The deacetylase HDAC6 is an essential component of stress granules and plays a critical role in the cellular response to stress

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Abbreviation

AD	Alzheimer's disease
Ago	Argonaute
AIF	Apoptosis-inducing factor
AMIs	Arginine methyltransferase inhibitors
ARE	AU-rich element
BCoR	Bcl-6-interacting co-repressor
CaMK	Ca^{+2}/calmodulin-dependent kinase
CK	Casein Kinase
Co-IP	Co-immunoprecipitation
CtBP	Carboxyl-terminal Binding Protein
CRS	Cytoplasmic retention sequence
DUBs	Deubiquitinating enzymes
dsRBMs	dsRNA-binding motifs
EF-1α	Elongation factor-1α
EHNA	Erhthro-9-[3-(hydroxynonyl)]adeninde
eIF2α	Eukaryotic initiation factor 2α
ER	Endoplasmic reticulum
eRF3	Eukaryotic releasing factor 3, translation termination factor 3
FAST	Fas-activated serine/threonine phosphoprotein
FL	Firefly luciferase
FXMR	Fragile X mental retardation protein
G3BP	Ras-GTPase activating protein SH3 domain binding protein
GAR	Glycine and arginine-rich
GR	Glucocorticoid receptor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HD^m	Cells re-expressing catalytic dead HDAC6 mutant
hnRNP H	Heterogeneous nuclear ribonucleoprotein H
HRE	Hypoxic response element

HRI	Heme-regulated kinase
HPTMs	Histone posttranslational modifications
HSF1	Heat shock factor 1
HSPs	Heat shock proteins
HSR	Heat shock response
ISR	Integrated stress response
KIF-11	Kinesin-like protein 11
MAP	Microtubule-associated protein
MEF2	Myocyte enhancer factor 2
MEFs	Mouse embryonic Fibroblasts
mRNP	messanger ribonucleoprotein
MTs	Microtubules
MTOC	Microtubule organizing center
N-CoR	Nnuclear receptor co-repressor
NES	Nuclear export signal
NLS	Nuclear localization signal
NuRD	Nnucleosome remodelling and deacetylating
ODD	Oxygen dependent degradation domain
PABP-1	Polyadenyl-binding protein-1
PBS	phosphate-buffered saline
PBs	Processing bodies (P-bodies or GW182 bodies)
PHD	Prolyl hydroxylase domain
RBPs	RNA binding proteins
PERK	PKR-like endoplasmic reticulum kinase
RISC	RNA-induced silencing complex
PKR	RNA-dependent protein kinase
RL	Ranilla luciferase
PRMT	Protein arginine methyltransferases
RRMs	RNA recognition motifs
SAHA	Suberoylaninide hydroxamic acid
SGs	Stress granules

SMA	Spinal muscular atrophy
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
SPs	Stress proteins
TIA-1/TIAR	T cell internal antigen-1/TIA-1 related protein
TCR	T cell receptor
TTP	Tristetraprolin
Ub^m	Cells re-expressing non-ubiquitin binding HDAC6 mutant
UIPS	Ubiquitination-independent proteasomal system
UPR	Unfolded protein response
UPS	Ubiquitin Specific Protease
UTR	Untranslated region
VEGF	vascular endothelial growth factor

Abstract

The reversible acetylation of histones has a critical role in transcriptional regulation. Likewise reversible acetylation of non-histones proteins is also important for other cellular processes. Acetylation and deacetylation of histones and other proteins are catalyzed by opposing histone acetyl transferases (HATs) and deacetylases (HDACs) respectively. Among three classes of histone deacetylases, HDAC6 is a very unique class II HDAC enzyme which possesses two independent deacetylase domains and a Zn-UBP ubiquitin binding domain at the C-terminus. HDAC6 has been shown to interact with nuclear proteins as well as cytoplasmic proteins such as tubulin and HSP90. However, the physiological function of HDAC6 is not fully understood yet. Therefore, to further define the cellular function of HDAC6, an identification of novel interacting proteins has been undertaken.

The first section of this thesis describes the identification of one novel HDAC6 interacting protein and the role of HDAC6 in stress granule (SG) formation in response to environmental stress. First of all, we identified new HDAC6 interacting proteins using proteomic affinity trap approach. Here, we focused that HDAC6 interacts and co-localizes with a previously identified stress granule component, G3BP (RasGAP associated endoribonuclease) *in vitro* and *in vivo*. We first discovered that HDAC6 is a stable and critical component of stress granules. Further experimental data suggested that HDAC6 can regulate the assembly of SGs via recruiting SG components to the microtubule system. Because of this HDAC6 may have an impact on various processes involoving RNA metabolism and we provide initial evidence that the miRNA pathway is indeed influenced by HDAC6 function.

The second section of this thesis examines the role of HDAC6 in response to various stresses. The involvement of HDAC6, a multi-functional cytoplasmic deacetylase, in processes such as the clearance of cytotoxic aggregated misfolded proteins and the deacetylation of HSP90 chaperone, has prompted us to investigate a role for HDAC6 in cellular protection under stress condition. In hypoxia, HDAC6 regulated stability of HIF-1α by controlling its deacetylation. Indeed, loss of HDAC6 rendered cells more sensitive

to programmed cell death. Moreover, depletion of HDAC6 affected the recovery of cells from stress as well as the direct stress response, suggesting a significant role of HDAC6 as a cellular regulator of the stress response.

In addition, a number of other putative HDAC6 interactors are presented, which were identified in the initial mass spectrometry screens. Several of these proteins encode cytoplasmic factors that have a role in RNA metabolism, protein translation or in cytoskeletal regulation. Therefore, it appears likely that at least some of these may turn out to be relevant partners contributing to HDAC6 function.

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1. Chapter 1: INTRODUCTION

1.1. Epigenetics

1.1.1. Concept of epigenetics and epigenetic regulation

"Epigenetics" is defined as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by change in DNA sequence" (Shemer et al., 1996). Epigenetic mechanisms are responsible for putting in place and maintaining the patterns of gene expression that specify the many different cell types required to make a higher eukaryote (Turner, 2007). Epigenetic mechanisms would include DNA methylation or histone modifications. At a molecular level, epigenetic mechanism needs co-operation of a variety of regulatory proteins including DNA methyltransferase, methyl CpG binding proteins, histone-modifying enzymes, chromatin remolding factors, transcriptional factors and chromosomal proteins. Moreover, chromosomes structures such as centromere, kinetochores, and telomeres enter into the category of epigentics even though they are or are not connected directly to gene function. Epigenetic phenomena have major economic and medical relevance, and several, such as imprinting and mutation, violate Mendelian principles. Epigenetic control of gene expression can be considered from the standpoint of normal development, which requires stable repression of genes not required in specific cell types. Dysregulation at the epigenetic states cause human disease phenotypes, especially developmental defects and tumorigenesis Many

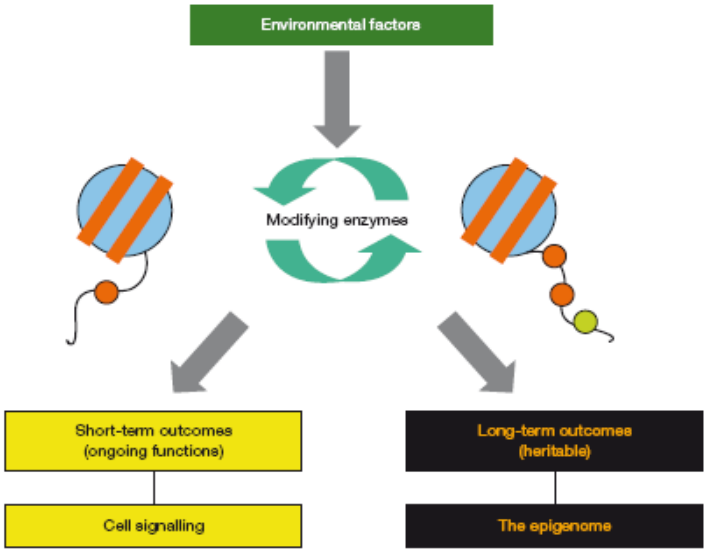

Figure 1. Histone modifications can generate both short-term and long-term outcomes Histone tail modifications are put in place by modifying and demodifying enzymes, whose activities can be modulated by environmental and intrinsic signals. Adapted from Turner, 2007.

epigenetic effects, however, are observed in unusual circumstances. Therefore, epigenetics will become a major target for emerging biological and medical discoveries (Nakao, 2001).

1.1.2. Chromatin and Histone modification

The nucleosome is the fundamental unit of eukaryotic chromosomes. It consists of a core of eight histone proteins (copies each of H2A, H2B, H3, and H4) around which 147 base pairs of DNA are wraped in 1.75 superhelical turn. Histones have a mass roughly equal to that of the DNA which they are associated with (Fig. 2). Each of the core histones exhibits a similar structural feature called the "histone fold," which consists of a long central α-helix flanked by shorter helices and loops that interact with DNA. Core histone octamer proteins are evolutionally conserved. They consist mainly of flexible N-terminal tails that protrude outward from the nucleosome and control the folding of nucleosomal arrays into higher order structure and of globular C-terminal domains that comprise the nucleosome scaffold mediating histone-histone interaction. Each nucleosome is separated by 10-60 bp of 'linker' DNA, and the resulting nucleosomal array constitutes a chromatin fiber of ~10 nm in diameter. This simple 'beads-on-a-string' arrangement is folded into more condensed ~30 nm thick fibers that are stabilized by binding of a linker histone to each nucleosome core. Such 30 nm fibers are then further condensed *in vivo* to form 100-400 nm thick interphase fibers or the more highly compacted metaphase chromosome structures. These local or extended structural changes in chromatin play an essential role in the control of gene expression and are governed by complexes that remodel chromatin and by enzyme that posttranslationally modify histones (Peterson and Laniel, 2004).

The amino-terminal tails of core histones are subject to various post-translational modifications modulating chromatin structure and function. Post-translational modifications of histones divide into two goups. First three are the small chemical groups, including acetylation of lysine residues, methylation of lysines and arginines, phosphorylation of serines and threonines. Second there are lager chemical modification including ubiquitination of lysine, sumoylation of lysines, and the poly-ADP-ribosylation of glutamic acid. Theae modifications decorate the nucleosome surface with an array of chemical information. Different combinations of histone posttranslational modifications

has been proposed to a "histone code" which is established and maintained in particular region of chromatin to specify unique downstream functions. The best understood histone modifying enzymes, histone acetylases (HATs) and histone deacetylases (HDACs) play important roles in physiological and aberrant gene regulation.

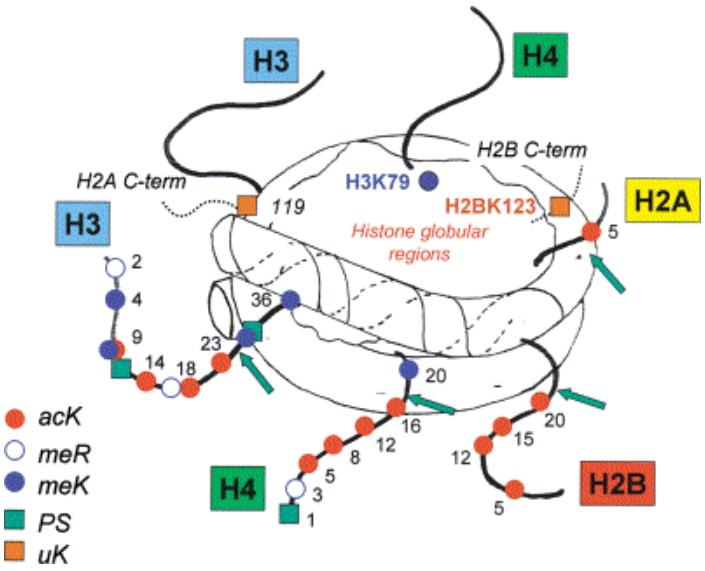

Figure 2. Histone modifications on the nucleosome core particle The nucleosome core particle showing 6 of the 8 core histone N-terminal tail domains and 2 C-terminal tails. Sites of posttranslational modification are indicated by colored symbols that are defined in the key (lower left). Sites marked by green arrows are susceptible to cutting by trypsin in intact nucleosomes. Adapted from Turner, 2002.

Over the years, many different types of HDAC inhibitors (HDACi) have been developed, ranging from complicated structures of bacterial or fungal origin (trichostatinA (TSA), trapoxin) to the very simple butyrate. HDACi are capable of inhibiting HDACs with varying efficiency (at nanomolar to millimolar concentrations). Inhibition of HDACs can result in a general hyperacetylation of histones, which is followed by the transcriptional activation of certain genes through relaxation of the DNA conformation. Generally, HDACi are known to be able to induce growth arrest, differentiation or apoptosis of cancer cells *in vitro and in vivo*. DNA micro-arrays using malignant cell lines cultured in the presence of a HDACi indicated that a specific small number of genes (1±7%) showed altered expression. In addition, HDACi also leads repression of a number of genes. Thus the effect of HDACi on gene expression is believed not to be a universal one, but rather involves alteration of the transcription of a specific subset of genes (Dangond and Gullans, 1998).

1.1.2.1. Histone posttranslational modifications and histone code hypothesis

Histone posttranslational modifications (HPTMs)

Covalent posttranslational modifications of histones play key roles in controlling the capacity of the genome to store, release, and inherit biological information. Histone modifications may function in both short term, ongoing processes (such as transcription, DNA replication and repair) and in more long-term functions (as determinants of chromatin conformation, for example, heterochromatin formation, or as heritable markers that both predict and are necessary for future changes in transcription). Short-term modifications are transient and show rapid turn over in response to external stimulation. Long-term, heritable modifications need not necessarily be static (Turner, 2007). Histones modifications can be highly reversible, such as histone acetylation, and histone phosphorylation, histone ubiquitination and sumoylation, ribosylation, or more stable, such as histone methylation. Recently it has been found that although methylation was considered a stable modification, recent several demethylations at aginine or lysine residue have been identified. Furthermore, each lysine residue and arginine residue can be either mono-, di-, or tri-methylated (Fig. 3). The majority of these post-translational marks occurs on the amino terminal and carboxy terminal histone tail domains, although more and more examples of modifications within the central domains of histones have been identified. A wide range of histone and chromatin-based regulatory options is available. These include rapid adjustments of gene expression in response to physiological and environmental stimuli as well as transmission of inheritable expression patterns to the next generation. Fundamental cellular mechanisms are manifested in the genetic and epigenetic regulatory circuits that control the post-translational modification of histone (Fischle et al., 2003).

The histone code hypothesis

Posttranslational modifications of histones constitute a code that allows specific interactions or reactions with chromatin-associated components to take place in a chromosomal context. This idea refers as the "histone code hypothesis". The code is generated by histone-modifying enzymes of defined specificity and read by nonhistone proteins in a modification-sensitive manner (Fig.4) (Strahl and Allis, 2000). The theory

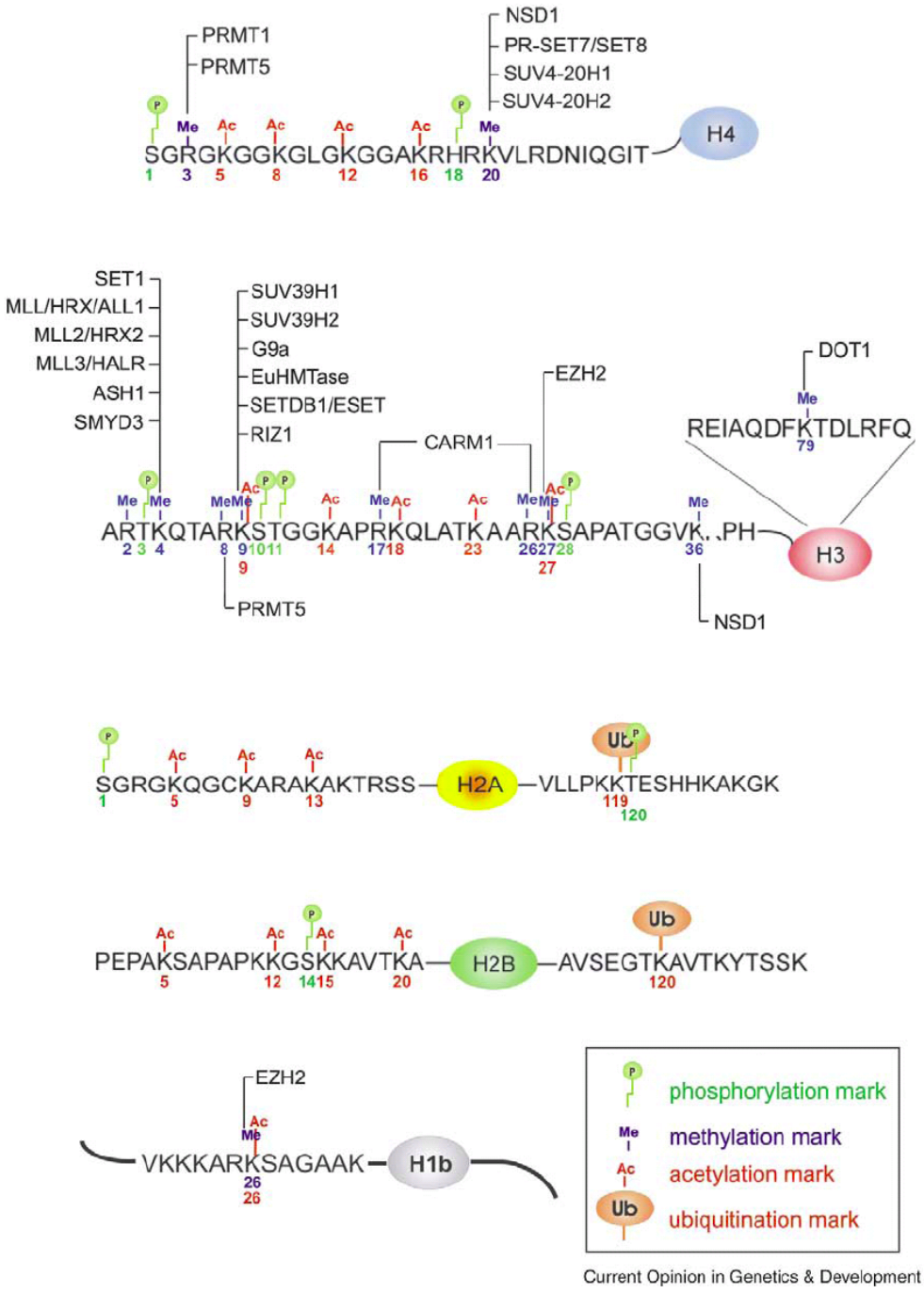

Figure 3. Histones are subjected to a variety of post-translational modifications

The modifications on human histones include acetylation (Ac, red), methylation (Me, blue), phosphorylation (P, green) and ubiquitination (Ub, brown). The enzymes responsible for methylation of mammalian histones are listed above or below their target sites. Note that there are several redundant enzymes specific for methylation of histone H3-K4 and H3-K9. Adapted from Margueron et al., 2005.

postulates that different combinations of post-translational histone modifications are established and maintained in particular regions of chromatin to specify unique downstream functions. Histone code would be a binary relationship between posttranslational histone modifications and either gene activation or repression, and distinct HPTMs for other precesses. The mechanism is likely to be also functioning in reactions other than transcription which are regulated by post-translational modification of histones, such as DNA replication, repair and recombination (Fig. 4) (Peterson and Laniel, 2004).

The histone codes are decoded by proteins that interact with histones in modification-dependent manners (Fig. 4). One group, the bromodomain proteins of various proteins have been reported to interact with acetylated histones in a lysine-specific manner *in vitro* and *in vivo* (Dhalluin et al., 1999). These bromodomain-containing proteins are components of nucleosome-modulating complexes that also include ATPases and HATs. Acetylation at specific lysines is thought to stabilize these

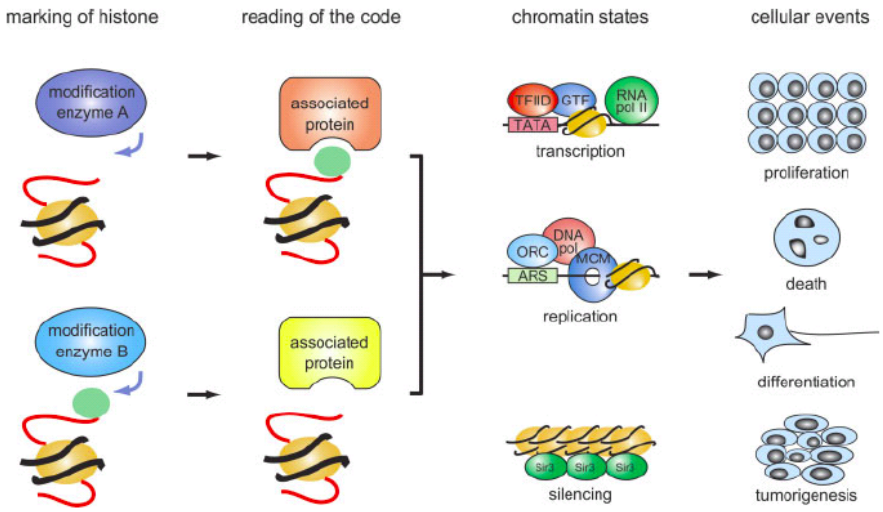

Figure 4. The histone code hypothesis

Schematic of the histone code hypothesis. Histones are labeled with "codes" by histone modifying enzymes ("marking of histone" in the figure). These post-translational modifications are recognized by proteins that interact with histones in modification-dependent manners ("reading of the code"). Recruitment of these histone-interacting proteins triggers subsequent reactions on chromatin ("chromatin states"), which cause various changes ("cellular events"). Adapted from Kimura et al., 2005.

complexes through bromodomain interaction and to stimulate nucleosome remodeling, further acetylation, or the recruitment of TFIID. Initial recruitment of a HAT to chromatin may require nucleosome remodeling. In these cases, HAT is proposed to be required for the subsequent stable binding of the ATPase complex. But, the HAT may also be recruited to chromatin before the ATPase complex and recruit ATPases in some instances (Hassan et al., 2001).

Sir3 and Tup1 are proposed to interact with hypoacetylated histones, and both repress gene expression in *S. cerevisiae*. Sir3 spreads along chromatin and contributes to gene repression over a range of several kilobases. Deacetylation of H4-K16 by an HDAC, Sir2, stimulates binding of Sir3 to chromatin and thus gene silencing, whereas acetylation of this lysine by a MYST-HAT, Sas2, prevents Sir3 from spreading on chromatin and contributes to anti-silencing (Suka et al., 2002). In contrast, Tup1 represses gene expression in a promoter-specific manner. The local recruitment of Tup1 is accomplished by sequence-specific DNA binding proteins such as α2/Mcm1, Mig1 and Sko1. Tup1 acts

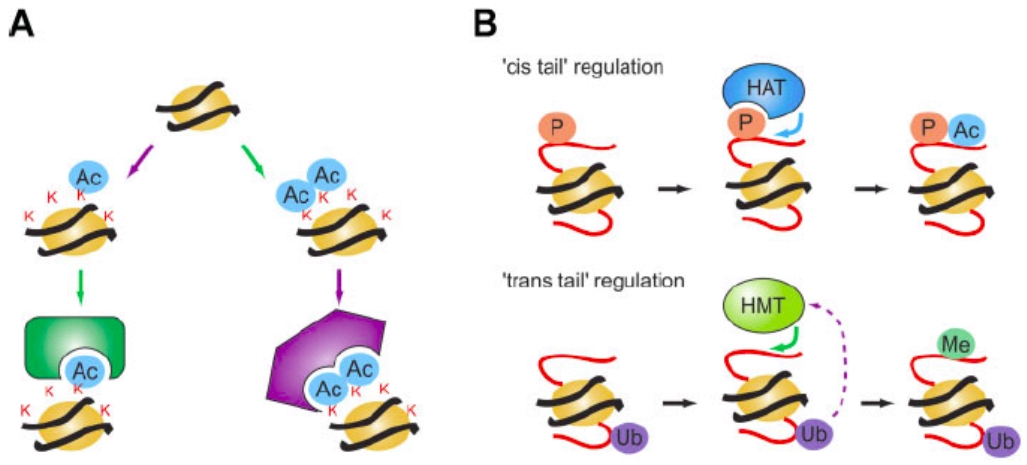

Figure 5. Reading histone codes

(A) Depending on specific patterns established by various histone-modification enzymes, distinct proteins are recruited to chromatin, with specific results. (B) Schematic of "chromatin crosstalk." The efficiency of modification at particular residues depends on pre-existing histone modification patterns. Such interdependency might involve residues in the same histone-tail ('cis tail' regulation) or those in different histone tails ('trans tail' regulation). Adapted from Kimura et al., 2005.

in concert with a histone H2B/H3-specific HDAC, Hda1, to repress gene expression, possibly by binding to hypoacetylated histones (Wu et al., 2001). Other modifications of histones also regulate their interaction with proteins, and these alterations also function as codes. For example, methylation of H3-K9 is known to stimulate the binding of chromodomain-containing proteins such as HP1 and Swi6 to chromatin, leading to gene repression (Lachner et al., 2001).

Cross-talk of DNA methylation and Lys methylation in Histone

Modification of histones also influences other histone modifications, a phenomenon called cross talk. In *cis*-tail crosstalk, a given modification affects modification of neighboring residues by physically stabilizing or inhibiting interaction between enzymes and substrates. For example, phosphorylation of serine 10 of histone H3 (H3-S10) enhances acetylation of H3-K14 by Gcn5 (Clements et al., 2003). In *trans*-tail crosstalk, a given modification affects modifications on other histone tails too. For example, ubiquitination of H2B-K123 by Rad6/Ubc2 is required for methylation of H3-K4 and H3-K79 (Fig. 5) (Sun and Allis, 2002). The interdependency of histone modifications proposes that histone modifications function as binary switches (Fischle et al., 2003).

For many years, DNA methylation, namely the 5 methylcytosine (5mC) modification at CpG islands of the genome, has been the main focus of the epigenetic gene regulation field (Feinberg and Tycko, 2004; Jaenisch and Bird, 2003) The finding that histone modifications can regulate DNA methylation patterns suggest that histone modifications, particularly Lys methylation, are important regulatory mechanism of epigenetic phenomena such as X-chromosome inactivation, imprinting, and cancer. In *Neurospora crassa*, DIM5, a methyltransferase of histone H3 Lys9, mediates DNA methylation (Tamaru et al., 2003). In *Arabidopsis thaliana*, KRYPTONITE, another histone H3 Lys9 methyltransferase, also is required for DNA methylation mechanism (Jackson et al., 2002). These studies suggest a regulatory mechanism whereby DNA methylation is targeted by histone methylation.

While the above data support that histone methylation guides DNA methylation, other reports suggest that DNA methylation may regulate histone methylation as well.

For example, DNA hypomethylation causes defects in H3 Lys9 methylation in Arabidopsis thaliana (Soppe et al., 2002). Biochemical studies found that the methyl-DNA binding protein (MeCP2) interacts with H3 Lys9 mehtyltransferase (Fuks et al., 2003). These results suggest that there may be continous interplay between histone methylation and DNA methylation in certain biological systems. The possibility that DNA methylation may guide histone methylation is especially intriguing in the context of maintaining histone modification patterns following DNA replication.

1.1.2.2. Overview of histone acetyltransferases (HATs) and histone deacetylases (HDACs)

Histone acetylation was first discovered by Allfrey et al. in 1964 and proposed to regulate gene expression. This idea was supported by the observation that hyperacetylation of histones correlates with transcriptional activation. Turner et al. observed acetylation of distinct lysine residues in specific chromosomal regions in *Drosophila melanogaster* polytene nuclei. For example, histone H4 Lys 5 (H4-K5) or H4-K8 is frequently acetylated in euchromatic regions, where transcription is potentially active. In contrast, acetylation of H4-K12 is increased in heterochromatic regions, where transcription is potentially inactive. Acetylation of H4-K16 is found along the transcriptionally hyperactive male X chromosome (Turner et al., 1992). Furthermore, the first histone deacetylase (HDAC) enzyme was isolated via biochemical purification (Taunton et al., 1996). In this case, the enzyme was purified from cell extracts using inhibitor, trapoxin, which physically bound to the catalytic site of the enzyme. These observations suggested that residue-specific acetylation, rather than bulk neutralization of electrostatic charge, is important in regulation of gene expression through histone modification. They further suggested that histone modifications mediate not only promoter-specific gene expression but also longer-range (and even chromosome-wide) gene expression.

All core histone proteins are reversibly and dynamically acetylated at multiple sites in their N-terminal tails. Hyperacetylated histones are generally found in transcriptionally active genes and hypoacetylated histones in transcriptionally silent regions, such as heterochromatin. The level of histone acetylation at a particular locus in chromatin reflects the competing activities of HATs and HDACs. The identification and

characterization of numerous transcriptional regulators recruiting HAT or HDAC has validated the prediction that histone acetylation plays a critical role in transcriptional regulatory mechanisms (Cheung et al., 2000).

In addition to histones, many nuclear and cytoplasmic proteins have recently been shown to be reversibly acetylated on lysine residues. Acetyl-lysine is now known to be present in at least 80 other proteins, including ~ 40 sequence-specific transcription factors, ~10 transcriptional co-regulators, several viral proteins, p53, tubulin, Hsp90, and Ku70. Lysine acetylation is controlled by the opposing actions of HATs and HDACs, and regulates the functional activity of these proteins. In several cases, the level of acetylaion was shown to have a critical impact on actitivy of the protein. (Yang and Gregoire, 2005).

Classification of HDACs

HDACs are conserved from yeasts to mammals. Eighteen distinct human HDACs are grouped into three classes based on their primary homology to three *Saccharomyces cerevisiae* HDACs. Class I HDACs (HDAC1, -2, -3, -8 and -11) are homologous to yRPD3, share a compact structure and a conserved deacetylase domain (hdac); they are predominantly nuclear proteins expressed in most tissues and cell lines (de Ruijter et al., 2003; Fischle et al., 2001b). Class II HDACs are homologous to yHDA1 and are subdivided into two subclasses, IIa (HDAC4, -5, -7 and -9 and its splice variant MITR) and IIb (HDAC6 and HDAC10), based on sequence homology and domain organization. The class IIa HDACs, HDAC4, -5, -7 and -9, share an N-terminal domain of ~450–600 amino acids. Domains in this region mediate interactions with the transcriptional co-repressor CtBP, the MEF2 family of transcription factors, and other proteins. HDAC6 and HDAC10 are unique in having two catalytic sites. However, in HDAC10, one of two catalytic domains is not functional. HDAC6 contains two independent HDAC domains, both of which are required for deacetylase activity. The expression pattern of class II HDACs is more restricted, suggesting that they might be involved in cellular differentiation and developmental processes. Whereas class I and II HDACs, all share some degree of homology in their catalytic domain, class III HDACs are homologous to ySIR2 and show no homology to class I and II proteins. Class I and II HDACs are Zn^{2+}-

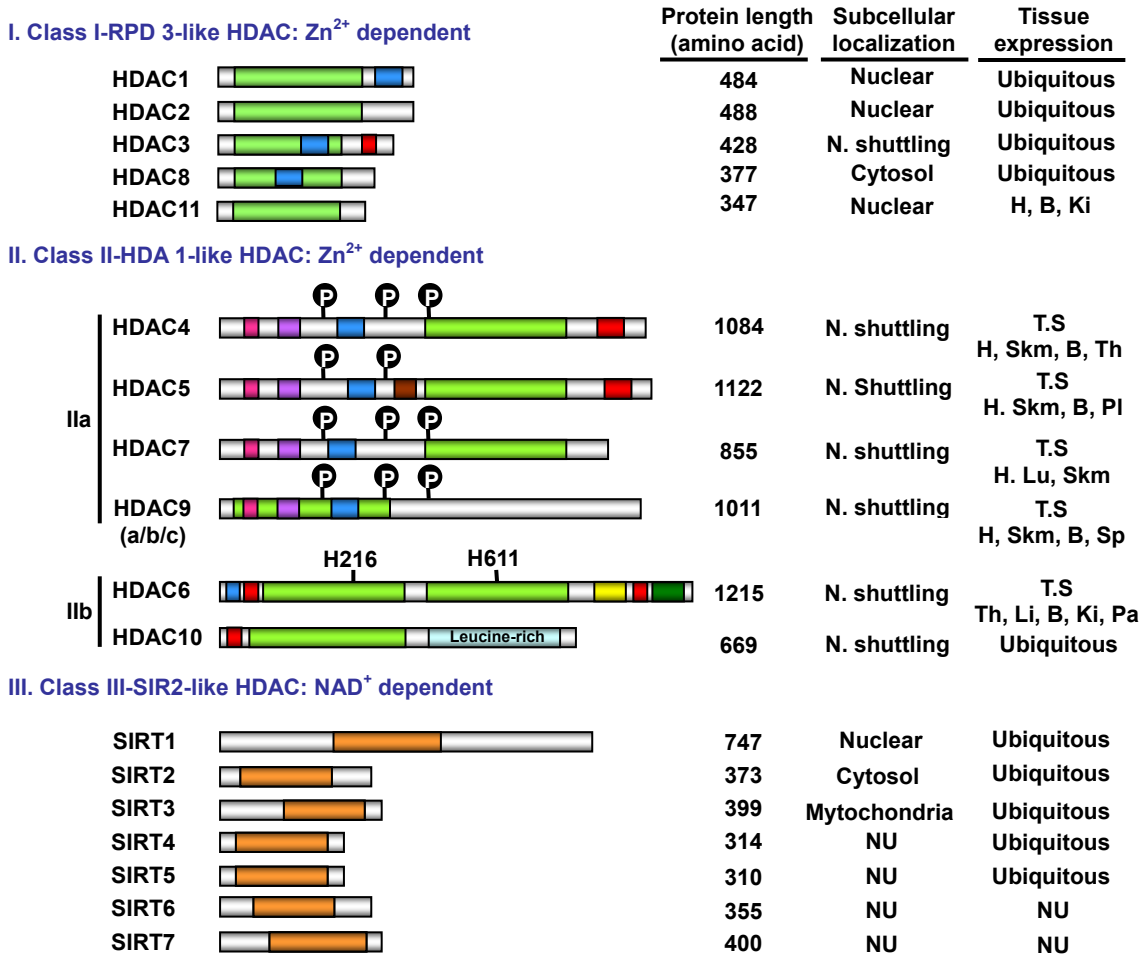

Figure 6. Schematic depiction of the different isoforms of histone deacetylase (HDAC)

HDACs are grouped into three classes - I, II and III - on the basis of their homology with three structurally and biochemically distinct yeast HDACs, Rpd3p, Hda1p and Sir2, respectively. Class II HDACs are unique in that they are expressed in a tissue restricted manner (H, heart; B, brain; Ki, kidney; Skm, skeletal muscle; Th, thymus; Pl, placenta; Lu, lung; Sp, spleen; Pa, pancreas; NU, not fully understood; N. shuttling, Nucleocytoplasmic shuttling. Domains are indicated by colored boxes; light green, and orange, HDAC domain; pink, CtBP binding domain; purple, MEF2 binding domain; blue, NLS; brown, HP1 binding domain; red, NES; yellow, SE14 (SerGlu-containing tetradecapeptide repeats); green, Zn-UBP; Leucine-rich, sky blue.

dependent enzymes, whereas the deacetylase activity of class III members is NAD+ dependent. In agreement with this, class I deacetylases function as transcriptional corepressors while Sir2p-related proteins appear to be involved in gene silencing. HDAC11 is most recently described HDAC (Gao et al., 2002; Voelter-Mahlknecht et al., 2005).

Localization of HDACs

To deacetylase histones, HDACs need to be in the nucleus, where their predominant substrate is found. The nuclear localization of HDACs occurs via a nuclear localization signal (NLS) or via colocalization together with other proteins/HDACs. Most HDACs contain a NLS, but some can be in the cytoplasm as well; this depends on other regulatory domains (de Ruijter et al., 2003). Class I HDACs are found almost in the nucleus. For example, the localization of HDAC1 and HDAC2 is exclusively nuclear, due to the lack of a nuclear export signal (NES) (Johnstone, 2002; Taplick, 2001). HDAC3 is largely localized in the nucleus even though it has both NLS and NES (Yang, 2002). HDAC8 is exclusively nuclear (Johnstone, 2002; Van den Wyngaert et al., 2000).

Class II HDACs are able to shuttle in and out of the nucleus in response to certain cellular signals. The predominant localization of HDAC6 is in the cytoplasm, although it can be partially found in the nucleus of osteobloasts and some cell lines (Bertos et al., 2004; Hubbert et al., 2002; Verdel et al., 2000). HDAC11 resides in the nucleus; however, in activity assays, HDAC11 colocalize with HDA6 in the cytoplasm (Bertos et al., 2001; Gao et al., 2002). HDAC10 can be localized in both the nucleus and the cytoplasm, although the function of the localization in the two compartments has not been clarified (Fischle et al., 2002; Kao et al., 2002). The subcellular localization of HDAC9 can be cytosolic as well as nuclear, depending on the splice variant (Zhou et al., 2001). The shuttling of HDAC4, -5 and -7 between the cytosol and the nucleus has been studied extensively in differentiating muscle cells (Fischle et al., 2001a; Fischle et al., 2002; Pflum et al., 2001). Due to a (pre-) differentiation signal, HDAC4 is phosphorylated by Ca^{+2}/calmodulin-dependent kinase (CaMK), resulting in the export of HDAC4 together with CRM1, a cellular export factor for proteins with a leucine-rich NES. 14-3-3 protein (a cytosolic anchor protein) binds the phosphorylated form of HDAC4 and thereby

retains HDAC4 in the cytosol. After fusion of muscle cells, terminal differentiation (post-differentiation) occurs, and HDAC4 is released from 14-3-3 due to a decrease in its phosphorylation status, and will consequently shuttle back to the nucleus (Grozinger and Schreiber, 2000; Kao et al., 2001). The localization of HDAC5 is regulated by phosphorylation signal and NLS in a similar manner to HDAC4 but shuttling direction of HDAC5 is opposite. Even though there is a large degree of similarity in function and localization between HDAC5 and HDAC7, both enzymes are found in the same or different cellular compartment in cell line dependent manner. Though the reason is not very clear, the presence of the NES domain on HDACs might have a dominant role in determining the localization of HDAC4 (NES), HDAC5 (one NES), or HDAC7 (no NES) (Bertos et al., 2001; Dressel et al., 2001; Kao et al., 2001).

Mechanism of action of HDACs

The mechanism of action of the HDAC enzymes involves removing the acetyl group from the histones, which leads to a decreased space between the nucleosome and the DNA that is wrapped around it. Tighter wrapping of the DNA diminishes accessibility for transcription factors, leading to transcriptional repression (Strahl and Allis, 2000). The catalytic domain of HDAC is formed by a stretch of ~ 390 amino acids consisting of a set of conserved amino acids. The active site consists of a gently curved tubular pocket with a wider bottom (Finnin et al., 1999). Removal of an acetyl group occurs via a charge-relay system consisting of two adjacent histidine residues, two aspartic residues (located

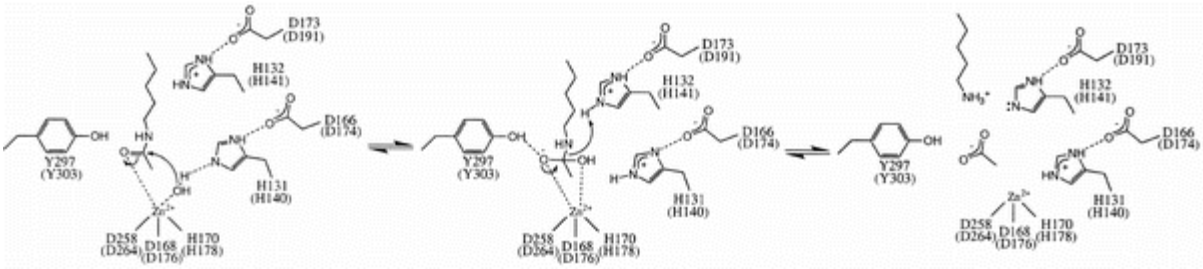

Figure 7. The proposed catalytic mechanism for the deacetylation of acetylated lysine

HDLP active-site residues and their proposed HDAC1 counterparts (in parenthesis) are labelled. Adapted from Grozinger et al, 2002.

approx. 30 amino acids from the histidines and separated by approx. 6 amino acids), and one tyrosine residue (located approx. 123 amino acids downstream from the aspartic residues (Buggy et al., 2000; Finnin et al., 1999). An essential component of the charge-relay system is the presence of a Zn^{2+} ion. This atom is bound to the zinc binding site on the bottom of the pocket. However, other cofactors are required for HDAC activity: most recombinantly expressed enzymes are found to be inactive. HDACi function by displacing the zinc ion and thereby rendering the charge- relay system dysfunctional. TSA, with its hydroxamic acid group and its five-carbon atom linker to the phenyl group, has the optimal conformation to fit into the active site (de Ruijter et al., 2003; Finnin et al., 1999).

1.1.2.3. Class I HDACs

1.1.2.3.1. HDAC1 and HDAC2

HDAC1 and HDAC2 are highly similar enzymes, with an entire sequence identity of approximately 82%. The catalytic domain on the N-terminus forms the major part of the protein (Cress and Seto, 2000; Kao et al., 2000; Li et al., 2002). HDAC1 gene is mapped to 1p34 and HDAC2 gene is mapped to 6q21 (NCBI SAGE database). HDAC1 and HADC2 are mainly localized in the nucleus and regulate general gene expression (Taplick, 2001). HDAC1 and HDAC2 are inactive when produced by recombinant techniques, implying that cofactors are necessary for HDAC activity. *In vivo*, HDAC1 and HDAC2 only display activity within a complex of proteins. These complexes consist of proteins necessary for modulating their deacetylase activity and for binding DNA, together with proteins that mediate the recruitment of HDACs to the promoters of genes (Zhang et al., 1999). HDAC1 and HDAC2 are generally found in stable, multicomponent complexes of proteins: the Sin3, NuRD, (nucleosome remodelling and deacetylating) and CoREST complexes. Both the Sin3 complex and the NuRD complex consist of a core complex containing HDAC1, HDAC2, Rb-associated protein 48 (RbAp48) and RbAp46. The core complex alone does not possess maximal HDAC activity, and additional cofactors are needed (Brehm et al., 1998; Galasinski et al., 2002; Heinzel et al., 1997; Zhang et al., 1999). Moreover, HDAC1 and HDAC2 can bind directly to DNA binding

proteins such as YY1, Rb binding protein-1 and Sp1 (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Yang et al., 1996; Yao et al., 2001; Yoshida et al., 1990).

In addition to the regulation of HDAC1 and HDAC2 activity by co-repressors, they are also regulated by several post-translational modifications. Both enzymatic activity and complex formation are regulated by phosphorylation. HDAC1 (Ser421 and Ser423) and HDAC2 (Ser394, Ser411, and Ser424) are phosphorylated by protein kinase CK2 *in vivo* and *in vitro* (Cai et al., 2001; Sun et al., 2002). Hyperphosphorylation of HDAC1 and HDAC2 leads to a slight but significant increase in deacetylase activity, and at the same time to disruption of complex-formation. When hypophosphorylation of HDAC1 and HDAC2 occurs, the activity of HDAC1 and HDAC2 decreases, but complex formation is increased. Phosphorylation status of HDAC1 or 2 determines and maintains HDAC activity at a certain optimal level (Galasinski et al., 2002; Pflum et al., 2001).

1.1.2.3.2. HDAC3

HDAC3 is evolutionarily most closely related to HDAC8 (34% identity). The HDAC3 protein is 50% identical in DNA sequence and 53% identical in protein sequence compared with HDAC1. Comparison of the HDAC3 and HDAC2 also yielded similar results, with 51% identity in DNA sequence and 52% identity in protein sequence (Yang, 1997). HDAC3 gene is mapped to 5q31 (NCBI SAGE database). Surprisingly, the non-conserved C-terminal region of HDAC3 is required for both deacetylase activity and transcriptional repression. HDAC3 has NLS as well as a NES (amino acids 180-313) but is nearly always in the nucleus. HDAC3 is ubiquitously expressed (Yang, 1997). HDAC3 shares structural and functional features with other class I HDACs and forms oligomers with other HDACs *in vivo* and *in vitro*, but it exists in multisubunit complexes that are different from other known HDAC complexes(Yang, 2002). Endogenous HDAC3 mostly associates with itself, and only a small fraction of HDAC3 interacts with HDAC4. Both co-repressors, SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) and N-CoR (nuclear receptor co-repressor) are activating cofactors for HDAC3 because they have a conserved deacetylase-activating domain for HDAC3 activation (Guenther et al., 2001). Recent evidence has shown that HDAC3 interacts with and deacetylates MEF2 transcription factors via the MADS box *in vivo* and *in vitro*. In addition, HDAC3

stimulated by SMRT associated with the acetyltransferases p300 and PCAF to reverse autoacetylation. This result suggests that HDAC3 represses MEF2-dependent transcription and inhibites myogenesis (Gregoire et al., 2006).

