minutes and stored at -80°C for two days. Then, they were transferred to liquid nitrogen for long storage.

## 2.2 RNA

### 2.2.1 Isolation of Total RNA

The cells were washed with ice-cold 1x PBS and harvested. After centrifugation at 4°C 1200rpm for 4 minutes, the pellet was resuspended in 1ml TRIZOL®Reagent (*GibcoBRL*) and centrifuged again at 4°C 14000rpm for 10 minutes. The supernatant was transferred into a new *Eppendorf* tube and incubated at RT for 5 minutes. 200µl chloroform was added and the sample was shaken vigorously for 15 seconds, followed by incubation at RT for 3 minutes. After centrifugation at 4°C 14000rpm for 15 minutes, the upper phase was transferred into a new *Eppendorf* tube and supplied with 500µl isopropyl alcohol, followed by incubation at RT for 10 minutes. The sample was centrifuged at 4°C 14000rpm for 10 minutes and the supernatant was discarded. The pellet was washed with 1ml of 75% ethanol and centrifuged at 4°C 14000rpm for 5 minutes. The pellet was air-dried and dissolved in sterile water. After incubation at 58-60°C for 10 minutes, the RNA was out on ice, and a 2.5 fold volume 96% ethanol and 0.1 volume 3.2M NaOAc were added to the RNA solution. The RNA was precipitated at -20°C for at least 2 hours and collected by centrifugation at 4°C 14000rpm for 30 minutes and washed, centrifuged as described before. The extracted RNA was resuspended with sterile water and the concentration of the isolated total RNA was measured at 260nm on a Nano-Drop spectrophotometer. The RNA concentration was adjusted to 1µg/µl by diluting with $H_2O$. The RNA is supplied with the RNA sample buffer and loaded on a MOPS/EDTA agarose gel to check the quality and concentration of the RNA. The RNA was stored at -20°C.

### 2.2.2 RNA Separation on MOPS/EDTA Agarose Gel

The MOPS/EDTA agarose gel was prepared. The RNA supplied with the RNA sample buffer was heated up to 65°C for 5 minutes before loaded on the gel. The gel was run at 70V. The samples on the gel were visualized under the UV light.

| **10x MOPS/EDTA** | |
|---|---|
| MOPS | 200mM |
| Na-Acetate | 50mM |
| EDTA | 10mM |
| pH 7,0 | |

| **RNA Sample Buffer (-20°C)** | |
|---|---|
| Formamide | 15ml |
| 10x MOPS | 3ml |
| 37% Formaldehyde | 4,8ml |
| $H_2O$ (RNAse free) | 2ml |
| Glycerol | 2ml |
| 10% (w/v) Bromphenol blue | 1,6ml |
| (5µl ethidium bromide) | |

| **MOPS/EDTA Gel** | | |
|---|---|---|
| Agarose | 0,6g | 1,2g |
| $H_2O$ | 42,5ml | 100ml |
| 10x MOPS | 5ml | 10ml |
| 37% Formaldehyde | 2,5ml | 5,2ml |

### 2.2.3 cDNA Synthesis

The RNA was reverse transcribed into the cDNA by using the iScript$^{TM}$cDNA synthesis kit (*Biorad*).

| **Reaction Mix** | |
|---|---|
| 5x iScript reaction Mix | 4µl |
| iScript Reverse Transcriptase | 1µl |
| Nuclease-free water | xµl |
| RNA Template | xµl |
| Total Volume | 20µl |

| **Reaction Condition** | |
|---|---|
| 25°C | 5´ |
| 42°C | 30´ |
| 85°C | 5´ |
| 4°C | ∞ |

### 2.2.4 Real Time PCR Analysis

5µl of cDNA was used to perform *SYBER* green Real Time PCR. The results were analyzed by *Biorad* iCycler iQ system.

| Gene | ID | Sequence | Temp. (°C) |
|---|---|---|---|
| p21 | mRT-p21 for | TCCAGACATTCAGAGCCACAG | 60-62 |
| | mRT-p21 rev | GAGACAGCCCGCCATGAG | |
| HDAC1 | mRT-HDAC for | AAGCAGCAGACGGACATCG | 60-62 |
| | mRT-HDAC rev | GCCTCTTCCACGCCATCG | |
| c-fos | mRT-c-fos for | ATCTGTCCGTCTCTAGTG | 57 |
| | mRT-c-fos rev | GCTTGGAGTGTATCTGTC | |
| Egr1 | mRT-Egr1 for | GGAGATGATGCTGCTGAG | 57 |
| | mRT-Egr1 rev | GAAGAGGTCGGAGGATTG | |
| PAI1 | mRT-PAI1 for | ACCGAGGACACGCCATAG | 58 |
| | mRT-PAI1 rev | ACAGCCAACAAGAGCCAATC | |
| Timp3 | mRT-Timp3 for | TTCACACGGAAGCCTCTG | 56-57 |
| | mRT-Timp3 rev | GCGTTGCTGATGCTCTTG | |
| OPN | mRT-OPN for | AACCAGCCAAGGACTAACTAC | 58 |
| | mRT-OPN rev | CATCGTCATCATCATCGTCATC | |
| uPA | mRT-uPA for | GGAAGCCTTGTTGGTCTAC | 57-58 |
| | mRT-uPA rev | CAGAACGGAGGTGTATGC | |
| Gels | mRT-Gels for | GGTTGCTGCTTCTGCCATC | 57-61 |
| | mRT-Gels rev | GGTCTTCTCCGCCTCACTG | |
| PTH | mRT-PTH for | GCTGGCAGTCTGTCTTCTTACC | |
| | mRT-PTH rev | TCTTGGTGGGCTTCTGGTGAC | |
| CD44 | mRT-CD44 for | CTCTTAGGTCACTCACTCTTTC | 57 |
| | mRT-CD44 rev | GTAGCGGCAGGTTACATTC | |
| Ifi44 | mRT-Ifi44 for | AATGCTCCAACTGACTGCTCGC | 55;61-65 |
| | mRT-Ifi44 rev | CTGGTGTGTGATGCTGCCCTTG | |
| Rnf44 | mRT- Rnf44 for | GCCGCCGCCACCATCTTACTAC | 55-65 |
| | mRT- Rnf44 rev | CAGCCGCTCAGCCAGGTTCAG | |
| Map2K7 | mRT-Map2k7 for | GCCGCTCACCATCCTCAG | 55-64 |
| | mRT-Map2k7 rev | ACCGCATCTTCCACACCTG | |
| Tor1aip | mRT-Tor1aip for | ACTCTCCGTCCTCCAGTG | 55-58 |
| | mRT-Tor1aip rev | CATCTTCGGTTTCTCCTTCTTC | |
| Pygo | mRT-Pygo for | CAGGAGGAGGACCCAGAG | 55-58 |
| | mRT-Pygo rev | GGCTTCCGAGTTGTATATTGG | |
| Acrbp | mRT-Acrbp for | GCAGCCCTCTCTCGTCCAC | 55-65 |
| | mRT-Acrbp rev | TCGGAGCAGACAGCACCATC | |
| Junb | mRT-Junb for | AACCTCAGCAGTTACTCC | 61 |
| | mRT-Junb rev | GTCTTCACCTTGTCCTCC | |
| Il6 | mRT-Il6 for | GTTCTCTGGGAAATCGTGGA | 60 |
| | mRT-Il6 rev | TGTACTCCAGGTAGCTATGG | |
| HPRT | mRT-HPRT for | GCTGGTGAAAAGGACCTCT | 60-62 |
| | mRT-HPRT rev | CACAGGACTAGAACACCTGC | |

## 2.3 PROTEINS

### 2.3.1 Isolation of Total Protein Extracts

The DMEM medium was aspirated from cultured cells and the cells were washed with 10ml of PBS, harvested in 10ml of PBS, followed by centrifugation at 4°C 1200 rpm for 5min. The cell pellet resuspended in 1ml PBS was transferred into 1.5ml *Eppendorf* tube and spun shortly down. The pellet was resuspended in suitable volume of Hunt Buffer supplied with protease inhibitors (Complete; *Roche*). It was frozen in liquid nitrogen shortly and thawed at 37°C. This step was repeated four times. After last thawing, the lysate was centrifuged at 4°C 14000rpm for 30min. The supernatant containing the proteins was transferred into a new *Eppendorf* tube and the protein was kept at -20°C.

| **Hunt Buffer** | |
|---|---|
| Tris-HCl pH 8.0 | 20mM |
| NaCl | 100mM |
| EDTA pH 8.0 | 1mM |
| NP-40 | 0.50% |

### 2.3.2 Histone Isolation

The medium of the cells was sucked off and the cells were washed once with 10ml of PBS, harvested in additional 10ml of PBS, centrifuged at 4°C 1200 rpm for 5min. The cell pellet was resuspended in 1ml of PBS and transferred into 1.5ml *Eppendorf* tube. Cells were collected by centrifugation at 4°C 2200rpm for 4min and resuspended in 1ml lysis buffer. This step was performed three times. The pellet was resuspended with 1ml wash buffer and centrifuged again. The nuclear pellet was resuspended in 100µl $H_2O$ supplemented with 2µl $H_2SO_4$ (0.4N) and incubated on ice for 1h. After centrifugation at full speed at 4°C, for 20 min, the supernatant was transferred into new 1.5ml *Eppendorf* tube and the histones were precipitated with 10-fold acetone overnight at -20°C. The next day, the precipitates were collected by centrifugation at full speed at 4°C for 20 minutes. The pellet was air-dried and resuspended in a suitable volume of $H_2O$.

| **Lysis Buffer** | |
|---|---|
| Tris | 100mM |
| Na-bisulfite | 50mM |
| $MgCl_2$ | 10mM |
| Na-Butyrate | 10mM |
| Triton X-100 | 1% |
| Sucrose | 8.60% |
| Adjust pH to 6.5 | |

| **Wash Buffer** | |
|---|---|
| Tris | 100mM |
| $Na_3EDTA$ | 13mM |
| Adjust pH to 7.4 | |

### 2.3.3 Isolation of Mitotic Histones

Cultured cells were incubated with nocodazole (1µM) for 20 hours. The cells were washed with the medium and scraped from the Petri dish by using a rubber policeman. The harvested cells were centrifuged at 4°C 1200rpm for 5 minutes. The pellet was washed with PBS. For the FACS analysis, the cells were added drop wise into 5ml of 80% ethanol under vortexing. After centrifugation again at 4°C 1200rpm for 5 minutes, the pellet was resolved in 100µl $H_2O$ supplied with 2µl $H_2SO_4$ and incubated on ice for 1 hour, followed by centrifugation at full speed at 4° for 20 minutes, the supernatant was transferred into a new *Eppendorf* tube and the histones were precipitated with 10-fold volume of acetone O.N. at -20°C. Next day, the precipitated histones were centrifuged at 4°C 140000rpm for 20 minutes, air-dried and resuspended with $H_2O$. The mitotic histones were kept at -20°C.

### 2.3.4 Determination of Protein Concentration

The proteins were diluted 1:1000 in Bio-Rad Protein Assay reagent which was already diluted 1:5. The concentration of proteins was measured at 595nm by using the Spectrophotometer. An $OD_{595}$ of 1 was equal to 10µg of protein.

### 2.3.5 SDS-PAGE

The SDS- Polyacrylamide gel electrophoresis was performed at 25mA.

| **Acrylamide Stock** | |
|---|---|
| Acrylamide | 30% |
| N,N´-methylene-bisacrylamide | 0.8% |

| **Separation Gel (16%)** | |
|---|---|
| 30% AA | 3.73ml |
| 1M Tris pH 8.8 | 2.6ml |
| $H_2O$ | 560µl |
| 20%SDS | 35µl |
| 20%APS | 56µl |
| TEMED | 5µl |

| **Stacking Gel** | |
|---|---|
| 30% AA | 510µl |
| 1M Tris pH 6.8 | 375µl |
| $H_2O$ | 2.1ml |
| 20%SDS | 15µl |
| 20%APS | 28µl |
| TEMED | 7.5µl |

| **SDS Sample Buffer** | |
|---|---|
| Tris-HCl pH 8.8 | 100mM |
| Glycerol | 20% |
| Bromphenol Blue | 0.01% |
| DTT | 200mM |
| SDS | 4% |

| **10x Running Buffer** | |
|---|---|
| Tris | 50mM |
| Glycine | 500mM |
| SDS | 0.2% |

### 2.3.6 Western Blot

The SDS-PAGE was performed to separate the proteins according their size. 2 sheets of 3MM filter papers were soaked in Anode I buffer, 2 in Cathode buffer und one sheet of 3MM filter paper and a nitrocellulose membrane were soaked in Anode II buffer. 2 sheets of 3MM (Anode I), 1 sheet of 3MM (Anode II) and the membrane were put on the transfer machine. The separation gel made wet in Cathode buffer was placed on the membrane and 2 sheets of 3MM (Cathode) were out on it. The transfer was performed at 200mA in a cold room for 40 minutes. After transfer, the membrane was stained with Ponceau for 1min and washed with distilled water.

| **Anode I (1l)** | |
|---|---|
| 0.3M Tris | 36.3 g |
| 20% Methanol | 200ml |
| Adjust pH to 10.4 | |

| **Anode II (1l)** | |
|---|---|
| 25mM Tris | 3.03 g |
| 20% Methanol | 200ml |
| Adjust pH to 10.4 | |

| **Kathode (1l)** | |
|---|---|
| 25mM Tris | 3.03 g |
| 40mM 6-Aminohexanoic-Acid | 5.25 g |
| 20% Methanol | 200ml |
| Adjust pH to 9.4 | |

| **10x Ponceau S** | |
|---|---|
| Ponceau S | 2% |
| Trichloracetic acid | 30% |
| Sulfosalicylic acid | 30% |

### 2.3.7 Dot Blot

The concentrations of the peptides modified on different amino acid residues were equalized and they were spotted on a nitrocellulose membrane. The membrane was dyed with Ponceau to check the presence of peptides and the equal concentrations.

### 2.3.8 Antibody Incubation

To prevent any unspecific binding with the antibodies, the membrane was blocked with blocking solution for 1h at RT under gentle agitation. After blocking, it was incubated with the primary antibody diluted in blocking solution at 4°C overnight. The next day, the blot was washed three times with 1x PBS supplemented with 0.1% Tween-20, for 10min each, at RT. The secondary antibody coupled to peroxidase was diluted in 1x PBS/ 0.1% Tween-20 and the incubation with the secondary antibody performed at RT for 1h under gentle agitation. The blot was washed again three times with the 1x PBS, 0.1% Tween-20.

| **Blocking Solution (1 l)** | |
|---|---|
| Milk powder | 1% |
| PVP | 1% |
| Tween20 | 0.1% |
| $NaN_3$ | 0.02% |
| PBS | 1x |
| Adjust pH to 7.4 | |

### 2.3.9 Immuno-Detection by ECL (Enhanced Chemo-Luminescence)

The ECL solution 1 and 2 were mixed equally and the blot was incubated with this mixture for 1min. The blot was packed in Saran wrap and developed to X-ray films for several minutes.

### 2.3.10 Western Blot Stripping

The blots have to be stripped to get rid of the antibodies used before. The blot was washed three times with 1x PBS/ 0.1% Tween-20 and incubated with the stripping buffer at 50°C for 30 minutes. The blot was washed again three times with 1x PBS/ 0.1% Tween-20. It had to be blocked again, before it was used for the incubation with a new primary antibody.

| **Stripping Buffer** | |
|---|---|
| 20% SDS | 10ml |
| 1M Tris-HCl pH 6.7 | 6.25ml |
| ß-Mercaptoethanol | 700µl |
| $H_2O$ | 83.05ml |

### 2.3.11 Immunoprecipitation

The proteins were isolated from the cultured cells as described in 2.3.1 and the protein concentration was determined as described in 2.3.4. A proper amount of proteins for the immunoprecipitation was supplied with Hunt buffer to a volume of 600µl and the tagged- proteins were bound to the appropriate beads by rotating at 4°C O.N. They were spun down at 1000rpm for 1 minute. The supernatant was kept as control. The proteins bound to the beads were washed once with each Hunt, RIPA and Hunt buffer, followed by centrifugation at 1000rpm for 1 minute. The proteins were eluted from the beads in SDS- sample buffer by heating up to 95°C for minutes and spun down shortly. They were used directly to load a polyacrylamide gel.

| **RIPA (150mM)** | |
|---|---|
| 4M NaCl | 15ml |
| 1M Tris pH 8.0 | 20ml |
| 10% SDS | 4ml |
| 10% NaDoc | 20ml |
| 10% NP40 | 40ml |
| Adjust to 400ml | |

### 2.3.12 Indirect Immunofluorescence experiments

The cells grown in on coverslips were washed once with 1x PBS and fixed with 1% PFA for 5 minutes. They were incubated with 2% PFA/ 0.1% Triton for 15 minutes to make the membrane permeable, followed by washing three times with 1x PBS for 2 minutes. The cells were incubated with the primary antibody prepared in 1x PBS/ 10% goat serum at 4°C O.N. The next day, they were washed three times with 1x PBS for 2 minutes and incubated with the fluorescence-labeled secondary antibody prepared in 1x PBS/ 10% goat serum at least for 45 minutes at RT. After washing three times with 1x PBS for 2 minutes, the coverslips were put upside-down onto the slides provided with (10-15µl) DAPI in Vectashield to stain the DNA. They were dried O.N. and fixed they on the slides with nail polisher, next day.

### 2.3.13 Affinity Purification of Antibodies

For the affinity purification of antibodies, the beads coupled to modified peptides with which the rabbits were immunized to produce antibodies were equilibrated with PBS. 1ml of serum supplied with phosphatase and protease inhibitors were incubated with beads coupled to modified peptides at 4°C o.n. on a shaker or roller. After centrifugation at 2000rpm for 1 minute, the supernatant was kept as control, and the antibodies bound to the beads were washed five to ten times with PBS. The antibodies were eluted from the beads three to five times with 100mM glycine pH 2-3. The eluted antibodies were neutralized with 1M Tris pH 8 and supplemented with BSA and 0.2% $NaN_3$. The beads were washed three to five times with PBS and neutralized to the pH of buffer and supplied with 0.2% $NaN_3$.

