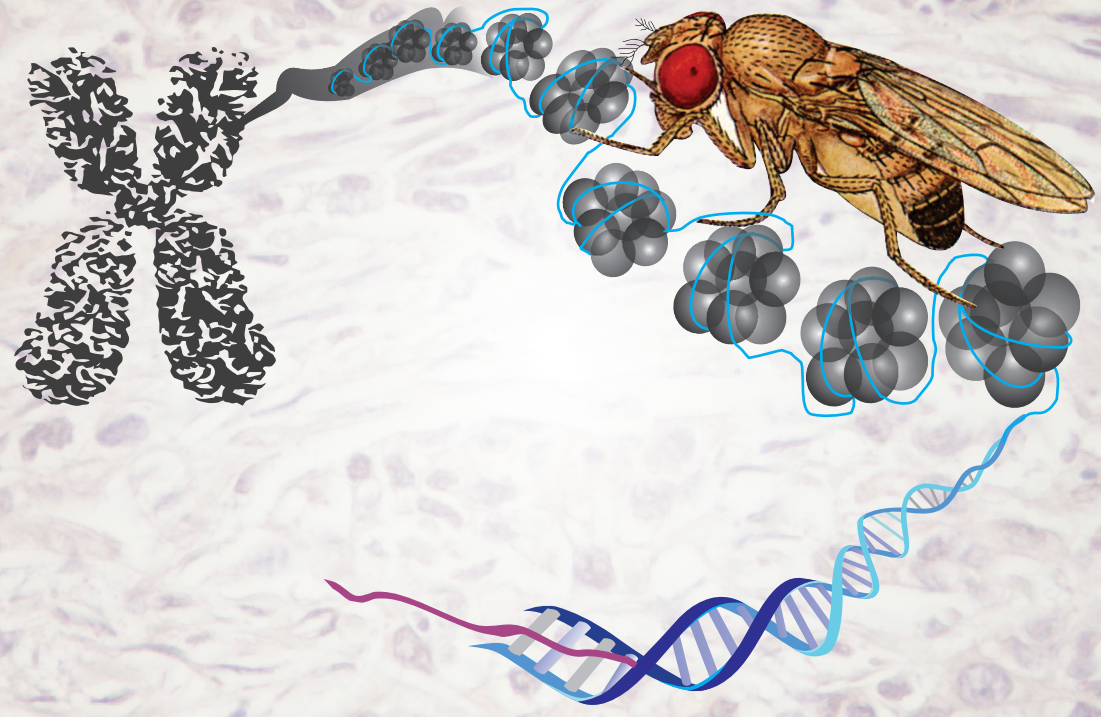
Gene Regulation by Metabolic Enzyme GMP Synthetase and Chromatin Remodeler NuRD



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Gene Regulation by Metabolic Enzyme GMP Synthetase and Chromatin Remodeler NuRD

Genregulatie door Metabolische Enzymen GMP Synthetase
en Chromatine Remodelleerder NuRD

Proefschrift

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Table of Contents

Scope of the Thesis

Spatial and temporal control of the gene expression is crucial for normal growth and development of an organism. Environmental stress factors pose a constant threat to normal development of an organism by causing altered gene expression. Cells have evolved counteractive mechanisms to overcome the stress caused by external factors. Among these, stabilization of p53 is the major stress responsive pathway. In response to diverse cellular insults including DNA damage, p53 gets stabilized and regulates the expression of genes that induce cell cycle arrest, senescence, and apoptosis. However, it is unclear how p53 is stabilized upon cellular stress. *Chapter 2* describes the identification of nucleotide biosynthetic enzyme GMPS and E3 ubiquitin-ligase TRIM21, as key players in regulation of p53 stability. Upon cellular stress, GMPS stabilizes p53 through USP7-mediated deubiquitylation. GMPS acts as a sensor of the nucleotide pool in the cell and activates p53-mediated replicative stress response to the nucleotides depletion. Thus, GMPS ensures the error free cell divisions by coordinating nucleotide synthesis and p53-mediated cell cycle regulation. TRIM21 negatively regulates p53 stability and function by targeting GMPS to cytoplasm. Since loss of p53 function is reported in more than 50% of human cancers targeting TRIM21 might serve as a therapeutic opportunity for cancer treatment.

Combination of transcription factors and specialized chromatin remodeling machines ensure tight control of gene expression. *Chapter 3* describes the combinatorial role of the transcription factor TTK69 and the chromatin remodeler NuRD complex in gene regulation. TTK69 is a transcriptional repressor of the neuronal cell fate in *Drosophila.* However, how exactly TTK69 represses its target genes still largely unknown. In this study we identified drosophila MEP1 as a *bona fide* subunit of the NuRD complex. Biochemical and genetic assays establish that TTK69 binds to MEP1 to recruit the NuRD complex for the transcriptional repression of its target genes. In addition, we also identified the fly homolog of human *Deleted in Oral Cancer 1* (DOC1) also known as CDK2-Associated Protein 1 (CDK2AP1) as a *bona fide* NuRD subunit. DOC1 is a potential tumor suppressor that is found deleted or down regulated in many cancers including oral cancer. *Chapter 4* addresses the role of human DOC1 in gene regulation. By proteomics analysis and functional assays, we establish that DOC1-NuRD complex inhibits the expression of TGF-β target genes those promote tumorigenesis. In this thesis, we identified GMPS and TRIM21 as key players in control of tumor suppressor p53 pathway and dMEP1 and DOC1 as *bona fide* subunits of the NuRD complex and their gene regulation networks in *Drosophila* development and cancer.

Chapter 1

Introduction

Chromatin structure and dynamics

In *eukaryotes*, DNA is packed into highly organized nucleoprotein complexes called *Chromatin*. Approximately 146 bp of the DNA is wrapped two times around an octamer of histones, H2A, H2B, H3 and H4. This is the fundamental repeating unit of eukaryotic chromatin structure, referred to as *Nucleosome* (Kornberg, 1977; Luger et al., 1997). Positively charged amino acid residues in histones contact the negatively charged phosphate backbone of DNA to form a stable nucleosome structure. The adjoining nucleosomes with linker DNA appear as structures similar to 'beads on a string'. With the addition of linker histone H1, 'beads on a string' structures coils into higher order structures ranging from 30 nm chromatin filaments to highly condensed mitotic chromosomes, see *Figure 1* (Li and Reinberg, 2011). Positioning of the nucleosomes along the DNA is determined by several factors, such as intrinsic DNA sequence properties, DNA binding proteins or transcription machinery and ATP-dependent chromatin remodeling complexes (Segal et al., 2006). Chromatin is largely divided into euchromatin and heterochromatin based on nucleosome density. Euchromatin is lightly packed where DNA is accessible to the transcription machinery and other chromatin regulators, whereas heterochromatin is tightly packed and not accessible to chromatin regulators and often associated with gene silencing (Simonis et al., 2006). Thus, nucleosomes act as a barrier for the factors that mediate DNA-dependent processes such as transcription, DNA replication, repair and recombination. Despite being rigid in its structure, chromatin is found to be remarkably dynamic. The dynamic chromatin structure is regulated by DNA packing proteins, such as histones and non-histone proteins, non-coding RNA, factors that mediate histone deposition and removal, histone modifying enzymes and a group of chromatin remodeling complexes.

Gene regulation by posttranslational modification of proteins

Histone modifiers directly add or remove posttranslational modifications to various amino acids in the histones. Lysine (K) residues are the target for acetylation, methylation, ubiquitylation or sumoylation whereas Arginines (R) can be either mono- or di-methylated. Serine (S) and Threonine (T) residues get phosphorylated (Strahl and Allis, 2000; Suganuma and Workman, 2011). The negatively charged acetyl moiety has been shown to disrupt the histone-DNA contacts, resulting in an open chromatin structure. Removal of acetylation on histones results in stabilization of histone-DNA contacts and a compact chromatin structure (Cheung et al., 2000). Unlike acetylation which disrupts histone-DNA contacts, methylation acts as a docking site for the chromatin regulatory proteins. Histone methylation is associated with either active or repressive chromatin states (Rea et al., 2000). Sumoylation of histones acts as a docking site for sumo-binding proteins and is implicated in transcriptional repression (Shiio and Eisenman, 2003; Yang and Sharrocks, 2004). Histone phosphorylation is linked to transcription regulation and

signalling (Berger, 2010). Phosphorylation at H3, S10 marks the mitotic stage of the cell cycle (Hsu et al., 2000). Similar to the histones, DNA also gets modified by methylation at cytosine residues. DNA methylation at gene promoters results in repression of the gene expression. DNA methylation also acts as a docking site for the methyl-DNA binding proteins, which are often associated with transcriptional repression and heterochromatin formation. (Jenuwein and Allis, 2001; Shilatifard, 2006; Strahl and Allis, 2000; Suganuma and Workman, 2011).

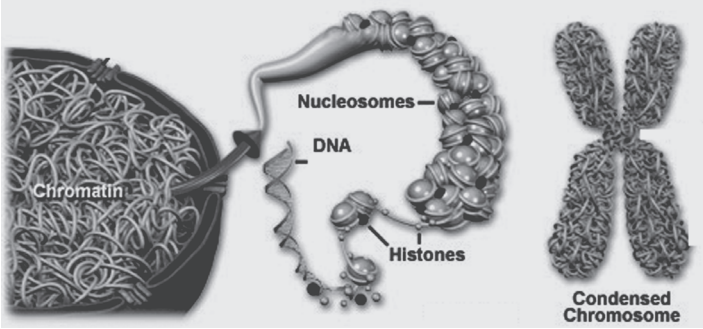


Figure 1; Schematic representation of different levels of chromatin organization (adopted from, http://micro.magnet.fsu.edu/cells/nucleus/chromatin.html)

Histone ubiquitylation is implicated in transcription regulation and DNA repair. Histone H2A and H2B has been shown to be monoubiquitylated (Shiloh et al., 2011; Wright et al., 2012). Ubiquitylation is a reversible post-translational modification that regulates stability, localization or function of a target protein. Protein ubiquitylation is a multi-step enzymatic process, wherein ubiquitin is conjugated to the lysine residues of a target protein by an E3 ubiquitin-ligase that is highly substrate-specific. In general, monoubiquitylation acts as a signal for protein localization, gene expression control or endocytosis. Polyubiquitylation, where in ubiquitin is linked by Lysine 48 (K48) is a signal for proteasome-mediated degradation of the target protein. K11-, K29- and K63-linked polyubiquitin acts as a signal for DNA damage response, kinase activation and vesicle trafficking, see *Figure 2* (Hershko and Ciechanover, 1998; Komander and Rape, 2012; Schlesinger and Goldstein, 1975). Ubiquitylation plays a crucial role in gene regulation not only through the direct modulation of gene expression by histone ubiquitylation but also by indirectly controlling stability and localization of transcription factors. Here, we discuss in detail the regulation of gene expression and transcription factor stability and localization by ubiquitylation-dependent mechanisms involving the metabolic enzyme GMP synthetase, the deubiquitinase USP7 and the E3 ubiquitin-ligase TRIM21.

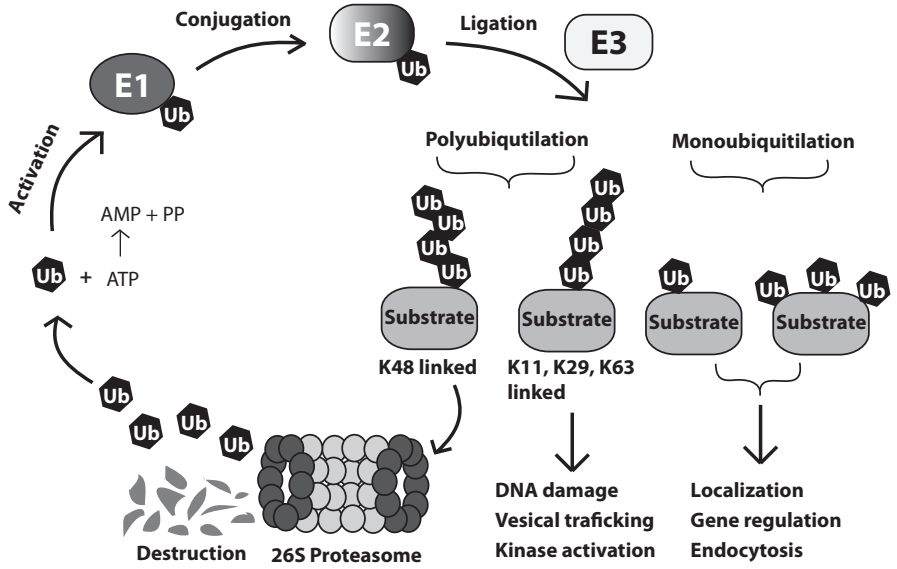


Figure 2; Overview of ubiquitin signalling; Ubiquitylation is a multistep enzymatic process wherein ubiquitin is ligated to the lysine residue of the target protein. In an activation step ubiquitin is transferred to enzyme E1 in an ATP-dependent manner. Next, in the conjugation step, ubiquitin is transferred to E2 enzyme. The E2 carries ubiquitin to E3 enzymes, also called ubiquitin-ligases. E3 ubiquitin-ligases covalently ligate ubiquitin to a lysine residue of the specific protein substrate. Transfer of multiple ubiquitin to the same lysine leads to polyubiquitylation. Ligation of polyubiquitylation has a specific effect on the target protein. For example, K48-linked polyubiquitin is a signal for the proteasome-mediated degradation of the target protein. K11-, K29- and K48-linked polyubiquitin acts as a signal for DNA damage response, kinase activation and vesical trafficking. Monoubiquitylation or multi-monoubiquitylation serves as a signal for protein targeting by sub-cellular localization, endocytosis and gene regulation (Komander and Rape, 2012).

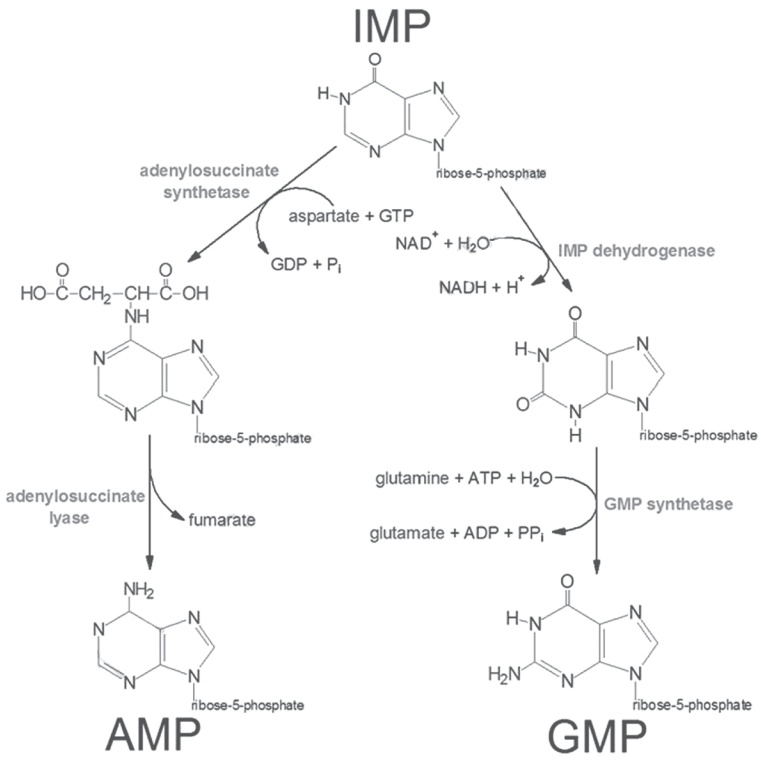
Guanine monophosphate synthetase (GMP *synthetase*)

GMP synthetase (EC 6.3.5.2) also known as GMPS, is a glutamine amidotransferase that catalyses the conversion of XMP to GMP, the final reaction in purine nucleotide biosynthesis in the *de novo* pathway, see *Scheme 1B*. GMPS contains N-terminal glutamine amidotransferase (GAT) domain with Cys-His-Glu as a catalytic triad for glutamine hydrolysis. The central ATP pyrophosphate (ATPP) domain is responsible for binding XMP and ATP, and catalyses the formation of O2-adenyl-XMP and PPi. The C-terminal dimerization domain (CTD) is essential for protein oligomerization, see *Scheme 1A* (Tesmer et al., 1996). The end products of purine metabolism such as AMP and GMP play a vital role in metabolic regulation and DNA biosynthesis. AMP and GMP are the precursors of ATP and GTP respectively, and are essential for DNA synthesis. Cyclic-AMP and cyclic-GMP act as secondary messengers and activate different protein kinases involved in cellular metabolism. Elevated levels of nucleotide biosynthetic enzymes were observed in rapidly growing

tissues and tumours. Therefore, nucleotide biosynthetic enzymes are considered prime targets for drug design for cancer (Weber, 1983). In contrast, a recent report suggests that nucleotide deficiency is the prime cause for the initiation of tumorigenesis. Oncogenic signals trigger cell proliferation without coordinate increase in nucleotide synthesis resulting in aberrant DNA replication and damage, contributing to carcinogenesis (Bester et al., 2011). Thus, nucleotide biosynthetic enzymes play a crucial role in the regulation of cell division by supplying nucleotides for error free DNA synthesis.

A)

B)



Scheme 1; A) Domain organisation of GMPS, GAT; glutamine amidotransferase domain, ATPP; ATP pyrophosphate domain and CTD; C-terminal dimerization domain
B) Schematic overview of the *de novo* purine nucleotide biosynthetic pathway. The end products are AMP and GMP. (adopted from, http://themedicalbiochemistrypage.org/nucleotide-metabolism.php)

In addition to functioning as a nucleotide synthetic enzyme, GMPS is also found in complex with the deubiquitinase *Ubiquitin Specific Protease 7* (USP7) in *Drosophila* (van der Knaap et al., 2005) and in mammalian cells (Sarkari et al., 2009). GMPS has been shown to stimulate the histone H2B deubiquitinase activity of USP7, a mechanism that is independent of GMPS nucleotide synthesis activity. Monoubiquitylation of histone H2A and H2B are implicated in gene expression regulation. In general, monoubiquitylation of histone H2B is associated with gene activation, whereas monoubiquitylation of H2A is associated with gene silencing. Therefore, GMPS/USP7, a histone H2B deubiquitinase complex, is implicated in the transcriptional repression of polycomb-responsive elements, ecdysone inducible genes and Epstein barr virus latent origin of replication (Frappier and Verrijzer, 2011; Sarkari et al., 2009; van der Knaap et al., 2010; van der Knaap et al., 2005).

Ubiquitin Specific Protease 7 (USP7)

USP7 also known as HAUSP and was originally identified as a binding partner of PML in nuclear bodies, herpes-simplex viral proteins, ICP0 (infected cell protein 0) and EBNA1 (Epstein-Barr nuclear antigen 1) (Everett et al., 1997b; Holowaty et al., 2003b; Meredith et al., 1994). USP7 structurally consists of an N-terminal TRAF domain, a central catalytic domain (CD) and a C-terminal ubiquitin like (Ubl) domain (*Figure 3A*). TRAF is a protein-protein interaction domain, CD is responsible for the deubiquitylation of proteins and the Ubl domain is essential for the regulation of USP7 catalytic activity (Faesen et al., 2012; Holowaty et al., 2003a). GMPS has been shown to activate USP7 catalytic activity by allosterically binding to the Ubl domain. USP7 consists of five Ubl's, Ubl4 and 5 are essential for activation of its catalytic activity. GMPS binds to Ubl1-3 and stimulates USP7 catalytic activity by stabilizing the Ubl4-5 dependent active state (Faesen et al., 2011). USP7 is implicated in the regulation of tumor suppressor p53 and E3 ubiquitin-ligase MDM2 stability by de-ubiquitylation (Cummins et al., 2004; Li et al., 2002). p53 and MDM2 competitively bind to the TRAF domain of USP7, MDM2 binds with a higher affinity to USP7 than p53 (Sheng et al., 2006). Death domain-associated protein, Daxx has been shown to form a complex with USP7 and MDM2. Daxx acts as a bridging factor between MDM2 and USP7 that result in MDM2 stabilization by deubiquitylation (Tang et al., 2006). EBNA1 binds to the TRAF domain of USP7 thereby preventing p53 binding. This results in destabilization of p53. Thus, EBNA1 favours cell survival in infected cells by lowering p53-mediated apoptosis (Saridakis et al., 2005).

USP7 is also implicated in regulation of tumor suppressors PML (*promyelocytic leukemia*), PTEN (*phosphatase and tensin homologue deleted in chromosome 10*) and INK4a. USP7 is found to be partially associated with PML nuclear bodies (PML-NB), which control several cellular functions including antiviral effects, cell cycle and apoptosis. EBNA1 binding to USP7 results in its stabilization which induces PML ubiquitylation and degradation (Everett et al., 1997a; Sarkari

et al., 2011; Sivachandran et al., 2008). Nuclear exclusion of tumor suppressor PTEN has been associated with cancer development. USP7 has been shown to induce the nuclear export of PTEN by deubiquitylation (Song et al., 2008). USP7 in complex with USP11 was shown to stabilize the mammalian polycomb repressive complex (PRC-1) subunits which results in initiation of PRC1-mediated silencing of the INK4a tumor suppressor gene (Maertens et al., 2010). USP7 has been shown to modulate oxidative stress. In response to oxidative stress, transcription factor FOXO (Forkhead box O) gets monoubiquitylated which induces its nuclear localization.

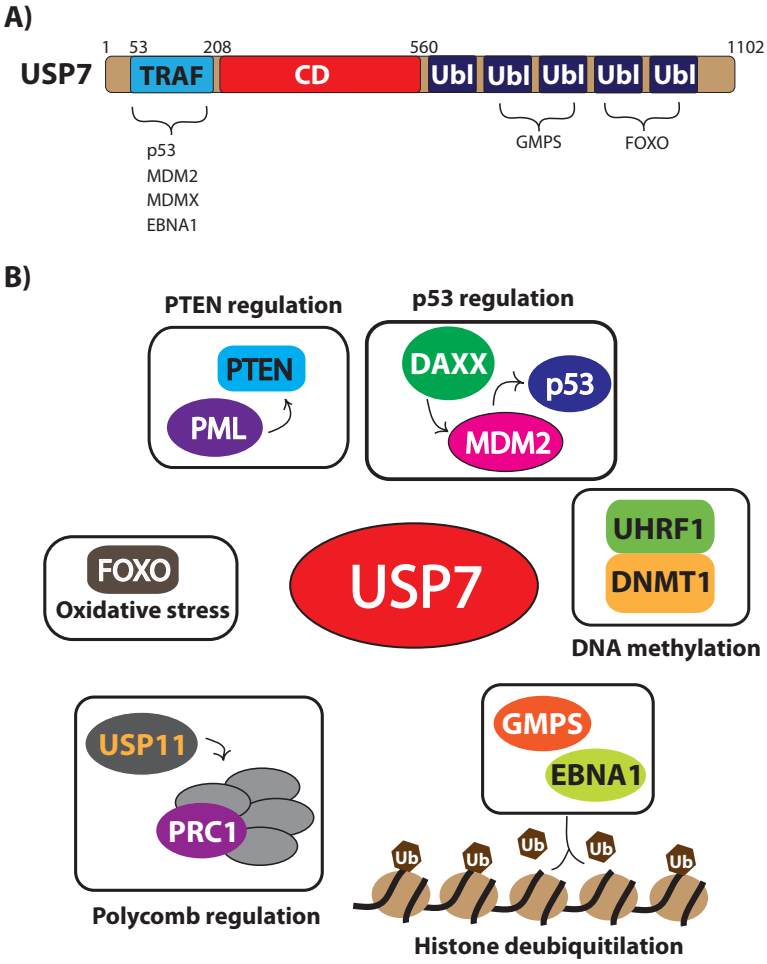


Figure 3; A) Domain organisation of USP7; TRAF (TNF-receptor associated factors) domain, CD (catalytic domain) and Ubl (ubiquitin like) domain.
B) Schematic representation of multifaceted roles of USP7 in the regulation of key tumor suppressors such as, p53, PTEN, PML and p16INK4a, oxidative stress and epigenetic regulation of gene expression.

USP7 deubiquitylates FOXO and negatively regulate its transcriptional activity (van der Horst et al., 2006). USP7 also has a role in epigenetic silencing through the modulation of DNA methylation. DNA methyltransferase 1 (DNMT1) is the primary enzyme that maintains DNA methylation. USP7 has been shown to stabilize DNMT1 by deubiquitylation. In colon cancers, overexpression of DNMT1 is correlated with expression of USP7 (Du et al., 2010). By stabilizing DNMT1, USP7 indirectly influences the increase of DNA methylation at the promoters of genes that results in silencing of expression of the downstream genes. For example, hyper-methylation of DNA at the *CDK2NA* locus which encodes two important tumor suppressors INK4a and ARF, resulted in the silencing of their expression in several cancers (Magdinier and Wolffe, 2001). Therefore, by negatively controlling tumor suppressors PML, PTEN, ARF and INK4a, USP7 actively participates in cancer development (*Figure 3B*).

Tumor suppressor p53/TP53

p53 is a sequence-specific DNA-binding transcription factor that functions in a complex signalling network to mediate cellular adaptation to stress (Levine, 1997). p53 mediates stress response to diverse cellular insults, such as oncogene activation, DNA damage, telomere erosion, metabolic stress, DNA replication stress, dysfunction of mitotic apparatus, hypoxia, and oxidative stress. Upon stress, p53 gets stabilized and activates the expression of genes that regulate cell cycle arrest, senescence, apoptosis, DNA repair, antioxidant production and metabolism. (Ryan et al., 2001; Vousden and Lane, 2007). Schematic representation of the mechanism of p53 activation and response is represented in *Figure 4*. p53 is also implicated in the regulation of diverse metabolic pathways including nucleotide biosynthesis and coordination of stress responses with changes in cell metabolism (Vousden and Prives, 2009). Recent reports suggest that alterations in cell metabolism plays a vital role in the cause of diseases such as cancer (Maddocks and Vousden, 2011). Early observations on altered energy metabolism in cancer cells were reported by *Otto Warburg* in 1920s, now popularly termed the "*Warburg effect*" (Warburg, 1956). p53 activation upon metabolic stress opposes the '*Warburg effect*' by inhibiting glycolysis and at the same time activating the oxidative phosphorylation pathway. Thus, in terms of metabolism, loss of p53 may provide a significant growth advantage to the cancer cells (Jones and Thompson, 2009). Therefore, either deletion of the p53 gene or downregulation of p53 protein levels or the loss of p53 function due to mutations in the DNA binding domain that abolishes its transcription function, altogether is the cause of more than 50% of the human cancers (Lane, 1992).

The tumor suppressive functional outcome is directly correlated to the p53 protein levels that are regulated by ubiquitylation, which is mainly catalysed by the E3 ubiquitin-ligase MDM2 (Oliner et al., 1992). USP7 has been shown to stabilize p53 by deubiquitylation in overexpression assays (Li et al., 2002). However, knockdown of USP7 does not decrease p53 levels as expected but rather than an increase in

p53 levels was observed. This is because of USP7's ability to stabilize MDM2 by deubiquitylation (Cummins et al., 2004). In fact, MDM2 was found to be a stronger binding partner for USP7 than p53 (Sheng et al., 2006). Thus, p53 stabilization by USP7 appears to be a rather complex process that still remains enigmatic. Stability of MDM2 is regulated by autoubiquitylation and proteasomal degradation. Therefore, under normal condition MDM2 is stabilized by USP7 and this in turn negatively regulates p53. Daxx has been shown to stabilize MDM2 by forming a complex with USP7. Upon induction of genotoxic stress, MDM2 disassociates from Daxx and USP7 resulting in its degradation by autoubiquitylation (Tang et al., 2006). Therefore, Daxx and USP7 negatively influence p53 stability through the stabilization of MDM2. While USP7 was shown to deubiquitylate p53 in the nucleus, recently USP10 has been reported as a deubiquitinase for p53 in the cytoplasm (Yuan et al., 2010).

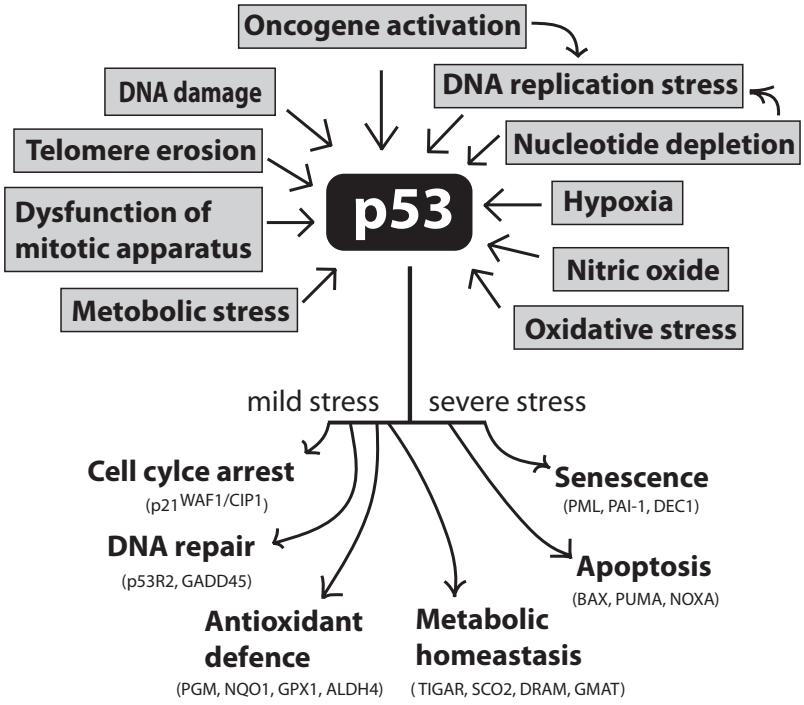


Figure 4; Mechanisms of p53 activation and response to the diverse cellular insults: p53 mediates the cellular adaptation to stress by transcriptional regulation of genes involved in cell cycle arrest (p21), DNA repair (p53R2, GADD45), antioxidant defence (PGM, NQO1, GPX1, ALDH4) and genes that regulates metabolic homeostasis (TIGAR, SCO2, DRAM, GMAT). Upon severe damage to the cell, p53 activates cell death pathways such as senescence (PML, PAI-1 and DEC1) and apoptosis (PUMA, BAX and NOXA), (Qian and Chen, 2010; Vousden and Prives, 2009).