1.1.2.3.3. HDAC8

HDAC8 consists largely of the catalytic domain with an NLS in the center (Buggy et al., 2000) and its genes are mapped to Xq21.2-Xq21.3 and to Xq13. HDAC8 is considered to be ubiquitously expressed. Using immunohistochemistry, in normal human tissues, HDAC8 is exclusively expressed by cells showing smooth muscle differentiation, including visceral and vascular smooth muscle cells, myoepithelial cells, and myofibroblasts, and is mainly detected in their cytosol. HDAC8 associates with the smooth muscle actin cytoskeleton and may regulate the contractility smooth muscle cells (Waltregny et al., 2005).

1.1.2.3.4. HDAC11

HDAC11 is most closely related to HDAC3 and HDAC8, but the classification of HDAC11 has not yet been determined clearly. However, recently HDAC11 has been classified as class IV HDACs. The human HDAC11 gene is localized to chromosome 3p25 (Gao et al., 2002; Voelter-Mahlknecht et al., 2005). HDAC11 contains a catalytic domain at the N-terminus. HDAC11 has not been found in any other known HDAC complexes, possibly implicating that HDAC11 might have distinct biological functions. AML blasts and cell lines, exposed to HDACis in culture, showed both histone hyperacetylation (H3 K4) and several inhibitors (valproic acid, butyrate, TSA, SAHA) caused strong induction of HDAC11 in all myeloid cells tested (Bradbury et al., 2005).

1.1.2.4. Class II HDACs

1.1.2.4.1. Class IIa HDACs: HDAC4, HDAC5, HDAC7 and HDAC9

HDAC4, -5, -7 and -9 contain a highly conserved catalytic domain (~420 amino acids) which corresponds to the C-terminal half of the protein. The NLS of these HDACs is situated close to the N-terminus; HDAC4, amino acids 251-272 and HDAC5, amino acids 264-285 (Wang and Yang, 2001). HDAC9 catalytic domain is located in the N-

terminus. There are three splice variants, HDAC9a, HDAC9b, and HDAC9c (Zhou et al., 2001). Binding domains for C-terminal binding protein (CtBP), myocyte enhancer factor 2 (MEF2) and 14-3-3 are conserved in all class IIa HDACs on the N-terminus (Bertos et al., 2001). HDAC4, -5, -7 are able to interact with SMRT/N-CoR, and the co-repressors BCoR (Bcl-6-interacting co-repressor) and CtBP. Also the N-termini of HDAC4, -5 and -7 interact specifically with and repress the myogenic transcription factor MEF2. When MEF2 is associated with HDAC4, -5 or -7, the function of MEF2 as a transcription factor is inhibited, thereby preventing muscle cell differentiation. CaMK activity overcomes this inhibition by dissociating the MEF2 and HDAC complex due to phosphorylation of HDAC4/5/7 and causes to export of the HDAC out of the nucleus by CRM1 (Grozinger and Schreiber, 2000; Kao et al., 2001; McKinsey et al., 2000a; McKinsey et al., 2000b). HDAC9 also interacts with MEF2/CaMK/14-3-3, indicating that HDAC9 may also function in muscle differentiation as the three class IIa HDACs (Zhang et al., 2001). Interestingly, HDAC4, -5 and -7 associate with HDAC3 through the SMRT/N-CoR co-repressors and become enzymatically active. This suggests that HDAC4, -5, and -7 functionally link between DNA-binding recruiters and the HDAC3-containing HDAC complex (Fischle et al., 2002). HDAC5 resides in the nucleus during pre-differentiation and is relocalized to the cytoplasm during differentiation. HDAC5 may be transported by CaMK into cytoplasm as it has a NES domain (amino acids 1086-1099) like HDAC4 (amino acid 1056-1069): however more research is needed to clarify this assumption. The subcellular localization of HDAC5 and HDAC7 differs from that of HDAC4 at the different stages of muscle cell differentiation, implicating that these HDACs might control the differential regulation of gene expression during the various stages of muscle cell differentiation. In cell types other than muscle cells, the regulation of the localization of HDAC5 and HDAC7 is less clear. Even though there is a large degree of similarity in function and localization between HDAC5 and HDAC7, both enzymes are found in the nucleus in some cell lines (CV-1, MCF7), whereas in other cell lines (HepG2) HDAC5 is located in the nucleus and HDAC7 is localized in the cytosol. The presence of the NES domain on HDAC5 might have an important role in determining the localization of HDAC5 in certain cell lines (Bertos et al., 2001; Dressel et al., 2001; Kao et al., 2001).

1.1.2.4.2. Class IIb HDACs: HDAC6 and HDAC10

Class IIb HDACs are characterized by duplicated HDAC domains, although this duplication is partial in the case of HDAC10.

HDAC6: HDAC6 structure

Different from class IIa members, class IIb HDACs are characterized by duplicated HDAC domains. HDAC6 is the largest member of the HDAC family and a very unique enzyme within the classical family of HDACs in that it contains two functional catalytic domains. The domain organization of HDAC6 is conserved in orthologs identified in *C.elegans and Drosophila*. The amino acid sequence alignment of the two catalytic domains of HDAC6 indicates that there is 61% similarity and 46% identity (Bertos et al., 2001)

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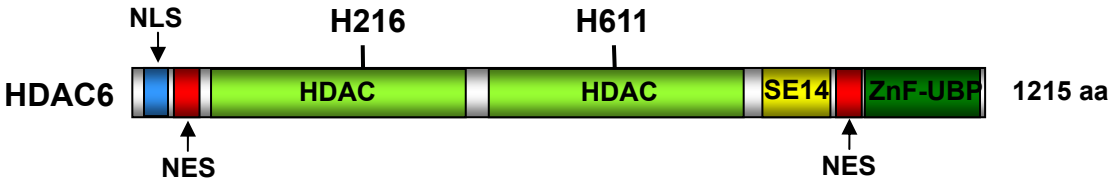


Figure 8. Schematic representation of HDAC6 domains

HDAC6 are unique class IIb HDAC, which possesses two catalytic domains and ZnF-UBF domain. Domains are indicated by colored boxes; light green, HDAC domain; blue, NLS; red, NES; yellow, SE14 (SerGlu-containing tetradecapeptide repeats); green, ZnF-UBP.

Two separate HDAC domains of HDAC6 are required for its catalytic activity *in vivo* and *in vitro* (Zhang et al., 2006). The catalytic domain of HDAC6 are most similar to the catalytic domain of HDAC9. Another unique feature of HDAC6 is the presence of ZnF-UBP domain on the C-terminus also called a HUB domain. Through this ZnF-UBP domain HDAC6 is able to bind ubiquitin as well as mono- or polyubiqutinated proteins (Boyault et al., 2006; Hook et al., 2002; Seigneurin-Berny et al., 2001). HDAC6 possesses sequences with nuclear import and export activities. It has two potent leucine-rich export signals, at residues 67-76 (NES1) and 1049-1058 (NES2) and one NLS,

which is two arginine/lysine-rich clusters, residues 14-58. NES1 is highly conserved in mHDAC6 (m55-104). In contrast, NES2 is less conserved in mHDAC6. NES2 but not NES1, is conserved in dHDAC6, so this region corresponding to dHDAC6 may function as an NES; accordingly dHDAC6 is mainly cytoplasmic (Barlow et al., 2001). The NLS of hHDAC6 is well conserved in mHDAC6 but not dHDAC6, suggesting that putative NLS of h/mHDAC6 is unique to HDAC6 protein from higher organisms. Human HDAC6 possesses a unique SE14-repeat domain, which is not intact or present in mouse, rat, *Drosophila, C.elegans* and *Arabidopsis thaliana* homologs (Bertos et al., 2001). This domain contains eight consecutive tetradecapeptide repeats and is important for the stable cytoplasmic retention of human HDAC6 (Brush et al., 2004). HDAC6 gene maps to Xp11.23 (Grozinger et al., 1999). The gene on X-chromosome raises the interesting possibility that HDAC6 can harbor special, different functions.

HDAC6 subcellular localization

HDAC6 can shuttle in and out the nucleus. In the absence of a stimulus, HDAC6 is predominantly localized in the cytoplasm, but cell cycle arrest causes to partially translocate of the protein to the nucleus (Verdel et al., 2000). The subcellular localization of HDAC6 is dependent on NES in the N-ternimus of the protein (NES1) (Bertos et al., 2001). mHDAC6 is actively shuttled between the nuclear and cytoplasmic compartments (Bertos et al., 2004; Bertos et al., 2001; Hubbert et al., 2002; Verdel et al., 2000). Even though HDAC6 is mainly cytosolic, expression and localization of this protein depends on tissue type. In differentiating osteoblasts, HDAC6 is localized in the nucleus where it interacts with Runx2 transcription factor (Westendorf et al., 2002).

HDAC6 expression in normal tissue and cancer

Class IIb HDACs show generally some degree of tissue-specific gene expression. HDAC6 is significantly expressed in testis, brain, and liver (Zhang et al., 2007). HDAC6 expression depends on cellular differentiation, especially osteoblast differentiation. HDAC6 level fluctuated early in differentiation but stabilized during the mineralization stage. HDAC6 is expressed at significantly lower levels in preosteoblast and osteoblast precursor cell lines, MC-3T3-E1 and C2C12, respectively. But HDAC6 is higher

expressed in differentiating and mature osteoblast (Westendorf et al., 2002). HDAC6 mRNA is expressed at higher levels in breast cancer patients with small tumors (< 2 cm), with low histological grade, and in estrogen receptor α and progesterone receptor positive tumors even though they did not find any correlation between HDAC6 mRNA and protein expression (Zhang et al., 2004). Patients expressing high levels of HDAC6 mRNA and protein have a better prognosis than those expressing low levels in disease-free survival rates and overall survival rates. HDAC6 was also overexpressed relative to adult, but not neo-natal cells in acute myeloma leukemia (AML) like SIRT1 (Bradbury et al., 2005). In addition, it has recently been reported that HDAC6 inhibitors are considered as an antitumor agent in multiple myeloma cells in combination with proteosome inhibitor (Hideshima et al., 2005).

HDAC6 functions

HDAC6 regulate various processes in the cytoplasm. Cytoplasmic HDAC6 functions as a specific tubulin deacetylase (Lys 40) *in vivo* and purified HDAC6 deacetylates α-tubulin in assembled *in vitro* (Zhang et al., 2003). It has been argued that tubulin acetylation by HDAC6 regulate cell motility (Hubbert et al., 2002), but this is still controversial (Palazzo et al., 2003; Palazzo et al., 2004). HDAC6 overexpression promotes chemotatic cell movement dependent on microtubule network. A subset of acetylated microtubules is necessary for proper organization of the immune synapse, a specialized cell-cell junction formed by antigen-presenting cells and T lymphocytes (Serrador et al., 2004). In addition to its deacetylase domains, HDAC6 also has a ZnF-UBP domain. For mammalian HDAC6, this finger binds to ubiquitin and E3 ligase activity (p97/VCP and a phospholipase A2 activating protein). HDAC6-interacting protein, p97/VCP, dissociates the HDAC6-ubiquitin complexes and blocks to accumulate polyubiquitinated proteins by HDAC6 (Boyault et al., 2006; Seigneurin-Berny et al., 2001). HDAC6 therefore makes a bridge between the protein acetylation and ubiquitin signaling pathways. HDAC6 also directly regulates aggresome formation. Cells where HDAC6 expression is reduced by siRNA do not form proper aggresome because of failure to load polyubiquitinated misfolded protein onto dynein motor for transport to aggresomes. Thereby HDAC6 may involve in cell viability and control cellular management of misfolded proteins

(Kawaguchi et al., 2003). Importantly, HDAC6 is present in Lewy bodies associated with neurodegenerative disorders, such as Parkinson's disease and dementia. Hsp90 chaperone activity is regulated by reversible acetylation and controlled by the deacetylase HDAC6. In HDAC6-deficient cells, HSP90-dependent maturation of the glucocorticoid receptor (GR) results in GR defective in ligand binding, nuclear translocation (Kovacs, 2005 #299; Zhang et al., 2007). Other client proteins of HSP90 are also affected, such as Bcr-Abl, c-Raf, and AKT (Murphy et al., 2005).

HDAC10

HDAC10 is one of the most recently discovered members of the class II HDACs. Two splice variants are observed for HDAC10, suggesting an additional level of regulation by RNA processing (Fischer et al., 2002). HDAC10 is most closely related to HDAC6 (37% overall similarity). HDAC10 has a catalytic domain and two putative NESs (1-349 and 339-669) on its N-terminus, and a putative second catalytic domain on the C-terminus. However, the C-terminal catalytic domain lacks the enzymatic activity. However, it has not been established wheter these putative NESs of HDAC10 are functional as true export signals. HDAC10 is primarily localized in the cytoplasm and nucleus (Tong et al., 2002). Also, two putative Rb binding domains have been found on HDAC10, suggesting a role in regulation of the cell cycle. HDAC10 is expressed in liver, spleen and kidney. Furthermore, HDAC10 is found to interact with HDAC1, -2 and -3 (and/or SMRT) and HDAC4, -5 and -7, but not with HDAC6. The fact that HDAC10 is able to associate with many other HDACs indicates that it might function as a recruiter rather than as a deacetylase. However, when expressed by recombination, HDAC10 alone does show deacetylating activity (Fischer et al., 2002; Kao et al., 2002; Tong et al., 2002).

1.1.2.5. Acetylation and deacetylation of non-histone proteins

Protein acetylation is a widespread phenomenon in eukaryotes. Acetylation and deacetylation likely have roles in cellular processes. Co-translational Nα-terminal acetylation is one of the most frequent protein modifications which occur on approximately 85% of eukaryotic proteins (Polevoda and Sherman, 2000). To a lesser extent, lysines in protein are posttranslationally acetylated at ϵ-amino group of. The addition of an acetyl group on lysines prevents positive charges from forming on the

amino group, and as a result, has a significant impact on the electrostatic properties of the protein. Although the exact number and variety of proteins that are posttranslationally lysine-acetylated in the cell is still unknown, it is clear that many proteins are modified by this mechanism. Dynamic acetylation of non-histone proteins has pleiotropic effects on cellular function. Early studies suggested that many lysine residues in histones are acetylated abundantly and that acetylated histones regulate gene transcription (Allfrey et al., 1964). HATs may have particular histone substrate specificity, and different HATs are specific with regard to which histone amino acids they will acetylate. HATs also have a wide range of protein substrates other than histones. Unlike Nα-terminal acetylation, post-translational ϵ-amino lysine acetylation of protein is highly reversible. Like HATs, HDACs may possess substrate specificity and can deacetylate non-histone proteins as well. However, the precise mechanism of non-histone deacetylation remains unclear. Specificity among different HDACs remains determined although the majority of non-histone proteins are deacetylated by class I HDACs so far.

Acetylation and deacetylation of transcription factors

Recent investigations have revealed that an increasing number of cellular and viral proteins subjected to lysine acetylation. Transcription factors (e.g., RUNX, SREBP, p53) comprise the largest known group for protein acetylation and deacetylation. Acetylation of transcription factors regulates the DNA binding activity, transcriptional activity, protein-protein interaction, and protein stability. For example, the tumor suppressor and sequence-specific DNA binding transcription factor p53 was the first reported non-histone target of HATs (Gu and Roeder, 1997). Acetylation of p53 by p300/CBP increases activation of its target genes (Espinosa and Emerson, 2001). p53 also interacts with HDAC1 through Sin3 or MTA2 proteins (Murphy et al., 1999). p53 acetylation serves to promote protein stability competing with ubiquitination at the same lysine residues and also enhanced its binding to DNA (Ito et al., 2002). YY1 is a protein with multiple biological roles, which has a dual function of transcription and required for co-activators and co-repressors to fully function (Thomas and Seto, 1999). YY1 interacts with HATs (CBP and p300) and with most class I HDACs (HDAC1, -2, and -3) in a phosphorylation dependent manner (Lee et al., 1995a; Yang et al., 1996). Acetylation of YY1 decreases

DNA binding affinity (Yao et al., 2001). CBP/p300 acetylates STAT3 on lysine 685 and HDAC3 deacetylates it. Acetylation enhances both DNA binding and transactivation (Yuan et al., 2005). Smad7 is acetylated by p300 and deacetylated by HDAC1 and -3. Acetylation causes an increased protein stability by preventing ubiquitination of overlapping lysines by the ubiquitin ligase Smurf1 (Gronroos et al., 2002). The c-MYC oncoprotein is acetylated by PCAF/GCN5 and TIP60 at three sites (lysines 149, 323, and 417) (Patel et al., 2004). Acetylation of Lys 323 and 417 has no effect on either the nuclear localization or dimerization. Acetylation leads to c-Myc protein stability prevention of ubiquitination, similar to what is seen with p53, but not necessarily activity. So far deacetylase for c-Myc remains unidentified. CBP acetylates and activates GATA1, which an important transcription factor in erythroid differentiation, at three sites: Lysine 218,220, and 214. Acetylation of GATA1 increases its DNA binding affinity and is critical for erythroid differentiation (Boyes et al., 1998). GATA-1 interacts with HDAC3,

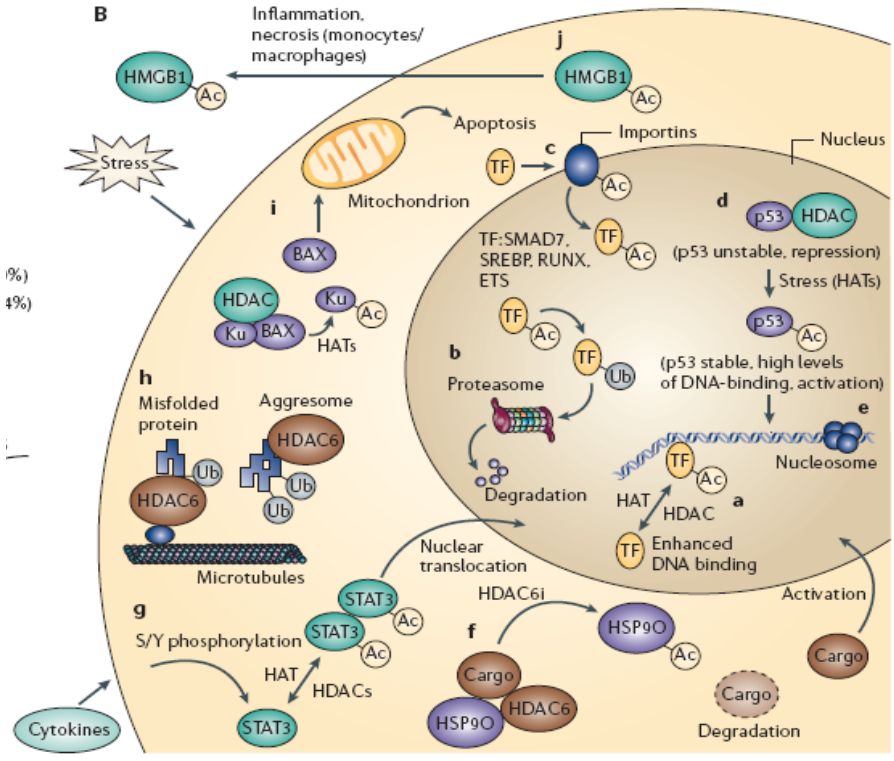


Figure 9. A schematic view of the acetylome

A partial list of biological processes that are regulated mechanistically by acetylation is sketched in a–j. Adpted from Minucci et al., 2006.

-4, and -5 (Watamoto et al., 2003). PCAF and p300/CBP acetylate MyoD at three sites (Lys 92,102, and 104) in differentiated muscle cells (Polesskaya et al., 2000) thereby activating it. HDAC1 deacetylates MyoD in undifferentiated cells. Therefore, HDAC1 can inhibit muscle cell conversion (Mal and Harter, 2003). Acetylation of NF-κB is complicated and controversial; p300/PCAF acetylates p65 subunit of NF-κB at lysine 218, 221 and 310 and acetylated p65 weakly binds to IκB. In contrast, HDAC3 deacetylates NF-κB promoting interaction with IκB (Chen et al., 2001). One subunit of Hypoxia inducible factor, HIF-α is acetylated by ARD1 at Lys 532 facilitating its ubiquitination via binding with VHL and proteasome-mediated degradation (Jeong et al., 2002) and deacetylated by HDAC4 and HDAC6 (Qian et al., 2006).

Acetylation and deacetylation of other cellular proteins

In addition to transcription factors, other cellular proteins are regulated by dynamic acetylation and deacetylation. Protein stability is influenced by HATs and HDACs, as lysines are subject to both acetylation and ubiquitination. The best characterized cytoskeletal protein is tubulin. Although acetylation of tubulin was found in mammalian cells more than 20 years ago(L'Hernault and Rosenbaum, 1985), the acetyltransferase is still unidentified today and the deacetylases HDAC6 and possibly Sir2 only were identified recently (North et al., 2003; Zhang et al., 2003) Furthemore, the biological role of this modification in microtubule function is still unclear. Stable microtubules are largely hyperacetylated whereas dynamic microtubules are hypoacetylated. But this may only be a correlation whose functional significance has not been established. HDAC6 might have impacts on cell motility and immune synapse reorganization by deacetylating tubulin (Serrador et al., 2004). Also HDAC6 binds and deacetylates Hsp90 and regulates molecular chaperone functions of Hsp90 including the maturation of glucocorticoid receptor (GR) or a subset of protein degradation. {Kovacs, 2005 #298; Kovacs, 2005 #299; Murphy, 2005 #300; Zhang et al., 2007}. Acetylation promotes the interaction of Importin-α with importin-β (Bannister et al., 2000), which leads to transport of bound cargo (e.g., HuR protein) through the nuclear pore complex. Acetylation of DNA damage associated protein Ku70 affects the translocation of BAX to mitochondria. When Ku70 is acetylated (Lys 539 and 542), BAX is free to move to the mitochondria. SIRT1

deacetylates Ku70 permitting it to sequester Bax away from mitochodria (Cohen et al., 2004).

1.1.2.6. Substrate specificity of HDACis

HDACis, which often inhibit most/all class I and II HDACs, usually relieve transcriptional repression and result in apoptosis or differentiation of cancer cells. Clinical studies on HDAC inhibitors as new anticancer agents are under way and show great promise. A relatively wild range of structures has been identified that inhibits the activity of class I and II HDACs nonspecifically. They derive from both natural source and from synthetic routes. With a few exception, they can all be divided into chemical classes including hydroxamic acid derivatives, carboxylates, benzamides, electrophilic ketones, and cyclic peptides (Minucci and Pelicci, 2006). Information on the subtype selectivity of available inhibitors is limited, and the consequences of such selectivity are unclear. No structural information on mammalian class I or II HDACs is available. However, the X-ray structure of a bacterial HDLP (from *Aquifex aeolicus*) has been resolved (Finnin et al., 1999) and used to construct a homology model of human HDAC1. In X-ray snapshot structure of human HDAC8 with TSA, a second molecule TSA is bound closely the active site and binding to an inhibitor to this second binding site alone might result in HDAC inhibition (Somoza et al., 2004). However, HDAC inhibitors would rather target class I and II HDACs nonspecifically. Although only a few molecules are emerging as preferential inhibitors of class I versus II HDACs, therapeutic potential of HDAC inhibitors is noteworthy. To date, the only known HDAC6-selective inhibitor is tubacin, which inhibits tubulin deacetylation. To find novel HDAC6 specific inhibitors, Miyata and colleagues designed inhibitors based on the structure of a small-molecular HDAC6-selective substrate. They have reported the first inhibitor that show significant HDAC6-selective inhibition in both western blot analysis with anti-acetylated H4 and anti-acetylated tubulin and enzyme assays. They discovered that the presence of a bulky alkly group in thiolate HDAC inhibitors is important for HDAC6-selective inhibition (Suzuki et al., 2006). Although they did not show clinical data yet, it is a good starting point to develop novel, specific HDAC inhibitors. If we can identify substrate specificity of each HDAC, HDAC inhibitors will be one of the strongest anticancer agents.

1.1.2.7. Clinical implication of HDACs and HDACis

Tumor cells can harbor abnormalities in histones as well as DNA. Now, with early clinical trials beginning to show promise, a whole new class of anticancer drugs, called HDACis have become the first to specifically target epigenetic abnormalities. New research is identifying a network of molecular interactions that link HDAC activity to diet, premalignant cell changes, aging, and development a variety of diseases, including cancer and autoimmunity. The first studies on the clinical use of HDACi have been published recently (Johnstone and Licht, 2003; Rosato and Grant, 2004). These studies are phase I/II trials. The most advanced of the HDAC inhibitors is a SAHA (suberoylaninide hydroxamic acid), which has completed phase II testing. While SAHA advances in clinical trials, investigators continue to explore HDAC inhibitor work. One question still to be determined is how and why the drugs preferentially target cancer cells instead of normal cells. It is interesting evident that the effect on histone and chromatin structure and in turn the alteration in transcription of specific genes is probably part of the anticancer effects of these agents but certainly no the whole story. It has been reported that relatively few genes are altered in their transcription by SAHA or TSA. SAGE (serial analysis of gene expression) data show that HDACs are generally expressed in almost all tissues investigated. Surprisingly, no major differences were observed between the expression pattern in normal and malignant tissues. HDAC inhibitors also appear to interfere with other proteins. For example, SAHA directly bind thioredoxin-binding protein (TBP2). This binding prevents TBP2 from detoxifying oxygen free radicals, which further facilitates cell death. This binding does not happen in normal cells, only in transformed cells. Even though we do not yet completely understand this phenomenon, acetylated histones in normal and cancer cells are accumulated, but the effect does not last in normal cells. Unfortunately, the relationship between the toxicity of HDACs and their phamacodynamic/pharmacokinetic properties is still largely unknown. We also do not know the key target(s) for HDACi action because genetic analysis of HDACs is still imcomplete. Therefore, the next step in the evolution of HDAC inhibitors will be to increases their specificity and efficacy as designing more specific inhibitors targeted to one particular HDAC (Hede, 2006). In addition, the combination of HDACi with other drugs acing on epigenetic mechanisms is being tested. DNA methylating agent in

combination with HDACi show potent responses *in vitro* and they are being tested in clinical trials (Cameron et al., 1999). HDACi might work synergistically with the HSP90 inhibitor, geldanamycin, which has been already in clinical trials. In this case, HDACi would inhibit the activity of HSP90 chaperone to oncoproteins required for tumor cells (Paschen, 2003).

1.2. Cellular stress response

The cellular stress response is a phylogenetically conserved protection mechanism from prokaryotes to humans and a phenomenon of adaptation of organisms. When cells are exposed to different stresses, they can react in two opposite ways. One way, they can activate defense mechanism to adapt to stressful condition, to repair damage and to restore normal cellular functions. Alternatively, they can activate programmed cell death. The choice between these two responses is decided by many factors such as the intensity of stress, or cell intrinsic parameters (Del Razo et al., 2001; Mosser et al., 2004). Altered patterns of protein synthesis, including stress proteins (SPs), may serve to monitor the impact of exposure to natural and anthropogenic stressors. SPs are synthesized in cells of most organisms in response to diverse circumstances such as physiological conditions and environmental stressors such as heat, ultraviolet light, and several chemicals (e.g. arsenite). The induction of SP synthesis is highly tissue-specific and is related to the damage induced by stress to specific proteins and protein complexes.

1.2.1. Effects of environmental stress on mRNA metabolism

Exposure of cells to environmental stresses can disrupt essential intracellular processes, which are extremely sensitive to disturbance by stress. The production of mature, translatable mRNAs is most sensitive to stress owing to the inhibition of messenger RNA splicing and alterations in the export of mRNA from the nucleus. Changes in the cytoplasmic pools of mRNAs also occur following exposure to stress conditions. Cytoplasmic mRNAs, especially translationally repressed, gather in specific particles during many different cellular processes. Cytoplasmic RNA granules in germ cells (polar and germinal cell granules (GCGs)), somatic cells (Stress granules (SGs) and processing

bodies (PBs)), and neurons (neuronal granules) play a critical role in the posttranscriptional regulation of gene expression (Krichevsky and Kosik, 2001; Leatherman and Jongens, 2003; Schisa et al., 2001). These different classes of RNA granules share protein components and may use similar mechanisms to regulate mRNA translation and decay. All RNA granules contain translationally silenced mRNA. GCGs and neuronal granules harbor highly specific mRNA cargo, whereas SGs and PBs are less discriminating. (Anderson and Kedersha, 2006). SGs contain the majority of polyadenylated mRNAs by stress-induced translational arrest but selectively exclude some mRNA encoding heat shock proteins as well as some transcription factors under these conditions. It allows the cell to repair the stress-induced damage and to aid in cellular recovery while conserving anabolic energy. Stress proteins, such as Hsp70 and Hsp 90, have been shown to play a direct role in the repair of intracellular damage involved in RNA metabolism in cells exposed to stress through their biological activities as molecular chaperones. Therefore, stress proteins help cells return to homeostasis (Mosser et al., 2004).

mRNA decay

Gene expression is initiated in the cell nucleus, where transcripts are produced and processed to mRNA. Mature mRNAs traverse nuclear pores and are translated in the cytoplasm. The abundance of an mRNA is determined by the balace between transcription and decay. Therefore, one of the important steps for regulation of gene expression is the degradation of mRNA. mRNA half lives are subject to control by changing intra- and extracellular conditions. How long an mRNA lives depends on how efficiently the mRNA degradation machinery is recruited to that mRNP. In general, the 3’ poly(A) tail is removed by deadenylases, which comprise three main enzymes, (CCR4/CAF1, PAN2/3, and PARN), while the 5’ cap is removed by specific decapping enzymes (Dcp1p/Dcp2p). Thereby the transcript is exposed to degradation by the 5’ to 3’ exonuclease, Xrn1p (Hilleren and Parker, 1999). A second mechanism of cytoplasmic mRNA degradation involves in the 3’ to 5’ exonuclease complex, the cytoplasmic exosome. In a 3’ to 5’ pathway of mRNA destruction, poly (A) tail is shortened followed by removal of poly (A) binding protein (PABP), and the exosome degrades mRNA.

Finally an oligonucleotide cap is hydrolyzed by the scavenger decapping enzyme, Dcp8. These two pathways represent the primary pathways for mRNA degradation. However, a number of specialized mRNA degradation pathways have also been described. These include the nonsense mediated mRNA decay pathway, which degrades aberrant mRNAs containing stop codons (nonsense mRNA) within the ORF, and the initiation-mediated mRNA decay pathway (nonstop mRNA), which acts under conditions when translation initiation is slowed down (Heikkinen et al., 2003). Of course, endonucleolytic degradation mechanisms also exist, most notably sequence-specific mRNA cleavage by the RNA-induced silencing complex (RISC) in association with endogenous siRNA (Sontheimer, 2005). Their efficient elimination is thought to protect cells from the potentially deleterious consequences of inappropriately terminated proteins.

1.2.2. Alterations in the cytoplasmic pools of mRNA in stressed cells: PBs and SGs in stressed cells

Translating and nontranslating pools of mRNAs are spatially segregated in the cytoplasm between polysome, SGs, and PBs. Cytoplasmic mRNAs dramatically redistribute in cells exposed to certain stress conditions. Cytoplasmic mRNAs are mostly associated with polysomes; however, under conditions where mRNA translation initiation is inhibited, the cytoplasmic pool of mRNA is redirected to granular cytoplasmic foci. These cytoplasmic foci function as the sorting place for sequestered mRNA: the mRNAs may be targeted for degradation or alternatively stored in a nontranslatable form until the stress is removed. Those mRNAs targeted for decay will be exported from the SG to sites of mRNA decay such as PBs (Kedersha et al., 2005).

1.2.3. Processing bodies (P-bodies (PBs) or GW182 bodies)

The discovery of P-bodies began about 10 years ago when researchers were studying a key step in mRNA degradation. In both yeast (Sheth and Parker, 2003) and mammalian cells (van Dijk et al., 2002), much of the mRNA decay machinery was found to be concentrated in discrete cytoplasmic foci termed processing bodies (PBs or GW182 bodies), which contain aggregates of nontranslatable mRNPs (Teixeira et al., 2005). Mammalian PBs are similar to yeast PBs, suggesting that the movement of mRNAs from

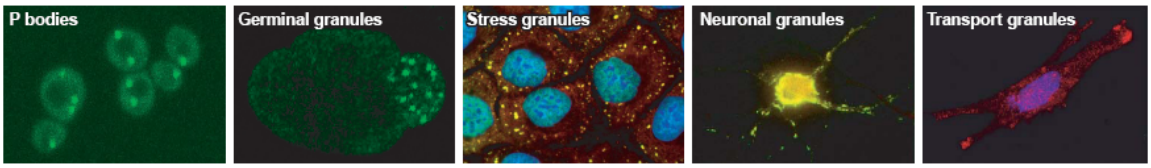


Figure 10. Particulate purgatory for mRNAs

In the yeast *S. cerevisiae*, the decapping protein Dhh1p (green) is localized together with mRNAs in P bodies. In the worm embryo, germinal granules (P granules) in the cytoplasm contain GLD-2, a poly(A) polymerase (green). Stress granules in the cytoplasm of cultured human cells contain the enzyme TIA-1/TIAR (green) and the translation initiation factor eIF3p116 (red), visualized as yellow spots due to colocalization of the proteins. Neuronal particles in cultured rat hippocampal neurons contain both Staufen1 protein and BC1 mRNA, which appear as small yellow spots in the thin neuronal processes. In chick fibroblasts, cytoskeletal actin mRNA (red) is contained in transport granules, and mRNA particles accumulate in the termini of lamellepodia (DNA is blue). Adapted from Wickens and Goldstrohm, 2003.

PBs to polysomes seems to be a fundamental property of eukaryotic cells. PBs have been shown to contain the Dcp1p/Dcp2p decapping enzymes, the cytoplasmic DExD/H-box helicase, Dhh1p, which stimulates mRNA decapping, the Topoisomerase II-associated deadenylation-dependent mRNA-decapping factor, Pat1p, the 5' to 3' exonuclease Xrn1p, Sm-like proteins 1-7 (Lsm 1-7), which enhance assembly of the decapping complex, and GW182. The presence of these factors in PBs has supported the notion that PBs are sites of mRNA degradation. Teixeira et al. (2005) have shown that PBs increase in number and in size in cells under stress. This stress leads to a concomitant decrease in protein synthesis. The number and the size of PBs increase when 5' to 3' decay is inhibited or translation initiation is stopped. In contrast, PBs virtually disappear when translation elongation or transcription is blocked with drugs (Cougot et al., 2004; Teixeira et al., 2005). Thus, it seems that PBs assembly requires mRNAs. Consistent with this result, RNase treatment results in dissociation of PBs (Teixeira et al., 2005). It has been reported that Argonaute (Ago) 1 and 2, which are key components of the RNAi machinery (RISC), interact with GW182 and concentrate in PBs, implicating the particles as a site of degradation. Consistent with this, nonsense-mediated decay and siRNA-mediated degradation of mRNA occur in PBs. miRNA mediated inhibition of translation can cause

mRNA accumulattion in PBs in a manner dependent on miRNA fuction (Pillai et al., 2005). In this regard, it is interest to transport from PBs to SGs in mammmlian cells (Kedersha et al., 2005). However, this process may not occur in yeast, since SGs have not been found in yeast. Parker et al. suggests that mRNA sequestered in PBs can move out of them and move to the polysomes following restoration of translation, suggesting that PBs serve as storage sites for mRNAs during certain physiological conditions (Brengues et al., 2005). But in mammalin cells, this finding has not been demonstrated yet. PBs resemble the granules that store the maternal mRNAs that function in very early embryo development. In *C. elegans*, a worm development control genes encode a protein that localize to PBs and interacts with the same Argonaut molecules involved in regulation by miRNAs (Lin et al., 2006).

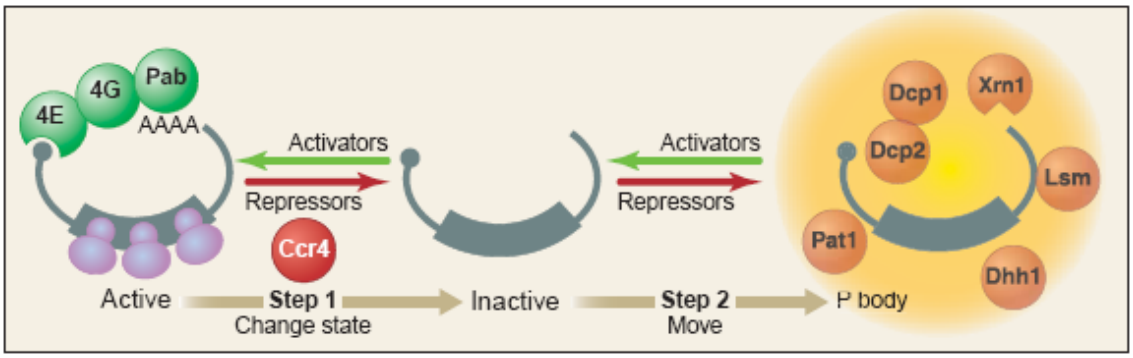


Figure 11. P bodies control mRNAs

Active mRNAs are bound to ribosomes (purple) and to the translation initiation factors eIF4E, eIF4G, and poly(A) binding protein (Pab) (all in green). (Step 1) The mRNA makes a transition to an inactive state induced by deadenylation, which is catalyzed by Ccr4p and other enzymes. An inactive intermediate is generated; the proteins associated with this intermediate are not yet known. (Step 2) The intermediate moves to the P body (yellow). There it can be degraded by the Xrn1p exonucleases (orange). Repressors and activators of mRNAs not only regulate the change in state transition (Step 1), but may also move mRNAs into and out of P bodies (Step 2). Adapted from Wickens and Goldstrohm, 2003.

Interestingly possible connections between PBs and disease are beginning to emerge. PBs or GW body was first visualized by using a patient-derived autoantisera reactive with GW182 (autoantigen), a 182-kD RNA-binding protein. GW bodies are prominent in actively growing unstressed cells (Eystathioy et al., 2002). Furthermore, it

has reported that primary biliary cirrhosis (PBC) is autoimmune disease and ~5% of PBC patients have antibodies directed against PBs (Bloch et al., 2005). Human PBs contain a protein called RCK that may help drive cancer development. Its concentration, along with the number of PBs, is increased in diverse cancers, including breast cancer (van Dijk et al., 2003). A disease link for PBs is necessary to more further works.

1.2.4. Stress granules (SGs)

1.2.4.1. Discovery

Other cytoplasmic granular bodies have been identified in higher eukaryotes exposed to stress. Many years after discovery of germ cell granules, another RNA granules were found in the cytoplasm of tomato cells in response to heat shock. In particular, discrete cytoplasmic phase-dense particles, referred to as stress granules (SGs), are observed in both plant and animal cells exposed to heat, oxidative, hyperosmolarity and UV stress (Kedersha et al., 1999; Nover et al., 1983). SGs are repositories of mRNA pools: SGs contain in particular mRNAs encoding constitutively expressed 'housekeeping' proteins, whereas they selectively exclude mRNAs encoding Hsps. Thus, stress granules store nontranslating mRNAs, enabling a redirecting of the translational machineary to produce Hsps predominantly. Selective recruitment of specific mRNAs into SGs is thought to regulate their stability and translation. So far, SGs have not been observed in budding yeast. In *S. cerevisiae*, cytoplasmic mRNAs can be visualized in cytoplasmic foci in certain genetic backgrounds, in particular in cells mutant in proteins that interact with the NPC. It is currently not clear whether these foci are similar or different in composition to the SGs identified in mammalian cells. However, one may predict, based on the different rates of mRNA decay in mammalian and yeast cells, that different mechanism may prevail in these two systems. Because yeast mRNAs appear to have much shorter half-lives compared with their mammalian counterparts, it may not be necessary to invoke SGs to sequester nontranslating but stable mRNAs in yeast cells. The turnover rate of mRNAs in yeast may simply be increased following stress by increasing the number of PBs in the cytoplasm.

1.2.4.2. Components

Core components of SGs are abortive 48S preinitiation complexes including small but not large ribosomal subunits as well as the early translation initiation factors eIF2, eIF3, eIF4E, and eIF4G. Also SGs contain many RNA binding proteins which regulate mRNA structure, stability, and function, such as TIA-1/TIAR, HuR, Staufen, Smaug, tristetraprolin (TTP), Fragile X mental retardation protein (FXMR), the RasGAP-associated endoribonuclease, G3BP, CPEB, and Survival motor neuron (SMN). Additionally, SGs possess PABP1, translational repressor p54/Rck helicase, the 5'-3' exonuclease XRN1, and Agos. In contrast, PBs contain proteins associated with mRNA degradation. SGs also include putative scaffold proteins such as Fas-activated serine/threonine phosphoprotein (FAST) and components with no obvious link to RNA metabolism such as TRAF2. According to the results of drug treatment (polysome stabilizer or destabilizer) and FRAP analysis, SGs are very dynamic structures. Although global translation arrest upon stress occurs, selective translation of heat shock proteins, as well as some transcription factors (e.g., GCN4 in yeast and ATF4 in mammals) allow the cell to recovery the stress-induced damage while conserving anabolic energy. When the stress is removed, SGs disassemble and the sequestered mRNAs either return to the translationally active pool or are targeted for degradation in PBs (Kedersha and Anderson, 2002; Kedersha et al., 2005). Therefore, SGs can serve triage centers that sort, remodel, store, or export specific mRNA transcripts for reinitiation, decay, or storage.

1.2.4.3. Assembly

SGs assembly is regulated by one or more RNA-binding protein, including TIA/TIAR (Gilks et al., 2004). G3BP (Tourriere et al., 2003), FXMR, and TTP (Stoecklin et al., 2004), Prion-like domains in TIA/TIAR are thought to self-oligomerize and promote SG assembly. In neurons, fragile X mental retardation protein promotes the assembly of neuronal granules that are structurally and functionally similar to SGs (Mazroui et al., 2003; Mazroui et al., 2002; Wickens and Goldstrohm, 2003). Survival motor neuron (SMN) protein forms granule-like aggregates in the cytoplasm of human fetal muscle cells and rat motor neurons (Burlet et al., 1998; Pagliardini et al., 2000). Moreover, SMN protein facilitates assembly of SGs, indicating that SGs may be involved in the pathogenesis of spinal muscular atrophy (Hua and Zhou, 2004).

Proposed mechanism for SG formation

In response to environmental stress, eukaryotic cells reprogram their translational machinery to allow the selective expression of proteins required for viability in the face of changing conditions. Both SG assembly (Kedersha et al., 1999) and translational arrest (Krichevsky and Kosik, 2001) are initiated by the phosphorylation of translation initiation factor eIF2α, which reduces the availability of the eIF2–GTP–tRNAMet ternary complex that is needed to initiate protein translation. Several proteins act downstream of phos-eIF2α. Self-aggregation of either TIA proteins or G3BP promotes SGs assembly. Stress-induced aggregation of TIA is mediated by a glutamine-rich prion-like domain that is regulated by HSP70. ATP is required for either SG assembly or disassembly. Therefore,

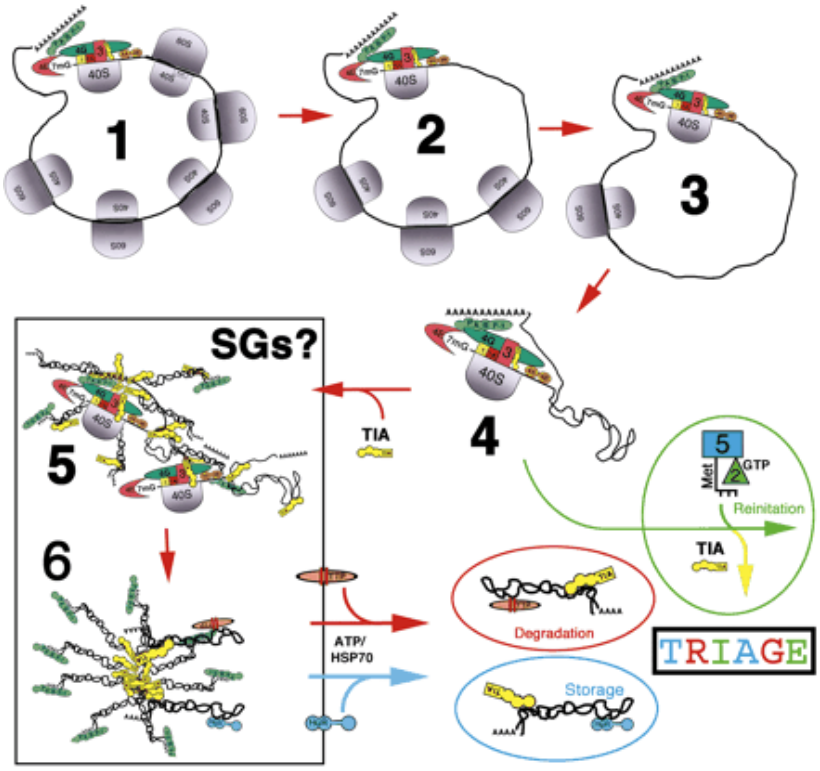


Figure 12. Proposed mechanism for the assembly of stress granules
Adapted from Anderson et al., 2002.