## 2.4 Chromatin Immunoprecipitation

### 2.4.1 Solutions

| **Wash I** | |
|---|---|
| 10% Triton X-100 | 10ml |
| 0.5M EDTA | 8ml |
| 0.5 EGTA | 400µl |
| 1m HEPES | 4ml |
| Adjust to 400ml | |

| **Wash II** | |
|---|---|
| 4M NaCl | 20ml |
| 0.5M EDTA | 800µl |
| 0.5 EGTA | 400µl |
| 1m HEPES | 4ml |
| Adjust to 400ml | |

| **Lysis Buffer** | **10ml** |
|---|---|
| $H_2O$ | 8.5ml |
| Proteinase Inhibitor (50x) | 200µl |
| 1M Butyrate (100x) | 100µl |
| 20% SDS | 500µl |
| 0.5M EDTA | 200µl |
| 1M Tris pH 8.1 | 500µl |

| **Dilution Buffer** | |
|---|---|
| 20% SDS | 500µl |
| 10% Triton | 55ml |
| 0.5M EDTA | 1.2ml |
| 1M Tris pH8.1 | 8.5ml |
| 4M NaCl | 20.88ml |
| Adjust to 500ml | |

| **Elution Buffer** | |
|---|---|
| 20% SDS | 500µl |
| 1M NaHCO3 | 500µl |
| DTT | 50µl |
| Adjust to 5ml | |

| **RIPA** | |
|---|---|
| 4M NaCl | 15ml |
| 1M Tris pH 8.0 | 20ml |
| 10% SDS | 4ml |
| 10% NaDoc | 20ml |
| 10% NP40 | 40ml |
| Adjust to 400ml | |

| **Hi-Salt** | |
|---|---|
| 4M NaCl | 50ml |
| 1M Tris pH 8.0 | 20ml |
| 10% SDS | 4ml |
| 10% NP40 | 40ml |
| Adjust to 400ml | |

| **LiCl** | |
|---|---|
| 1M LiCl | 100ml |
| 1M Tris pH 8.0 | 20ml |
| 10% NaDoc | 20ml |
| 10% NP40 | 40ml |
| Adjust to 400ml | |

| **TE** | |
|---|---|
| 1M Tris pH 8.0 | 4ml |
| 0.5M EDTA | 800µl |
| Adjust to 400ml | |

### 2.4.2 Preparation of Sepharose Bead Suspension

Proper amount of the G-beads, already as slurry, was filled up to 2ml with TE and the A-beads were swollen in 2ml TE, followed by incubation on ice for 30 minutes. The beads were washed three times with TE, followed by centrifugation at 3000rpm for 2 minutes. Then, they were blocked with a mixture of 2µl salmon sperm DNA, 10µl BSA,

5µl $NaNa_3$, and 84µl TE (for 100µl) by rolling at 4°C for 20 minutes. After centrifugation at 2000rpm for 3 minutes, the beads were stored at 4°C.

### 2.4.3 Formaldehyde Fixation and Isolation of Soluble Chromatin

The DMEM medium in the culture dish was supplemented with 540µl formaldehyde and incubated for 10 minutes under gentle agitation to crosslink the chromatin. The cross-linking was stopped with 1ml of 2.5M glycine by incubating for 5 minutes under gentle agitation. The cells were washed with 5ml of PBS twice and harvested in PBS by using a rubber policeman. They were centrifuged at 4°C 12000rpm for 5 minutes. PMSF was added to the freshly prepared Wash I and Wash II solutions. The pellets were resuspended with 1ml of Wash I and supplied with additional 4ml of Wash I, followed by incubation for 10 minutes on ice. After centrifugation at 4°C 1200rpm for 5 minutes, the pellets were resuspended with 1ml of Wash II and additional 4ml of Wash II was added. After incubation for 10 minutes on ice followed by centrifugation at 4°C 1200rpm for 5 minutes, the pellets were resuspended with 2ml of lysis buffer containing PMSF and the suspension were transferred into 2ml *Eppendorf* tubes. The chromatin was sonicated at 45% output and 90% dot cycles to achieve a proper fragment size for the further steps of experiment. After sonication, the lysate was centrifuged at 14000rpm 8°C 15 minutes twice. The supernatant was transferred into new *Eppendorf* tubes. The OD was measured by Nano-Drop by using two blanks, water and lysis buffer. The concentrations were calculated according to the amount that will be used for the immunoprecipitation. The samples were brought to equal volume with lysis buffer, whereas the input was prepared in a half volume. They were kept at 4°C.

### 2.4.4 Immunoprecipitation and Isolation of DNA Fragments

The dilution buffer was supplied with PI, 1M Butyrate and PMSF. The chromatins and inputs were diluted 10-fold in dilution buffer. To preclear the chromatin, 30µl of A/G-beads were added and incubated by rotating at 4°C at least for 2h. After centrifugation at 2000rpm 4°C for 5 minutes, the supernatant was incubated with the antibody by rotating at 4°C O.N. Next day, 30µl beads were added to harvest the immunoprecipitates by rotating at 4°C for 4-5 hours. After spinning down at 2000rpm 4°C for 3 minutes, the immunoprecipitates bound to the beads were washed with the each RIPA, HiSalt, LiCl, and TE buffer twice.

The immunoprecipitates were eluted with 200µl elution buffer by rocking at 14000rpm RT for 15min twice. The volume of input was adjusted to 400µl with elution buffer. The supernatant was transferred into new *Eppendorf* tubes and provided with 20µl NaCl to reverse the cross link. After incubation with NaCl at 300rpm 65°C O.N, they were incubated with a mixture of 8µl 0.5M EDTA, 16µl 1M Tris pH6.5, and 2µl Proteinase K at 300rpm 5°C for 1.5 hours to digest the proteins.

The DNA was extracted with 600µl PCI, a mixture of phenol-chloroform-isoamylalcohol and vortexed well. After centrifugation at 14000rpm 4°C for 15 minutes, the aqueous phase was transferred into new Eppendorf tube and the DNA was precipitated with 40µl 3M NaOAc, 1µl Glycogen, and 1ml 96% EtOH at -20°C for at least 30 minutes. After centrifugation at 4°C 14000rpm for 30 minutes, the DNA was washed with 1ml 70% ethanol and the pellet was air-dried and resolved in were 100µl $H_2O$. The DNA was then incubated with 1µl RNase at RT for 30 minutes.

### 2.4.5 Amplification of DNA Fragments by PCR

The immunoprecipitated DNA fragments of chromatin and the inputs diluted 1:20 or 1:40 were amplified by a PCR reaction. The primers used for the PCR were designed according to the gene of interest. A 2% agarose gel was performed with 20µl of PCR products at 100V.

| Gene | ID | Sequence | Temp. (°C) |
|---|---|---|---|
| p21 Seq | A for | CGACCTTGAATGCCTATTTC | 56 |
| | B rev | TTGCCTAACTTGCTGGAAG | |
| | C for | CTGCGTGACAAGAGAATAGC | 56-58 |
| | D rev | CCACTCCTTCACCGATCC | |
| | E for | TGCCCGCCAGAGTCACAG | 56-62 |
| | F rev | CGAAGCCTTCAGCCACCAC | |
| | G for | TGTTTCGGTTGGTTGGTGTTTAGC | 56-62 |
| | H rev | ACAGCACTCGGGAGGCAGAG | |
| | 3 for | TCGGAGACCAGCAGCAAAATCG | 60 |
| | 4 rev | GCAGCCCCACCTCTTCAATTCC | |
| | 5 for | GCCAAGCCCTTCCCAGACTTC | 56-62 |
| | 6 rev | CTAGAGATCGCTGCCCAGACATG | |
| p21 | 3 for | GGATACCTTGCAAGGCTGCATC | 56-62 |
| | 4 rev | CTCCACCACCCTGCACTGAAGC | |
| | 5 for | CCAAGCCCTTCCCAGACTTCCA | 60 |
| | 6 rev | CTGGAGTCTTAGTTTGAACGGA | |
| OPN | 5 for | CGGGATTCTAAATGCAGTCTA | 56 |
| | 6 rev | CAAGCAAGGATGCTTCTTCAG | |
| HD1 | G for | TGTTCCTTGCCATTCATTTCTC | 57 |
| | H rev | CAGCCCGCTTCACAGTTG | |

# 3 RESULTS

## 3.1 Part I: The Role of Phosphoacetylation Mark on Histone H3 in The Transcriptional Regulation of Target Genes

Although only 2% of interphase histones are phosphorylated, the number of genes regulated by this process is increasing. First, it was shown for the immediate early genes (IE genes) c-fos and c-jun, that phosphorylation of serine10 on histone H3 can influence the gene expression concomitant with acetylation of the neighboring lysine14 on histone H3 (Barratt, Hazzalin and Mahadevan 1994, Winter, Simboeck and Seiser 2007) and the term "phosphoacetylation" was formed to specify this dual modification on histone H3. For a long time, it has been believed that this regulatory role of phosphoacetylation mark at histone H3 tail is restricted to IE genes. However, our group could show that the transcriptional activation of the late inducible gene, HDAC1, correlates with phosphoacetylation of histone H3 (Hauser, et al. 2002). These results gave birth to a new idea that there could be other genes regulated by the dual modification mark on histone H3. This idea was tested by a screen for potential target genes using expression arrays.

The next step was to test whether individual genes are transcriptionally regulated by phosphoacetylated histone H3 and are therefore putative targets of dual modification.

### 3.1.1 Working Model

Serine10 phosphorylation of histone H3 is mediated by Msk1 and Msk2 kinases activated by ERK and stress induced p38 pathway (Cheung, Allis and Sassone-Corsi 2000). While growth factors are responsible for the activation of ERK pathway, the stress induced p38 pathway can be activated by the drug anisomycin. In low dosage anisomycin activates the MAP kinase pathway without affecting protein synthesis (Edwards and Mahadevan 1992).

Reversible lysine acetylation of histones is controlled by HATs and HDACs that normally keep the acetylation equilibrium of histones which is constant in the cell. The acetylation equilibrium can be shifted into the direction of hyperacetylation by inhibiting histone deacetylases with trichostatin A (Yoshida, et al. 1990).

Treatment with TSA alone has no effect on H3 phosphorylation, whereas anisomycin treatment leads to transient serine10 phosphorylation of H3. In combination, the activation of Msk1/ 2 kinases and the inhibition of HDACs result in a rapid and stable phosphoacetylation of histone H3 (Hauser, et al. 2002).

It has been observed that transient phosphoacetylation of histone H3 – upon activation of MAP kinase pathway is sufficient to induce the transcriptional induction of immediate-early genes (Mahadevan, Willis and Barratt 1991). However, other genes such as a late inducible gene HDAC1 require a stable phosphoacetylation of histone H3 to be activated (Hauser, et al. 2002).

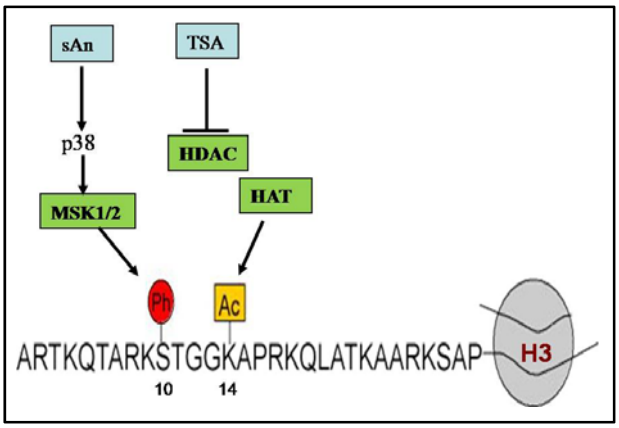



**Fig. 4: The model to achieve a stable and prolonged phosphoacetylation of histone H3.** Blue squares indicate the drugs and green squares represent the enzymes. MSK1/2, mitogen-stimulated kinase 1/2; HDAC, histone deacetylase; HAT, histone acetyltransferase.

Due to our aim to activate the nucleosomal response of target genes upon phosphoacetylation of histone H3, thus elucidating the role of this modification in transcription of target genes, we used both agents together in our experiments to achieve a stable phosphoacetylation mark at histone H3 tail (Fig. 4). Nearly all histone H3 molecules in mitotic cells are phosphorylated at serine10. Therefore, we arrested the fibroblasts by serum deprivation for up to 72 hours prior to drug treatment to avoid the mitotic phosphorylation of histones.

### 3.1.2 Characterization of System

Serines on position 10 and 28 and threonines on position 3 and 11 on N-terminal tails of histone H3 are strongly phosphorylated during mitosis (Polioudaki, et al. 2004). Besides the mitotic phosphorylation, serine10 of histone H3 is also found to be phosphorylated during the interphase. Stress or growth factor induced serine10 phosphorylation during interphase is suggested to play a role in transcriptional activation (Mahadevan, Willis and Barratt 1991) and is performed by the effector kinases of MAP kinase pathways

Msk1 and Msk2. These kinases are also responsible for phosphorylation of histone H3 at serine28 (Cheung, Allis and Sassone-Corsi 2000, Dyson, et al. 2005).
In contrast to serine phosphorylation, not much is known about the role of threonine phosphorylation of histone H3 in transcriptional activation or about the underlying mechanisms indicating how threonine phosphorylation is performed.
To test whether threonine phosphorylation is also activated in response to stress signaling, we analyzed in our system the specificity of histone H3 phosphorylation patterns upon TSA/anisomycin treatment.

#### 3.1.2.1 Serine and threonine residues on histone H3 show distinct phosphorylation patterns

As mentioned above, histone H3 can be also phosphorylated at threonine residues on position 3 and 11, besides serine residues on position 10 and 28. In order to see the specificity of the system for serine phosphorylation of histone H3, Western blot analysis was performed. Swiss 3T3 mouse fibroblasts were arrested by serum deprivation for 72 hours and treated either with anisomycin (188.5nm) alone or simultaneously with anisomycin (188.5nm) and TSA (165.5nM) for 0, 1, 3 and 6 hours. 2µg of isolated histones were loaded on a 16% polyacrylamide gel and analyzed onWestern blots. The phosphorylation state of histone H3 was analyzed by probing the Western blots with antibodies raised specifically against phosphorylated serine10, serine28, threonine3 and threonine11, and phosphoacetylated histone H3 (S10phK14ac) (Fig. 5A-E).
The activation of p38 stress pathway by anisomycin led to a transient increase in serine10 and serine28 phosphorylation as well as S10K14 phosphoacetylation of histone H3 with a peak at 1 hour (Fig. 5A i, B i and C i).
It was previously shown by our group that activation of p38 stress pathway by anisomycin and inhibition of HDACs by TSA led to increased phosphoacetylation levels of histone H3 when compared to the arrested, untreated cells (Hauser, et al. 2002). As shown in Figs. 5, additional treatment with TSA resulted in increase and stabilization of the S28ph and S10Kph14ac marks, while no induction could be observed for H3S10 phosphorylation. This is most likely due to impaired recognition of the H3S10ph mark upon additional K14 acetylation (Fig. 5A ii, B ii and C ii).