Tripartite motif 21 (TRIM21)

TRIM (*Tripartite motif*) proteins are the RING finger containing E3 ubiquitin-ligases. The presence of a RING, B-box and coiled-coil motif (RBCC) is characteristic of TRIM family proteins. There are more than 70 TRIM proteins identified so far in humans and mice (*Table1*). TRIMs are broadly classified into eleven groups based on the presence of additional domains. TRIMs are involved in the regulation of broad range of biological processes and their alterations are associated with diverse disease conditions, such as viral infections, neurological disorders, cancers and autoimmune diseases (Hatakeyama, 2011). TRIM21 is also called RO52 or SSA1. It was originally identified as an autoantigen commonly found in patients with *Sjogren's syndrome*, a chronic autoimmune disease characterized by the dryness of eyes and mouth. TRIM21 was later characterized as a RING finger protein with intrinsic E3 ubiquitin-ligase activity (Wada and Kamitani, 2006). The known substrates for TRIM21-mediated ubiquitylation include IRF3, IRF5, IRF7 and IRF8. TRIM21 is reported to be predominantly present in the cytoplasm but upon cellular exposure to different reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), nitric oxide (NO), as well as type I interferon (INFa) it translocates to the nucleus. Thus, TRIM21 plays a crucial role in the inflammatory responses and is reported to be upregulated at the sites of autoimmune inflammation (Oke and Wahren-Herlenius, 2012).

Subgroup	Domain structure	TRIM family Members
G-I	R B1 B2 CC	TRIM8, TRIM19, TRIM31, TRIM40, TRIM52, TRIM56, TRIM61, TRIM73, TRIM74
G-II	R B1 B2 CC MATH	TRIM37
G-III	R B1 B2 CC ARF	TRIM23
G-IV	R B1 B2 CC FIL	TRIM45
G-V	R B1 B2 CC TM	TRIM13, TRIM59
G-VI	R B1 B2 CC PHD BROMO	TRIM24, TRIM28, TRIM33
G-VII	R B1 B2 CC FIL NHL	TRIM2, TRIM3, TRIM32, TRIM71
G-VIII	R B1 B2 CC COS ACID	TRIM54, TRIM55, TRIM63
G-IX	R B1 B2 CC COS FN3	TRIM42
G-X	R B1 B2 CC PRY SPRY	TRIM4, TRIM5, TRIM6, TRIM7, TRIM10, TRIM11, TRIM15, TRIM17, TRIM21, TRIM22, TRIM25, TRIM26, TRIM27, TRIM34. TRIM35, TRIM38, TRIM39, TRIM41, TRIM43, TRIM47, TRIM48, TRIM49, TRIM50, TRIM58, TRIM60, TRIM62 TRIM64, TRIM65, TRIM68, TRIM69, TRIM72, TRIM75, TRIML1
G-XI	R B1 B2 CC COS FN3 PRY SPRY	TRIM1, TRIM9, TRIM18, TRIM36, TRIM46, TRIM67

Table1: Classification and domain overview of TRIM family protein

TRIM21 is also implicated in cell cycle regulation through the ubiquitylation and degradation of cell cycle inhibitor p27 during S-phase progression (Sabile et al., 2006). TRIM21 also regulates stability of the transcription factor CCAAT/enhancer-binding protein alpha (C/EBPa) that functions in cell differentiation. Downregulation of C/EBPa by TRIM21was found to be a key mechanism in the development of lung cancer (Grandinetti et al., 2011). Elevated levels of TRIM21 antibodies were also found in the serum of patients with oesophageal squamous cell carcinoma and colon cancer. Presence of serum TRIM21 antibodies correlated with tumor size and the disease progression (Kuboshima et al., 2006). Melanoma antigen (MAGE) proteins were identified in complex with the TRIM E3 ubiquitin-ligases. MAGE family proteins are cancer/testis-antigens that are highly expressed in cancers and play a crucial role in the tumorigenesis. MAGE proteins were shown to stimulate the ubiquitin-ligase activity of TRIM E3 ubiquitin-ligases. For example, MAGE-C2 was shown to be the stimulator of TRIM28 E3 ubiquitin-ligase activity towards ubiquitylation and degradation of tumor suppressor p53 (Doyle et al., 2010).

ATP-dependent chromatin remodeling complexes

Chromatin remodelers are multi-subunit enzyme complexes containing an ATPase subunit to slide nucleosomes along DNA, to remove/exchange histones, or to alter histone-DNA contacts by using energy derived from ATP hydrolysis. Currently, there are four major classes of chromatin remodeling complexes SWI/SNF, INO80, ISWI and the Mi2 or CHD family. These classes are named after the core ATPase subunit. All the four classes of remodelers differ in their subunit composition, specialized for particular purpose and biological context. The functional diversity of remodelers is determined by the specialized domains present in the ATPase subunit and also by the domains of the associated unique subunits (Clapier and Cairns, 2009; Smith and Peterson, 2005). The ATPase subunit of SWI/SNF, Swi2 or Brahma or Brg1, contains a bromodomain and an AT-hook regions. Bromodomains selectively recognize the acetyl-histone tails and the AT-hook domain binds to AT-rich DNA (Mohrmann and Verrijzer, 2005). The ISWI, ATPase contains SANT and SLIDE domains. The SANT domain structurally related to the cMYB DNA-binding domain, binds to histone tails and the SLIDE domain mediates DNA interactions (Langst and Becker, 2001). INO80 consists of a helicase-SANT domain (HSA) that mediates histone binding (Watanabe and Peterson, 2010). The Mi2/CHD4 ATPase consists of tandem chromodomains that bind to histone tails, particularly methylated histones and nucleosomal DNA (Brehm et al., 2004). Although remodelers are classified into four major groups, several sub-complexes were reported based on the combinatorial assembly of subunits. Sub-complexes perform common as well as unique functions depending on the cell type, developmental stage and chromatin targeting (Clapier and Cairns, 2009). Here, we discuss in detail about the NuRD chromatin remodeling complex.

Composition and functions of the NuRD complex

Nucleosome Remodeling and Deacetylase (NuRD or Mi2) complex contains enzymatic subunits such as an ATPase, chromatin-helicase-domain 3 (CHD3, also called as Mi2α) and CHD4 (Mi2β or *Drosophila* Mi2/dMi2) and histone deacetylase 1 (HDAC1 or RPD3) and HDAC2 (Tong et al., 1998; Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998). Mi2/CHD4 was originally identified as an autoantigen found in the connective tissue disease dermatomyositis (Ge et al., 1995). Mi2 is a chromatin helicase containing an ATPase domain, chromodomains, PHD fingers and a putative DNA binding domain (Woodage et al., 1997). HDACs were identified originally as proteins closely related to yeast RPD3, a known regulator of gene expression. Biochemical characterization of these proteins revealed that they possess histone deacetylase activity (Taunton et al., 1996). Thus, at the time of discovery NuRD complex was found to be unique as it couples both chromatin helicase and histone deacetylase activities in the same complex. Since histone deacetylation is implicated in gene silencing, the NuRD complex is thought to be a transcription repressor complex. Recently, lysine specific demethylase 1A (LSD1 or KDM1A) was also identified as a subunit of the NuRD complex (Wang et al., 2009). LSD1 is a histone H3K4 demethylase implicated in transcriptional repression (Wang et al., 2009). Other non-enzymatic subunits include metastasis-associated gene 1 (MTA1), MTA2 and MTA3, methyl-CpG-binding domain 2 (MBD2 or MBD like-A) and MBD3 (MBD like-B) and retinoblastoma-binding protein 4 (RBBP4 or RBAP48 or p55/CAF1) and RBBP7 (RBAP46). GATA-like transcription factors, GATAD2A (p66a) or GATAD2B (p66b) were also found in complex with NuRD (Brackertz et al., 2002; Feng et al., 2002; Wade et al., 1999). GATAD2A, GATAD2B, RBBP4 and RBBP7 were shown to interact with histone tails and thought to be the structural components of NuRD (Brackertz et al., 2006; Marhold et al., 2004). MTA and MBD subunits are implicated in gene regulation by targeting the NuRD complex to different genomic loci through association with transcription factors or methylated DNA, respectively (Fujita et al., 2004; Hendrich and Bird, 1998). A schematic overview of the subunit composition of *Drosophila* NuRD and human NuRD is given in *Figure 5* and the domain organization of the subunits is displayed in *Table 2*.

The formation of alternate and sub-complexes by combinatorial assembly of subunits determines the functional specificity of the NuRD. For example, in *Drosophila,* Mi2 and MEP1 form a two-subunit complex called dMec. dMec is shown to be functionally different from classical NuRD complex (Kunert et al., 2009). Although it has been reported that NuRD subunits are expressed ubiquitously in different cell types, some paralogues of NuRD subunits are expressed specifically in certain tissue types. For example, CHD5, a brain-specific paralogue of CHD4 is found in a NuRD-like complex with similar functions as canonical NuRD (Potts et al., 2011; Thompson et al., 2003). Similarly, MTA family members were also found in alternate NuRD complexes. For example, MTA3 is a B cell specific paralogue of

MTA. MTA3 interacts with transcription factor BCL6 and targets NuRD to different genomic loci to regulate B cell differentiation (Fujita et al., 2003). MBD2 and MBD3 were also shown to form mutually exclusive NuRD complexes with different biochemical and functional properties (Le Guezennec et al., 2006). MBD2 has been shown to bind methylated DNA through its MBD domain and this function of MBD2 is evolutionarily conserved throughout higher eukaryotes. In contrast, MBD3 cannot bind to the methylated DNA because of amino acid mutation in its MBD domain. Instead, the MBD domain of MBD3 is shown to participate in protein-protein interactions (Hendrich and Tweedie, 2003; Saito and Ishikawa, 2002). Some of the NURD subunits were also found in multiple protein complexes. For example, HDAC1 and HDAC2 were found in the Sin3A and CoREST complexes as enzymatic subunits (Grozinger and Schreiber, 2002; Yang and Seto, 2008; You et al., 2001). It remains unclear whether these multiple HDAC complexes are targeted to different genomic loci or to the same genomic loci to work synergistically. Some NuRD subunits were also reported in complexes other than NuRD. For example, RBBP4 and RBBP7 were found in a complex with histone chaperones. They act as structural components to provide stability to the complex by protein-protein interactions (Loyola and Almouzni, 2004).

Subunit	Domain structure	Description
Mi2 / CHD3, CHD4, CHD5	PHD PHD CHROMO CHROMO ATPase	CHD3, CHD4 and CHD5 core subunit consits of PHD fingers for recognition of histone modification. The chromo domains display DNA binding activity and ATPase domain carry ATP hydrolysis required for chromatin remodeling
KDM1A	SWIRM Oxidase Oxidase	Histone H3 K4 demethylase, contains SWIRM, helix loop helix domain and binds to DNA. Oxidase domain mediates demethylation.
MTA1, MTA2, MTA3	BAH ELM SANT Zn	MTA contains BAH domain required for protein-protein interactins, SANT and Zinc finger domain for DNA binding The function of ELM domain is not known.
p66/68 / GATA2A, GATA2B	MBDbd Zn	GATA transcription factors contains MBD binding domain and Zinc finger interacts with histones and recognises specific DNA elements
RPD3 / HDAC1, HDAC2	CD RB binding	Histone deacetylases remove acetyl mark from histones, involved in control of gene expression. Also contains RB binding domain at C-terminus.
p55 / RBBP4, RBBP7	WD WD WD WD WD WD	Histone binding proteins with WD repeats binds to histone H4
MBD (A) / MBD2	GR MBD TRD	MBD2 binds to methylated DNA and contains GR repeats and TRD domain that reqruits HDACs
MBD (B) / MBD3	MBD E	MBD domain of MBD3 does not bind to binds to methylated DNA. MBD3 contains glutamic acid (E) repeats at C-terminus

Table 2; Domain overview of sub units of the NuRD complex

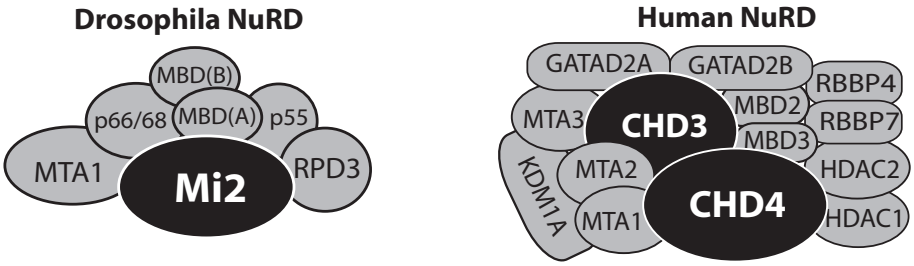


Figure 5; Cartoon representing the subunit composition of Drosophila NuRD and human NuRD complex.

Chromatin remodeling and gene regulation by NuRD complex

NuRD is a unique chromatin remodeling complex comprising of different chromatin directed enzymatic activities, such as chromatin helicase activity, histone deacetylation and histone demethylation. By combining these three enzymatic activities NuRD plays a crucial role in the control of gene expression. The fundamental property of the chromatin remodeler is to mobilize the nucleosomes by utilizing energy derived from ATP hydrolysis. The outcome of remodeling could be equal spacing of nucleosomes or disassembly and repositioning of nucleosomes (Hota and Bartholomew, 2011). The ATPase activity of Mi2 is stimulated by nucleosomes but not by free histones or by free DNA. Both *in vitro* and *in vivo* studies have demonstrated that Mi2 pulls the nucleosomes towards the center of its binding site on the chromatin (Brehm et al., 2000; Moshkin et al., 2012). Nucleosome remodeling by Mi2/CHD4 has been implicated in Pol-I mediated transcriptional activation of rRNA genes (Xie et al., 2012). However, the precise role of chromatin helicase activity of NuRD in Pol-II mediated transcription remains unclear. Histone deacetylation by HDACs leads to stabilization of histone-DNA contacts which results in compact chromatin and repression of gene expression (Bowen et al., 2004; Lai and Wade, 2011). LSD1 is a histone H3K4 specific demethylase (Shi et al., 2004). H3K4 methylation is considered to be hallmark of transcription activation. Therefore, H3K4 demethylation by LSD1-NuRD results in transcriptional repression (Wang et al., 2009). Thus, NuRD couples the histone deacetylation and histone H3K4 demethylation activities for silencing its target genes. Other than having enzymatic subunits, MDB2 has been shown to bind methylated DNA to establish repressive chromatin state (Zhang et al., 1999). The combination of enzymatic and non-enzymatic subunits of NuRD favors the formation of repressive chromatin that results in silencing of gene expression (A schematic representation of the transcriptional roles of NuRD is given in *Figure 6*).

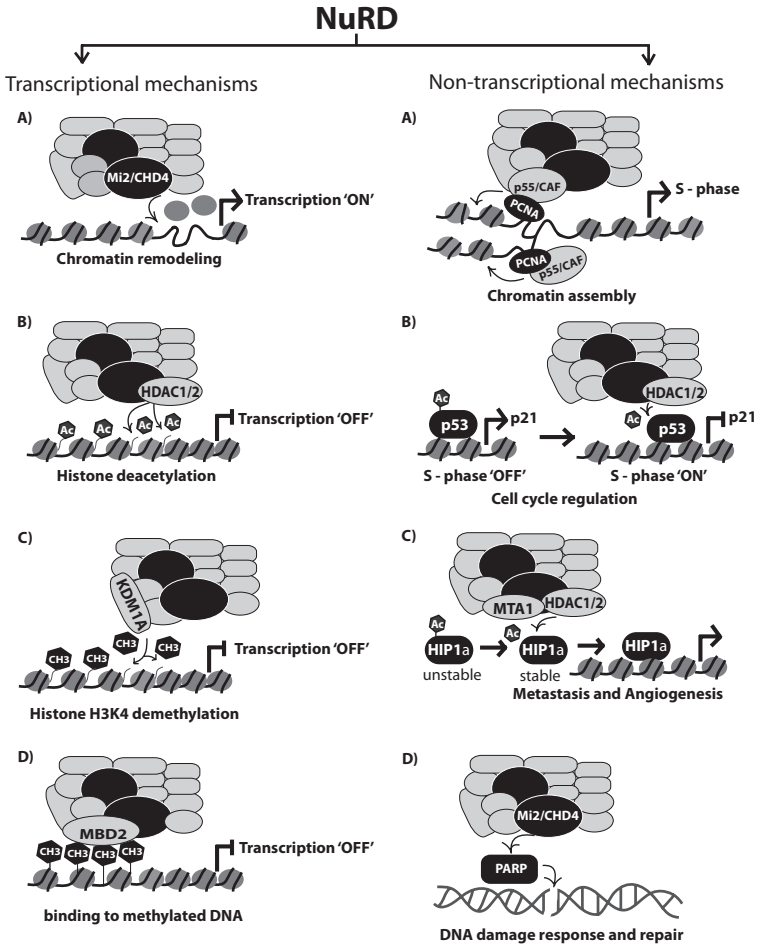


Figure 6; Schematic representation of transcriptional and non-transcriptional roles of NuRD complex

Non-transcriptional roles of NuRD complex

The NuRD complex is also implicated in chromatin assembly, cell cycle regulation, maintenance of genome stability and aging (Chou et al., 2010; Pegoraro et al., 2009; Polo et al., 2010; Smeenk et al., 2010). For example, the p55 (CAF1 or RBBP4) subunit assembles chromatin in a replication-dependent manner during the S-phase of cell division (Verreault et al., 1996). NuRD is found to be associated with the pericentric heterochromatin during S-phase of the cell cycle in rapidly proliferating cells. This association is linked to the DNA replication and chromatin assembly. Therefore, the p55-NuRD complex is thought to be involved in assembly of chromatin during S-phase (Helbling Chadwick et al., 2009). NuRD also has a role in DNA damage response and cell cycle control. Poly ADP-ribose polymerase (PARP) binds to CHD

to recruit NuRD to the sites of DNA damage for efficient repair of the DNA double strand breaks (Polo et al., 2010). HDAC1, a subunit of NuRD, was shown to deacetylate p53 which is essential for the transition of G1/S phase of the cell cycle (Polo et al., 2010). Knockdown of CHD4 and MTA2 leads to accumulation DNA double strand breaks (DSB) and the cells become sensitive to ionizing radiation. It has been shown that CHD4 mediates DSB repair and check point activation in response to ionizing radiation (Smeenk et al., 2010). Therefore, NuRD plays a crucial role in DNA damage response and repair. Loss of several of the NuRD subunits is found to be associated with the premature aging. Silencing of the individual NuRD subunits resulted in chromatin defects associated with aging through the accumulation of DNA DSBs. Therefore, NuRD is considered to be essential for the maintenance of chromatin structure to prevent the DNA damage and premature aging (Pegoraro et al., 2009). The HDAC1 subunit of NuRD has been shown to stabilize HIF-1a (*hypoxia inducible factor 1 alpha*) by deacetylation. Stabilization of HIF-1a is found to be the key factor in initiation of tumor angiogenesis (Yoo et al., 2006). Thus, NuRD plays a crucial role in regulation of chromatin assembly, genome stability, aging and angiogenesis (see *Figure 6* for an overview of non-transcriptional roles of the NuRD complex).

Role of NuRD complex in development and disease

Several NuRD subunits play a significant role in normal development of the organism. For example, in *C. elegans*, MEP1 in association with LET-418 (orthologue of Mi2) and HAD-1 (orthologue of HDAC1) are essential for the maintenance of germ line-soma distinctions (Unhavaithaya et al., 2002). NuRD also plays an important role in the differentiation of haematopoietic stem cells into lymphoid and myeloid cells (Yoshida et al., 2008). NuRD is also required for development of T-lymphocytes (Williams et al., 2004). MBD3-NuRD is found to be essential for the maintenance of pluripotency and initiation of differentiation programme in embryonic stem cells (Kaji et al., 2006; Zhu et al., 2009). Knockout and transgenic mouse models of NuRD subunits reveal that it has a role in normal developmental processes. For example, the *Mbd3* knockout mouse is embryonic lethal, in contrast to *Mbd2* knockout mice that are viable with mild defects (Hendrich and Tweedie, 2003). NuRD also plays a crucial role in peripheral nerve myelination by Schwann cells. Mice with CHD4 knockout in Schwann cells showed delayed hypomyelination and persistence of promyelinating Schwann cells (Hung et al., 2012). Thus NuRD plays an important role at different developmental stages of an organism.

Mi2 was originally identified as an autoantigen found in patients with dermatomyositis (Ge et al., 1995). About 15-25% of patients with dermatomyositis eventually develop malignancies (Callen and Wortmann, 2006). However, the link between presence of Mi2 autoantibodies and development of cancer remains unclear. MTA family members have been implicated in the development of several cancers, including breast cancer (Kumar et al., 2003; Toh and Nicolson, 2009; Zhang

et al., 2006). Overexpression of MTA1 is observed in tumors derived from several tissue origins in humans, including breast, colon, gastric, endometrial, esophageal, pancreatic, ovarian, prostate, liver, B-lymphocytes and non-cell-lung cancer (Nicolson et al., 2003). MTA1-NuRD is a potent repressor of oestrogen receptor (ER)-dependent transcription resulting in disruption of oestrodial responsiveness, thus contributing to progression to more invasive forms of breast cancer (Mazumdar et al., 2001). More specifically, MTA1-NuRD represses the expression of tumor-suppressor BRCA1 which results in chromosomal instability and development of malignancy (Molli et al., 2008). In contrast, MTA3-NuRD acts as a tumor suppressor in ER-negative breast cancers. MTA3 is induced in an oestrogen-dependent manner and represses the expression of transcriptional repressor Snail, a key promoter of epithelial-to-mesenchymal transition (EMT). Therefore, absence of either ER or MTA3 results in aberrant expression of Snail which results in repression of the cell adhesion protein E-cadherin. Loss of E-cadherin expression is the cause for EMT and invasive growth in breast tumors (Fujita et al., 2003). Therefore, the balance between MTA1-NuRD and MTA3-NuRD complexes might be a determining factor in breast cancer development.

Aberrant DNA methylation at gene promoters is the most commonly observed phenomenon in human cancers. Promoter hyper-methylation leads to repression of downstream target genes, such as key tumor suppressor genes including Retinoblastoma protein (RB), INK4a and BRCA1 (Costello et al., 2000; McCabe et al., 2009). The mechanism of gene silencing by promoter hyper-methylation involves the recruitment of MBD proteins and their associated repressor complexes leading to formation of a repressive chromatin state (Zhang et al., 1999). MBD2 was found at the hyper-methylated promoter region of the *CDK2NA* locus which encodes the tumor suppressors ARF and INK4a. Expression of these proteins is repressed in colon cancers (Magdinier and Wolffe, 2001). Consistent with this observation MBD2 has been shown to recruit the co-repressor complexes to the promoters of hyper-methylated DNA for efficient initiation of tumorigenesis in the mouse intestine. Systemic deletion of *Mbd2* results in the suppression of tumor formation in these colon cancer mouse models (Sansom et al., 2003). Some of the NuRD subunits are found deleted in certain type of cancers. For example, CHD5, a brain specific paralouge of Mi2/CHD4 is present at chromosome 1p36. Deletion of 1p36 locus is the most frequent genetic lesion in human cancers of epithelial, neural and hematopoietic origin. In mouse models, CHD5 suppresses the tumor growth by activating the ARF/p53 pathway in mouse models (Bagchi et al., 2007). NuRD as a complex has the potential to either induce or suppress the development of tumors by forming alternate complexes. Therefore, understanding the composition and functional diversity of the NuRD complex might shed light towards identifying therapeutic targets for cancer.

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Chapter 2

Metabolic Enzyme GMP Synthetase is a TRIM21-controled Relay of Stress-induced stabilization of p53

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Summary

Functional outcomes of tumor suppression by p53 are tightly coupled to its nuclear levels, which are controlled predominantly through ubiquitylation by MDM2 and deubiquitylation by ubiquitin specific protease 7 (USP7). Here, we show that biosynthetic enzyme guanosine 5'-monophosphate synthetase (GMPS) is required for stress-induced stabilization of p53. GMPS predominantly resides in the cytoplasm, but following genotoxic- or replicative stress GMPS accumulates in the nucleus. Here, it drives the formation of a GMPS-USP7-p53 complex, in which p53 is deubiquitylated and stabilized. Stabilization of endogenous p53 by USP7 requires GMPS. The cytoplasmic E3 ubiquitin-ligase TRIM21 controls GMPS subcellular localization, and depletion of TRIM21 leads to GMPS-dependent induction of p53. Normally, TRIM21 binds and ubiquitylates GMPS to retain it in the cytoplasm, but stress triggers the release of GMPS and its nuclear accumulation. Our results delineate a TRIM21-GMPS-USP7 molecular cascade that mediates p53 stabilization in response to cellular stress.

Introduction

p53 is a sequence-specific transcription factor that is a central regulator of cellular homeostasis and tumor suppression (Lane and Levine, 2010). p53 can direct a variety of responses ranging from cell cycle arrest, cellular senescence, cell death, cell survival, DNA repair and metabolic adaptation. Reflecting this myriad of cellular outcomes, p53 itself is regulated at multiple levels, involving a plethora of different post-transcriptional modifications (Bieging and Attardi, 2012; Brown et al., 2009; Jayaraman and Prives, 1999; Maddocks and Vousden, 2011; Sullivan et al., 2012; Vogelstein et al., 2000). The levels of p53 expression need to be tightly controlled as loss of p53 predisposes to cancer, but over-expression of p53 can promote accelerated aging or even lethality. Normally, p53 protein levels are kept low as a consequence of its high turnover due to continuous ubiquitylation followed by proteasomal degradation. Upon cellular stress there is a dramatic drop in p53 ubiquitylation, leading to its accumulation (reviewed in Brooks and Gu, 2011; Coutts et al., 2009; Hock and Vousden, 2010).

Briefly, the RING domain protein MDM2 is the major E3 ubiquitin-ligase for p53 (Kubbutat et al., 1997; Hock and Vousden, 2010). MDM2 mediates lysine 48-linked polyubiquitylation of p53, targeting it for proteasomal degradation. In addition, monoubiquitylation by MDM2 stimulates its nuclear export to the cytoplasm. Studies in mice have established that MDM2 and p53 functions are intricately linked, and that the E3 ligase activity of MDM2 is crucial for *in vivo* control of p53 (Itahana et al., 2007; Montes de Oca Luna et al., 1995; Ringshausen et al., 2006). Stabilization of p53 following cellular stress involves both a loss of ubiquitylation

by MDM2 and its deubiquitylation. USP7 (a.k.a. HAUSP) has emerged as the major deubiquitylating enzyme (DUB) controlling the MDM2-p53 pathway. Originally, cotransfection over-expression studies showed that USP7 can bind and stabilize p53 by deubiquitylation (Li et al., 2002). Paradoxically, knockout of the *USP7* gene or RNAi-mediated depletion of endogenous USP7 also resulted in p53 stabilization (Cummins et al., 2004; Li et al., 2004). In the absence of deubiquitylation by USP7, MDM2 becomes unstable and is degraded, leading to reduced p53 ubiquitylation. Thus, USP7 plays a central, but dualistic, role in the regulation of p53. Complicating matters further, USP7 regulates other proteins that cross-talk with p53, and its activity is regulated by a variety of factors (Epping et al., 2011; Frappier and Verrijzer, 2011; Khoronenkova et al., 2012; Meulmeester et al., 2005; Saridakis et al., 2005; Sarkari et al., 2009; Song et al., 2008; van der Knaap et al., 2010; van der Knaap et al., 2005).

One intriguing partner protein that stimulates USP7 activity is the nucleotide biosynthetic enzyme GMPS (van der Knaap et al., 2005). GMPS mediates the final step of the *de novo* synthesis of guanine nucleotides, converting xanthosine 5'-monophosphate (XMP) into GMP, a building block of DNA and RNA. Purification of *Drosophila* USP7 identified GMPS as its major associated protein (van der Knaap et al., 2005). Biochemical and genetic evidence established that GMPS strongly stimulates histone H2B deubiquitylation by USP7 in *Drosophila* and human cells (Sarkari et al., 2009; van der Knaap et al., 2005; van der Knaap et al., 2010). GMPS-USP7 can be targeted to specific genomic loci to direct gene expression. For example, GMPS-USP7 binds Polycomb response elements and enhances Polycomb-directed silencing of homeotic genes *in vivo* (van der Knaap et al., 2005). In addition, GMPS-USP7 mediates H2B deubiquitylation and transcription repression at ecdysteroid regulated genes (van der Knaap et al., 2010). GMPS stimulates USP7 activity independent of its own catalytic activity (van der Knaap et al., 2010; van der Knaap et al., 2005). GMPS activates USP7 allosterically by inducing a conformational change (Faesen et al., 2011). In summary, GMPS does double duty as a biosynthetic enzyme and as a USP7-associated chromatin regulator.