SG assembly/disassembly is regulated by many signaling pathways acting downstream of phos-eIF2α. Destabilizing elements such as TTP are proposed to direct selected stress granule mRNAs to sites of degradation, whereas stabilizing elements such as HuR are proposed to direct selected mRNAs to sites of storage and/or reinitiation. By this triage

process, the SG may monitor the structure and integrity of mRNP complexes and determine the fate of specific RNAs.

eIF2α kinases and translation initiation inhibition

Translation is normally initiated when the small ribosomal subunit and its associated initiation factors are recruited to a capped mRNA transcript to form a 48S complex. Hydrolysis of eIF2 bound GTP by eIF5 displaces the early initiation factors, allowing binding of the large ribosomal subunit. Repeated cycles of successful initiation convert an mRNA into a polysome. In stressed cells, activation of one or more eIF2α kinases (e.g. PKR, PERK/PEK, GCN2, HRI, see below) results in the phosphorylation of eIF2α (Williams, 2001), which consequently inhibits eIF2B, the GTP/GDP exchange factor that charges the eIF2 ternary complex. Depletion of eIF2-GTP-tRNAMet prevents productive translation initiation. TIA-1 and TIAR promote the assembly of an eIF2/eIF5-deficient preinitiation complex that is directed to SGs. Mutant MEFs expressing only the nonphosphorylatable form of eIF2α (S51A) do not assemble SGs in stress whereas expression of a phosphomimetic mutant of eIF2α (S51D) is sufficient to induce the assembly of SGs. Thus, phos-eIF2α is essential for SG assembly (McEwen et al., 2005).

The eukaryotic translation factor eIF2 consists of three subunits, α, β, γ, specifically binds the initiator methionyl-tRNA in a GTP-dependent manner and delivers this essential component of translation initiation to the small ribosomal subunit. eIF2 is exquisitely sensitive to regulation by phosphorylation of its α subunit. The γ subunit of eIF2 is responsible for GTP binding. eIF2 cycles between its GTP-bound state and its GDP-bound state by the guanine nucleotide exchange factor, eIF2B. When as little as 20% of eIF2α is phosphorylated on serine 51, protein translation initiation may be inhibited. Four mammalian eIF2α kinases has been identified, namely double-stranded RNA-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), heme-regulated kinase (HRI), and amino acid regulated kinase (GCN2). The conserved eIF2α kinase domain in these four proteins share approximately 22-37% amino acid sequence identity and they have distinct regulatory domains as well. PKR is member of a small family of evolutionarily conserved eIF2α kinases distinguished by the presence of a signature amino acid sequence that constitutes part of the eIF2α binding site. In human

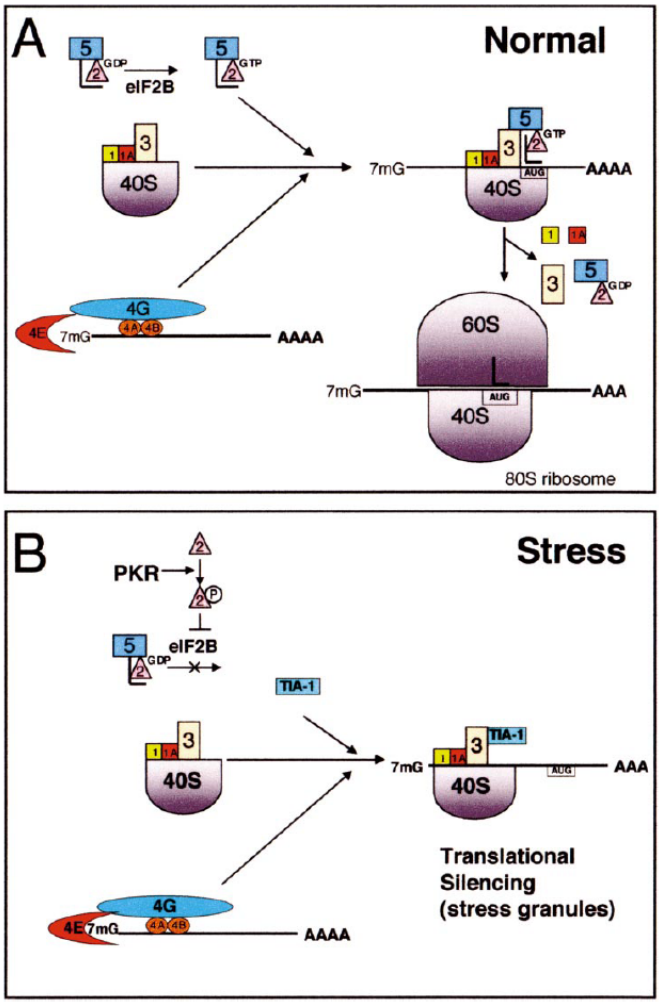
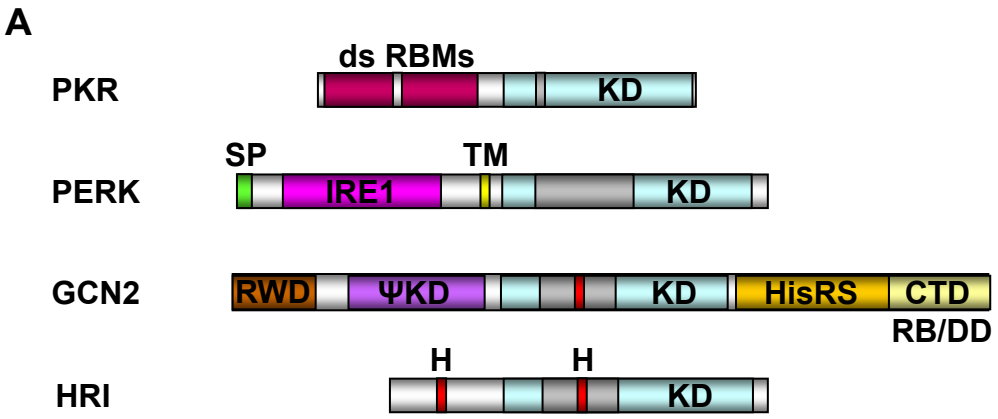


Figure 13. Translationa in the absence or presence of stress

(A) Normal: when eIF2-GTP transfers ribonucleic acid for methionine ($tRNA^{Met}$) ternary complex is available, a canonical 48S preinitiation complex is assembled at the 5' end of capped transcripts and scanning begins. Upon recognition of the initiation codon by the anticodon of $tRNA^{Met}$, eIF5 promotes GTP hydrolysis, and early initiation factors are displaced by the 60S ribosomal subunit. (B) In stressed cells the phosphorylation of eIF2α prevents GDP-GTP exchange by eIF2B, which lowers the effective concentration of eIF2-GTP-tRNAMet. Under these conditions, TIA-1 is included in a noncanonical preinitiation complex. TIA-1 self-aggregation then promotes the accumulation of these complexes at SGs. Adapted from Anderson et al., 2002.

PKR, two dsRNA-binding motifs (dsRBMs) located between residues 6-79 and 96-169 precede the protein kinase domain (residues 258-551). Highly structured RNAs, including viral RNAs, viral dsRNA genomes, and mRNAs with extensive secondary structures, can bind to dsRBM of PKR and this binding cause a conformational change exposing Ser 51 of the kinase domain. Binding of two PKR molecules to the same molecule of dsRNA enable PKR dimerization and kianse activation. PERK (PKR-like ER kinase) is activated under ER stress conditions. However, in unstressed cells, PERK interacts with the ER chaperone BiP (GRP78) and GRP94. Upon ER stress, unfolded proteins accumulate in the ER and titrate the chaperones away from PERK, enabling kinase dimerization and activation (Bertolotti et al., 2000). The heme-regulated inhibitor (HRI) is the main kinase in erythroid cells where it is activated by autophosphorylation

under heme deprivation condition (Lu et al., 2001). HRI has two heme binding sites within and N-terminal to the kinase domain. In the presence of heme, ATP binding by HRI is decreased, inhibiting autophosphorylation and activation (Kaufman, 2004), and HRI is maintained in an OFF state. However, in the absence of heme, the heme dissociates from the kinase domain, the interdomain interactions as well as the intermolecular disulfide bonds are broken, and the kinase converts to its ON state (Yun et al., 2005). Yeast GCN2 regulates the translation of the transcriptional activator GCN4.



B

	Activation	Source
PKR	dsRNA	vertebrates
PERK	ER Stress	metazone
GCN2	Uncharged tRNA	All eukaryotes
HRI	low heme, NO heat shock	Vertebrates *A. Gambiae* *S pombe*

Figure 14. Dipiction of the eIF2α kinases

(A) Conserved kinase domain, sky blue; kinase insert, gray; Also shown are the dsRBMs in PKR; the signal peptide (SP), green; IRE1 homology region, pink; transmembrane domain (TM) in PERK, yellow; the amino-terminal RWD domain, brown; pseudo-kinase domain (ΨKD), purple; histidyl-tRNA synthetase-related domain (HisRS), orange; the ribosme-binding (RB) and dimerization domain (DD) in the carboxy-terminal domain (CTD) of GCN2, light yellow; and the two heme, red.

GCN2-mediated phosphorylation of eIF2α in response to amino acid starvation results in the translation of GCN4. Activation of GCN2 occurs through an autophosphorylation reaction (Dong et al., 2000; Kaufman, 2004).

1.2.4.4. Function

SGs also are found in tissues from stressed animals. In chicken treated with gentamycin, SGs appear in cochlear cells several hours before onset of apoptosis. Whole-animal radiotheraphy induces SGs within the individual tumor cells. The radiation-induced translation of hyoxia-inducible factor-1α (HIF-1α) regulated transcripts is delayed pending SG disassembly during recovery, suggesting that the expression of these transcripts is inhibited by their retention in SGs (Moeller et al., 2004). Similar results have been described in animal model of stroke, in which SGs may regulate protein translation in neurons during ischemia. Furthermore, after initial priming of T cells, naïve T helper cells express cytokine mRNA but do not secrete effector cytokine proteins such as interleukin-4 (IL-4) or interferon-γ (IFN-γ) without additional T cell receptor (TCR) stimulation. Stefanie et al. explained phenomenon of this uncoupled production of cytokine mRNA and protein that primed T cells contain more phosphorylated eIF2α and accumulated SGs which store untranslated transcripts in stress, and only increased expression of stress-response genes. After re-stimulation of T help cells with TCR, these cells cause rapid dephosphorylation of eIF2α, mRNA translation reinitiation, and cytokine secretion (Scheu et al., 2006). This suggests that SGs might regulate a quality control of protein synthesis during T cell differentiation. These studies indicate that SGs are not *in vitro* artifacts but are an *in vivo* physiological part of the organism response to stress.

1.2.5. Relationship between PBs and SGs

PBs and SGs in mammalian cells are physically distinct and spatially separate. Anderson et al. have proposed that SGs are sites of mRNA triage in which individual transcripts are sorted for storage, reinitiation, or degradation. This model suggests that those mRNAs targeted for decay will be exported from the SG to sites of mRNA decay such as PBs.

The close juxtaposition between SGs and PBs may allow mRNA to move from the SG to the PB. Two lines of evidence suggest the direction of this process. First, arsenite induces the formation of juxtaposed SGs and PBs, and subsequent emetine treatment forces the disassembly of SGs before the disassembly of PBs. Second, heat shock induces SG formation before PB formation. Initially, eIF4E is concentrated at SGs in cells lacking PBs, but in the continued presence of heat, SGs are disassembled, and PBs containing eIF4E are concomitantly assembled. These results imply that eIF4E is first incorporated into SGs and later translocates into PBs. SGs and PBs share some proteins and mRNA components as well as some functional properties. Both structures are induced by stress, although PBs are continuously present in cells, but are regulated by distinct signaling pathways, and each can exist without the other. As eIF3, eIF4G, PABP-1, small ribosomal subunits, and G3BP are found in SGs but not in PBs, these proteins must be removed from mRNA before its export from the SG. Because eIF4G and PABP-1 are directly involved in mRNA circularization, it is probable that mRNAs exported from SGs into PBs are decircularized before translocation, which is concurrent with their deadenylation.. Finally, as eIF4E and TTP are components of both SGs and PBs, these RNA-binding proteins may remain with mRNA as it moves from the SG to the PB.(Kedersha et al., 2005). Importanly, SGs and PBs are induced by different mechanism and are able to function independently. SGs need phosphorylation of eIF2α and contain small ribosome units while PBs do not. SGs possess translationally terminating and polyadenylated mRNA whereas PBs possess mRNAs subject to general-, nonsense-, adenine/uridine rich element-mediated decay as well as mRNAs targeted by miRNAs and siRNAs. PBs and SGs exhibit a high degree of motility when independent but appear less motile when they are tethered together, and their interaction is promoted by the mRNA destabilizing protein TTP. SGs induced by stress are likely to contain a mixture of transcripts, but SGs induced by the overexpression of different RNA-binding proteins (e.g., TIA, G3BP, TTP) are likely to differ in their mRNA composition. TTP seems to serve to deliver its mRNA to PBs by interacting with stable components of these particles because TTP itself is not likely to be a stable component (Lykke-Andersen and Wagner, 2005). FAST has the properties of a putative scaffold protein that might stabilize SG-PB interactions. It has a very slow exchange rate, lacks known RNA binding motif, nucleate both SGs and PBs

upon overexpression, and interacts with TIA-1. It is possible that TTP or TTP-associated proteins promote SG-PB fusion by interacting either directly or indirectly with FAST to remodel the SG-PB scaffold (Kedersha et al., 2005).

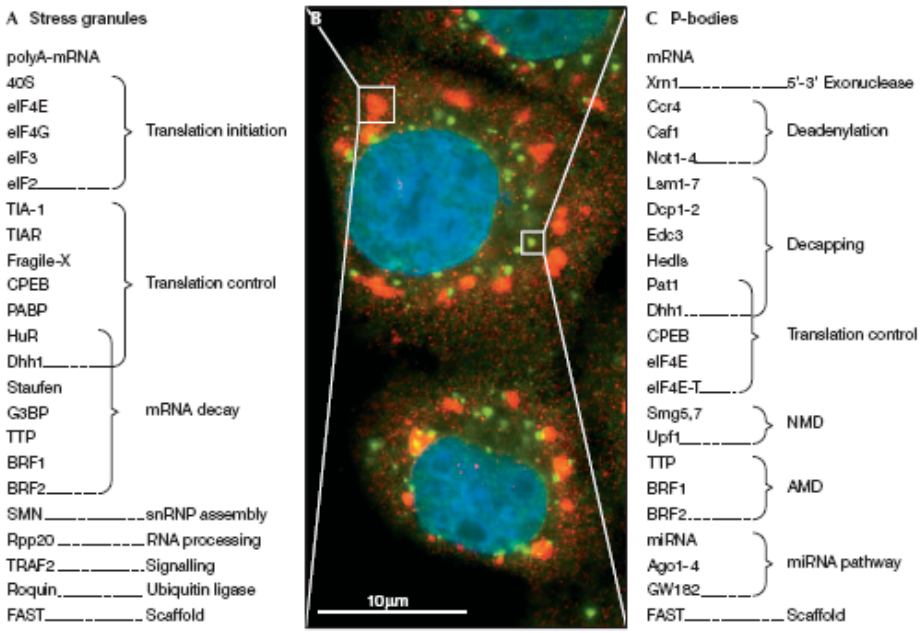


Figure 15. Stress granules and processing bodies have a central role in controlling messenger RNA translation and stability

(A)Stress granules components (B) Immunofluorescence micrograph of human HeLa cells that were subject to oxidative stress by treatment with arsenite. Fixed cells were stained in red with a polyclonal eIF4E antibody and in green with a human auto-antiserum that recognizes GW182. Nuclei were stained in blue with Hoechst dye.Areas delineated by boxes show a stress granule (red) and a processing (P)-body (green,with partial yellow overlap). (C) P-bodies components. Adapted from Newbury et al., 2006.

1.3. Ras-GTPase activating protein SH3 domain binding protein (G3BP)

The first G3BP family member to be discovered, G3BP1, was isolated in a screen for proteins that bind the SH3 domain of Ras GTPase Activating Protein (RasGAP) (Parker

et al., 1996). In the year 2000, the *Drosophila* homologue of G3BP was reported and named Rasputin/Rin because of its genetic interactions with members of the Ras signalling pathway. The G3BP family of proteins is evolutionarily conserved throughout eukaryotes. *Drosophila* homologue of G3BP, Rin shares 40% amino acid identity and 60% homology with human G3BP1 and G3BP2 over their entire lengths. The *S. pombe* protein is less closely related to the others (26% identity and 36% homology to Rin). Mammals have three G3BPs: G3BP1, 2a and 2b which are the products of two distinct genes (Kennedy et al., 2001). The proteins are relatively ubiquitously expressed and seem to play important roles in several biological processes, but their actual physiological functions still remain unclear.

1.3.1. G3BP structure

G3BP1 and 2 are encoded by distinct genes on human chromosomes 5 and 4 (5q 14.2, 4q 12-4q 24) and mouse chromosomes 11 and 5 respectively. G3BP2b is a splice isoform of G3BP2a, lacking 33 amino acids in the central region (Kennedy et al., 2001). There is 65% identity and 74% similarity between G3BP1 and G3BP2a protein sequences across the mouse and human species. hG3BP1 shares 94.4% sequence identity with mG3BP1. hG3BP2 and mG3BP2 show 98.5% identity of protein sequence.

G3BPs have four domains. First, the most highly conserved domain, both between species and within the mammalian G3BP family members, is the N-terminal Nuclear Transport Factor 2-like (NTF 2-like) domain. NTF2 is a small protein involved in RanGTP-dependent nuclear import of proteins through the nuclear pore complex (Ribbeck et al., 1998). As expected from the sequence homology, G3BPs might play a role in nuclear transport and, like NTF2, G3BPs may also bind Ran or other small GTPases. Second, G3BPs possess an acid-rich domain containing a serum dependent phosphorylation site at Ser 149. Third, G3BPs' central regions consist of varying numbers of proline rich (PxxP) motifs. PxxP motifs represent the minimal consensus sequence for SH3 domain binding (Booker et al., 1993; Saksela et al., 1995). All three G3BPs have been shown to bind the SH3 domain of RasGAP (Kennedy et al., 2001; Parker et al., 1996). But, the NTF2-like domain of these proteins, rather than the PxxP motifs, was responsible for RasGAP binding based on *in vitro* binding assay (Kennedy et

al., 2001). Fourth, G3BP C-termini comprise two motifs traditionally associated with RNA binding. These are a canonical RNA Recognition Motif (RRM) and a loosely conserved RGG (arginine-glycine rich) box (Birney et al., 1993). The RRM-containing family of proteins is the largest family of RNA-binding proteins in mammals. The domain consists of two short, loosely conserved motifs, RNP1 and RNP2. RGG domains are often found in RNA-binding proteins and may confer cooperative binding to RRM motifs (Burd and Dreyfuss, 1994). RGG domains have also been shown to influence nuclear translocation.

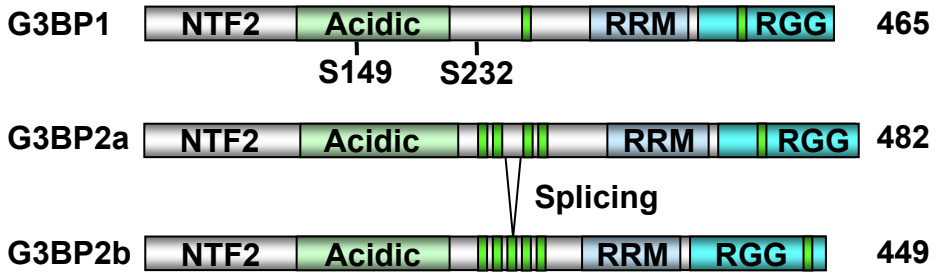


Figure 16. Schematic representation of human G3BPs domains

The position of the alternative splicing which remove 33 amino acids from G3BP2a to generate G3BP2b, thereby creating an additional PxxP motif in G3BP2b is indicated. Green box indicates PxxP motif.

1.3.2. G3BP subcellular localization

G3BP1 and G3BP2a are primarily cytoplasmic proteins (Parker et al., 1996; Prigent et al., 2000), but both proteins have been observed in the nucleus in several studies (Costa et al., 1999; French et al., 2002; Tourriere et al., 2001). Tourriere et al. reported partially nuclear localisation of phosphorylated G3BP1 in quiescent mouse embryonic fibroblasts. Nuclearly localized G3BP1 has been shown a functional DNA and RNA helicase in HeLa cells (Costa et al., 1999). By contrast, Parker and colleagues did not detect G3BP1 in the nucleus of Epidermal Growth Factor (EGF)-transformed fibroblasts at any stage of the cell cycle (Parker et al., 1996). At least one G3BP2 isoform has also been shown to shuttle between the nucleus and the cytoplasm in a cell cycle dependent manner. In

contrast to G3BP1, G3BP2 is exclusively cytoplasmic in quiescent fibroblasts and rapidly enter the nucleus upon serum stimulation (French et al., 2002).

1.3.3. G3BP expression in normal tissues and cancer

Some tissues express abundant levels of G3BP1 and include lung, kidney, and colon. Heart, liver, and spleen also express lower levels of G3BP1. Some tissues are shown to express both isoforms of G3BP2 including lung, liver, kidney, stomach, and colon, pancreas, and testis and others such as spleen express only G3BP1. Other tissues are restricted to only expressing G3BP2a including brain, muscle, and heart. All G3BPs are expressed during development with G3BP1 and G3BP2b rapidly down-regulated at birth. However, histochemical data would suggest that G3BP1 is not expressed in mature neurons (Kennedy et al., 2001).

Both G3BP1 and G3BP2 are dramatically overexpressed in human cancers such as breast, head, neck, colon and thyroid cancer. (Barnes et al., 2002; French et al., 2002; Guitard et al., 2001). Barnes and co-authors have reported that growth factor heregulin b1 (HRG), induced expression (mRNA and protein), phosphorylation, ATPase activity, and nuclear localization of G3BP in parallel with HER2 overexpression in an estrogen-independent manner, in eight human breast cancers and patients (Barnes et al., 2002). HER2 is also frequently overexpressed in breast cancer and this is associated with poor prognosis and malignancy (Yarden, 2001). Over-expression of G3BP2 has been demonstrated in 88% of 56 breast tumours, whilst G3BP2 expression was rarely detectable in surrounding normal tissue (French et al., 2002). It is not yet known whether G3BPs function in tumour progression and by what mechanism, or whether they are simply up-regulated as a consequence of cancer. However, French et al. have observed G3BP2 overexpression in early *in situ* ductal carcinomas, suggesting that the high expression of G3BP2 occurs in parallel with tumour progression rather than as a consequence of cancer formation (French et al., 2002). It is also noteworthy that reduced G3BP1 mRNA was found in metastatic human lung carcinoma (Liu et al., 2001b). Although this result is not consistent with G3BP overpression in cancer, it is noteworthy of the fact that G3BP may differently function in various pathways including Ras signalling (Malumbres and Pellicer, 1998), NFκB signalling (Chen and Li, 2002) and the

ubiquitin proteasome system (Gray et al., 1995). Growing evidence suggests that deregulated RNA processing is often associated with cell proliferation and cancer (Sonenberg et al., 1998; Sueoka et al., 1999). Pathological stabilisation of ARE mRNA is often seen in tumour cells and TTP which acts to destabilise ARE mRNA, has been shown to be a potent tumour suppressor (Stoecklin et al., 2003). The observation that G3BPs are specifically overexpressed in several cancers and involved in RNA metabolism and cell cycle, makes them potential candidate targets for anti-cancer therapeutics.

1.3.4. G3BPs and invertebrate development

Drosophila encodes only one G3BP: Rasputin (Rin). Pazman and colleagues studied the effects of Rin mutants on ommatidial polarisation during eye development in *Drosophila* (Pazman et al., 2000). They reported that Rin mutants are viable and display defects in photoreceptor recruitment and ommatidial polarity in the eye development, resembling phenotype of Ras1 and RhoA mutants as well as other polarity genes such as *frizzled (fz)* and *dishevelled (dsh)*. Additional experiments using constructs driven by the *sevenless* promoter led them to conclude that the Rin mutation genetically interacts with RhoA.

1.3.5. G3BPs and vertebrate development

Recently evidence emerged that several downstream effectors of planar polarisation in *Drosophila* are involved in Convergence and Extension (CE) in vertebrates and that the processes are broadly similar (reviewed in Strutt, 2003). Zebrafish has three G3BP homologues (α, β, and γ). Knock down of G3BPβ with specific morpholinos showed a phenotype, which was defects in CE and/or cell tracking with no apparent changes in cell fate. Another aspect of polarisation is that asymmetric distribution of the core proteins, involved in downstream pathways, is common within cells (Strutt, 2003) and this was also shown for Rin (Pazman et al., 2000). It is interesting to note that an asymmetric distribution of G3BP1 was observed in breast cancer cells (French et al., 2002). The classification of G3BP1 as a cell-cycle regulated transcript peaking in mitosis would also seem to support a role for G3BP in morphological remodeling and adhesion (Whitfield et al., 2002).

1.3.6. G3BP functions

1.3.6.1. G3BP and Ras signaling

G3BP1 was first co-immunoprecipitated with the RasGAP SH3 domain from fibroblasts overexpressing the EGF Receptor and subsequently shown to bind to full length RasGAP (Parker et al., 1996). Interaction of RasGAP and G3BP occurred only in proliferating cells when Ras is in an active form (Gallouzi et al., 1998; Parker et al., 1996). Gallouzi and colleagues reported that G3BP1 was heavily serine phosphorylated in quiescent cells and this cell-cycle regulated phosphorylation of Ser149 is RasGAP dependent (Tourriere et al., 2001). Ser149 is in a consensus Casein Kinase II phosphorylation site2 and is conserved between G3BP1 and the G3BP2 isoforms. G3BP1 phosphorylation and Ras-GTPdependent RasGAP association are consistent with the possibility that G3BP1 is regulated in response to external signals. Nevertheless, G3BP1 was able to interact with a purified RasGAP SH3 domain regardless of its phosphorylation status, implying that G3BP phosphorylation affects downstream functions rather than RasGAP interaction (Gallouzi et al., 1998). However, G3BP1 phosphorylation on Ser149 affects a number of functions, including RNase activity and stress granule recruitment/assembly (Tourriere et al., 2003; Tourriere et al., 2001). Also, G3BP1 overexpression in fibroblasts was found to increase S-phase entry and this was dependent on an intact RNA-binding domain (Guitard et al., 2001). G3BP1 may bind and regulate *c-myc* mRNA degradation. c-Myc is an important transcription factor in cell cycle progression (Dang, 1999; Dang et al., 1999). These data suggest that G3BP may be involved in regulation of cell cycle.

1.3.6.2. G3BP2 and NFκB signalling

IκB, in addition to its cytoplasmic role, also functions in the nucleus to dissociate NFκBs from DNA and re-export them. IκBα contains an N-terminal cytoplasmic retention sequence (CRS). G3BP2a was identified as a CRS-binding protein. G3BP2a interacted with both IκBα and IκBα/NFκB complexes by coimmunoprecipitation experiments and these interactions promote cytoplasmic retention of IκBα in HeLa cells. The IκBα interacting domain on G3BP2a was mapped and the central acid-rich domain of G3BP2a (aa 117-223) was sufficient to promote cytoplasmic retention (Prigent et al., 2000). This interaction provides a functional link between G3BP2a and NFκB signalling, implicating

that G3BP2a may play a role in regulating the nucleocytoplasmic NFκB/IκBα equilibrium and therefore NFκB activity. However, the suggestion that G3BP2a interacts with both IκBα and IκBα/NFκB omplexes is somewhat paradoxical. G3BP2a retention of IκBα positively influences on NFκB activation whereas G3BP2a-mediated cytoplasmic retention of IκBα/NFκB complex negatively affects on NFκB activation. Interestingly, IκBα Tyr 42 phosphorylation dramatically decreased CRS function (Prigent et al., 2000). These observations imply that signal induced modification of the IκBα CRS could affect the affinity of CRS-binding partners, such as G3BP2a, and therefore cytoplasmic retention of IκBα. Thus, it suggests that the real consequence of the interaction between IκBα and G3BP2a could well depend on the type of activating signal and post-translation modifications.

1.3.6.3. G3BPs and ubiquitin-mediated activity

Ubiquitin-mediated protein degradation is a fundamental process in cells to regulate protein turnover. Interestingly, in the first case an interaction between G3BP1 and Ubiquitin Specific Protease-10 (USP10) was discovered using a yeast-two-hybrid system and confirmed in human cells. G3BP1 did not appear to be a real substrate of USP10. Rather it inhibited USP10 de-ubiquitinating activity on a linear ubiquitin construct *in vitro* (Soncini et al., 2001). Soncini et al. suggested that G3BP1 might function *in vivo* to restrict de-ubiquitinating activity to appropriate substrates. With regard to the potential function/s of G3BP1 in the ubiquitin metabolism, it is noteworthy that several studies have demonstrated roles for ubiquitination and the proteasome in mRNA degradation. In one study, degradation of an ARE-containing reporter mRNA was regulated by the level of ubiquitin-conjugating activity in the cell and inhibition of a cytokine-inducible de-ubiquitinating enzyme enhanced mRNA decay (Laroia et al., 2002). Whether G3BP1's involvement in ubiquitin metabolism is linked to its involvement in mRNA metabolism, or other ubiquitin-mediated activities such as signal transduction, is unknown. G3BP1 may interact with de-ubiquitinating enzymes other than USP10. USP10 may have substrates other than the vesicle transport proteins, or protein transport and mRNA metabolism could be mechanistically linked in a way that is not yet appreciated.

1.3.6.4. G3BPs and RNA metabolism

The putative RNA-recognition and binding domains of G3BPs led to be speculation that these proteins are involved in signal-regulated mRNA metabolism. Although all G3BPs bind homopolymeric ribonucleic acid sequences *in vitro* and G3BP1 associated with a heterogenous pool of polyA mRNAs (Tourriere et al., 2001), *c-myc* is the only specific mRNA for a phosphorylation-dependent endoribonuclease activity of G3BP1 *in vitro* (Gallouzi et al., 1998; Tourriere et al., 2001). Also, G3BP2a and G3BP2b cleave *c-myc in vitro*, although with much lower efficiency than G3BP1 (reviewed in (Irvine et al., 2004). *c-myc* mRNA decay is delayed in *RasGAP-/-* fibroblasts which contain a phosphorylation-deficient form of G3BP1. Subsequent to the initial identification of G3BP1 as an RNase, Tourriere et al. showed specific cleavage of *c-myc* between CA di-nucleotides, which was dependent on RNA-binding; and determined an affinity binding sequence for G3BP1 using the SELEX technique (Tourriere et al., 2001). But the biological significance of G3BP1-mediated *c-myc* decay is not clear. Recently it has reported that PKC phosphorylated G3BP1 was isolated with mRNP complex containing tau mRNA, HuD and insulin-like growth factor mRNA-binding protein IMP-1, which was formed during retinoic acid induced differentiation of P19 neuronal cells (Atlas et al., 2004). The interactions within the mRNP were RNA-dependent and the complexes precipitated with polysomal proteins. This data not only shows that G3BP1 is a polysome-associated protein which plays a role in mRNA metabolism, but its presence in an mRNP containing tau mRNA raises the possibility that it is through association with specific mRNAs that G3BP may participate in cytoskeletal re-modelling. Also the G3BP2 isoform binds an mRNP complex associated with actively translated mRNAs in neuronal synapses (Angenstein et al., 2002). Thereby, it suggests that G3BPs play important roles in RNA metabolism and mRNP complex.

1.3.6.5. G3BP and Stress granules (SGs)

One of particularly intriguing properties of G3BP1 is its assembly in SGs under stress conditions (Tourriere et al., 2003). SGs are translationally incompetent mRNP complexes (Kedersha and Anderson, 2002). G3BP1 was recruited to stress granules after arsenite treatment in mammalian cells and over-expression of G3BP1 like TIA-1 was sufficient to induce SG formation. Dephosphorylation of G3BP1 (S149) result in SG recruitment and

assembly owing to facilitating self-aggregation after arsenite treatment (Tourriere et al., 2003). G3BP at Ser 149 is phosphorylated and dephosphorylated by downstream of RasGAP (Gallouzi et al., 1998), thereby implicating Ras activation in stress granule formation for the first time. Thus, it has been suggested that G3BP1 might function to determine the fate of mRNAs during cellular stress. It has been reported that ARE mRNAs stabilized in stress and the evidence suggests that this may be mediated through changes in ubiquitination state, localization and interactions between diverse RBPs (Irvine et al., 2004).

2. Chapter 2: Materials and Method

2.1. Materials

2.1.1. Plasmids

pcDNA3-FLAG-tagged hHDAC6 mutants that have point mutations in either or both of the hdac domains (H216A, H611A, and DM) were generated as previously described (Grozinger et al., 1999). HA-tagged full length mHDAC6 or mHDAC6 deletion mutants were generated as described (Zhang et al., 2006). Myc-tagged full length hG3BP or G3BP deletion fragments were generated by PCR, verified by sequencing, and inserted in pcDNA3 myc-tagged vector.

2.1.2. Antibodies

The following antibodies were used in this study: anti-cleaved Caspase-3 (1:1000; Cell signaling), anti-DCP1a (1:100 (IS), kindly provided by W. Filipowicz), anti-eIF2α (FL-315) (1:500; Snata Cruz), anti-phos-eIF2α (1:1000; Cell signaling), anti-eIF3ή (N-20) (1:1000, 1:100 (IS); Santa Cruz), anti-FLAG M2 (1:2000; Sigma), anti-G3BP (1:1000, 1:200 (IS); BD Transduction Laboratories), anti-HA (1:1000, Santa Cruz), anti-HDAC6 (H-300) (1:1000, 1:100 (IS); Santa Cruz), anti-HIF-1α (1:1000; NOVUS biologicals), anti-HSP70 (1:1000; Stressgen), anti-HSP90 (1:500; Stressgen), anti-Ace-Lys (1:1000; Cell signaling), anti-c-myc (9E10), anti-cleaved PARP (1:1000; Upstate), anti-alpha tubulin (DM1A) (1:1000; Santa Cruz), anti-beta tubulin (TUB 2.1) (1:1000, 1:200 (IS); Santa Cruz), anti-gamma tubulin (GTU-88) (1:1000, 1:200 (IS); Santa Cruz), anti-Ace-tubulin (TU6-1) (1:1000; Snata Cruz), anti-TIA-1/TIAR (H-120) (1:1000, 1:100 (IS); Santa Cruz), anti-Ubiquitin (1:100 (IS), Santa Cruz), or mHDAC-6 (Verdel et al., 2000)(1:1000, 1:100 (IS)). IS indicates immunofluorescence.

2.1.3. Reagents

The following reagents were obtained from company respectively: Okadaic acid, Alexis; EHNA (Erhthro-9-[3-(hydroxynonyl)]adeninde), Biomol; Proteaosme inhibitor (Z-Leu-Leu-Leu-CHO (MG132), Boston Biochemicals; Latrunculin B, Calbiochem; Sodium arsenite ($NaAs_2O_3$), Fluka; λ phosphatase, NEB; Cobalt chloride ($CoCl_2$), Colchicine, Cytochalsin D, Nocodazole, Sodium butyrate, Sodium Vanadate, Trichostatin A (TSA),

Sigma.

2.2. Methods

2.2.1. Cell lines and Transfection

All cells (HEK 293, HEK 293T, HeLa, NIH 3T3 cells) were obtained from the American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and antibiotics at 5% CO2. Transfections were performed with FuGENE6 (Roche) following the manufacturer's protocol or by the calcium phosphate method. HEK 293 overexpressing HDAC6 were made by transfection with a CMV-FLAG-hHDAC6 vector and selection for neomycin resistance.

2.2.2. HDAC6 -/- cell line and rescue by HDAC6 wild type or mutants

Mice with invalidated with HDAC6 gene were generated (Zhang et al., 2003), and mouse embryo fibroblasts were isolated from E13.5. Sex genotyping was used to select only male embryos. HDAC6 knockout and wild type lines were further identified by PCR genotyping and western blot. 3T3 cell lines were established following a standard protocol. The cells were plated at a density of $3x10^5$ per 5 cm plate and split every 3 days for about 20 passages. Wild type and mutant HDAC6 cDNA were cloned into a pMSCV.EGFP vector. The retrovirus was made from Phoenix cells following standard protocol. The HDAC6-/- 3T3 cells were infected. The infected cells were kept in culture for 2 weeks and single GFP positive cells were sorted into 96 well plates. The positive cells were identified by western blot and PCR (Zhang et al., 2006).

2.2.3. Preparation of Cell Extracts and Western Blotting Analysis

Cells were rinsed twice with ice-cold PBS and were then extracted NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl pH 7.4, 120 mM NaCl, 25 mM NaF, 25 mM glycerol phosphate, 1 mM EDTA, 5 mM EGTA, and protease inhibitor cocktail tablet (Roche)). Cells were collected by pipetting, and centrifuged at 15,000 × g for 15 min at 4 °C. Protein concentration was measured by the method of BCA kit (Pierce) with bovine serum albumin as the standard. Aliquots of the supernatant were stored at −80 °C until use. Cell lysates containing 50 μg of total protein were subjected to SDS-PAGE on 8-

12% slab gels, and proteins were transferred to nitrocellulose membranes. Membranes were blocked for 1 hour in PBS containing 0.1% Tween 20 and 10% (v/v) horse serum and incubated overnight with primary antibody. The membranes were then washed with 0.1% Tween 20/PBS and incubated for 1 hour with an anti-rabbit secondary antibody or anti-mouse secondary antibody labelled to HRP, and bound antibodies were detected with ECL western blotting analysis system (Amersham bioscience).

2.2.4. Co-immunoprecipitation assay

For co-immunoprecipitation, ~500 µg of extracts from either NIH-3T3 cells or HEK 293T cells transfected by FuGENE6 (Roche) were incubated overnight with the primary antibody at 4°C with gentle agitation. After this, 25 µl of protein A–Sepharose slurry or protein G-agarose slurry were added and samples were incubated for 3 hours at 4°C with gentle agitation. Beads were washed three times with NP-40 lysis buffer and subsequently resuspended and boiled in 20 µl of loading buffer for SDS–PAGE.

2.2.5. Translation *in vitro* and binding assay

In vitro binding assay: GST and GST-G3BP were produced using standard methods. Briefly, protein expression was induced by addition of 0.1 mM IPTG to a bacteria culture at OD600 0.7. The bacteria were lysed after 4 hours of growth at 37°C, in 1% Triton in PBS plus 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin. After French pressure cell press and centrifugation, the supernatant was incubated with glutathione sepharose beads (Pharmacia) rotating for 1 h at 4°C. After three washes in ice-cold PBS, bound proteins were eluted with 10 mM glutathione in 50 mM Tris HCl pH 8.0. Full-length human HDAC6 was *in vitro* translated using the TNT T7 Coupled TNT reticulocyte lysate (Promega) following the instructions of the manufacturer. Binding reactions were performed in 600 µl of 20 mM HEPES pH 7.4, 150 mM NaCl, 0.05% NP40, 10% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride; for 3 hours at 4°C, using from 10 µg of GST fusion proteins and 10 µl of the in vitro translated product. 25 µl of glutathione sepharose slurry was added and the reactions continued for 1 hour with rotation. The beads were pelleted, washed three times in the binding buffer, resuspended in 4x Laemmli sample buffer and heated at 95°C for 5 min.

After separation on a SDS PAGE the gel was dried and exposed. Fluorography of ^{35}S was performed to detect co-precipitated HDAC6. Generally, in lanes labeled 'input' 1/10 of the material used for the binding reaction was loaded.

2.2.6. Mass Spectrometry

Ten 10 cm dishes of the stable cell lines expressing FLAG-hHDAC6 proteins (S-HDAC6 293) were harvested and lysed. Total extracts were analyzed by immunoprecipitation using anti-FLAG M2 agarose (Sigma), bound proteins were eluted from the beads using 10 mM 3xFLAG peptide, and were separated on SDS-PAGE, and stained with coomassie blue staining. The HDAC6 interacting proteins were identified by LC-MS/MS spectrometry.

2.2.7. Immunofluorescence and Micoroscopy

Exponentially growing cells were plated on 8 chamber slide and incubated overnight. After drug treatment, cells were washed in PHEM buffer (60 mM PIPES pH6.9, 25 mM HEPES pH 7.5, 10 mM EGTA pH 7.5, 4 mM $MgCl_2$ pH 6.9) and fixed with 4% formaldehyde/PBS for 15 min at room temperature or with methanol at –20°C for 10 min. Slides were then rinsed three times with 0.2% Triton X-100/PHEM buffer, and cells were permeabilized with 1% Triton X-100/PBS for 5 min at room temperature. Slides were then incubated in blocking solution (3% BSA/PBS) for 1 hour at room temperature for reducing non-specific binding of the antibody. Incubation with the primary antibodies was carried out for 3 hours at room temperature or at overnight at 4°C, and the slides were then washed three times with 0.2% Triton X-100/PHEM buffer. Alexa-Fluor anti-mouse and Alexa-Fluor anti-rabbit immunoglobulin antibodies were used as secondary antibodies. After the slides had been washed three times with 0.2% Triton X-100/PHEM buffer and once with deionized water, they were observed under a confocal LSM 510 meta microscope (Zeiss Axioplan 2). Pictures were deconvolved with the theoretical PSF calculated within the software Huygens (Scientific Volume Imaging, http://www.svi.nl).

2.2.8. Quantification of stress granules-containing cells and large stress granules

For quantification, nine fields of each sample were randomly selected. The occurrence of SGs was estimated as the average number of SG-containing cells. For quantification of large SGs, 100 cells of each sample were randomly selected. The number of large SG was counted using confocal microscopy software (LSM viewer, Carl Zeiss).

2.2.9. Proportional Distribution of HDAC6 in stress granules

After obtaining a granule pellet by centrifugation of cytoplasm at 12.000 X g for 10 min, the pellet was lysed and aliquoted into two samples; one was saved as the "total granules" sample and the other was used for immunoprecipitation to collect stress granules. To quantify protein levels, nitrocellulose membranes were scanned by Odyssey infrared imaging system (LI-COR Bioscience) and relative optical densities were determined using Odyssey .For each lane, the backgraound-subtracted value for each band was the divided by the value of the background-correct tubulin band from the same lane to obtain a normalized value. Each protein samples was run in triplicate on three separate blots, and normalized values from each set of replications were averaged to obtain a final value for each sample.

2.2.10. Polysome Analysis

MEFs were plated and used within 24 hours of plating, at ~ 70% confluence. Briefly, cells were treated with 100 μg/ml of cycloheximide for 5 min, followed by washes with PBS and a hypotonic buffer (5 mM Tris-HCl, pH 7.5, 1.5 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml cycloheximide). Lysates were prepared by scrapping the cells in lysis buffer [hypotonic buffer containing 0.5% deoxycholate, 0.5% Triton X-100, 1 mM dithiothreitol, protease inhibitor cocktail tablet (Roche) and 120 U/ml of Rnasin (Promega)]. Lysates were centrifuged for 8 min at 3,000 x g at 4°C and supernatants were then layered onto 10-50% sucrose gradients and spun in an SW40 rotor (Beckman) at 36,000 rpm for 2 hours at 4°C. Gradients were eluted from the top by using a Brandel elution system (Brandel, Gaithersburg, MD). The eluate was continuously monitored at 260 nm using an ISCO UA5 UV monitor (ISCO, Lincoln, NE). Fractions were collected from the top of

the gradient. Aliquots of individual fractions were acetone precipitated to remove sucrose and to concentrate the samples, resuspended in SDS sample buffer, and processed for Western blot analysis.