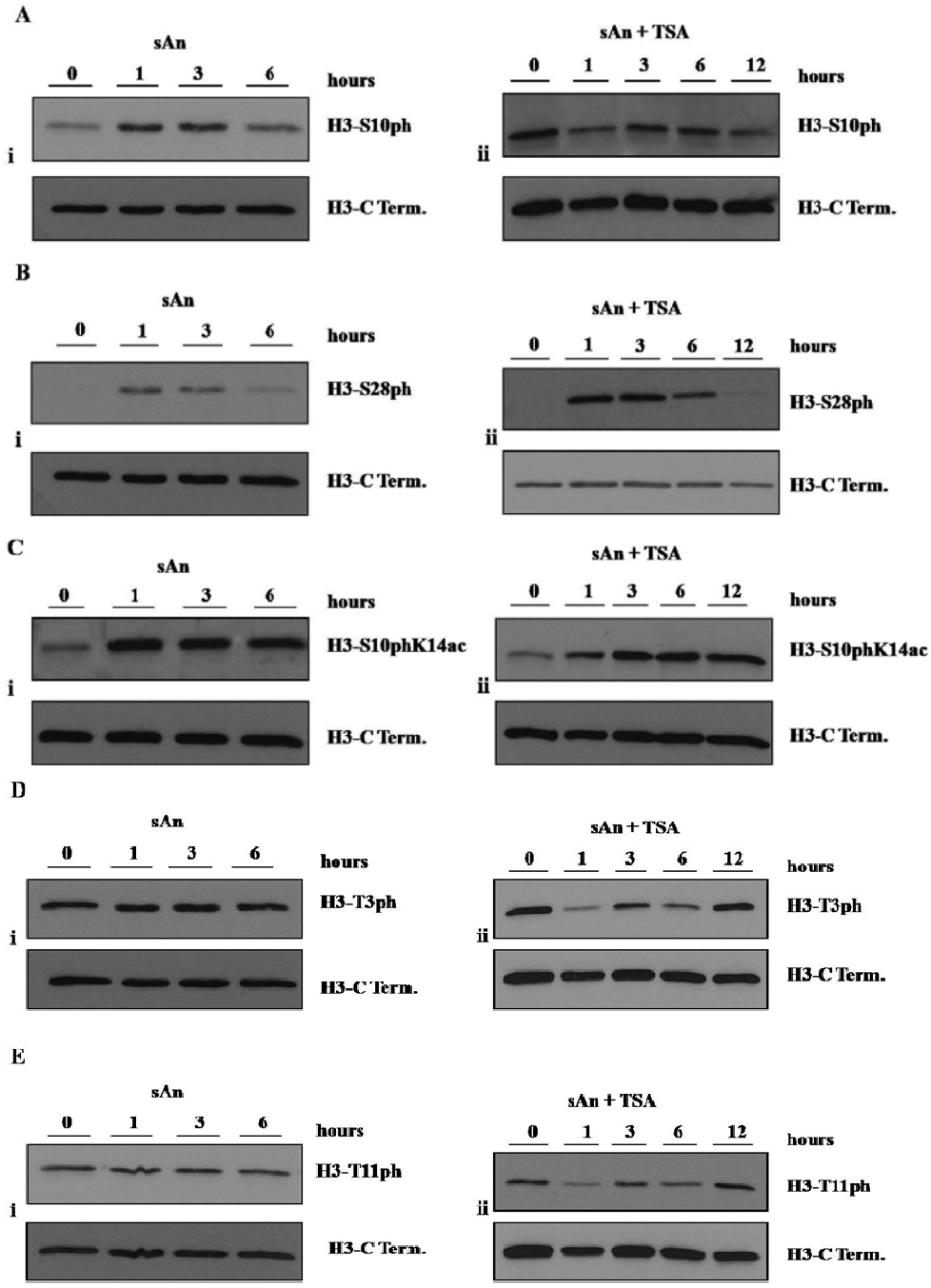


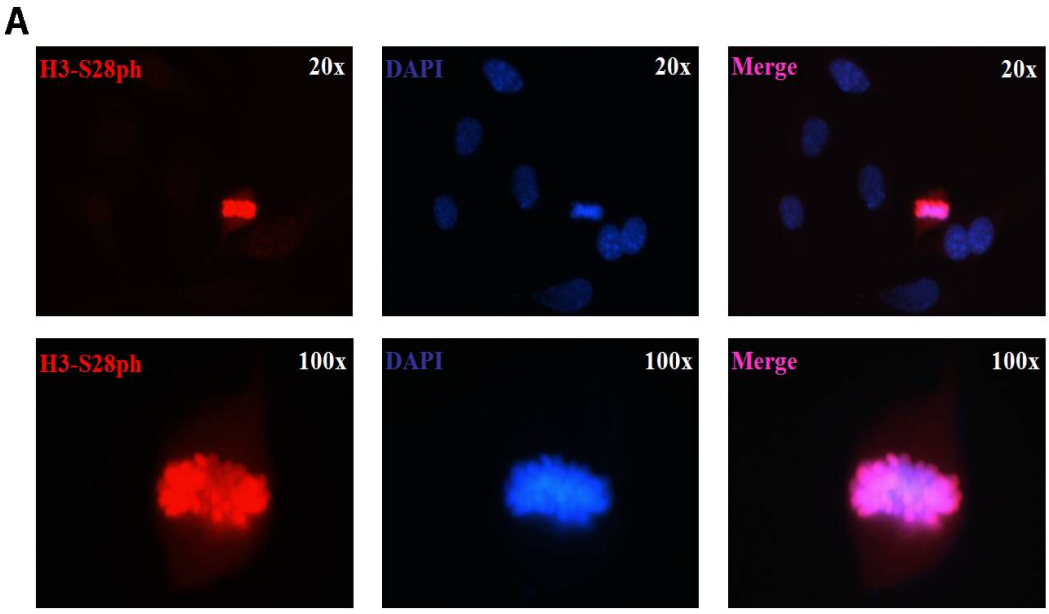

**Fig.5 : Different response of histone H3 to the single (sAn) and combine treatment (sAn/ TSA):** Swiss 3T3 mouse fibroblasts were serum-starved for 72 hours and treated either only with anisomycin (188.5nM) **(i)** or simultaneously with anisomycin (188.5nM) and TSA (165.5nM) **(ii)** in a time-course dependent manner. Histones were extracted from the cells and separated on a 16% SDS-PAGE. Different phosphorylation marks on histone H3 were tested by probing the Western blots with specific antibodies. The antibodies used here were: **(A)** histone H3S10ph, **(B)** histone H3S28ph, **(C)** histone H3S10phK14ac, **(D)** histone H3T3ph and **(E)** histone H3T11ph. The Western blots were re-probed with an antibody specific for the C-terminus of the histone H3 to verify the equal loading of histones.

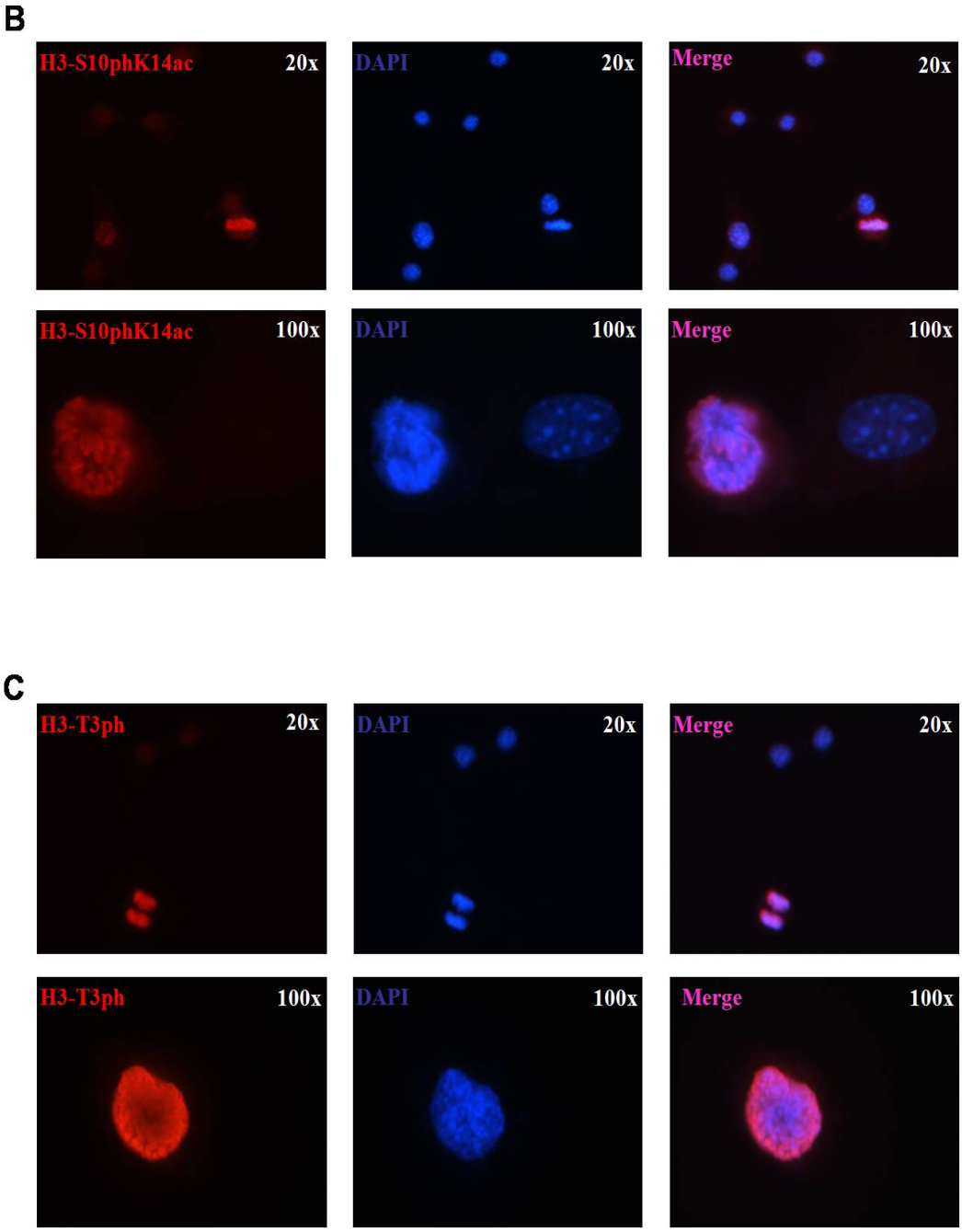
In contrast, phosphorylation of threonine residues 3 and 11 of histone H3 was not induced upon MAP kinase pathway stimulation by anisomycin (Fig. 5D i and E i). Also combinatorial treatment with anisomycin/TSA did not stimulate histone H3 phosphorylation at T3 and T11 (Fig. 5D ii and E ii).

Taken together, our results show us that activation of MAP kinase pathway by stress specifically activates H3S10 and H3S28 phosphorylation but not H3T3 and H3T11 phosphorylation.

### 3.1.2.2 Histone H3 phosphorylated at serine and threonine residues distributed differently in nucleus

Next, we investigated by an indirect immunofluorescence analysis how histone H3 phosphorylated at different serine and threonine residues is localized in mouse fibroblasts. Logarithmically growing Swiss 3T3 mouse fibroblasts were grown on cover slips, cross-linked with paraformaldehyde, hybridized with antibodies raised against phosphorylated serine28, threonine3, threonine11, and phosphoacetylated S10/K14 histone H3. As the cells were not arrested, there was an increasing population of them, each growing at different stages of cell cycle (Fig. 6A-D). Chromosomes being in different phases of mitosis were visible which reveals that phosphorylated serine28, threonine3, threonine11 as well as S10/K14 phosphoacetylated histone H3 were subjected to mitotic phosphorylation.




B
H3-S10phK14ac
20x
DAPI
20x
Merge
20x
H3-S10phK14ac
100x
DAPI
100x
Merge
100x
C
H3-T3ph
20x
DAPI
20x
Merge
20x
H3-T3ph
100x
DAPI
100x
Merge
100x

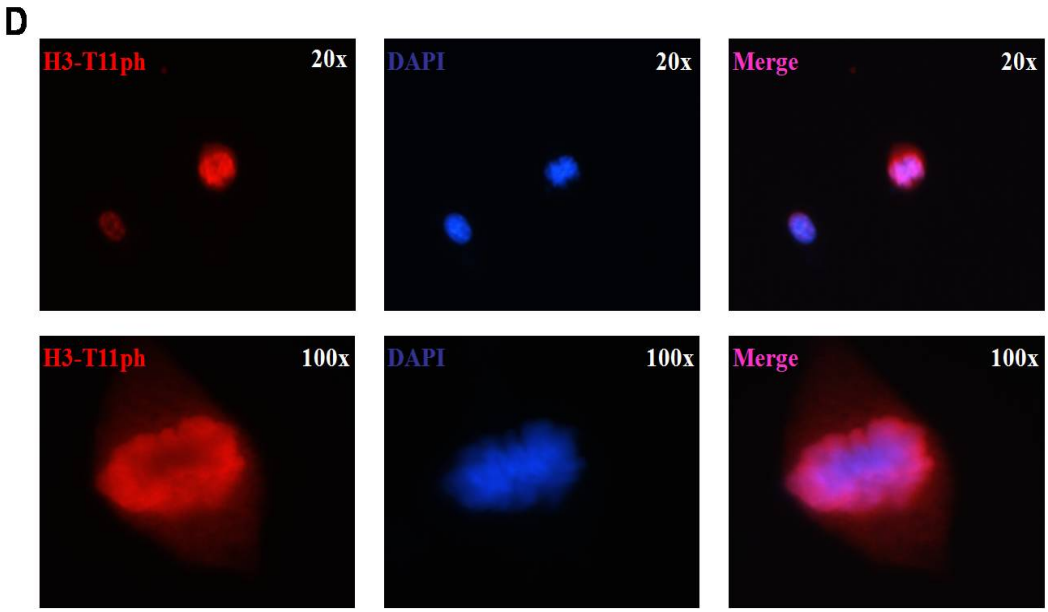
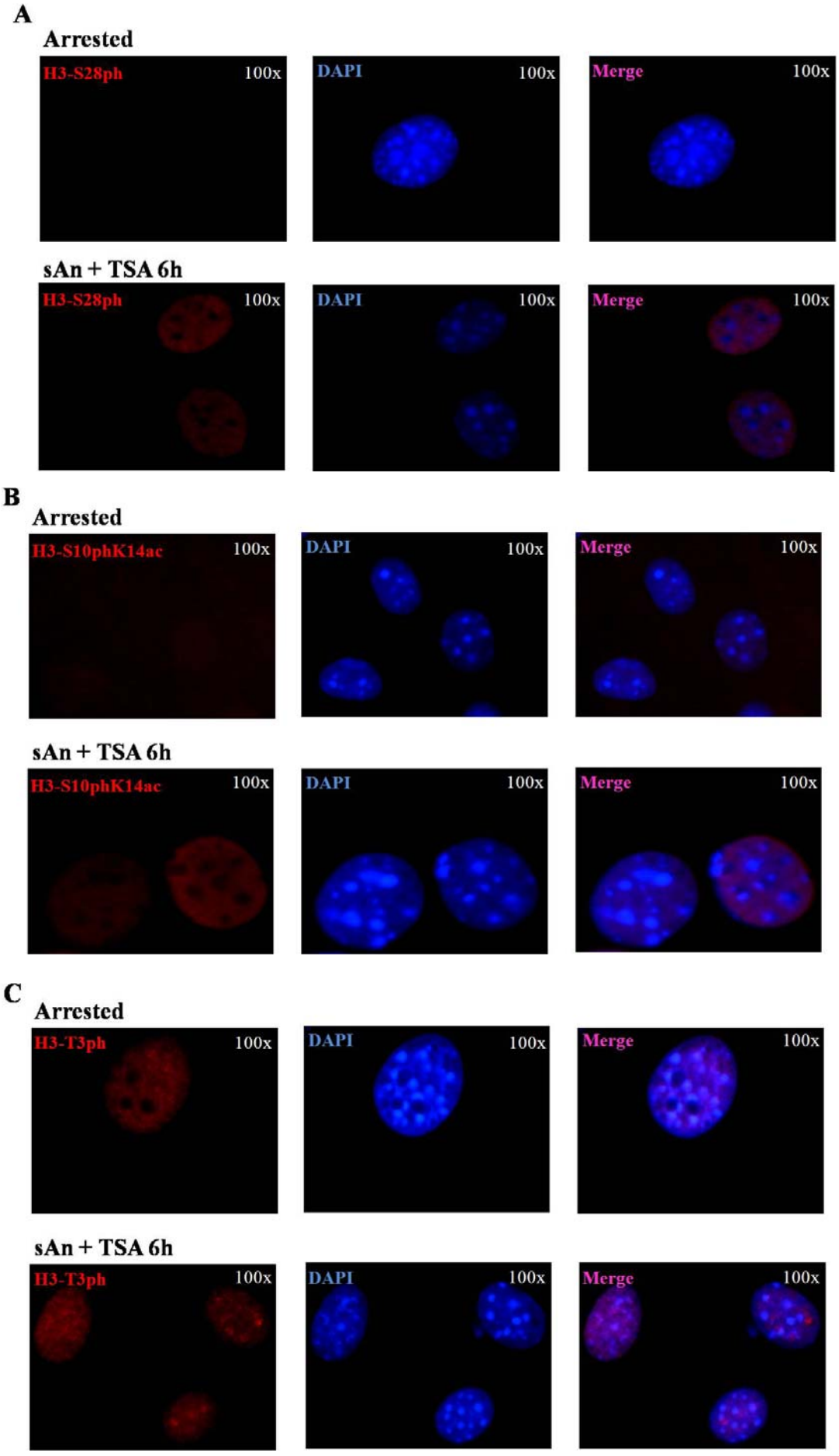



**Fig. 6: Indirect immunofluorescence of mouse fibroblasts showing mitotic staining according to different phosphorylation marks on histone H3.** The logarithmically growing Swiss 3T3 mouse fibroblasts were stained with DAPI (DNA staining, blue, middle panel) and antibodies (red, left panel) against different phosphorylation marks on histone H3. The right panel indicates the overlay picture of left and middle panels. The pictures were taken with 20x and 100x objective. **(A)** Histone H3S28ph (0562), **(B)** histone H3S10phK14ac (GP5), **(C)** histone H3T3ph (Abcam), **(D)** histone H3T11ph (Abcam).

We also analyzed the distribution of phosphorylated histone H3 isoforms in resting Swiss 3T3 mouse fibroblast that were either left untreated or stimulated with anisomycin (188.5nM) and TSA (165.5nM) for six hours (Fig. 7A-D).

The histone H3 was detected with antibodies raised against phosphorylated serine28, threonine3, threonine11 and phosphoacetylated S10/K14.

In case of phosphorylated serine28 and phosphoacetylated S10/K14 of histone H3, the nuclear staining of cells could be observed upon stress induction whereas the resting cells gave no signal. This modified histone H3 distributed in entire nucleus except heterochromatic regions. In comparison to serine residues of histone H3, the activation of MAP kinase pathway by anisomycin and inhibition of HDACs did not affect the nuclear staining of phosphorylated threonine3 and threonine11 of histone H3 as both resting and treated cells gave the same signal.