To maintain homeostasis, cells have to coordinate their level of nucleotide synthesis and proliferation (Tong et al., 2009). GMPS's close functional link with USP7 made us wonder if it may also be involved in p53 regulation. Suggestively, we found previously that *Drosophila* GMPS *in vitro* can stimulate deubiquitylation of p53 by USP7 (van der Knaap et al., 2005). However, the physiological relevance of this observation remains unclear. Here, we show that GMPS is part of a critical switch that controls p53 induction. USP7-mediated deubiquitylation- and stabilization of p53 is strictly dependent on GMPS, which is regulated by the E3 ubiquitin-ligase TRIM21. Thus, GMPS, a biosynthetic enzyme associated with cell proliferation, plays a crucial role in regulation of the p53 tumor suppressor.

Results

GMPS binds USP7 and p53

To identify GMPS-associated factors in human cells, we immunopurified endogenous GMPS from whole cell extract (WCE) prepared from U2OS cells (*Figure 1A*). In addition, we expressed a flag-tagged version of GMPS in HEK-293T cells, followed by protein isolation from WCE with anti-flag antibodies (*Figure 1B*). Purified proteins were resolved by SDS polyacrylamide electrophoresis (SDS-PAGE) and visualized by coomassie staining. Protein identities were determined by massspectrometric analysis, which uncovered the association of both endogenous GMPS and flag-GMPS with USP7 and p53. In addition, we identified nucleotide biosynthetic enzymes inosine monophosphate dehydrogenase 2 (IMPDH2) and CAD, the DUB USP11, the MRN complex that is involved in DNA repair, and TRIM21, a RING domain protein that belongs to the tripartite (TRIM) family. A full overview of GMPS-associated proteins is provided in *Table S1*.

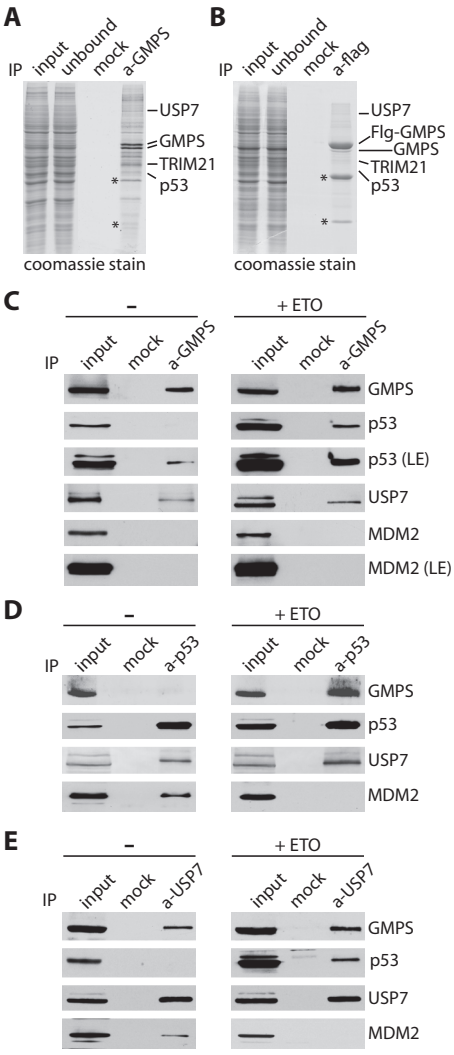

Figure 1; GMPS interacts with USP7 and p53 (**A**) GMPS-associated proteins immunopurified from U2OS whole cell extracts (WCE). Cell extracts were incubated with Protein A Sepharose beads coated with either control anti-GST (mock) or affinity purified anti-GMPS antibodies. Input, unbound material and IPed proteins were resolved by SDS-PAGE and visualized by coomassie staining. GMPS-associated proteins were identified by nanoflow LC-MS/MS mass spectrometry. A full list of GMPS-bound proteins is provided in *Table S1*. * indicates background proteins and immunoglobins. (**B**) IPs using anti-flag antibodies were performed using WCEs prepared from 293T cells transiently transfected with a vector expressing flag-GMPS. Analysis as described above. (**C-E**) Genotoxic stress triggers the formation of a GMPS-USP7-p53 complex. CoIPs of GMPS (**C**), p53 (**D**) or USP7 (**E**) from WCEs prepared from U2OS cells that were either mock-treated or incubated with 10 μM etoposide (+ETO). Mock indicates IP with anti-GST antibodies. Associated proteins were resolved by SDS-PAGE and analyzed by immunobloting with antibodies against the proteins indicated.

Next, we investigated the effect of genotoxic stress on the interactions between GMPS, USP7 and p53. We immunoprecipitated (IPed) these proteins from WCEs from U2OS cells that were either mock treated (-) or incubated with the DNA-damaging drug etoposide (+ETO). Protein immunoblotting revealed a dramatic change in the pattern of protein-protein interactions after addition of etoposide (*Figure 1C-E*). In the absence of ETO, p53 is predominantly associated with MDM2, which in turn is bound by USP7. GMPS binds USP7, but does not associate substantially with p53. Long exposure (LE) of the blots revealed weak binding of GMPS to p53. We could not detect any interaction between GMPS and MDM2, either by mass spectrometry or by immunoblotting analysis. Upon addition of etoposide, p53 dissociated from MDM2 and instead bound GMPS and USP7. Like p53, USP7 no longer bound MDM2. Thus, genotoxic stress induces the transfer of p53 from an ubiquitylating enzyme, MDM2, to a complex comprising GMPS and the DUB USP7.

GMPS is required for p53 stabilization

To test their role in the regulation of p53 protein levels, we used shRNAs to deplete cellular USP7 or GMPS. We monitored endogenous p53 by immunoflurescence using highly specific antibodies (*Figure 2A and Figure S1A-F*). In non-stressed cells, loss of USP7 caused an increase in p53 levels. This is consistent with the notion that the main effect of USP7 in these cells is to bind and stabilize MDM2, thereby promoting p53 degradation (Cummins et al., 2004; Li et al., 2004). In contrast, knockdown of GMPS gave a modest reduction in the level of p53. As expected, addition of etoposide caused a strong increase in p53 levels. The induction of p53 was completely reversed by knockdown of either GMPS or USP7. Moreover, a substantial portion of the remaining p53 in these cells appears to be cytoplasmic. These observations were confirmed by protein immunoblotting analysis of cell extracts (*Figure 2B*). Confirming results of others (Li et al., 2002; Maertens et al., 2010), knockdown of USP11, which also binds GMPS, did not affect p53 levels (data not shown). We note that the high level of mutant p53 protein in MDA-231 cells was not affected by either GMPS or USP7 depletion (*Figure S1G-H*). Thus, the inherent stability of mutant p53 does not appear to depend on GMPS and USP7. We conclude that GMPS is required for the induction of wild type p53 upon genotoxic stress. USP7 plays a dualistic role: in unstressed cells it is a negative regulator of p53, whereas after genotoxic stress USP7 stabilizes p53.

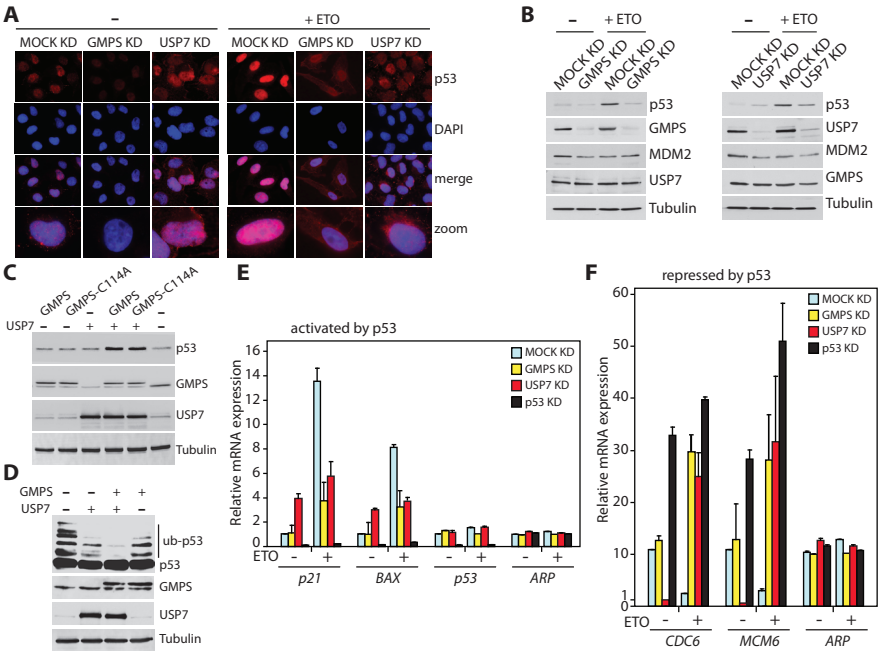

Figure 2; GMPS and USP7 are required for p53 induction after genotoxic stress
(A) Indirect immunofluorescence of U2OS cells, cultured in either the absence (-) or presence of 10 μM etoposide (+ETO), after knockdown (KD) of GMPS or USP7. Cells were fixed and stained with antibodies directed against p53 (red). Nuclei were visualized by DAPI staining of DNA (blue). Images were capture by using fluorescent microscope. (**B**) Immunobloting analysis of WCEs of cells treated as described above, using the indicated antibodies. Tubulin serves as a loading control. (**C**) U2OS cells were transfected with vectors expressing flag-GMPS, flag-GMPS^{C114A} or HA-tagged USP7 (HA-USP7) in the indicated combinations. Endogenous p53 and the transfected proteins were detected by immunoblotting with the indicated antibodies. (**D**) GMPS stimulates deubiquitylation of p53 by USP7. H1299 cells were transiently transfected with vectors expressing p53, MDM2 and His$_6$-ub in combination with HA-USP7 or flag-GMPS in the indicated combinations. After 48 hours, cells were treated with the proteasome inhibitors MG132 (50 μM) and Lactacystine (5μM) for 4 hours. After preparation of WCEs, proteins were detected by immunoblotting with the indicated antibodies. (**E**) Depletion of GMPS or USP7 affects p53 target gene expression. Following lentiviral shRNA-mediated knockdown of GMPS, USP7 or p53 and etoposide treatment, as described above, total RNA was isolated and gene expression determined by RT-qPCR. GAPDH mRNA levels were used for normalization. Changes in expression are represented as fold change relative to mock-treated cells. p21 and BAX are activated by p53, ARP is a p53-independent control gene. Note that p53 mRNA levels are not affected by GMPS or USP7 depletion. Mean and standard deviations were derived from three independent biological replicates. (F) Analysis of CDC6 and MCM6 expression, genes that are repressed by p53.

These results can be explained by our protein-protein interaction analysis that showed that genotoxic stress induces the transfer of p53 from MDM2 to GMPS-USP7 (*Figure 1*). When bound by GMPS-USP7, p53 can be deubiquitylated and stabilized (*Figure 2C*). The enzymatic activity of GMPS is not required, because the

catalytically impaired mutant GMPS^{C114A} also mediated p53 stabilization by USP7. This result is reminiscent of our observation that histone H2B deubiquitylation by USP7 required GMPS but not its enzymatic activity (van der Knaap et al., 2010; van der Knaap et al., 2005), and supports an allosteric mechanism of USP7 activation by GMPS (Faesen et al., 2011). In agreement with earlier *in vitro* results using *Drosophila* GMPS (van der Knaap et al., 2005), co-transfection experiments showed that human GMPS stimulates p53 deubiquitylation by USP7 (*Figure 2D*).

p53 is a transcription factor that elicits its cellular response by controlling gene expression. Therefore, we determined the effect of GMPS- or USP7 depletion on the expression of a number of p53 target genes involved in cell cycle control or apoptosis. Reverse transcription quantitative PCR (RT-qPCR) revealed that knockdown of GMPS or USP7 caused a reduced induction of p21 and BAX after addition of etoposide (*Figure 2E*). Whereas loss of GMPS did not significantly affected p53 target gene expression in unstressed cells, knockdown of USP7 caused increased expression of p21 and BAX. Thus, the effects on transcription are consistent with the observed changes in p53 levels. Examination of two genes that are repressed by p53, CDC6 and MCM6, yielded a similar result. Knockdown of USP7 or GMPS caused a dramatic loss of p53-dependent transcriptional repression. In the absence of etoposide, loss of USP7 caused a loss of CDC6 and MCM6 expression (*Figure 2F*). The expression of a p53-independent control gene, ARP, remained unaffected by knockdown of GMPS, USP7 or p53. Importantly, neither GMPS- nor USP7 depletion affected p53 mRNA levels, confirming that they control p53 post- translationally (*Figure 2E*). We conclude that changes in the expression of these p53 target genes reflect the effects of GMPS and USP7 on p53 protein levels.

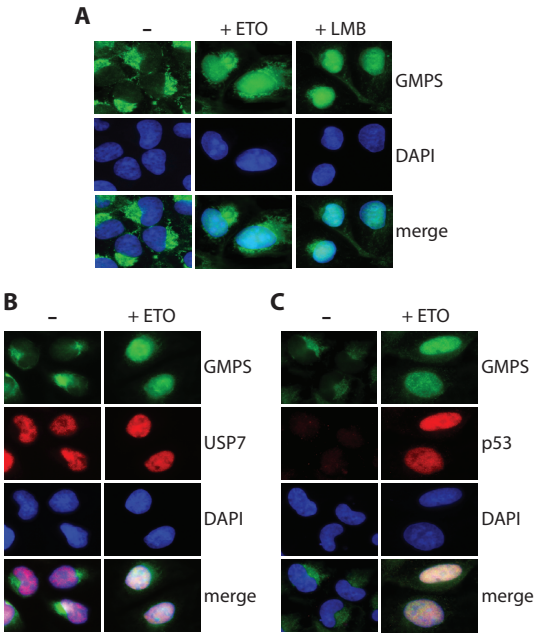

Figure 3; Genotoxic stress induces nuclear accumulation of GMPS
A) Indirect immunofluorescence of U2OS cells that were either untreated (-), treated with 10 μM etoposide (+ETO) for 24 hours, or with 50 nM Leptomycin B (LMB) for 5 hours. Cells were stained with antibodies against GMPS (green), and nuclei were visualized by DAPI staining of DNA (blue). (**B**) Costaining of GMPS (green) and USP7 (red). (**C**) Costaining of GMPS (green) and p53 (red). Images were capture by using fluorescent microscope.

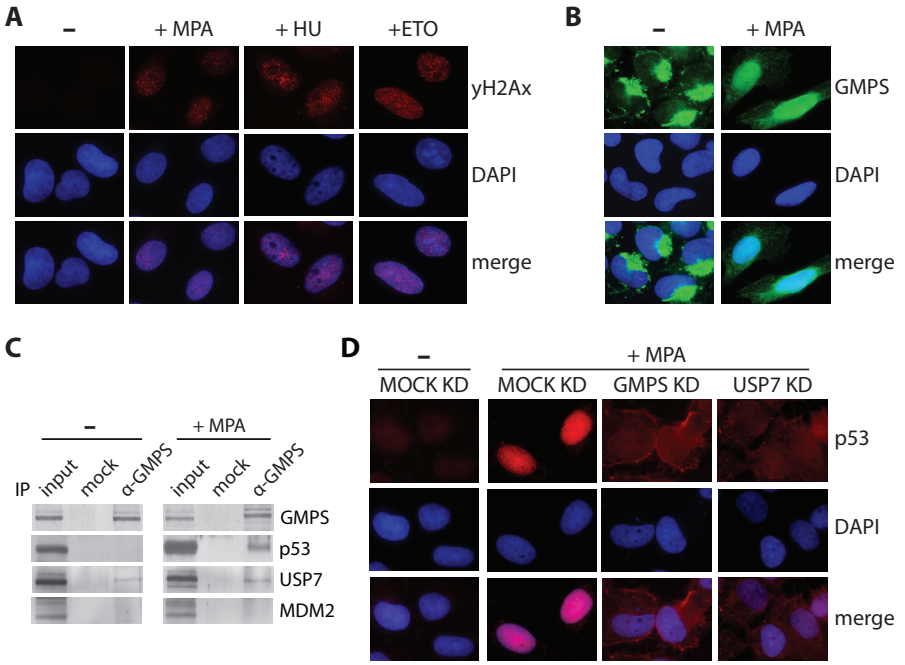

Figure 4; GMPS is required for p53 induction in response to nucleotide depletion
(**A**) Blockage of GMP synthesis by Mycophenolic acid (MPA) leads to the formation of γH2Ax foci. Indirect immunofluorescence of U2OS cells were either untreated (-), treated with 5 μg/ml MPA, 4 mM hydroxy urea (HU) or 10 μM etoposide (+ETO). Cells were stained with antibodies against γH2Ax (red), and nuclei were visualized by DAPI staining of DNA (blue). Images were capture by using fluorescent microscope. (**B**) MPA induces accumulation of nuclear GMPS. Cells cultured in the absence (-) or presence of 5 μg/ml MPA were stained with antibodies against GMPS (green). Images were capture by using fluorescent microscope. (**C**) Replicative stress drives formation of a GMPS-USP7-p53 complex. CoIPs of GMPS from WCEs prepared from cells that were cultured in the absence (-) or presence of 5 μg/ml MPA. (**D**) GMPS and USP7 are required for p53 induction by MPA. Indirect immunofluorescence of p53 (red) in U2OS cells cultured in the presence of 5 μg/ml MPA, following knockdown (KD) of GMPS or USP7. Images were capture by using fluorescent microscope.

Genotoxic stress induces the accumulation of nuclear GMPS

We recently established that cytoplasmic-nuclear partitioning is part of the mechanism by which nucleotide biosynthetic enzyme IMPDH regulates transcription of its target genes (Kozhevnikova et al., 2012). This observation prompted us to investigate the subcellular localization of GMPS in the absence or presence of etoposide. Immunofluorescence with antibodies directed against GMPS (green), in parallel with DAPI staining of DNA (blue), showed that the majority of GMPS is normally cytoplasmic (*Figure 3A*). In the presence of etoposide, however, there is a strong increase in the amount of nuclear GMPS. Addition of the nuclear export inhibitor Leptomycin B (LMB) to unstressed cells caused a transfer of GMPS from the cytoplasm to the nucleus. This result suggests that GMPS shuttles in and out of the nucleus, but that active export normally outcompetes import. Upon

genotoxic stress, this balance shifts, resulting in the accumulation of GMPS in the nucleus. Here, GMPS can bind USP7 (red, *Figure 3B*) and p53 (red, *Figure 3C*), which are both nuclear proteins. These results suggest that induction of p53 by genotoxic stress may be controlled by the subcellular localization of GMPS.

GMPS mediates the p53 response to replicative stress

A recent study suggested that nucleotide deficiency promotes genomic instability in early stages of cancer development due to DNA replication stress (Bester et al., 2011). An attractive idea is that GMPS not only catalyzes a key step in guanine nucleotide synthesis, but in case of deficiencies in this process, also mediates a p53 response. To test this hypothesis, we treated cells with Mycophenolic acid (MPA), an inhibitor of IMPDH. IMPDH is the enzyme directly upstream of GMPS that catalyzes the oxidation of IMP, generating XMP, the substrate of GMPS. The depletion of guanine nucleotides caused by MPA leads to replicative stress, as indicated by the formation of γH2Ax foci (*Figure 4A*). This effect was similar to the well-established response to the inhibition of ribonucleotide reductase by hydroxy urea (HU), which also causes replicative stress or the induction of DNA breaks by etoposide.

Next, we tested if GMPS subcellular localization changed in response to depletion of its substrate XMP. Immunoflurescence showed that the addition of MPA caused a dramatic increase in the amount of nuclear GMPS (*Figure 4B*). Co-IPs confirmed the association of GMPS with p53 and USP7 following the addition of MPA (*Figure 4C*). Knockdown of GMPS or USP7 caused a strong reduction in p53 levels (*Figure 4D*). Moreover, in the absence of GMPS or USP7, a substantial portion of the remaining p53 is cytoplasmic. These results suggest that GMPS not only produces GMP, but also mediates the induction of p53 in response to imbalances in the GMP synthesis pathway. In other words, GMPS is not only a biosynthetic enzyme allowing cell proliferation, but also part of a checkpoint that prevents genomic instability as a result of nucleotide deficiency.

TRIM21 retains GMPS in the cytoplasm, preventing p53 induction

Our results thus far suggest that the accumulation of nuclear GMPS promotes p53 stabilization. However, the molecular mechanism by which the bulk of GMPS is retained in the cytoplasm of unstressed cells is unclear. Therefore, we set out to identify factors that might control the subcellular localization of GMPS. Our analysis of GMPS-associated proteins identified TRIM21 (*Table S1*), an autoantigen associated with *Sjögren's syndrome* and *systemic lupus erythematosus* (Yoshimi et al., 2012). TRIM21 is a RING-finger E3 ubiquitin-ligase that function in innate and acquired immunity. We IPed TRIM21 from either unstressed cells, or cells treated with etoposide or MPA. Immunoblotting revealed that in unstressed cells TRIM21 binds GMPS, but not USP7 or p53 (*Figure 5A*). Cellular stress, however, caused TRIM21 to dissociate from GMPS. We also note that TRIM21 is a cytoplasmic protein

(*Figure 5B*). Thus, TRIM21 may retain GMPS in the cytoplasm of unstressed cells.

To test its effect on the subcellular localization of GMPS, we used shRNAs to deplete endogenous TRIM21. In the absence of stress, knockdown of TRIM21 caused translocation of GMPS from the cytoplasm to the nucleus, and strong p53 induction (*Figure 5B*). p53 induction upon loss of TRIM21 is strictly dependent on GMPS, because the effect is reversed after concomitant knockdown of TRIM21 and GMPS. These observations were confirmed by quantification of p53 immunofluorescence (*Figure 5C*). Depletion of USP7 or p53 did not affect GMPS subcellular localization (*Figure S2*). We note that knockdown of TRIM21 does not induce a DNA damage response, as indicated by the absence of γH2Ax foci (*Figure 5D*). Collectively, these results suggest that TRIM21 regulates p53 negatively by binding and retaining GMPS in the cytoplasm. In other words, the cytoplasmic-nuclear partitioning of GMPS by TRIM21 appears to be a switch controlling p53 levels.

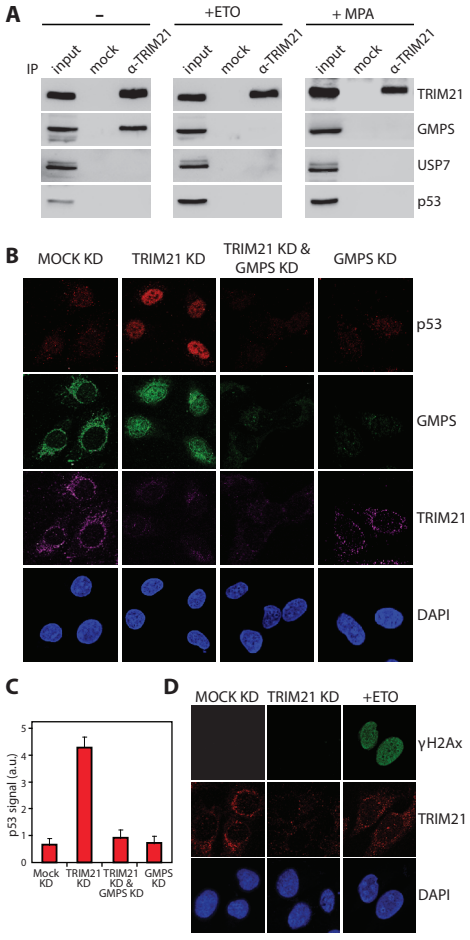

Figure 5; TRIM21 counteracts p53 stabilization through cytoplasmic retention of GMPS

(**A**) TRIM21 binds GMPS, but dissociates upon genotoxic- or replicative stress. CoIPs of TRIM21 from WCEs prepared from cells that were cultured in the absence (-) or presence of 10 μM etoposide or 5μg/ml MPA.

(**B**) Depletion of TRIM21 suffices for, GMPS-dependent, induction of p53. Indirect immunofluorescence analysis of U2OS cells after KD either TRIM21 or GMPS or both TRIM21 and GMPS. Cells were fixed and stained with antibodies directed against p53 (red), GMPS (green) or TRIM21 (turquoise). Images were capture by using confocal microscope.

(**C**) Quantification of p53 immunofluorescence using fiji-win32 software, plotted as fold change relative to mock-treated cells. Mean and standard deviations were derived from three independent biological replicates. In each case 20 nuclei were used for quantification.

(**D**) KD of TRIM21 does not induce a DNA damage response. Indirect immunofluorescence of U2OS cells after KD of TRIM21 or after addition of 10 μM etoposide. Cells were stained with antibodies against γH2Ax (green). Images were capture by using confocal microscope.

Ubiquitylation by TRIM21 controls GMPS subcellular localization

Because TRIM21 is a RING domain E3 ubiquitin-ligase, we decided to investigate the potential role of ubiquitylation in controlling GMPS localization. First, we co-expressed flag-GMPS, TRIM21 and His_6-tagged ubiquitin (ub) in cells, followed by the preparation of nuclear and cytoplasmic fractions. Western immunoblotting revealed the ubiquitylation of cytoplasmic, but not nuclear GMPS (*Figure 6A*). Like PTEN (Trotman et al., 2007), efficient ubiquitylation of GMPS required overexpression of ubiquitin, and was enhanced by additional TRIM21 (*Supplementary Figure 3A*). We note that, by itself, ectopically over-expressed flag-GMPS mainly localizes in the nucleus (*Supplementary Figure 3B*). Only in the presence of extra TRIM21 and ubiquitin does recombinant GMPS, like its endogenous counterpart, localize in the cytoplasm (*Supplementary Figure 3B*). Knockdown of endogenous TRIM21 led to a loss of GMPS ubiquitylation, showing it is the responsible ubiquitin-ligase (*Figure 6B*). We note that the migration of ubiquitylated GMPS suggests it is mainly monoubiquitylated by TRIM21.

To identify the ubiquitylated residues in GMPS we affinity purified flag-GMPS, followed by SDS-PAGE and coomassie staining (*Figure 6C*). Mass spectrometric analysis revealed the di-glycine hallmark of ubiquitylation on lysine (K) residues K182, K389 and K607 (*Figure 6D*). To test whether ubiquitylation of these lysines is important for the subcellular localization of GMPS we mutated them to arginine (R) residues, which cannot be ubiquitylated. Transfection studies revealed that in the presence of extra TRIM21 and ubiquitin wild type GMPS was mainly cytoplasmic. However, the GMPS K-to-R mutants were nuclear (*Figure 6E*). We conclude that ubiquitylation of these residues by TRIM21 is required for the cytoplasmic targeting of GMPS.

We wondered if USP7 could counteract TRIM21 and deubiquitylate GMPS, thereby shifting the balance towards nuclear retention. Indeed, concomitant over-expression of USP7, TRIM21 and GMPS led to a nuclear localization of GMPS (*Figure 6F*). This was dependent on deubiquitylation by USP7, because the catalytically inactive mutant $USP7^{C223S}$ could change the cytoplasmic localization of GMPS. Reflecting their effect on the subcellular localization of GMPS, wild type USP7, but not $USP7^{C223S}$, deubiquitylated GMPS in cells (*Figure 6G*). We conclude that (de) ubiquitylation is an essential part of the mechanism that controls the subcellular localization of GMPS.