2.2.11. Construction of let-7 plasmids

The RL and FL constructs bearing let-7 sites

To obtain Renilla luciferase (RL) expression from a CMV promoter, the RL coding region was released from phRLTK (Promega) as a NheI-BamHI fragment and inserted into similar sites in the pCIneo vector (Promega) to get pRL-Con. Note that this RL gene contains optimized codon usage (humanized) for better expression in mammalian cells. To make RL reporters whose expression is controlled by endogenous let-7a RNA, we inserted annealed primers into the XbaI-NotI sites of pRL-Con to get constructs having one perfectly base-pairing (ACTATACAACCTACTACCTCA; pRL-Perf), one bulged (GCACAGCCTATTGAACTACCTCA; pRL-1xBulge), three bulged (GGACAGCCTATTGAACTACCTCACTCGGAGCACA GCCTATTGAACTACCTCAGGCCTGCACAGCCTATTGAACTACCTCA; pRL-3xBulge) and three mutated bulged (GCACAGCCTATTGAACTACCCCTCACTCGAGCACAGCCTATTGAACTACCCCT CAGGCCTGCACAGCCTATTGAACTACCCCTCA: pRL-3xBulgeMut) let-7 sites in the 3'UTR. The mutated bulged binding sites have a 2-nt insertion (underlined) in the seeding region of the bulged let-7 complementary site. We verified that this mutation abolishes the repressive effect of endogenous let-7 RNA on translation of the RL reporter To obtain firefly luciferase (FL) constructs with let-7 complementary sequences, we initially modified the pGL3 Promoter plasmid (Promega) by insertion of a T7 RNA polymerase promoter into the HindIII site upstream of the FL ORF. The resulting plasmid was named pFL-Con. To obtain pFL-Perf and pFL-3xBulge, containing one perfect and three bulged let-7 sites, respectively, the corresponding XbaI-HpaI fragments were excised from the 3'UTR regions of pRL-Perf and pRL-3xBulge and inserted to corresponding sites of pFL-Con (Pillai et al., 2005).

2.2.12. Transient Transfection and Luciferase assay

MEFs were seeded into 6 well plates and allowed to reach ~50% confluence. Cells were cotransfected with 1 μg of MTV-GRE-luciferase reporter and 50 ng of renilla vector as the control. Dexamethatsone (100 nM) was added to the culture medium for the last 4 hours of transfection. After 48 hour transfection, the cells lysates were analyzed with a Dual Luciferase Reporter Assay kit activity (Promega). Relative luciferase activity was determined by the ratio of firefly to renilla.

2.2.13. Cell Viability

MEFs were seeded at 105 cells/well in 6-well plate. Cell viability was determined by Vi-Cell (Beckman Coulter) at 0, 6, 12, 24, and 48 hours by trypan blue exclusion from each individual culture.

2.2.14. Cell Death Assay

Cells treated with 5 μM of MG132 or 1 mM of arsenite as stressor. Annexin V staining was performed exactly as described by the manufacturer (BD Bioscience), and samples were analyzed via flow cytometry. Apoptotic cells were detected by western blotting with an anti-cleaved PARP or anti-cleaved Caspase-3 antibody (apoptosis marker).

2.2.15. DNA Fragmentation Assay

The cells were rinsed with ice-cold PBS and harvested by trypsination. The cell pellets were resuspended and incubated in 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5% SDS, and 0.1 μg/ml proteinase K at 50°C overnight. The digested cells were extracted for DNA with phenol/chloroform (1:1) and chloroform/isoamylalcohol (1:24). The extracted DNA was precipitated and digested in 10 mM Tris-HCl (pH 5.0) containing 1 mM EDTA and 10 μg/RNase for 1 hour at 37°C. Ten micrograms of DNA per sample was resolved by electrophoresis in a 1.8% agarose gel impregnated with ethidium bromide (0.5 μg/ml), and the DNA pattern was examined by ultraviolet transillumination.

3. Chapter 3: RESULTS

3.1. The histone deactylase 6 (HDAC6) interacts with G3BP and regulates stress granule formation

3.1.1. Abstract

HDAC6 is a mostly cytoplasmic deacetylase that has been shown to interact with and deacetylate tubulin. HDAC6 also has a ZnF-UBP domain through which it interacts with high affinity with mono- or poly-ubiquitin and ubiquitinited proteins and facilitates the accumulation of ubiquitinated misfolded proteins in an aggresome. Here we have identified RasGAP associated endonuclease (G3BP), a component of stress granules, as a novel HDAC6 interacting protein. Stress granules (SGs) are formed in the cytoplasm in response to environmental stresses and play a critical role in the regulation of mRNA metabolism during stress. Phosphorylation of G3BP -which regulates its localization to stress granules- also modulates its capacity to interact with HDAC6. Pharmacological inhibition of HDAC6 activity in cultured cells leads to impaired SG assembly, and overexpression of HDAC6 facilitates the formation of SGs. Indeed, MEFs deficient in HDAC6 cannot form SGs properly, although they exhibit normal phosphorylation of eIF2α in response to stress; in agreement with this, HDAC6 was found to be a stable and critical component of stress granules. The deacetylase domains of HDAC6 were necessary and sufficient to target the protein to SGs. Intriguingly, the ubiquitin binding domain of HDAC6 appears to be particularly important and intact HDAC6 function is required for SG assembly. Furthermore, disruption of microtubule arrays with nocodazole treatment or impairment of motor proteins with a dynein inhibitor abolishes arsenite-induced formation of SGs. Taken together, our results show that HDAC6 regulates the assembly of SGs. We propose that HDAC6 might facilitate SG formation by the motor-protein driven movement of individual SG components along microtubules.

3.1.2. Introduction

Reversible protein acetylation has emerged in recent years as one of the major forms of protein modifications. The importance of acetylation and deacetylation has been particularly well documented in the case of the N-terminal histone tails, and of a few transcription factors such as p53 and STAT3. Acetylation and deacetylation are catalyzed by (histone) acetylases (HATs) and (histone) deacetylases (HDACs). HDAC6 is a unique class II deacetylase that contains two catalytic domains and also a C-terminal domain binding with high affinity free ubiquitin as well as mono- and polyubiquitinated proteins (Boyault et al., 2006). Like other class II HDACs, HDAC6 can shuttle between nucleus and cytoplasm; however, owing to a nuclear export signal in the N-terminus of the protein, HDAC6 localizes predominantly to the cytoplasm, where it is found partly associated with the microtubule network (Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003). We and others have shown that HDAC6 can deacetylate tubulin as well as the microtubule network *in vivo*. HDAC6 also associates with the chaperone-like AAA ATPase p97/VCP, a protein that is critical for proteasomal degradation of misfolded proteins. Thereby, the ratio of HDAC6 and p97/VCP modulates the levels of polyubiquitinated aggregates (Boyault et al., 2006). HDAC6 also facilitates the clearance of misfolded ubiquitinated proteins, promoting their accumulation in an aggresome and protects cells from apoptosis following misfolded proteins stress (Kawaguchi et al., 2003). Furthermore, HDAC6 can deacetylate the chaperone Hsp90 and regulate its activity (Bali et al., 2005; Kovacs et al., 2005). Consequently, these different biochemical functions of HDAC6 impinge on diverse cellular processes. For example, HDAC6 function was found to be necessary for the formation of an immune synapse between antigen presenting cells and T lymphocytes (Serrador et al., 2004) and also for nuclear translocation and transcription activation by the glucocorticoid receptor (Kovacs et al., 2005). Mice lacking HDAC6 are viable and have greatly elevated tubulin acetylation in multiple organs; in addition, they exhibit a moderately impaired immune response and also show a slight phenotype in the bone (Zhang et al., 2007).

One of the most immediate responses to cellular stress is a block of mRNA translation, triggered by phosphorylation of the translation initiation factor eIF2α under the action of

several stress sensing kinases (Bertolotti et al., 2000; Kaufman, 2004; Lu et al., 2001; Williams, 2001). Thereby, translationally stalled mRNAs are sequestered in dynamic cytoplasmic structures called stress granules (SGs). These granules represent a complex assembly of various initiation factors, such as eIF3 or, eIF4E proteins involved in translation control, such as TIA-1 or Fragile X mental retardation protein (FMRP) and proteins implicated in RNA remodelling or degradation, such as HuR, tristetraproline or Staufen as well as 40S ribosome subunits (reviewed in (Anderson and Kedersha, 2006; Newbury et al., 2006)). In addition, SGs also contain various polyadenylated mRNAs whose translation has been arrested.,It is thought that SGs are sites where triage takes place in order to direct RNAs to degradation in processing bodies or to recycle mRNAs for translation.. In addition, very recent evidence suggests that parts of the micro RNA pathway may also take place in SGs which contain Argonaute proteins and also miRNAs such as let-7 (Leung et al., 2006).

Here, we report the identification of G3BP (RasGAP associated endoribonuclease), a stress granule component, as a novel protein interacting with HDAC6 *in vivo* and i*n vitro*. This protein is conserved between species, and orthologues are found in Drosophila, humans, and mice. G3BP has been implicated in modulating Ras activity and the cell cycle, by binding to the RasGAP protein (Guitard et al., 2001; Kennedy et al., 2001; Pazman et al., 2000). The precise function of G3BP is not understood yet, but it appears to be an essential gene in the mouse: inactivation of the G3BP gene leads to embryonic lethality and growth retardation (Zekri et al., 2005). Furthermore, G3BP has attracted attention recently as it was found to have endoribonuclease activity and to localize to SGs (Tourriere et al., 2003). We show that HDAC6 is recruited to SGs and that pharmacological HDAC inhibition leads to impaired SG assembly, while overexpression of HDAC6 facilitates the formation of SGs. Indeed, HDAC6 deficient MEFs fail to form SGs, although they exhibit normal phosphorylation of eIF2α in response to stress. Furthermore, inactivating mutations in the catalytic domains or ubiquitin binding domain of HDAC6 significantly reduce SG assembly. Interestingly, the ubiquitin binding domain of HDAC6 seems to be particularly important for SG formation. Moreover, SG formation is abolished by disruption of microtubule arrays or by impairment of dynein motor proteins. Based on these results, we propose that HDAC6 is a central component of the

stress response, regulating SG formation and potentially contributing to the control of RNA metabolism and translation.

3.1.3. Results

HDAC6 interacts with G3BP (RasGAP-associated endoribonuclease) *in vivo* and *in vitro*

We set out to identify novel proteins associating with HDAC6 and which might help explain the regulation and cellular role of this enzyme. To this end, we established stable cell lines expressing FLAG-tagged HDAC6 and used these for co-immunoprecipitation assays: cell extracts were analyzed by immunoprecipitation using anti-FLAG antibodies, bound proteins were eluted from the beads, separated by SDS-PAGE and analyzed by mass spectrometry. By this approach, we identified G3BP-1, Ras-GTPase activating protein SH3 domain binding protein 1, as a prominent HDAC6 interacting partner. To verify the interaction between G3BP and HDAC6 a variety of assays were established. HEK 293T cells were transiently cotransfected with constructs encoding epitope-tagged G3BP and HDAC6 proteins and a co-immunoprecipitation assay was performed. The precipitated material was separated on SDS–PAGE, and western blot analysis was done with specific antibodies. As shown in Figure 1A, immunoblot with the FLAG M2 antibody verified HDAC6 precipitation and G3BP was found to efficiently co-precipitate. The experiment gave the same result when done in the reverse order, by first precipitating G3BP and testing the co-immunoprecipitation of HDAC6. Interaction between these two proteins could be demonstrated using an alternative *in vitro* binding assay. Bacterially expressed GST-G3BP fusion protein was used for pull-down assays with either extracts from HDAC6-transfected HEK 293T cells or *in vitro* translated HDAC6 protein. HDAC6 also interacted with G3BP under either of these conditions (Fig. 1B and 1C). We next attempted to detect an interaction between the endogenous G3BP and HDAC6 proteins. As shown in Figure 1D, co-immunoprecipitation assays with HEK 293T cell extracts demonstrated a specific interaction between endogenous HDAC6 and G3BP, irrespective of the precipitation order. To determine whether G3BP interacts specifically with HDAC6, or more generally with deacetylases, we transfected FLAG-tagged class I or class II HDACs and HA-tagged G3BP into HEK 293T cells and performed

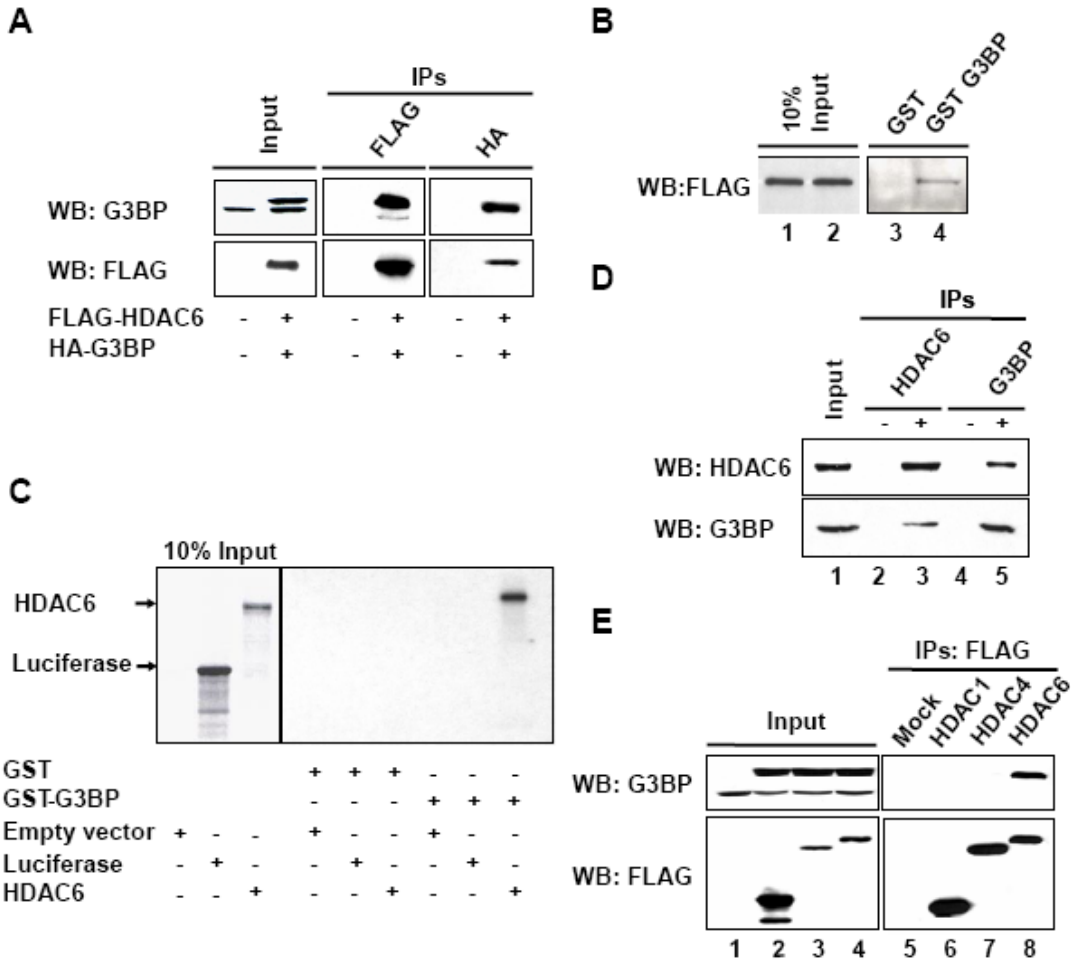


Figure 1. HDAC6 associates with G3BP

(A) Co-immunoprecipitation assay. 293T cells were co-transfected with either mock or the FLAG-tagged HDAC6 and HA-tagged G3BP. Interaction was measured by immunoprecipitation with an anti-HA or anti-FLAG antibody, followed by immunoblotting with an antibody to detect G3BP or HDAC6, as indicated. 10% of total cell lysates used in immunoprecipitation are shown as input. (B) GST Pull down assay. Equal amounts of extract from 293T cell transiently transfected with expression vector for FLAG-HDAC6 were incubated with beads loaded with GST alone or GST-G3BP fusion protein. After washing, bound proteins were run on SDS-PAGE and retained HDAC6 was detected by western blotting using an anti-FLAG M2 antibody. (C) In vitro binding assay. Recombinant GST or GST-G3BP was incubated with in vitro translated 35S-radiolabelled HDAC6 or luciferase and binding was allowed to proceed. The presence of HDAC6 protein was detected by fluorography. (D) Co-immunoprecipitation assay for endogenous proteins. HEK 293T cells extracts were immunoprecipitated with an anti-HDAC6 or anti-G3BP antibody, followed by mmunoblotting with antibodies as indicated. (E) HEK 293T extracts transfected with FLAG-tagged HDACs and GFP-tagged G3BP were immunoprecipitated with an anti-FLAG antibody and immunoblotted for G3BP.

coimmunoprecipitation assays. As presented in Figure 1E, G3BP co-immunoprecipitated with HDAC6, but not with the class I enzyme HDAC1, or the class II HDAC4. Taken together, these results indicate that G3BP is a bona fide novel specific interaction partner of deacetylate HDAC6.

Identification of the domain(s) required for interaction between HDAC6 and G3BP

To define the domain(s) required for interaction between HDAC6 and G3BP, truncated proteins were expressed by transfection in 293T cells and interaction was tested by co-immunoprecipitation assays. First, a series of HA-tagged HDAC6 deletion mutants were analyzed. As shown in Figure 2A and B, the full length protein as well as deletion mutants containing at least one HDAC domain co-immunoprecipitated efficiently with G3BP. The N-terminal region of HDAC6 bound weakly to G3BP, whereas the C-terminal did not bind appreciably to G3BP. These results demonstrate that the HDAC domain is both necessary and sufficient for G3BP binding. Next, a series of G3BP deletion constructs were tested in a similar manner. The results presented in Figure 2C show that the acidic-rich domain of G3BP (labelled B), containing the serum dependent phosphorylation site (see below), is required for binding to HDAC6, whereas the N-terminal NTF2-like domains (labelled A) and the C-terminal half of the protein (labelled CD) are dispensable. In addition, we also tested HDAC6 proteins with point mutations in the catalytic site of either or both of the HDAC domains (Grozinger et al., 1999). Similar to what had been seen previously with tubulin (Zhang et al., 2003), both the single and double mutants could be co-precipitated with G3BP as efficiently as the wild type enzyme (Fig. 2D). This indicates that the interaction between HDAC6 and G3BP is mediated by the HDAC domain, but does not depend on integrity of its catalytic center.

Dephosphorylation of G3BP at S149 enhances interaction between HDAC6 and G3BP

G3BP can be phosphorylated at Ser149 and Ser232 (see Fig. 3A). Phosphorylation at Ser149 has been shown to dominantly inhibit stress granules formation (Tourriere et al., 2003); furthermore, arsenite or heat-shock treatment of cells, as well as H-ras activation induce dephosphorylation of G3BP at S149, which can then assemble in SGs

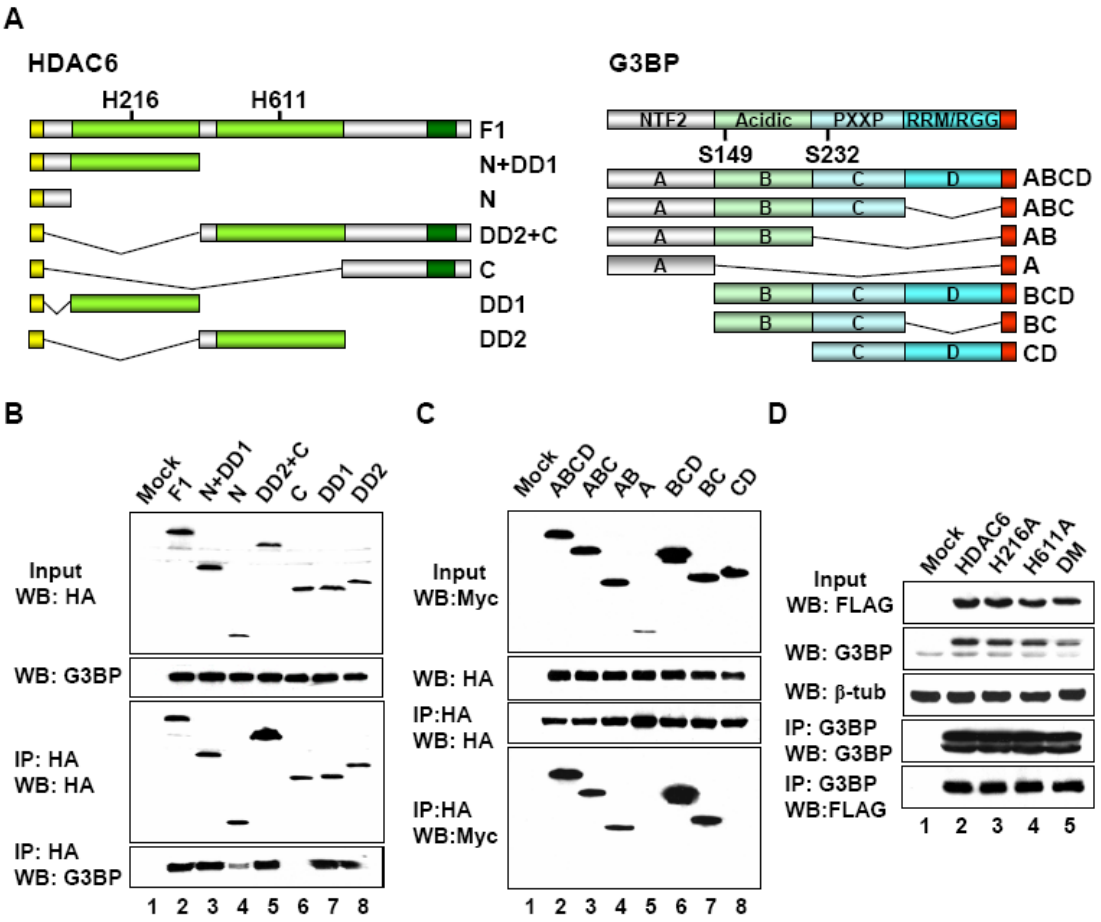


Figure 2. HDAC-6 interacts with G3BP via its HDAC domains

(A) Schematic representation of the N-terminally HA-tagged HDAC6 deletion constructs and C-terminally Myc-tagged G3BP truncated mutants used in this study. The two G3BP serine residues that can be phospshorylated are indicated. One representative experiment is presented (n=3-4). (B-D) Co-immunoprecipitation assay. (B) HDAC6 interacts with G3BP through the HDAC domains. 293T cells were co-transfected with the indicated HDAC6 expression vectors together with a G3BP full-length expression vector (lanes 2 to 8), and cellular extracts were prepared. Expression of HDAC6 or G3BP was measured by western blot with an anti-HA or anti-G3BP antibody. Association with HDAC6 was measured by performing an immunoprecipitation with an anti-HA antibody, followed by analysis of the precipitate by western blotting with the anti-G3BP antibody. (C) G3BP interacts with HDAC6 through the acidic B domain. The domain in G3BP required to interact with HDAC6 was identified by testing extracts from cells transfected with full-length HDAC6 and deletion mutants of G3BP. Analysis was done as in (B). (D) The catalytic domains of HDAC6 are not critical for interaction with G3BP. Extracts from 293T cells co-transfected with HA-tagged G3BP and the indicated FLAG-tagged HDAC6 point mutants were immunoprecipitated with an anti-HA antibody. In the HDAC6 mutants proteins, the histidine at position 216 or 611 were mutated to alanine (H216A, H611A). DM., double mutant protein.

(Tourriere et al., 2003; Tourriere et al., 2001). To test whether phosphorylation of G3BP at Ser149 influences interaction with HDAC6, we transiently co-transfected constructs encoding wild type or phosphomutant GFP-G3BP fusions and FLAG-tagged HDACs into HEK 293T cells and performed co-immunoprecipitation assays. Both the WT and the S149A non-phosphorylatable G3BP mutant co-precipitated with HDAC6 efficiently, however the phosphorylation-defective mutant co-precipitated more effectively than the WT (Fig 3B). In contrast, the phosphomimetic G3BP S149E mutant completely failed to co-precipitate, although it was expressed at equivalent levels to the other proteins. Identical results were also obtained when the experiment was performed in the reverse order (data not shown). We next assessed whether treatments that affect phosphorylation of G3BP would influence its association with HDAC6. Dephosphorylation of G3BP, induced by treatment of cells with arsenite or incubation of extracts with λ phosphatase, led to increased interaction with HDAC6 (Fig. 3C, lanes 2 and 4; Fig. 3D, lane 5). In contrast, the interaction was weaker when phosphorylation of G3BP was increased by treatment of the cells with a phosphatase inhibitor such as okadaic acid or vanadate (Fig. 3D, lanes 3 and 4). These results therefore indicate that the interaction of G3BP and HDAC6 is modulated by the phosphorylation status of G3BP.

HDAC6 and G3BP co-localize and are recruited to stress granules

The biochemical interaction between HDAC6 and G3BP prompted us to test whether these two proteins co-localize in the cell. For this, exponentially growing HeLa cells were fixed and G3BP as well as HDAC6 were visualized by immunostaining and confocal microscopy. In untreated cells, both proteins are diffusely distributed throughout the cytoplasm, as had been observed previously (Gallouzi et al., 1998; Parker et al., 1996; Zhang et al., 2003), and showed significant co-localization in the perinuclear region (Fig. 4A). In mammalian cells, exposure to environmental stress results in the formation of transient cytoplasmic structures known as “stress granules” (SGs, (Kedersha et al., 2002; Kedersha et al., 1999)). SGs contain among other components: mRNA, translation initiation factors, mRNA binding proteins such as TIA-1 and TIAR, and 40s ribosome subunits (reviewed in Anderson and Kedersha, 2006; Newbury et al., 2006). It has been recently reported that G3BP is recruited to SGs in cells exposed to stress

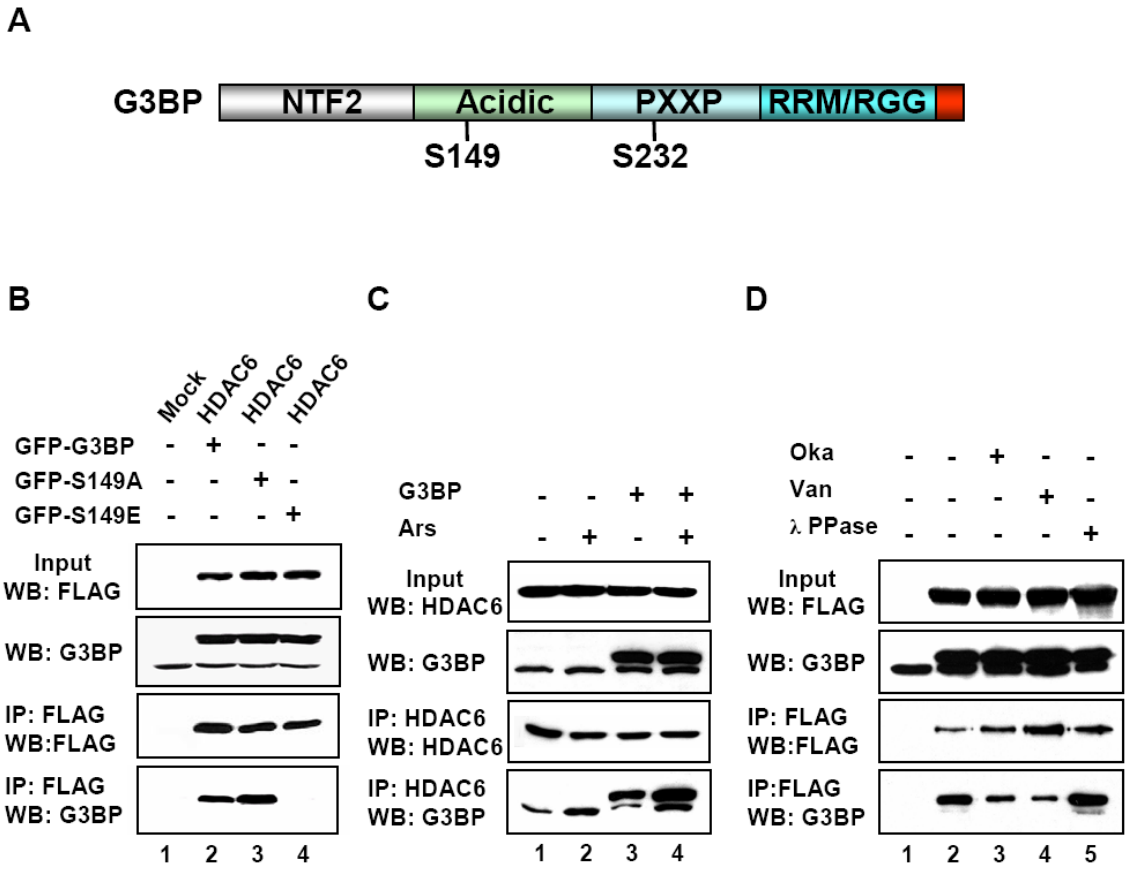
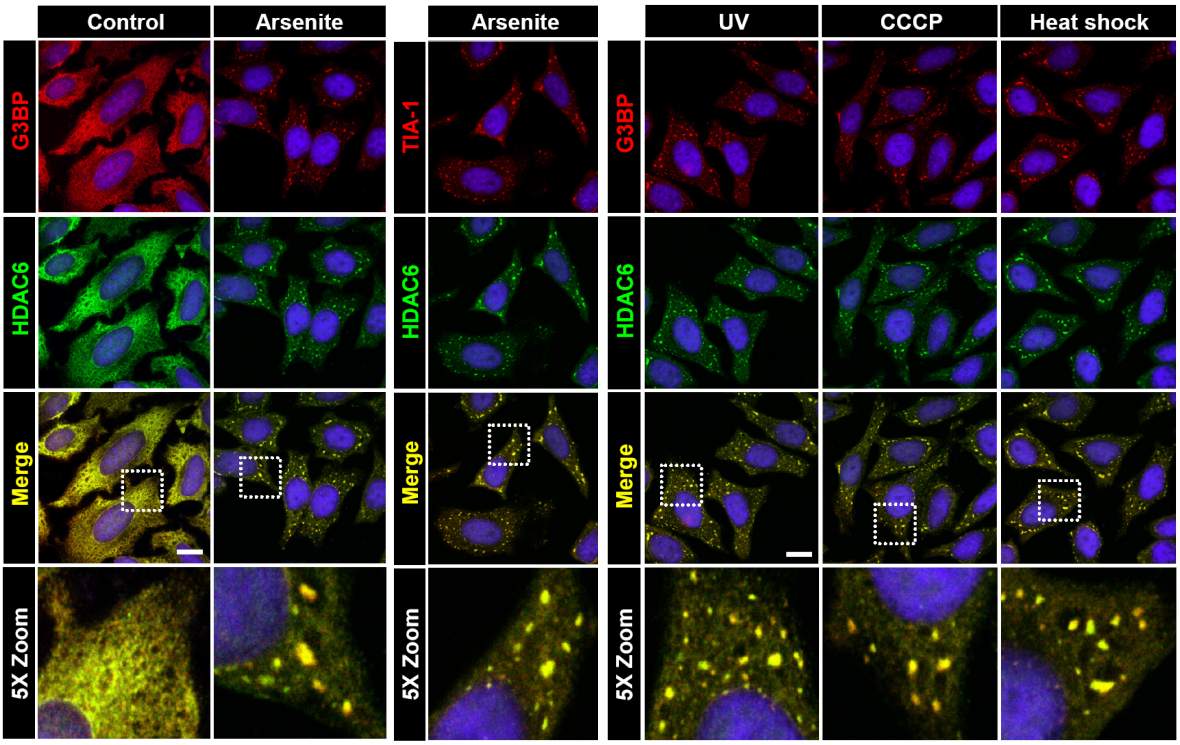


Figure 3. Phosphorylation of G3BP modulates the/its interaction with HDAC6

(A) Schematic representation of the subdomains within the G3BP protein. (B) G3BP phosphorylation at Ser149 modulates interaction with HDAC6. GFP fused wild type or GFP fused mutants G3BP and FLAG-tagged HDAC6 co-transfected into the HEK 293T cells. The lysates were immunoprecipitated with an antibody for FLAG, followed by immunoblotting with an anti-FLAG antibody and then reblotted for G3BP. 10% of total cell lysates used in immunoprecipitation are shown as input. (C) Arsenite-induced G3BP dephosphorylation promotes interaction with Hdac6. 293T cells were mock-transfected or transfected with HA-tagged G3BP and were treated with 1 mM arsenite for 1 hr prior to lysis (lanes 2 and 4); extracts were then used for co-immunoprecipitation with an anti-G3BP antibody and analysis by immunoblotting, as indicated. (D) Phosphatase inhibition reduces the interaction between G3BP and HDAC6. 293T cells were co-transfected with HA-tagged G3BP and FLAG-tagged HDAC6 and treated with phosphatase inhibitors (lanes 3 and 4); alternatively, cell lysates were incubated in vitro with λ phosphatase. Subsequent analysis was carried out as in (B). Oka; Okadaic acid, Van; Orthovanadate, λ PPase; λ phosphatase.

(Tourriere et al., 2003). To test whether HDAC6 is also recruited to SGs, we examined the localization of endogenous HDAC6 or G3BP, following treatment of the cells with arsenite, an oxidative stress inducing agent. As shown in Figure 3, G3BP and HDAC6 were both found in smallcytoplasmic foci and the merged picture shows good co-localization of the two proteins. To confirm this result, arsenite-treated cells were also stained for TIA-1 (T cell internal antigen-1), another robust marker of SGs, and HDAC6. In this case as well, endogenous HDAC6 was concentrated at discrete cytoplasmic foci and co-localized with TIA-1. The perfect colocalization of HDAC6 and G3BP or TIA-1 at SGs therefore indicates that HDAC6 is a novel component of SGs. Previous studies have shown that the components of SGs vary with the stimulus used to elicit their assembly; e.g., heat shocked-induced SGs contain HSP27, whereas arsenite-induced SGs do not (Kedersha et al., 1999) and SGs containing G3BP have been described as lacking TIA-1 (Tourriere et al., 2003). To address whether the recruitment of HDAC6 into SGs is restricted to a specific form of stress, HeLa cells were treated with different SG-inducing stimuli such as arsenite, UV irradiation, CCCP (mitochondrial stress), or heat shock, and were stained with antibodies against G3BP or HDAC6. As shown in Figure 4, G3BP localized to SGs under all conditions tested; remarkably, HDAC6 was also found in SGs irrespective of the induction stimulus. When the stress stimulus is removed and cells are returned to normal culture conditions, SGs disassemble rapidly (Anderson and Kedersha, 2002). We therefore performed experiments to examine the kinetics of SGs assembly and disassembly, using G3BP and HDAC6 as markers. We found that both proteins behaved identically also under these conditions (Fig. 4C). These results altogether indicate that HDAC6 and G3BP are recruited to the same SGs in response to stress, with identical kinetics, and that HDAC6 is an integral (stable) component of SGs. Under the stress conditions, such as oxidative stress, TIA-1 and PABP-1 rapidily and continuously shuttle in and out of SGs (Kedersha and Anderson, 2002). However, in lysed cells, the conformational chamges which drive SG assembly *in vivo* may be altered as a result of decreased levels of chaperones such as HSP70. Interestingly, it has reported quantification analysis, using immunopurified SGs, of translocation of FMRP between polyribosomes and SGs after arsenite stress (Kim et al., 2006). We investigated whether chemical stress would cause HDAC6 to shift between cytoplasm and SGs. After induced

A


Control
Arsenite
Arsenite
UV
CCCP
Heat shock
G3BP
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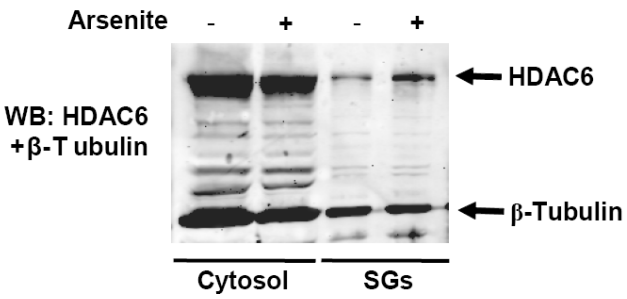
B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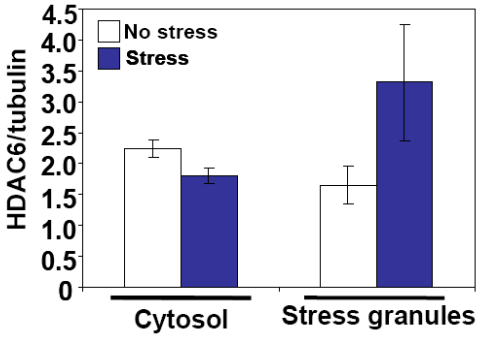
No stress
Stress
HDAC6/tubulin
4.5
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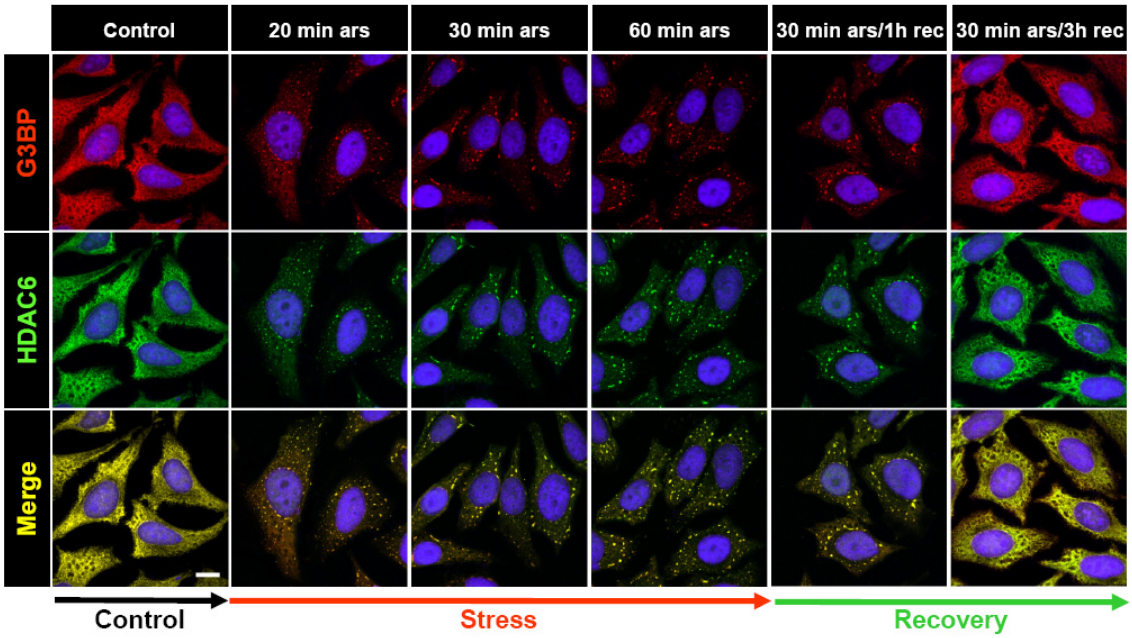


Figure 4. HDAC6 localizes to stress granules

(A) Exponentially growing HeLa cells were control-treated or stressed by exposure to 1 mM arsenite for 1 hr, 100 mJ UV irradiation, 1 µM CCCP for 90 min, or heat (44°C) for 1 hr. Subsequently cells were fixed and stained for G3BP, HDAC6 or TIA-1. Double immunofluorescence experiments were performed using anti-HDAC6 and anti-G3BP or anti-TIA-1/TIAR antibody and labelled secondary antibodies (Alexa Fluor 488, green) and (Alexa Fluor 594, red). Nuclei were counterstained using DAPI (blue). Localization of proteins was monitored by confocal microscopy. Yellow represents co-localization. (B) Proportional distribution of HDAC6 in stress granules. To quantify protein levels, nitrocellulose membranes were scanned by Odyssey infrared imaging system (LI-COR Bioscience) and relative optical densities were determined using Odyssey. (C) Assembly and disassembly of arsenite-induced SGs. HeLa cells were cultured in the absence (control) or presence of arsenite (1 mM) for 1 hr (Stress), washed and allowed to recover for from 1 hr to 3 hr (Recovery) before processing for two colour immunofluorescence microscopy. Enlargements of boxed regions are shown on the bottom. Scale bar = 10 µm

stress, HDAC6 levels in SGs and cytoplasm were measured on Western blots. Arsenite caused a rapid redistribution of HDAC6 into SGs and away from cytoplasm as shown in Figure 4B. HDAC6 distribution did not significantly change in control experiments without added arsenite. This result suggests that HDAC6 can move into subcellular compartments in response to stress.

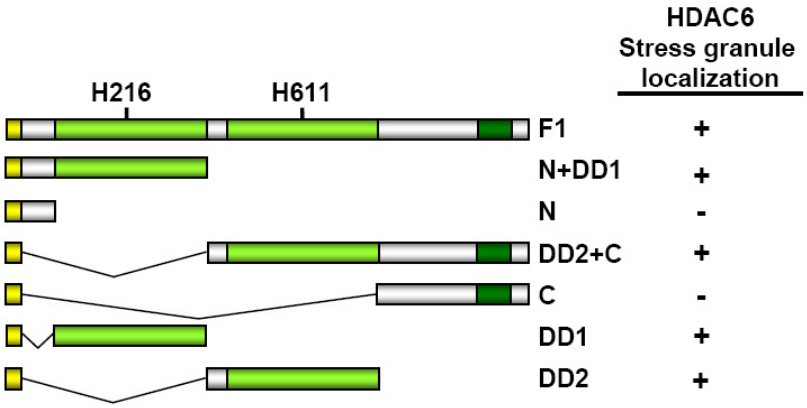
The deacetylase activity of HDAC6 can regulate SG formation

To identify the domain(s) of HDAC6 that direct the protein to SGs, expression vectors encoding tagged HDAC6 deletion mutants and G3BP were transiently cotransfected into HeLa cells and the subcellular localization of these proteins was determined under control or oxidative stress conditions. The results are summarized in Figure 5A and representative photomicrographs are shown in Figure 5B. All of the HDAC6 deletion mutants were localized in the cytoplasm, in agreement with our earlier results. Consistent with the immunoprecipitation results defining the interaction with G3BP, the full length HDAC6 and deletion mutants containing at least one HDAC domain were recruited to SGs; in contrast, mutants containing only the N- or the C-terminal portion of HDAC6 failed to localize to SGs. Thus, either of the two HDAC domains is necessary and sufficient to direct HDAC6 to SGs. To examine whether the deacetylase activity of HDAC6 is required for SG formation, we first treated HeLa cells with the HDAC inhibitor, TSA, which inhibits all known HDACs, or with butyrate, which inhibits HDACs with the exception of HDAC6 (Guardiola and Yao, 2002). As shown in Figure 5C, cells treated with TSA and arsenite exhibited a hyperacetylated microtubules network and formed fewer or no HDAC6 positive SGs. In contrast, treatment of the cells with butyrate did not alter SG formation, suggesting that the deacetylase activity of HDAC6 might be important for formation of these structures.

HDAC6 KO MEFs exhibit impaired ability to form stress granules

To examine the importance of HDAC6 in the regulation of SG assembly, we used wild-type or HDAC6 KO MEFs to test their capacity to assemble SGs following stress. Whereas wild-type MEFs readily assembled SGs in response to arsenite treatment, HDAC6 KO MEFs failed to exhibit morphologically discrete SGs, as determined by using antibodies against two independent markers of SGs, TIA-1 and G3BP (Fig. 6A).