A
Arrested
H3-S28ph 100x
DAPI 100x
Merge 100x
sAn + TSA 6h
H3-S28ph 100x
DAPI 100x
Merge 100x
B
Arrested
H3-S10phK14ac 100x
DAPI 100x
Merge 100x
sAn + TSA 6h
H3-S10phK14ac 100x
DAPI 100x
Merge 100x
C
Arrested
H3-T3ph 100x
DAPI 100x
Merge 100x
sAn + TSA 6h
H3-T3ph 100x
DAPI 100x
Merge 100x

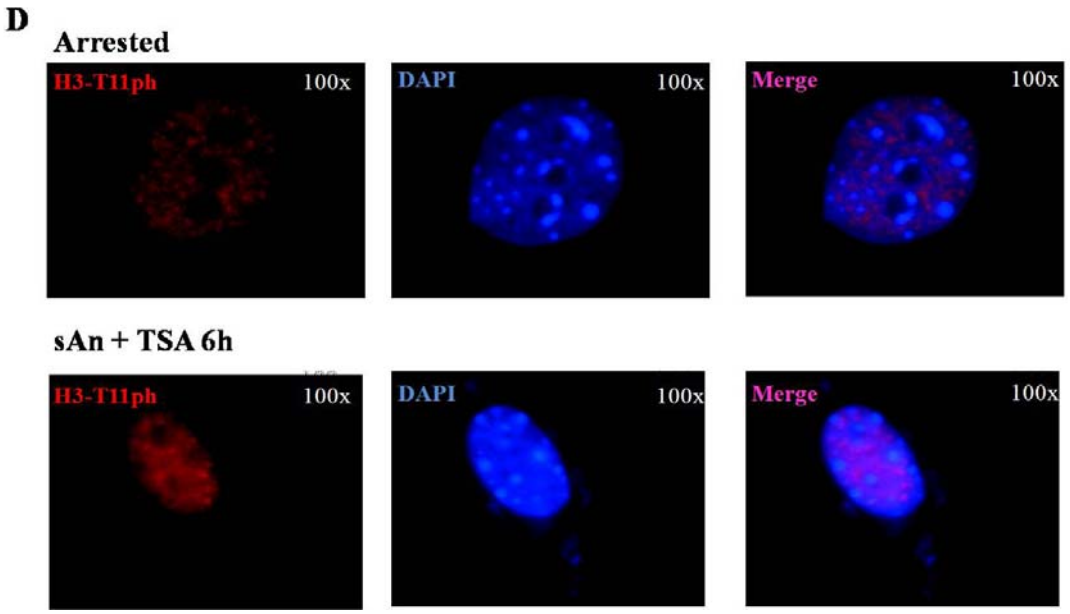



**Fig. 7: Indirect immunofluorescence microscopy of mouse fibroblasts showing distinct patterns after staining with different phospho-specific antibodies against histone H3.** Swiss 3T3 mouse fibroblasts were arrested for 72h and left either untreated or treated simultaneously with anisomycin (188.5nM) and TSA (165.5nM) for 6 hours. The DNA staining with DAPI (blue) is shown in the middle panel, whereas the staining of different phosphorylation mark on histone H3 with different antibodies is shown in red in left panel. The right panel indicates the overlay picture of left and middle panels. **(A)** Histone H3S28ph (0562), **(B)** histone H3S10phK14ac (GP5), **(C)** histone H3T3ph (Abcam), **(D)** histone H3T11ph (Abcam).

### 3.1.3 The activation of specific target genes correlated with phosphoacetylation of histone H3

Next we asked whether stress induction of specific genes correlates with phosphoacetylation of histone H3 in mouse fibroblast.

Stress induced genes were previously identified by a gene expression screen. Resting Swiss 3T3 mouse fibroblasts were either left untreated or treated with anisomycin or TSA alone or with both drugs given together for six hours. Total RNA isolated from these cells was reverse-transcribed in radiolabelled cDNAs that were used to hybridize the cDNAs spotted on array (C. Hauser, PhD thesis). The Atlas array covered 1185 genes. 65% of cDNAs could be measured, out of which 18% of genes showed two fold induction upon treatment. To extend this analysis a genome wide expression screen was performed. This time resting Swiss 3T3 fibroblasts were stimulated with anisomycin and TSA simultaneously for 0, 3, 6, and 9 hours. Some of the genes showed superinduction and were found in both arrays. These genes were defined as candidates regulated by phosphoacetylation of histone H3.

| **Abbr.** | **Name of Gene** |
|---|---|
| c-fos | FBJ osteosarcoma oncogene |
| Egr1 | Early Growth Response 1 |
| p21 | Cyclin-dependent Kinase Inhibitor 1A |
| PAI1 | Serpine-1 |
| uPA | Urokinase-type Plasminogen Activator |
| Timp3 | Tissue Inhibitor of Metalloproteinases 3 |
| HDAC1 | Histone Deacetylase 1 |
| OPN | Secreted Phosphoprotein 1 |

**Table 1: The genes obtained from Atlas array**

According to their biological roles in the cell and to their kinetics of induction, eight genes were chosen for validation (Table 1). The data obtained from the Atlas array was validated by Northern blot analysis (C. Hauser and E. Simboeck, unpublished). ChIP assays revealed that the promoter regions of target genes are subjected to histone H3 phosphoacetylation (Fig. 8) (Simboeck, E., unpublished data).

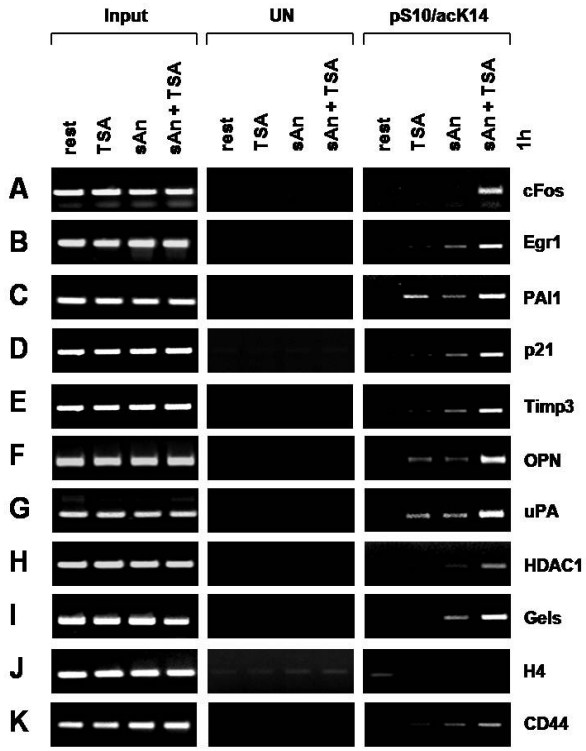



**Fig. 8: The promoter-associated histone H3 phosphoacetylation was verified by ChIP assays for target genes.** Chromatin immunoprecipitation analysis from resting Swiss 3T3 mouse fibroblasts either untreated or treated with anisomycin (188.5nM) and TSA (165.5nM) either alone or together for one hour. An unspecific antibody and an antibody specific for histone H3 phosphorylated at serine10 and acetylated at lysine14 were used to precipitate chromatin associated with phosphoacetylated histone H3. The precipitated DNA fragments were examined with the promoter specific primers by semi-quantitative PCR. **(A)** c-fos, **(B)** Egr1, **(C)** PAI1, **(D)** p21, **(E)** Timp3, **(F)** OPN, **(G)** uPA, **(H)** HDAC1, **(I)** Gels, **(J)** H4, **(K)** CD44 (Simboeck, E. PhD Thesis).

All of the listed genes were originally identified as anisomycin/ TSA-inducible genes. The main question of this part of the study was whether TSA or anisomycin alone would also increase their expression.

Genes listed in the Table 1 and two additional genes were taken for further investigations. To this end, genes listed in Table 1 were tested by quantitative Real Time PCR upon treatment of resting Swiss 3T3 mouse fibroblasts with anisomycin or TSA alone, or simultaneously with both drugs. Dependent on their kinetics of induction genes were grouped into three groups, immediate early, early and late genes. In general, immediate early genes show a transient activation upon a rapid response to extracellular

stimuli, whereas late genes can be activated only, after the protein synthesis by early genes occurs.

The immediate early gene c-fos is one of the first-identified genes regulated by phosphoacetylation of histone H3 (Winter, Simboeck and Seiser 2007). It is a proto-oncogene and involved as a transcription factor in the upregulation of transcription of many genes. The promoter-associated histone H3 phosphoacetylation of c-fos gene had been observed as a rapid and transient event (Barratt, Hazzalin and Mahadevan 1994). The stimulation of stress-inducible p38 pathway by anisomycin seemed to be enough to trigger the induction of c-fos gene (Fig. 9A). The transient phosphoacetylation of histone H3 upon anisomycin treatment correlated with a 20-fold induction in gene expression within one hour, but it lost its impact in three hours dramatically and the expression reached the same expression level as in control cells within six hours. In contrast TSA treatment led only to weak induction with late gene kinetics. If TSA was added to cells simultaneously with anisomycin, the gene expression reached a 35-fold induction immediately in one hour. The acetylation of histone H3 did not only increase the induction, but it also stabilized the expression of c-fos gene, so that a 15-fold induction could still be observed after three hours and even there was more than 10-fold induction after six hours.

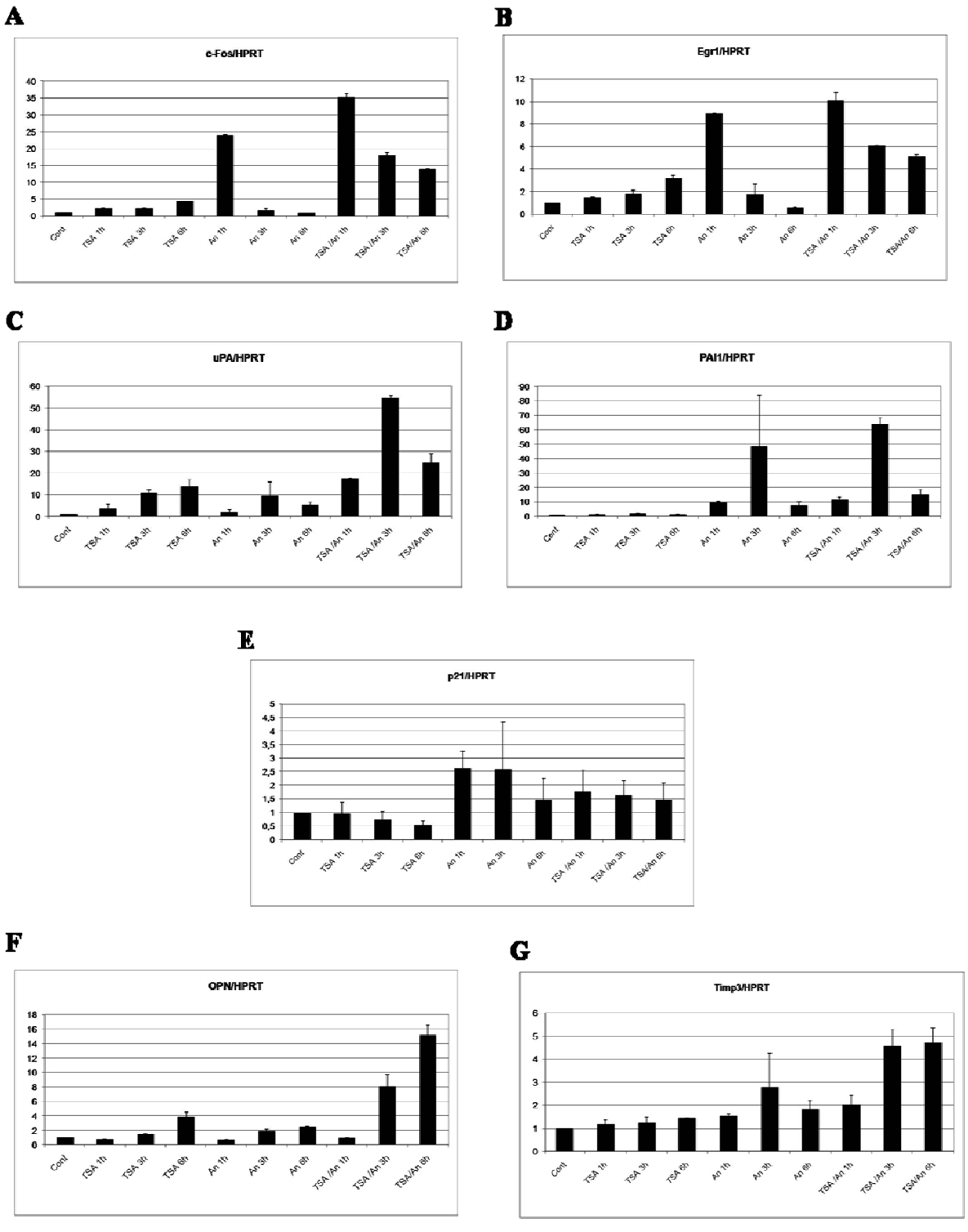
A similar expression pattern was observed for Egr1, a gene that also belongs to the group of IE genes. Egr1 plays a role in the regulation of transcription of genes that are responsible for differentiation. The expression profile of Egr1 correlated well with the expression profile of c-fos gene (Fig. 9B). Like in the case of c-fos gene, activation of MAP kinase pathway seemed to be the main trigger for activation because an 8-fold induction was achieved within one hour after anisomycin treatment alone. Similarly, anisomycin in combination with TSA prolonged the activation of the Egr1 gene, whereas TSA treatment alone had no string impact on Egr1 gene expression (2-fold induction). Taken together, c-fos and Egr1 showed the kinetics of immediate early genes upon stress induction by anisomycin. Simultaneous treatment with TSA led to enhanced and prolonged expression of these genes.

The early genes differ from IE genes by their delayed response upon activation of signaling cascades. In the context of histone H3 phosphoacetylation, the best example for an early gene would be uPA gene, a serine protease that plays a crucial role in invasion and metastasis of cancer. The expression of uPA gene by anisomycin together with TSA rose up to 50-fold within 3 hours and decreased to 20-fold levels after 6 hours

(Fig. 9C). Compared to the combinatorial treatment with anisomycin and TSA, treatment with each agent alone gave only a relatively moderate response. The expression of uPA gene upon activation of p38 stress pathway alone peaked at 10-fold levels after 3 hours, while TSA treatment led to continuous increase in uPA expression up to 13-fold after 6 hours (Fig. 9C).

PAI1, identified as a novel target gene of the stress induced MAP kinase pathway, encodes an inhibitor of uPA and is found in many cancers. The inhibition of HDACs by TSA alone did not trigger the gene expression, whereas the activation of p38 pathway by anisomycin was sufficient to give rise to a 50-fold induction within 3 hours. The simultaneous treatment with both agents increased the expression level of PAI1 gene up to 60-fold within 3 hours, followed by a decrease to 10-fold levels after 6 hours. In conclusion, uPA and PAI1 showed the kinetic of an early gene (Fig. 9D).

p21, another target gene of the nucleosomal response, encodes a cyclin-dependent kinase inhibitor and is the most studied HDAC target gene. The transcriptional induction of p21 gene was not observed upon treatment with TSA for any of indicated time points, whereas the activated p38 pathway alone could increase the gene expression up to 1.5-fold levels within one hour. Although the combination of both drugs did not cause to a significant change in expression, the activation of gene seemed to be stabilized (Fig. 9E).


A
c-Fos/HPRT
B
Egr1/HPRT
C
uPA/HPRT
D
PAI1/HPRT
E
p21/HPRT
F
OPN/HPRT
G
Timp3/HPRT
Cont
TSA 1h
TSA 3h
TSA 6h
An 1h
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TSA/An 6h

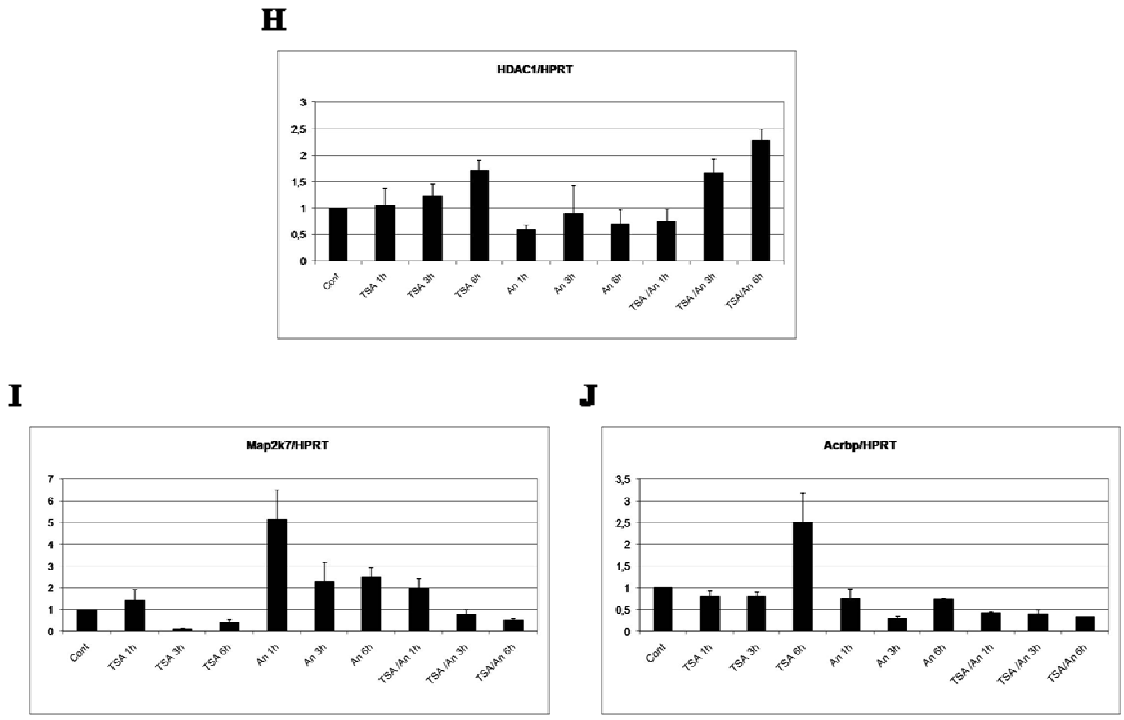


**Fig. 9: Target genes showed different expression patterns.** The response of induction of target genes was measured by RT-PCR. The resting Swiss 3T3 mouse fibroblasts were treated with anisomycin (188.5nM) or TSA (165.5nM) either alone or together for 1, 3 and 6 hours. Control indicates the untreated cells. The RNA was isolated from cells and reverse-transcribed into cDNAs. RT-PCR was performed with primers specific for the analyzed genes. HPRT was used for normalization. The error bars indicate duplicated data. **(A)** c-fos, **(B)** Egr1, **(C)** p21, **(D)** PAI1, **(E)** uPA, **(F)** HDAC1, **(G)** Timp3, **(H)** OPN, **(I)** Map2k7, **(J)** Acrbp.