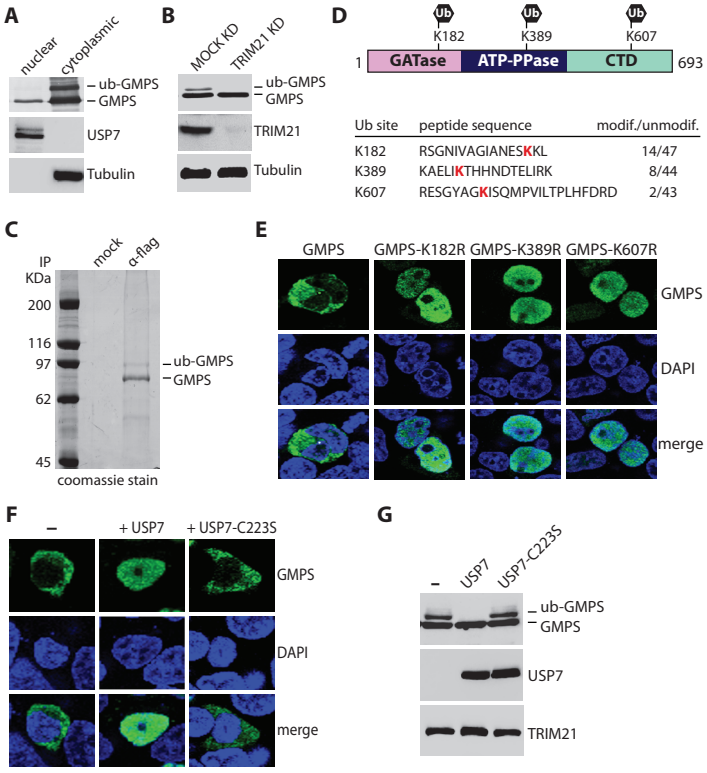

Figure 6; Ubiquitylation by TRIM21 determines the subcellular localization of GMPS
(**A**) Cytoplasmic, but not nuclear, GMPS is ubiquitylated. Nuclear and cytoplasmic fractions were prepared from HEK-293T cells that were transfected with vectors expressing flag-GMPS, His_6-ub and HA-TRIM21. Cell fractions were analyzed by immunoblotting with the indicated antibodies. See also *Figure S3A*. (**B**) TRIM21 is required for GMPS ubiquitylation. Immunoblotting analysis of WCEs of HEK-293T, following KD of endogenous TRIM21 cells transfected with vectors expressing flag-GMPS and His_6-ub. (**C**) flag-GMPS IPed from HEK-293T cells transfected with vectors expressing flag-GMPS, His_6-ub and HA-TRIM21, using anti-flag M2 agarose beads. Bound proteins were eluted with flag peptides, resolved by SDS-PAGE and visualized by coomassie staining. Bands corresponding to GMPS were excised and analyzed by mass spectrometry. (**D**) Ubiquitylated lysine residues of GMPS, K182, K389 and K607, identified by mass spectrometry. The glutamine amidotransferase (GATase) and ATP pyrophosphatase (ATP-PPase) catalytic domains of GMPS, and its C-terminal domain (CTD), are indicated. The table depicts the peptides identified with the target K in red and the ration between modified and unmodified peptides. (**E**) Mutation of ubiquitylated lysines to arginines (K to R) causes the transfer of GMPS from the cytoplasm to the nucleus. Indirect immunofluorescence of HEK-293T cells transfected with plasmid vectors expressing HA-TRIM21, His_6-Ub and either wild type flag-GMPS or GMPS-K182R, -K389R or K607R mutants. flag-GMPS (green) was detected with anti-flag antibodies. Images were capture by using confocal microscope. (**F**) USP7 promotes nuclear accumulation of GMPS. Indirect immunofluorescence of HEK-293T cells transfected with plasmid vectors expressing flag-GMPS, HA-TRIM21, His_6-ub, no additional factors (-), USP7 or the catalytically inactive $USP7^{C223S}$ mutant. Flag-GMPS (green) was detected with anti-flag antibodies. Images were capture by using confocal microscope. (**G**) USP7 deubiquitylates GMPS. Immunoblotting analysis of GMPS in WCEs prepared from cells treated as described above.

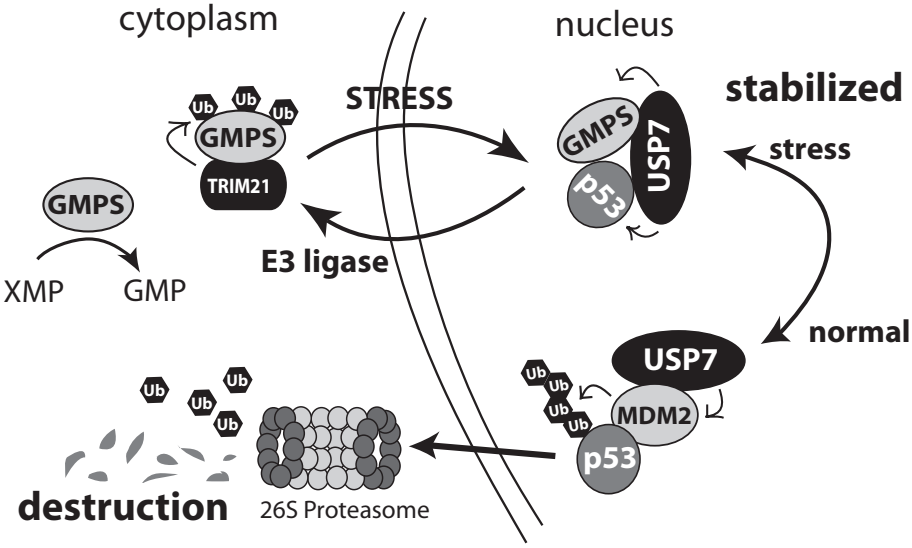

Figure 7; A TRIM21-GMPS-USP7 cascade controls p53 stability
Model summarizing our results. GMPS is essential for cell proliferation as it converts XMP into GMP, a precursor of DNA and RNA. Under homeostatic conditions TRIM21 binds and ubiquitylates GMPS to keep it predominantly cytoplasmic. In the nucleus, USP7 binds and stabilizes MDM2. MDM2 ubiquitylates p53, thereby marking it for degradation by the proteasome. Genotoxic- or replicative stress triggers the release of GMPS from TRIM21 and its accumulation in the nucleus, where it is deubiquitylated by USP7. Nuclear GMPS drives the transfer of p53 from MDM2 to a GMPS-USP7-p53 complex, resulting in p53 deubiquitylation by USP7. Our results suggest that nuclear-cytoplasmic trafficking of GMPS, controlled through ubiquitylation by TRIM21, is a key switch of p53 stabilization by USP7. See main text for details.

Discussion

In this study, we established that the nucleotide biosynthetic enzyme GMPS is a crucial relay of p53 induction after DNA damage or nucleotide depletion (*Figure 7*). Under homeostatic conditions, TRIM21 binds and ubiquitylates GMPS to keep it predominantly excluded from the nucleus. In the nucleus, USP7 deubiquitylates and stabilizes MDM2, thereby promoting p53 ubiquitylation and degradation. Genotoxic - or replicative stress triggers the release of GMPS from TRIM21 and its accumulation in the nucleus. Nuclear GMPS drives the formation of a GMPS-USP7-p53 complex, and stimulates p53 deubiquitylation by USP7. These results implicate a classic biosynthetic enzyme, associated with cell proliferation, in p53 control.

Several additional factors modulate p53 ubiquitylation and stability. Phosphorylation of p53 by ATM or Chk2 inhibits MDM2 binding and p53 degradation (Banin et al., 1998; Shieh et al., 1997). In parallel, DNA damage

induced phosphorylation of MDM2 and MDM4 blocks binding of USP7, causing their destabilization (Maya et al., 2001; Meulmeester et al., 2005; Stommel and Wahl, 2004). Dephosphorylation of the specific isoform USP7S by PPM1G causes its downregulation and reduced levels of MDM2 (Khoronenkova et al., 2012). Another protein, TSPYL5 has been reported to with p53 for USP7 binding (Epping et al., 2011). Finally, USP10 deubiquitylates and recycle p53 from the cytoplasm which has been exported from the nucleus (Yuan et al., 2010). These alternate factors notwithstanding, our knockdown experiments showed that they do not bypass the role of GMPS in p53 stabilization. GMPS does not appear to interact with these factors or MDM2 but target USP7 and p53 directly (*Figure 1, Table S1*).

Recently, USP7 has emerged as a potential target for anti-cancer therapy. However, the role of USP7 in cancer is context-dependent and impinges on multiple tumor suppression pathways. Inactivation experiments in mice confirmed that regulation of p53 is a crucial, but not the only, function of USP7 (Kon et al., 2010; Kon et al., 2011). Targeting of USP7 by the viral EBNA1 protein (Saridakis et al., 2005) or by cellular TSPYL5 (Epping et al., 2011) represses p53 function. In breast cancer, USP7 activity appears to help tumor suppression (Epping et al., 2011). In contrast, USP7 is overexpressed in prostate cancer and has been implicated in the inactivation of tumor suppression by PTEN (Song et al., 2008). Through its cooperation with Polycomb repressors, USP7 stimulates silencing of the *p16INK4a* tumor suppressor (Maertens et al., 2010). Inhibition of USP7 overcomes resistance to the proteasome inhibitor bortezomib in multiple myeloma cells (Chauhan et al., 2012). Finally, our results suggest that genotoxic drugs or nucleotide depletion can trigger, GMPS-dependent, stabilization of wild type p53 by USP7. Thus, when designing therapeutic strategies it is pertinent to take into account that USP7 might either inhibit or stimulate different tumor pathways, depending on the type of cancer or drugs used.

Cytoplasmic-nuclear partitioning of GMPS is a crucial part of the switch for p53 stabilization. Ubiquitylation by TRIM21 determines GMPS localization in the cytoplasm. However, addition of the nuclear export inhibitor Leptomycin B to unstressed cells leads to strictly nuclear GMPS. This observation suggests cytoplasmic-nuclear shuttling of GMPS in which active export outcompetes import. Stress shifts the balance towards the accumulation of non-ubiquitylated GMPS in the nucleus, where it can bind USP7 and p53. TRIM21 is associated with Sjögren's syndrome and systemic lupus erythematosus autoimmune diseases (Yoshimi et al., 2012). In the immune system, TRIM21 controls interferon and pro-inflammatory cytokine expression via ubiquitylation of interferon regulating transcription factors and the NF-κB inhibitor IKKβ. Our results suggest that TRIM21 may provides a connection between autoimmune diseases and p53 function.

A cell's proliferative ability is limited by its nucleotide pool. To support cell growth and rapid proliferation, cancer cells redirect glucose and glutamine

towards *de novo* nucleotide synthesis (Levine and Puzio-Kuter, 2010; Tong et al., 2009). Enzymes such as GMPS and IMPDH are frequently upregulated in rapidly proliferating- and cancer cells (Jackson et al., 1975; Su et al., 2004; Weber, 1983). However, early oncogenic transformation may stimulate DNA replication in the absence of coordinate nucleotide synthesis, causing replicative stress and genomic instability (Bester et al., 2011). We now show that GMPS represents a direct molecular link between nucleotide biosynthesis and activation of the p53 tumor suppressor. Depletion of the GMPS substrate XMP triggers GMPS-mediated p53 stabilization. Likewise, it will be interesting to explore the roles of GMPS and p53 in the addiction of cancer cells to glutamine, another GMPS substrate. We found recently that *Drosophila* IMPDH, like GMPS, has a double function. In addition to XMP synthesis, IMPDH is a transcription factor that couples expression of *E2f* and *histone* genes to cellular state (Kozhevnikova et al., 2012). Our results suggest that selective biosynthetic enzymes that enable cell proliferation are enlisted to safeguard against unbalanced proliferation. This connection may provide novel opportunities for cancer therapy.

Experimental procedures

Antibodies: Polyclonal antibodies were generated by immunizing the rabbits or guinea pigs with GST-tagged proteins expressed in *E. coli* and affinity purified as described previously (Chalkley and Verrijzer, 2004). The following antigens were used, 1) USP7; mixture of peptides containing Full-length protein, N-terminus, amino acids (aa) 24-329 and C-terminus, aa 428-704, 2) GMPS; mixture of peptides N-terminus, aa 1-312 and C-terminus, aa 281-693, 3) p53; Full length protein, 4) MDM2; Full length protein and 5) TRIM21; Full length protein. The following antibodies were purchased as indicated. Anti-p53 (DO-1), anti-p53 (DO-7) and anti-TRIM21 (D-12) from Santa Cruz, anti-TRIM21 (Atlas antibodies), anti-Flag M2 antibodies (Sigma), anti-HA antibodies (Abcam) and anti-phospho H2A.x antibodies (JBW-301 clone, Millipore)

Imuunoprecipitations and massspectrometry (IP-massspec) and Co-Imuunoprecipitations and Western blotting (Co-IPs): Immunopurifications were essentially carried out by using standard procedures as described earlier (Chalkley and Verrijzer, 2004). Briefly, whole cell extracts were prepared from U2OS or HEK-293T cells either untreated or treated with 10 μM etoposide or 5 μg/ml MPA for 24 hours. Cells were lysed in NET 0.1% buffer (*50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P40, freshly added 1 mM Phenyl Methyl Sulphonyl fluride, protease inhibitors, phosphatase inhibitors*) and sonicated with a Bioruptor for 5 min, 30-s 'on' and 1-min 'off' cycles. After removal of cell debris by centrifugation, cell lysates were incubated with affinity purified antibodies cross linked to Protein A-sepharose beads for three hours at 4^0 C. Beads were then subjected to extensive

washes for 5x5min with NET 0.1% buffer. Affinity purified proteins were then eluted with Glycine buffer pH 2.5 (*100 mM Glycine, 150 mM NaCl*). Eluted samples were TCA precipitated and dissolved in 1xSDS loading buffer and resolved by SDS-PAGE and proteins were identified by nanoflow liquid chromatography-tandem mass spectrometry. Similarly, Co-IPs were carried out by incubating 1 mg of cell lysates with 2-3 μg of antibody cross-linked to Protein A-agarose beads. Followed by extensive washes with NET 0.1% buffer, affinity purified proteins were then eluted with Glycine buffer pH 2.5. Eluted samples were TCA precipitated and dissolved in 1x SDS loading buffer. Protein samples were resolved by SDS-PAGE and subjected to Western blotting analysis.

Cell culture and lentiviral procedures: Cell culture was essentially carried out according to the standard procedures. Lentivirus expressing specific shRNA were purchased form shRNA library (*Erasmus Centre for Biomics, Erasmus MC, Rotterdam, The Netherlands*). High-titre viral stocks were produced in HEK-293T cells by cotransfection of shRNA vector with packaging constructs by using standard transfection procedures. U2OS cells were then transduced with lentivirus expressing specific shRNA against GMPS, USP7, p53 and TRIM21 (See Supplementary *Document S1* for shRNA sequences). Knockdowns were carried out for 4 days and the knockdown efficiency was analyzed either by immunobloting or by immunoflurescence.

Cloning and transfection procedures: For transient expression in mammalian cells full-length cDNA of GMPS, USP7, p53 and TRIM21 were cloned into pQCXIP vector with flag-tag sequence or HA-tag sequence flanking at 5' end. $GMPS^{C114A}$, GMPS-K182R, K389R and K607R were generated by PCR based, site-directed mutagenesis. MDM2, His_6-Ubiqutin and $USP7^{C223S}$ clones were as described earlier (van der Knaap et al., 2005). Transient transfections were carried out by using Fu-Gene reagent, Roche diagnostics or PEI (Polyethylenimine) reagent, Sigma-Aldrich. Procedures followed were according to the manufacturer's instructructions.

Real time quantitative PCR: Total RNA isolation, RT-qPCR, microarray experiments and data analysis were all performed by using standard procedures. See Supplementary *Document S2* for primers details.

Immunofluorescence: Immunoflurescence was essentially carried out by using standard procedures. Briefly, cells grown as monolayer on cover slips were fixed by using 2% *Para formaldehyde* (PFA) or methanol and permeabalized by 0.2% *Triton-X-100* solution for one hour on shaker incubator at room temperature. Then cells were incubated with primary antibody overnight at 4^0C on shaker incubator followed by three time washes. Then cells were incubated with secondary antibody for 30 minutes at room temperature followed by three time washes. Then cover slips were mounted by using mounting solution with DAPI and images were captured by using fluorescent microscope or confocal microscope.

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Supplementary

Protein identity (MW in kDa)	GMPS MS/ ES./ UP/ % C	flag-GMPS MS/ ES./ UP/ % C	comments
GMPS (76)	2680 / 24.27 / 40 / 64.9	3581 / 66.5 / 52 / 77.3	de novo purine nucleotide biosynthesis
USP7 (128)	302 / 0.25 / 10 / 10.3	2258 / 3.83 / 47 / 50.8	ubiquitin specific protease 7
TP53 (44)	50 / - / 2 / 6.4	249 / 0.5 / 7 / 17.6	tumor suppressor p53
CAD (242)	2667 / 1.2 / 55 / 28	400 / 0.1 / 10 / 5.3	de novo pyrimidine nucleotide biosynthesis
IMPDH2 (55)	1162 / 3.6 / 20 / 43	167 / 0.2 / 6 / 16	de novo purine nucleotide biosynthesis; enzyme directly upstream of GMPS
TRIM21 (52)	1685 / 8.8 / 26 / 50.3	358 / 0.58 / 9 / 20.2	tripartite motif 21; E3 ubiquitin ligase; autoantigen implicated in lupus erythematosus & Sjögren's syndrome autoimmune diseases
SSB (46)	387 / 1.1 / 9 / 21	182 / 0.2 / 6 / 10	Sjögren's syndrome antigen B
MAGEA1 (34)	408 / 1 / 8 / 20.7		melanoma antigen A-1
MAGEA4 (37)	233 / 0.6 / 5 / 14.5		melanoma antigen A-4
MAGED4 (81)		182 / 0.2 / 6 / 10	melanoma antigen D-4
MAGED2 (64)		117 / 0.12 / 4 / 7.6	melanoma antigen D-2, MAGE proteins are cancer antigens; selective MAGE proteins bind and stimulate RING ubiquitin ligases
USP11 (110)	2087 / 2.8 / 32 / 42	126 / 0.1 / 4 / 4.9	ubiquitin specific protease 11
HDAC6 (132)	1402 / 1.8 / 21 / 28.1		Histone deacetylase 6, cytoplasmic deacetylase; binds ubiquitinated proteins
WDR92 (84)	874 / 1 / 13 / 18		WD repeat domain 92
WDR82 (35)	630 / 4.5 / 10 / 33	354 / 1.7 / 9 / 24	WD repeat domain 82
EBNA1BP2 (34)	563 / 4.1 / 13 / 40		Epstein-Barr nuclear antigen 1 binding protein 2; negative regulator of p53
PDRG1 (15)	273 / 4.9 / 6 / 36	337 / 0.37 / 7 / 11	p53 and DNA-damage regulated 1
MRE11A (81)	191 / 0.2 / 5 / 8.4	337 / 0.3 / 7 / 11	subunit MRN complex; double-strand break repair
RAD50 (138)	189 / 0.1 / 5 / 8.4	188 / 0.1 / 6 / 5.5	subunit MRN complex; double-strand break repair
NBS1 (85)	44 / 0.04 / 1 / 1.5		Nibrin, subunit MRN complex; double-strand break repair

Table S1; Massspecrtometric analysis of GMPS immunopurifications, Related to Figure 1

Details of Massspecrtometric analysis such as, Mascot score (MS), emPAI score (ES), Unique peptides (UP) and % coverage (%C) are given in the table**.** GMPS immunopurifications identified USP7, p53 and TRIM21 as interacting proteins. We also found Melanoma antigen A, 1 (MAGE A1) and A, 4, (MAGE A4), MAGE proteins were shown to be in complex with TRIM E3 ubiquitin ligases (Doyle et al., 2010). We also found Sjogren syndrome antigen B is an autoantigen found in patients with Sjogren syndrome (Hennig et al., 2008). Among others, we found deubiquitinase USP11, USP7 and USP11 are implicated in polycomb-mediated regulation of INK4a tumor suppressor locus. However, USP11 was shown to have no effect on p53 stability or deubiquitylation (Li et al., 2002; Maertens et al., 2010). Also, we found that USP11 does not have any effect on GMPS deubiquitylation or subcellular localization (*data not shown*). We also found nucleotide biosynthetic enzymes such as, CAD, and IMPDH2. Also, histone deacetylase, HDAC6, WD repeat proteins WDR82 and WDR92. Apart from this, we also found Epstein bar virus nuclear antigen 1 binding protein 2 (EBNA1BP2), it has been shown that EBNA1 recruits GMPS-USP7, complex to repress Epstein bar virus latent origin of replication. We also found DNA damage response proteins such as p53 and DNA damage regulated 1 (PDRG1), MRE11, RAD50 and Nibrin, MRN complex.

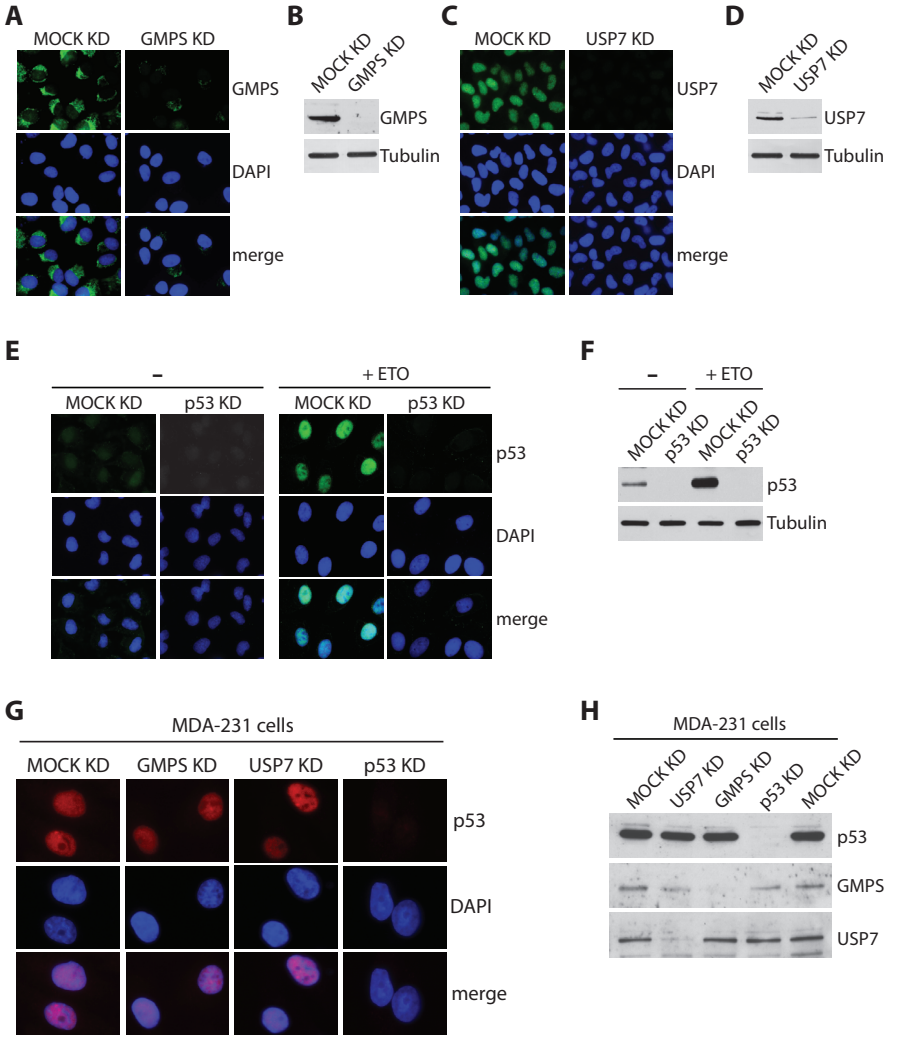

Figure S1; Lentiviral-mediated knockdown of GMPS, USP7, p53 to check antibody specificity, Related to Figure 1, 2, 3 & 4

A-F) U2OS cells were transduced with lentivirus encoding specific shRNA against GMPS or USP7 or p53. Followed by cells were either untreated or treated with 10μM etoposide for 24 hours as indicated. Cells were then subjected to either Western blotting analysis or immunofluoroscence using the antibodies as indicated. Images were captured by using fluorescent microscope.

G&H) MDA-231 cells were transduced with lentiviral encoding specific shRNA against GMPS or USP7 or p53. Followed by cells were subjected to either Western blotting analysis or immunofluoroscence using the antibodies as indicated. Images were captured by using fluorescent microscope.

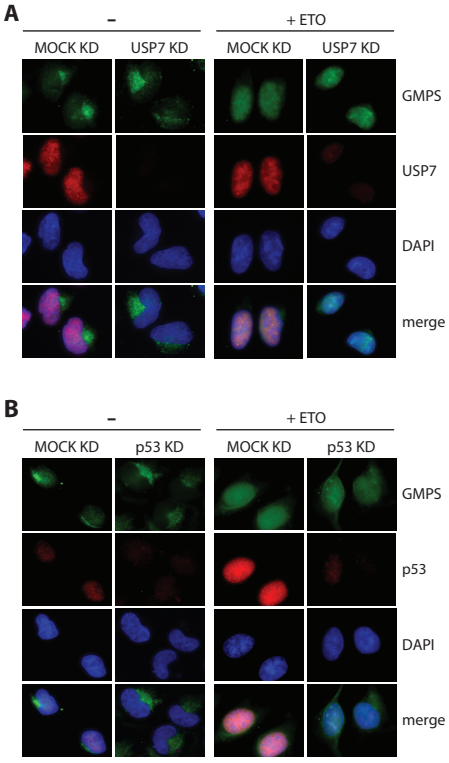

Figure S2; USP7 and p53 does not affects GMPS sub-cellular localization, Related to Figure 5

U2OS cells grown as a monolayer on cover slips were transduced with lentivirus expressing either non-targeted shRNA (mock) or shRNA against USP7 (**A**) or p53 (**B**) for 72 hours. Followed by cells were either untreated (UNT) or treated with 10μM etoposide (ETO) for 24 hrs. Cells were stained by using specific antibodies as indicated. Images were captured by using fluorescent microscope.

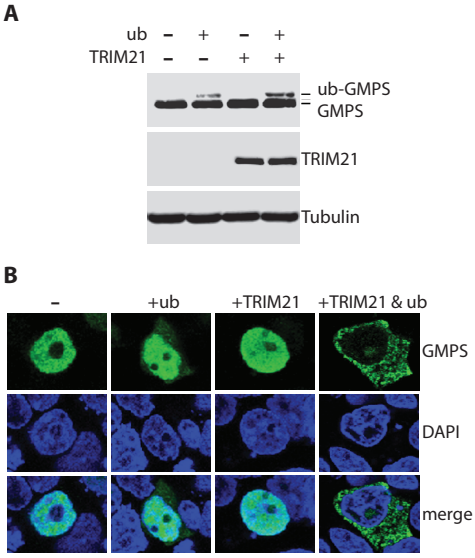

Figure S3; Ubiquitylation by TRIM21 is essential for GMPS cytoplasmic localization, Related to Figure 6

HEK-293T cells were transiently transfected with flag-GMPS with His_6-Ub or with TRIM21 or with His_6-Ub and HA-TRIM21 for 48 hours. Followed by cells were either subjected to Western analysis (**A**) or immunofluoroscence (**B**) using the antibodies as indicated. Images were captured by using confocal microscope.

Document S1; shRNA target sequences, Related to Figure 2 & 4:

USP7: CCAGCTAAGTATCAAAGGAAA, CGTGGTGTCAAGGTGTACTAA
GMPS: GCATTTGCTATAAAGGAACAA, CCTACAGTTACGTGTGTGGAA
p53: GTCCAGATGAAGCTCCCAGAA
TRIM21: TGAGAAGTTGGAAGTGGAAAT, GAGTTGGCTGAGAAGTTGGAA

Document S2; Primers used for RT-qPCR, Related to Figure 2:

p53: CCTCCTCAGCATCTTATCCGA & CATAGGGCACCACCACACTA
p21: TGTGGACCTGTCACTGTCTTG & CGGCGTTTGGAGTGGTAGAA
BAX: GCTTCAGGGTTTCATCCAGG & GCTCAGCTTCTTGGTGGA
CDC6: GCTGTCTCGGGCATTGAACAA & CATAGGTTGTCATCGCCCAG
MCM6: GAGTTTCAGAGCAGCGATGG & GGTGGTGGAAAGTTGCTGGTT
GAPDH: GGATTTGGTCGTATTGGGCG & TCCTGGAAGATGGTGATGGGA

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Chapter 3

Drosophila Transcription Factor Tramtrack69 Binds MEP1 to Recruit the Chromatin Remodeler NuRD

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Drosophila Transcription Factor Tramtrack69 Binds MEP1 To Recruit the Chromatin Remodeler NuRD▽†

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ATP-dependent chromatin-remodeling complexes (remodelers) are essential regulators of chromatin structure and gene transcription. How remodelers can act in a gene-selective manner has remained enigmatic. A yeast two-hybrid screen for proteins binding the *Drosophila* transcription factor Tramtrack69 (TTK69) identified MEP1. Proteomic characterization revealed that MEP1 is a tightly associated subunit of the NuRD remodeler, harboring the Mi2 enzymatic core ATPase. In addition, we identified the fly homolog of human Deleted in oral cancer 1 (DOC1), also known as CDK2-associated protein 1 (CDK2AP1), as a *bona fide* NuRD subunit. Biochemical and genetic assays supported the functional association between MEP1, Mi2, and TTK69. Genomewide expression analysis established that TTK69, MEP1, and Mi2 cooperate closely to control transcription. The TTK69 transcriptome profile correlates poorly with remodelers other than NuRD, emphasizing the selectivity of remodeler action. On the genes examined, TTK69 is able to bind chromatin in the absence of NuRD, but targeting of NuRD is dependent on TTK69. Thus, there appears to be a hierarchical relationship in which transcription factor binding precedes remodeler recruitment.

Chromatin is the natural template of the eukaryotic transcription machinery. Consequently, regulation of gene expression involves the interplay between sequence-specific transcription factors, the basal machinery, coregulators, and enzymes that modulate chromatin structure. ATP-dependent chromatin-remodeling factors (remodelers) constitute one class of enzymes that target chromatin. The basic biochemical activity of remodelers is to use the energy of ATP hydrolysis to move or eject nucleosomes (8). Although their *in vitro* activity might suggest that remodelers act in a generic way, it has become clear that different remodelers perform distinct, nonredundant functions. An early example of functional specialization was our finding that the Brahma (BRM) remodeling complexes, but not ISWI remodelers, act as chromatin-specific coactivators for the transcription factor ZESTE (15). Conversely, unlike ISWI, the BRM remodelers were unable to order a nucleosomal array. Moreover, several studies have demonstrated that different remodelers control distinct biological processes (4, 8, 24).

