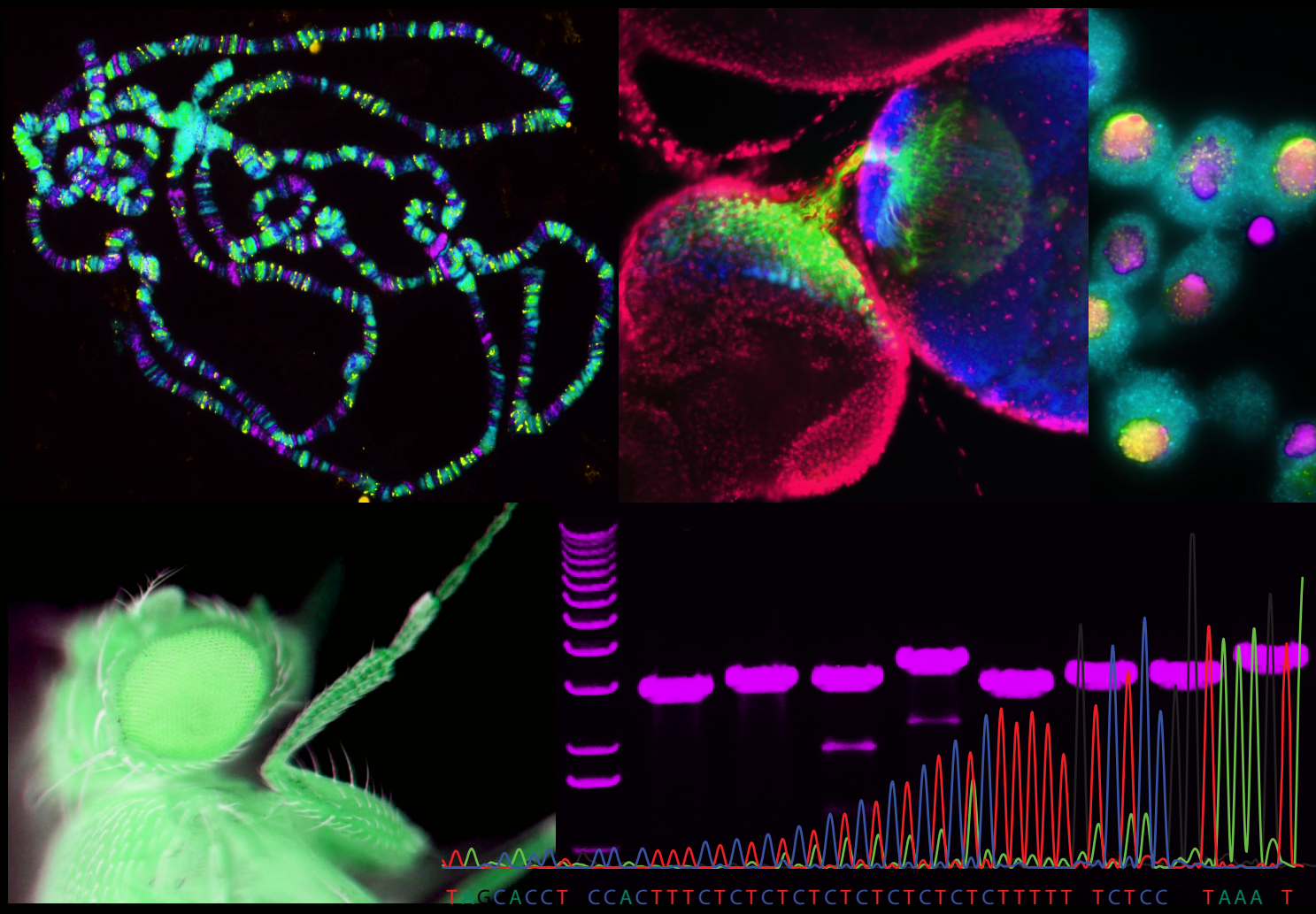
Metabolic enzymes in gene control



Elena Kozhevnikova

Metabolic enzymes in gene control

Metabolische enzymen in genregulatie

Thesis

To obtain the degree of Doctor from the
Erasmus University of Rotterdam
By command of the
Rector magnificus