OPN is another gene identified as a target gene of the p38 MAP kinase pathway. It codes for a protein involved mainly in bone remodeling and immunological functions. Upon treatment with anisomycin or TSA alone, it was observed that the p38 pathway as well as HDAC inhibition could trigger the activation of OPN gene at low levels. If both drugs were added simultaneously, a continuous increase in gene expression was seen which reached 14-fold levels within 6 hours (Fig. 9F).

Timp3, another target gene found in expression screen, codes for one of the metalloproteinase inhibitors. Upon simultaneous treatment with anisomycin and TSA, the expression of Timp3 gene was induced up to 4-fold levels within 3 hours and a 5-fold induction could be still observed after 6 hours. Activation of the p38 pathway caused only a weak induction after 3 hours and TSA alone had no effect at all on the expression of Timp3 gene (Fig. 9G). The kinetics of induction seen for OPN and Timp3 genes were similar to the kinetics of late genes whose induction usually needs hours.

The HDAC1 gene, the last member of the late genes discussed here, is responsible for the deacetylation of histones and other proteins. It was shown that the HDAC1 gene is transcriptionally repressed by HDAC1 itself which sits on the HDAC1 promoter (Schuettengruber, et al. 2003). A synergy between phosphorylation and acetylation of histone H3 is necessary (Hauser, et al. 2002) to fully overcome this inhibitory effect of HDAC1 and possibly other deacetylases. Hence, TSA treatment led to a 2-fold induction in gene expression after 6 hours. TSA in combination with the MAP kinase inducer anisomycin prolonged the phosphoacetylation of histone H3 and caused an increase in expression of the HDAC1 gene which peaked at 2.5-fold levels after 6 hours, whereas the activation of MAP kinase pathway alone did not affect the transcription of HDAC1gene (Fig. 9H).

Map2k7 and Acrbp genes were taken as an example for genes whose transcriptional induction was not regulated by the dual modification of histone H3. Map2k7 expresses a mitogen-activated protein kinase kinase and Acrbp codes for a proacrosin binding protein. The MAP kinase pathway seemed to be the main trigger for the transcriptional induction of Map2k7 gene whose expression peaked at 5-fold levels within one hour (Fig. 9I), whereas the Acrbp gene was activated only by TSA treatment after six hours (Fig. 9J). In both cases, the gene expression was downregulated by combinatorial treatment of anisomycin and TSA.

### 3.1.4 The Agilent whole mouse genome 44K array referred to another subset of genes regulated via the nucleosomal response

Next, we performed another gene expression screen to expand the number of genes whose stress induction can be associated with phosphoacetylated histone H3 (Zupkovitz, G., Simboeck, E.). For this purpose we used an Agilent whole mouse genome 44K array covered app. 28000 genes and transcripts. The RNA used in this screen was gained from resting Swiss 3T3 mouse fibroblasts that were either left untreated or treated simultaneously with anisomycin and TSA for 1, 3 and 6 hours. The genes that showed 2-fold or higher induction were estimated as potential target genes of the nucleosomal response. Out of 28000 genes, 2500 were induced to 2-fold levels upon stimulation with anisomycin and TSA. 18% of 2500 genes responded within one hour, 37% in three hours and 36% in six hours. 825 of them were protein coding genes and grouped into three groups due to their kinetics of induction; immediate-early (Table 2),

early (Table 3) and late (Table 4). 75 of 825 genes were listed as IE, 254 as early and 496 as late genes (Fig. 10). The eight genes listed in Table1 were also found in this expression screen.

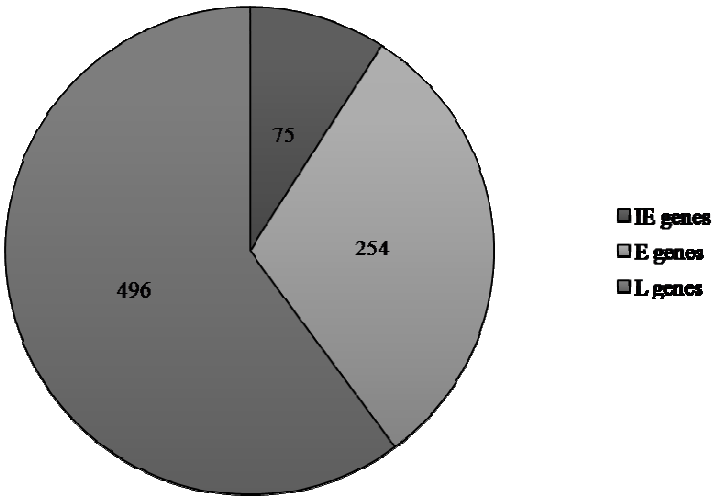


**Fig. 10: The numeric distribution of genes according to three gene clusters.** IE indicates immediate-early, E early and L late.

| Gene Name | ID | Gene Name | ID |
|---|---|---|---|
| Fos | A_52_P262219 | Smad7 | A_51_P403636 |
| Dusp1 | A_51_P430900 | Socs3 | A_51_P474459 |
| Zfp36 | A_52_P80944 | Phlda1 | A_51_P195958 |
| Btg2 | A_52_P31543 | Nuak2 | A_51_P504073 |
| Ier2 | A_51_P481325 | Csf2 | A_51_P171075 |
| Plat | A_52_P405772 | Dlx2 | A_51_P480202 |
| Cyr61 | A_51_P252859 | Gfod1 | A_51_P104162 |
| Nr4a1 | A_51_P239654 | Spna2 | A_51_P140000 |
| Egr1 | A_51_P367866 | Ier5 | A_51_P456941 |
| Jun | A_51_P325914 | Map3k14 | A_52_P364130 |
| Egr3 | A_52_P440836 | Hic2 | A_52_P234354 |
| Axud1 | A_52_P604629 | Bzw2 | A_52_P392467 |
| Egr2 | A_51_P173043 | Hrbl | A_51_P232384 |
| Gadd45g | A_51_P315904 | Tnfaip3 | A_52_P412417 |
| Junb | A_51_P159194 | Tob2 | A_52_P301103 |
| Mt1 | A_52_P423810 | Irf1 | A_51_P146103 |
| Id3 | A_51_P380178 | Lin54 | A_52_P199280 |
| Ier3 | A_51_P286488 | Taok3 | A_52_P278489 |
| Snai1 | A_51_P165087 | Metrnl | A_52_P249937 |
| Id1 | A_51_P385786 | Baz2a | A_52_P191135 |

| | | | |
|---|---|---|---|
| Nfkbia | A_51_P295192 | Lpin2 | A_51_P483081 |
| Anxa6 | A_52_P434670 | Bmf | A_52_P384394 |
| Nfkbiz | A_51_P387591 | Zbed4 | A_51_P199832 |
| Obfc2a | A_51_P499254 | Sfrs10 | A_52_P320822 |
| Dusp6 | A_51_P502614 | Sgk | A_51_P118237 |
| Scx | A_51_P380432 | Etf1 | A_52_P585350 |
| Tgif2 | A_51_P456657 | Il17c | A_51_P350005 |
| Sertad3 | A_52_P71105 | Gdap5 | A_51_P144014 |
| Enah | A_52_P282000 | Jhdm1d | A_51_P245718 |
| Klf6 | A_51_P503162 | Erf | A_52_P326354 |
| Setd3 | A_52_P236777 | Prmt10 | A_52_P203143 |
| Usp36 | A_52_P627925 | Tmem49 | A_52_P413448 |
| Fn1 | A_52_P237357 | Tet3 | A_52_P173108 |
| Spag9 | A_52_P465171 | Hmgcs1 | A_52_P119039 |
| Atp6v0a1 | A_52_P515336 | D3Ertd254e | A_52_P328597 |
| Gyk | A_52_P1020291 | Stau1 | A_52_P65136 |
| Hsf5 | A_52_P1027170 | Kcnk7 | A_51_P354018 |
| Flnb | A_51_P446856 | | |

**Table 2: List of immediate early genes**

| Gene Name | ID | Gene Name | ID |
|---|---|---|---|
| Zfpm2 | A_52_P633571 | Vegfa | A_52_P638895 |
| Zfp697 | A_51_P202574 | Vasn | A_51_P487298 |
| Zfp623 | A_51_P162760 | Utp14b | A_51_P153693 |
| Zfp566 | A_51_P437478 | Usp38 | A_52_P235479 |
| Zfp553 | A_51_P152773 | Unc5b | A_51_P294535 |
| Zfp526 | A_52_P580145 | Unc13a | A_51_P188704 |
| Zfp509 | A_51_P194224 | Ubc | A_51_P292097 |
| Zfp398 | A_51_P493587 | Tyki | A_52_P186937 |
| Zfp36l2 | A_51_P406265 | Trio | A_52_P521507 |
| Zfp36l1 | A_51_P235798 | Trim59 | A_52_P512561 |
| Zfp354c | A_51_P273705 | Trim25 | A_52_P654161 |
| Zfp319 | A_52_P604515 | Trib1 | A_52_P573552 |
| Zfp217 | A_51_P404779 | Traf5 | A_51_P473154 |
| Zfp169 | A_52_P196743 | Top1 | A_51_P276404 |
| Zfand5 | A_51_P256003 | Tnfsf14 | A_51_P208240 |
| Zcchc6 | A_51_P225761 | Tnfrsf1b | A_52_P228621 |
| Zbtb10 | A_52_P495553 | Tnfaip2 | A_51_P364485 |
| Ythdf1 | A_52_P394767 | Tiparp | A_51_P514961 |
| Wsb1 | A_52_P66107 | Ticam1 | A_51_P294020 |
| Vps37b | A_52_P541833 | Thbs1 | A_52_P337086 |

| | | | |
|---|---|---|---|
| Thbd | A_51_P291417 | Ptger4 | A_51_P207988 |
| Tank | A_52_P241917 | Prickle2 | A_52_P360241 |
| Taf1a | A_51_P516057 | Prdm2 | A_52_P25812 |
| Stard13 | A_51_P237915 | Ppp2r5e | A_52_P448936 |
| Spty2d1 | A_51_P375509 | Ppargc1a | A_51_P279038 |
| Spsb1 | A_51_P423737 | Plk3 | A_51_P375201 |
| Sphk2 | A_52_P8059 | Plk2 | A_51_P290576 |
| Spdef | A_51_P421790 | Plekhq1 | A_51_P293982 |
| Spata2 | A_51_P110699 | Plekhh3 | A_51_P228792 |
| Snx30 | A_51_P494481 | Plekhf1 | A_51_P418725 |
| Snf1lk2 | A_52_P575296 | Plau | A_52_P302433 |
| Snf1lk | A_51_P328725 | Pim3 | A_51_P189746 |
| Snai2 | A_51_P305547 | Pim1 | A_52_P530291 |
| Smad3 | A_51_P441022 | Pik3r1 | A_52_P307938 |
| Slc25a37 | A_52_P507688 | Phf16 | A_51_P318731 |
| Slc25a33 | A_51_P156438 | Pfkp | A_52_P223083 |
| Slc25a29 | A_51_P288479 | Per1 | A_52_P79889 |
| Slc25a28 | A_52_P295533 | Pdk4 | A_51_P350453 |
| Slc25a25 | A_51_P193176 | Pde4d | A_51_P120990 |
| Slc10a6 | A_51_P173678 | Pank3 | A_52_P572197 |
| Skil | A_52_P597461 | Osgin1 | A_52_P576854 |
| Six1 | A_51_P268069 | Nuak1 | A_52_P306065 |
| Sh3gl1 | A_51_P517094 | Ngfb | A_51_P381208 |
| Sfrs3 | A_51_P417016 | Nfkbie | A_51_P235123 |
| Serpine1 | A_51_P183571 | Nfil3 | A_51_P111492 |
| Sdk2 | A_52_P570543 | Nfe2l2 | A_52_P396436 |
| Scyl1bp1 | A_52_P648573 | Mylip | A_51_P324303 |
| Rtn3 | A_52_P271725 | Myd116 | A_51_P302503 |
| Rsad2 | A_52_P670026 | Myc | A_51_P102096 |
| Rrs1 | A_52_P579640 | Mobkl2a | A_52_P374961 |
| Rrp1b | A_52_P574214 | Mmp28 | A_51_P392408 |
| Rnd3 | A_52_P392456 | Mizf | A_52_P391081 |
| Rhou | A_51_P227392 | Midn | A_52_P324566 |
| Rhob | A_52_P89567 | Mfsd2 | A_51_P279437 |
| Rhbdf2 | A_51_P412160 | Mesdc1 | A_51_P220884 |
| Rgs3 | A_51_P223583 | Mdga2 | A_52_P207001 |
| Rgs2 | A_51_P419768 | Mcl1 | A_52_P93933 |
| Rgs16 | A_51_P249286 | Mars2 | A_51_P396385 |
| Rg9mtd1 | A_51_P301994 | Map2k3 | A_51_P272817 |
| Rbm38 | A_51_P262670 | Mafk | A_51_P306183 |
| Rasl11b | A_51_P293938 | Maff | A_52_P608322 |
| Rara | A_51_P389724 | Lrig3 | A_51_P428134 |
| Rab3a | A_52_P276397 | Lipa | A_52_P553471 |
| Rab20 | A_51_P417854 | Lims1 | A_52_P206836 |
| Ptx3 | A_51_P374726 | Lfng | A_52_P187940 |
| Ptgs2 | A_51_P254855 | Ksr1 | A_52_P114414 |

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|---|---|---|---|
| Klhl28 | A_52_P86143 | Fbxo42 | A_51_P492595 |
| Klhl25 | A_51_P208121 | Fbxo30 | A_51_P223036 |
| Klhl21 | A_51_P477833 | Fbxl5 | A_52_P39533 |
| Klhl15 | A_52_P317524 | Exoc4 | A_52_P107717 |
| Klf4 | A_51_P213544 | Etv6 | A_52_P597800 |
| Klf10 | A_51_P347547 | Ets2 | A_52_P658122 |
| Kif21b | A_52_P282500 | Errfi1 | A_51_P382393 |
| Jund1 | A_51_P357744 | Ereg | A_51_P171215 |
| Jmjd6 | A_52_P428721 | Epm2aip1 | A_52_P37522 |
| Jmjd1a | A_51_P489406 | Ephb3 | A_51_P334199 |
| Ivns1abp | A_52_P584058 | Epha2 | A_52_P518997 |
| Itpkc | A_51_P507622 | Enc1 | A_51_P178692 |
| Irf2bp2 | A_51_P424221 | Elavl1 | A_52_P429534 |
| Ing2 | A_52_P266459 | Efna1 | A_51_P266683 |
| Il6 | A_51_P217218 | Dusp8 | A_51_P263246 |
| Il4ra | A_51_P464478 | Dusp2 | A_52_P399934 |
| Il1b | A_51_P212782 | Dus1l | A_52_P172312 |
| Ifit2 | A_52_P542388 | Dot1l | A_52_P324965 |
| Id2 | A_52_P240542 | Dnajb4 | A_52_P308863 |
| Ibrdc3 | A_51_P475156 | Dlgap4 | A_51_P343739 |
| Hk2 | A_51_P204080 | Ddi2 | A_51_P152115 |
| Hist1h1d | A_51_P501260 | D8Ertd82e | A_52_P23413 |
| Hist1h1b | A_51_P270949 | Cxcl10 | A_51_P432641 |
| Hes1 | A_51_P498093 | Cxcl1 | A_51_P363187 |
| Hbegf | A_51_P181565 | Cttnbp2nl | A_52_P481423 |
| Grasp | A_51_P425642 | Ctdp1 | A_51_P313467 |
| Gpr146 | A_51_P316379 | Csf1 | A_51_P195506 |
| Gem | A_51_P318192 | Chst12 | A_51_P464023 |
| Gdnf | A_51_P147777 | Chka | A_52_P61903 |
| Gdf15 | A_52_P532982 | Ch25h | A_51_P112966 |
| Gcnt2 | A_52_P363216 | Cenpl | A_51_P195808 |
| Gcc1 | A_51_P507851 | Cebpb | A_52_P605846 |
| Gba2 | A_51_P261359 | Cdkn1c | A_51_P339540 |
| Gadd45b | A_51_P500984 | Cdkn1a | A_51_P363947 |
| Gadd45a | A_51_P296608 | Cdc42se1 | A_52_P340700 |
| Fzd7 | A_52_P552665 | Cdc42ep3 | A_51_P267494 |
| Frs2 | A_51_P413991 | Cdc42ep2 | A_52_P155554 |
| Foxo3a | A_51_P145507 | Cdc42ep1 | A_52_P573255 |
| Fosl2 | A_51_P507242 | Ccrn4l | A_51_P189777 |
| Fosl1 | A_51_P308796 | Ccnl1 | A_52_P590474 |
| Fosb | A_51_P138378 | Ccnd3 | A_51_P238448 |
| Fkbpl | A_51_P515452 | Ccl7 | A_52_P208763 |

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|---|---|---|---|
| Ccl11 | A_51_P331752 | Angptl4 | A_51_P338443 |
| Ccdc86 | A_51_P108901 | Alkbh5 | A_51_P295215 |
| Cbx4 | A_51_P133582 | Akap11 | A_52_P633481 |
| Calcrl | A_52_P58949 | Adora2b | A_52_P419455 |
| C79267 | A_51_P217465 | Adm | A_51_P265571 |
| Btbd4 | A_52_P652885 | Adcy4 | A_51_P229911 |
| Brpf1 | A_51_P271354 | Adamts16 | A_52_P424231 |
| Bcl2l11 | A_52_P158527 | Aak1 | A_51_P284686 |
| Bcar1 | A_51_P375227 | Vegfa | A_52_P229471 |
| Atp7a | A_51_P473179 | Fam13a | A_52_P40975 |
| Atf3 | A_52_P452689 | D10Wsu102e | A_52_P140663 |
| Arrdc3 | A_52_P164136 | Mfsd7b | A_52_P181748 |
| Armc7 | A_51_P162676 | Fam110c | A_52_P476731 |
| Arl5b | A_52_P38228 | Fam46b | A_51_P511250 |
| Arid5a | A_51_P482990 | Filip1l | A_51_P142153 |
| Arhgap17 | A_51_P359983 | Secisbp2l | A_51_P386964 |
| Arc | A_51_P503494 | Fam123b | A_52_P11395 |
| Ankrd57 | A_52_P21837 | Mex3a | A_52_P706060 |
| Ankrd34 | A_52_P262462 | | |