A


HDAC6
Stress granule
localization
H216
H611
F1
+
N+DD1
+
N
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DD2+C
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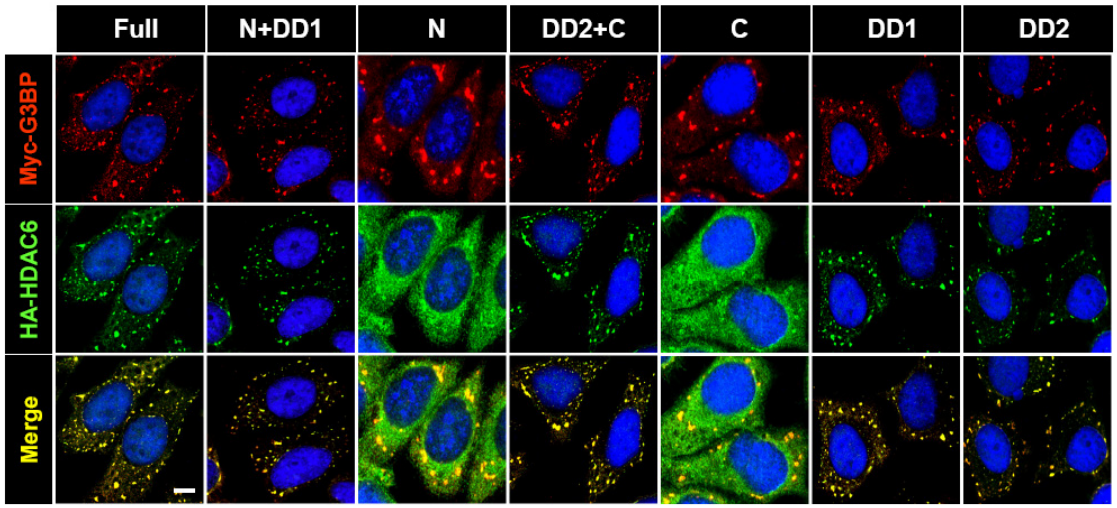
Full
N+DD1
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HA-HDAC6
Merge

C

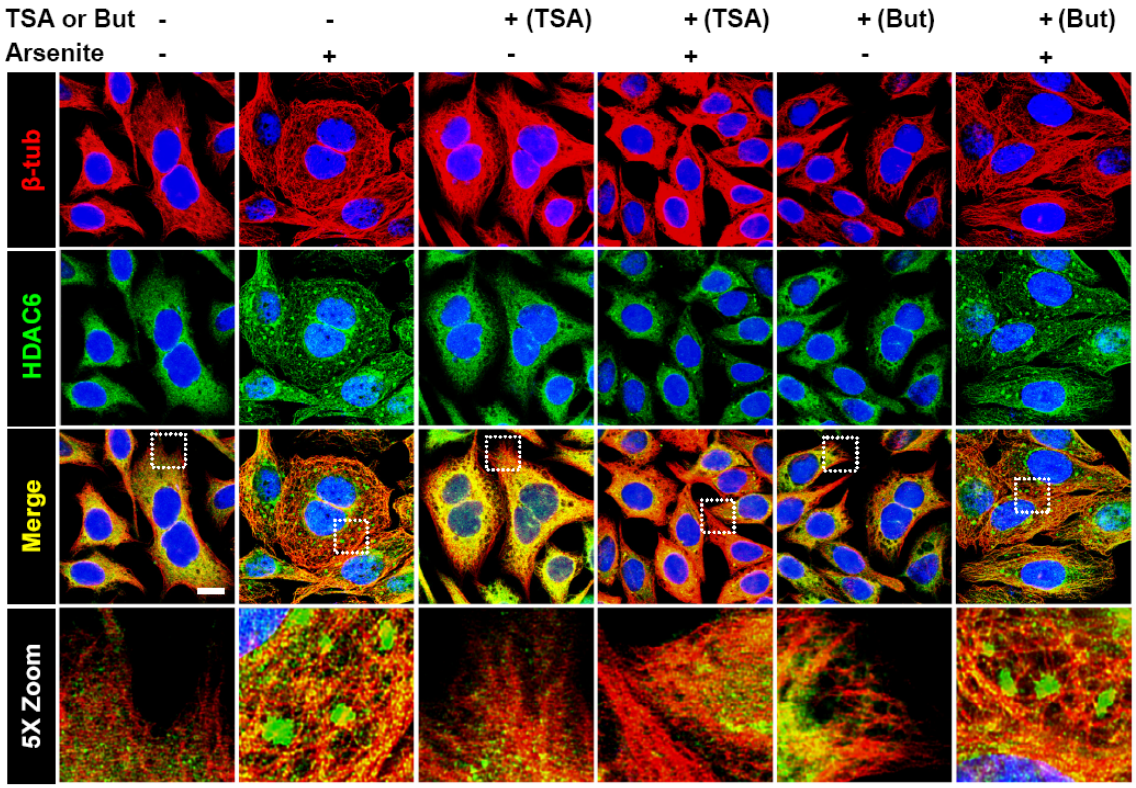


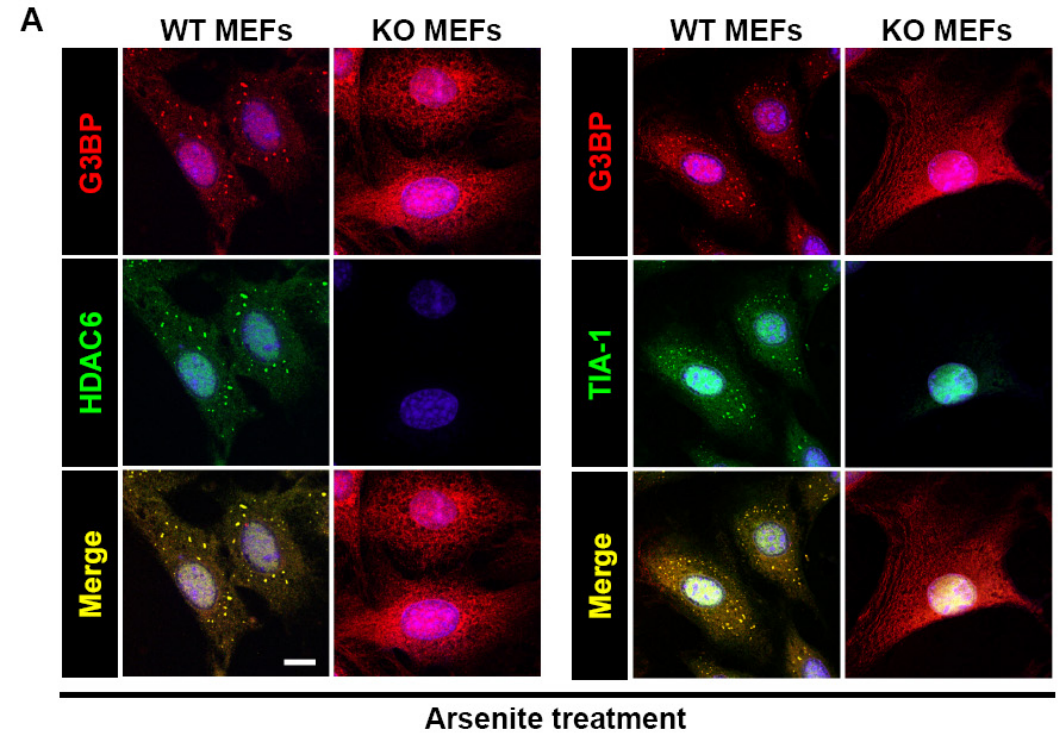
Figure 5. The deacetylase activity of HDAC6 is critical for assembly of stress granules

(A and B) Subcellular localization of HDAC6 deletion mutants and their stress granule localization. The N-terminally HA-tagged HDAC6 deletion mutants and Myc-tagged G3BP construct were transiently co-transfected into HeLa cells. 24 hr after transfection, the cells were treated 1 mM arsenite for 1 hr and and fixed. The subcellular localization of HDAC6 truncated proteins and G3BP was analyzed by a confocal microscopy. Stress granule localization of constructs was classified into two categories: positive localization (+) and negative localization (-).The staining for Myc-G3BP represents a control for the formation of SGs. Scale bar = 5 μm. (C) HDAC inhibition impairs formation of stress granules. HeLa cells were control-treated or treated with 500 nM TSA or 5 mM Butyrate (But) for 4 hr prior to treatment with arsenite for 1 hr and fixation. Double immunostaining experiments were carried out with anti-Ac-α-tubulin and anti-HDAC6 or anti-G3BP antibodies and analysis was done by confocal microscopy. Enlargements of boxed regions are shown on the bottom. Scale bar = 10 μm.

The same results were obtained using other stress forms such as heat shock, UV, or CCCP (Fig. 6E). We also quantified the percentage of cells forming SGs, as well as the kinetic of their assembly. As shown in Figure 6B, in wild-type MEFs up to ca. 80% of the cells were positive for SGs after 1 hr arsenite treatment, as in HeLa cells (not shown); in contrast, in HDAC6 deficient cells, no more than ca. 20-25% of the cells were positive. Furthermore, while wild-type cells exhibited SGs already after 5 to 10 min of arsenite treatment, and reached a plateau after 30 min, HDAC6 deficient cells showed a much delayed response (Fig. 6C). One of the earliest steps in the formation of SGs is the phosphorylation of eIF2α by various stress-activated kinases, such as PKR or PERK, which leads to inhibitionof translation initiation (Williams, 2001). To test whether loss of HDAC6 influences eIF2α phosphorylation in response to stress, wild-type or *HDAC6*$^{-/-}$ MEFs were treated with different concentrations of arsenite and protein extracts were analyzed by using a phospho-specific antibody. As presented in Fig. 6D, both wild type (lanes 2-4) and HDAC6 KO MEFs (lane 6-8) exhibited a similar increase in phospho-eIF2α upon arsenite treatment. In agreement with the above observations, we also found that global translation was not altered in absence of HDAC6 as polysomal profiles were identical in wild-type or *HDAC6*$^{-/-}$ MEFs (see Fig. 11). Thus, the effect of HDAC6 on SG formation is clearly downstream of eIF2a phosphorylation and protein translation arrest.

Intact HDAC6 function is required for SG formation

To better define the role of HDAC6 in controlling SG formation, we made use of *HDAC6*$^{-/-}$ MEFs in which wild-type or mutant forms of HDAC6 have been re-expressed by retroviral transduction. As seen in Figure 7A, these cells all express wild type levels of HDAC6 and behave as expected with respect to tubulin acetylation: the cells expressing WT HDAC6 have a tubulin acetylation level similar to WT MEFs (lane 3), while the cells expressing a deacetylase mutant form of HDAC6 have hyperacetylated tubulin (lane 4), just like *HDAC6*$^{-/-}$ MEFs. In addition, cells expressing a non-ubiquitin binding mutant HDAC6 also have a WT level of tubulin acetylation (lane 5). These different cell lines, as well as wild-type control MEFs, were used to examine SG formation following stress induction. As shown in Figure 7B, expression of HDAC6 in *HDAC6*$^{-/-}$ MEFs largely restored SG formation; in contrast expression of a deacetylase mutant HDAC6 failed to


A
WT MEFs
KO MEFs
WT MEFs
KO MEFs
G3BP
HDAC6
Merge
G3BP
TIA-1
Merge
Arsenite treatment
B
% of SGs containing cells
WT MEFs
KO MEFs
HDAC6
G3BP
TIA-1
C
% of SGs containing cells
WT MEFs
KO MEFs
Time (min)
D
W.T MEFs
K.O MEFs
Arsenite
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E

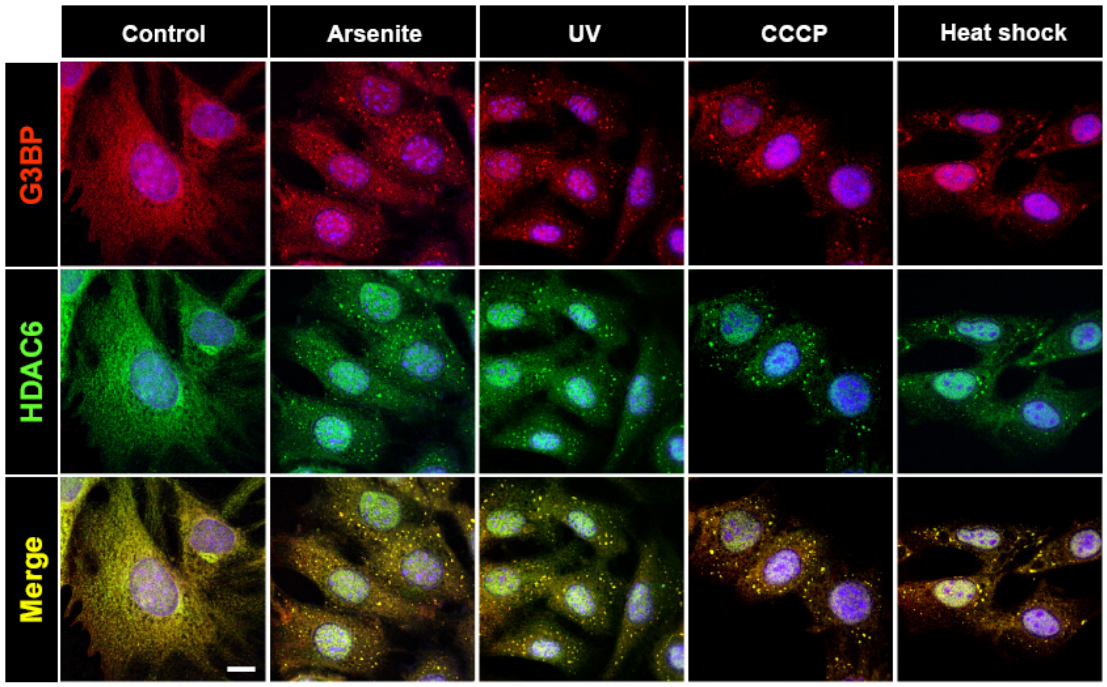


Figure 6. MEFs lacking HDAC6 exhibit impaired SG assembly

(A) Absence of SGs in MEFs lacking HDAC6. Wild-type or HDAC6-/- were exposed to 1 mM arsenite for 1 hr prior to fixation and immunostaining for G3BP (red) and HDAC6 or TIA-1 (green). (B-C) Quantification of percentage of cells contains stress granules in WT and KO MEFs under stress. The average number of SG-containing cells with the indicated antibodies is indicated. For the time-course experiment presented in (C) MEFs were treated with arsenite for the indicated times and SGs were determined on the basis of G3BP staining. Error bars represent the standard deviation (SD) calculated from 200 cells in 9 random fields. Student's t test was used for statistical analysis; * indicates $p < 0.01$ versus wild type control. (D) HDAC6 is required for SG assembly downstream of eIF2□ phosphorylation. Wild-type or KO MEFs were control-treated (lanes 1 and 5) or treated for 1 hr with 0.25 mM (lanes 2 and 6), 0.5 mM (lanes 3 and 7), or 1 mM arsenite (lanes 4 and 8) and extracts were prepared. Blots were probed for phospho-eIF2α, eIF2α, TIA-1/TIAR, HDAC6, G3BP Ac-α-tubulin, and α-tubulin (as a loading control). (E) Wild type MEFs were exposed to different stress inducing stimuli and performed immunofluorescence staining. Scale bar = 10 μm.

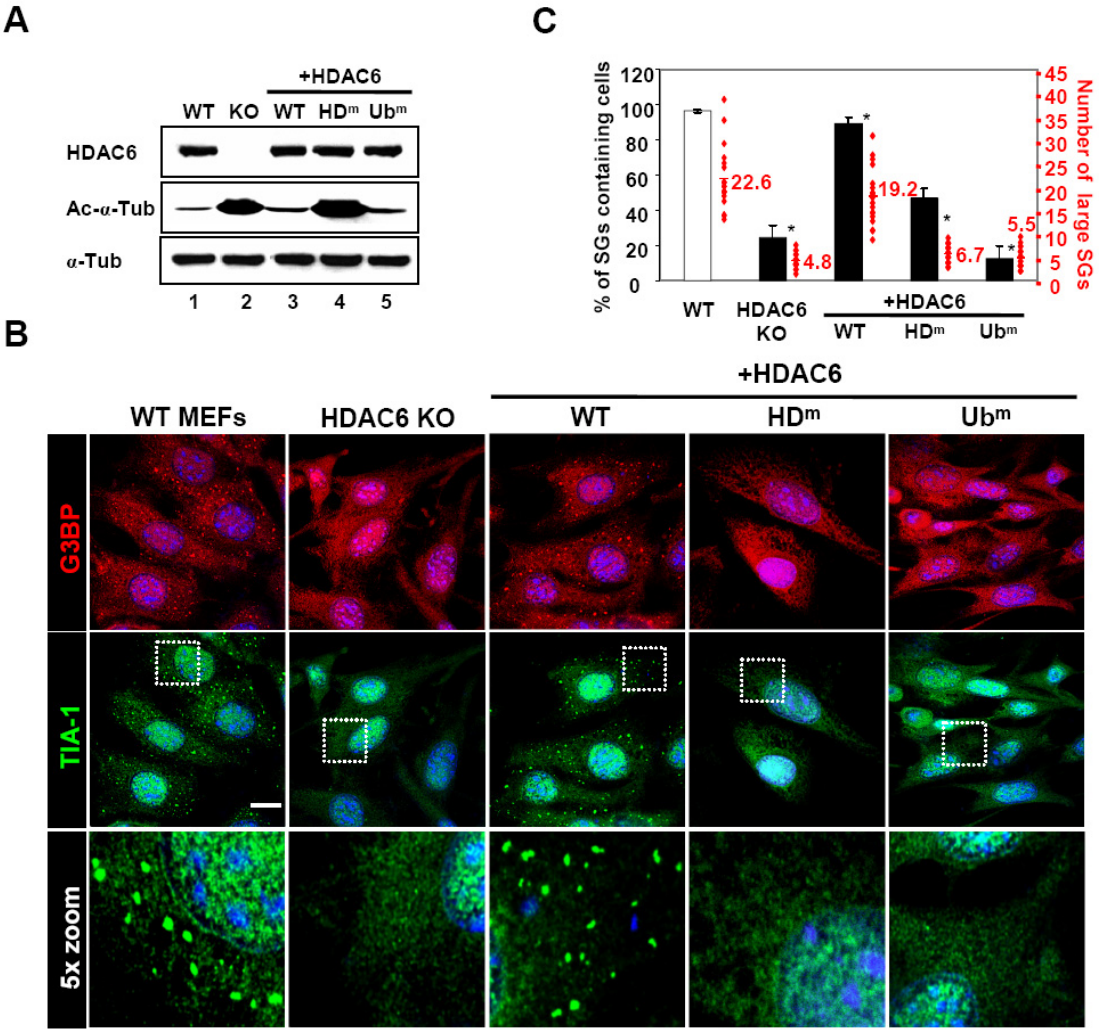


Figure 7. SG formation requires both the deacetylase and the ubiquitin-binding activity of HDAC6

(A) HDAC6-/- MEFs (lane 2) were used to establish cell lines expressing wild type HDAC6 (WT, lane 3), a catalytically dead (HDm , lane 4) or a non ubiquitin-binding mutant of HDAC6 (Ubm, lane 5). Extracts from these cells as well as from wild type MEFs (WT, lane 1) were used to monitor expression of HDAC6, Ac-α-tubulin and tubulin. (B) SGs formation requires intact HDAC6 function. The five cell lines described above were arsenite-treated for 1 hr and SG formation was assessed by immunostaining with an antibody against G3BP or TIA-1/TIAR, as indicated. (C) Quantification of the percentage of cells positive for SGs and number of large SGs per cell. Black labeling: percentage of cells containing SGs. Error bars represent the standard deviation (SD) calculated from 200 cells in 9 random fields. Student's t test was used for statistical analysis; * indicates $p < 0.01$ versus wild type control. Red labeling: number of large (size >1 μm) SGs per cell. Twenty cells are presented and the median in indicated. The occurrence of SGs was determined on the basis of G3BP (or TIA-1) staining. Enlargements of boxed regions are shown on the bottom. Scale bar = 15 μm

do so. Moreover, cells expressing the non-ubiquiting binding HDAC6 mutant appeared to completely lack SGs. To further solidify these results, we quantified in the different cell lines both the percentage of cells positive for SGs, as well as the number of large (>1.0 μm, see legend) SGs per cell. From the data presented in Figure 7C, it is clear that both the deacetylase activity as well as the ubiquitin binding activity of HDAC6 are required for promoting SG assembly. In fact, the cells expressing the non-ubiquitin binding mutant HDAC6 are more impaired than the *HDAC6*$^{-/-}$ MEFs, indicating that the mutant protein may act as a dominant negative. Because of this observation, we tested whether ubiquitin is present in SGs. As shown in Figure 21B, in non-treated HeLa cells, immunostaining for ubiquitin shows a diffuse pattern in the cytoplasm as well as in the nucleus. In contrast, arsenite treatment of the cells leads to the appearance of a punctate staining in the cytoplasm, that perfectly colocalizes with HDAC6 or other SG markers (data not shown). In addition, the same results have been obtained in MEFs; we therefore conclude that SGs contain ubiquitinated proteins and that staining for ubiquitin can be used to monitor the presence of these structures.

SG formation depends on the microtubules system and is mediated by motor protein function

Formation of SGs is very rapid and within minutes microscopically visible structures assemble in WT cells (see Fig. 6). We have shown here that HDAC6 is a critical factor for SG assembly, yet it is not clear what other mechanisms are involved. We have therefore re-investigated of the role of the cytoskeleton in SG formation by testing the effect of drugs that alter the microtubules or the actin network. As shown in Figure 8A upper panel, we found that the microtubule network destabilizing drug Nocodazole prevented the appearance of SGs following induction by arsenite; similar to what was seen in HDAC6 knockout cells, when cells were treated with Nocodazole no more than ca. 20% of cells were ever positive and showed very small SGs. When cells were treated with the vehicle control, DMSO, SGs appeared normally. In addition, when another microtubule-disruptin drug was used, colchicine, SGs also did not appear (data not shown). In contrast, disruption of the actin network by latrunculin B did not impair SG formation though it was associated with significant contraction of the cell body

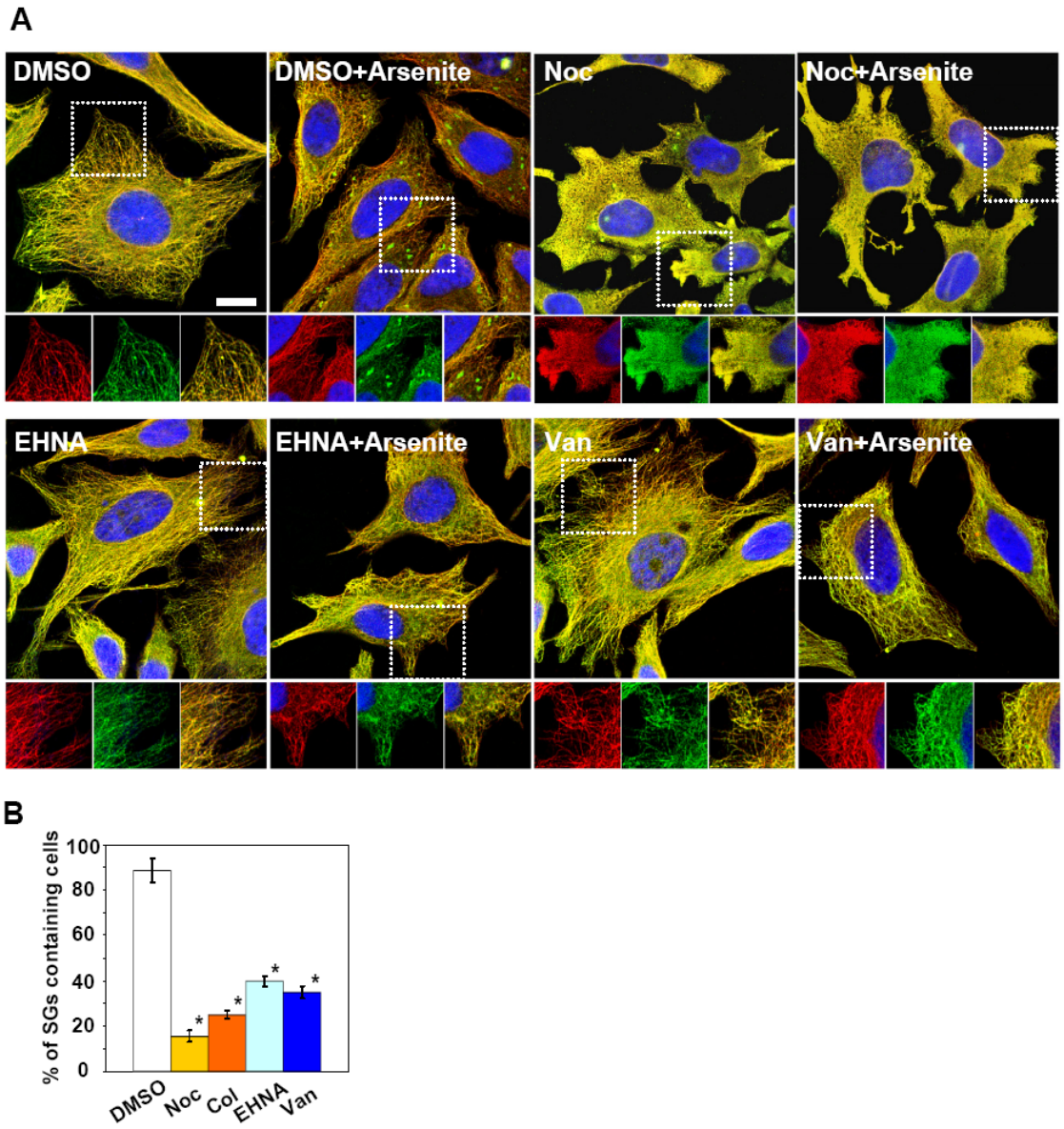


Figure 8. An intact microtubule network and dynein function are required for SG assembly

(A) Hela cells were treated with 0.1% DMSO (control) for 4 hr, 6.6 μM Nocodazole (Noc) for 2 hr, 1 mM EHNA for 1 hr or 0.5 mM vanadate (Van) for 4 hr, prior to treatment for 30 min with arsenite, as indicated. Cells were fixed and immunostained for β-tubulin and HDAC6. The inset presents a higher magnification showing SG on the microtubule network. Scale bar = 10 μm (B) Quantification of the percentage of HeLa cells containing SGs under conditions of microtubule disruption or inhibition of dynein ATPase activity. The occurrence of SGs was estimated based on HDAC6 immunostaining. Error bars represent standard deviation (SD) calculated from 200 cells in 9 random fields. Student's t test was used for statistical analysis; * indicates $p < 0.01$ versus wild type control. Noc; Nocodazole, Col; Colchicine, EHNA; erythro-9-[3-(2-Hydroxynonyl)]adenine, Van; Orthovanadate.

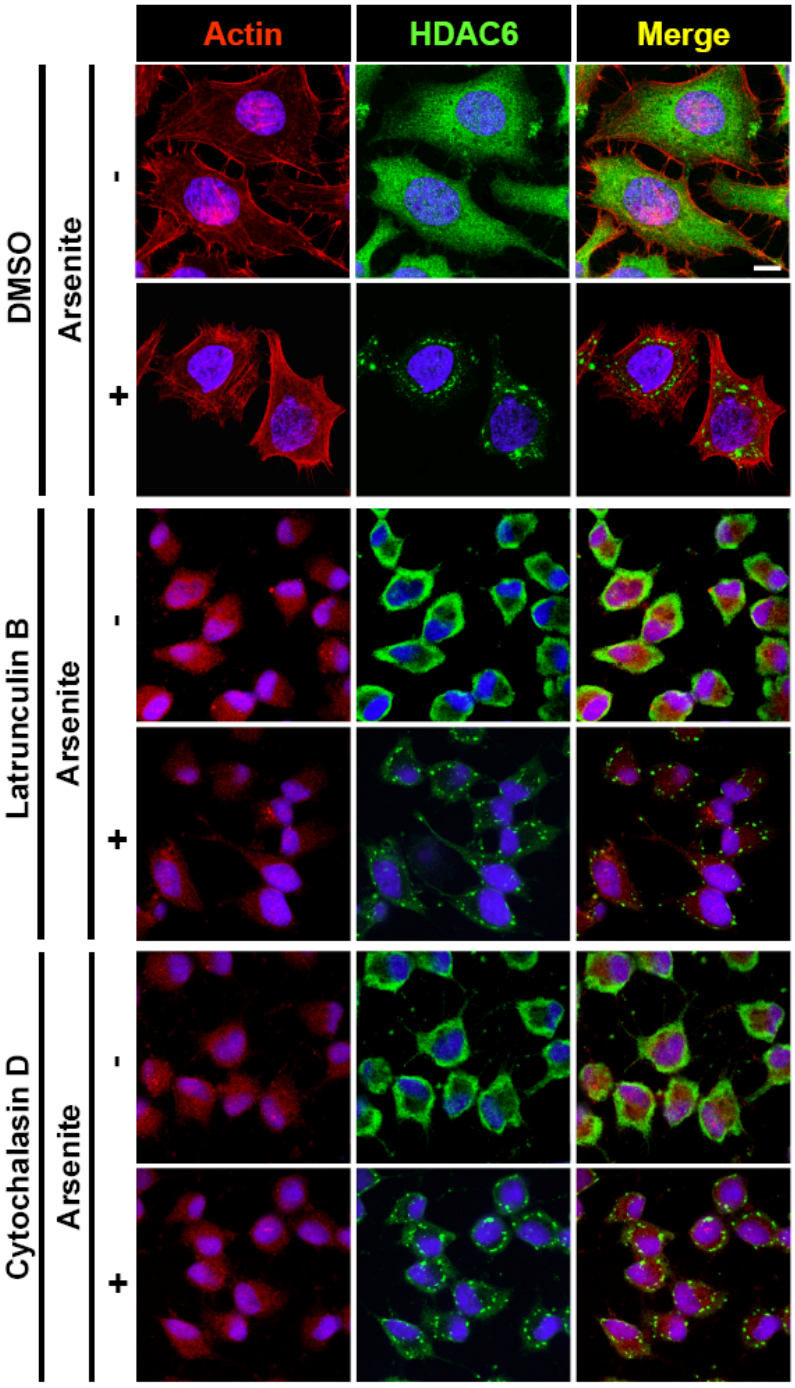


Figure 9. Effect of actin destroying drugs to stress granule formation

HeLa cells were treated with latrunculin B or Cytochalasin D for 30 min and then 0.5 mM of arsenite was added for 30 min (together with latrunculin B or Cytochalasin D). Double immunostaining was performed with anti-HDAC6 antibody and rhodamine-phalloidin. Nuclei are counterstained using DAPI (blue). Localization of proteins was monitored by a confocal microscopy. Scale bar =10 μm.

(Fig.9). Furthermore, stabilization of the microtubules network with taxol (Paclitaxel) also did not impair the formation of SGs. (data not shown). Thus, mictrotubules provide a necessary scaffold for the assembly of stress granules components.

HDAC6 interacts with MTs and dynein, and thereby facilitates the formation of the aggresome (Hubbert et al., 2002; Kawaguchi et al., 2003; Zhang et al., 2003). The capacity to associate with both HDAC6 and microtubule provides hits to understand how HDAC6 regulates SG formation. We thus inveestigated whether motor proteins could of dynein ATPase function, EHNA or vanadate, in conjunction with arsenite. In both cases, the microtubule network remained intact, and the localization of HDAC6 was not impaired; however, cells that were arsenite-treated in the presence of these inhibitors did not exhibit SGs (Fig. 8A, lower panel and 8B). Thus, dynein motor proteins are required, in conjunction with HDAC6, to assemble stress granules along the microtubules.

HDAC6 does not influence assembly of processing bodies

It has recently been shown that processing bodies (PBs), site of mRNA degradation and storage (Sheth and Parker, 2003; Teixeira et al., 2005; van Dijk et al., 2002), are dynamically linked to SGs (Kedersha et al., 2005) and found in close juxtaposition to SGs. We examined whether HDAC6 also localizes to PBs. As shown in Fig. 10A, PBs visualized with the antibody against decapping enzyme 1 (Dcp1a), a robust marker for PBs, are well visible in HeLa cells following stress induction with arsenite, and also in control-treated cells, in agreement with previous results (Kedersha et al., 2005; Teixeira et al., 2005). Furthermore, many PBs are indeed found next to SGs, that are evidenced here by HDAC6 staining, but the same results are obtained when SGs are detected by G3BP or TIA staining (data not shown). Remarkably, no colocalization of HDAC6 to the P bodies was observed. We next examined formation of PBs in wild type MEFs or in MEFs lacking HDAC6. As shown in Figure 10B, arsenite-treated wild type MEFs exhibit numerous PBs, many of which are adjacent to HDAC6- or G3BP-positive SGs. Strikingly, in HDAC6 knockout MEFs G3BP fails to form SGs, as shown above, but PBs are present in normal number and size. Thus, we conclude that HDAC6 is not required for the formation of PBs and that they can form independently of SGs.

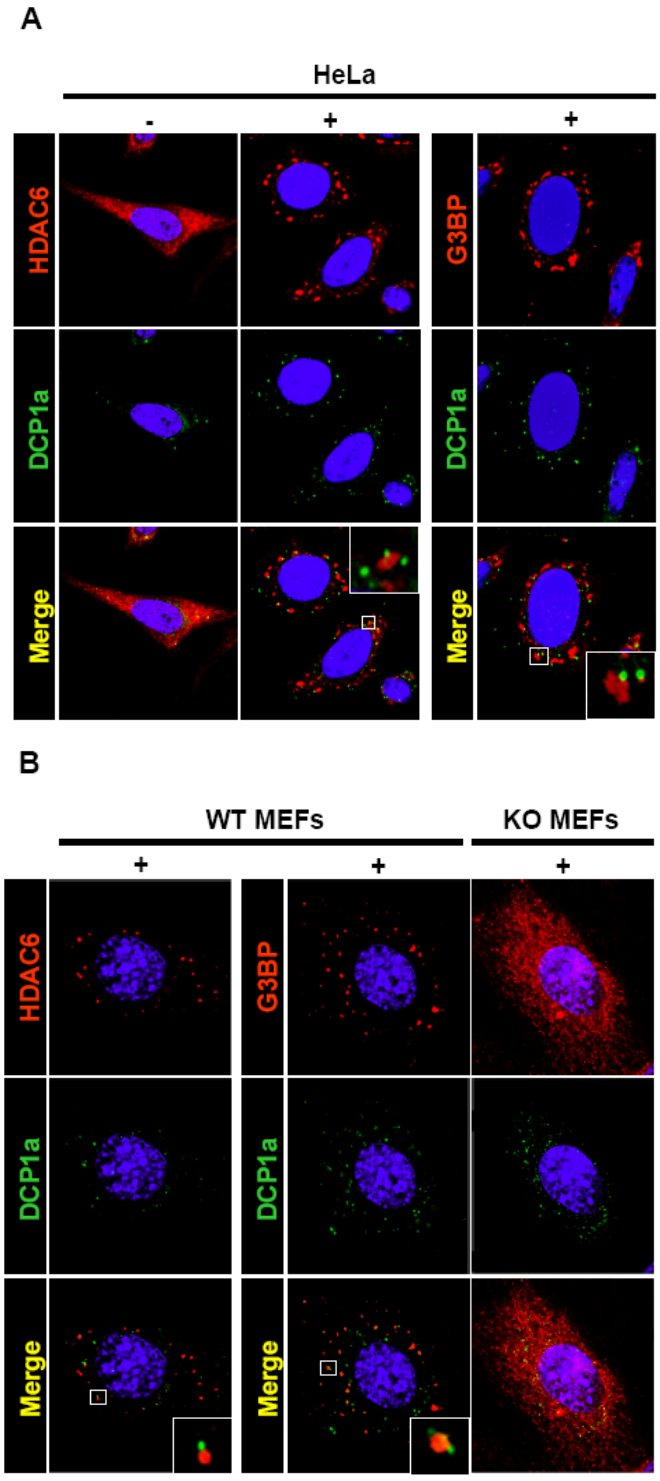


Figure 10. HDAC6 is not required for processing bodies formation

(A) HeLa cells and (B) MEFs (wild-type or HDAC6-/-) were control-treated or treated with 1 mM arsenite for 1 hr. Cells were then fixed, immunostained for HDAC6 or G3BP (red) and DCP1a (green) and analyzed by confocal microscopy. The squared inset presents a high magnification in the corner of the merged pictures. Scale bar = 5 μm.

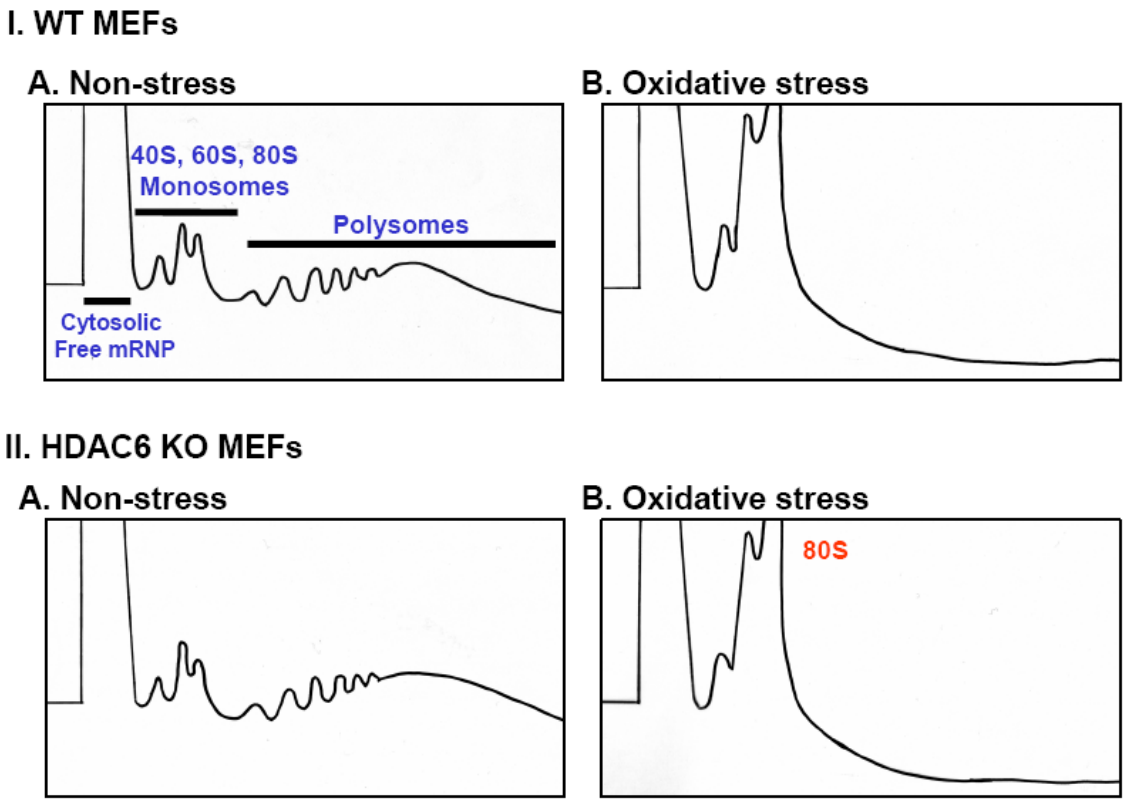


Figure 11. HDAC6 does not influence on global translation

(A) Polysome profiles of control and treated wild type or HDAC6 KO MEFs. MEFs were untreated (A and C) or treated with 1 mM of arsenite for 30 min (B and D), resolved on 10-50% sucrose gradient, eluted from the top, and the elution profile at OD260 was recorded. Monosomes are found in fraction 2-5, and polysomes are found in fractions 7-12. Fractions were collected.

Loss of HDAC6 results in translation derepression by let-7 miRNA

TIA/TIAR have "prion-like" domains that seem to promote aggregation of nontranslating mRNAs under stress conditions (Gilks et al., 2004). In addition, TIA-1/TIAR facilites AU-rich element (ARE)-mediated translational silencing of tumor necrosis factor-α (TNF-α) mRNA in immune cells (Piecyk et al., 2000). Indeed, two other ARE-binding proteins, HuR, and TTP have also been localized to SGs (Kedersha et al., 2002; Stoecklin et al., 2004), suggesting that ARE-mediated effects on translation and mRNA turnover may be initiated in SGs. Recently it has been reported that Argonaute proteins localize quantitatively to SGs in addition to PBs and that miRNA-mediated repression takes place in SGs. miRNAs are required for the Argonaute protein localization to SGs but not PBs (Leung et al., 2006). These observations prompted us to test the hypothesis that HDAC6 has a role in miRNA mediated repression.. To test this idea, we used a luciferase reporter assay. In this system, the luciferase gene is under the control of a CMV promoters and it contains in the 3' untranslated region (UTR) binding sites for the endogenous let-7 miRNA. Specifically, we used a Renilla luciferase reporters containing either a perfect let-7 site (Perfect), three copies of a let-7 site with a mismatched (3xBulge), or three copies of an imperfect let-7 site. This plasmids allow to score for translational repression (imperfect match) as well as for slicing of the target mRNA (perfect match). In addition a firefly luciferase reporter lacking any miRNA binding site is co-transfected as an internal reference. Normalized luciferase activity is represented as Renilla/Firefly ratio with the ratio measured in transfections with pRL-Con set to one. In agreement with the results of Pillai et al., expression of RL-Perfect and RL-3xBulge was inhibited ca 50-60% in WT cells. This reflects inhibition of translation or cleavage of the mRNA, mediated by the endogenous let-7 miRNA. The same result was obtained in HDAC6 KO cells reexpressing WT HDAC6 or a ubiqutin binding mutant of HDAC6 (Ub^m). In contrast, HDAC6 KO MEFs or MEFs expressing a deacetylase mutant form of HDAC6 both showed a strong derepression of the reporters carrying let-7 binding sites. These results indicate that HDAC6 impact on the miRNa pathway, either through its regulation of SG formation, or by another unidentified mechanism.

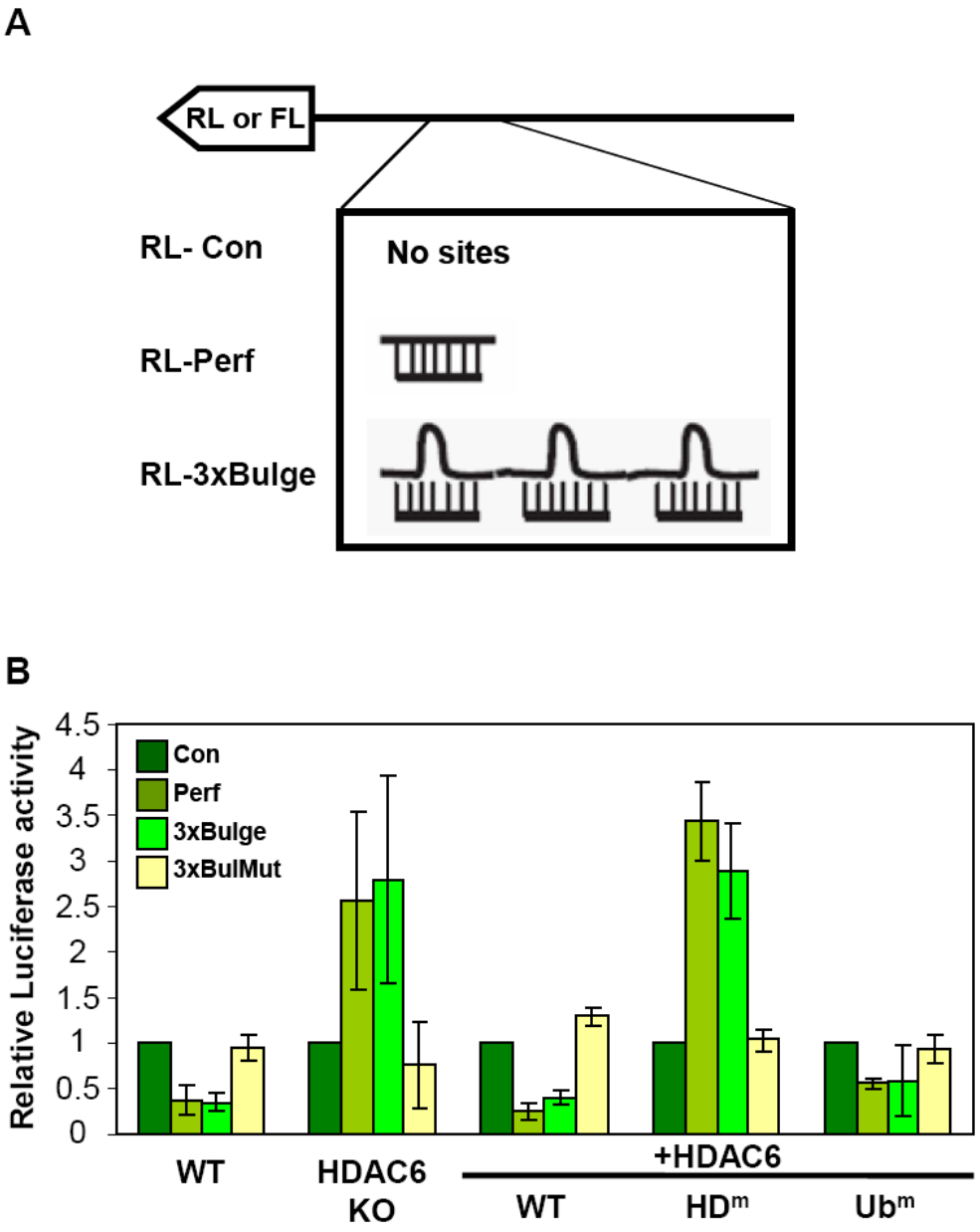


Figure 12. HDAC6 inhibits miRMA-mediated repression

(A) Schematic representation of RL reporter mRNAs containing let-7 sites in the 3'UTR used for experiments (adapted from Pillai et al, 2005). (B) MEFs were cotransfected with indicated RL reporters and pFL-Con luciferase vector as the control. After 48 hr, dual luciferase activity was measured and the relative luciferase activity was represented as the ratio of RL/FL with the ratio measured in transfections with pRL-Con set to one. The mean and standard deviation based on three independent transfection are shown. 3xBulgeMut: the mutated bulge binding sites have a 2-nt insertion in the seeding region of the bulged let-7 complementary site.