Prof.Dr.H.G.Schmidt

and in accordance with the decision of the Doctorate Board

The public defence shall be held on
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By

Elena Kozhevnikova

Born in Pavlodar, Kazakhstan

Doctoral Committee

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CHAPTER 1

Introduction

Summary

Cellular compartments are believed to serve functional separation within the cell and allow it to perform multiple metabolic processes at the same time. Processes are divided between compartments with respect to the physiological requirements for a certain process. Thus, regulatory processes involving chromatin organization and control of gene activity are performed in the nucleus while metabolic processes are believed to be performed in the aqueous cytoplasm and cytoplasmic organelles. Numerous reports demonstrate that transcriptional modulation has a strong impact on metabolism, as expression levels of metabolic enzymes are limited by signaling pathways and the transcriptional machinery (Blanchet et al., 2011; Desvergne et al., 2006; Dufour et al., 2011). On the other hand, there should be mechanisms allowing feedback control from metabolism to gene expression that would tune the transcriptional program of the cell in accordance with its physiological needs (McKnight, 2010; Ray, 2010). Essentially, metabolic flux, including metabolic enzymes, small metabolites, nutrients and redox state, should impose reciprocal control upon nuclear events. However, there is little knowledge on mechanisms that allow such regulation. In recent years, several groups addressed this question and discovered that concentrations of intermediate metabolites indeed affect gene control (Shi, 2004). In all cases, metabolic compounds such as NADH, Acetyl-CoA and poly-ADP ribose bind to chromatin and change gene activity. Potentially many more metabolites should touch upon the gene expression machinery. These “metabolic” regulatory complexes might, in turn, affect expression of the transcriptional regulators of metabolism and close the feedback loop.

Metabolism

Intermediary metabolism comprises the intermediate steps within the cells in which the nutrient molecules are metabolized and converted into cellular components catalysed by enzymes. Metabolic processes are grouped into two major classes: catabolism and anabolism. In the course of catabolism large molecules such as sugars, fats and proteins are processed into smaller compounds, like Acetyl-CoA. Through the citric acid cycle, acetyl-CoA is oxidised producing water, CO2 and energy, stored in the form of reduced nicotinamide adenine dinucleotide (NADH). Finally, the electron

transport chain utilizes NADH to transform energy into ATP. In contrast, anabolism involves a number of reactions directed to build cellular compounds using energy obtained from catabolism. Most of these reactions are performed by specialized metabolic enzymes and are regulated through the cell cycle and development at the level of gene expression (Dang and Lewis, 1997; Kowaloff et al., 1976). Certain features of metabolism are characteristic for specific cell states. For instance, stem cells have recently been shown to over-express threonine dehydrogenase and critically depend on threonine (Wang et al., 2009). Another example comes from cancer: tumor cells predominantly use anaerobic glycolysis for energy production even in the presence of oxygen, the so-called Warburg effect (Lopez-Lazaro, 2008; Warburg, 1956). Moreover, metabolic disturbances underlie many human disorders including diabetes and atherosclerosis (Hotamisligil and Erbay, 2008).