Currently, four major classes of remodelers are recognized, based on their ATPase and accessory subunits (8). These comprise the SWI/SNF, ISWI, CHD, and INO80 families. Mi2 (also known as CHD4) is the founding member of the CHD family of remodelers, characterized by the presence of a tandem chromodomain in the ATPase subunit (9). A unique aspect of NuRD is its coupling of remodeling and histone deacetylase (HDAC) activities in one complex. Although there is some variability between the various vertebrate NuRD complexes described so far, the key subunits of NuRD are the Mi2 ATPase, the protein deacetylases HDAC1 and HDAC2, metastasis-associated proteins MTA1, MTA2, and MTA3, the retinoblastoma-associated histone binding proteins RBP46 and RBP48, p66/68, and MBD2 and MBD3, harboring a methyl CpG binding domain (9). These vertebrate proteins have highly conserved *Drosophila* counterparts. However, date, *Drosophila* NuRD has not been purified as a defined entity. Instead, a two-subunit complex comprising Mi2 and the fly homolog of the *Caenorhabditis elegans* Mog interacti ectopic P granulocyte 1 (MEP1) protein has been isolated from *Drosophila* Kc cells (17). Thus, the identity of *Drosoph* NuRD has remained unclear.

Mi2 and NuRD play important roles in cell fate control during development. For example, the *C. elegans* homologs Mi2 and MEP1 cooperate to maintain the distinction between germ line and soma in developing embryos by inhibiting the expression of germ line-specific genes in somatic cells (32). During lymphocyte development in mammals, NuRD acts as a corepressor for the BTB/POZ domain and the zinc finger transcription factor BCL6, repressing plasma cell-specific genes so as to promote differentiation toward B cells (11). In *Drosoph ila*, Mi2 has been implicated in the repression of homeotic genes by the GAP protein Hunchback (16). Moreover, the repression of proneural genes by the transcription factor Tramtrack69 (TTK69) so as to block a neuronal cell fate is dependent on Mi2 (27, 40). Indeed, Mi2 was identified as an

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interacting partner of TTK69 in a two-hybrid screen, and the two proteins colocalize on many loci on third-instar larval polytene chromosomes (27). Thus, Mi2 appears to act as transcriptional corepressor for TTK69.

TTK is an important *Drosophila* transcription factor, which is involved in various aspects of development and cell differentiation. As a result of alternative splicing, the *ttk* locus encodes two isoforms, TTK69 and TTK88. These proteins have different C-terminal zinc finger DNA-binding domains but share an N-terminal BTB/POZ protein-protein interaction domain (5, 14, 28, 39). TTK was first identified as a repressor of the pair-rule genes *even skipped* and *fushi tarazu* (5, 14) but is involved in numerous additional processes. For example, TTK controls selective cell fate decisions in the developing embryonic nervous system, in photoreceptor differentiation, and in sensory organ precursor differentiation (1, 3, 12, 13, 18, 21, 30, 37–39). During eye development, TTK blocks the differentiation of precursor cells to photoreceptors and promotes specific nonneuronal fates, such as cone cells. TTK is regulated posttranslationally through ubiquitin-mediated degradation triggered by the E3 ubiquitin ligase SINA, which is itself controlled by RAS-mitogen-activated protein (MAP) kinase signaling (21, 30). This ubiquitin-dependent developmental switch depends on the balance between the antagonistic activities of the deubiquitylating protease UBP64 and SINA (2).

Here we explored the interplay between the sequence-specific transcription factor TTK69 and the remodeler NuRD. A yeast two-hybrid screen identified MEP1 as a TTK69 partner. Our proteomic characterization of NuRD established MEP1 and fly CDK2AP1/DOC1 as *bona fide* subunits. TTK69 interacts genetically and functionally with both MEP1 and Mi2. We performed a genomewide expression analysis to determine the transcriptional circuitries controlled by TTK69 and NuRD. Their substantial overlap suggests that TTK is an important factor recruiting NuRD to its targets. Our analysis of chromatin association suggests a hierarchical relationship in which TTK69 recruits NuRD rather than the remodeler facilitating TTK69 binding.

MATERIALS AND METHODS

Protein-protein interaction assays. Yeast two hybrid screens were performed as described previously (27) using a full-length TTK69 cDNA cloned into pLexA202 to screen a 0- to 24-h *Drosophila* embryonic cDNA library fused to the B42 activation domain in plasmid pJG4-5. Domain mapping was performed by using TTK69 deletion constructs cloned into pLexA202 and tested for their interaction with MEP1 residues 257 to 536 fused to the B42 activation domain in plasmid pJG4-5. The strength of interaction was tested by both colony growth and β-galactosidase assays (27). For glutathione *S*-transferase (GST)-tagged interaction assays, radiolabeled proteins were produced using the TnTQuick system (Promega) with the pLinkT7β vector. Glutathione beads containing 5 to 10 μg of the appropriate GST fusion protein made up to a 30-μl volume were mixed with 25 μl of the TnT reaction mixture and 50 μl of 2× pulldown buffer (40 mM Tris-HCl [pH 8], 200 mM NaCl, 800 mM KCl, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], protease inhibitors), and the mixture was incubated at 4°C for 2 h. The beads were transferred to MobiCol columns (MoBiTec/VH Bioscience) and were washed 4 times with 1× pulldown buffer. Bound proteins were eluted by addition of 20 μl 3× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and boiling. A 10-μl volume of the resulting mixture, along with the input material, was analyzed by SDS-PAGE.

Antibodies, immunological procedures, protein purification, and mass spectrometry. Polyclonal antibodies were generated by immunizing guinea pigs with GST fusion proteins expressed in *Escherichia coli* and were affinity purified as described previously (7). The following antigens were used: Mi2 amino acids (aa) 1290 to 1533, MTA1-like aa 132 to 476, MEP1 aa 681 to 800, TTK69 aa 333 to 546, and the full-length CG18292 (CDK2AP1/DOC1) protein. Immunization and affinity purification were carried out as described previously (7). Rabbit polyclonal antibodies against TTK69 (27) and ISWI (6) have been described previously. Coimmunoprecipitations (coIPs), immunoblotting, and immunolocalization on 3rd-instar larval salivary gland polytene chromosomes were performed as described previously (6). Embryo nuclear extracts were prepared from 0- to 12-h-old *Drosophila* embryos. Immunopurification procedures using affinity-purified antibodies directed against Mi2 or MEP1 and mass spectrometric analysis were all performed as described previously (6, 7). After affinity purification, beads were washed twice with HEMG buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, and a cocktail of protease inhibitors) containing 200 mM KCl (HEMG/200), 5 times with HEMG/500, once with HEMG/200, and finally once with HEMG/200 lacking NP-40. Typical contaminants, also present in the products of immunopurification using beads coated with preimmune serum or antibodies directed against unrelated proteins, were omitted from Table 1. Immunodepletion was performed essentially as described previously (7), with the following adaptations. The nuclear extract was diluted with HEMG/100 to a final total protein concentration of 5 mg/ml and was then cleared by centrifugation. This extract was then incubated with protein A beads cross-linked with either anti-CDK2AP1/DOC1 (α-CDK2AP1/DOC1) antibodies or preimmune serum (mock control). After 2 h, the beads were removed by centrifugation. These steps were repeated 3 more times. Supernatants were then resolved by 8% SDS-PAGE and were analyzed by immunoblotting.

***Drosophila* genetics.** All fly stocks were maintained under standard conditions, and crosses were performed using standard procedures. RNA interference (RNAi) lines (10) for MEP1 (strain 24533), Mi2 (strain 10766), and TTK69 (strain 10855) were obtained from the Vienna *Drosophila* RNAi Centre (http://stockcenter.vdrc.at). The GMR-Gal4 enhancer line was obtained from the Bloomington *Drosophila* Stock Center at Indiana University (http://flystocks.bio.indiana.edu/). All crosses were performed at 25°C and were repeated several times. Representative examples are shown in Fig. 3.

RNAi, genomewide expression analysis, and ChIP-qPCR. RNAi in *Drosophila* S2 cells, RNA isolation, reverse transcription-quantitative PCR (RT-qPCR), microarray experiments, and data analysis were all performed as described previously (26). Details are available upon request. Chromatin immunoprecipitation (ChIP)-qPCR assays were performed, and results were quantified, as described previously (22, 25). Immunoprecipitations were performed with the following antibodies: α-Mi2, α-MEP1, α-TTK69, and α-BAP111 (6). Briefly, *Drosophila* S2 cells were cultured in Schneider's medium (catlog no. 21720-024; Invitrogen) and treated with double-stranded RNA (dsRNA) for 4 days. Double-stranded RNAs were synthesized using an Ambion Megascript T7 kit. RNA samples from three fully independent experiments were prepared and analyzed by RT-qPCR. Cross-linked chromatin was prepared from S2 cells and was sheared by sonication to an average length of 0.5 kbp. Chromatin was then incubated with the antibodies indicated in Fig. 6. Background ChIP levels, subtracted during data processing, were determined by using beads lacking specific antibodies. Following IP, the recovered DNA was analyzed by qPCR with SYBR green I, using the MyiQ single-color real-time PCR detection system (Bio-Rad). The data presented are the results of three independent biological replicate experiments.

ChIP primers were as follows: for ARK, 5′-ACCTGGCAGTGAATACCTTTGTTG-3′ and 5′-GTGTGACCATATTGAGCCGTATCG-3′; for Hairy, 5′-GAGCCGCAGATACACAGTACACAG-3′ and 5′-GCCGTTCGTGGTTTGCTGATTC-3′; for Engrailed, 5′-GAGCCACTGATTCTTCTG-3′ and 5′-TGTCGGAACAACAGTTGC-3′; for Sk1, 5′-AAAGCAAAGGCAAAAGCAACAG-3′ and 5′-GAGGGTGAACTAACCTTATTTTCC-3′; and for KCNQ, 5′-CGTTGTGGGCGGGTCAGG-3′ and 5′-TATTTGGGTTGTTGGGGTATGGC-3′. All other primer sequences will be made available upon request.

Microarray data accession number. Raw expression data have been submitted to the ArrayExpress database (Microarray Informatics Team, EMBL) under accession no. E-TABM-1010 (http://www.ebi.ac.uk/microarray-as/ae/).

RESULTS

Transcription factor TTK69 binds the NuRD subunit MEP1. Previously, we published a yeast two-hybrid screen that resulted in the identification of Mi2 as a TTK69-binding factor (27). Here we report an extension of this screen. Using full-length TTK69 as the bait, we isolated a partial cDNA encoding aa 257 to 536 of *Drosophila* MEP1, a 1,152-aa protein harbor-

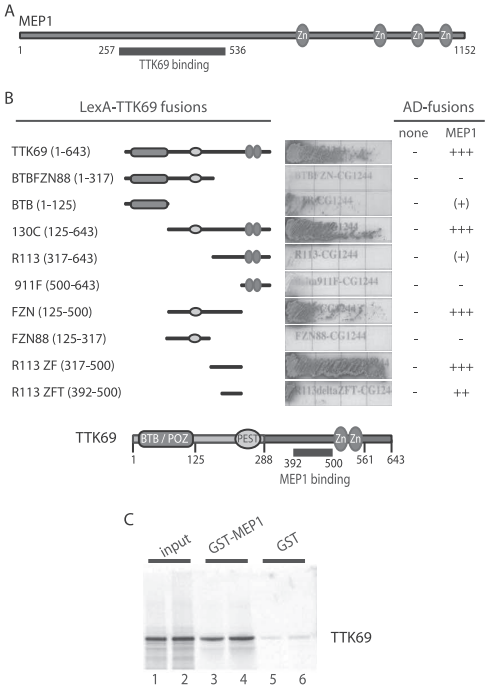

FIG. 1. TTK69 binds *Drosophila* MEP1. (A) A yeast two-hybrid screen identified *Drosophila* MEP1 (CG1244) as a TTK69-interacting protein. TTK69 interacts with MEP1 aa 257 to 536, encoded by a partial cDNA that was expressed fused to the activation domain (AD) in the original screen. The potential C2H2 zinc fingers are indicated. (B) A series of TTK69 truncation constructs fused to the LexA DNA-binding domain were used to map the MEP1-binding domain. The BTB/POZ domain, the double zinc finger DNA-binding domain, and the PEST domain are diagramed. Dark green indicates the TTK69 sequence; light green, the sequence shared with TTK88. A region spanning aa 317 to 500 retained full MEP1-binding activity. Amino acids 392 to 500 bound somewhat more weakly but still displayed robust binding to MEP1. The same constructs have been used previously to delineate the Mi2 binding domain (27), revealing an overlap between the MEP1 and Mi2 binding regions of TTK69. Representative streaks of yeast expressing the two-hybrid fusions are shown. The relative interaction strengths are indicated as follows: −, no detectable interaction; (+), very weak; +, weak; ++, strong; +++, very strong. (C) TTK69 binds MEP1 directly. [^{35}S]methionine-labeled full-length TTK69 was incubated with either GST-MEP1 aa 257 to 536 or GST alone. Following washes, bound material was resolved by SDS-PAGE and visualized by autoradiography. Duplicate samples and 10% of the input were loaded.

ing multiple potential C2H2 zinc fingers and SUMO-interacting motifs (Fig. 1A). To delineate the MEP1-binding domain of TTK69, we again utilized the two-hybrid assay. Surprisingly, we found that the same region that mediates Mi2 binding also suffices to recruit MEP1 (Fig. 1B) (27). A minimal binding region of about 100 amino acids (aa 392 to 500) is located directly upstream of the zinc fingers of TTK69 and is absent in TTK88. The other portions of TTK69 do not display robust binding to either MEP1 or Mi2. As an independent assay, we tested the ability of recombinant ^{35}S-labeled TTK69 to bind GST-tagged MEP1 (Fig. 1C). TTK69 associates efficiently with immobilized MEP1 but does not bind GST. Together, our results suggest that TTK69 binds MEP1 directly.

To determine the interaction network of MEP1, we took a proteomics approach to make an inventory of its associated proteins in *Drosophila* embryo nuclear extracts. Nuclear extracts were incubated with protein A Sepharose beads coated with affinity-purified antibodies directed against MEP1. Following extensive washes with a buffer containing 500 mM KCl and 0.1% NP-40, bound and unbound material was resolved by SDS-PAGE, followed by Coomassie staining (Fig. 2A). Mass spectrometric analysis suggested that MEP1 is tightly associated with the full NuRD complex and with TTK69 (Fig. 2A and Table 1). No binding to the control beads occurred. Thus, in contrast to MEP1 isolated from Kc cells, which binds only Mi2 (17), our purification from embryo nuclear extracts suggests that MEP1 is part of NuRD. In parallel, we used affinity-purified antibodies directed against Mi2 to purify NuRD (Fig. 2B). Again, our mass spectrometric analysis revealed the presence of MEP1, all core NuRD subunits, and TTK69 (Fig. 2B and Table 1). No peptides unique for TTK88 were identified in the MEP1 or Mi2 purification. Our results suggest that *Drosophila* NuRD comprises Mi2, MEP1, MTA1, p66/68-like, RPD3, CAF1 p55 (the homolog of RbAp46/48), MBD-like protein isoforms A and B, and the fly homolog of CDK2-associated protein 1 (CDK2AP1). CDK2AP1 is a potential tumor suppressor also known as DOC1 (deleted in oral cancer 1) (31). Although not generally listed as such, CDK2AP1/DOC1 was identified previously as a protein associated with mammalian NuRD (19). Our independent identification of CDK2AP1 in *Drosophila* NuRD suggests that it might, in fact, be an overlooked subunit of NuRD. Finally, in our immunopurifications, we detected TTK69, confirming its binding to NuRD. However, based on its modest scores in the mass spectrometric analysis, we consider TTK69 a substoichiometric interacting factor and not a NuRD subunit.

As an additional comparison of MEP1- and Mi2-associated factors, we performed coimmunoprecipitations (coIPs) from embryo nuclear extracts (Fig. 2C). Western immunoblotting confirmed the stable association of Mi2, MEP1, MTA1, CDK2AP1/DOC1, RPD3, and TTK69. BRM and ISW1 acted as negative controls, demonstrating the specificity of the coIPs. To obtain additional evidence that CDK2AP1/DOC1 is a core NuRD subunit, we performed coIPs using antibodies directed against this protein (Fig. 2D). Indeed, anti-CDK2AP1/DOC1 antibodies efficiently purified the NuRd complex, as illustrated by the copurification of Mi2, MEP1, MTA1, and RPD3. To investigate whether the majority of NuRD would be associated with CDK2AP1/DOC1, we immunodepleted an embryo nuclear extract using antibodies directed against this protein. Inspection of the CDK2AP1/DOC1-depleted extract revealed the concomitant loss of Mi2, MEP1, MTA1, and, to a somewhat lesser extent, RPD3 (Fig. 2E). In contrast, ISWI levels were not affected.

Collectively, our proteomic analysis, coIPs, and immune depletion experiments provide compelling evidence that MEP1 and CDK2AP1/DOC1 are tightly associated subunits of *Dro-*

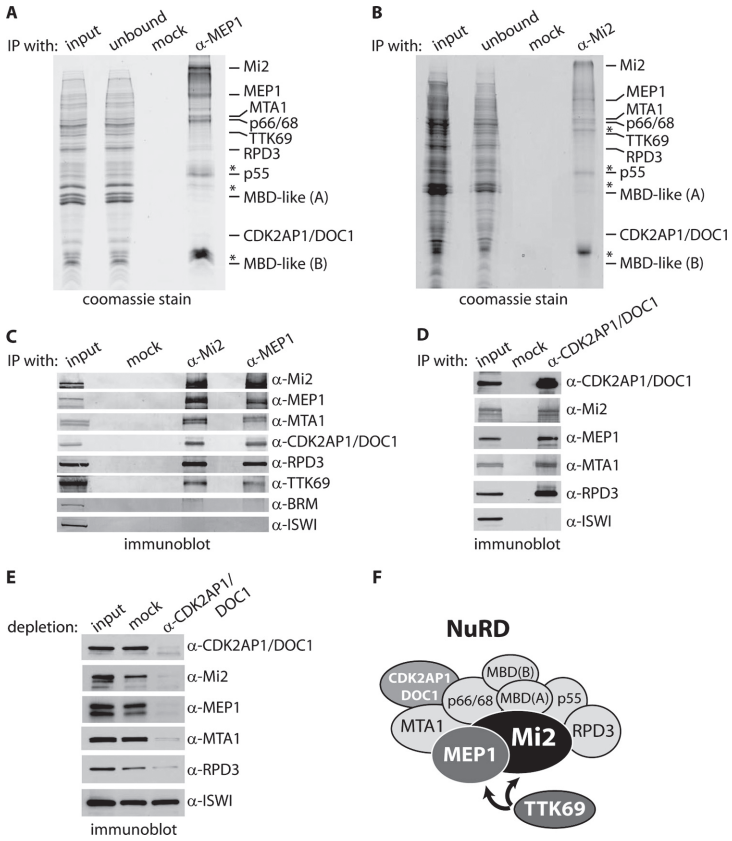

FIG. 2. MEP1 and CDK2AP1/DOC1 are *Drosophila* NuRD subunits. (A and B) Immunopurification (IP) of MEP1 (A) and Mi2 (B) from embryo nuclear extracts. Embryo nuclear extracts were incubated with protein A-Sepharose beads coated with either a control (anti-GST) antibody (mock) or an affinity-purified anti-MEP1 or anti-Mi2 antibody. Input, unbound material, and proteins retained on the beads after extensive washing were resolved by SDS-PAGE and visualized by Coomassie staining. Bands were excised, and proteins were identified by nanoflow liquid chromatography-tandem mass spectrometry. The mass spectrometry scores of the indicated proteins are listed in Table 1. (C) Coimmunoprecipitations of NuRD and TTK69 with MEP1. Embryo nuclear extracts were incubated either with preimmune serum (mock) or with an anti-Mi2 or anti-MEP1 antibody. Immunopurified proteins were resolved by SDS-PAGE and analyzed by immunoblotting using the indicated antibodies. Ten percent of the input material was loaded for reference. (D) CDK2AP1/DOC1 is a NuRD subunit. Results of coIPs using anti-CDK2AP1/DOC1 antibodies are shown. (E) The majority of NuRD is stably associated with CDK2AP1/DOC1. Nuclear extracts were immunodepleted with beads that were coated either with preimmune serum (mock) or with an antibody directed against CDK2AP1/DOC1. The supernatants were then resolved by SDS-PAGE and analyzed by Western immunoblotting with the indicated antibodies. Whereas NuRD subunits were strongly depleted, ISWI remained unaffected. Because RPD3 is part of multiple complexes, its depletion is expected to be less complete. (F) Cartoon summarizing our proteomic results. *Drosophila* NuRD comprises the ATPase Mi2, the HDAC RPD3, MTA1-like, CAF1 p55, p66/68-like, MBD-like isoforms A and B, MEP1, and CDK2AP1/DOC1. We note that mammalian CDK2AP1 was identified previously as a mammalian NuRD-associated protein by Le Guezennec et al. (19). We failed to detect additional proteins that have been reported incidentally as binding to NuRD. We do not consider TTK69 a NuRD subunit, although this cannot be formally concluded from the proteomics results. Rather, we view TTK69 as a transcription factor that interacts with NuRD by binding MEP1 and Mi2.

sophila NuRD. The composition of fly NuRD, based on our analysis, is diagramed in Fig. 2F. The results from our two-hybrid screen, *in vitro* interaction assay, proteomic survey, and coIPs all support the conclusion that TTK69 binds MEP1 directly. These findings suggest that MEP1 might act as a bridging factor between the DNA-binding transcription factor TTK69 and NuRD.

TTK69, MEP1, and Mi2 interact genetically. To complement our biochemical results and establish the *in vivo* significance of the interactions between TTK69, MEP1, and Mi2, we

TABLE 1. Mass spectrometric analysis of MEP1- and Mi2-associated proteins purified from *Drosophila* embryo nuclear extracts

Protein identity	Mol mass (kDa)	MEP1				Mi2				Comments[a]
		Mascot score	emPAI score	No. of unique peptides	% Sequence coverage	Mascot score	emPAI score	No. of unique peptides	% Sequence coverage	
Mi2 (CG8103)	225	8,311	11.87	98	54.1	9,323	14.83	112	62.1	ATPase, NuRD enzymatic core
MEP1 (CG1244)	175	3,817	9.97	40	53.2	3,315	6.02	37	46.3	Zinc fingers, SUMO interaction motifs
MTA1-like (CG2244)	97	2,610	3.94	39	55	3,047	6.77	43	59.1	Metastasis-associated protein
p66/68-like (CG32067)	95	2,229	2.33	22	48.1	2,480	2.24	24	48.8	Transcriptional corepressor
RPD3 (CG7471)	58	1,617	7.80	20	51.2	1,279	3.1	17	44.9	Histone deacetylase
p55 CAF1 (CG4236)	48	1,316	2.95	14	52.3	1,354	3.04	15	50.9	Histone binding
MBD-like A (CG8208-PA)	36	888	4.2	11	36.6	461	0.8	8	41.9	Methyl-CpG binding domain-like isoform A
MBD-like B (CG8208-PB)	25	481	2.69	7	46.5	300	0.92	5	31.4	Methyl-CpG binding domain-like isoform B
CDK2AP1/DOC1 (CG18292)	29	815	3.91	8	52	816	1.98	9	44.1	CDK2-associated protein 1; deleted in oral cancer 1
TTK69 (CG1856)	69	478	0.28	7	21.8	70	0.08	1	5.4	POZ domain, Zn finger DBD, sequence-specific transcriptional repressor
MAD (CG2662)	50	681	1.09	9	34.5					SMAD transcription factor
dCtBP (CG7583)	42	891	2.03	12	41.5					Transcriptional corepressor
Nejire/CBP (CG15319)	343	1,044	0.16	17	9.8					Histone acetyltransferase and coactivator

[a] DBD, DNA binding domain.

employed a genetic assay. We took advantage of the availability of fly lines expressing interfering RNA (RNAi) targeting TTK69, MEP1, or Mi2 (10). RNAi expression in these lines is under the control of the GAL4-upstream activation sequence (UAS) system, allowing the use of specific drivers to direct tissue-specific knockdowns. To lower TTK69, MEP1, or Mi2 levels in the developing eye, we used glass multiple reporter (GMR)-GAL4 to drive RNAi expression. Depletion of TTK69 (GMR>TTK69^{RNAi}), MEP1 (GMR>MEP1^{RNAi}) or Mi2 (GMR>Mi2^{RNAi}) alone had only a slight effect on eye development and ommatidial arrangement (Fig. 3A to D). However, the combined reduction of TTK69 and MEP1 (Fig. 3E) strongly affected ommatidial organization and caused a clear rough-eye phenotype. Likewise, the combined reduction of MEP1 and Mi2 synergistically affected eye development (Fig. 3F). These genetic interactions demonstrate that TTK69, MEP1, and Mi2 interact and cooperate *in vivo*.

TTK69, MEP1 and Mi2 control overlapping transcriptomes. To compare the genomewide binding pattern of TTK69 versus the NuRD subunits MEP1 and Mi2, we determined their distributions on larval salivary gland polytene chromosomes (Fig. 4). TTK69 colocalizes with MEP1 and Mi2 on a significant portion of their chromosomal binding sites. However, it is also clear that there are NuRD sites that lack TTK69 and, vice versa, that there are loci bound by TTK69 that are devoid of NuRD. We conclude that whereas TTK69 and NuRD also occupy unique loci, they colocalize on a substantial number of their binding sites.

To investigate the level of transcriptional coregulation by TTK69, MEP1 and Mi2, we combined RNAi-mediated depletion with genomewide expression analysis. We treated S2 cells with dsRNA directed against TTK69, MEP1, or Mi2. Immunoblot experiments showed that loss of TTK69 did not affect the stability of the NuRD subunit Mi2, MEP1, or RPD3 (Fig. 5A). Likewise, depletion of Mi2 or MEP1 left TTK69 levels unchanged. However, depletion of MEP1 did cause a reduction in Mi2 levels, whereas loss of Mi2 did not affect MEP1. RPD3 levels were unaffected by depletion of Mi2, MEP1, or TTK69. ISWI and tubulin acted as loading controls. Next, we extracted RNA from these cells or mock-treated cells. For each subunit, we performed three fully independent RNAi-mediated depletion experiments using distinct cell batches. For comparison, we used the expression analysis of cells depleted of ISWI, SNR1, Moira (MOR), or BRM (26). BRM, MOR, and SNR1 are three common core subunits of the BAP and PBAP remodelers, which represent the SWI/SNF class in *Drosophila*. The ISWI ATPase forms the enzymatic core of the ISWI class of remodelers, comprising NuRF and ACF/ChRAC.

The extracted RNA was labeled and hybridized with Affymetrics *Drosophila* Genome 2 arrays. Analysis of the expression data was performed as described previously (26). We used an unbiased statistical analysis of the whole data set to compare the impacts of the various proteins on gene expression. Hierarchical agglomerative clustering derived from Spearman's correlation analysis revealed a striking correlation between the effects of MEP1, Mi2, or TTK69 depletion. In contrast, neither ISWI depletion nor loss of (P)BAP subunits correlated well with the TTK69/NuRD cluster. Another way to uncover relationships between the gene expression profiles of different regulators is the application of principal-component analysis (PCA). PCA is a linear transformation that finds and projects original variables to the minimal principal components (PCs) that account for the maximal variance in the data set. About 76% of the variance in transcriptomes obtained after depletion of the 7 proteins analyzed here is explained by PC1 to PC3. Figure 5C shows the close clustering of the TTK69, Mi2, and MEP1 expression profiles, reflecting their high degree of correlation. The profiles of the (P)BAP core subunits and of ISWI were clearly separated from the TTK69/NuRD cluster, reflecting the fact that each regulates a specific set of genes.

Venn diagram analysis of genes that were affected significantly by the knockdowns demonstrates the substantial overlap between the TTK69-, MEP1-, and Mi2-dependent transcrip-

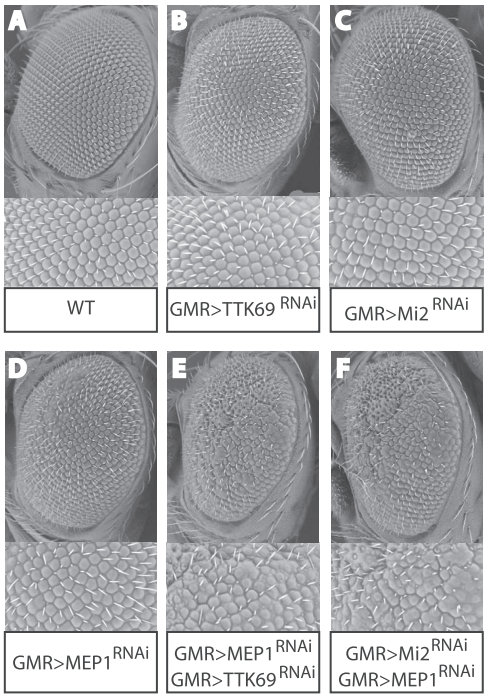

FIG. 3. TTK69, MEP1, and Mi2 interact genetically. Concomitant reduction of MEP1 and TTK69 or MEP1 and Mi2 causes an enhancement of the rough-eye phenotype. Representative scanning electron micrographs of adult eyes from flies with the indicated genotypes are shown. The GMR driver was used to direct the expression of specific interfering RNAs targeting TTK69 (B), Mi2 (C), or MEP1 (D), which had only a slight effect on eye development and the arrangement of ommatidia. However, a combined reduction in the levels of TTK69 and MEP1 (E) or Mi2 and MEP1 (F) strongly enhanced the rough-eye phenotype.

tomes (Fig. 5D). Consistent with the well-established repressive functions of TTK69 and NuRD, roughly twice as many genes were upregulated as downregulated following depletion of TTK69, MEP1, or Mi2. Nonetheless, our results raise the possibility of a role for TTK69 and NuRD in gene activation as well as repression, although the latter function clearly appears to be more prevalent. Alternatively, the activating role of TTK69 and NuRD might be indirect, i.e., due to repression of anther repressor. Gene Ontology (GO) analysis revealed the strong overrepresentation of developmental signaling in processes regulated by TTK69 and NuRD (Fig. 5E). A striking feature of the GO category grouping is that whole classes of genes appear to be either mostly repressed or mostly activated, suggesting coordinate regulation. In conclusion, our genome-wide analysis revealed substantial overlap between the TTK69, MEP1, and Mi2 transcriptomes, confirming that these factors collaborate closely.