3.1.4. Discussion

The cellular response to stress entails a very rapid reversible suppression of mRNA translation accompanied by relocalization of mRNPs into discrete cytoplasmic stress granules, where they are remodelled and protected from degradation (reviewed in (Anderson and Kedersha, 2006)). Here we show that the deacetylase HDAC6 is an essential factor for SG assembly. Our results demonstrate that HDAC6 regulates SG formation by integrating different cellular processes, all of which we show to be important: protein acetylation, ubiquitination, integrity of the microtubule network and function of motor proteins.

G3BP is a novel interacting partner of HDAC6 *in vivo*

In this report, we have identified Ras GTP activating protein (GAP)-binding protein as a novel protein interacting specifically with HDAC6. We found that the hdac domains of HDAC6 are necessary and sufficient for interaction with a central domain of G3BP rich in acidic residues. It had been demonstrated that phosphorylation of G3BP at Ser149 plays a key role in regulating protein–protein interactions, subcellular localization, its endoribonuclease activity, and localization to stress granules (reviewed in Irvine et al., 2004). Also, the arsenite-induced phosphorylation of TTP, a SG-associated RNA-destablizing factor, promotes its rapid exit from SGs and concurrently inhibits its ability to promote ARE-mediated mRNA decay (Stoecklin et al., 2004). We found that phosphorylation of G3BP at Ser149 also regulates the interaction with HDAC6, as only non-phosphorylated G3BP can associate with HDAC6. Interestingly, HDAC6 has been shown to also bind to protein phosphatase 1 (PP1) and to be potentially recruited as a HDAC6-PP1 complex to microtubules (Brush et al., 2004). Thus, it is possible that under some conditions, an HDAC6-PP1 complex could also contribute to dephosphorylation of G3BP. Tubulin and HSP90 are established substrates of HDAC6, that can be deacetylated by this enzyme *in vitro* and *in vivo*. Whether HDAC6 could also deacetylate G3BP is not known yet. Analysis of the acetylation level of G3BP with acetyl-lysine specific antibodies revealed a very faint signal, which was however not influenced by the presence or absence of HDAC6 (data not shown).

HDAC6 is a novel integral component of stress granules, but not processing bodies

G3BP is one of a few proteins that are used as robust markers for the identification of stress granules. Under normal conditions, and in agreement with previous reports, we observed by immunostaining that both HDAC6 and G3BP are predominantly cytoplasmic proteins. However, we found that under stress conditions the two proteins co-localize and that HDAC6 is a novel component of SGs. The presence of HDAC6 in SGs was confirmed in several cell lines (data not shown) as well as under different stress conditions. Remarkably, we found that HDAC6 is essential for the assembly of SGs: both pharmacological and genetic inactivation of HDAC6 dramatically impair the assembly of SGs, as can be monitored by different markers. For example, TIA-1 is an established marker for SGs, which under normal conditions is mostly in the nucleus and moves into the cytoplasmic SGs following stress: however, in cells lacking HDAC6, TIA-1 fails to relocalize under stress conditions and remains largely nuclear. Intact deacetylase activity of HDAC6 is necessary for facilitating SGs assembly, indicating that acetylation of some protein(s) needs to be maintained at a low level in order for the granules to form efficiently. Whether HSP90, which is deacetylated by HDAC6, may be implicated in this process remains to be tested. In addition, we demonstrated the importance of the microtubules network and motor proteins for SG formation (see below); it is therefore possible that the level of MT acetylation, controlled by HDAC6, is also important. Further experiments will be required to address this issue.

Surprisingly, we found that also the ZnF-UBP domain of HDAC6, which binds ubiquitin, is critical. So far the role of ubiquitin or ubiquitinated proteins for SG formation is not known, but we discovered that SGs can be very efficiently detected by staining for ubiquitin (data not shown). Thus, stress granules also contain ubiquitinated proteins whose interaction with HDAC6 appears to be necessary for their formation. Furthermore, Roquin, a RING-type ubiquitin ligase required to repress follicular helper T cells and autoimmunity, also localizes to SGs (Vinuesa et al., 2005). It is possible that HDAC6 binds to specific ubiquitinated proteins and recruits to the SGs, in a manner analogous to the recruitment of ubiquitinated misfolded proteins into an aggresome.

Unlike stress granules, processing bodies are not affected by the absence of HDAC6 or by the chemical inhibition of HDAC activity (data not shown). Although the

two structures are often found next to each other, and may in fact be in direct contact - possibly exchanging mRNAs or RNPs-, PBs are present in normal numbers and shape in cells totally lacking HDAC6. This is also in line with the notion that PBs, where nonsense-mediated mRNA decay takes place, are constantly present in the cell, while SGs are only present (or visible) following stress. Furthermore, PBs are found in yeast, but SGs are not; in this context, it is interesting to note that there is no orthologue of HDAC6 in yeast. Absence of HDAC6 had no effect on global translation under control or stress conditions. In agreement with this finding, HDAC6 did not affect eIF2α phosphorylation which is the initial step of stress-induced translational arrest. These data demonstrate that HDAC6 controls SG formation downstream of eIF2α phosphorylation. A recent study has shown that inhibition of ribosome recruitment by eIF4A inhibitors (hippuristanol and pateamine) induces SG formation independently of eIF2α phosphorylation (Mazroui et al., 2006). We predict that also in this case HDAC6 is required for SG formation, but this remains to be tested.

HDAC6 links the microtubule system and SG formation

It is well known that cellular mRNPs can be transported along MTs by motor proteins. In *Xenopus* oocytes, the translocation of the *veg-1* RNA to the vegetal axis requires intact microtubules (Yisraeli et al., 1990), and in *Drosophila* oocytes, plus-end-directed motor protein kinesin I is required for the posterior localization of *oskar* mRNA and Staufen protein (Micklem et al., 2000). Likewise, Staufen may mediate the recruitment of the motor proteins required for SG aggregation in neurons (Thomas et al., 2005). In somatic cells, mRNA anchors to actin filaments in fibroblasts (Bassell et al., 1994; Taneja et al., 1992) and translocation of *myelin* mRNA is necessary for microtubules and kinesin in neurons (Carson et al., 1997). However, this active transport has been only poorly studied in fibroblasts. Also, it is not known whether SGs contain proteins associated with microtubules. Considering these previous reports, it is possibly that the participation of tubulin-dependent motors to gather the otherwise disperse RNPs quickly.

We observed that microtubules integrity, as well as action of dynein motor proteins, are required for SG assembly. These data suggest that minus-end-directed transport on microtubules is a mechanism used by cells to enhance the efficiency and

selectivity of SGs assembly. Interestingly, it has been previously reported that HDAC6 co-localizes with the $p150^{glued}$-containing motor complex and controls microtubule motor-based cargo transport; in the case of ubiquitinated misfolded proteins this promotes their accumulation in an aggresome.

The relationship that we observed between MTs and SG dynamics can be supported by additional observations. First, disruption of MT network causes to inhibition of SG formation in mammalian cells (Ivanov et al., 2003). Second, dDcp 1 of the oskar mRNP complex in Drosophila oocytes mislocates in mutants in which microtubule organization is abnormal (Lin et al., 2006). Third, the Agonaute 1 homolog from sea urchins, Seawi, has been identified as a microtubule-associated protein which localizes in cytoplasmic puncta (Rodriguez et al., 2005). It has very recently been demonstrated that Argonaute proteins and miRNAs quantitatively localize into SGs, suggesting that these structures may be relevant for pathways involving miRNA function. While a precise function of microtubules in PB formation in mammalian cells remains undetermined, these reports support our result that cytoplasmic mRNP granules, including SGs and possibly PBs, are influenced by the microtubule network in cells.

Furthermore, some translation factors (e.g., eIF3, eEF1α and eEF2) can bind to the microtubule network and actin in mammalian cells (Liu et al., 2002; Shanina et al., 2001; Shestakova et al., 2001). Together, our data suggest that HDAC6 may be at the center of an important node regulating cytoplasmic transactions and also point to an unexpected crosstalk between the microtubule network and the stress response.

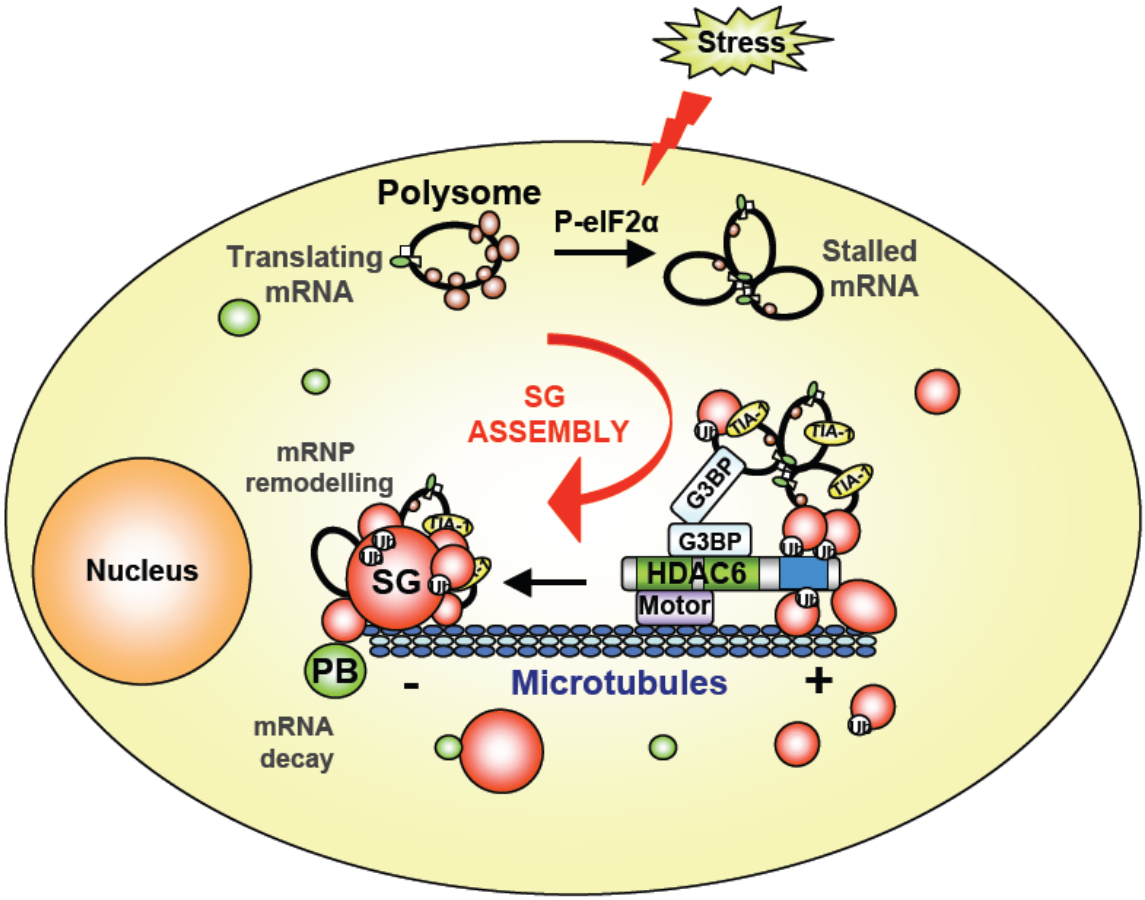


Figure 13. Model for HDAC6-dependent SG formation

Model summarizing the findings. Following cellular stress, eIF2α becomes phosphorylated and leads to stalled polysomes. HDAC6 then nucleates formation of SGs by interacting directly and indirectly with microtubules and motor proteins, G3BP and ubiquitinated proteins that are components of SGs. SGs and their precursors are depicted by red circles of various sizes, some of which are positive for ubiquitin (Ub). PBs are represented by green circles. The blue box in HDAC6 depicts the ZnF-UBP domain. See text for details.

3.2. HDAC6 functions as a stress regulator as well as a stress sensor

3.2.1. Abstract

The cellular stress response is a phylogenetically conserved protection mechanism from prokaryotes to humans and a phenomenon of adaptation of organisms. The cytoplasmic deacetylase HDAC6 has previously been showen to a key role in stress response to misfolded protein accumulation and also in translational arrest-induced stress granules. Here, we demonstrate that HDAC6 is also involved in hypoxic and oxidative stress response. Indeed, our data reveal that HDAC6 deacetylates HIF-1α, upregulates stability and subsequently activates its function in hypoxia. Intriguingly, both deacetylase and ubiquitin binding activities of HDAC6 contribute to stabilization of HIF-1α as independent functions: the deacetylase activity posttranslationally modifies HIF-1α, whereas the ubiquitin binding activity blocks polyubiquitination of HIF-1α and both activities increase stability of HIF-1α. Depletion of HDAC6 leads to hypersensitivity to cell death in oxidative stress and post-stress recovery. Therefore, HDAC6 can serve as a critical stress regulator in response to different stresses.

3.2.2. Introduction

Cells have evolved a variety of stress response pathways to cope with exogenous stresses, including the heat shock response (HSR), the unfolded protein response (UPR), the integrated stress response (ISR), the interferon response, and the ER overloaded response. This group of stress response pathways is activated in response to damage of intracellular protein systems. A large variety of exogenous stresses cause cells to transiently suppress protein synthesis as a means of coping with the stress. Reversible translation arrest is recognized as a general response of eukaryotic cells to exogenous stresses. Example of stresses that induce translation arrest are among others, hyperthermia (heat shock), heavy metal poisoning (e.g., arsenite), ER stresses (e.g., depleting ER Ca^{2+} stores, inhibiting ER-mediated post-translation modification), viral infection, nutrient (amino acid, glucose) deprivation, excessive free radial production, and ethanol intoxication (Degracia and Hu, 2006).

Generally stress responses include two major parallel pathways of activity. First, there is a transient suppression of protein synthesis. Second, there is activation of transcriptional inducers that will upregulate transcription of a subset of mRNAs (e.g., stress proteins, HSP70, ATF4, GADD34…). Transient translational arrest has a dual role. First, it prevents further damage to cellular proteins by shutting off accumulation of newly synthesized proteins that could potentially be damaged by the stress. Second, shut off of the translation of constitutive (of housekeeping) proteins allows the cell to only translate the mRNAs encoding the stress proteins. Selective synthesis of stress proteins therefore provides a mechanism for cells to buffer and repair stress-induced damage. The balance between the intensity of stress-induced damage and the activity of translated stress proteins set a decision point, determining whether the consequence is cell survival or cell death.

Hypoxia-inducible factor 1 (HIF-1) plays a key role in the cellular adaptive response to the lack of oxygen supply. HIF-1 is transcriptional regulator of angiogenesis, erythropoiesis, energy metabolism, and cell survival in mammals (Semenza, 2003). HIF-1 is a heterodimer that consists of a constitutively expressed HIF-1β subunit and a HIF-1α subunit, the expression of which is highly regulated. The activity of HIF-1 is

predominantly regulated via stability of its α-subunit. HIF-1α is constitutively expressed and rapidly degraded by the ubiquitin-proteasome system at the same time under normoxia, thereby keeping the steady state level at an undetectable level. In contrast, HIF-1β is continually present. Under hypoxia conditions, the oxygen-dependent prolyl hydroxylase domains (PHD) of HIF-1α are inactive and not hydroxylated; this impairs its interaction with the E3 ubiquitin ligase VHL and consequently prevents its degradation. Stable HIF-1α translocates into nucleus, dimerizes with HIF-1β, and activates the transcription of target genes containing hypoxia response element (HRE).

It has been reported that HDACis inhibit HIF expression and activity in tumor cells through yet unidentified pathways (Kim et al., 2001; Kong et al., 2006; Zgouras et al., 2003). HDACis has anti-cancer and anti-angiogenic features thus they are in clinical trials for caner therapy (Johnstone and Licht, 2003). TSA, FK228, butyrate, and LAQ824 were found to repress angiogenesis and expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) which is a target of HIF-1α. TSA and FK228 have been found to induce HIF-1α degradation in most tumor cell lines tested, including Caki, Hep3B, DU145, PC3, U87, BT20, MCF7 and VHL-/- cells such as RCC4 and C2. Also TSA repressed HIF-1α levels in HCT116 cells (p53+/+) and an isogenic p53-/- HCCT116-derived cells. These date suggest that HDACi-mediated destabilization of HIF-1α is independent of VHL and p53 function (Bunz et al., 1998; Kong et al., 2006). Moreover, inhibition of HSP90 function leads to VHL-independent destabilization of HIF-1α (Isaacs et al., 2002). HDACi-induced hyperacetylation of HSP90 repressess its chaperone function and allows its client proteins to be degraded by a ubiquitination-independent proteasomal system. In agreement with this, HDACis affects the HSP70/HSP90 chaperone complex and its activity thereby controlling stability of HIF-1α. Also two groups identified HDAC6 as a regulator of HSP90. The acetyltransferase for HSP90 has not yet been identified (Bali et al., 2005; Kovacs et al., 2005).

Intriguingly, recent studies showed that class II HDAC6 and HDAC4 are associated with HIF-1α and inhibition of both HDACs reduces HIF-1α protein level in cancer cell lines (Kong et al., 2006; Qian et al., 2006). One paper showed that HDAC6 and HDAC4 interacts with HIF-1α directly and control acetylation level and degradation

of HIF-1α in a VHL-independent manner (VPA and LAQ824) (Qian et al., 2006). The other paper reported that HDAC6 controls HSP90 chaperone function and indirectly regulates HIF-1α stability in a VHL- and p53-independent manner (TSA and SAHA). Inhibition by TSA was equally effective with hypoxia as well as with the hypoxic mimics Cobalt chloride ($CoCl_2$) (Kong et al., 2006). Therefore, These data suggest that HDAC6 might manage HIF-1 expression and function.

In the current study, we investigated the posttranslational modifications, protein stability, and transcriptional activity of HIF-1α in WT, and HDAC6 knockout MEFs in hypoxia and reoxygenation. Also, we examined how HDAC6 manages cellular stress response during different stresses and after releasing stress.

3.2.3. Results

HDAC6 deficient MEFs are defective in GR translocation and GR-mediated transcription activation

Kovacs et al. showed by siRNA knockdown of HDAC6 that this deacetylation is important for the activity of the chaperone HSP90 (Kovacs et al. 2005). We therefore tested whether this could also be observed in cells that constitutively and completely lack HDAC6. For this, we used five MEF lines described in Figure 6. First, we have showed that HSP90 is hyperacetylated in MEFs lacking HDAC6 (Zhang et al., 2007). A GR-dependent luciferase reporter was transiently transfected into the different cell lines, together with a reference plasmid. As shown in Figure 14A, upon hormone treatment wild type cells showed a ten fold activation of the reporter, while cells lacking HDAC6 only exhibited ca. 3.5 fold activation; furthermore, cell reexpressing wild type HDAC6 showed nearly the same activation as wild type cells. Remarkably, cells expressing a deacetylase defective or a non-ubiquiting binding HDAC6 were at least as much impaired in activation as the knockout cells. These results do not reflect altered GR levels in the different cell lines and the western blot presented in Figure 14B demonstrates that GR protein expression in not modulated by HDAC6. We therefore examined hormone-induced nuclear translocation of the GR; as shown in Figure 14C, hormone addition leads to nuclear translocation of the GR in wild type, but not in HDAC6 deficient cells. Together, these data indicate that HSP90 deacetylation by HDAC6 is necessary for the activation of the GR and possibly other proteins.

HDAC6 controls protein stability of HIF-1α

It has been reported that HIF-1α is acetylated by ARD1 in mammalian cells. ARD1-mediated acetylation enhances the interaction of HIF-1 with pVHL and increases the degradation of HIF-1α (Jeong et al., 2002). In contrast, two other groups found that ARD1 could not acetylate Lys532 in HIF-1α *in vitro* (Arnesen et al., 2005; Murray-Rust et al., 2006). Moreover, the ARD1 expression status was shown not to affect the expression of HIF-1α or HIF-regulated genes (Bilton et al., 2005; Fisher et al., 2005). To

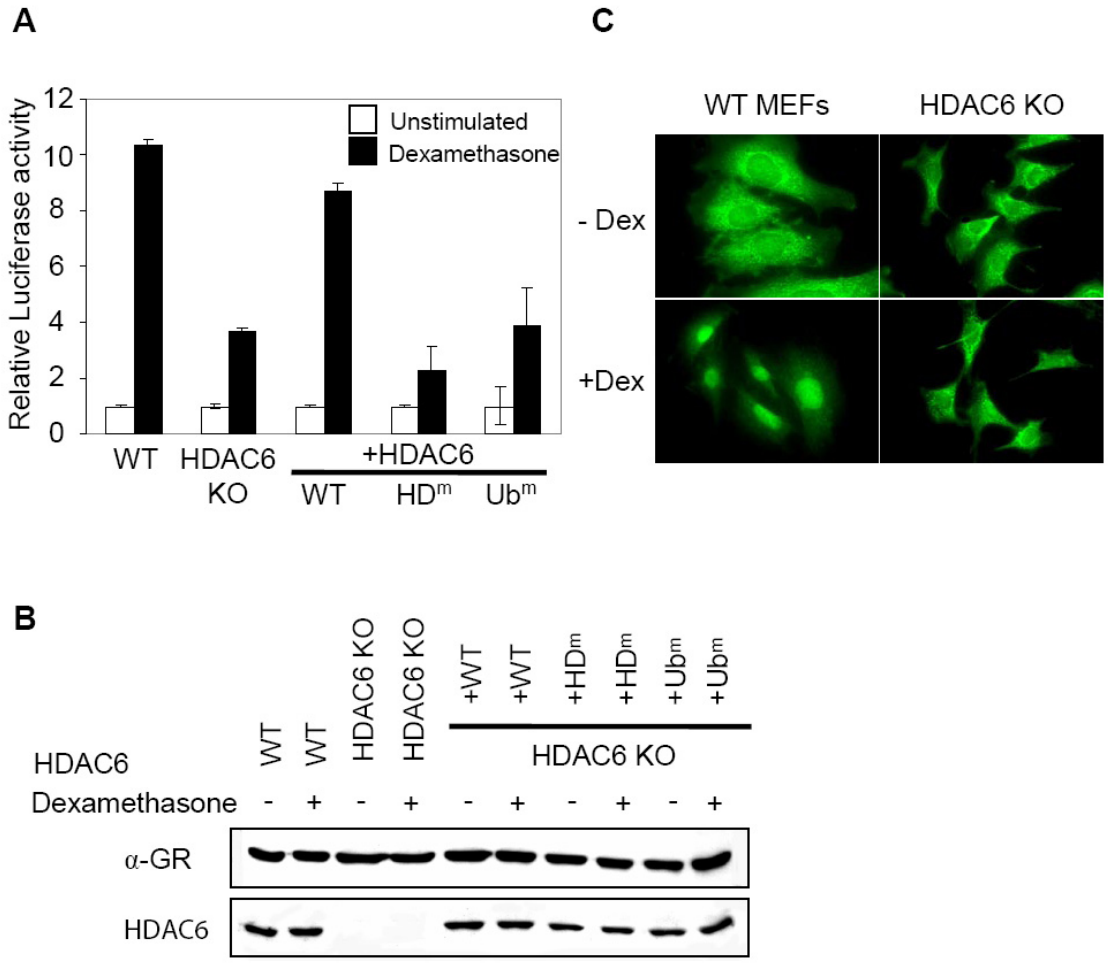


Figure 14. HDAC-6 is required for GR translocation, and transcriptional activity

(A) Wild type, HDAC-6 deficient MEFs or mutated HDAC-6 derivatives were transiently cotransfected with an MTV-GRE-luciferase reporter and Renilla vector as the control. Dual luciferase activity was measured after 4 hr treatment with dexamethasone and the ratio of firefly to Renilla was used as the relative luciferase activity. $p < 0.005$. Data are the mean of experiments repeated in triplicate. (B) The protein levels of GR and HDAC-6 are determined by immunoblotting with anti-GR and anti-HDAC6 antibodies, respectively. (C) Wild type and HDAC-6 deficient MEFs were cultured in hormone free media for 24 hr and then stimulated with dexamethasone for 30 min. The localization of GR was determined by immunostaining with an α-GR antibody.

examine whether HDAC6 influences the acetylation status and stability of HIF-1α, we studied the protein expression level of HIF-1α in WT MEFs and HDAC6 KO MEFs in hypoxia and hypoxic mimic, cobalt chloride ($CoCl_2$). As shown in Figure 15 and 16, the protein levels of HIF-1α were decreased in HDAC6 KO cells compared to control WT cells. Upon $CoCl_2$ treatment, HDAC6 deficient MEFs degraded more rapidly HIF-1α than WT MEFs (Fig. 16A). To determine which functional domain of HDAC6 affects HIF-1α stability, we examined its stability in different MEF cell lines which lack HDAC6 or express mutant forms of this enzyme. For this, cells were kept under hypoxia or hypoxia-mimic condition and protein levels were determined by western blotting. WT cells and rescuant with WT HDAC6 had in higher hypoxic levels of HIF-1α than the KO cells or the cells expressing mutant HDAC6. Conversely, HDAC6 KO cells and catalytically dead mutant of HDAC6 showed inhibition of HIF-1α protein in response to hypoxia (Fig. 15A and 15B). These results demonstrate that HDAC6 is involved in the stability of HIF-1α by affecting its acetylation status in hypoxia.

Recently it has been reported that HDAC6 and HDAC4 interact with HIF-1α directly and control the acetylation level and degradation of HIF-1α in a VHL-independent manner in cancer cells (Qian et al., 2006). To investigate whether HIF-1α is acetylated and deacetylated by HDAC6, we immunoprecipitated HIF-1α and blotted with an anti-acetylated Lysine antibody. HIF-1α acetylation was readily detected after its immunoprecipitation (shown in Figure 15). As expected, HIF-1α was found hyperacetylated in HDAC6 KO cells as well as in cells expressing the catalytically dead HDAC6 (HD^m) (Fig. 15A and B, lane 2 and 4), even though hypoxic expression and immunoprecipitation level of HIF-1α were much lower. Interestingly, hypoxic expression level of HIF-1α was dramatically lower in non-ubiquitin binding HDAC6 mutant cells as well as HDAC6 KO cells and HD^m compared with WT cells. Taken together, these data suggest that HDAC6 deacetylates HIF-1α and controls its stability through its acetylation and ubiqutination.

Inactivation of HDAC6 gene inhibits transcriptional activation mediated by HIF-1

To further show the functional consequence of HDAC6 loss on HIF-1, we transiently

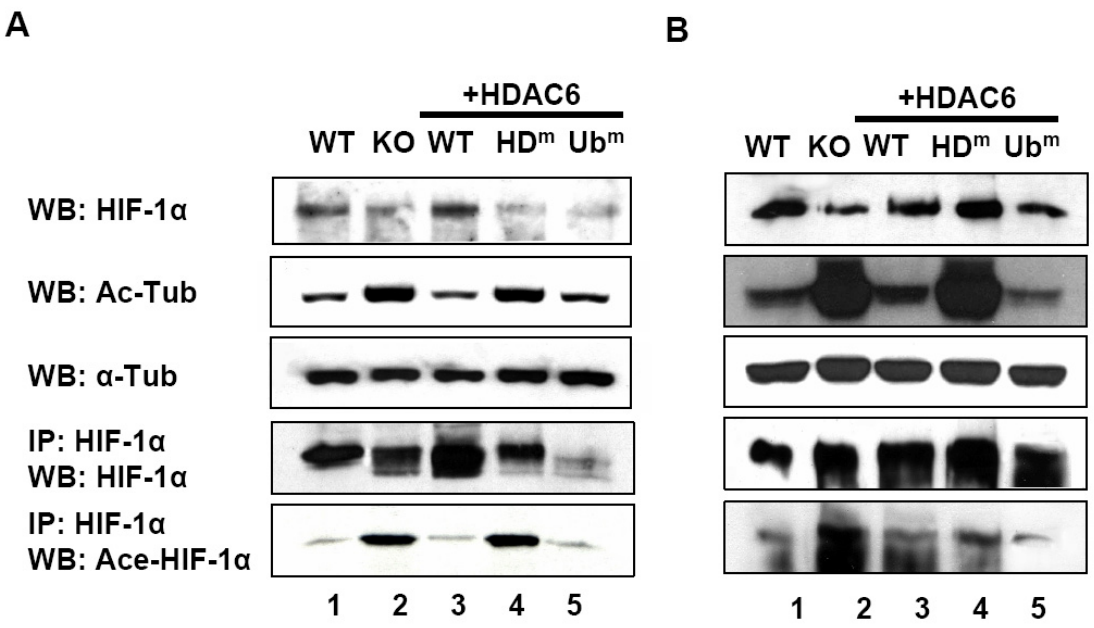


Figure 15. HDAC6 deacetylates and regulates degradation of HIF-1α in hypoxia

MEFs were incubated for 24 hr at 1% O2 (A) or for 6 hr with 500 μM of $CoCl_2$ to mimic hypoxia (B). Whole cell lysates were immunoprecipitated with anti-HIF-1α antibody. Immunocomplexes were either probed for anti-HIF-1α or anti-acetylated lysine antibody. 10 % of whole cell lysates was used as input control.

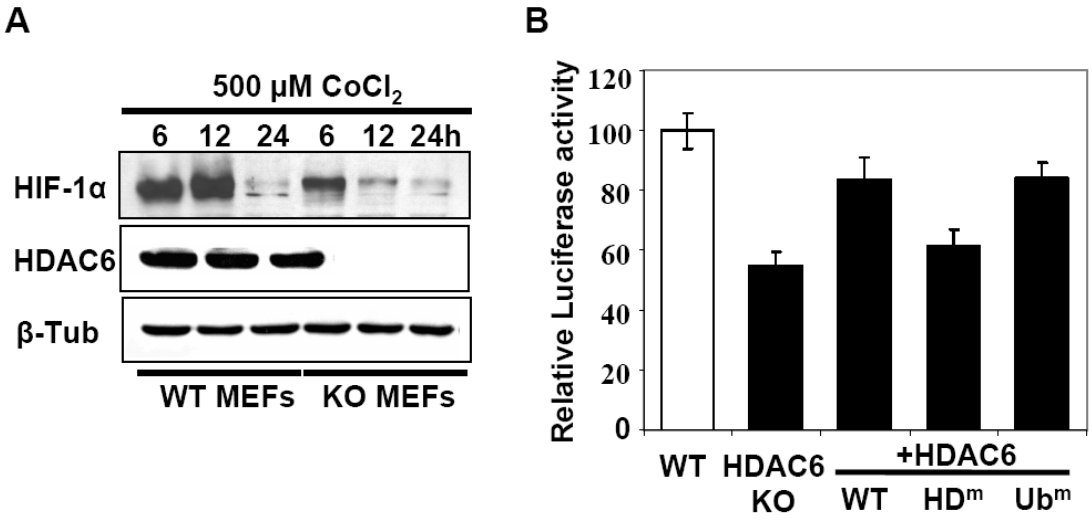


Figure 16. HDAC6 regulates stability and transcriptional activity of HIF-1α

(A) MEFs were incubated for 6, 12, or 24 hr with 500 μM of $CoCl_2$ to mimic hypoxia. HIF-1α protein levels were examined by Western blot analysis. The same blot was probed with HDAC6 as confirming HDAC6 KO cell lines and with β-tubulin as equal loading control. (B) MEFs were cotransfected with HRE-firefly luciferase vector and renilla vector as the control. Transfected cells were incubated for 24 hr at 21% O_2 and then incubated for an additional 6 hr with 500 μM of $CoCl_2$ or at 1% O_2. Dual luciferase activity was measured and the ratio of firefly to renilla was used as the relative luciferase activity. The mean and standard deviation based on three independent transfection are shown. Student's t test was used for statistical analysis. Indicates *, $p < 0.001$ versus wild type control.

transfected with a hypoxic response element (HRE)-driven luciferase reporter gene whose expression is based on the availability of HIF-1α. As a transfection control, a renilla luciferase vector was cotransfected. 24 hours after transfection, $CoCl_2$ was added to the culture medium for the last 6 hours of transfection and extracts were assayed for luciferase activity. Interestingly, activity of the HRE reporters was reduced ca. 2 fold in cells lacking HDAC6 or in cells expressing the HD^m HDAC6 protein (Fig. 16B). Increased acetylation level of HIF-1α protein caused to reduction of transcriptional activity as well as induction of degradation of HIF-1α in HDAC6 KO cells and cells expressing the catalytically dead mutant of HDAC6. Thus, these results suggest that HIF-1α acetylation also compromises its transcriptional activity.

The interaction between HIF-1α and HSP70 is enhanced by inhibition of HIF-1α deacetylation

HIF-1α protein stability can also be affected by the heat shock proteins HSP90 and HSP-70, because HDAC inhibitors have been reported to induce HSP90 acetylation and cause the disassociation of client proteins (Kovacs et al., 2005). Previously it has been observed that oxygen dependent degradation domain (ODD) of HIF-1α interacts with HSP70 (Zhou et al., 2004) and HIF-1α needs the HSP70/90 chaperone complex to complete its maturation (Kong et al., 2006). These observations prompted us to study whether HDAC6 also affects the association of HIF-1α with HSP90 and HSP70 in hypoxia and hypoxic mimics. Immunoprecipitation studies showed that the interaction between HIF-1α and HSP70 is enhanced in HDAC6 KO and catalytically dead mutant of HDAC6 under hypoxia, even though expression level of HIF-1α was much lower in these cells. It has been reported that HDAC6 is involved in proteasomal degradation of several proteins by inducing hyperacetylation and inhibition of HSP90 chaperone. As HIF-1α is a client protein of HSP90, we investigated the effect of HDAC6 on the regulation of HIF-1α. Depletion of HDAC6 did not alter the HIF-1α/HSP90 association as indicated by coimmunoprecipitation experiments (Fig. 17). These results implicate that HDAC6 might directly affects the stability of HIF-1α or indirectly affects stability of HIF-1α by controlling the chaperone activity of HSP90.

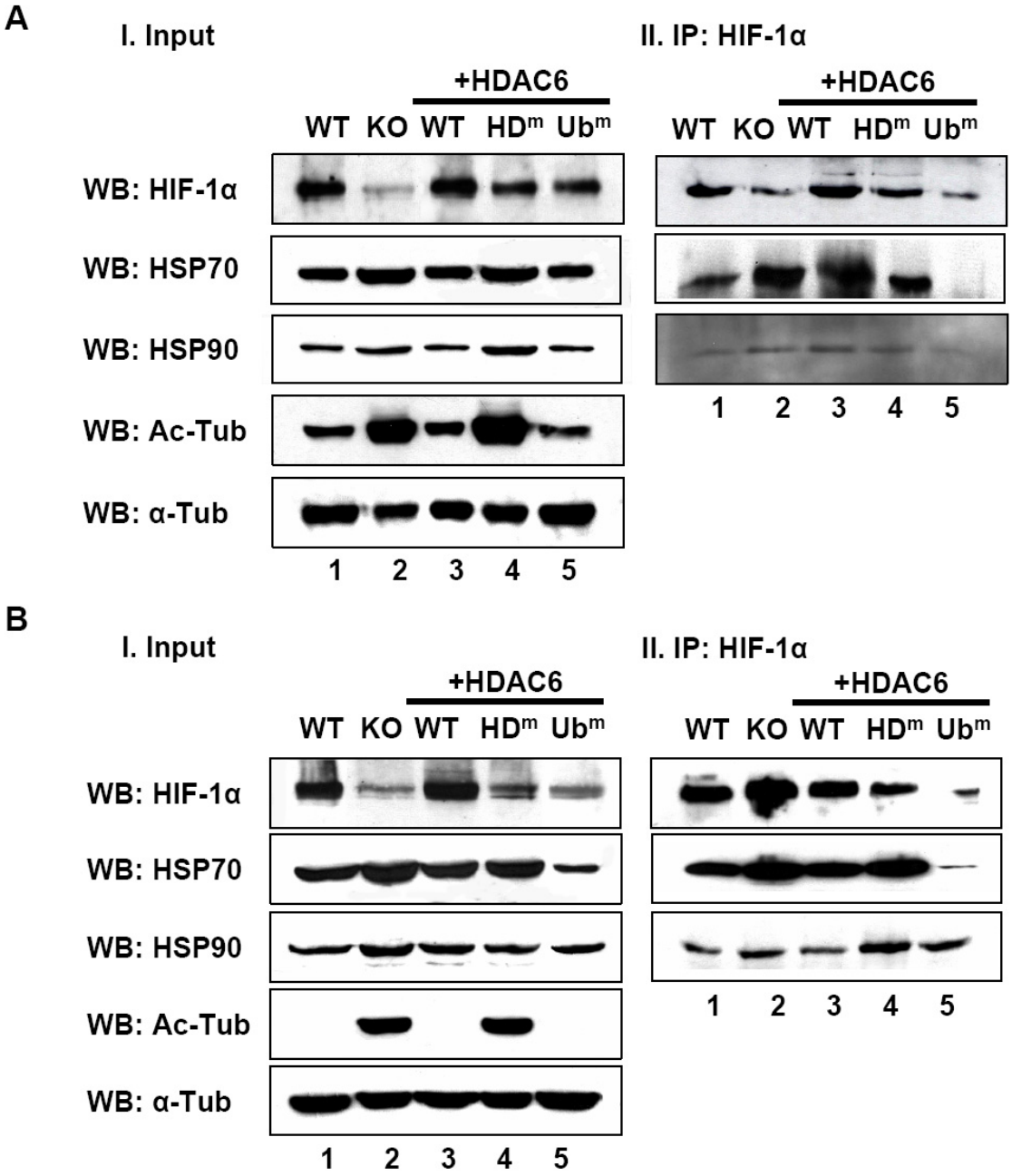


Figure 17. Deacetylation of HIF-1α by HDAC6 weakens the interaction between HIF-1α and HSP70

MEFs were incubated for 24 hr at 1% O_2 (A) or for 6 hr with 500 μM of $CoCl_2$ to mimic hypoxia (B). Whole cell lysates were immunoprecipitated with anti-HIF-1α antibody. The precipitate was immunoblotted with anti-HIF-1α, anti-HSP70 or anti-HSP90.

Loss of HDAC6 causes increased apoptosis in response to hypoxia

HDAC6 senses misfolded ubiquitinated proteins and facilitates the transport of these toxic proteins to aggresome (Kawaguchi et al., 2003). In addition, we have shown here that HDAC6 regulates SG formation in various stresses. Therefore, HDAC6 can be considered as a stress sensor responding specifically to different stresses. It has been reported that SGs form in tumor cells after hypoxia and that the granules disappear upon reoxygenation (Moeller et al., 2004). We investigated how HDAC6 functions in response to hypoxia and reoxygenation. Under hypoxic condition, HDAC6 KO MEFs showed hypersensitivity to apoptosis. HD^m cells showed no significant effects on apoptosis as HDAC6 KO cells. Reoxygenation of hypoxic cells resulted in a complete recovery in WT MEFs. Conversely, HDAC6 KO cells and HD^m, and to a lesser extent Ub^m, cells did not properly recover following reoxygenation (Fig. 18). Ub^m cells did not show significant defect in these conditions.

Loss of HDAC6 leads to hypersensitivity to cellular stress

Mammalian cells have evolved a variety of mechanisms to facilitate cellular recovery from environmental stresses. The failure to response stress results in cell death. Stress defense and apoptotic destruction tend to occur in a mutually exclusive manner. Interestingly, AIF (apoptosis-inducing factor) functions as a negative regulator of SGs (Cande et al., 2004). In addition to hypoxia, to generalize a role of HDAC6 for the cellular stress response under different stress conditions, we examined cell viability in the five cell lines described above in response to γ-irradiation and heat shock. As shown in Figure 19B and C, the absence of HDAC6 only has minor impact on the cell response to either γ-irradiation or heat shcok.

Next, to test for a role of HDAC6 in the oxidative stress response, we compared the recovery from stress of wild type cells with that of HDAC6 KO cells. To do this, we assessed the ability of the five cell lines described above to recover from oxidative stress. As shown in Figure 19B and C, viability assays and Annexin-V assays revealed that after recovery from oxidant exposure, *HDAC6* -/- MEFs showed a significantly higher percentage (~40-50%) of apoptosis compared to control MEFs (~5%) over time. In

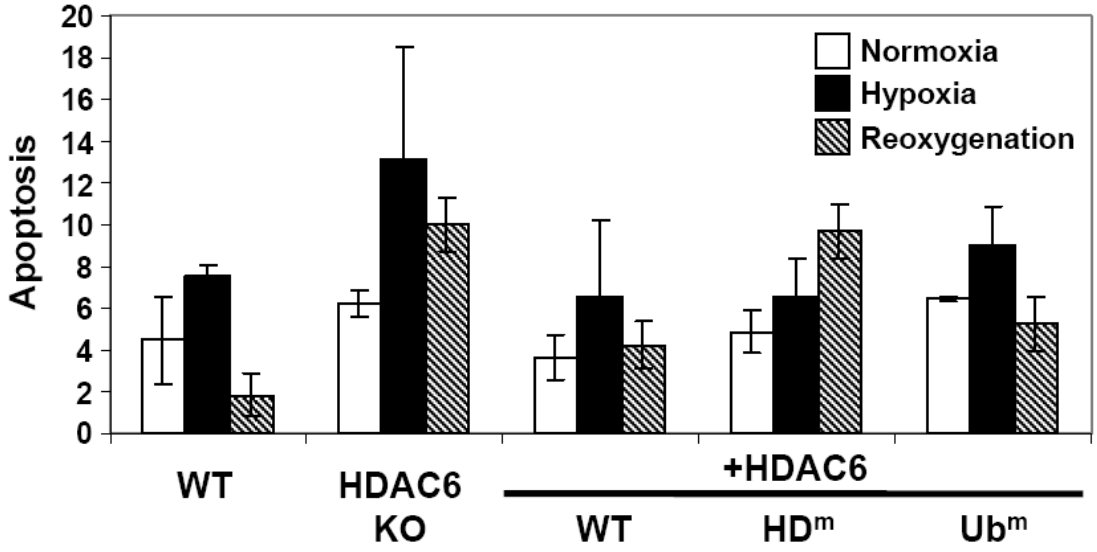


Figure 18. Loss of HDAC6 causes hypersensitivity to apoptosis in response to hypoxia

MEFs were exposed to normoxia, hypoxia (1% O_2, 24 hr) or reoxygenation (1% O_2, 24 hr and 21% O_2, 48 hr). The number of apoptotic cells in these cells was counted and graphed by Annexin-V assay. Error bars represent standard deviation (SD) calculated from 3 independent experiments.