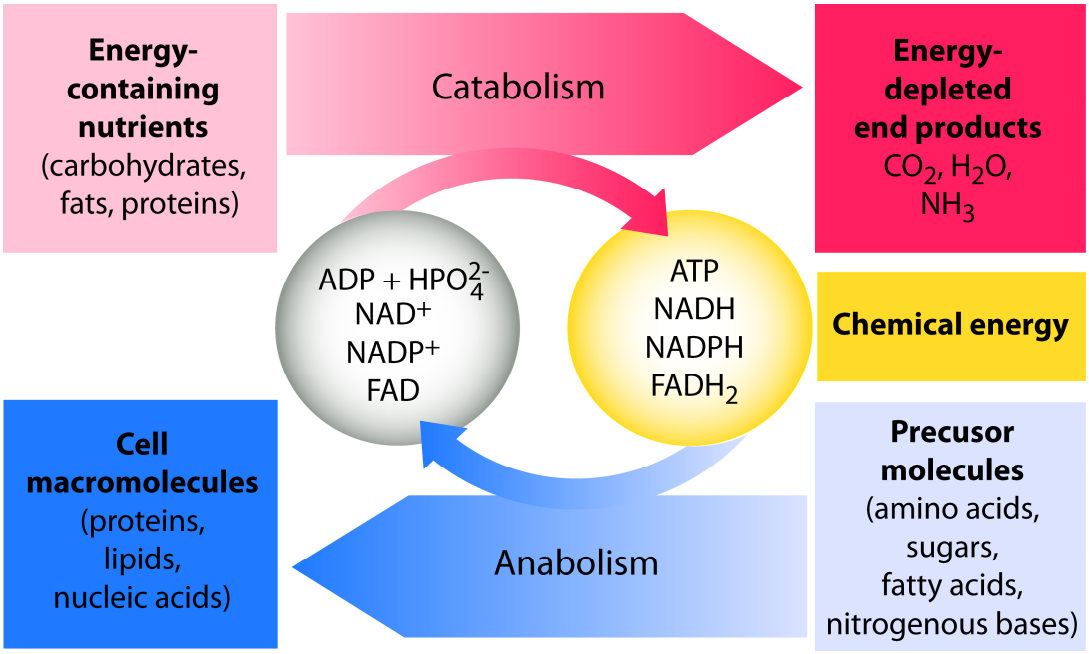

Figure 1. Overview of the cellular metabolism.Catabolic reactions produce energy stored in the form of NADH, NADPH, $FADH_2$ and ATP. This energy is used in the course of anabolic reaction that produce cellular macromolecules from small metabolic compounds.
Adapted from Lehninger's «Principles of biochemistry», 4th edition.

Transcription

Several levels of compaction organize large eukaryotic genomes into chromatin (Li and Reinberg, 2011). Basic chromatin unit is a nucleosome that comprises DNA sequence of 147 bp wound around a histone octamer. Histone proteins: H2A, H2B, H3 and H4 serve to wrap DNA into a “bead-on-a-string” structure. Essentially, this form of compaction

represents actively transcribed chromatin, or euchromatin (Dorigo et al., 2004). DNA is compacted further by addition of the linker histone H1 and the whole structure coils into a 30nm fibre (Wu et al., 2007). The 30nm chromatin is believed to be less transcriptionally active and can be further compacted by scaffold proteins (Figure 2). The highest order of chromatin compaction is achieved in mitotic chromosomes, although the exact mechanisms of this process are not completely understood (Bazile et al., 2010).