TTK69 recruits NuRD to selective loci. Does NuRD first open up chromatin to allow sequence-specific DNA-binding by TTK69? Or does TTK69 bind chromatin independently, followed by NuRD recruitment? To distinguish between these two scenarios, we combined RNAi-mediated knockdown in S2 cells with chromatin immunoprecipitations quantified by real-time PCR (ChIP-qPCR). All our ChIP data are the results of at least three fully independent biological replicates. Chromatin was extracted from S2 cells that were either mock treated or depleted of TTK69, MEP1, or Mi2 (Fig. 5A). For ChIP-qPCR analysis, we examined binding to the regulatory regions of three representative TTK69 target genes: *Hairy*, *Engrailed* (*En*), and *Apaf1-related-killer* (*ARK*; CG6829). The first two are classic TTK69-regulated genes, and *ARK* was identified as one of the potential targets of TTK69 and NuRD in our genome-wide expression analysis.

First, we confirmed the derepression of *Hairy*, *En*, and *ARK* after TTK69, MEP1, or Mi2 knockdown by qPCR on mRNA isolated from S2 cells (Fig. 6A). ChIPs using antibodies against TTK69 revealed that depletion of either MEP1 or Mi2 did not affect TTK69 binding (Fig. 6B). As expected, TTK69 knockdown led to a loss of the TTK69 ChIP signal. The (P)BAP target *SK1* served as a negative control and was not bound by TTK69. These results show that the binding of TTK69 to the DNA loci tested is independent of chromatin remodeling by NuRD. ChIPs using antibodies against Mi2 revealed strongly reduced promoter binding due to a loss of TTK69, MEP1, or Mi2 itself (Fig. 6C). Because knockdown of MEP1 also caused a reduction in Mi2 protein levels (Fig. 5A), we cannot distinguish between loss of Mi2 and loss of recruitment. Like Mi2 binding, MEP1 binding was strictly dependent on TTK69 (Fig. 6D). However, loss of Mi2 only modestly affected MEP1 recruitment, suggesting that TTK69 can recruit MEP1 directly and independently of Mi2. As a control, ChIPs against BAP111 showed that BAP binding to its targets *SK1* and *KCNQ* was unaffected by the knockdown of TTK69, MEP1, or Mi2 (Fig. 6E).

In summary, our ChIP results showed that NuRD recruitment required TTK69. In contrast, TTK69 binding was independent of NuRD. Loss of Mi2 caused only a modest reduction of MEP1 recruitment, suggesting that TTK69 binding to MEP1 suffices for promoter tethering. We conclude that, on the loci examined, TTK69 binds first and then recruits NuRD (Fig. 7).

DISCUSSION

Here we have studied the cooperation between the sequence-specific transcription factor TTK69 and the ATP-dependent chromatin-remodeling factor NuRD. One prevalent view of remodeler action is that remodelers act randomly to open up chromatin, creating a window of opportunity for sequence-specific transcription factors to bind their cognate DNA recognition sequences. A drawback of such a scenario is that it does not readily explain the functional specialization of remodelers and how they act in a gene-selective manner. In this study, we provide an example of transcription factor binding preceding remodeler recruitment through selective protein-protein interactions. In addition, we provide a detailed characterization of *Drosophila* NuRD.

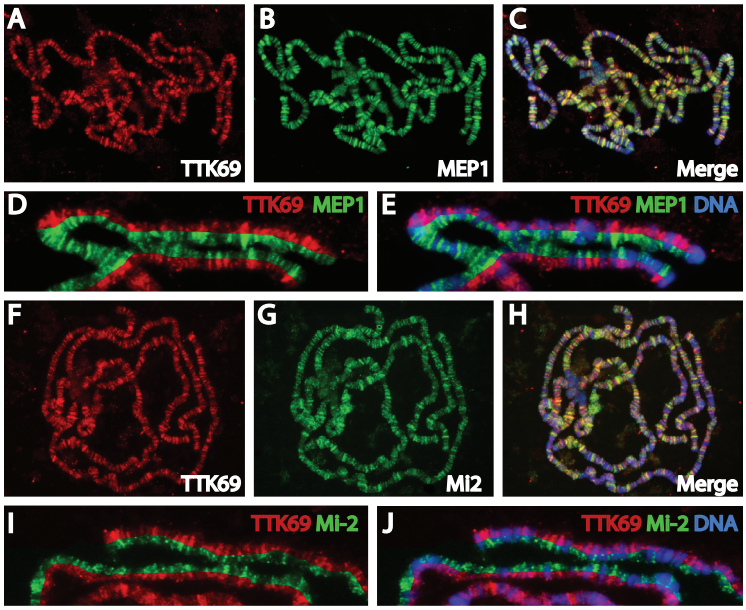

FIG. 4. TTK69 colocalizes with MEP1 and Mi2 on many loci. (A to E) The distributions of TTK69 (red) and MEP1 (green) on *Drosophila* salivary gland chromosomes were determined by immunostaining. (F to J) Likewise, the distributions of TTK69 (red) and Mi2 (green) were determined. DNA stained blue (with DAPI) in the merged panels. The colocalization of TTK69 and NuRD on many sites is demonstrated by the yellow staining in the merged panels and the similar patterns in split polytenes. However, TTK69 and NuRD also occupy unique loci.

As part of our efforts to understand the molecular mechanism underpinning transcription control by TTK69, we identified MEP1 as a TTK69-interacting protein. Subsequent proteomic and biochemical analyses established MEP1 and CDK2AP1/DOC1 as *bona fide* NuRD subunits. For example, the sequence coverage and emPAI scores (identification scores corrected for protein sequence length) of NuRD subunits in our Mi2 and MEP1 purifications were remarkably comparable (Table 1). Immunodepletion of CDK2AP1/DOC1 concomitantly removes key NuRD subunits, including Mi2, MEP1, and MTA1 (Fig. 2E), confirming that CDK2AP1/DOC1 is stably associated with the majority of NuRD complexes in the extract. Recently, the dMec complex, comprising solely MEP1 and Mi2, was isolated from Kc cells (17). Thus, as seen more commonly for chromatin-regulatory factors, MEP1 and Mi2 appear to be part of alternate assemblages. However, our results indicate that in embryo nuclear extracts, the majority of Mi2 and MEP1 exists as part of NuRD.

In addition, our biochemical analysis confirmed the association of MEP1 and TTK69. Genetic interaction assays provided independent functional evidence for cooperation between TTK69 and NuRD. TTK69 and NuRD colocalize on a substantial fraction of their binding sites but also occupy unique loci. Genomewide expression analysis revealed that the transcriptomes of TTK69, MEP1, and Mi2 overlap significantly. In contrast, the TTK69 gene expression profile correlated poorly with remodelers other than NuRD, reflecting their functional differentiation. Notably, some GO classes were largely repressed by TTK69 and NuRD, whereas others were mainly activated. Thus, it appears that some sets of functional gene classes are coordinately regulated by TTK69 and NuRD. Because both TTK69 and NuRD are commonly considered transcriptional repressors, their apparently positive role might be due to indirect effects. However, we note that a potential positive role for HDACs has been raised as well (36). Likewise, NuRD might directly activate certain target genes. Our ChIP analysis established that TTK69 could bind its targets independently of NuRD. Binding of NuRD, however, was critically dependent on TTK69. We conclude that TTK69 recruits NuRD to selective loci, not the other way around (Fig. 7). Of course, interactions of NuRD itself with DNA and histones are likely to contribute significantly to its targeting. Transcription factors other than TTK69 will also mediate NuRD recruitment to target loci. Conversely, there is no reason to assume that NuRD is the only transcriptional cofactor of TTK69.

Our work suggests that MEP1 is a genuine subunit of *Drosophila* NuRD. What is the relationship between MEP1 and NuRD in other organisms, including humans? Interestingly, previous studies have shown that in *C. elegans*, MEP1 associates with the homologs of Mi2 (LET-418) and RPD3 (HDAC-1) and functions in the repression of germ line genes in somatic cells (32). These observations are fully consistent with the notion that worm MEP1 is part of NuRD. Because

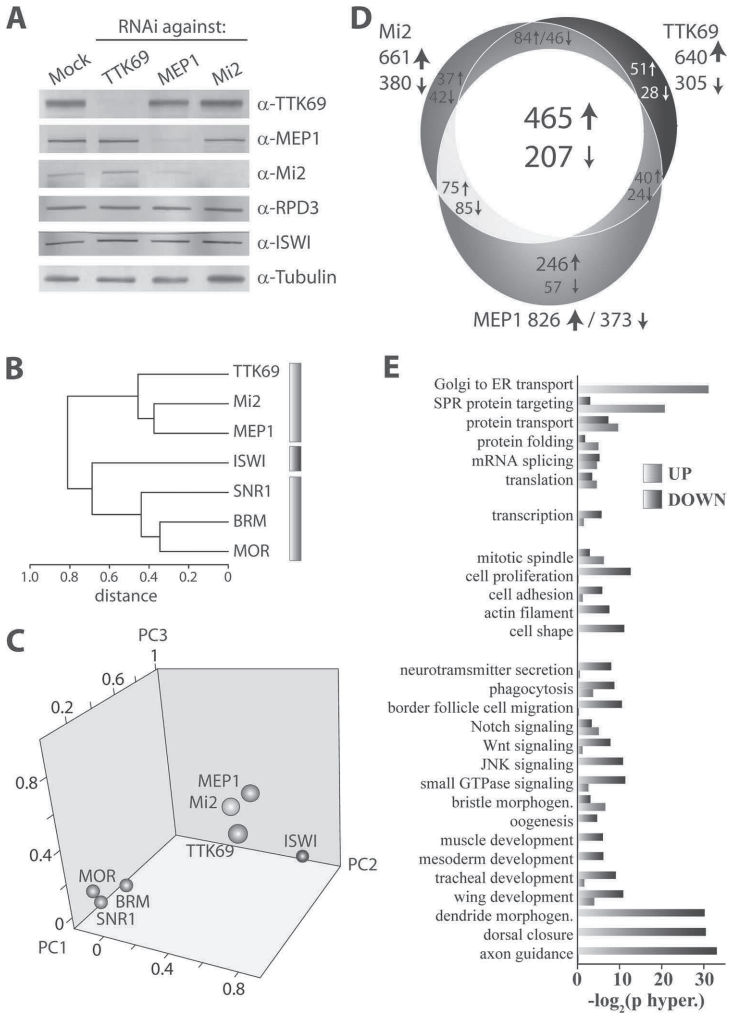

FIG. 5. Genomewide expression profiling reveals that TTK69, MEP1, and Mi2 control overlapping transcriptomes. (A) Selective depletion of TTK69, MEP1, and Mi2. S2 cells were either mock treated or treated with dsRNA against TTK69, MEP1, or Mi2. Whole-cell extracts were analyzed by Western immunoblotting using the indicated antibodies. ISWI and α-tubulin served as loading controls. (B) The TTK69 transcriptome correlates well with that of NuRD, but not with that of ISWI or (P)BAP. Agglomerative hierarchical cluster analysis of the expression profiles of TTK69, NuRD, ISWI, and the (P)BAP core subunits SNR1, BRM, and MOR was performed. Clustering is based on Spearman *R* values. TTK69 and NuRD (green), ISWI (blue), and (P)BAP (red) clusters are indicated. (C) Principal-component (PC) analysis of gene expression profiles reveals the close clustering of TTK69 with Mi2 and MEP1, but not with ISWI or the (P)BAP core subunits. (D) Venn diagram depicting the numbers of genes that are coordinately regulated by TTK69, Mi2, and MEP1. Arrows indicate either upregulation or downregulation following depletion. The numbers of genes affected are given. (E) GO analysis and biological pathway clustering of genes coordinately regulated by TTK69, Mi2, and MEP1. morphogen., morphogenesis.

MEP1 is critical for Mi2 function in flies (this study) and worms (32), we wondered whether there might also be a human ortholog. Although straightforward database inspection did not reveal a mammalian homolog, a search using the zinc finger domain of fly MEP1 revealed homology with human ZFHX1B/SIP1/ZEB2. A direct Clustal 2.0 alignment of ZFHX1B (35), *Drosophila* MEP1, and *C. elegans* MEP1 demonstrated a modest but suggestive homology between the

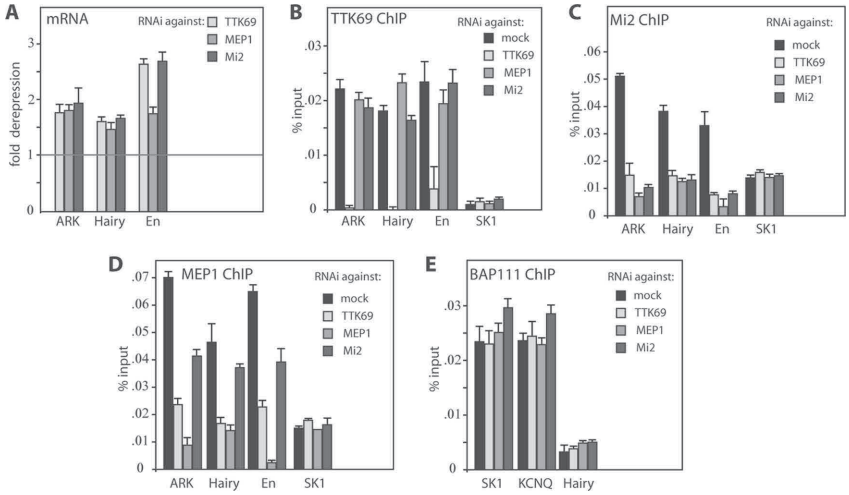

FIG. 6. NuRD binding to selective promoters depends on TTK69, whereas TTK69 binding is independent of NuRD. (A) Upregulation of *ARK*, *Hairy*, and *En* after TTK69, MEP1, or Mi2 knockdown. RNA was extracted and quantified by RT-qPCR using appropriate primers. mRNA levels were normalized against those of CG11874, a gene whose expression did not change in our microarray experiments. Normalized mRNA levels were expressed relative to those in mock-treated cells. Graphs represent the results of three independent biological replicate experiments. Error bars represent the standard errors of the means. (B to E) ChIP-qPCR analysis of the binding of TTK69 (B), Mi2 (C), and MEP1 (D) to *ARK*, *Hairy*, and *En*. The BAP target *SK1* is not bound by TTK69 or NuRD and serves as a negative control. ChIPs were performed on chromatin from mock-treated or RNAi-depleted cells for TTK69, MEP1, or Mi2. (E) The binding of BAP111 to its targets SK1 and KCNQ is not affected by RNAi against TTK69, MEP1, or Mi2. Cross-linked chromatin was prepared from mock-treated or RNAi-treated cells. All ChIP data are the results of at least 3 independent biological replicates. Error bars represent the standard errors of the means.

3 proteins (see Fig. S1 in the supplemental material). Importantly, it was recently reported that ZFHX1B interacts physically and functionally with NuRD (35). Thus, not only does ZFHX1B display structural similarity to MEP1; it also interacts with NuRD, making it an extremely attractive candidate mammalian ortholog. Additionally, ZFHX1B has been reported to interact with the SMAD transcription factors, the corepressor CtBP, and the acetyltransferase p300 (34). Suggestively, we identified the *Drosophila* homologs of these three proteins in our MEP1, but not Mi2, purifications (Table 1). These observations provide additional support for the notion that ZFHX1B and MEP1 might be orthologs; in addition, they indicate that MEP1 might participate in additional interactions, separate from NuRD.

Loss of heterozygosity of ZFHX1B/SIP1/ZEB2 has been implicated in the etiology of Mowat-Wilson syndrome (MWS), characterized by severe mental retardation and a range of additional defects. Mice lacking ZFHX1B display defects in early neurogenesis (33). Strikingly, a MWS-associated mutant form of ZFHX1B is unable to bind and recruit NuRD (35). In conclusion, we propose that MEP1 is an evolutionarily conserved NuRD subunit that is critical for targeting. In this sense, its function is reminiscent of that of selected signature subunits within the SWI/SNF class BAP and PBAP remodelers (6, 23, 24, 26).

Mi2 and MEP1 were identified in a screen for SUMO-dependent transcriptional repression (29). Both factors act downstream of SUMOylation of the transcription factor Sp3. MEP1 and Mi2 each interact with SUMO *in vitro*, and their recruitment to an integrated reporter gene is dependent on the SUMOylation of Sp3 (29). Extrapolating these observations made for Sp3, it is an attractive idea that TTK69 SUMOylation (20) will also modulate NuRD-dependent repression by TTK69. Indeed, we also observed an interaction between SUMO and *Drosophila* MEP1 and Mi2 (A. A. Travers and A. Bassett, unpublished results). However, thus far, we were unable to establish a direct function for SUMO in TTK69-directed silencing. We note that previous studies revealed a critical role for ubiquitylation/deubiquitylation of TTK in cell fate control (2, 21, 30). Likewise, it will be important to explore the role of SUMO signaling in TTK69/NuRD repression during development.

We identified the *Drosophila* homolog of the human CDK2-interacting protein CDK2AP1/DOC1 as a NuRD subunit. This small protein is a potential tumor suppressor (31), which thus far has received little attention. However, its identification in both mammalian (19) and fly NuRD strongly suggests that it is a conserved subunit, which may play a regulatory role. In our Mi2 and MEP1 purifications, we did not observe homologs of any of the other proteins incidentally reported as NuRD subunits in other organisms. Thus, based on the results of others and on this study, we surmise that the composition of NuRD as depicted in Fig. 2F represents its conserved core.

In conclusion, we present here a biochemical characteriza-

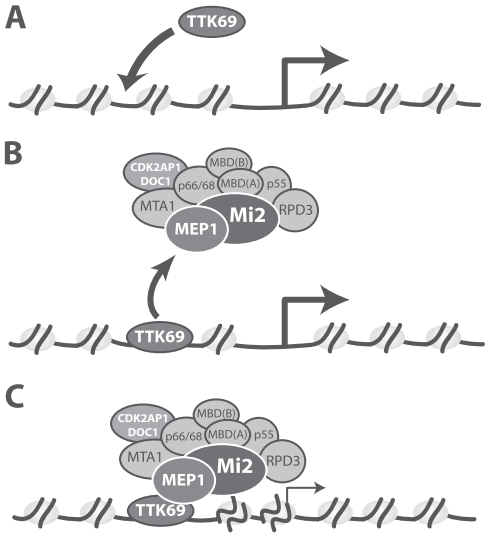

FIG. 7. TTK69 recruits NuRD to selective target loci. The models depict the hierarchical relationship between the sequence-specific transcription factor TTK69 and the ATP-dependent chromatin remodeling factor NuRD. (A) NuRD cannot bind TTK69 loci by itself, whereas TTK69 can bind its binding site independently of NuRD. (B) TTK69 can target NuRD through direct protein-protein interactions with its MEP1 and Mi2 (27) subunits. (C) TTK69 tethers the NuRD complex to specific loci, where it attenuates target gene transcription by modulating the local structure of chromatin. We note that the precise molecular nature of NuRD remodeling *in vivo* is still unclear. Our results support the notion that TTK69 recruits NuRD to selective loci, not the other way around. See the text for details.

tion of *Drosophila* NuRD. Our results showed that MEP1 and CDK2AP1 are two novel NuRD subunits. MEP1 plays a key role in linking NuRD to TTK69. Genomewide analysis of transcription revealed that TTK69 is critically required for a significant portion of NuRD-regulated genes. However, we note that TTK69 and NuRD also regulate genes independently of each other. Our results support a model in which TTK69 recruits NuRD to selective loci, rather than one in which NuRD-mediated chromatin remodeling is required for DNA binding by TTK69. We suggest that TTK69 belongs to a group of transcription factors, sometimes referred to as pioneer proteins, that bind chromatin independently of remodelers (41). We propose that, rather than acting in a generic fashion, specific remodelers cooperate with selective transcription factors in the control of gene expression.