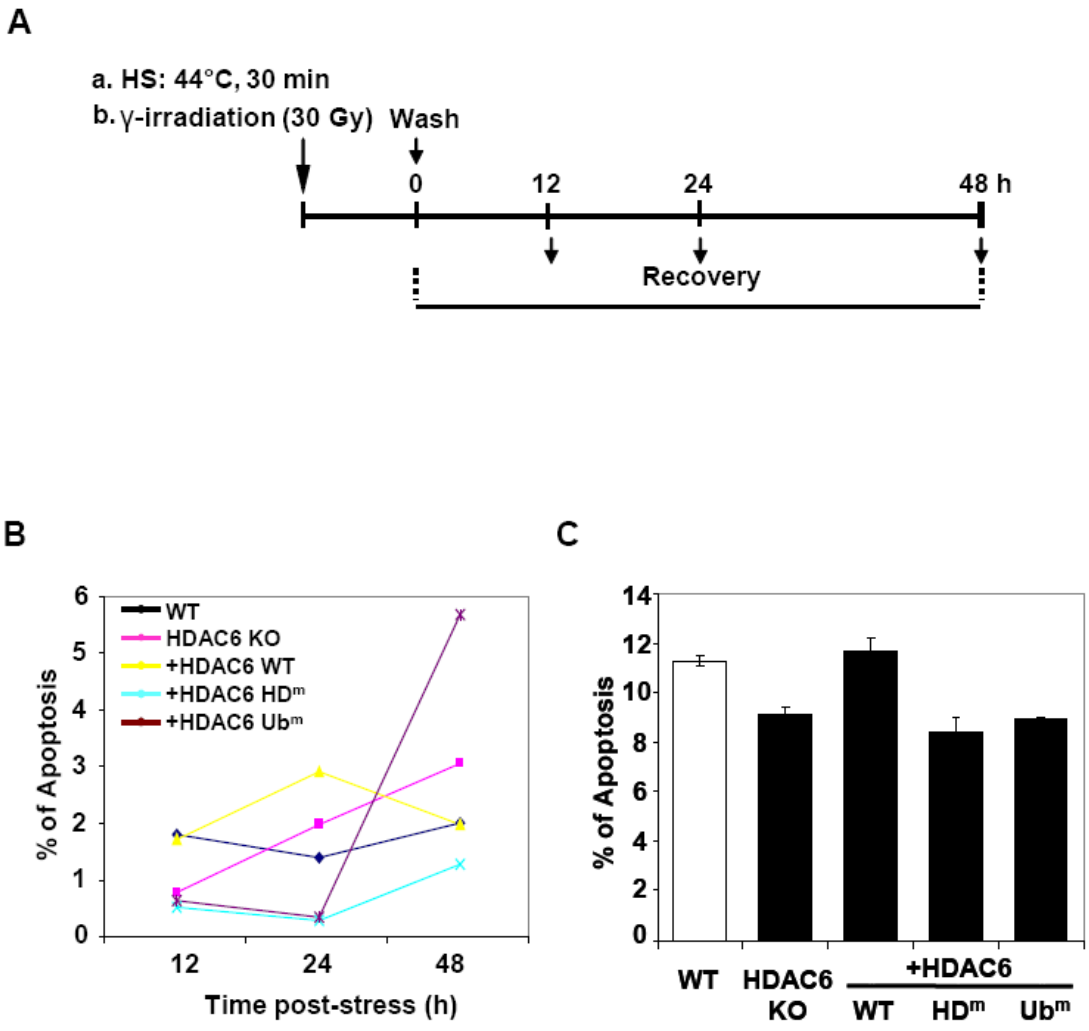


Figure 19. MEFs lacking HDAC6 leads to be more sensitive to stress

(A) Schematic representation of experiment. MEFs were exposed to heat shock at 44 ^{o}C for 30 min and recovered at the indicated times after stress (B) or to γ-irradiation (30 Gy) and recovered for 24 hr after stress (C). The number of apoptotic cells in these cells was counted and graphed Annexin-V assay.

contrast, even though knock out of HDAC6 had no measurable effect on viability of cells prior to the induction of stress, cells lacking HDAC6 recovered poorly after stress release. To convincingly demonstrate that this poor recovery was due to the lack of HDAC6, we used complemented MEFs that rescued of wild type, catalytically dead or non-ubiquitin binding mutant. Importantly, the hypersensitivity of HDAC6 KO cells to stress can be significantly alleviated by the reintroduction of wild type, but not catalytically inactive or ubiquitin binding-deficient, HDAC6. In agreement with this result, cleaved PARP and cleaved caspase-3 as well as DNA fragmentation were detected in only HDAC6 KO cells, HD^m and Ub^m cells (Fig. 20D and E). In the absence of deacetylase activity or ubiquitin binding activity of HDAC6, cells are dramatically impaired in their capacity to recover after stress. This result suggests that loss of HDAC6 render cells more sensitive to stress and post-stress recovery. However, in contrast to oxidative stress, we did not observe a clear role for HDAC6 in response to heat shock and γ-irradiation (Fig. 19). Therefore, we conclude that the role of HDAC6 in stress response is depended on the type of stress stimuli. It could be postulated that HDAC6 modulates the cell's capacity to respond to environmental challenges and to adaptive response against stress.

Stress granules and aggresome are distinct cytoplasmic structures mediated by HDAC6 in response to different stresses

HDAC6 is a component of aggresomes and cells deficient in HDAC6 cannot form the aggresome properly, apparently because of a failure to load polyubiquitinated misfolded protein onto dynein motor for transport to aggresomes (Kawaguchi et al., 2003). We determined whether HDAC6 differently responds to different stresses and made a comparison of aggresomes and SGs. To do this, we performed double immunostaining with anti-γ-tubulin or anti-ubiquitin antibody, as markers of aggresome and anti-HDAC6 antibody in misfolded protein-induced stress and oxidative stress. We treated cells with a proteasome inhibitor, MG132, to induce aggresomes or with arsenite to induce SGs. Aggresomes are formed around the microtubule organizing center (MTOC) and HDAC6 colocalizes with γ-tubulin or ubiquitin to aggresome. However, γ-tubulin was not recruited to SGs after arsenite or proteasome inhibitor plus arsenite treatment (Fig. 21A). Cells treated with MG132 for 24 hours and then arsenite did not form SGs properly in

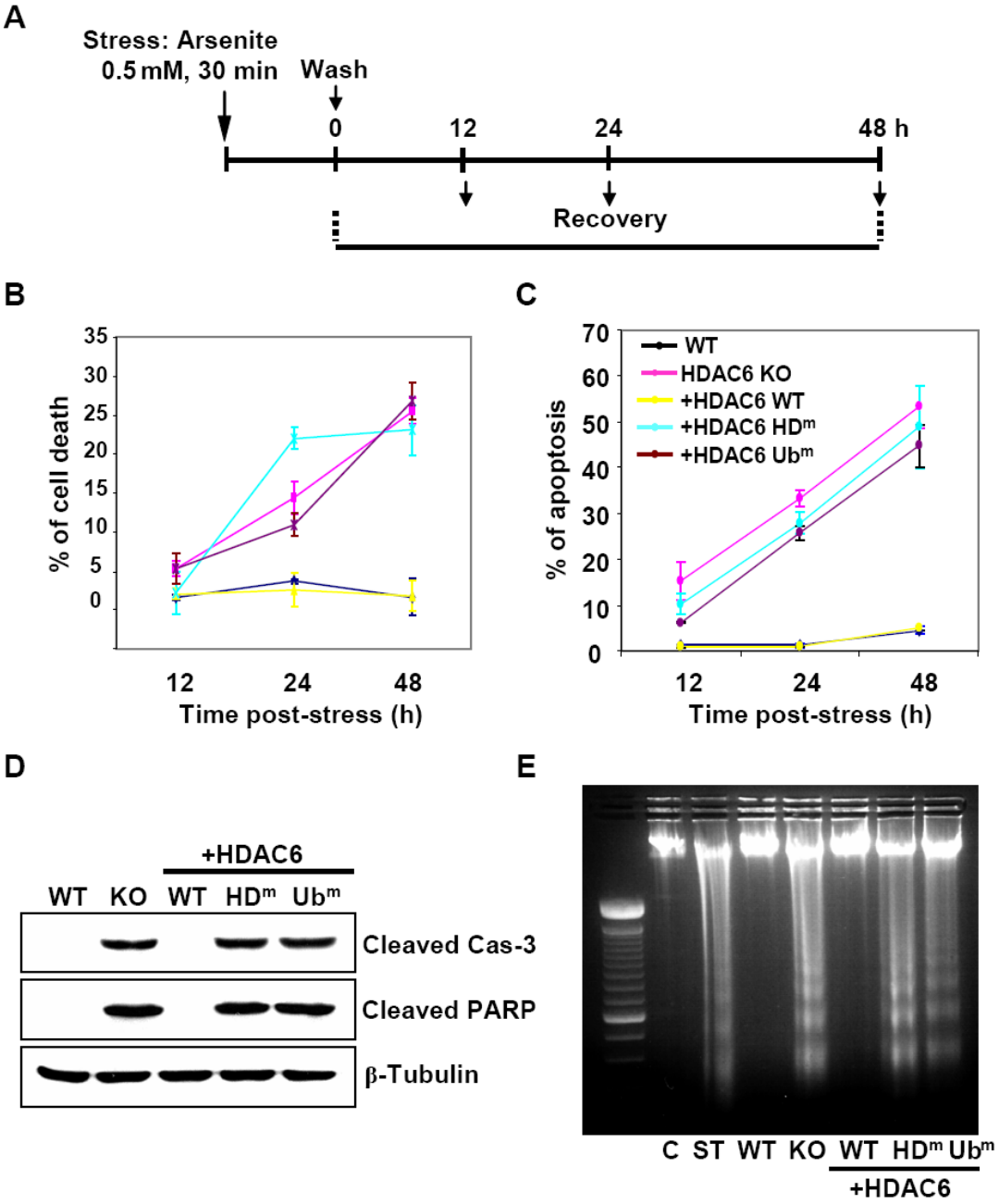


Figure 20. Loss of HDAC6 leads to hypersensitivity to stress

(A) Schematic representation of experiment. MEFs were treated with 0.5 mM of arsenite for 30 min and recovered at the indicated times after stress. The number of apoptotic cells in these cells was counted and graphed by trypan blue staining (B) or Annexin-V assay (C). Error bars represent standard deviation (SD) calculated from 4 experiments. After treatment with arsenite and recovery for 48 hr, the cleaved PARP and Caspase-3 were examined by western blot analysis (D) and apoptotic DNA fragmentation was visualized by ethidium bromide staining (E). C, control group; ST, 500 μM staurosporine-treated group.

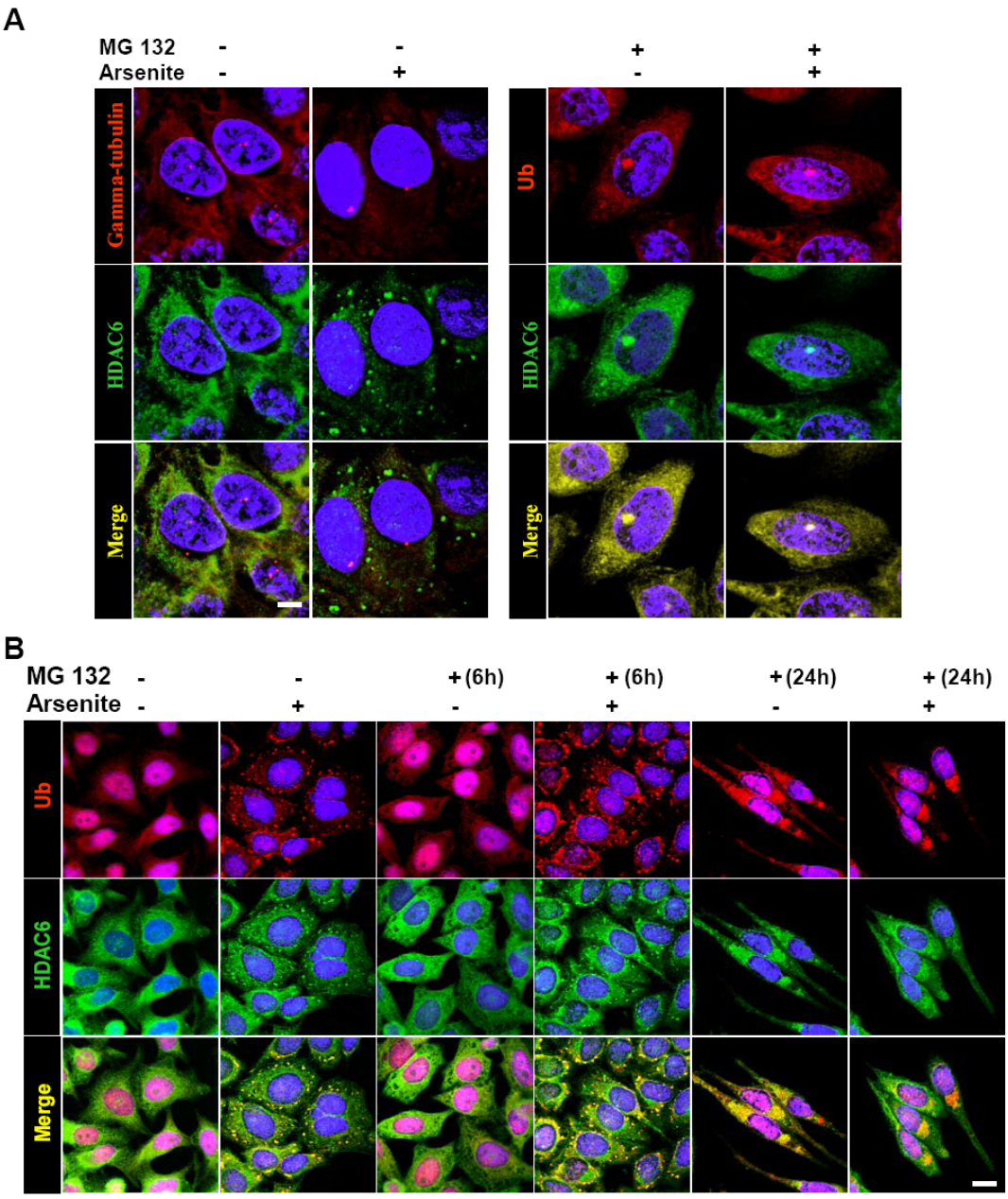


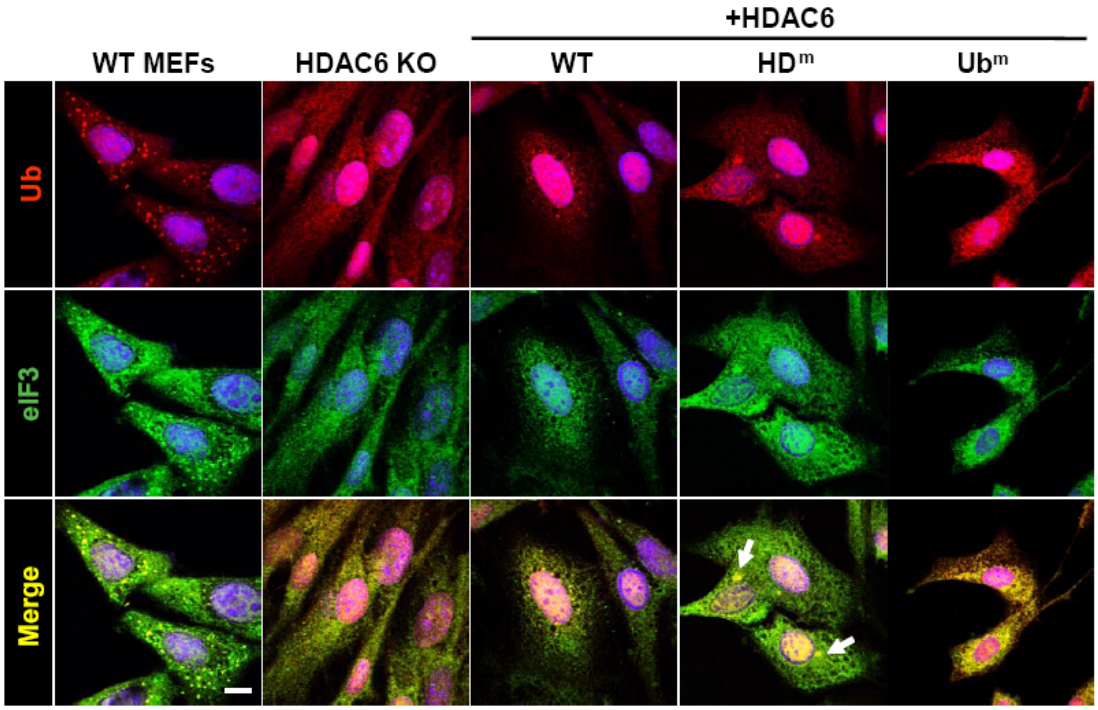
Figure 21. SGs and an aggresome are discrete cytoplasmic structures from which distinctly respond in different stresses

(A) HeLa cells were untreated; exposed to 5 μM of MG 132 for 24 hr and added 0.5 mM of arsenite for 30 min. Double immunofluorescence experiments were performed using anti-HDAC6 (green) and anti-γ-tubulin (red) antibodies or (B) HeLa cells were untreated; exposed to 5 μM of MG 132 for indicated times and added 0.5 mM of arsenite for 30 min. Double immunofluorescence experiments were performed using anti-HDAC6 (green) and anti-Ubiquitin (red) antibodies and secondary antibodies against rabbit (Alexa Fluor 488, green) and mouse (Alexa Fluor 594, red). Nuclei are counterstained using DAPI (blue). Localization of proteins was monitored by a confocal microscopy. Yellow represents co-localization. Scale bar = 7 μm (A), 10 μm (B).

HeLa cells (Fig. 21B). This data suggests that aggresome and SGs are discrete cytoplasmic structures with distinct pathways in response to different cellular stresses.

We showed that the ubiquitin binding domain of HDAC6 is important for SG formation because SG formation is very significantly impaired in the non-ubiquitin binding HDAC6 mutant cell line. Based on the biochemical interaction assays, this domain of HDAC6 does not interact with G3BP. This indicates that this domain might have a different function. This prompted us to investigate how HDAC6 through its ubiquitin binding activity can control SG assembly. To do this, we examined whether SGs can form in the accumulation of heavily ubiquitinated cellular proteins. We treated MEFs with the proteasome inhibitor, MG132 for 6 hours and then with arsenite for 30 min. Under these conditions polyubiquitinated protein accumulation, but no aggresome is visible yet. Ubiquitin was partially recruited to SGs responding oxidative stress as presented in Figure 22A. In this condition, cells formed SGs which are mostly large but an aggresome was not detected in WT MEFs. Until this intensity of stress, SG formation might facilitate to protect cells and HDAC6 quickly responds to SG formation. In contrast, we could see a small aggresome in catalytically dead HDAC6 mutant cells. In HDAC6 KO MEFs and non-ubiquitin binding HDAC6 mutant cells, we could not observe either an aggresome or SGs. When cells were treated with proteasome inhibitor for 12 hours, Ubiquitin and eIF3 were recruited to SGs as well as aggresome in WT HDAC6 rescue and WT MEFs. But under this conditions SGs were smaller in size and less numerous than those observed in arsenite only treatment or arsenite and proteasome treatment for 6 hours (Fig. 22A and B). Also aggresome is smaller in the rescuant of WT MEFs and WT MEFs compared to HD^m cells. We thought that HDAC6 protein moved more to aggresome and could participate to control misfolded protein stress at this intensity of stress. Up to 12 hours treatment with proteasome inhibitor, neither SGs nor aggresome formed in the HDAC6 KO cells and rescue of non-ubiquitin binding HDAC6 mutant. Interestingly, aggresome formation seems facilitate in the rescue of catalytically inactive HDAC6 mutant (Fig. 22B). After 24 hours treatment with proteasome inhibitor, all of the cells formed aggresome and many aggregates accumulated in the intracellular membrane compartment, though HDAC6 KO cells and Ub^m cells assembled clearly smaller aggresomes (Fig. 22C). In the absence of HDAC6 or ubiquitin binding activity,

A



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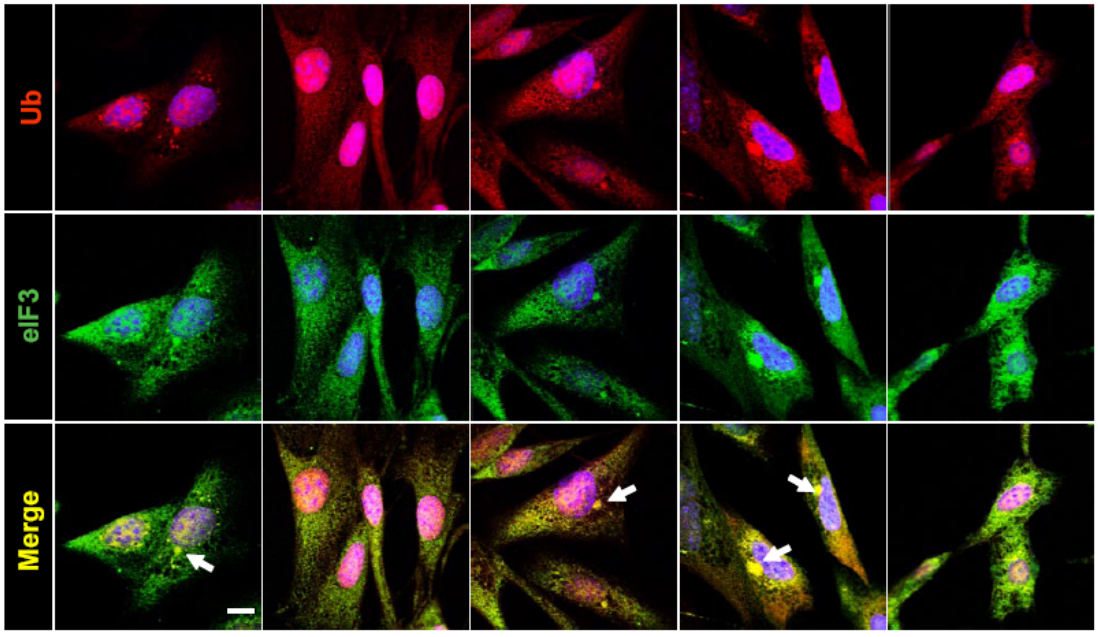

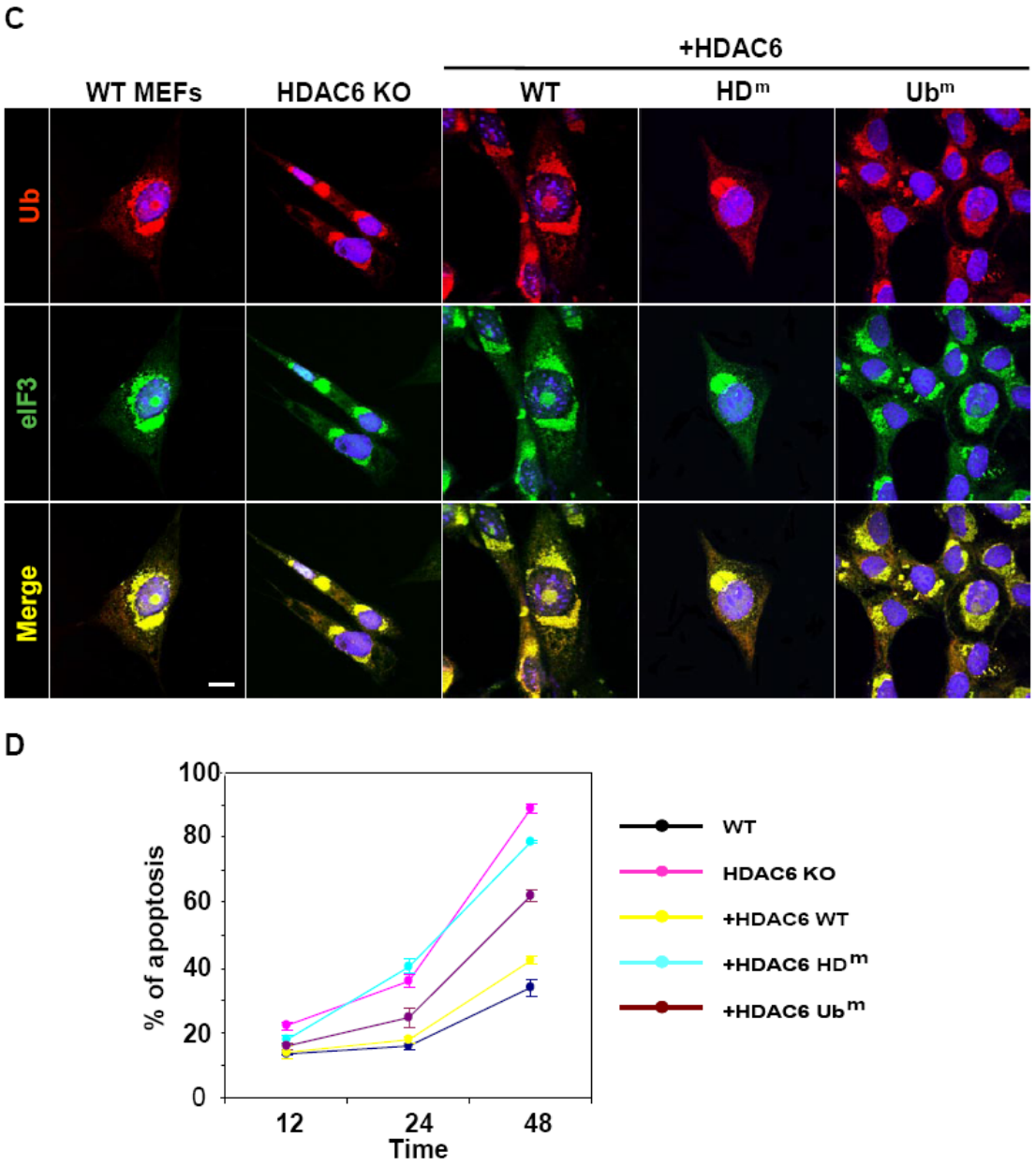

Figure 22. Depletion of HDAC6 results in reduced cell viability after stress

(A) MEFs were treated with 5 μM of MG 132 for 6 hr (A), 12 hr (B), or 24 hr (C) and added 1 mM of arsenite for 30 min. Double immunofluorescence experiments were performed using anti-Ubiquitin and anti-eIF3 antibodies and secondary antibodies against rabbit (Alexa Fluor 488, green) and mouse (Alexa Fluor 594, red). Nuclei are counterstained using DAPI (blue). Localization of proteins was monitored by a confocal microscopy. Yellow represents co-localization. (D) MEFs were treated with 5 μM of MG 132 for 24 hr and added 1 mM of arsenite for 30 min and analyzed apoptosis by Annexin V apoptosis assay. White arrow indicates aggresome. Scale bar = 10 μm.

aggresome formation delayed but was not prevented completely because HDAC6 KO MEFs can assemble aggresomes. We could not detect any SGs under these harsh stress conditions. To determine whether HDAC6 is critical for response to accumulation of misfolded protein-induced stress, we examined the viability of HDAC6 KO cells in response to misfolded protein stress induced by inhibition of proteasome activity. As shown in Fig. 22C and D, in HDAC6 KO MEFs MG132 treatment did not induce the aggresome properly and caused cells to undergo higher percentage of apoptosis than in wild type MEFs. The rescue with HDAC6 led to form aggresome and protected them from cell death. In contrast, deacetylase or ubiquitin-deficient HDAC6 cells had higher apoptosis. This result indicates that indeed HDAC6 is important for cell viability in response to misfolded protein stress. According to the literature, AIF inhibits SG formation (Cande et al., 2004). HDAC6 possesses the potential to protect cells against the environmental stress. We assume that apoptosis induced by heavily misfolded protein stress may prevent SG formation and lead to cell death. These results implicate that HDAC6 might act as an essential regulator of SGs in response to stress and link ubiquitin signaling pathway and cellular protection against different stresses.

3.2.4. Discussion

Effects of histone deacetylase 6 (HDAC6) on HIF-1

HDACi-induced hyperacetylation of HSP90 repress its chaperone function and allows its client proteins to be degraded by an ubiquitination-independent proteasomal system (UIPS) (Isaacs et al., 2002). It is not clear whether the UIPS pathway is specifically triggered or enhanced by HDACis, but it seems to be part of a quality control system of protein synthesis which protects the cell in response to misfolded proteins induced stress. Interestingly, HSP90 K294 is acetylated in its middle domain (Scroggins et al., 2007) and deacetylated by HDAC6 (Bali et al., 2005; Kovacs et al., 2005). Histone deacetylase inhibitors and konockout of HDAC6 induce HSP90 acetylation and inhibit its activity (Zhang et al 2007). HIF-1α is one of the HSP90 chaperone clients. We showed that HDAC6 deacetylates HIF-1α and promotes HIF-1α degradation in hypoxia and hypoxic mimic condition. Based on our data, we propose the putative model presented in Figure 22. HDAC6 binds to and deacetylates HIF-1α, protects its ubiquitin binding sites and recuits deubiquitinating enzymes to these sites, thereby allowing HIF-1α stabilization in hypoxia (Fig. 22C). Intriguingly, the hypoxic expression level of HIF-1α was dramatically lower in non-ubiquitin binding HDAC6 mutant as well as HDAC6 KO cells and HD^m compared with WT cells. Based on this data, we hypothesize that the Ub^m HDAC6 still binds to and decactylates HIF-1α and a conformation change of the HDAC6 mutant allows HIF-1α to expose its ubiquitin binding sites, to be ubiquitinated and degraded by the 26S proteasome in hypoxia (Fig. 22C). This result indicates that these phenomena are mediated by both the deacetylase and the ubiquitin binding activities of HDAC6. Taken together, these results support that HDAC6 plays a key role in quality control mechanism of HIF-1α in hypoxia. Interestingly, non-ubiquitin binding HDAC6 mutant had an impact on HIF-1α stability but no impact on its transcriptional activity. It implys that HIF-1α is required for deacetylation to fully function in transcription. Furthermore, it suggests that a selective inhibitor of HDAC6 can specifically block HIF-1α activity through either controlling HSP90 cheperon function or by directly affecting HIF-1α stability through its acetylation and degradation.

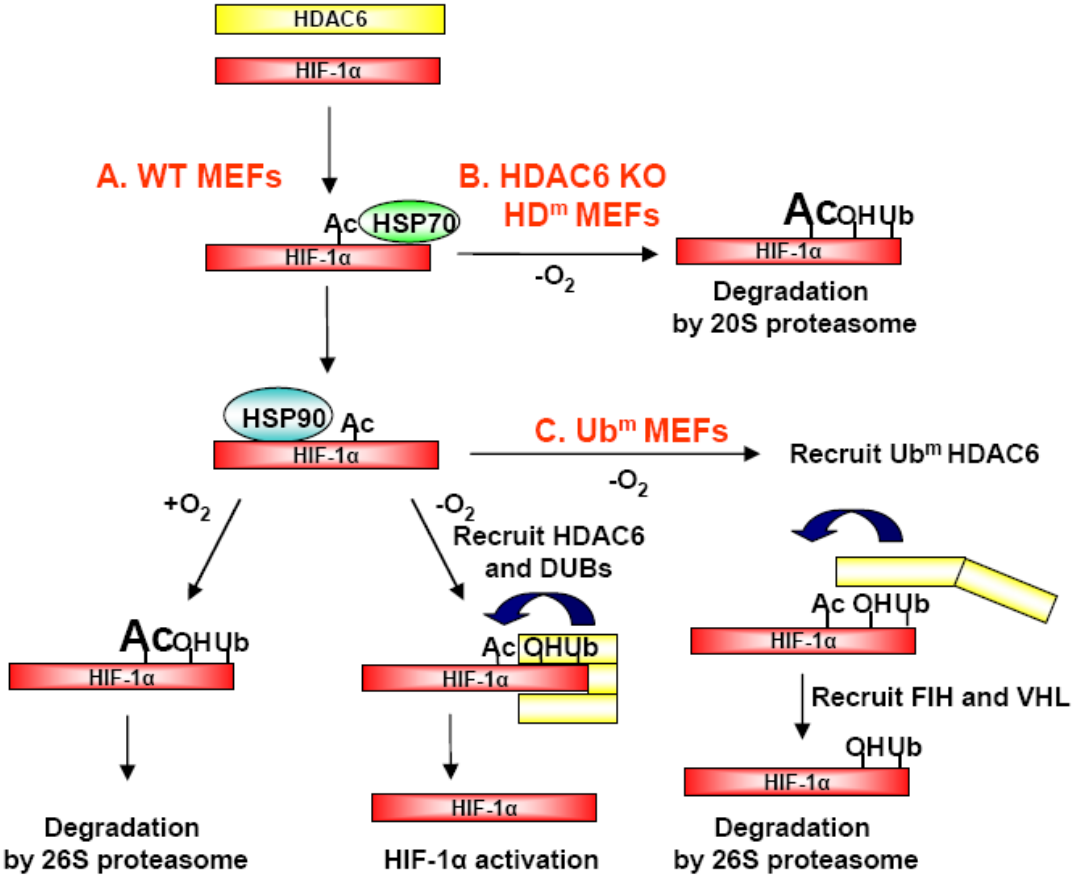

Figure 23. Schematic representation of mechanism of HIF-1α degradation by HDAC6

(A) In the WT MEFs, newly synthesized HIF-1α molecules interact with HSP70 and HSP90 to complete its maturation. Under normoxic conditions ($+O_2$), the mature protein is hydroxylated, ubiquitinated, and degraded by 26S proteasome. In contrast, under hypoxic conditions ($-O_2$), HIF-1α recruits HDAC6. HDAC6 deacetylases HIF-1α, hides its ubiquitinating sites, thereby recruiting deubiquitinating enzymes (DUBs) and protecting HIF-1α. Survivalal HIF-1α interacts with HIF-1β and binds hypoxia response element (HRE) sequences to initiate transcription. (B) In the HDAC6 KO and catalytically dead HDAC6 mutant (HD^m), hyperacetylation of HIF-1α results in enhanced interaction with HSP70 and accumulation of immature HIF-1α/HSP70 complex, and subsequent degradation of HIF-1α by the 20S proteasome. (C) In the non-ubiquitin binding HDAC6 mutant (Ub^m), this mutant HDAC6 can still recruit to and deacetylate HIF-1α protein in hypoxia. However, comformation of this HDAC6 mutant allows HIF-1α to be ubiquitinated by VHL and to be degraded by 26S proteasome.

Effects of histone deacetylase inhibitors on HIF-1

Growing evidence supports that HDACis repress the function of HIF in tumor cells throug yet unidentified pathways (Kim et al., 2001; Mie Lee et al., 2003; Zgouras et al., 2003). HDACis were a broad range of activities including anti-cancer (Johnstone and Licht, 2003). TSA, FK228, butyrate, and LAQ824 were found to repress angiogenesis and expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF). TSA and FK 228 were found to induce HIF-1α degradation in most tumor cell lines tested, including Caki, Hep3B, DU145, PC3, U87, BT20, MCF7 and VHL-/- cells such as RCC4 and C2. Also TSA repressed HIF-1α levels in HCT116 cells (p53+/+) and an isogenic p53-/- HCCT116-derived cells. These data indicate that HDACi-mediated destabilization of HIF-1α is independent of VHL and p53 function (Bunz et al., 1998; Kong et al., 2006).

HIF-1α expression in human cancer and manipulating HIF-1 activity

HIF-1α is overexpressed in many human cancers (Zhong et al., 1999). Significant association between HIF-1α overexpression and patient mortality has been shown in brain, breast, cervix, oropharynx, ovary and uterus cancers. Association between HIF-1α overexpression and apoptosis was correlated with increased patient survival in ovarian cancer. However, in ovarian cancers that overexpressed both HIF-1α and p53, apoptosis levels were low and were associated with significantly decreased overall patient survival (Birner et al., 2001). Therefore, the effect of HIF-1α overexpression is dependent on the cancer type and the presence or absence of genetic alterations that influence the balance between pro- and anti-apoptotic factors (Semenza, 2003).

Expression of VEGF, xenograft growth, and angiogenesis were remarkedly increased in HCT116 colon cancers that were transfected with an expression vector encoding HIF-1α (Ravi et al., 2000). HIF-1α overexpression in PCI-10 pancreatic cancer cells was also associated with an increased xenograft growth and survival rate under glucose and oxygen deprivation (Akakura et al., 2001). Two strategies have been used to inhibit HIF-1 activity. The first approach the expression of a HIF-1α form lacking the DNA binding and transactivation domain, which results in a dominant negative form of

HIF-1α that can bind to HIF-1β (Jiang et al., 1996). The second approach is a fusion protein that consists of GAL4 fused to TAD-C (Transactivation domain at C-terminus); this inhibits the interaction with other coactivators such as CBP/p300 and blocks HIF-1 dependent transcription (Jiang et al., 1997). Based on these studies, we conclude that increased HIF-1α or HIF-2α expression is associated with increased tumor xenograft growth, while inhibition of HIF-1 activity impaires tumor growth. In pancreatic cancer cells, both gain- and loss-of-function experiments highlighted the role of HIF-1 activity in regulating glucose metabolism and cell survival (Akakura et al., 2001). However, in colon cancer cells HIF-1α expression, angiogenesis and tumor growth were correlated (Ravi et al., 2000). These results emphasize that the specific outcomes of increased HIF-1 activity differ depending on cell type (Semenza, 2003).

HIF-1 targeted therapies

A subset of therapeutic agents were been identified that inhibit HIF-1 activity such as inhibitors of signal-transduction pathways, or small molecule inhibitors of HIF-1. Inhibitors of signal-transduction pathway have an anti-angiogenesis effect. It seems to be due to the fact at least partly that these inhibitors led to a decrease HIF-1α levels. At present screen for small molecule inhibitors are underway in different places. Topoisomerase inhibitors (Camptothecin, Topotecan) block HIF-1α expression via an unclear mechanism (Rapisarda et al., 2002). HIF-1α interacts with the chaperone HSP90, and the HSP90 inhibitor, 17-allyl-aminogeldanamycin (17-AAG) in clinical trials induces HIF-1α degradation in a VHL-independent manner (Isaacs et al., 2002). Disrupting agent of microtubule polymerization, 2-methoxyoestradiol (2ME2), has been shown to result in decreased HIF-1α levels, tumor growth and vascularization (Mabjeesh et al., 2003). These small molecule inhibitors share common properties, which are to decrease HIF-1α levels, to inhibit the expression of VEGF and other HIF-1 target genes, and to impair tumor growth and vascularization. But these drugs do not specifically target HIF-1, although these are potential anticancer agent. It would be useful to identify more selective HIF-1 inhibitors in the near future. In addition to discovery of drugs, we should be developed high techniques and biomarkers to monitor response of a drug target to therapy in patient.

HDAC6 serve as a stress protector of cellular stress response in stress condition

Translational arrest is a subroutine of certain classes of endogenous cellular stress responses. Exogeous stresses induce damage to protein synthesis. Cellular damage-detection mechanisms activate in response to accumulation of damaged cell components. This mechanism leads to either translation arrest or selective transcriptional activation of e.g. stress proteins. Translational arrest is initiated by phosphorylation of eIF2α, which is readily reversible (via kinases and phosphatase), maintained in SGs to elicit genetic repair; it is terminated after successful execution of the stress response by stress proteins and phosphatase activity of GADD34. Success to stress response leads to repair of cell damage and recovery of general protein synthesis. However, if cell damage overwhelms the capacity of the cell's to cope with stress,, cell death mechanisms are triggered, and general protein synthesis never fully recovers (Degracia and Hu, 2006).

SG formation is now recognized as a general response that occurs during stress-induced translational arrest. SGs form on stress induction and persist for the duration of the stress.. In reversible models of cellular stress, the SGs decrease in the cytoplasm as the stresses disappear. In cells treated with lethal stressors, SGs persist until the cells die (Anderson and Kedersha, 2006; Kedersha et al., 2002). Recently, aberrant protein synthesis or proteotoxicity have been demonstrated to play a causal role in irreversible translation inhibition. For example, the importance of protein misfolding or aggregation and proteotoxicity in ischemic vulnerable neuron is under investigation.. Ischemia and reperfusion (I/R) alter cellular protein synthesis system and cause delayed neuronal death (DND); neuron shows a persistent translation arrest until they die. Postischemic and reperfused neuron shows two features of stress-induced translation arrest: co-translational protein misfolding and aggregation and dysfunction of SGs. First, ischemia, as a stress stimulus, induces irreversible misfolding of protein and traps the protein synthesis machinery into functionally inactive protein aggregates. Second, I/R causes to modification of SGs that sequester functionally inactive 48S preinitiation complexes to maintain translation arrest and later sequester SGs in protein aggregates. This situation has been well studied in neurons but not in other cell types. Interestingly, HDAC6 is highly expressed in brain and can control both aggresome and SGs in response to stress.

Also HDAC6 is present in Lewy body associated with neurodegenerative disorders, such as Parkinson's disease and dementia. HDAC6 deficient cells are severely impaired in cellular stress response such as aggresome or SGs and are hypersensitive to cell death. Translational protein aggregates and SGs are separate and different phenomena. The formation of co-translational protein aggregates is an irreversible process caused by misfolded proteins in which 40S, 60S ribosomal subunits, and ubiqutin proteins are present. In contrast, SGs are dynamic reversible process including 48S preinitiation complexes, and RNA binding proteins. In agreement with this finding, we showed that aggresome and SGs are discrete structures induced in different stresses at the different time point. However, we did not test whether the same stressor (e.g. ER stress) can induce both cytoplasmic structures simultaneously. However, it is not easy to test this idea as aggresomes and SGs display different kinetics; SG formation is faster event whereas aggresome formation is slower. Recently, intriguing paper has been published. In this case, as similar to the stroke animal model, SGs are formed for protein quality control of effector cytokines (e.g. IL4) during T cell differentiation as part of an ISR without exogenous stresses (Scheu et al., 2006). However, it has not been reported whether HDAC6 impact on T cell differentiation by affecting SG formation. Therefore, in the near future, we need to elucidate by what mechanism HDAC6 might control two separate cellular stress response programs distinctly. The knowledge of the relationship between HDAC6 and cellular stress response may offer new insight for the development of therapies in neurodegenerative disorders.

4. Chapter 4: DISCUSSION

4.1. Possible novel HDAC6 interacting proteins identified by proteomic approach

In order to identify new HDAC6 interacting partners, we first established stable cell line expressing FLAG-tagged human HDAC6, performed coimmunoprecipitation assays, and analyzed bound protein by mass spectrometry. By these affinity trap approaches, we identified a number of new proteins associated with HDAC6 as well as recovered previously identified HDAC6 binding proteins, including tubulin, polyubiquitin, PLAP, HSP90, and dynein. Possibly the most interesting novel putative candidate is Ras-GTPase activating protein SH3 domain binding protein 1 (G3BP1) which we focused mainly in this study. Other potential candidates that were identified in proteomics analysis are described below in order of frequency of identification. Interestingly, most of the target proteins are involved in diverse cytoplasmic events as we expected; RNA metabolism (e.g., SGs, and translation machinery), cytoskeletal functions, ubiquitin proteasome pathway, and cytoplasmic stress response. None of the proteins described below has been validated by independent methods yet. Nevertheless, several/most of them appear promising, as they were identified in multipul independent mass spectrometry experiments following independent enrichment procedures (e.g., co-immunoprecipitation or GST pull down assays).

Elongation factor-1α (EF-1α)

Elongation factor-1α (EF-1α), also known as eukaryotic elongation factor 1A (eEF 1A), is a highly abundant, cytoplasmic, ubiquitous G-protein named for its role in protein translation. It directs aminoacyl-tRNA to the A-site of the ribosome. However, EF-1α is also a multifunctional protein (Durso and Cyr, 1994). In addition to numerous translationally-related functions, non-canonical functions of EF-1α have been described reportedly including oncogenic transformation, microtubule severing, actin filament bundling, and ubiquitin-dependent proteolysis of N-terminally-blocked proteins (Moore and Cyr, 2000; Moore et al., 1998; Shestakova et al., 2001; Yang and Boss, 1994). As a mediator of signal transduction, EF-1α activates phosphotidyl inositol kinase (PI 4-kinase), interacts with the zinc finger protein, ZPR1, and is part of the Rho G-protein

signal pathway. As a cytoskeletal-associated protein, EF-1α binds and bundles actin in a pH-dependent manner and is also a microtubule-associated protein (MAP). Like many structural MAPs, EF-1α can bind, bundle, stabilize, and promote the assembly of microtubules in plant cells *in vitro* and *in vivo*. EF-1α's association with other proteins is affected by a number of regulatory molecules, particularly calcium/calmodulin, phosphorylation, and pH. EF-1α has two microtubule-binding domains: one within domain I, which binds conditionally to microtubules in vivo, and the other in domain III, which is competent to bind microtubules under normal cellular condition (Moore and Cyr, 2000; Moore et al., 1998). Interestingly, EF-1α aggregates are observed in microtubule organizing granules isolated from sea urchin eggs or plant culture cells (Kumagai et al., 2003; Kuriyama et al., 1990). Furthermore, it has been reported that EF-1α indeed plays an important role in the apoptotic program. Chen et al revealed that upregulation of EF-1α protein level was an immediate early event during oxidative stress (H_2O_2)-induced apoptosis in cardiac myocytes and may be essential to the *de novo* protein synthesis needed for execution of apoptosis (Chen et al., 2000).

EF-1α is a very interesting molecule that interacts with cytoskeletal proteins such as microtubule and actin. HDAC6 also associated with tubulin, microtubules and MT motor protein. Based on our proteomic research, HDAC6 may interact with actin as well. We do not know whether HDAC6 can indirectly bind to EF-1α through interaction with microtubules. It has been reported that EF-1α might be required for synthesis of new pro-apoptotic proteins in the apoptosis process following oxidative stress. In the absence of HDAC6, cells are rendered more sensitive to cell death in stress. These data indicate that HDAC6 may negatively regulate translational function of EF-1α or facilitate movement of EF-1α from translational machinery to cytoskeleton, but this remains to be tested experimentally.