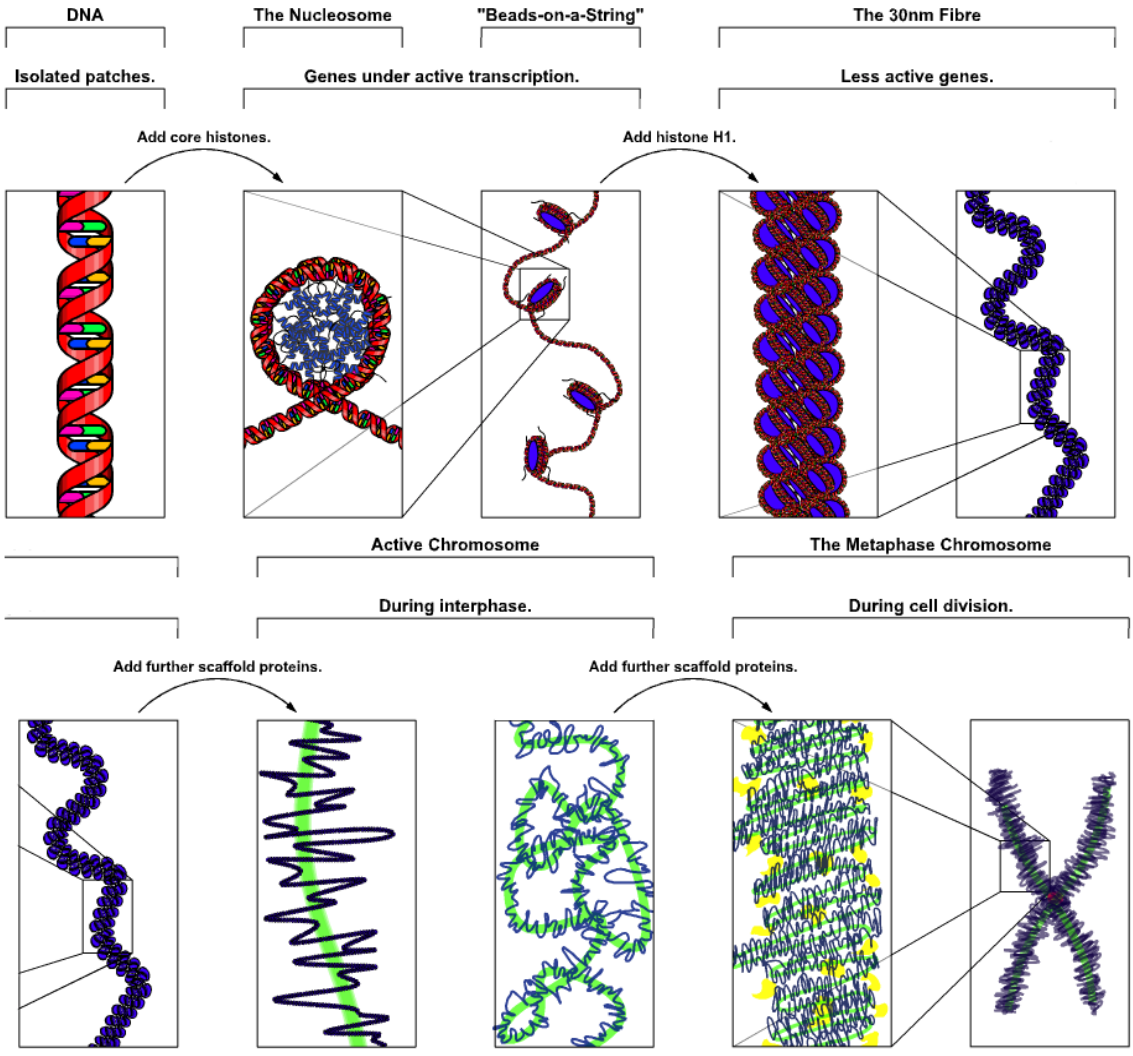

Figure 2. Chromatin compaction.Schematic representation of the major compaction levels of chromatin. DNA wraps around nucleosome and form the 10 nm beads-on-the-string structure. Upon addition of Histone H1 the structure coils and form the 30nm fiber. Scaffold proteins further compact it into the interphase chromatin that can be organized into mitotic chromatin by condensation proteins.
Adapted from Richard Wheeler (http://en.wikipedia.org/wiki/File:Chromatin_Structures.png).

Compaction of DNA into chromatin restricts its accessibility to regulatory proteins and the transcription machinery (Bell et al., 2011). Chromatin remodeling complexes utilize ATP to facilitate dynamic rearrangements of nucleosomes and

promote DNA accessibility (Lusser and Kadonaga, 2003). Histone modifying enzymes catalyze the addition or removal of covalent modifications that serve to change chromatin structure and recruit regulatory complexes (Marmorstein and Trievel, 2009). Major histone modifications include methylation, acetylation, phosphotylation and ubiquitination (Kouzarides, 2007). Combinatorial effects imposed by histone modifications and nucleosome remodeling determine transcriptional output of a given gene (Beck et al., 2010).

DNA-binding sequence-specific transcription factors orchestrate selective gene regulation by recruiting either co-activator (Lemon and Tjian, 2000) or co-repressor complexes (Huisinga et al., 2006). Signaling cascades and hormonal cues trigger specific transcription factors to initiate tissue-specific gene expression programs (Gordon and Nusse, 2006; Honda and Taniguchi, 2006). In this way, transcription factors regulate numerous developmental and signaling events including stem cell differentiation, body segmentation, formation of the nervous system, memory consolidation, etc (Coppola et al., 2010; Cordes and Barsh, 1994; Hawk and Abel, 2011).