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Reddy et al.
Supplementary Figure 1. **Clustal 2.0 Alignment of cMEP1, dMEP1 and ZEB2**

```
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dMEP1           MTEVDVVLPEGVAEPTTKLASASSSPKQSKPSADDVEQQVTTVEDDEEQVVEEQQEEDVH 60
ZEB2            -------MKQPIMADGPRCKRRKQANPRRKNVVNYDNVVDTGSETDEEDKLHIAEDDGIA 53
                        : :    ..    .      :   :         . .* : :.  : :

cMEP1           VMADAAN---------GSEVNGNQENGKEEAASADVEVIEIDDTEESTDPSPDGSDENGD 100
dMEP1           LPEAPSNEKLELLSAGGKDISKEDVAQASPQAPVADEIMEVDGTEDVEENEVENDDEDSQ 120
ZEB2            NPLDQETS--------PASVPNHESSPHVSQALLPREEEEDEIREGGVEHPWHNNEILQA 105
                      .           .:  .:       *    *  * :  *   :   ...:

cMEP1           AASTSVPIEEEARKKDEGASEVTVASSEIEQDD--DGDVMEITEEPNGKSEDTANGTVTE 158
dMEP1           ITSSSS-KEPKLDEEDEEATTNGDGDHEPEDED--DAQKIGSTAENSCEAEDPLGAVTVP 177
ZEB2            SVDGPEEMKEDYDTMGPEATIQTAINNGTVKNANCTSDFEEYFAKRKLEERDGHAVSIEE 165
                 .. .   : .    .  *:     .    .:    .:      : . : .*

cMEP1           EVLDEEEPEPSVNGTTEIATEKEPEDSSMPVEQNGKGVKR-------------------- 198
dMEP1           DDELASGKKAHLDGEESVASEGVVEEEPPAKQENGFGASPNQKENGQASIAEARAAIAPS 237
ZEB2            YLQRSDTAIIYPEAPEELSRLGTPEANGQEENDLPPGTPDAFAQLLTCPYCDRGYKRLTS 225
                     .      :.  .::     * .    ::   *.

cMEP1           PVECIELDDDDDDE--IQEISTPAPAKKAKIDDVKATSVP---------------------- 236
dMEP1           DGGVVNLDEDSDEE--MEDITEHKEPAKPTEKAAKPASSSGGESSDDAVLIASDSDTESA 295
ZEB2            LKEHIKYRHEKNEENFSCPLCSYTFAYRTQLERHMVTHKPG-------------------- 266
                    ::  .:.::*     :     . :.  .    :  .

cMEP1           ----------------------EEDNNEQ------------------AQKRLLDKLEEY 255
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ZEB2            ----------------TDQHQMLTQGAGNRKFKCTECGKAFKYKHHLKEHLRIHSGEKPY 310
                                  :. .:                    :    .  :

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dMEP1           QPSHKRQRSSTPSGMGQLTIKDARSLMPLDGSPSSVSGPRDSIYPVTITNAVTGAATNLG 415
ZEB2            ECPNCKKRFSHSGSYSSHISSKKCIGLISVNGRMRNNIKTGSSPNSVSSSPTNSAITQLR 370
                   :  :  * .                                      ..  .* .

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dMEP1           VVPPKLVPMAGGMSASPVQLNPPTLSLTGLPSMTNNANLL------------PGLTDDMF 463
ZEB2            NKLENGKPLSMSEQTGLLKIKTEPLDFNDYKVLMATHGFSGTSPFMNGGLGATSPLGVHP 430
                             ..    :.   * :     :  . .:             ..

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                  . *        * *             : *.::        .  :..  :  .:   :

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ZEB2            AKACLQSLTTDSRRQISNIKKEKLRTLIDLVTDDKMIENHNISTPFSCQFCKESFPGPIP 595
                                                  ::   :.  : :*     :
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dMEP1           RLQKRKMRKAQVQNSKEFEMAINALSG-------------NLQASKTKNAPFKFRMKRCE 684
ZEB2            LHQHERYLCKMNEEIKAVLQPHENIVPNKAGVFVDNKALLLSSVLSEKGMTSPINPYKDH 655
                  ::        :: :        :                 .. . *. .  :.  : .

cMEP1           VCGFQTESKLVMSTHKENLHFTGSKFQCTMCKETDTSEQRMKDHYFETHL-VIAKSEEKE 483
dMEP1           FCNFKSESAMAMANHYETPHMNGVLYKCNFCTFEIRNATEIVYHMEAVHN-IKARLIKPL 743
ZEB2            MSVLKAYYAMNMEPNSDELLKISIAVGLPQEFVKEWFEQRKVYQYSNSRSPSLERSSKPL 715
                .. :::   : *  : :     .                .   :    :     :  :

cMEP1           SKYPCAICEEDFNFKGVREQHYKQCKKDYIRIRNIMMPKQDDHLYINRWLWERPQLDPSI 543
dMEP1           PYHQCPNCGFEDNGKAKLARHQPVCAKKFRPELNLAPPND----------WEAPAKIPRI 793
ZEB2            APNSNPPTKDSLLPRSPVKPMDSITSPSIAELHNSVTNCDPPLRLTKPSHFTNIKPVEKL 775
                .    .    .   :.          .     *    :          :       :

cMEP1           LQQQQQAALQQAQQKKQQQLLHQQQAAQAAAAAQLLRKQQLQQQQQQQQQARLREQQQAAQ 603
dMEP1           KPRHG-----LVGTATAYQAMAAQAAAQKAALANIQQQQAAAQARNNLQAAALAAQNAAK 848
ZEB2            DHSRSNTPSPLNLSSTSSKNSHSSSYTPNSFSSEELQAEPLDLSLPKQMKEPKSIIATKN 835
                   :           .  :    .  :  :  ::  : :      :          : :

cMEP1           FRQVAQLLQQQSAQAQRAQQNQGNVNHNTLIAAMQASLRRGGQQGNSLAVSQLLQKQMAA 663
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ZEB2            KTKASSISLDHNSVSSSSENSDEPLNLTFIKKEFSNSNNLDNKSTNPVFSMNPFSAKPLY 895
                  : :    .:.   . :          :    . .   .          :  .

cMEP1           LKSQ-QGAQQLQAAVNSMRSQNSQKTPTHRSSKLVTTPSHATVGSSSAPTFVCEICDASV 722
dMEP1           QPSISITPLPRQSSVGAGAGASSSKAPQAAAG---MKPGQSPSGNNKAQFVICEICDGYI 958
ZEB2            TALPPQSAFPPATFMPPVQTSIPGLRPYPGLDQMSFLPHMAYTYPTGAATFADMQQRRKY 955
                       .    : : .     .   *    .     *  :    . *  .

cMEP1           QEKEKYLQHLQTTHKQMVG-------------KVLQDMSQGAPLACSRCRDRFWTYEGLE 769
dMEP1           KDLEQLRNHMQWMHKVKIH-------------PKMIYNRP--PLNCQKCQFRFFTDQGLE 1003
ZEB2            QRKQGFQGELLDGAQDYMSGLDDMTDSDSCLSRKKIKKTESGMYACDLCDKTFQKSSSLL 1015
                :  :    .:    :  :                                *. *   * . ..*

cMEP1           RHLVMSHG------------LVTADLLLKAQKKEDGGR---CKTCGKNYAFNMLQHLVAD 814
dMEP1           RHLLGSHG------------LVTSSMQEAANKGKDAGR---CPVCGRMYQWKLLNHVSRD 1048
ZEB2            RHKYEHTGKRPHQCQICKKAFKHKHHLIEHSRLHSGEKPYQCDKCGKRFSHSGSYSQHMN 1075
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dMEP1           HHMTLKP-------------------------AHLSYKCTVCTATFGMYKQFETHVYTAH 1083
ZEB2            HRYSYCKREAEEREAAEREAREKGHLEPTELLMNRAYLQSITPQGYSDSEERESMPRDGE 1135
                *: .                               .  *   : .   .  :  *:     .

cMEP1           PKGDKKTS-------------------------------TPAKKDDCITLDD-------- 870
dMEP1           STVARKAMDSKKNSAQSSGSGSGAGMSRSSLGAANDSLLKPLKINDEITIIPQPASKPRI 1143
ZEB2            SEKEHEKEGEDGYGKLGRQDGDEEFEEEEEESENKSMDTDPETIRDEEETGDHSMDDSSE 1195
                .   ::                                  * .  *

cMEP1           -------------------
dMEP1           TNMESHVID---------- 1152
ZEB2            DGKMETKSDHEEDNMEDGM 1214
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Chapter 4

Gene Regulation by Tumor Suppressor DOC1

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Abstract

DOC1 (*deleted in oral cancer 1*) is a potential tumor suppressor that is either deleted or down regulated in several cancers including oral cancer. DOC1 has been proposed as a tumor suppressor in light of its role in inhibition of Cyclin dependent kinase 2 (CDK2) function and cell cycle regulation. We earlier reported DOC1 as a subunit of the Nucleosome Remodeling and Deacetylase (NuRD) complex in *Drosophila* (*Chapter 3*). The precise mechanism of tumor suppression by DOC1 in the context of the NuRD complex remains enigmatic. Re-expression of DOC1 in deleted oral cancer cell lines resulted in cell growth inhibition. Proteomics analysis identified SMAD4, a transducer of transforming growth factor-beta (TGF-β) signalling as a DOC1 interacting protein. RNAi-mediated knockdown of DOC1 and Chromatin-helicase-DNA-binding-protein-4 (CHD4) (ATPase subunit of NuRD) resulted in de-repression of SMAD4 target genes involved in tumorigenesis. Our results suggest that DOC1-NuRD complex functions as a repressor of TGF-β regulated genes, which are involved in promoting cell proliferation, metastasis and angiogenesis.

Introduction

DOC1 is a highly conserved, ubiquitously expressed gene located on chromosome 12q24. It encodes 115-aa nuclear polypeptide that is down regulated or deleted in ~70% of oral cancers (Wong et al., 2012). DOC1 was originally identified as a novel tumor suppressor using chemically-induced oral cancer in hamster. Reintroduction of DOC1 into the malignant hamster oral keratinocytes and *in vivo* mouse models of head and neck cancer resulted in the tumor growth inhibition, suggesting reversal of malignant transformation (Figueiredo et al., 2005; Wong et al., 1996). DOC1, also called CDK2AP1 (CDK2-associated protein 1), is a CDK2 inhibitor implicated in cell cycle regulation through G1/S transition (Shintani et al., 2000). DOC1 inhibits CDK2-mediated phosphorylation of DNA-polymerase α/primase that resulting in inhibition of DNA synthesis and cell growth (Matsuo et al., 2000). Therefore, execution of tumor suppressive activity by DOC1 is thought to be through regulation of the cell cycle (Shintani et al., 2000; Wong et al., 2012). Targeted deletion of *DOC1* in mice resulted in embryonic lethality (Kim et al., 2009b). DOC1 is also implicated in embryonic stem cell differentiation through the control of RB phosphorylation and epigenetic regulation of OCT4 expression (Deshpande et al., 2009; Kim et al., 2009a). DOC1 has also been identified as a subunit of the NuRD chromatin remodeling complex in *Drosophila* (*Chapter 3*) and also in human cells (Le Guezennec et al., 2006; Spruijt et al., 2010).

TGF-β family members play an important role in cell growth, differentiation and apoptosis. SMAD proteins, a family of eight members (SMAD1-8) are the intracellular mediators of canonical TGF-β family signalling (ten Dijke and Hill,

2004). SMAD4 is a central mediator of transcriptional responses to TGF-β-induced transcriptional responses (Hahn et al., 1996; Lagna et al., 1996). SMAD4 is the key factor in the TGF-β pathway and can function as a tumor suppressor. Germline mutations in the *SMAD4* cause *Juvenile Polyposis Syndrome* (JPS) with high risk of developing colorectal cancer. Homozygous deletion or somatic mutation of *SMAD4* gene occurs most frequently in pancreatic, gastrointestinal, and skin cancers (Waite and Eng, 2003). Conditional knockout of *SMAD4* gene in mouse skin resulted in the development of squamous cell carcinoma (Qiao et al., 2006). Mouse models with tissue specific deletion of *SMAD4* gene revealed that the loss of SMAD4 accelerates tumor formation (Yang and Yang, 2010). Mutations or loss of heterozygosity of other TGF-β core components were also observed in several human cancers (Levy and Hill, 2006). TGF-β acts as a tumor suppressor through the regulation of cell cycle and apoptosis by inhibiting the expression of the MYC oncogene and activating the expression of cell cycle inhibitors, such as p21 and p15. In contrast, TGF-β also actively participates in tumor development through the initiation of processes such as tumor cell invasion, epithelial-to-mesenchymal transition (EMT) and metastasis (Meulmeester and Ten Dijke, 2011). Thus, at the early stages of tumor development TGF-β acts as a tumor suppressor and later stages it may actively promote tumorigenesis.

Since DOC1 is a potential tumor suppressor having implications in human cancers, we shifted our focus from *Drosophila* DOC1 to human DOC1. Here we investigate in detail the gene regulation by human DOC1. We propose a novel function for DOC1 in gene regulation through its interaction with SMAD4.

Results

DOC1 inhibits the cell growth in squamous cell carcinoma cell lines

DOC1 is a potential growth suppressor which is found deleted in many cancers including oral cancer. Therefore, we tested the effect of re-expression of DOC1 in deleted squamous carcinoma cell lines SCC4 and SCC9. Expression of DOC1 is analyzed by Western blotting analysis (*Figure 1A*). Reintroduction of DOC1 in both SCC4 and SCC9 cells resulted in inhibition of cell proliferation in a time-dependent manner compared to the cells expressing a control plasmid (*Figure 1B*). Hence, DOC1 acts as a suppressor of growth in these cancer cell lines. These results are in agreement with earlier reports that DOC1 elicits cytostatic effects through the regulation of cell cycle by inhibition of CDK2 (Matsuo et al., 2000; Shintani et al., 2000). However, the role of DOC1 as a tumor suppressor, being a part of NuRD complex still remains enigmatic. Therefore, we took an unbiased proteomic approach to unravel the gene regulatory networks of the DOC1-NuRD complex.

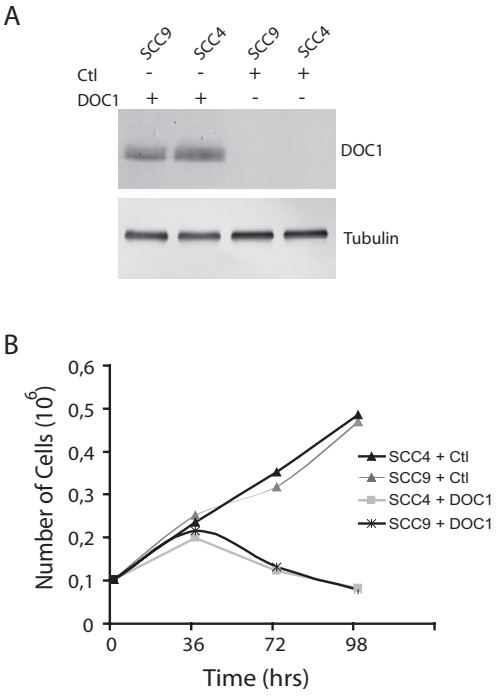

Figure 1; Re-expression of DOC1 inhibits cell growth in SCC4 and SCC9 cells: SCC4 or SCC9 cells were transiently transfected with either control plasmid (Ctl) or plasmid expressing DOC1. Expression of DOC1 is analysed by Western blotting analysis Tubulin serves as a loading control (**A**). Cells were counted by using heamocytometer at 36, 72 and 96 hours of post-transfection. The average number of cells from three independent experiments is plotted against the time as indicated (**B**).

DOC1 interacts with the TGF-ß intracellular mediator SMAD4

We took a proteomics approach to make an inventory of DOC1 associated proteins in *HeLa* nuclear extracts. Nuclear extracts were incubated with protein A-Sepharose beads coated with affinity-purified antibodies directed against DOC1. Following extensive washes, bound and unbound material was resolved by SDS-PAGE followed by coomassie staining (*Figure 2A*). Massspectrometric analysis revealed that DOC1 is tightly associated with the NuRD complex and with SMAD4 a key intracellular mediator of TGF-β signalling (*Figure 2A and Table 1*). No binding of NuRD complex or SMAD4 is observed to the control beads coated with anti-glutathione-S-transferase (GST) antibodies. We found all known subunits of the NuRD complex in DOC1 immunopurifications (IPs) including chromodomain helicases CHD3 and CHD4 histone deacetylases HDAC1, HDAC2 and HDAC3, metastasis tumor antigens MTA1, MTA2 and MTA3, histone binding proteins RBBP4 and RBBP7, GATA-like transcription factors, GATAD2A/2B and methyl-DNA binding proteins MBD2 and MBD3. These results are comparable with the *Drosophila* DOC1 IPs carried out on embryo nuclear extracts (*Table S1*). These findings suggest that DOC1 is an evolutionally conserved subunit of the NuRD complex. In addition, we also found CHD5 (*chromodomain helicase 5*), a paralouge of Mi2/CHD4 as a subunit of the NuRD complex.

In parallel, we used affinity-purified antibodies directed against CHD4 to purify NuRD complex. Massspectrometric analysis revealed the presence of

DOC1 and all core NuRD subunits (*Figure 2B and Table 1*). These results suggest the NuRD complex comprises: CHD3/4/5, HDAC1/2/3, MTA1/2/3, RBBP4/7, GATA2A/2B, MBD2/3 and DOC1. We previously reported MEP1 as a subunit of *Drosophila* NuRD complex. MEP1 targets the NuRD complex to different genomic loci by binding to the BTB-POZ domain transcription factor *Tramtrack 69* (TTK69) (*Chapter 3*). However, there are no homologues of MEP1 or TTK69 identified so far in vertebrates. MEP1 is a zinc finger and homeobox domains containing protein. Incidentally we found a similar zinc finger protein, zinc finger and homeobox 3 (ZFXH3) in CHD4 IPs. Therefore, ZFHX3 could be a functional homologue of MEP1 in humans. Similarly we also found ZBTB2 (zinc finger and BTB-POZ domain 2) in both DOC1 and CHD4 IPs. This could be the functional homologue of TTK69 (Supplementary Table 1). Additionally we also found LSD1/KDM1A in both DOC1 and CHD4 IPs. LSD1 is a subunit of NuRD complex (Wang et al., 2009). However, we do not find LSD1 in *Drosophila* NuRD IPs (*Chapter 3 and Table S1*). We also found CDK2, a known interactor of DOC1 in both DOC1 and CHD4 IPs. To our surprise, we did not find SMAD4 in CHD4 IP's which we had found in DOC1 IP's (*Figure 2A, 2B, 2C, Table 1*). These findings suggest that SMAD4 is not a subunit of NuRD complex but interacts with the NuRD through the DOC1 subunit.

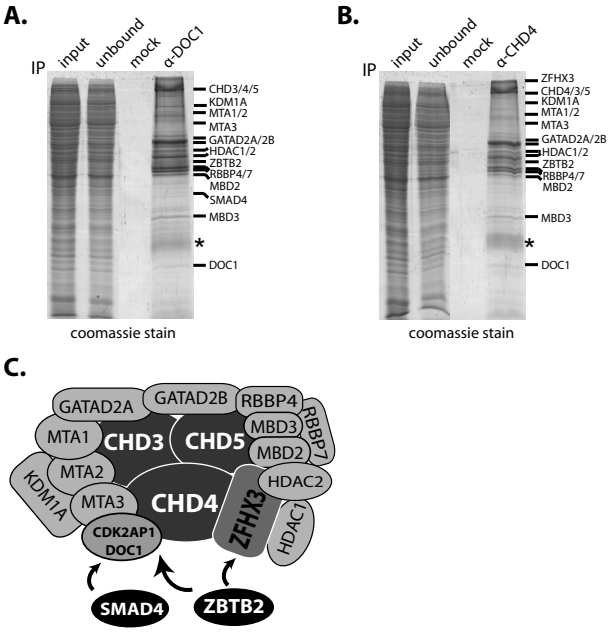

Figure 2; Immunopurification of DOC1 and CHD4: Immunopurification of DOC1 (**A**) and CHD4 (**B**) from *HeLa* nuclear extracts. *HeLa* nuclear extracts were incubated with Protein A-Sepharose beads coated with either control (α-GST) antibodies (mock) or affinity-purified α–DOC1 or α-CHD4 antibodies. Input, unbound material and proteins retained on the beads after extensive washing were resolved by SDS-PAGE and visualized by Coomassie staining. Bands were excised and proteins were identified by nanoflow LC-MS/MS mass spectrometry. The various mass spectrometry scores of the indicated proteins are listed in *Table 1*. **C**) Cartoon summarizing our proteomic results. The core NuRD complex comprises: the ATPase's CHD3/4/5, HDAC1/2/3, MTA1/2/3, RBBP4/7, GATA2A/2B, LSD1 (KDM1A), MBD2/3 and DOC1. SMAD4 interact with NuRD through DOC1 subunit. ZBTB and ZFHX3 are NuRD interacting proteins.

DOC1 is a repressor of SMAD4 target genes

Next we asked whether DOC1-NuRD cooperates with SMAD4 to regulate the TGF-β responsive gene expression. To test this, we performed RNAi-mediated knockdown of DOC1 and CHD4 followed by RT-qPCR analysis of SMAD4 target genes in TGF-β responsive *HaCaT* cells. The knockdown efficiency is analyzed by RT-qPCR (*Figure 2A*). Knockdown of DOC1 and CHD4 resulted in the up-regulation of SMAD4 target genes, such as MMP9, SNAIL and EpCAM. Similarly, up-regulation of these target genes was observed upon stimulation by TGF-β (*Figure 2B, 2C & 2D*). MMP9 is a matrix metalloproteinase which promotes breast cancer invasion in a SMAD4- and TGF-β- dependent manner (Wiercinska et al., 2011). SNAIL is a TGF-β inducible gene implicated in EMT and tumor invasion (Xu et al., 2009). EpCAM promotes metastasis by inhibiting the expression of E-cadherin, a cell-cell adhesion molecule (van der Gun et al., 2010). Surprisingly, the expression of other SMAD4 target genes, such as p21 and p15 were not affected by DOC1 or CHD4 knockdowns (*Figure 2E & 2F*). These results suggest that the DOC1-NuRD complex regulates the expression of specific set of TGF-β responsive genes that promote tumor development. Whether the DOC1-NuRD complex directly controls the expression of these genes still needs to be addressed.

Protein identity	MW kDa	DOC1		CHD4		Comments
		MS	ES	MS	ES	
CHD4	218	8132	40.57	7603	21.72	Chromodomain-helicase-DNA-binding protein 4 (Mi-2ß)
CHD3	233	4661	4.06	1457	0.68	Chromodomain-helicase-DNA-binding protein 4 (Mi-2α)
CHD5	223	1992	1.1	1688	0.83	Chromodomain-helicase-DNA-binding protein 4 (Mi-2 related)
ZFHX3	404			1826	0.34	Zinc finger homeobox protein 3, drosophila MEP1
MTA1	79	2898	13.28	2419	8.42	Metastasis-associated protein 1
MTA2	75	3153	20.7	2970	16.36	Metastasis-associated protein 2
MTA3	66	2001	7.21	1747	7.67	Metastasis-associated protein 3
GATAD2A	68	2642	14.25	2496	15.78	Transcriptional repressor p66-alpha
GATAD2B	66	2154	17.72	2025	12.07	Transcriptional repressor p66-beta
HDAC1	55	1953	92.67	1738	64.16	Histone deacertrylase 1
HDAC2	55	2028	146.6	1823	129.9	Histone deacertrylase 2
RBBP4	47	1491	6.63	1385	5.63	Histone-binding protein RBBP4
RBBP7	47	1668	15.29	1474	12.21	Histone-binding protein RBBP7
MBD2	43	1366	9.05	1499	10.73	Methyl-CpG-binding domain protein 2
MBD3	32	1575	68.57	1314	21.9	Methyl-CpG-binding domain protein 2
KDM1A	92	808	0.66	1003	0.99	Lysine-specific histone demethylase 1 A
DOC1	12	378	9.16	281	4.24	Deleted in Oral Cancer 1
ZBTB2	51	303	0.42	393	0.59	Zinc finger and BTB domain-containing protein 2, Tramtrack69
SMAD4	48	622	1.81			SMAD transcription factor, TGF-ß signalling
CDKN2	30	215	0.63	88	0.28	Cyclin dependent kinase 2

MS - Mascot score ES - emPAI score

Table1; Massspecrtometric analysis of DOC1 and CHD4 immunopurifications

Discussion

Here we explore the physical and functional relationship between chromatin remodeler NuRD and TGF-β signalling. TGF-β plays a dual role in tumorigenesis. In the initial stages of cancer development, TGF-β acts as a tumor suppressor by inhibiting the cell growth and accelerating apoptosis. However, at later stages, TGF-β promotes tumor growth by facilitating migration, invasion and angiogenesis (Elliott and Blobe, 2005). Our results suggest that DOC1-NuRD inhibits the expression of TGF-β-induced genes, such as MMP9, SNAIL and EpCAM which promote tumor invasion, EMT and metastasis. Our findings are in agreement with Zolochevska *et al*., who showed that reintroduction of DOC1 in deleted cancer cell lines resulted in inhibition of tumor invasion, metastasis and angiogenesis (Zolochevska and Figueiredo, 2010). However, the molecular mechanism of inhibition of tumorigenesis by DOC1 is not clear. It is possible that SMAD4 binds to DOC1 to recruit the NuRD complex for establishing transcriptional repression of TGF-β responsive genes involved in tumorigenesis. Further research is warranted to address these issues.

DOC1 is a growth inhibitor that executes its function by inhibiting the cell cycle through the regulation of CDK2 (Matsuo et al., 2000; Shintani et al., 2000). Therefore, deletion or down regulation of DOC1 is found in many cancers including oral cancer (Wong et al., 2012). Apart from cell cycle regulation, DOC1 is also tightly associated with the NuRD complex in *Drosophila* and in mammalian cells. What could be the relevance of the association of DOC1 with NuRD complex in functioning as a tumor suppressor? Our findings suggest that the DOC1-NuRD complex inhibits the expression of TGF-β responsive genes involved in tumorigenesis. Thus, DOC1 might execute its tumor suppressor activity by multiple mechanisms, such as inhibition of cell cycle and inhibition of the tumorigenesis activities of TGF-β.

Experimental procedures

Antibody production, Immunopurification and massspectrometry analysis: Polyclonal antibodies were generated by immunizing the rabbits or guinea pigs with GST-tagged proteins expressed in *E. coli* and affinity purified as described previously (Chalkley and Verrijzer, 2004). DOC1; Full-length protein and CHD4: N-terminus, 1 to 419 aa (amino acids). Immunopurification was essentially carried out by using standard procedures as described earlier (Chalkley and Verrijzer, 2004). Briefly, HeLa nuclear extracts were incubated with Protein A-Sepharose beads cross-linked with either anti-GST (mock) or anti-DOC1 or anti-CHD4 antibodies. Following the affinity purification beads were subsequently washed with HEMG buffer (*25mM HEPES-KOH pH 7.6, 0.1mM EDTA, 12.5mM $MgCl_2$, 10% glycerol, 0.1% NP-40 and a cocktail of protease inhibitors*) containing 300 mM KCl. Input, unbound and

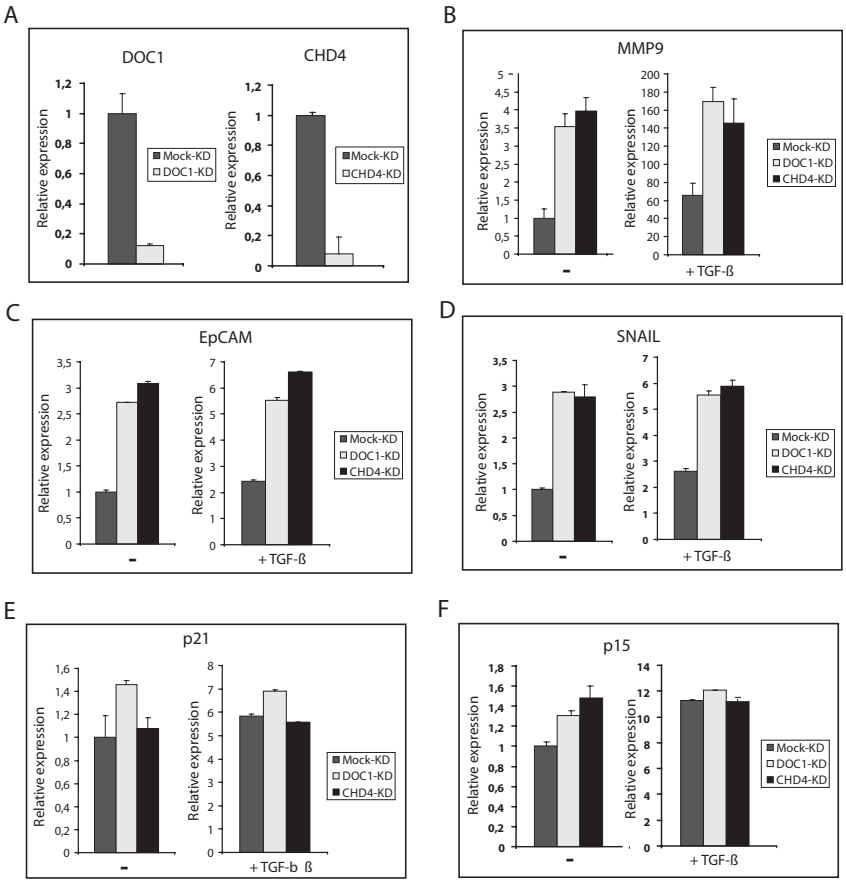

Figure 3; DOC1 represses the SMAD4 target genes: *HaCaT* cells were transduced with lentivirus encoding either non-targeted shRNA (Mock-KD) or shRNA against DOC1 or CHD4. Cells were either untreated (UNT) or treated with 5 ng/ml of TGF-β for 24 hours. cDNA prepared from the total RNA was used for the expression analysis of indicated genes by RT-qPCR. Error bars indicate the standard deviation derived from three independent experiments. Knockdown efficiency is accessed by RT-qPCR analysis of DOC1 and CHD4 (**A**). The expression of following TGF-β target genes were analyzed, MMP9 (**B**), EpCAM (**C**), SNAIL (**D**), p21 (**E**) and p15 (**F**).

immunoprecipitated protein samples were resolved by SDS-PAGE and proteins were identified by nanoflow liquid chromatography-tandem massspectrometry.

Cell growth assay: SCC4 or SCC9 cells were seeded into 6 well plates. Followed by cells were transfected with either control plasmid or plasmid expressing DOC1. Cells were counted at 36, 72 and 96 hours post-transfection using heamocytometer.

Cell culture and lentiviral procedures: Cell culture was essentially carried out according to the standard procedures. Lentivirus expressing specific shRNA were obtained form shRNA library (*Erasmus Centre for Biomics, Erasmus University*

Medical Centre, Rotterdam, The Netherlands). High-titer viral stocks were produced in HEK293T cells by cotransfection of shRNA vector with viral packaging constructs using standard transfection procedures. *HaCaT* cells were then transduced with lentivirus expressing specific shRNA against DOC1 and CHD4 (*see supplementary for shRNA sequences*). Knockdowns were carried out for 4 days.

Cloning and transfection procedures: For transient expression in mammalian cells full-length DOC1 cDNA was cloned into pQCXIP vector with Flag-tag sequence or HA-tag sequence flanking at the 5' end. Transient transfections were carried out using Fu-Gene transfection reagent (Roche diagnostics) and procedures were followed essentially according to the manufacturer's instructructions. Transfections were carried out for 48 hours and cell lysates were made in SDS-PAGE loading buffer and further analyzed by SDS-PAGE and immunobloting.

Real time quantitative PCR: RNA isolation, RT-qPCR, microarray experiments and data analysis were performed using standard procedures. Details of primers are given in the supplementary section.

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Supplementary information:

Primers used for q-PCR:

SNAIL: ACCACTATGCCGCGCTCTT & GGTCGTAGGGCTGCTGGAA
MMP9: TACTGTGCCTTTGAGTCCG & TTGTCGGCGATAAGGAAG
EpCAM: GGACTAGTATGGCGCCCCCGCAGG & CCTGATCATTATGCATTGAGTTCCC
ARP: CACCATTGAAATCCTGAGTGATGT & TGACCAGCCGAAAGGAGAAG
p15: ATGCGCGAGGAGAACAAG & CTAGGTTCCAGCCCCGAT
p21: TGTGGACCTGTCACTGTCTTG & CGGCGTTTGGAGTGGTAGAA
DOC1: CTTACAAACCGAACTTGGC & GTTGCTGGGTGTAGCCTAG
CHD4: CGCAAGAAACTCCGAACCACT & GCACACCTCGCAATAGTCCT

shRNA sequences:

DOC1:
CATGGCAACGTCTTCACAGTA
CTTGGCAGAAACGGAACGGAA
CGCGGCATCATTCACGCTAGA
GAAGGAGATCAGACCCACGTA
CCAAAGCAAATACGCGGAGCT

CHD4:
CCTTACTAGAATTGGTGTTAT
CGAAGGTTTAAGCTCTTAGAA
GCTGCTGACATCCTATGAATT
GCTGACACAGTTATTATCTAT
GCGGGAGTTCAGTACCAATAA

Protein identity	MW kDa	hDOC1 MS	hDOC1 ES	Protein identity	MW kDa	dDOC1 MS	dDOC1 ES	Comments
CHD4	218	8132	40.57	dMi2	225	8658	17.12	Chromodomain-helicase-DNA-binding protein 4 (Mi-2ß)
CHD3	233	4661	4.06					Chromodomain-helicase-DNA-binding protein 4 (Mi-2α)
CHD5	223	1992	1.1					Chromodomain-helicase-DNA-binding protein 4 (Mi-2 related)
ZFHX3	404			MEP1	175	3725	7.1	Zinc finger homeobox protein 3, drosophila MEP1
MTA1	79	2898	13.28	MTA1-like	97	4034	16.78	Metastasis-associated protein 1
MTA2	75	3153	20.7					Metastasis-associated protein 2
MTA3	66	2001	7.21					Metastasis-associated protein 3
GATAD2A	68	2642	14.25					Transcriptional repressor p66-alpha
GATAD2B	66	2154	17.72	p66/68-like	95	2297	2.72	Transcriptional repressor p66-beta
HDAC1	55	1953	92.67	RPD3	58	1993	12.62	Histone deacertrylase 1
HDAC2	55	2028	146.6					Histone deacertrylase 2
RBBP4	47	1491	6.63	p55 CAF1	48	1375	4.84	Histone-binding protein RBBP4
RBBP7	47	1668	15.29					Histone-binding protein RBBP7
MBD2	43	1366	9.05	MBD-like A	36	1168	16.56	Methyl-CpG-binding domain protein 2
MBD3	32	1575	68.57	MBD-like B	25	667	10.81	Methyl-CpG-binding domain protein 2
KDM1A	92	808	0.66					Lysine-specific histone demethylase 1 A
DOC1	12	378	9.16	DOC1	29	615	3.05	Deleted in Oral Cancer 1
ZBTB2	51	303	0.42	TTK69	69	1421	2.06	Zinc finger and BTB domain-containing protein 2, Tramtrack69
SMAD4	48	622	1.81					SMAD transcription factor, TGF-ß signalling
CDKN2	30	215	0.63	CDC2c	36	91	0,19	Cyclin dependent kinase 2

hDOC1- human DOC1, dDOC1- drosophila DOC1 MS - Mascot score, EM - emPAI score

Table S1; Comparison of massspecrtometric analysis of hDOC1 and dDOC1 immunopurifications

Chapter 5

General discussion

Genetics and epigenetics play a crucial role in the normal growth and development of an organism. Alteration of the key cell growth regulatory pathways is the major cause of developmental disorders and diseases including cancer (Hanahan and Weinberg, 2011). Among these, misregulation of transcription and cell cycle regulatory proteins are the major determining factors. Thus, unravelling transcription and cell cycle regulatory pathways is crucial to identify the therapeutic targets. This thesis has studied the gene regulatory pathways of a metabolic enzyme GMPS and the chromatin remodeler NuRD and their involvement in development and disease.