Myosin 9 and 10 (Non-muscle myosin heavy chain type A and B)

Myosin is a hexameric protein that consists of 2 heavy chain subunits (MHC), 2 alkali light chain subunits (MLC) and 2 regulatory light chain subunits (MLC-2). The rodlike tail sequence is highly repetitive, showing cycles of a 28-residue repeat pattern composed

of 4 heptapeptides, characteristic for alpha-helical coiled coils. It is specifically expressed in the kidney and leukocyte. Cellular myosin appears to play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping. Defects in myosin 9 are the cause of May-Hegglin anomaly (MHA), Sebastian syndrome (SBS), and Fechtner syndrome (FTNS), which are an autosomal dominant macrothrombocytopenia characterized by thrombocytopenia, giant platelets and leukokyte inclusions. FTNS is distinguished by Alport-like clinical features of sensorineural deafness, cataracts and nephritis. Abnormality in myosin 9 lead to Alport syndrome with acrothrombocytopenia (APSM), and Epstein syndrome (EPS) which are an autosomal dominant disorder characterized by the association of ocular lesions, sensorineural hearing loss and nephritis (Alport syndrome) with platelet defects (Heath et al., 2001).

Heat shock 70 kDa protein 1 (HSP70-1/HSP-1, HSP70.1)

In higher eukaryotes, the stress signal leads to the elevated expression of heat shock genes; stress induced transcription requires activation of heat shock factors (HSFs) that bind to the heat shock promoter element. In unstressed cells, HSFs are maintained in an inactive non–DNA-binding state. Upon exposure of cells to stress conditions, HSFs become activated to a DNA-binding competent, transcriptionally active state, which results in preferential transcription of the heat shock genes. In stress conditions, Hsp70 stably associates with the heat shock factor 1 (HSF1), and so it functions as a repressor of transcriptional activity of the heat shock genes (Shi et al., 1998). Heat-shock proteins (HSPs) are known to serve as protein chaperones and assist protein folding, assembly, degradation, and translocation in the cytosol as well as within organelles. Chaperones of HSP70 participate in all these processes through their ability to recognize nonnative conformations of other proteins. Hsp70-1 is the stress inducible member of the HSP70 family which binds TCS2 (Nellist et al., 2005). HSP70 is for example upregulated in response to hypoxia and is involved in cell protection and survival. The 70 kDa family of HSPs, HSP70, is up-regulated in response to hypoxia and involved in cell protection and survival.

The HSP70 family protein is one of the most interesting HDAC6 interactors we

have identified. HSP70 proteins may be one of the very essential molecules associated with HDAC6 in stress response. HSP90 protein is HDAC6's substrate as well as HDAC6 interacting partner. These results suggest that HDAC6 can regulate functions of other heat shock or stress proteins in stress condition.

Probable ubiquitin carboxyl terminal hydrolase FAX (Deubiquitinating enzyme FAF-X, Ubiquitin-specific protease 9, X chromosome)

Attachment of ubiquitin or polyubiquitination chain to proteins is a crucial step in many cellular regulatory mechanisms and contributes to numerous biological processes, including embryonic development, the cell cycle, growth control, and prevention of neurodegeneration. In these diverse regulatory settings, the most widespread mechanism of ubiquitin action is protein degradation by the proteasome. Deubiquitinating enzymes (DUBs) catalyze the removal of ubiquitin from ubiquitin-conjugated substrate proteins as well as from its precursor proteins. Selectivity of proteolysis depends on the combination of ubiquitinating and deubiquitinating enzymes. Deubiquitinating enzyme FAF-X may function as a ubiquitin-protein hydrolase. It may play an important role at the level of protein turnover by preventing degradation of proteins through the removal of conjugated ubiquitin. Inactivation of the USP9X gene may result in the gonadal degeneration observed in Turner syndrome where there is a failure of oocytes to proliferate and develop, leading to the degeneration of the developing ovary into a streak gonad (Noma et al., 2002).

Deubiquitinating enzyme FAF-X (USP9x) is one of the exciting HDAC6 interacting proteins we identified. HDAC6 has Zn-UBP domain at the C-terminus which mediates the highest known affinity for ubiquitin monomer and the ability of HDAC6 to negatively control the turnover of cellular polyubiquitin chain (Boyault et al., 2006). Another HDAC6-interacting protein, p97/VCP, dissociates the HDAC6-ubiquitin complexes and blocks to accumulate polyubiquitinated proteins by HDAC6. Another study showed that HDAC6 may not itself be a DUB enzyme, but it associated with DUB enzymes independent of the HDAC6 ZnF-UBP (Hook et al., 2002). According to these results, selective ability of HDAC6 to finely tune ubiquitin turnover and protein

degradation might depend on competition between p97/VCP and deubiquitinating enzyme 9 (USP9x) and this will be the object of future studies.

Elongation factor 2 (EF2)

Eukaryotic elongation factor 2 (eEF-2) mediates the translocation step of elongation. eEF-2 has three posttranslational modifications that contribute to its function and regulation. First, a conserved histidine residue (H714) is modified to diphtalmide and this modification inhibits the activity of eEF2 (Jorgensen et al., 2006). Second, diphtalmide is a substrate for ADP ribosylation by diphtheria toxin and this explains the highly toxic effect of diphtheria toxin. Third, eEF-2 phosphorylated at Thr 56, this prevents its binding to ribosones and thus inactivates EF2. It has been reported that several components of the protein synthetic machinery such as EF-1α or EF-2 can also bind to actin microfilaments. EF-2 is colocalized with actin microfilament bundles in mouse embryo fibroblasts although EF-2 was not observed in cell edges or in actin microfilament junctions (Shestakova et al., 2001). EF-2 bound to F-actin. The interaction eEF-2 with F-actin appeared to be inhibited competitively by EF-1α and non-competitively by G-actin. Both G-actin and F-actin forms of actin appeared to be inhibitory on the action of eEF1 and eEF2 in polyphenyalanie synthesis (Bektas et al., 2004; Bektas et al., 1994). These data suggest a possible regulatory link between the protein translation machinery and the cytoskeleton.

EF-2 is an interesting molecule that interacts with cytoskeletal protein, actin. According to our proteomic analysis, HDAC6 can also bind to actin. Therefore, HDAC6 could indirectly bind to EF-2 through interaction with actin or *vice versa*. This information implicates that HDAC6 may bridge between translation machinery and cytoskeleton.

Protein arginine N-methyltransferase-5 (PRMT5)

Arginine is a positively charged amino acid and the nitrogens of arginine can be posttranslationally modified to contain methyl groups, a process termed arginine methylation. Protein arginine methylation results in addition of one or two methyl groups

to arginine residues in glycine and arginine-rich (GAR) motifs. Eight mammalian protein arginine methyltransferases (PRMT) have been identified. Six have been shown to catalyze the transfer of a methyl group from S-adenosylmethionine (AdoMet) to a guanidino nitrogen of arginine, resulting in S-adenosylhomocysteine and methylarginine. No activity has been demonstrated for PRMT2 and PRMT8. Arginine methyltransferases have been identified in yeast, *Drosophila melanogaster*, plants, *C. elegans*, and fish (Hung and Li, 2004). PRMTs are classified as either type I or type II enzyme. PRMTs are ubiquitously espressed. RNA binding proteins (RBPs) are major-substrate of PRMTs because most hnRNPs (A1, A2, K, R, and U) harbor GAR motifs in yeast and mammalian cells. PRMTs facilitate the nucleocytoplasmic shuttling of RBPs through their methylation. They impact interaction and recruitment of mRNA processing and export factor thereby globally affecting transcription. Methylated arginines have also been shown to block some interactions and to promote others in signal transduction pathway. PRMT5 is found in at least three different protein complexes - two nuclear and the third one cytoplasmic. In the cytoplasm, PRMT5 is found in the "methylosome," where it is involved in the methylation of Sm proteins, thus implicating PRMT5 in snRNP biogenesis (Friesen et al., 2001). Nuclear PRMT5 associates with the regulator of transcriptional elongation, SPT4 and SPT5, and pICln, which is also a component of the methylosome. Nuclear PRMT5 also complexes associate with the hSWI/SNF chromatin remodelers BRG and BRM, this association enhances PRMT5 methyltransferase activity (Pal et al., 2004). Intriguingly, one component of the methylosome, pICln is one of the putative HDAC6 interacting proteins. PRMTs, which possess a duel role in transcription, are involved in a subset of diseases including breast and prostate cancer, cardiovascular disease, and viral infection, and spinal muscular atrophy (SMA). Small molecule inhibitors of PRMT were recently identified, (Cheng et al., 2004). AMIs (arginine methyltransferase inhibitors) selectively inhibite PRMTs, not lysine methyltransferases. However, the AMIs display no specificity for individual PRMTs, so further study are required to discover each PRMT-specific inhibitor.

PRMT5 is an intrigue candidate for an interacting with HDAC6. Interestingly, one component of the methylosome, pICln is one of the putative HDAC6 interacting proteins as well as a PRMT5's substrate. Another interesting thing is that PRMT5 interacts with

Lsm4, one subunit of the decapping activators in RNA decay. Based on our initial result, we speculate that HDAC6 may play a role of miRNA-mediated translation repression and SGs. In this context, PRMT5 is a very interesting protein associated with HDAC6 and future analysis probably reveals unexpected findings.

Polyadenyl-binding protein-1 (PABP-1)

The poly(A) tail is a common control site during translational initiation. Changes in poly(A) tail length are a hallmark of translational regulation. Typically, long poly(A) tails are associated with increased translation, and short poly(A) tails are associated with decreased translation. These changes in poly(A) length are triggered in specific mRNAs by sequence specific 3' UTR-binding proteins. The poly(A) tails also represent a critical cis-acting element for translation initiation. The trans-acting factors for poly (A) tail function are the poly(A) binding protein (PABPs). PABPs exist in cytoplasmic and nuclear forms, which resemble little each other. Human has four PABP genes: the ubiquitously expressed PABPC1 (also called PABP1), PABPC3, PABPC4, and PABPC5. The N-terminal region of all these PABPs contains four conserved RNA recognition motifs (RRMs) and PABPC1, -3, and -4 possess a conserved carboxyl- terminal domain (PABC). Several molecules of PABP can bind to poly(A) tails with a periodicity of about 25 adenosine residues, although 12 adenosines are sufficient for binding. RRMs1 and 2 bind to poly(A) with high affinity and specificity, whereas RRMs 3 and 4 exhibit more generic RNA-binding activity. eIF4E bounds to the cap structure and PABP bound to the poly(A) tail jointly recruit eIF4G and the 43S preinitiation complex to the mRNA. The poly(A) tail also stimulates the 60S joining step at the start codon. The PABC domain of PABP interacst with the translation termination factor 3 (eRF3) and this interaction terminates translation and mediates mRNA decay (Gorgoni and Gray, 2004).

HDAC6 can bind to PABP1 and small ribosomal subunits (S3, S18, and S24). Based on our proteomics research, interestingly also PABP1 and small ribosomal subunits are components of SGs. HDAC6 has several putative interacting partners involved in mRNA metabolism (EFs, PABP1, hnRNP H, ribosome proteins and splicing factor). Although HDAC6 is not known to play any role in protein synthesis yet, these

interactions might provide a hit about a potential role of HDAC6 in translation.

Heterogeneous nuclear ribonucleoprotein H (hnRNP H)

Heterogeneous nuclear ribonucleoprotein H (hnRNP H) protein is a component of the heterogeneous nuclear ribonucleoprotein (hnRNP) complexes which provide the substrate for the processing that pre-mRNAs undergo before becoming functional, translatable mRNAs in the cytoplasm. It contains 3 RRM domains. hnRNP H inhibits nuclear export of mRNA containing expanded CUG repeats and a distal branch point sequence. hnRNP H plays a suppressive role in visceral myogenesis. (Liu et al., 2001a) hnRNP H is a component of a splicing enhancer complex that activates a c-src alternative exon in neuronal cells. (Chou et al., 1999).

Kinesin-like protein 11 (KIF-11)

Kinesin-like protein 11 (KIF-11) belongs kinesin-like protein family and contains kinesin-motor domain. KIF-11 is a motor protein required for establishing a bipolar spindle (Blangy et al., 1995) Blocking of KIF11 prevents centrosome migration and arrest cells in mitosis with monoastral microtubule arrays. KIF-11 interacts with the thyroid hormone receptor in the presence of thyroid hormone (Lee et al., 1995b). It becomes phosphorylated exclusively on serine during S phase, but on both Ser and Thr-926 during mitosis, so controlling the association of KIF11 with the spindle apparatus.

KIF-11 is also an interesting candidate. HDAC6 interacts with dynein motor complex, minus end directed MT motor complex, and facilitates cell motility. Recently, it has been reported that microtubule acetylation promotes kinesin-1 binding and transport (Reed et al., 2006). The ability of HDAC6 to bind to kinesin like protein suggests that HDAC6 can selectively interact with opposing retrograde motor protein and anterograde motor protein and modulate in protein transport based on microtubule network in certain condition.

Splicing factor, proline-glutamine rich

Splicing factor is DNA- and RNA binding protein, which is involved in several nuclear

processes. It is an essential pre-mRNA splicing factor required early in spliceosome formation and for splicing catalytic step II, probably as a heteromer with NONO. It binds to pre-mRNA in the spliceosome C complex, and specifically binds to intronic polypyrimidine tracts. It may be involved in a pre-mRNA coupled splicing and polyadenylation process as component of a snRNP-free complex with SNRPA/U1A. Transcriptional repression is probably mediated by an interaction of SFPQ with SIN3A and subsequent recruitment of HDACs.

4.2. HDAC6 is a novel stress granule regulator

4.2.1. G3BP is a new specific HDAC6 interacting protein

Although a couple of HDAC6 interacting partners have been reported, little is known on the physiological functions of HDAC6. Therefore, to this end, we tried to identify new partners for HDAC6 using a proteomic approach. In this report, we have identified G3BP (Ras GTP activating protein (GAP)-binding protein) as a protein interacting specifically with HDAC6. The G3BP protein family modulates Ras activity and the cell cycle, by binding to the RasGAP protein (Guitard et al., 2001; Kennedy et al., 2001; Pazman et al., 2000). G3BP-1 has been shown to have a phosphorylation dependent RNase activity (Tourriere et al., 2001), a regulatory activity of ubiquitin protease (USP10) (Soncini et al., 2001), a transcriptional cofactor function during vaccine virus late replication (Katsafanas and Moss, 2004) and an endoribonuclease activity in stress granules (Tourriere et al., 2003). Furthermore, mice lacking G3BP led to embryonic lethality and growth retardation. Using indirect immunostaining method, we observed colocalization of HDAC6 and G3BP either to the cytoplasm in non-stressed cells or to the SGs in stress. Interestingly, posttranscriptional modification of G3BP impacted on interaction with HDAC6 through hdac domain.

In addition to microtubule, HDAC6 has an enzymatic activity which is able to deacetylate other proteins as well as histones. As might be expected from the HDAC6 localization, most of its substrates are found in the cytoplasm. Amidst them tubulin and

HSP90 are well-characterized as a HDAC6 substrate. G3BP could be likely candidate for HDAC6 substrate because it is largely localized in the cytoplasm. To address whether G3BP can be a substrate for deacetylase HDAC6, we examined acetylation level of G3BP using acetylated-lysine antibodies. We could detect acetylated G3BP protein but the acetylation signal was very weak (data not shown) and more work will be needed to fully examine this issue.

4.2.2. HDAC6 is a pivotal SG regulator as well as a new component of SGs in response to stress

Stress granules are considered as an essential cytoplasmic structure in sorting of individual transcripts for storage, reinitiation, or degradation in stressed cells (Kedersha and Anderson, 2002). Despite the importance of SGs in managing stress response, few protein factors critical for SG formation including RNA-binding proteins, translation initiation factors, and small ribosome subunits, has been identified. In addition, it remains unclear what proteins regulate SG assembly. Herein we reported the identification of HDAC6 as a stress regulator as well as a stable component of SGs. HDAC6 was observed in SGs responding to various environmental stresses. In contrast TIA-1 and G3BP, which induce SG formation upon overexpression, forced expression of HDAC6 did not affect the rate of SG assembly. Importatnly, we showed that HDAC6 regulates SG assembly. For this, intact HDAC6 functions, which are deacetylase and ubiquitin binding activity, is necessary.

Cytoplasmic RNA granules contain motors responsible for translocating the RNA particle along microtubules or actin filaments in especially neuron. However, this active transport was poorly studied in fibroblast. Also, little is kwon that SGs contain proteins associated with microtubule and how SG constituents quickly congregate to local site within few minutes. In this study, we showed that SG formation depends on the HDAC6 through dynein motor protein-driven MT system. MT could serve as a scaffold as well as a track bringing and transporting SG components together. It is feasible that HDAC6 has a role in chaperone and mediator for the rapid formation of SGs. Much remains to be determined about how individual mRNAs are specifically recognized and transported and how localization and translation are coordinately regulated. Although the exact

mechanism by which HDAC6 regulates SG formation remains to be established and the execution of cytoskeleton-dependent active transport and SG formation are mechanically unrelated, our findings suggest that HDAC6 can play an important role in the intersection of the two phenomena. Together, these data point to unexpected crosstalk between microtubule network and the stress response. But, our work with indirect immunostaining gives us a static view of the steady-state distribution of SG components, but does not describe the rate or path of movement of mRNA molecules or SG components. It is little known about the signaling pathways and molecular mechanisms governing formation and disassembly of SGs. Therefore, further efforts will be required to demonstrate the complete linkage between specifically localized RNA, RNA-binding proteins and a relevant motor.

In addition to SGs, other fascinating cytoplasmic aggregates are PBs, which are found in both yeast and mammals. SGs and PBs share some proteins but are to have distinct functions. SGs are considered as a sorting place of mRNP complexes, whereas PBs are considered as RNA place for degradation. Intriguingly, HDAC6 remained absent from PBs and had no effect on PB formation and global translation in both non-stress and stress conditions. In agreement with this finding, HDAC6 did not affect eIF2α phosphorylation which is initiation step for stress-induced translational arrest. Taken together, the identification of G3BP as a target for HDAC6 provides further support for broad functions of HDAC family members in important biological processes beyond histone and chromatin remodeling in general.

4.2.3. eIF2α phosphorylation and SGs in cellular stress response and disease

The phosphorylation of eIF2α and downstream signaling represent conserved adaptation to cell stress referred to as the integrated stress response (ISR). The ISR influences the balance of precursor and mature proteins in higher eukaryotes in regulating the protein–folding environment in the ER. Signaling in the ER has biphasic and tissue-specific effects on cell survival under various stress conditions. The ISR is important for homeostasis and failure of this homeostasis in the ISR can cause to such common human diseases as diabetes mellitus, the metabolic syndrome, and osteoporosis,

neurodegeneration, and demyelination (Harding et al., 2001). A number of environments in which ISR activation induces correlated with cell death. PKR, an eIF2α kinase activated by viral infection, is critical to vertebrate innate immunity. Most animal cell viruses have evolved specific mechanisms for blocking eIF2α phosphorylation in their host cells. However, it appears that PKR provides its benefit to the organism by promoting cell death of virally infected cells (Srivastava et al., 1998). In this regards, translational repression synergizes with other signals to promote apoptosis. PERK -/- cells in mice and human have higher levels of ER stress and they develop a syndrome form of diabetes mellitus in infancy, exocrine pancreatic dysfunction, and a severe bone defects (Delepine et al., 2000; Harding et al., 2001; Zhang et al., 2002). It has been reported that impaired recovery of protein synthesis during the reperfusion phase of ischemic injury compromises neuronal survival (Paschen, 2003). A rare human genetic syndrome, childhood ataxia with cerebral hyomyelination (CACH), is a severe disorder of the white matter associated with abnormalities in the myelin-producing oligodendrocytes caused by mutation of eIF2B. The CACH-associated mutations mimic the consequences of eIF2α phosphorylation. It is possible that eIF2α phosphorylation contributes directly to the pathophysiology of the CACH syndrome (Kantor et al., 2005). The severe consequences of the CACH mutations point to the danger of ISR hyperactivation as these cells are hypersensitive to both mild defects in increasing an ISR and to excess in eIF2α phosphorylation (Southwood et al., 2002). Therefore, the ISR is a potential drug target for the treatment of a variety of common disorders.

4.2.4. HDAC6 has a potential role of miRNA mediated mRNA decay in SGs

TIA-1/TIAR promote aggregation of nontranslating mRNAs in stress (Gilks et al., 2004) and facilite AU-rich element (ARE)-mediated translational silencing of tumor necrosis factor-α (TNF-α) mRNA in immune cells (Piecyk et al., 2000). Indeed, two other ARE-binding proteins, HuR, and TTP have also been localized to SGs (Kedersha and Anderson, 2002; Stoecklin et al., 2004), suggesting that ARE-mediated effects on translation and mRNA turnover may be initiated in SGs. Recently it has been reported that Argonaute proteins quantitatively accumulate to SGs as well as PBs. The majority of Ago2 is found

diffused thoughout the cytoplasm, with only 1.3% localized to microscopically visible PBs. Under stress condition, Ago2 also accumulate in SGs. Interestingly, Argonaute proteins displayed distinct kinetics at different structures: exchange faster at SGs and much slower at PBs. Further, miRNAs are required for the Argonaute protein localization to SGs but not PBs. These data provide insights into miRNA-mediated repression process and suggest that part of it take place in SGs (Leung et al., 2006). Using the reporter system from Pillai and colleaque we showed that let-7 mediated translation repression (3xBulge) and miRNA cleavage (Perfect) are altered in HDAC6 KO MEFs and catalytically dead mutant. The fact suggests that HDAC6 has no effect at the level of the RISC complex or above. It is tempting to speculate that Ago proteins, or perhaps dicers, could be novel HDAC6 targets. Therefore, this result suggests that miRNA-mediated repression and degradation may occur in SGs and HDAC6 may play a role of miRNA mediated mRNA turnover in SGs but not PBs.

Active repression of protein synthesis protects cells against protein malfolding during endoplasmic reticulum stress, nutrient deprivation and oxidative stress. We hypothesized that HDAC6 plays a role in translation recovery. During stress, cells require translation repression except stress-induced proteins. Cells lacking HDAC6 showed derepression of let-7 mediated translation and did not recover properly from stress. In the absence of HDAC6, cells may correctly not perform translation recovery following stress release, resulting in cell death. It is not clear which translation step may be influenced by HDAC6 and by what mechanism cells undergo apoptosis in stress and stress recovery. Nevertheless, these data imply that HDAC6 may influence cell viability via controlling translation machinery and programmed cell death.

4.2.5. HDAC6 may have a potential role of recruitment of ubiquitinated SG-associated proteins to SGs.

Surprisingly, we found that ubiquitin or ubiquitinated proteins were very clearly detected in SGs. In agreement with this, non-ubiquitin binding HDAC6 mutant cells showed a drastically impaired SG formation. So far it is not known whether the ubiquitin proteasome system functions in SGs or has inter-relation with these structures. It is interesting to note that phosphorylation of SG-associated protein regulates their

recruitment to SGs. For example, the arsenite-induced phosphorylation of TTP, a SG-associated RNA-destablizing factor, protmotes its rapid exit from SGs and concurrently inhibits its ability to promote ARE-mediated mRNA decay (Stoecklin et al., 2004). Similarly, the phosphorylation of serine 149 in G3BP, another SG-associated protein, prevents its targeting to SGs (Tourriere et al., 2003). Wherein, it suggests that posttranslational modification might control recruitment of SG's components to SG. Particularly, HDAC6 interacts with mono- and polyubiquitinated proteins and transports them to aggresome via the MT system in misfolded protein stress. In this regard, HDAC6 might bind to and recruit ubiquitinated SG-associated protein to SGs efficiently. Supporting this hypothesis, a RING-type ubiquitin ligase, Roquin localizes to SGs and is required to repress follicular helper T cells and autoimmunity (Vinuesa et al., 2005). Roquin mutation fails to repress diabetes-causing T cells and develop high titers of autoantibodies and pathology consistent with a lupus phenotype. Furthermore, G3BP1 interacts with de-ubiquitinating enzymes, USP10 and inhibits the ability of USP10 to de-ubiquitinate. HDAC6 might also interact with or recruit deubiquitinating enzymes (data not shown). Whether G3BP1's and HDAC6's involvement in ubiquitin metabolism is linked to its involvement in mRNA metabolism, is unknown. It has not yet been reported whether SG components are ubiquitinated and whether this modification is a prerequisite for recruitment to SGs. However, these data suggest that interaction between HDAC6 and G3BP may mediate ubiquitin proteasome system and RNA metabolism, especially SG formation. Therefore, our results are highly related and will open up novel research direction.

4.3. HDAC6 plays a role in the cellular stress response

4.3.1. HDAC6 deacetylates and regulates stability of HIF-1α by controlling of its acetylation and ubiquitination status

Acetylation of the ε amino group of lysine residues has emerged as an important posttranslational modification regulating protein functions. The degree of acetylation of a given protein depends on the dynamic balance of the activity of specific acetylase and deacetylase enzymes. Although HDACs are mostly known to deacetylate histones, they

also interact with other proteins. In this regard, HDAC6 is one of the potential deacetylases that can specifically deacetylate cytoplasmic proteins. Knockdown of HDAC6 or HDACis result in increased acetylation levels and decreased protein level of HIF-1α (Kong et al., 2006; Qian et al., 2006). Indeed, HDAC6 deacetylated HIF-1α and promoted HIF-1α degradation in hypoxic condition, though its deacetylated lysine residues were not found precisely. WT cells and rescuant of WT HDAC6 resulted in higher hypoxic levels of HIF-1α. Conversely, HDAC6 KO cells and catalytically dead mutant of HDAC6 showed inhibition of HIF-1α protein in response to hypoxia. We showed that HIF-1α is a substrate of HDAC6. Unlike Kong et al. we found that HIF-1α interacted stronger with HSP70, a family member of the chaperone class of proteins; however, interaction between HIF-1α and HSP90 did not decrease in hypoxia. Knockout of HDAC6 might result in hyperacetylation of HSP90, accumulation of immature and acetylated HIF-1α protein/HSP70 complex, and degradation of HIF-1α by the 20S proteasome. Whereby, hyperacetylation of HIF-1α may reinforce the interaction with HSP70 and interfere with interaction with HSP90, thus promoting degradation in hypoxia. These results support that HDAC6 is involved in stability of HIF-1α by affecting its acetylation status in hypoxia. However, it is unclear whether direct acetylation of HSP70 is involved in HIF-1α degradation. Interestingly, we identified HSP70-1 as one putative HDAC6 interacting protein. We need to investigate more detailed mechanism by which HDAC6 regulates HIF-1α stability through HSP70/ HSP90 chaperone system.

Hypoxia leads to activation of PERK, phosphorylation of eIF2α, and SG formation, a modification that was readily reversed upon reoxygenation (Koumenis et al., 2002; Moeller et al., 2004). eIF2α was found to be phosphorylated in response to hypoxia in both *HIF-1α* +/+ and *HIF-1* -/- MEFs, with similar kinetics. This result indicates that the signal for eIF2α phosphorylation under hypoxia is independent of HIF-1α accumulation and subsequent downstream events. In this respect, it is notable that the translation of the α-subunit of HIF1 and its target gene (VEGF) has been reported to be mediated by IRES element in response to hypoxia (Lang et al., 2002; Stein et al, 1998). Addtionally, depletion of HDAC6 did not have an effect on phosphorylation of eIF2α in stress. HDAC6 regulates SG formation downstream of eIF2α phosphorylation and affects HIF-1α transcriptional activity. It implicates that HDAC6 control downstream hypoxia

response of phosphorylation of eIF2α in hypoxia.

4.3.2. HDAC6 acts as a modulator of cytoprotective response in stress

The cellular stress response is a phylogenetically conserved protection mechanism from prokaryotes to humans. When cells are exposed to different stresses, cells can activate defense mechanisms to adapt to stressful conditions, to repair damage and to resume normal cellular functions or rather activate the apoptosis depending upon the intensity of physical or chemical stresses. One of the possible defense mechanisms responding to stress is the formation of cytoplasmic "Stress granules" that manipulate an arrest of mRNA translation. Although translation arrest upon stress is widespread, selective translation of heat shock proteins, as well as of some transcription factors, under these conditions allows the cell to repair the stress-induced damage while conserving anabolic energy. When stress is relieved, SGs disassemble and the stalled mRNAs either return to the active translation machinery or are targeted for degradation in PBs. The other possible defense mechanism in stress is the assembly of aggresome that eliminate the accumulation of toxic misfolded proteins. Cells can resist to cell death and activate stress defense mechanism to repair damage until certain stress threshold. But reaching apoptotic threshold, cells activate apoptosis. Depending on the type of stress stimulus, the multiple events associated with HDAC6 activation might be affected differently. HDAC6 can regulate SGs as well as aggresome upon stresses. Conversely, the same stress has different impacts on cellular responses. For example, thapsigargin, an ER-stress-inducing agent, cause aggresome and a prominent relocalization of HDAC6 (Kawaguchi et al., 2003). Also, like arsenite, thapsigargin inhibites protein synthesis, enhances eIF2α phosphorylation, and promotes stress granules formation. Here, HDAC6 deficiency resulted in impaired stress responses (aggresome or SGs) triggered by different stresses and post-stress recovery. Strikingly, the hypersensitivity of HDAC6 KO MEFs to stress can be significantly alleviated by reintroduction of wild type, but not two functional domain mutants of HDAC6. The physiological significance of HDAC6 in the stress is underscored by the observation that cells lacking HDAC6 exhibit decreased viability rates, both during stress and following release from stress conditions. Therefore, HDAC6 might play a critical role in regulating the cellular management of different stress

response.

4.4. Closing remarks

Since histone acetyltransferases have been identified, we have understood a role of HATs and HDACs. Mammal has 18 distinct histone deacetylases which possess discrete functions and regulate many proteins. Recently evidence has emerged that many non-histone protein, also cytoplamic, are modulated by acetylation. HDAC6 is mostly cytoplasmic and has a distinct cellular function. HDAC6 is a specific cytoplasmic tubulin deacetylase and a regulator of polyubiquitinated protein turnover togegher with p97/VCP. Intriguingly, HDAC6 is recruited to SGs when cells are exposed to environmental stresses. HDAC6 mediates SG components to congregate and to transport together to SGs along microtubules through dynein motor complex, thereby facilitating SG formation. In addition, HDAC6 function as a stress regulator under stress conditions such as hypoxia, or oxidative stress. Indeed, a critical physiological role of HDAC6 emerges in stress condition. Depleting of HDAC6 causes to hypersensitivity cell death during stress and impaired stress recovery. To more clarify a role of HDAC6 in stress, we need to identify detailed mechanism by which HDAC6 regulates cellular stress response upon a subset of stress. Also, future studies are bound to elucidate the mechanism by which specific mRNAs or miRNAs are sorted into different cytoplasmic compartments such as SGs or PBs, which function in mRNA metabolism. We also need to reveal the mechanism by which components of SGs congregates orderly in stress. Furthermore, we investigate how the cell distinguished between transcripts destined for decay and those that are to be reinitiated translation machinery.

5. Chapter 5: BIBLIOGRAPHY

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6. Chapter 6: APPENDIX

6.1 Possible other HDAC6 interacting proteins

	Putative HDAC6 interacting proteins			
	Protein name & Description	Gene name	Acess Num	Frequency
1	Elongation factor-1α-1 (eEF-1α-1/eEF1A-1) (EF-Tu) (Leukocyte receptor cluster member 7)	EEF1A1, EF1A, LENG7	P04720	6
2	Myosin 9 (nonmuscle myosin heavy chain type A)	MYH9	P35579	5
3	Myosin 10 (nonmuscle myosin heavy chain typeB)	MYH10	P35580	5
4	Heat shock 70 Kda protein 1 (HSP70-1/HSP70-2) (HSP70.1)	HSPA1A, HSPA1B	P08107	4
5	Cofilin-1, non-muscle isoform 18 kDa phosphoprotein p18	CFN-1	P23528	4
6	Dihydrolipoamide succinyltransferase component of, of 2-oxoglutarate dehydrogenase complex, E2, E2K	DLST	P36957	4
7	Elongation factor-2 (EF2)	EEF2	P13639	3
8	40S ribosomal protein S3	RPS3	P23396	3
9	40S ribosomal protein S18 (KE-3) (KE3)	RPS18	P25232	3
10	Actin, cytoplasmic 1 (Beta-actin)	ACTB	P02570	3
11	G3BP (Ras-GTPase-activating protein binding protein 1)	G3BP	Q13283	3
12	Nucleoin (Protein C23)	NCL	P19338	3
13	Hetrogeneous nuclear rionucleoprotein H (hnRNP H)	HNRNP 1	P31943	3
14	Probable ubiquitin carboxyl terminal hydrolase FAX (Deubiquitinating enzyme FAF-X, Ubiquitin-specific protease 9, X chromosome)	USP9X	Q93008	3
15	Kinesin-like protein KIF11	KIF11	P52732	2
16	Polyadenyl-binding protein-1 (PABP1)	PABPC1	P11940	2
17	Splicing factor, proline-and glutamine-rich	SFPQ	P23246	2
18	ADP/ATP translocase 2 (Adenine nucleotide translocator 2) ADP/ATP carrier protiein fibroblast isoform (ANT 2)	SLC25A5	P05141	2
19	ATP synthase alpha chain, mitochondrial precurso	ATP5A1	P25705	2
20	Calmodulin (CaM)	CALM1, CAM, CALM,CAM1	P02593	2
21	Filamin A (α-filamin, Filamin 1, Actin-binding protein 280)	FLNA	P21333	2
22	Methylosome subunit pICln	CLNS1A	P54105	2
23	Stress-70 protein, mitochondrial precursor, GRP 75	HSPA9B	P38646	2
24	Interleukin enhancer-binding factor 3	ILF3	Q12906	2

6.2 Effect of overexpressed HDAC6 and eIF2α phosphorylation in stress

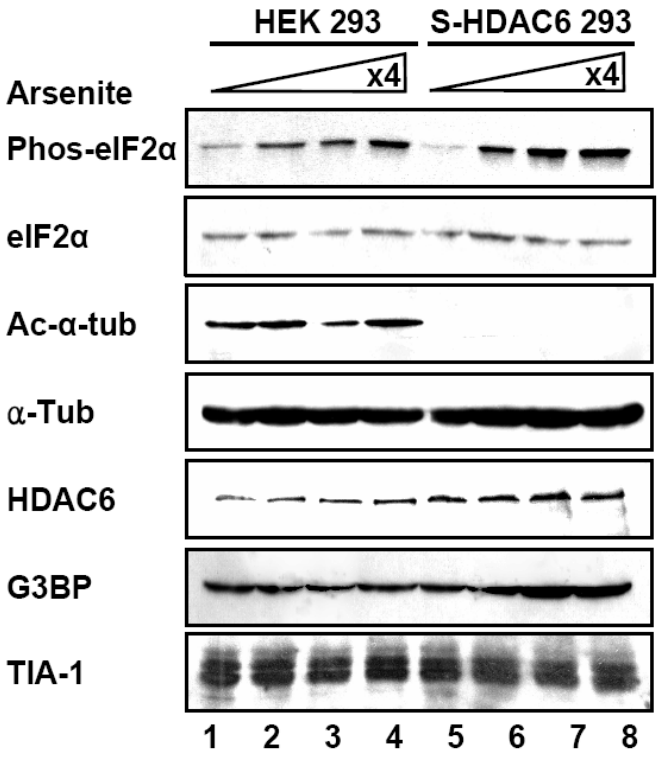
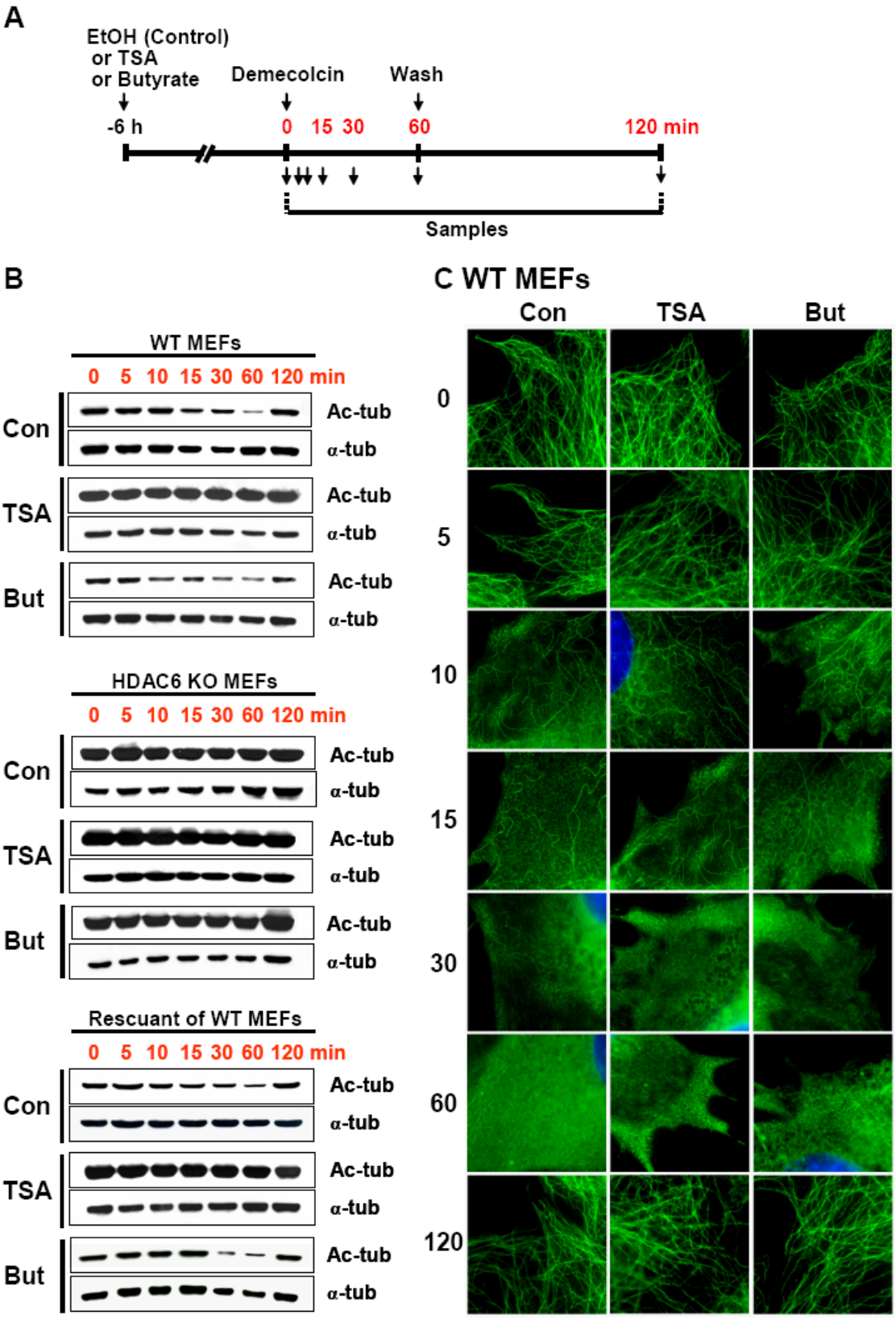


Figure S1. HDAC6 regulates SG formation downstream of eIF2α phosphorylation

Parental HEK 293 cells or a derivative cell line stably overexpressing HDAC6 (S-HDAC6 293) were control-treated (lanes 1 and 5) or treated for 1 hr with increasing amounts of arsenite: 0.25 mM (lanes 2 and 6), 0.5 mM (lanes 3 and 7), or 1 mM (lanes 4 and 8). Extracts were analyzed by western blotting and probed for phospho-eIF2α, eIF2α, TIA-1/TIAR, HDAC6, G3BP Ac-α-tubulin, and α-tubulin (as a loading control).

6.3 Effect of HDAC6 on microtubule stability



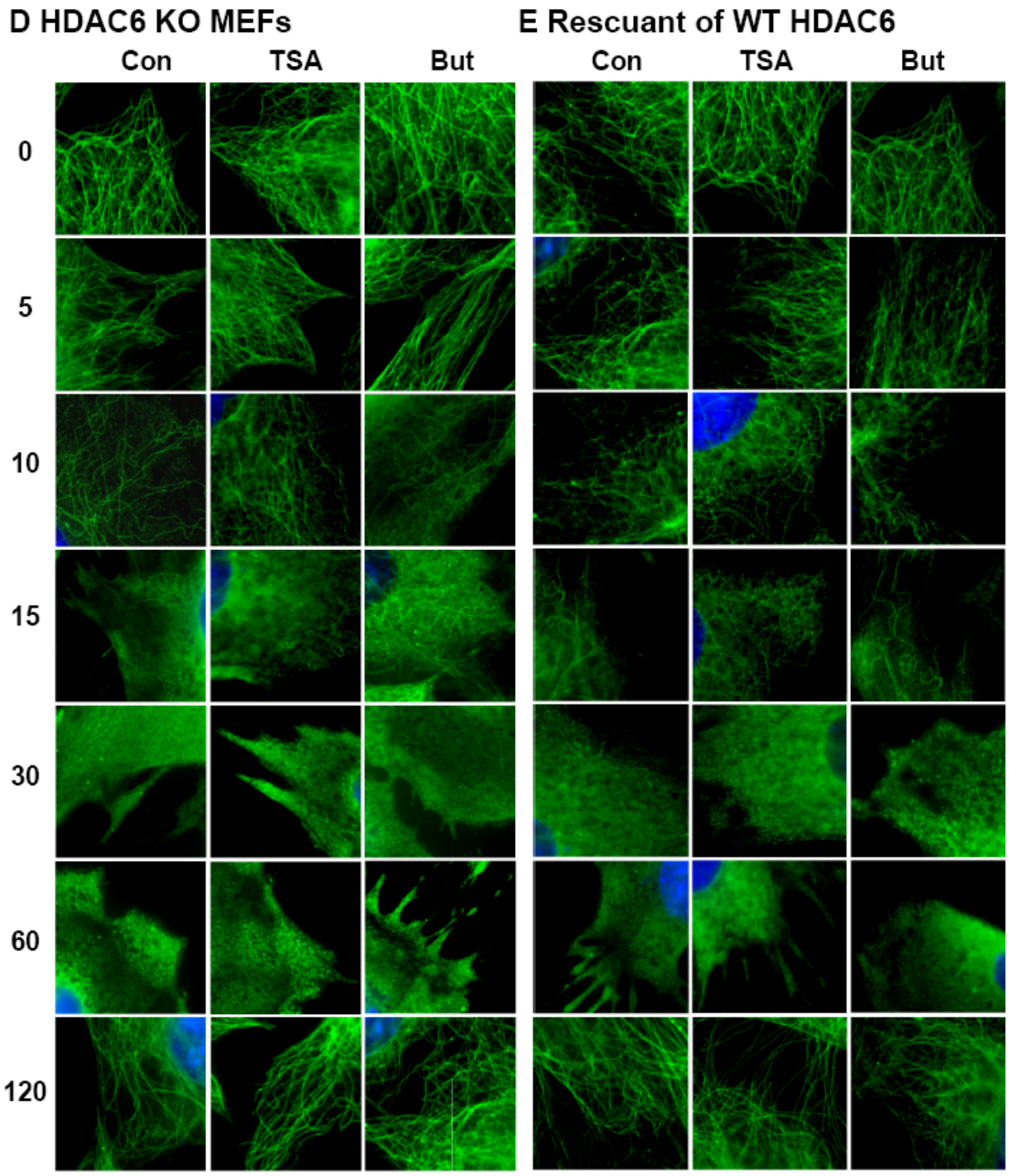


Figure S2. Polymerization and depolymerization of microtubules in MEFs.
(A) Schematic representation of experimental procedures. Bars indicates the periods during which the cells were treated with drugs. Arrows indicates the time-points at which cells were taken for immunoblot analysis (B) and immunofluorescent microscopy (C-E). (B) Cellular acetylation levels of tubulin in the time-course experiments. The amount of acetylated and total tubulin in the cells treated with various drugs in the time-course experiments shown in (A) were determined by immunoblotting with anti-acetylated α-tubulin and anti-α-tubulin antibodies. (C-E) Depolymerization of microtubule during demecolcin treatment and removal. Microtubules were immunostained with the anti-α-tubulin antibody and observed under fluorescent microscope at the time-points indicated in (A).

PUBLICATIONS

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