**Table 3: List of early genes**

| Gene Name | ID | Gene Name | ID |
|---|---|---|---|
| Zswim6 | A_52_P663005 | Zc3h10 | A_52_P405525 |
| Zswim3 | A_52_P59228 | Zbtb11 | A_52_P377299 |
| Zscan20 | A_51_P242446 | Wdr70 | A_52_P685445 |
| Zrsr1 | A_51_P407675 | Wdr37 | A_52_P273787 |
| Zkscan6 | A_51_P337755 | Wdfy2 | A_52_P122822 |
| Zkscan14 | A_51_P399614 | Vil2 | A_51_P413348 |
| Zfp9 | A_52_P315910 | Vdr | A_52_P334562 |
| Zfp78 | A_51_P448091 | Vcpip1 | A_52_P210206 |
| Zfp7 | A_52_P588997 | Uvrag | A_52_P153265 |
| Zfp69 | A_52_P478550 | Utp18 | A_51_P177118 |
| Zfp667 | A_52_P532957 | Usp53 | A_51_P358423 |
| Zfp655 | A_51_P280085 | Usp49 | A_52_P428673 |
| Zfp654 | A_51_P413255 | Usp34 | A_52_P172453 |
| Zfp609 | A_52_P646066 | Usp33 | A_52_P625748 |
| Zfp593 | A_51_P106474 | Usp28 | A_51_P216595 |
| Zfp516 | A_52_P244030 | Usp20 | A_51_P122085 |
| Zfp422-rs1 | A_52_P323658 | Usp2 | A_51_P172663 |
| Zfp39 | A_52_P600535 | Ugdh | A_51_P137953 |
| Zfp184 | A_51_P143528 | Ube2h | A_52_P304798 |
| Zfand2a | A_52_P15536 | Ube2f | A_52_P362611 |
| Zcchc2 | A_51_P274667 | Ube1l2 | A_52_P92490 |

| | | | |
|---|---|---|---|
| Txnip | A_51_P438805 | Syde2 | A_51_P362538 |
| Tubb2b | A_51_P514256 | Surf6 | A_52_P283119 |
| Tubb2a | A_51_P490023 | Surf5 | A_52_P17194 |
| Tuba1c | A_52_P479099 | Stom | A_51_P294807 |
| Ttrap | A_51_P169047 | Stmn4 | A_51_P194249 |
| Tssc4 | A_51_P179241 | Stk38l | A_51_P519984 |
| Tspyl3 | A_52_P144297 | Stk17b | A_51_P214197 |
| Tsc22d1 | A_51_P341918 | Star | A_51_P274436 |
| Trp53bp2 | A_52_P537663 | Sssca1 | A_52_P287052 |
| Trove2 | A_52_P97451 | Ss18l1 | A_52_P612537 |
| Trim62 | A_51_P221677 | Srp54 | A_52_P279184 |
| Trim32 | A_52_P458742 | Srfbp1 | A_52_P257827 |
| Trim13 | A_51_P114714 | Spp1 | A_51_P358765 |
| Tram2 | A_51_P246224 | Sox4 | A_51_P105178 |
| Tpst2 | A_52_P180752 | Sox11 | A_51_P297434 |
| Tomm34 | A_51_P320876 | Sntb2 | A_51_P221291 |
| Tob1 | A_51_P293339 | Snapc1 | A_52_P686405 |
| Tnrc15 | A_52_P166846 | Snag1 | A_52_P73134 |
| Tnnc2 | A_51_P353232 | Smc6 | A_52_P503857 |
| Tnfrsf19l | A_51_P439299 | Smad6 | A_51_P427926 |
| Tnfaip6 | A_51_P315785 | Slc9a3r1 | A_51_P502150 |
| Tmod1 | A_51_P297069 | Slc5a5 | A_51_P115738 |
| Tmem24 | A_51_P342835 | Slc5a3 | A_51_P112762 |
| Tmc7 | A_52_P213889 | Slc35e4 | A_51_P268934 |
| Tm2d2 | A_51_P504588 | Slc30a1 | A_51_P300572 |
| Tkt | A_52_P624447 | Slc25a44 | A_51_P488768 |
| Tiprl | A_51_P216157 | Slc25a2 | A_51_P285279 |
| Tinf2 | A_51_P506695 | Ski | A_51_P301508 |
| Timp3 | A_51_P463765 | Six4 | A_52_P420340 |
| Tigd5 | A_51_P276452 | Sirt7 | A_52_P138727 |
| Thy1 | A_51_P420731 | Shc3 | A_51_P399223 |
| Thsd1 | A_52_P22001 | Sh3bp1 | A_51_P342906 |
| Tgoln2 | A_52_P134639 | Sgtb | A_51_P433769 |
| Tgoln1 | A_52_P143477 | Sfrs2 | A_51_P262432 |
| Tgfbr1 | A_51_P280137 | Sesn3 | A_51_P246773 |
| Terf2ip | A_51_P400335 | Sdc4 | A_52_P93467 |
| Tceb3 | A_51_P517126 | Scyl2 | A_52_P643415 |
| Tbcc | A_51_P388801 | Sat1 | A_51_P348494 |
| Tbc1d10a | A_51_P222386 | Sap30 | A_51_P432199 |
| Taf7 | A_52_P520940 | Rsbn1 | A_52_P447500 |
| Taf5 | A_52_P272915 | Rps6kb2 | A_52_P277335 |
| Taf3 | A_52_P577497 | Rpia | A_51_P157472 |
| Synpo2 | A_51_P119776 | Rnf38 | A_51_P214755 |

| | |
|---|---|
| Rnf24 | A_52_P421364 |
| Rnf122 | A_52_P566396 |
| Rnf12 | A_52_P450045 |
| Rnd1 | A_52_P947137 |
| Rmnd5a | A_52_P78659 |
| Riok2 | A_51_P255329 |
| Rgs4 | A_52_P43326 |
| Rffl | A_52_P399175 |
| Rbm27 | A_52_P54297 |
| Rbbp6 | A_51_P285413 |
| Rassf1 | A_51_P435023 |
| Rasgef1b | A_52_P188827 |
| Ranbp6 | A_52_P450123 |
| Ranbp10 | A_52_P215278 |
| Rad9 | A_51_P281024 |
| Rabgef1 | A_51_P109923 |
| Rabgap1 | A_51_P188085 |
| Rab8b | A_52_P477286 |
| Rab40c | A_52_P355075 |
| Rab3il1 | A_51_P451759 |
| Rab33b | A_51_P502026 |
| Rab30 | A_52_P319361 |
| Pxn | A_51_P214859 |
| Pvt1 | A_52_P76184 |
| Pvr | A_52_P162697 |
| Ptpn1 | A_51_P241705 |
| Ptges | A_51_P312327 |
| Psd3 | A_52_P682330 |
| Pscd1 | A_52_P350111 |
| Prtg | A_52_P239246 |
| Prosapip1 | A_52_P120022 |
| Prkrip1 | A_52_P214897 |
| Prkab2 | A_52_P126266 |
| Prelid2 | A_51_P480119 |
| Pqlc1 | A_51_P341369 |
| Pptc7 | A_51_P210560 |
| Ppp2r5b | A_51_P386058 |
| Ppp1r10 | A_51_P243609 |
| Ppme1 | A_52_P69867 |
| Ppm2c | A_52_P157402 |
| Ppm1k | A_51_P130057 |
| Ppm1d | A_52_P26155 |
| Ppat | A_51_P296905 |
| Ppargc1b | A_52_P293222 |
| Pou3f1 | A_51_P511270 |
| Por | A_51_P207921 |
| Pop5 | A_51_P490204 |
| Polr2l | A_51_P331246 |
| Polr2e | A_52_P973604 |
| Polh | A_52_P411716 |
| Pnpla3 | A_51_P389265 |
| Pnpla2 | A_51_P197213 |
| Pmaip1 | A_51_P477121 |
| Plcxd2 | A_51_P476492 |
| Pitx3 | A_51_P477614 |
| Pik3c2a | A_52_P563489 |
| Piga | A_52_P405465 |
| Phtf2 | A_51_P225105 |
| Pgpep1 | A_51_P363801 |
| Pfkfb3 | A_52_P138126 |
| Pfkfb2 | A_52_P31125 |
| Per2 | A_51_P282760 |
| Peli1 | A_51_P232889 |
| Peg3 | A_52_P235861 |
| Pdxp | A_52_P609120 |
| Pdxk | A_51_P498640 |
| Pdlim7 | A_52_P21574 |
| Pdhx | A_51_P149946 |
| Pcf11 | A_52_P323991 |
| Pard6b | A_51_P374101 |
| Paqr3 | A_52_P341016 |
| Papolg | A_51_P478003 |
| Papd5 | A_52_P239645 |
| Pag1 | A_52_P389484 |
| Paf1 | A_51_P183995 |
| P4ha2 | A_52_P617707 |
| Oxsr1 | A_52_P347705 |
| Oxr1 | A_52_P585509 |
| Ovgp1 | A_51_P337708 |
| Otub2 | A_51_P336544 |
| Orc6l | A_51_P296416 |
| Orc4l | A_52_P261020 |
| Oaz3 | A_51_P157698 |
| Oaz2 | A_51_P311945 |
| Nudt22 | A_51_P436189 |
| Nrbf2 | A_51_P284988 |

| | | | |
|---|---|---|---|
| Nr4a2 | A_52_P494622 | Kalrn | A_51_P215828 |
| Nppb | A_51_P426195 | Jundm2 | A_51_P254646 |
| Npat | A_51_P463784 | Jrkl | A_51_P380848 |
| Notch4 | A_51_P195875 | Josd3 | A_52_P470916 |
| Notch1 | A_51_P508510 | Jarid1b | A_52_P584874 |
| Nkiras1 | A_51_P448391 | Jarid1a | A_51_P249689 |
| Nek1 | A_52_P560990 | Jag1 | A_52_P634090 |
| Nedd9 | A_52_P418884 | Iqcb1 | A_51_P307567 |
| Ndnl2 | A_52_P196721 | Ippk | A_52_P99531 |
| Nat12 | A_52_P144399 | Ing1 | A_52_P598 |
| Narf | A_51_P335981 | Ifrg15 | A_51_P111936 |
| Mxd1 | A_51_P490795 | Ifrd1 | A_52_P124352 |
| Mtl5 | A_52_P454127 | Id4 | A_51_P494430 |
| Mtap7d1 | A_51_P189343 | Hyou1 | A_52_P375076 |
| Mt2 | A_51_P246317 | Hspa5 | A_51_P395098 |
| Mrpl49 | A_51_P261351 | Hsf2 | A_52_P479683 |
| Mrpl17 | A_52_P71249 | Hsd17b7 | A_51_P250934 |
| Mms19l | A_52_P214936 | Hrh1 | A_52_P295712 |
| Mlstd2 | A_52_P164172 | Hpxn | A_51_P325836 |
| Mlph | A_51_P272323 | Homer1 | A_51_P387160 |
| Mll3 | A_52_P448569 | Hivep3 | A_51_P100021 |
| Mknk2 | A_52_P376135 | Hist4h4 | A_52_P299703 |
| Med18 | A_51_P141104 | Hist3h2bb | A_52_P527400 |
| Mdm1 | A_51_P396163 | Hist3h2ba | A_51_P338664 |
| Mcam | A_51_P268439 | Hist3h2a | A_52_P615375 |
| Mapk8ip1 | A_52_P349477 | Hist2h4 | A_51_P426975 |
| Mapk14 | A_51_P325343 | Hist2h3c1 | A_51_P359462 |
| Map3k2 | A_51_P182993 | Hist2h2bb | A_51_P361620 |
| Map2k6 | A_52_P395242 | Hist2h2aa1 | A_52_P64609 |
| Mad2l1bp | A_51_P425632 | Hist1h4i | A_51_P505521 |
| Ltb4r1 | A_51_P109449 | Hist1h4f | A_52_P52964 |
| Lrig2 | A_52_P228491 | Hist1h4d | A_51_P482070 |
| Lnpep | A_52_P302395 | Hist1h4a | A_51_P412050 |
| Lin7b | A_51_P325223 | Hist1h3d | A_51_P492488 |
| Leng1 | A_51_P167513 | Hist1h3a | A_51_P518621 |
| Lct | A_52_P164797 | Hist1h2bm | A_52_P176361 |
| Kremen1 | A_52_P143056 | Hist1h2bk | A_52_P388224 |
| Klf2 | A_51_P144264 | Hist1h2bc | A_51_P462311 |
| Klf16 | A_51_P215097 | Hist1h2ba | A_51_P180140 |
| Kitl | A_52_P572571 | Hist1h2ae | A_51_P286460 |
| Kin | A_51_P436483 | Hist1h1t | A_51_P312615 |
| Kif1b | A_51_P264956 | Hist1h1e | A_52_P67892 |
| Kctd7 | A_52_P553000 | Hist1h1c | A_51_P516133 |

| | | | |
|---|---|---|---|
| Herc1 | A_51_P167426 | Eif2c3 | A_52_P126513 |
| Hdhd3 | A_51_P465582 | Efna3 | A_51_P186203 |
| Hdac5 | A_51_P278893 | Efcab4a | A_52_P552879 |
| Hcn3 | A_52_P655508 | Eef1d | A_51_P449886 |
| Hal | A_51_P172155 | Dyrk3 | A_52_P232580 |
| H3f3b | A_52_P217875 | Dynll2 | A_51_P203878 |
| H2afx | A_51_P245275 | Dusp14 | A_52_P297724 |
| H1f0 | A_52_P442234 | Dusp10 | A_51_P104418 |
| Gzf1 | A_51_P491095 | Dos | A_51_P436817 |
| Gtf2b | A_51_P502054 | Dok4 | A_51_P415475 |
| Gse1 | A_52_P489979 | Dnase2a | A_51_P380129 |
| Grpel1 | A_52_P156158 | Dnajb1 | A_51_P153486 |
| Gprc5b | A_51_P173709 | Dgke | A_51_P410744 |
| Gnat1 | A_52_P211185 | Defcr20 | A_52_P249336 |
| Gm288 | A_52_P255959 | Dedd2 | A_52_P204393 |
| Gm129 | A_52_P63343 | Deb1 | A_51_P119715 |
| Glt8d3 | A_52_P572702 | Ddx21 | A_52_P279143 |
| Glce | A_52_P673978 | Ddit3 | A_52_P533146 |
| Ghrl | A_52_P675556 | Dcun1d4 | A_52_P483768 |
| Gch1 | A_52_P298002 | Dcun1d3 | A_51_P192979 |
| Gas2l3 | A_52_P328492 | Dbr1 | A_52_P255841 |
| Fzd5 | A_52_P224125 | Dact2 | A_52_P619911 |
| Fzd4 | A_51_P361220 | Dact1 | A_52_P141417 |
| Frat2 | A_52_P162957 | D5Wsu178e | A_51_P104891 |
| Foxj1 | A_51_P456870 | Cx3cl1 | A_51_P205129 |
| Fnip1 | A_51_P163876 | Ctgf | A_51_P157042 |
| Fndc7 | A_52_P551987 | Csrp1 | A_52_P209184 |
| Flrt3 | A_52_P476935 | Cspp1 | A_51_P428595 |
| Fkbp5 | A_52_P481493 | Csnk1e | A_51_P429197 |
| Fgfr2 | A_51_P432403 | Cry2 | A_52_P248378 |
| Fgd6 | A_51_P401673 | Crsp7 | A_51_P199567 |
| Fem1b | A_51_P352924 | Crsp2 | A_52_P574685 |
| Fbxw7 | A_51_P506683 | Creb3l2 | A_52_P343856 |
| Fbxo46 | A_51_P357877 | Creb3 | A_52_P135707 |
| Fbxo33 | A_51_P261324 | Cpne9 | A_51_P147766 |
| Fbxo25 | A_52_P268434 | Cplx1 | A_52_P465980 |
| Fbxl14 | A_51_P113722 | Cpeb4 | A_52_P323415 |
| Evpl | A_51_P324838 | Cpeb3 | A_52_P535212 |
| Ets1 | A_52_P258291 | Coq10b | A_52_P650553 |
| Esrra | A_51_P248580 | Cobll1 | A_52_P631356 |
| Erc1 | A_52_P598912 | Cnksr3 | A_51_P184728 |
| Ell2 | A_52_P13305 | Clp1 | A_51_P407193 |
| Ell | A_51_P292943 | Cln8 | A_51_P224983 |
| Elavl3 | A_51_P305230 | Cited2 | A_51_P478548 |