Ubiquitin in gene regulation

Ubiquitination is well known as a part of the protein degradation system: polyubiquitin chains serve as signals for proteosome-mediated cleavage of a target protein (Hochstrasser, 2004; Murata et al., 2009). In the past three decades numerous reports demonstrated that monoubiquitination can modify protein function or facilitate alternative protein interactions (Woelk et al., 2007). Therefore, ubiquitin-modifying enzymes came into focus as essential regulators of numerous cellular events (Marfany and Denuc, 2008). With respect to gene regulation, the most prominent ubiquitination targets are histones (Weake and Workman, 2008). These marks serve as important signals to attract other histone modifying enzymes in order to establish appropriate chromatin landscapes for gene regulatory complexes (Bell et al., 2011; Frappier and Verrijzer, 2011).

Ubiquitination of Histone H2A has been generally associated with gene repression (Goldknopf et al., 1975; Higashi et al., 2010). It is essential for heterochromatin maintenance, Polycomb-mediated silencing and X-chromosome inactivation (Higashi et al., 2010). In contrast, ubiquitinated Histone H2B (Ub-H2B) is associated with actively transcribed genes and is largely involved in positive regulation

of gene expression (Minsky et al., 2008; West and Bonner, 1980). The mechanism of gene activation by Ub-H2B involves stimulation of Histone H3 lysine 4 methylation (Me-H3K4) (Kim et al., 2009) that, in turn, recruits co-activator complexes such as COMPASS and NURF (Lee et al., 2007). Bre1 orthologues are the only ubiquitin ligases responsible for H2B ubiquitination *in vivo*. Bre1 associates with active transcription, and its depletion results in the loss of Ub-H2B and Me-H3K4 (Bray et al., 2005). *In vitro* experiments suggest that Ub-H2B assists the passage of RNA polymerase II and might be important for transcriptional elongation (Pavri et al., 2006).

Ubiquitination of H2B is counteracted by ubiquitin specific proteases (USPs), which play an important role in chromatin organization and gene regulation (Chandrasekharan et al., 2010). For instance, yeast UBP10 is required for telomeric silencing (Ingvarsdottir et al., 2005). In contrast, UBP8 within the SAGA co-activator complex deubiquitinates H2B as it goes along with elongating RNA polymerase II and facilitates transcription (Henry et al., 2003). Thus, rapid ubiquitination/deubiquitination seems to be important for active transcription. *Drosophila* USP7 counteracts Bre1 and reduces the overall level of Ub-H2B *in vivo* (van der Knaap et al., 2010). In contrast to Bre1, USP7 does not co-localize with actively transcribing RNA polymerase II; moreover, it dissociates from inducible genes upon their activation. USP7 is involved in Polycomb silencing and in repression of ecdysone-inducible genes in the absence of this hormone (van der Knaap et al., 2010; van der Knaap et al., 2005). The human homologue of USP7 (HAUSP) was first identified as a factor stabilizing the ICP0 protein of herpes simplex virus (Everett et al., 1997). Later, HAUSP was shown to bind and stabilize p53 and MDM2 – major regulators of apoptosis and cancer development (Brooks and Gu, 2004; Li et al., 2002).

Metabolism goes transcription

The metabolic state of the cell and extracellular environment have long been known to affect the regulation of yeast and bacterial metabolic genes. The presence of galactose and glucose in the medium drastically changes the expression of *GAL* genes in yeast (Johnston, 1987). Switching carbon source from glucose to maltose in yeast causes transcriptional changes in *alpha-glucosidase* and *glucose transporter* genes (Bordel et al., 2010). This suggests that the metabolic state affects the transcriptional machinery in order to adjust cellular metabolic activities to the needs imposed by the environment.

In recent years, significant advances have been made in understanding the principles of metabolic regulation of transcription in higher eukaryotes (Shi, 2004). Zhang and colleagues demonstrated that a metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acts as a transcriptional co-factor. GAPDH was co-purified with the OCA-S co-activator complex and was essential for H2B transcription *in vitro* (Zheng et al., 2003). Strikingly, its co-activator activity was dependant on the NAD+/NADH ratio (Dai et al., 2008), suggestive for its sensitivity to the redox state of the cell. Another enzyme, poly ADP ribose polymerase (PARP), binds chromatin and facilitates gene activation (Tulin and Spradling, 2003). Guanosine monophosphate synthetase (GMPS) co-operates with USP7, stimulates H2B ubiquitylation and contributes to the Polycomb-mediated silencing (van der Knaap et al., 2005). All these studies uncovered metabolic enzymes moonlighting as transcriptional co-factors that can potentially bridge metabolism and gene regulation.