Chapter 2 describes the metabolic enzyme GMPS as a positive regulator of the p53 stability and function upon cellular stress. Several indirect mechanisms were proposed for p53 activation upon stress; p53 is phosphorylated by ATM/ATR kinases resulting in its dissociation from MDM2 and similarly, ATM/ATR-mediated phosphorylation of MDM2 leads to its disassociation from USP7, thus resulting in MDM2 autoubiquitylation and degradation. It has also been proposed that dephosphorylation of USP7 by ATM activated phosphatase PPM1G results in the disruption of USP7 and MDM2 interaction that eventually leads to MDM2 destabilization and p53 activation (Khoronenkova et al., 2012). However, stabilization of p53 directly by deubiquitylation upon stress is still an unresolved issue. Our findings suggest that upon stress GMPS translocates into the nucleus and forms a stable complex with USP7 and p53. GMPS binds to USP7 at the C-terminal Ubiquitin like-domain (Ubl) where as p53 binds to USP7 at the N-terminal TRAF-like domain. Ubl domains are critical for USP7 catalytic activity. GMPS binds to Ubl domain and allosterically hyperactivates USP7 deubiquitilase activity (Faesen et al., 2011; Sheng et al., 2006). Thus, GMPS might act as a bridging factor between p53 and USP7 resulting in p53 stabilization by deubiquitylation. GMPS/USP7 complex is also implicated in transcriptional repression by deubiquitylation of histone H2B. p53 mediates the cellular stress response by transcriptional activation or repression of genes. RNAi-mediated depletion of GMPS/USP7 results in de-repression of p53 repressed genes. Therefore, it is the interesting topic for future study to address whether p53 recruits the GMPS/USP7 complex to promoters of its target genes for establishing transcription repression.

GMPS plays a dynamic role in the regulation of error-free cell division. GMPS resides in the cytoplasm and synthesizes nucleotides to support cell growth. However, upon cellular stress GMPS acts as a first responder to the stress and translocates to the nucleus to stabilize p53. GMPS acts as a sensor of the nucleotide pool in the cell and responds to replicative stress caused by the depletion of nucleotides. Replicative stress and the resulting genomic instability has been proposed to be the key driving force in early stages of cancer development. Oncogenic signals triggering cell proliferation without concomitant elevation in

nucleotide synthesis results in replicative stress which eventually leads to DNA damage and carcinogenesis (Bester et al., 2011). Therefore, GMPS might play a crucial role to maintain cellular homeostasis by coordinating cell division with nucleotide synthesis. Thus, GMPS act as guardian of the cell cycle to safeguard normal proliferation of the cells.

Ubiquitylation and deubiquitylation of GMPS is catalysed by TRIM21 and USP7 respectively. GMPS exists as two isoforms in the cells; monoubiquitinated form resides in the cytoplasm and unmodified form in the nucleus. Then, what is the relevance of monoubiquitylation of GMPS to its cytoplasmic function? Posttranslational modification of metabolic enzymes has been shown to influence their catalytic activity, oligomerization and subcellular localization. Phosphorylation and acetylation have been shown to have antagonistic effects on the catalytic activity of Phosphoenolpyruvate carboxylase (PEPC), an enzyme that functions in plant C4-metabolism. Interestingly, monoubiquitylation of PEPC has been shown to activate its catalytic function by sensitizing the enzyme to its allosteric activators (O'Leary et al., 2011; Uhrig et al., 2008). It is interesting to note that monoubiquitylation of GMPS takes place in the glutamine amidotransferase, ATP hydrolysis, and C-terminal dimerization domain (*Chapter 2, Figure 6*). Therefore, monoubiquitylation might play a crucial role in activating GMPS nucleotide biosynthetic function. GMPS is deubiquitinated by USP7 in the nucleus. The relevance of deubiquitylation of GMPS is unclear at this moment. It is possible that deubiquitylation of GMPS is essential for binding and activation of USP7 deubiquitilase activity. Clearly, additional research is warranted to investigate the influence of monoubiquitylation on GMPS functions.

TRIM21 binds to GMPS in unstressed cells. This retains GMPS in the cytoplasm. Upon stress GMPS dissociates from TRIM21 resulting in GMPS nuclear translocation. The cause for the dissociation of GMPS and TRIM21 is not clear at this moment. It is possible stress response kinases such as ATM/ATR or check point kinases phosphorylate either TRIM21 or GMPS leading to their disassociation. However, in depth studies are required to address these issues. GMPS is a positive regulator of the tumor suppressor p53. Therefore, GMPS could also be acting as tumor suppressor by regulating p53. However, elevated levels of nucleotide biosynthetic enzymes, including GMPS have been reported in several cancers (Weber, 1983). GMPS is also reported as a poor prognostic marker for patient survival in breast cancers (van 't Veer et al., 2002). How can this paradox be explained? Our findings suggest that GMPS nuclear localization is crucial for p53 regulation. Monoubiquitylation of GMPS catalysed by TRIM21 results in its cytoplasmic retention that prevents p53 stabilization. TRIM21 is also found to be over-expressed in several cancers including colorectal, breast, ovarian, lung and cervical cancers (http://www.proteinatlas.org/ENSG00000132109/cancer). Therefore, TRIM21 might play a crucial role in carcinogenesis by targeting GMPS

to the cytoplasm and forcing it to synthesize nucleotides to fuel cancer cell growth and at the same time preventing GMPS-mediated stabilization of the tumor suppressor p53. However, in detail investigation has to be carried out in future to address the role of TRIM21 in cancer development.

Chapter 3 describes the purification and functional characterization of the *Drosophila* NuRD complex. Through a proteomics approach we identified *Drosophila* MEP1 (CG1244) and DOC1 (CG18292) as *bona fide* subunits of the NuRD complex. MEP1 is a Kruppel-type-zinc finger protein with putative SUMO-interacting motif. MEP1 form a NuRD-like complex in *C. elegans* by interacting with LET-418 (Mi2) and HAD-1 (HDAC1) to repress the expression of germline specific genes in somatic cells during embryogenesis (Unhavaithaya et al., 2002). In this study, we found a similar complex in *Drosophila* comprising of MEP1, Mi2, RPD3 (HDAC1) and other subunits of NuRD. MEP1 mediates transcriptional repression of neuronal specific genes by TTK69 through recruitment of the NuRD complex. TTK69 is an important transcription factor which plays a crucial role in neuronal cell fate decision and eye development in *Drosophila*. Thus, role of MEP1 in transcription regulation and developmental processes appears to be conserved in *C. elegans* and *Drosophila*.

In contrast to our findings, a two-subunit complex comprising MEP1 and Mi2 called dMec, was isolated from *Drosophila* KC cells. dMec is functionally different from classical Mi2-NuRD complex (Kunert et al., 2009). Thus, multiple MEP1 containing complexes appear to exist in *Drosophila*. Therefore, it is the future area of interest to investigate specific roles of different MEP1 complexes in *Drosophila* development. MEP1 is also implicated in SUMO-dependent transcriptional repression. MEP1 binds to sumoylated Sp3 through its SUMO-interacting motif and recruits Mi2 for Sp3-dependent transcriptional repression of target genes (Stielow et al., 2008). Thus, MEP1 participate in transcription regulation by multiple mechanisms.

Additionally, we also identified DOC1 as a *bona fide* subunit of the *Drosophila* NuRD complex. DOC1, also called as CDK2AP1, is found deleted in several cancers including oral cancer. DOC1 is also reported as a subunit of human NuRD complex (Le Guezennec et al., 2006; Spruijt et al., 2010). Thus, DOC1 is an evolutionally conserved subunit of the NuRD complex. Therefore, it is interesting to investigate the role of *Drosophila* DOC1 in gene regulation.

In this study, we investigated the mechanism of TTK69-mediated transcription regulation in detail. TTK69 is a transcriptional repressor implicated in the neuronal cell fate determination (Okabe et al., 2001), eye development (Li et al., 1997; Xiong and Montell, 1993) and regulation of mitosis (Baonza et al., 2002). TTK69 is tightly regulated at the protein level by ubiquitin-mediated degradation. UBP64 has been shown to stabilize TTK69 by deubiquitylation, which is an important pathway in *Drosophila* eye development (Bajpe et al., 2008). Our findings suggest

that TTK69 and the NuRD complex work together in gene regulation. Therefore, it is interesting to address in future whether the NuRD complex also participates in a wide range of developmental processes regulated by TTK69.

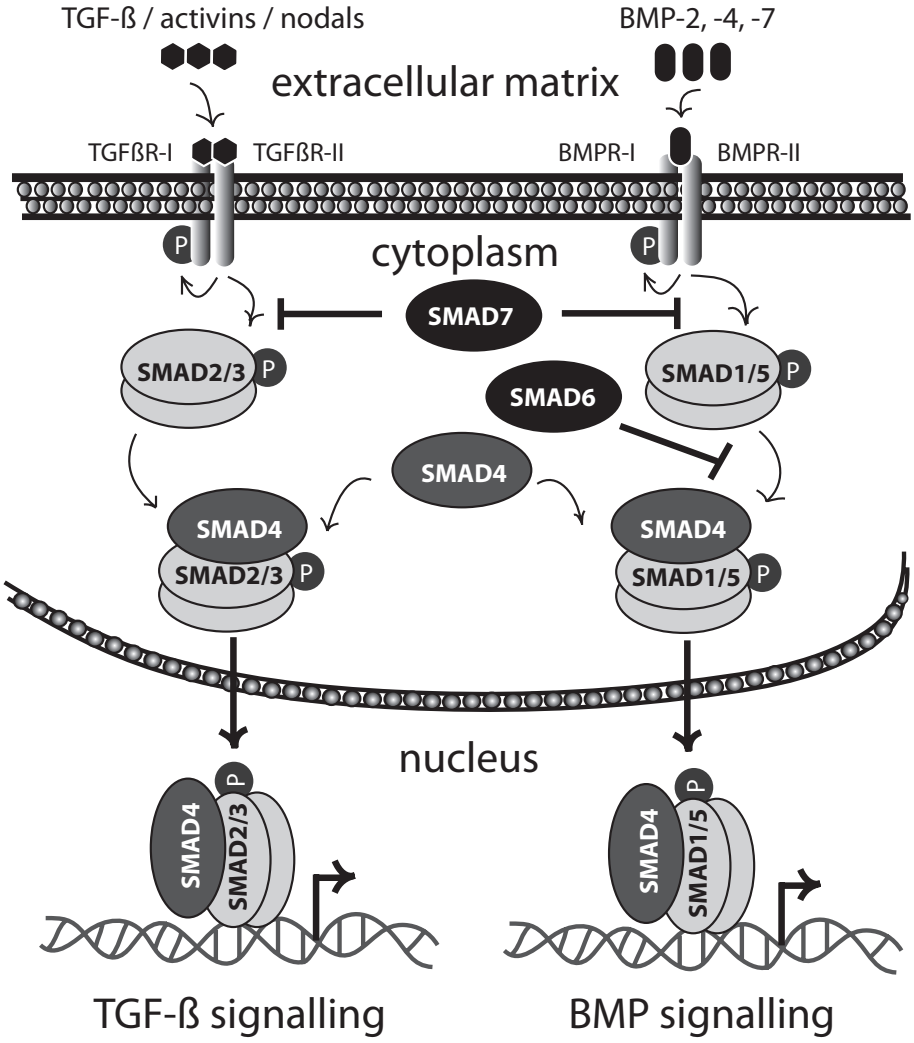

Figure 1; Overview of TGF-ß signalling: TGF-β is a cell surface receptor with serine/threonine kinase activity. TGF-β superfamily consists of more than 40 structurally related proteins. They can be subdivided into several groups, including TGF- β, bone morphogenic proteins (BMP) and activins/inhibins. Functionally, SMADs are classified into three groups, receptor-associated SMADs (R-SMADs: SMAD1, SMAD2, SMAD3, SMAD5), which are phosphorylated by receptor kinases, common mediator SMAD (Co-SMAD: SMAD4) and inhibitory SMADs (I-SMADs: SMAD6, SMAD7). SMAD4 in association with SMAD2 and SMAD3 mediates TGF-β signalling and together with SMAD1 and SMAD5 mediates BMP signalling. SMAD7 inhibits phosphorylation of R-SMADs, whereas, SMAD6 disrupts SMAD1 and SMAD4 interaction.

Chapter 4 describes gene regulatory network of human DOC1. Here we explore the precise role of DOC1 as a tumor suppressor through its association with the NuRD complex. We found SMAD4 as a DOC1 interacting protein. SMAD4 is a common intracellular mediator of TGF-β- and BMP-signalling (ten Dijke and Hill, 2004), see *Figure 1*. Our findings suggest that SMAD4 and the DOC1-NuRD complex works together in the transcriptional repression of TGF-β target genes, which are involved in the tumorigenesis. The systemic deletion of SMAD4 in mouse skin results in the development of squamous cell carcinoma (Qiao et al., 2006). Deletion of DOC1 also results in the development of squamous cell carcinoma. Therefore, DOC1 and SMAD4 function as tumor suppressors of squamous cell carcinoma. However, the *in vivo* relevance of interaction between DOC1 and SMAD4 in tumor suppression needs to be addressed.

Our proteomics analysis identified ZFHX3 and ZBTB2 as NuRD interacting proteins. ZFHX3 and ZBTB2 have similar domains as *Drosophila* MEP1 and TTK69 respectively. ZFHX3 is an AT-rich DNA binding protein and implicated in transcriptional repression (Morinaga et al., 1991). ZBTB2 is a BTB-POZ domain containing transcription factor (Jeon et al., 2009). We established functional cooperation between TTK69, MEP1 and NuRD complex in transcription regulation in *Drosophila (Chapter3)*. Therefore, it is an interesting topic for future investigation to address whether similar functional cooperation exists between ZBTB2, ZFHX3 and NuRD complex in transcription regulation and developmental processes in humans.

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Summary

Controlled expression of genes is essential for normal growth and development of an organism. Alteration in gene expression by either mistargeting, deletion or degradation of important transcription factors or chromatin regulators can lead to changes in cellular physiology which ultimately can cause developmental disorders and diseases. Therefore, understanding the underlying mechanisms of gene regulation is crucial for identifying therapeutic targets.

Chapter 2 describes the role of metabolic enzyme GMPS in regulation of p53 stability and function. Stabilization of p53 is essential to carry out cellular adaptation to stress. In response to diverse cellular stresses p53 gets stabilized and regulates the expression of genes that control cell cycle, apoptosis, senescence and cell metabolism. Thus, cells gain a significant growth advantage in the absence of p53. Indeed loss of p53 function is reported in more than 50% of the human cancers. p53 is mainly regulated at the protein level by ubiquitylation catalysed by E3 ubiquitin-ligase MDM2 which induces p53 degradation. We found GMPS as a major player in the regulation of p53 stability upon various cellular stresses. GMPS stabilizes p53 by forming a complex with the deubiquitilase USP7. GMPS predominantly resides in the cytoplasm in unstressed cells but translocates into the nucleus upon induction of stress and stabilizes p53 through USP7-mediated deubiquitylation. GMPS acts as a sensor of the cellular nucleotide pool and activates the p53-mediated replicative stress response to the nucleotides depletion. GMPS nuclear exclusion is regulated by mono-ubiquitylation mainly catalyzed by the E3 ubiquitin-ligase TRIM21. Thus, TRIM21 negatively regulates p53 stability by preventing GMPS nuclear translocation. Upon induction of stress, GMPS dissociates from TRIM21 and translocate into the nucleus where it is deubiquitinated by USP7. Thus, monoubiquitylation forms a switch which controls GMPS sub-cellular localization and determines p53 stability. Therefore, TRIM21-GMPS-USP7 forms a novel regulatory circuit of the tumor suppressor p53 stabilization pathway.

Chapter 3 describes the purification and functional characterization of the *Drosophila* NuRD complex. NuRD is one of the four major ATP-dependent chromatin remodeling complexes, which regulates chromatin structure and gene expression. The subunit composition of the remodeling complex determines its functional specificity by targeting to different chromatin loci. We identified MEP1 and DOC1 as *bona fide* subunits of the *Drosophila* NuRD complex. In addition, we found transcription factor Tramtrack69 (TTK69) as a NuRD interacting protein. TTK69 has been implicated in various aspects of *Drosophila* development, such as neuronal differentiation and photoreceptor and tracheal development. TTK69 interacts with the NuRD complex via the MEP1 subunit. TTK69 binds to promoters of target genes and recruits the NuRD complex to establish transcriptional repression. Therefore, there appears to be a hierarchical relationship in which transcription factor binding

precedes chromatin remodeler recruitment. Thus, TTK69 and the NuRD complex participate together in gene expression control.

Chapter 4 describes the gene regulatory network of human DOC1. DOC1 is a potential tumor suppressor protein that is found deleted or down regulated in several cancers. Re-expression of DOC1 in deleted oral cancer cell lines results in cell growth inhibition. The cell growth inhibitory effect of DOC1 has been attributed to inhibition of CDK2-mediated cell proliferation. Apart from CDK2 regulation, DOC1 has also been identified as a subunit of the NuRD complex. However, the precise mechanism of tumor suppression by DOC1 through gene regulation is not well understood. Proteomic analysis of DOC1 associated proteins identified SMAD4, an intracellular mediator of the TGF-β signalling, as a DOC1 interacting protein. RNAi-mediated depletion of DOC1 and CHD4 (ATPase subunit of NuRD) results in the de-repression of SMAD4 target genes. Therefore, our preliminary results suggest that DOC1 together with the NuRD complex functions as an inhibitor of TGF-β target genes which are involved in promoting cell proliferation, EMT (epithelial-to-mesenchymal transition), metastasis and angiogenesis. However, additional research is warranted to better understand the role of DOC1 in gene regulation.

Nederlandse Samenvatting

Gereguleerde genexpressie is essentieel voor de normale groei en ontwikkeling van een organisme. Verandering in genexpressie door verkeerde lokalisatie, deletie, of degradatie van belangrijke transcriptiefactoren of chromatine regulators kan leiden tot veranderingen in de cellulaire fysiologie, wat uiteindelijk weer kan resulteren in ontwikkelingsstoornissen en ziektes. Begrip van genregulatiemechanismes is daarom essentieel voor het ontdekken van therapeutische doelen.

In *hoofdstuk 2* wordt de rol van het metabolische enzym GMPS in de regulatie van de stabiliteit en functie van p53 beschreven. Stabilisatie van p53 is essentieel voor de cellulaire aanpassing aan stress. Als gevolg van verschillende cellulaire stresssignalen wordt p53 gestabiliseerd, waarna p53 de expressie van genen reguleert die betrokken zijn bij de controle van de celcyclus, apoptose, senescentie en celmetabolisme. Hieruit volgt dat bij afwezigheid van p53, cellen een aanzienlijk groeivoordeel hebben. Er is inderdaad gerapporteerd dat er in 50% van de humane kankers verlies van p53 functie is. p53 wordt voornamelijk op eiwitniveau gereguleerd door ubiquitylering, wat wordt gekatalyseerd door het E3 ubiquitine-ligase MDM2. Dit leidt tot degradatie van p53. Wij hebben ontdekt dat GMPS een belangrijke speler is in de regulatie van p53 stabiliteit na verscheidene cellulaire stresssignalen. GMPS stabiliseert p53 door, als gevolg van stress, een complex te vormen met het deubiquitylase USP7. In niet-gestresste cellen zit GMPS voornamelijk in het cytoplasma, maar na inductie van stress verplaatst het naar de kern en stabiliseert het p53 via deubiquitylering door USP7. GMPS werkt als een sensor van de cellulaire nucleotide voorraad en activeert de p53-afhankelijke stress respons na nucleotide depletie. Wering uit de kern van GMPS wordt gereguleerd door monoubiquitylering, wat voornamelijk wordt gekatalyseerd door het E3 ubiquitine ligase TRIM21. Hieruit volgt dat TRIM21 de stabiliteit van p53 negatief reguleert door de verplaatsing van GMPS naar de kern te verhinderen. Bij stress inductie dissocieert GMPS van TRIM21 en verplaatst het naar de kern waar het wordt gedeubiquityleerd door USP7. Aldus vormt monoubiquitylering een schakelaar die de subcellulaire lokalisatie van GMPS reguleert en de stabiliteit van p53 bepaalt. TRIM21-GMPS-USP7 vormt daarom een nieuw regulerend circuit in de stabilisatie route van de tumor suppressor p53.

Hoofdstuk 3 beschrijft de zuivering en functionele karakterisering van het *Drosophila* NuRD complex. NuRD is één van de vier belangrijke ATP-afhankelijke chromatine remodellerende complexen, wat de chromatine structuur en genexpressie reguleert. De samenstelling van de subeenheden van het remodellerende complex bepaalt zijn functionele specificiteit door binding aan verschillende chromatine loci. Wij hebben MEP1 en DOC1 geidentificeerd als *bona fide* subeenheden van het *Drosophila* NuRD complex. Bovendien vonden we dat transcriptiefactor Tramtrack69 (TTK69) een NuRD bindend eiwit is. TTK69 is

geacht betrokken te zijn bij verscheidene aspecten van *Drosophila* ontwikkeling, zoals neuronale differentiatie, en fotoreceptor- en tracheale ontwikkeling. TTK69 bindt aan het NuRD complex via de MEP1 subeenheid. TTK69 bindt aan de promotors van specifieke genen en trekt het NuRD complex aan, wat leidt tot transcriptionele repressie. Bijgevolg blijkt er een hiërarchische relatie te bestaan, waarin transcriptiefactor binding vooraf gaat aan trekking van de chromatine remodelleerder. Aldus werken TTK69 en het NuRD complex samen in genexpressie regulatie.

Hoofdstuk 4 beschrijft het genregulatie netwerk van het humane DOC1. DOC1 is een mogelijk tumor suppressor eiwit, wat gedeletet, of omlaag gereguleerd is in verscheidene tumoren. Herexpressie van DOC1 in *deleted oral cancer* cellijnen leidt tot remming van celgroei. Dit effect van celgroeiremming door DOC1 wordt toegewezen aan de inhibitie van CDK2-afhankelijke celproliferatie. Afgezien van zijn rol in CDK2 regulering is DOC1 ook geïdentificeerd als een subeenheid van het NuRD complex. Het precieze mechanisme van tumor suppressie door DOC1 via genregulatie wordt nog niet goed begrepen. Proteomische analyse van DOC1-geassocieerde eiwitten leidde tot de identificatie van SMAD4 als een DOC1-bindend eiwit. SMAD4 is een intracellulair regeleiwit van TGF-β-afhankelijke signaaltransductie. RNAi-afhankelijke depletie van DOC1 en CHD4 (een ATPase subeenheid van het NuRD complex) resulteert in de derepressie van SMAD4 gereguleerde genen. Onze voorlopige resultaten suggereren derhalve, dat DOC1, samen met het NuRD complex, functioneert als een remmer van TGF-β gereguleerde genen, welke betrokken zijn bij de stimulering van celproliferatie, EMT (epitheel naar mesenchyme transitie), metastase, en angiogenese. Echter, verder onderzoek is noodzakelijk om de rol van DOC1 in genregulatie beter te begrijpen.

PhD Portfolio

Summary of PhD training and teaching

Name of PhD student:	Ashok Bandi Adinarayana Reddy
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PhD period:	August 2007 to December 2012
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PhD training — Year

Courses/Classes

In Vivo Imaging from Molecule to Organism' Optical Imaging Centre	2007
Molecular and Cell Biology course	2008
Photoshop and Illustrator CS5 for PhD student and other researchers	2011

Permits / Experience

Radiation worker 5B	2009
ML-I (Work permit for GMOs)	2007
ML-II (Biosafety level II work experience; Adeno- and Lentivirus)	2008

Seminars and workshops

MGC symposia	2008
MGC symposia	2009
MGC PhD workshop	2009
MGC symposia	2010
MGC symposia	2012

International conferences

CGC/CBG meeting on "Molecular Mechanisms and Mouse Models in Cancer" Amsterdam	2008
2nd International symposium "Stem cells, Development and Regulation", Amsterdam	2008
3rd International symposium "Stem cells, Development and Regulation", Amsterdam	2009
CGC/CBG meeting on "Molecular mechanisms in cancer", Amsterdam	2010

Supervising Bachelor's / Master's theses

Supervision of Master's student, Ivar Schut, Faculty of Earth and Life sciences, Graduate school of Health and Life Sciences, University of Amsterdam, The Netherlands	2011
Supervision of Bachelor's student, Melitza Elizabeth, Life sciences, Hogeschool Rotterdam, The Netherlands	2012

Presentations

Reddy BA, Bajpe PK, Bassett A, Moshkin YM, Kozhevnikova E, Bezstarosti K, Demmers JA, Travers AA, Verrijzer CP. Drosophila transcription factor Tramtrack69 binds MEP1 to recruit the chromatin remodeler NuRD.
16th MGC PhD workshop, Bruges, Belgium, June 2009

Reddy BA, Knaap JA, Bot AGM, Dekkers HW, Demmers JA and Verrijzer CP. Metabolic enzyme GMP synthetase relays control of p53
22nd MGC symposium, Leiden, The Netherlands

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Education:

1997 to 2001 – **BSc (Ag)**, UAS, GKVK, Bangalore, INDIA
2002 to 2004 – **MSc Biotechnology**, UAS, GKVK, Bangalore, INDIA
2004 to 2007 – **Junior Research Fellow**, JNCASR, Bangalore, INDIA
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Work experience:

Master's thesis (2002 to 2004): UAS, Bangalore, INDIA
Cloning and characterization of a novel Bt-toxic gene from *Bacillus thuringiensis* bacteria and development of transgenic crops by using Agrobacterium-mediated transformation

Junior Research Fellow (2004 to 2007): JNCASR, Bangalore, INDIA.
Worked on identification of small molecule modulators of chromatin modifying enzymes, particularly histone acetyl transferase, p300/CBP to probe their role in transcription regulation and therapeutics

PhD thesis (2007 to 2012): Erasmus MC, Rotterdam, Netherlands.
Study on gene regulatory roles of metabolic enzyme GMP synthetase and NuRD chromatin remodeler. Mainly purification and characterization of NuRD complex and studied the role of metabolic enzymes GMP synthetase in p53 regulation.

Fellowships and Medals:

- Awarded CSIR-Junior Research Fellowship, Govt. of India, 2004-2005
- University Merit Scholarship (MSc), 2002-2004
- Awarded 3 gold medals (BSc) for securing highest marks among the graduates of the University, 2002
- SJ Jindal Trust Merit Scholarship (BSc), 1997-2001

Publications:

1. Moshkin YM, Chalkley GE, Kan TW, **Reddy BA**, Ozgur Z, van Ijcken WF, Dekkers DH, Demmers JA, Travers AA, Verrijzer CP (2012). Remodelers organize cellular chromatin by counteracting histone-DNA sequence preferences in a class specific manner. Mol Cell Biol; 32(3): 675-88.
2. **Reddy BA**[†], Bajpe PK[†], Bassett A, Moshkin YM, Kozhevnikova E, Bezstarosti K, Demmers JA, Travers AA, Verrijzer CP (2010). Drosophila transcription factor Tramtrack69 binds MEP1 to recruit the chromatin remodeler NuRD. Mol Cell Biol. 30(21): 5234-44.

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3. Ravindra KC, Selvi BR, Arif M, **Reddy BA**, Thanuja GR, Agrawal S, Pradan SK, Nagashayana N, Dasgupta D and Kundu TK (2009). Inhibition of lysine acetyltransferase KAT3B/p300 activity by a naturally occurring hydroxynaphthoquinone, plumbagin. J Biol Chem; 284(36): 24453-64.
4. Kishore AH, Vedamurthy BM, Mantelingu K, Agrawal S, **Reddy BA**, Roy S, Rangappa KS and Kundu TK (2008). Specific small-molecule activator of Aurora kinase A induces autophosphorylation in a cell-free system. J Med Chem; 51(4): 792-7.
5. Mantelingu K[†], **Reddy BA**[†], Swaminathan V, Kishore AH, Siddappa NB, Kumar GV, Nagashankar G, Natesh N, Roy S, Sadhale PP, Ranga U, Narayana C and Kundu TK (2007). Specific inhibition of p300-HAT alters global gene expression and represses HIV replication. Chem Biol; 14(6): 645-57.

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6. Swaminathan V[†], **Reddy BA**[†], Ruthrotha Selvi B[†], Sukanya MS and Kundu TK (2007). Small molecule modulators in epigenetics: implications in gene expression and therapeutics. Subcell Biochem; 41:397-428. Book Chapter "Chromatin and Disease".

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7. Kumar GV, **Reddy BA**, Arif M, Kundu TK, Narayana C (2006). Surface-enhanced Raman scattering studies of human transcriptional coactivator p300. J Phys Chem B. 110(33):16787-92.

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"ఎందరో మహానుభావులు... అందరికి వందనములు"
(Endaro Mahanu Bhavulu…Andariki vandanamulu) which means, "My salutations to all great people".
Tyagaraju (1767-1847), composer of Carnatic music (classical South Indian music)

If I missed anyone, sorry guys…
Ashok

List of Abbreviations:

ALDH4	Aldehyde dehydrogenase 4
AMP	Adenosine monophosphate
BMP	Bone morphogenetic protein
BTB-POZ	BR-C, ttk and Bab – Poxy virus and Zinc finger
CDK2NA	Cyclin-dependent kinase inhibitor 2A
Daxx	Death-domain associated protein
DEC1	Deleted in esophageal cancer 1
DNMT1	DNA methyltransferase 1
DOC1	Deleted in oral cancer 1
DRAM	Damage-regulated autophagy modulator
EBNA1	Epstein-Barr nuclear antigen 1
GADD45	Growth Arrest and DNA Damage 45
GMAT	Guanidinoacetate methyltransferase
GMP	Guanosine monophosphate
GMPS	Guanosine monophosphate synthetase
GPX1	Glutathione peroxidase 1
HAUSP	Herpes-simplex virus associated ubiquitin specific protease
ICP0	Human Herpes Virus Infected Cell Polypeptide 0
IMPDH	Inosine-5′-monophosphate dehydrogenase
IRF	Interferon regulatory factor
MDM2	Murine double minute 2
MEP1	Mog-interacting and ectopic P-granules 1
NuRD	Nucleosome remodeling and deacetylase
NQO1	NAD(P)H: quinone oxidoreductase 1
PAI-1	Plasminogen activator inhibitor-1
PGM	Phosphoglycerate mutase
PML	Promyelocytic leukemia protein
SCO2	Synthesis of Cytochrome C Oxidase 2
SMAD	SMA and MAD related family
SSA 1	Sjogren's syndrome autoantigen 1
TGF-β	Transforming growth factor - beta
TIGAR	TP-53- induced glycolysis and apoptosis regulator
TNF	Tumor necrosis factor
TRIM21	Tripartite motif 21
TTK69	Tramtrack 69
Ubl	Ubiquitin-like domain
USP7	Ubiquitin specific protease 7
USP10	Ubiquitin specific protease 10
USP11	Ubiquitin specific protease 11
XMP	Xanthosine monophosphate