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|---|---|---|---|
| Chic2 | A_52_P574291 | Arhgef7 | A_52_P26357 |
| Chd4 | A_51_P454280 | Arhgef12 | A_52_P390227 |
| Chd1 | A_52_P547965 | Arhgap29 | A_51_P367947 |
| Chchd7 | A_52_P364970 | Arhgap26 | A_51_P119932 |
| Cep192 | A_52_P427194 | Arhgap12 | A_52_P527106 |
| Cep152 | A_52_P657800 | Apol2 | A_51_P115471 |
| Centb5 | A_52_P129114 | Apoe | A_51_P171999 |
| Centb2 | A_52_P217986 | Amigo3 | A_51_P442933 |
| Cdkn2d | A_51_P122356 | Amhr2 | A_51_P466221 |
| Cdkn2aip | A_52_P406036 | Amd2 | A_52_P337297 |
| Cdan1 | A_52_P339703 | Amd1 | A_51_P381763 |
| Cd3eap | A_51_P317443 | Alkbh7 | A_52_P637338 |
| Cd248 | A_52_P5454 | Alg13 | A_52_P453417 |
| Ccnk | A_51_P157866 | Akap13 | A_51_P452702 |
| Ccne1 | A_51_P408946 | Akap1 | A_52_P556328 |
| Ccdc85b | A_51_P477768 | Ahr | A_51_P449133 |
| Ccdc38 | A_51_P127507 | Afap1 | A_51_P291210 |
| Ccdc32 | A_51_P259848 | Adra2a | A_51_P434567 |
| Cbx8 | A_52_P89273 | Adarb1 | A_51_P156882 |
| Cbx2 | A_52_P13730 | Adamts2 | A_52_P510592 |
| Cbara1 | A_51_P288514 | Acvr2a | A_52_P575854 |
| Casp3 | A_52_P117576 | Abhd2 | A_52_P664506 |
| Capza2 | A_52_P477836 | Abcg4 | A_51_P376560 |
| Btg1 | A_51_P288193 | Abcf3 | A_51_P468531 |
| Bscl2 | A_52_P256584 | Pde12 | A_52_P49406 |
| Brwd3 | A_52_P247679 | Nt5dc3 | A_51_P507023 |
| Brdt | A_52_P70787 | Fam118b | A_52_P499967 |
| Bdp1 | A_51_P316575 | Ippk | A_52_P69615 |
| Bcorl1 | A_52_P91757 | Lipt1 | A_52_P104562 |
| Bcl6 | A_52_P161495 | Gm5188 | A_52_P100381 |
| Bcl10 | A_51_P209527 | Gm5124 | A_51_P437538 |
| Bat4 | A_51_P136650 | Zfp867 | A_52_P334808 |
| Baiap2 | A_52_P38436 | Fam126b | A_52_P41545 |
| Bai2 | A_51_P284079 | Csrnp2 | A_52_P342620 |
| Bace1 | A_51_P167971 | Tmem194 | A_51_P241645 |
| Azin1 | A_52_P117313 | Tmem82 | A_52_P112110 |
| Atxn3 | A_52_P308681 | Urb2 | A_52_P484118 |
| Atp10d | A_51_P488211 | Pwwp2a | A_52_P1124217 |
| Atg3 | A_51_P143855 | Srd5a1 | A_52_P577084 |
| Atg12 | A_52_P579719 | Tomm6 | A_51_P288277 |
| Ate1 | A_52_P60760 | Tmem189 | A_51_P452506 |
| Asb1 | A_51_P111757 | Map3k2 | A_52_P424197 |
| Arl5c | A_52_P203691 | Hsd17b6 | A_51_P399277 |
| Arl4d | A_51_P402378 | Napepld | A_52_P292853 |

| | | | |
|---|---|---|---|
| Six4 | A_52_P607702 | Fam46c | A_51_P447976 |
| Zfp800 | A_51_P212840 | Urb1 | A_51_P151820 |
| Fam179b | A_52_P177988 | Fam107b | A_52_P528456 |
| Fam109a | A_51_P466829 | Fam53c | A_51_P121432 |
| Tmem198 | A_52_P277128 | Smg1 | A_51_P371695 |
| Zfp871 | A_52_P33163 | Mettl11a | A_51_P176517 |
| Gpr137c | A_51_P281912 | Fbrsl1 | A_51_P363525 |
| Akirin1 | A_52_P142208 | Zfp763 | A_51_P250123 |
| Fam117a | A_51_P148314 | Rsph9 | A_51_P435922 |
| Prkab2 | A_51_P264781 | Frmd8 | A_52_P219266 |

**Table 4: List of late genes**

Next, we asked the question whether the activation of some of 254 early genes is linked to transcription factors that were expressed by immediate-early genes identified in the screen. We also tried to find out whether the late response of late genes is a consequence of early genes transcribed before. Some of the genes from our screen showed this relationship among each other and are listed in Table 5. For example, the transcription of Egr1 gene, an IE gene, is required for the activation of Id2, Atf3 and Gem genes that belong to the group of early genes.

| **IE** | **Early Genes** | **Late Genes** |
|---|---|---|
| Nfkbia | Il6 | - |
| Fos | Fosl1 | - |
| Egr1 | Id2,Atf3,Gem | Spp1,Apoe,Thy1,Id4,Sox11 |
| Irf1 | Cdkn1a,Myc,Il6 | - |

**Table 5: The genes whose activation is triggered by genes activated before.**

We also clustered the genes from screen according to their biological roles. In general, all three groups of genes were found to play diverse roles from gene expression to apoptosis. For example, IE genes were implicated 72 cellular processes. 25 of them are players in the control of gene expression, whereas 21 perform important functions during transcription (Fig. 11A). 183 of the early genes are linked to the cellular processes and 63 to signal transduction (Fig. 11B). In total, 136 different processes were allocated to late genes. 365 of them are active in cellular processes, whereas 4 are involved in the cell cycle checkpoint (Fig. 11C).

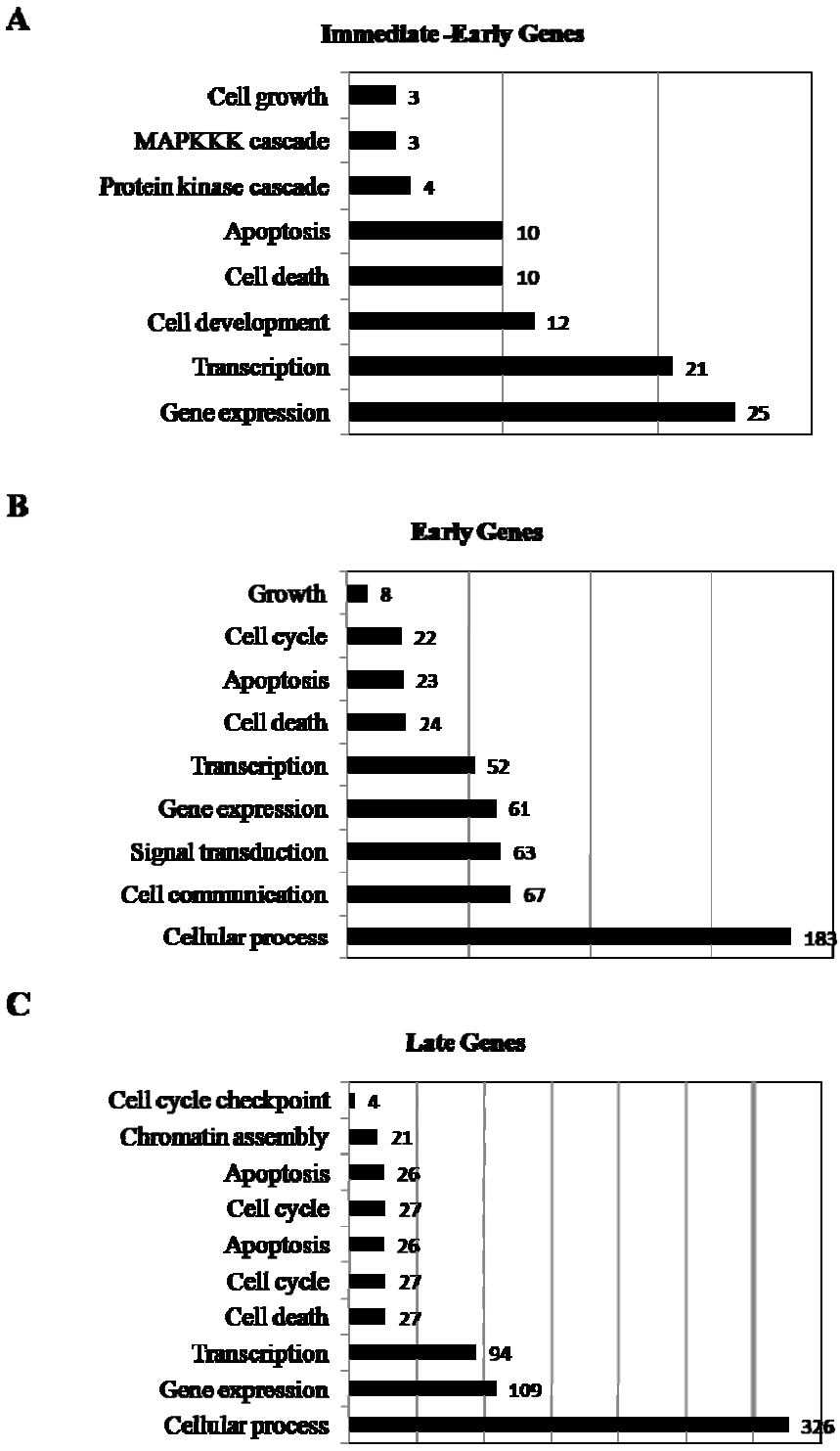


**Fig. 11: The numeric distribution of each group of genes due to their biological roles.** The analysis was done by using the program DAVID Bioinformatics Resources. **(A)** Immediate-early genes**, (B)** early genes, **(C)** late genes

### 3.1.5 The preparation of samples for ChIP sequencing technique

Next, we wanted to determine the distribution and binding sites of phosphoacetylated histone H3 on entire genome. Therefore, we used the new technique called ChIP sequencing (ChIP-Seq). This technique is an alternative technique to ChIP-on-Chip and covers the entire genome. In this technique, DNA fragments gained from chromatin immunoprecipitation assays are sequenced and aligned to a reference sequence to map the localization of binding sites of protein of interest.

Chromatin was isolated from resting Swiss 3T3 mouse fibroblasts that were either left untreated or treated with anisomycin alone or simultaneously with anisomycin and TSA for 1, 3 and 6 hours and sheared to a fragment size between 100 and 300bp to increase the resolution of ChIP-Seq (Fig. 12A). The fragments were then precipitated with an antibody raised against phosphoacetylated histone H3 (H3-S10phK14ac). The DNA fragments were amplified with primers that were specific for promoter region of p21 gene.

Our group could show that the promoter region of p21 gene is associated with phosphoacetylated histone H3 and its induction by stress also correlates with this dual modification mark on histone H3 tail. Therefore, p21 gene was considered as positive control for our chromatin immunoprecipitation assays that were sent to be sequenced.

Activation of MAP kinase pathway by anisomycin led to an association between phosphoacetylated histone H3 and promoter region of p21 gene after 3hours (Fig. 12B), whereas this dual-modified histone H3 appeared on promoter of the gene within one hour and could be still observed after 6 hours if anisomycin was used in combination with TSA (Fig. 12C).

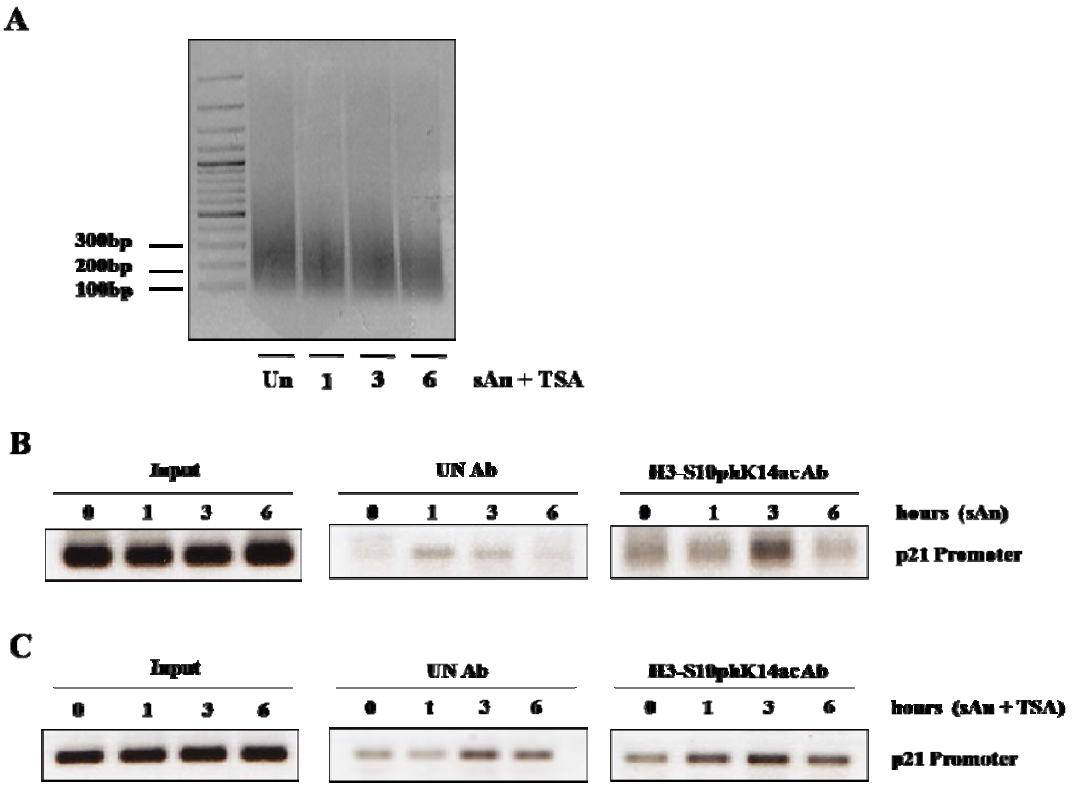


**Fig. 12: Promoter regions of p21 gene are associated with phosphoacetylated histone H3.** Chromatin is isolated from resting Swiss 3T3 mouse fibroblasts either left untreated or treated with anisomycin (188.5nM) alone and anisomycin (188.5nM) and TSA (165.5nM) for 1, 3 and 6 hours, sheared to a fragment size between 100 and 300bp **(A)**, and precipitated with an unspecific antibody (UN Ab) and an antibody against histone H3 phosphorylated at serine10 and acetylated at lysine 14 (H3-S10phK14ac Ab). The DNA fragments were examined with a promoter specific primer of p21 gene by semi-quantitative PCR **(B, C)**.

## 3.2 Part II: Characterization of a potential interaction partner of 3.3 14-3-3 proteins

14-3-3 proteins are phosphoserine/-threonine binding proteins and take a part in various cellular processes and signal transduction pathways (Aitken 1995, Muslin, et al. 1996, Yaffe and Smerdon 2001). They have more than hundred interacting partners among which histone proteins are also listed as 14-3-3 targets. The 14-3-3 isoforms ε, γ and ζ have been identified to bind specifically to histone H3 phosphorylated at serine10 (Macdonald, et al. 2005). It has been also observed that the binding affinity of 14-3-3 proteins is increased, when the neighboring lysine14 at histone H3 tail was acetylated (Winter, et al. 2008, Walter, et al. 2008). The recruitment of 14-3-3 proteins to phosphoacetylated histone H3 also correlated with the transcriptional induction of immediate-early genes (Macdonald, et al. 2005) as well as of a late inducible gene, HDAC1 (Winter, Simboeck and Seiser 2007). Therefore, it is suggested that these proteins can play a more general role for regulation of gene expression that is targeted by phosphoacetylation of histone H3.

The fact that 14-3-3 proteins are required for trans-activation of HDAC1 gene raised the question whether there is a relationship between these proteins and transcription machinery. To answer this question, we performed immunoprecipitation assays by considering the P-TEFb as a potential interaction partner of 14-3-3 proteins. We also tried to establish a cell line producing TAP- tagged 14-3-3ζ protein to use it for screen for interaction partners and for a genome-wide analysis of the distribution of 14-3-3 in stress induced cells.

### 3.3.1 P-TEFb is a Putative Interaction Partner of 14-3-3 Proteins

14-3-3 proteins act as detectors for phosphorylated histone H3 and their recruitment as well as the recruitment of RNAP II to promoter regions of HDAC1 gene associated with phosphoacetylated histone H3 correlates with transcriptional induction (Winter, Simboeck and Seiser 2007).

Like serine10 phosphorylation of histone H3 is detected by 14-3-3 proteins, it was reported for *Drosophila* that this modification mark is also required for the P-TEFb recruitment (Ivaldi, Karam and Corces 2007). These observations raised the question whether there is a link between 14-3-3 proteins and the transcription machinery. To answer the question, we performed coimmunoprecipitation assays where a Hek293 cell

line producing double tagged CycT (Flag and HA) and an untransfected Hek293 cell line were used to precipitate specifically 14-3-3ζ protein, because this isoform was identified by our group to induce expression of HDAC1 gene (Winter, Simboeck and Seiser 2007). To trigger the nucleosomal response in context of phosphoacetylation of histone H3, the activation of MAP kinase pathway and inhibition of HDACs were done by treating the cells with anisomycin and TSA, respectively. The double tagged CycT protein was captured from total protein extract either with Flag or HA beads. The eluted protein was loaded on polyacrylamide gel and western blot analysis was performed. During this protocol, different washing conditions and different percentage of polyacrylamide gel were also tested to optimize the investigation.

First, we detected CycT protein with an antibody raised against Flag-tag to show that the immunoprecipitation assay was successful. Since P-TEFb protein consists of CycT and Cdk9, the existence of Cdk9 was also tested to show that coimmunoprecipitation assay functioned. An antibody recognizing specifically 14-3-3ζ was used to check, whether 14-3-3ζ was co-precipitated with double tagged CycT.

Out of seven coimmunoprecipitation assays, we could observe only two times that 14-3-3ζ was precipitated in small amounts and most of the protein remained in supernatant after coprecipitation (Fig.13A and B). Although CycT and Cdk9 were also found in supernatant, the precipitates of both proteins were highly rich compared to precipitated 14-3-3ζ protein. These results showed us that the interaction between 14-3-3ζ and P-TEFb can be indirect meaning that there can be other proteins mediating a bridge between 14-3-3ζ and P-TEFb, because 14-3-3 proteins are able to mediate the scaffolding within protein complexes due to their homo- or heterodimerization (Tzivion, Shen and Zhu 2001).