Rutter and colleagues demonstrated that circadian rhythm regulators, the transcription factors NPAS2 and BMAL1, exhibit NAD-dependent DNA binding activity (Rutter et al., 2001). One of the target genes for NPAS2 and BMAL1 is *lactate dehydrogenase* (LDHA). This enzyme catalyses conversion of pyruvate to lactate, using NADH as a co-factor. The authors reasoned that LDHA levels or food intake during ultradian period might influence the NAD(H) redox state and thus affect NPAS2/BMAL1 transcriptional activity. NAD+/NADH dependence was also observed for CtBP and sirtuins, suggesting that the redox state is an essential component of the gene regulatory systems (Shi, 2004). Moreover, high Acetyl coenzyme A concentrations, indicative for the high overall energy metabolism, facilitate gene activation by promoting histone acetylation (Takahashi et al., 2006). Thus, metabolic enzymes, redox state and biosynthesis intermediates feedback to the regulatory state of the cell.

GMPS

GMP synthetase catalyses the final step of *de novo* guanosine 5'-monophosphate (GMP) biosynthesis. GMPS is a glutamine-dependant amidotransferase, and it consists of a glutaminase domain, responsible for glutamine hydrolysis, and an ATP pyrophosphatase domain, catalyzing GMP conversion from ATP, xantosine 5'-monophosphate (XMP), and ammonia (Hartman and Buchanan, 1959). Essentially, the formation of GMP involves the transfer of ammonia, generated by hydrolysis of L-

glutamine, to XMP (Figure 3). GMPS is conserved from prokaryotes to human and is absolutely essential for guanine nucleotide metabolism and cell viability. Eukaryotic homologues of bacterial GMPS contain an extra domain in the C-terminus of the protein, suggesting that GMPS might have acquired a new function in the course of evolution (Hirst et al., 1994).