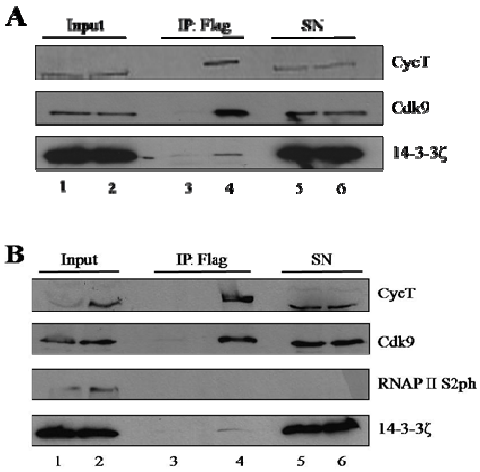


**Fig. 13: Coimmunoprecipitation assay revealed hints for the interaction between 14-3-3ζ and P-TEFb. (A, B)** Hek293cells either untransfected or transfected with a vector producing Flag-CycT-HA were treated with anisomycin (188.5nM) and TSA (165.5nM) for one hour. The CycT was precipitated with Flag beads and seperated on a 12.5% SDS-PAGE . The proteins of interest were detected with specific antibodies on Western blots; α-CycT, α-Cdk9 (Santa Cruz), α-14-3-3ζ (aff. Purified) and α-RNAPII S2ph (Bethyl). 1, 3 and 5 are controls obtained from untransfected Hek293 cells. 2, 4 and 6 indicate the transfected Hek293 cells.

### 3.3.2 Establishment of a Single Clone producing TAP-tagged 14-3-3ζ

Next, we tried to establish a cell line producing TAP-tagged 14-3-3ζ protein. We concentrated mainly on ζ isoform, because it has been found to trigger the induction of HDAC1 gene (Winter, Simboeck and Seiser 2007). To elucidate the underlying mechanisms how 14-3-3 proteins participate on transcriptional regulation of stress-induced genes, further investigations are required. As 1% of interphase histones are subjected to the phosphoacetylation of histone H3 that is recognized by 14-3-3ζ isoform, the amount of this nuclear protein is not sufficient for such experiments. Therefore, we wanted to use TAP system to enrich the protein amount that can be used for coimmunoprecipitation assays, ChIP, screens to identify the interaction partners or a genome-wide analysis to see the distribution of 14-3-3ζ in stress induced cells.

TAP is a tandem affinity purification system that uses an expression vector with two tags. The CBP tag is gained from a C terminal fragment of muscle myosin light-chain kinase and has high affinity for calmodulin, whereas the SBP tag is a synthetic sequence belonging to a random peptide library and shows high affinity for streptavidin (InterPlay

Mammalian System, Instruction Manual). One tag is used after the other one to enable a tandem purification of protein of interest.

The 14-3-3ζ gene was cloned into this expression vector and Swiss 3T3 mouse fibroblasts and HeLa cells were transfected with this vector in different concentrations. After the protein was extracted from cells, the transient expression (Fig. 14A) as well as the stable expression of the protein was examined by Western blot analysis (Fig. 14B). The antibodies recognizing specifically TAP-tag and 14-3-3ζ were used to detect the tagged and the endogenous protein.

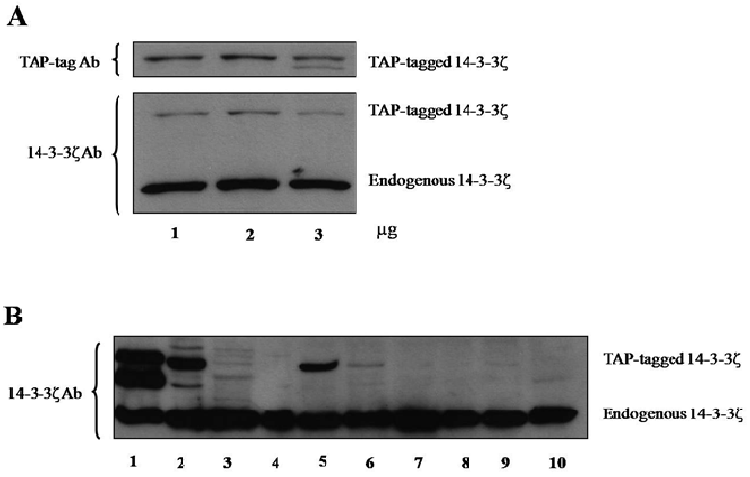


**Fig. 14: Transient and stable expression of TAP-tagged 14-3-3ζ protein**. The Swiss 3T3 mouse fibroblasts were transfected with pNTAP-14-3-3ζ construct. Endogenous and TAP-tagged 14-3-3ζ was detected on Western blots with antibodies against TAP-tag (Upstate) and 14-3-3ζ (aff. Purified); **(A)** Transient expression of 14.3.3ζ transfected with different concentrations of vector (1, 2, 3µg), **(B)** stable expression of protein after transfection with 3µg vector. 1 and 2 are controls whereas 2-10 indicate the number of different single clones.

Out of approximately hundred clones, we were able to establish one clone producing TAP-tagged 14-3-3ζ protein (Fig. 14B) which can then be used for experiments such as mass spectrometry and immunoprecipitation assay or ChIP-Seq to identify the interaction partners of 14-3-3ζ protein and to see its distribution on entire mouse genome, respectively.

# 4 DISCUSSION

It has been shown that activation of the IE genes, c-fos and c-jun by the nucleosomal response correlates with rapid and transient phosphoacetylation of histone H3 (Clayton and Mahadevan 2003, Cheung, Tanner, et al. 2000, Thomson, Clayton and Mahadevan 2001, Clayton, Rose, et al. 2000). The activation by the nucleosomal response is not restricted to IE genes and other genes such as HDAC1 can be also activated upon phosphoacetylation of histone H3 (Hauser, et al. 2002, Winter, Simboeck and Fischle, et al. 2008). Compared to IE genes, these genes require a stable phosphoacetylation to be activated. This stable phosphoacetylation mark on histone H3 tail can be induced by using agents such as anisomycin and TSA. Anisomycin as stress inducer activates the MAP kinase pathway that is responsible for histone H3 phosphorylation, whereas TSA inhibits many HDACs and increases the acetylation of histones.

Our group performed expression screens to identify other genes that can be putative targets of the nucleosomal response. The activation of some of these target genes upon stress induction was examined by quantitative Real-time PCR analysis. Another important question was to clarify whether phosphoacetylation of histone H3 is a relevant modification mark for the transcriptional regulation of these genes.

## 4.1 The phosphorylation of serine and threonine residues at N-terminal tail of histone H3 is mediated by different pathways

Since we are interested in the impact of H3 phosphoacetylation on transcriptional regulation, we used a system that leads to this modification at histone H3 tail. We stimulated serine10 phosphorylation at histone H3 by treating cells with an agent that activates the stress inducible p38 pathway and with another agent to inhibit HDACs for hyperacetylation of histones.

It has been already known that serine28, theronine3 and threonine11 of histone H3 can also be phosphorylated besides serine10 (Polioudaki, et al. 2004). Therefore, we asked the question, whether the signaling cascade that is triggered by stress also causes the phosphorylation of threonine residues.

Western blot analysis showed that the phosphorylation of serine10 and serine28 as well as S10K14 phosphoacetylation of histone H3 was transiently increased within 1 hour upon activation of MAP kinase pathway (Fig. 5A i, B i and C i). The stimulation of the MAP kinase pathway together with inhibition of HDACs increased and stabilized serine28 phosphorylation and S10K14 phosphoacetylation of histone H3. However,

serine10 phosphorylation signals were not affected due to the impaired recognition of this modification mark upon lysine14 acetylation (Fig. 5A ii, B ii and C ii).

Compared to serine phosphorylation, the phosphorylation of threonine3 and threonine11 at histone H3 tail was not induced by MAP kinase pathway stimulated by anisomycin (Fig. 5D i and E i). Also, the simultaneous inhibition of HDACs by TSA together with anisomycin did not induce threonine3 and threonine11 phosphorylation (Fig. 5D ii and E ii). These results verify that the activation of MAP kinase pathway specifically triggers serine10 and serin28 phosphorylation, but not affects threonine3 and threonine11 phosphorylation of histone H3.

When this work had been started, the threonines 3 and 11 were the only known threonines of histone H3 that undergo phosphorylation. During the progress of this study, it has been reported that threonine6 and threonine45 can also be phosphorylated by different kinases. PKCα, $\beta_I$ and $\beta_{II}$ kinases phosphorylate specifically threonine6 and are not responsible for phosphorylation of threonine3, threonine11 and serine10 of histone H3 (Metzger, et al. 2010), whereas threonine45 is phosphorylated by PKCδ (Hurd, et al. 2009).

Today, it is also known that threonine11 of histone H3 is phosphorylated by PRK1 kinase in androgen-dependent manner and correlates with gene expression. Therefore, it has been claimed that threonine11 phosphorylation of histone H3 can be another modification mark for transcriptional regulation (Metzger, Yin, et al. 2008).

The fact that serine and threonine residues on histone H3 tail are phosphorylated during mitosis (Hendzel, et al. 1997, Gurley, et al. 1978, Goto, et al. 1999, Dai, et al. 2005, Preuss, Landsberg and Scheidtmann 2003) has been also verified by our immunofluorescence analysis that showed strong signals for H3 serine28, threonine3 and 11 in each phase of mitosis (Fig. 6A-D). Additionally, mitotic S10K14 phosphoacetylation of histone H3 was also seen in this analysis. Compared to mitotic phosphorylation, stress induction led also to modification of serine residues at histone H3 tail, whereas no difference between resting and stress-induced cells was observed in case of threonine3 and threonine11 phosphorylation of histone H3 (Fig. 7A-D). These data are in accordance with the Western blot results showing that the stimulation of MAP kinase pathway does not trigger threonine3 and threonine11 phosphorylation of histone H3.

## 4.2 A subset of genes were identified as targets of phoshphoacetylated histone H3

Phosphoacetylation of histone H3 correlates not only with the activation of IE genes but also other genes such as HDAC1. Based on the idea of a more general gene regulation via nucleosomal response, gene expression screens were performed and a number of novel genes were identified as potential target genes. Eight of them were chosen for further investigations such as ChIP analysis and Real-time PCR assays (Table 1). The ChIP analysis gave us the information that the promoter regions of these genes are associated with phosphoacetylated histone H3 in response to activation of the nucleosomal response in the presence of TSA (Fig. 8). The transcriptional induction under these conditions of these genes was examined by RT-PCR.

In addition, the impact of single treatment with either TSA or anisomycin on expression of these genes was examined.

The genes were divided into three gene clusters according to their kinetics of induction. The first gene cluster consisted of immediate-early genes whose nucleosomal response was a rapid and transient event. Due to the kinetic of expression, c-fos and Egr1 were considered as IE genes. It was observed that the genes were activated within one hour upon stimulation of MAP kinase pathway by anisomycin. Compared to anisomycin, TSA showed no strong effects on transcription of these genes. However, upon combination of both agents, the induction of gene expression was increased and stabilized for a period of six hours (Fig. 9A, B).

The second gene cluster exhibits the kinetic of early genes and the genes uPA, PAI1 and p21 were listed in this group. The transcriptional induction of the genes uPA and PAI1 was triggered by activation of MAP kinase pathway within 3 hours. Indeed, the highest gene expression was observed upon simultaneous treatment with both agents (Fig. 9D, E). Compared to uPA and PAI1 gene, the MAP kinase pathway seemed to be the main regulator for the activation of p21 gene (Fig. 9E).

In general, late genes representing the third cluster of genes give a delayed response to extracellular stimuli, because they require the synthesis of transcription factors encoded by IE or early genes to be expressed. We allocated the genes OPN, Timp3 and HDAC1 to this gene cluster due to their kinetic of induction. OPN and HDAC1 were moderately activated by TSA alone whereas Timp3 was slightly induced by anisomycin. Upon combinatorial treatment all three genes showed enhanced transcriptional induction compared to single treatments (Fig. 9F, G).

In addition to putative target genes of the nucleosomal response, Acrbp and Map2k7 genes were tested as negative controls. It was observed that combinatorial treatment with TSA and anisomycin did not result in activation of these genes whereas stimulation by anisomycin alone induced Map2k7 and TSA treatment alone activated Acrbp expression (Fig. 9I and 9J).

We know that less than 2 % of histones are phosphorylated and only a subset of genes is activated upon MAP kinase pathway induction. Therefore, gene activation by the nucleosomal response seems to be a well-regulated process. Additionally, our results support the idea that the H3 phosphoacetylation might be an important chromatin mark for the regulation of these target genes.

From our previous investigations, we have already known that the promoter regions of genes, discussed here, are associated with phosphoacetylated histone H3 (Fig.8). Next question that we asked was whether other genomic regions such as exons and introns of these and other genes from screen provide binding sites for phosphoacetylated histone H3. To answer this question and to map the phosphoacetylation mark on entire mouse genome, we prepared samples for a deep sequencing technique called ChIP sequencing that combines regular chromatin immunoprecipitation with DNA sequencing. We used an antibody raised against phosphoacetylated histone H3 to perform ChIP. After the quality of samples was tested, they were sent to be sequenced.

In addition to our expression screen, we also performed two other expression screens with resting Swiss 3T3 mouse fibroblasts treated either with anisomycin or TSA by using Agilent array. The next step of this study will be to combine all three expression profiles with each other and with the genome-wide map of phosphoacetylated histone H3. This will give us the information about which genes are transcriptionally induced by simultaneous activation of the MAP kinase pathway and HDAC inhibition. Furthermore, we will be able to correlate the presence of the H3 phosphoacetylation mark with activated genes. Finally, we will learn how this modification mark is localized on entire mouse genome and which DNA sequences of these target genes are associated with it.

## 4.3 Interaction between 14-3-3ζ isoform and P-TEFb can be indirect

14-3-3 proteins are phosphoserine binding proteins and their isoforms ε, γ and ζ act as “detectors” for histone H3 phosphorylated at serine 10 and 28. The binding of 14-3-3 proteins to phosphorylated serine10 on histone H3 is supported by acetylation of

neighboring lysine14 on histone H3 (Winter, Simboeck and Fischle, et al. 2008, Winter, Fischle and Seiser 2008, Walter, et al. 2008). They also mediate transcription of genes whose promoter regions are associated with phosphoacetylated histone H3 (Winter, Simboeck and Fischle, et al. 2008, Winter, Fischle and Seiser 2008). Although there are more than hundred interaction partners of 14-3-3 proteins, their interaction partners, in context of transcriptional regulation by the nucleosomal response, are not known. However, 14-3-3 proteins and RNAP II were found on promoter region of p21 gene (Simboeck, E. PhD Thesis). That gave rise to the idea that there can be link between 14-3-3 proteins and transcription machinery. This idea was supported with the finding that in *Drosophila* the P-TEFb recruitment to RNAP II requires the phosphorylation of serine10 at histone H3 tail (Ivaldi, Karam and Corces 2007). Therefore, we focused on the question whether there is an interaction between 14-3-3 proteins and P-TEFb complex consisting of CycT and Cdk9. We asked whether 14-3-3 proteins when bound to phosphorylated histone H3 can provide a docking site for P-TEFb to target P-TEFb to RNAP II. The phosphorylation of serine2 on CTD of RNAP II by recruited P-TEFb initiates elongation and causes gene expression. In that sense, 14-3-3 proteins can serve as mediators between phosphorylated or phosphoacetylated histone H3 and the P-TEFb complex (Brés, Yoh and Jones 2008, Ivaldi, Karam and Corces 2007).

To test our hypothesis, we tried to precipitate specifically the ζ isoform during coimmunoprecipitation assays. The 14-3-3ζ was chosen, because we have already know that 14-3-3ζ isoform binds to phosphorylated and phosphoacetylated histone H3 and triggers the induction of HDAC1 gene

Out of seven IP assays, we could precipitate 14-3-3ζ with Flag/HA tagged CycT only two times. The amount of precipitated 14-3-3ζ observed on Western blots was low compared to precipitated Cdk9. Based on these results, we propose that the interaction between 14-3-3ζ and P-TEFb can be indirect and mediated by other proteins. Our argument was supported by recent publications. The group of Zippo could show a crosstalk between serine10 phosphorylation of histone H3 and lysine16 acetylation of histone H4. They observed that 14-3-3 proteins bound to phosphorylated serine10 on histone H3 is recognized by MOF, a histone acetyltransferase. MOF can acetylate lysine16 on histone H4, which is recognized by the bromodomain of BRD4. The BRD4 brings the P-TEFb to RNAP II and P-TEFb can initiate transcriptional elongation (Zippo, et al. 2009). This finding shows the crosstalk among proteins associated with histones and might also indicate an indirect link between 14-3-3 proteins and P-TEFb.

In parallel, we established a single clone of Swiss 3T3 mouse fibroblasts producing TAP-tagged 14-3-3ζ protein. The TAP system is based on a tandem affinity purification (TAP) of protein of interest. The protein of interest can be purified by two steps and sent to mass spectrometry to identify its interacting partners. We wanted to use this system because the available antibodies are not sufficient quality for immunoprecipitation and ChIP assays.

Besides the interacting partners of 14-3-3ζ, it is also interesting to know how the protein is distributed on DNA sequences of our target genes. For that purpose, the purified protein can be also used for genome-wide analysis.

In the future, the combination of data from expression screens with the data from ChIP-Seq done for 14-3-3ζ and phosphoacetylated histone H3 can give us the information whether 14-3-3ζ plays a more general role for the transcriptional regulation of target genes of the nucleosomal response. Additionally, the identification of interaction partners of 14-3-3ζ will enable us to understand the link between histone phosphoacetylation, recruitment of the 14-3-3ζ protein and subsequent activation of transcription.

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# 6 CURRICULUM VITAE

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