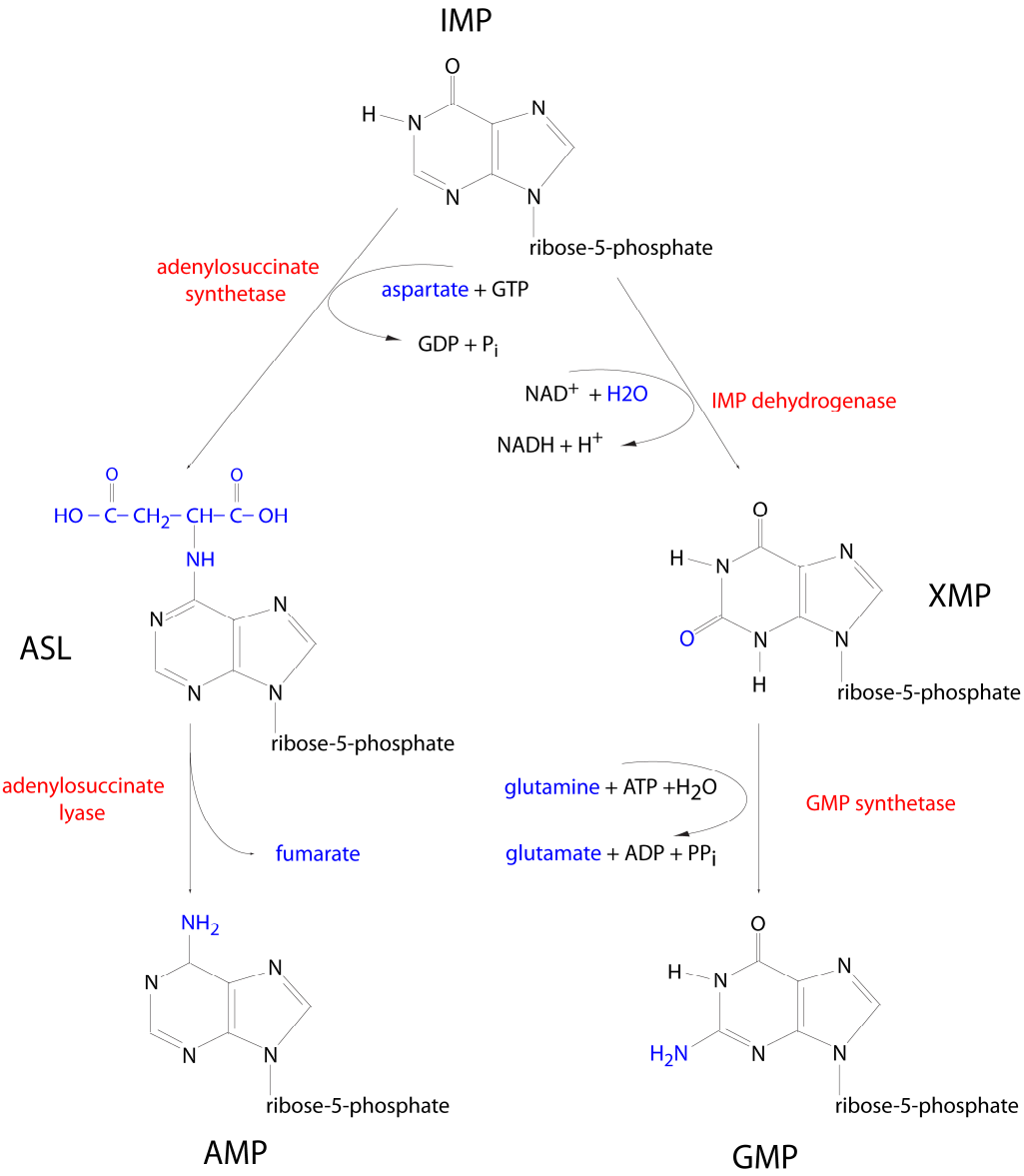

Figure 3. Overview of *de novo* purine synthesis.
Adapted from Lehninger's «Principles of biochemistry», 4th edition.

Recently, an absolutely new activity of GMPS was discovered in *Drosophila* (van der Knaap et al., 2005). van der Knaap and colleagues performed a multistep purification of USP7 protein from *Drosophila* embryo nuclear extracts, and identified GMPS as the strongest interactor of USP7. Next, they demonstrated that GMPS stimulates USP7-mediated deubiquitination of H2B and p53 *in vitro*. Catalytic mutants of GMPS also stimulate USP7's activity suggesting that GMPS has a function independent

of GMP synthesis. Genetic experiments revealed that USP7 and GMPS enhance the Polycomb phenotypes and are involved in repression of homeotic genes. Both USP7 and GMPS localize to chromatin and share a number of common target sites, e.g. the *bxd* Polycomb response element. Thus, GMPS is the first nucleotide biosynthetic enzyme with a chromatin function.

Mutations in the gene encoding for GMPS (*burgundy*) result in defects of photoreceptor axon guidance in the *Drosophila* visual system (Long et al., 2006). In the same study, the authors showed that mutations in inosine monophosphate dehydrogenase (IMPDH), another metabolic enzyme of the GMP biosynthesis branch, result in a similar phenotype.

IMPDH

IMPDH catalyses conversion of IMP to XMP at a rate-limiting step of *de novo* guanine nucleotide biosynthesis. IMPDH is a NAD-dependent dehydrogenase and consists of a catalytic domain, responsible for NAD^+ and IMP binding, and a cystathionine beta synthase (CBS) domain, the function of which is under debate. IMPDH-mediated catalysis includes a dehydrogenase reaction with the transfer of hydrogen to NADH followed by hydrolysis and release of XMP (Figure 3) (Hedstrom, 2009). IMPDH is conserved within all species except for *Giardia lamblia* and *Trichomonas vaginalis*, and is absolutely essential for cell proliferation and viability (Liu et al., 1998). Mammals have two IMPDH homologues, IMPDH1 and IMPDH2, with minor differences in amino acid sequence. IMPDH1 predominates in retina, spleen and blood mononuclear cells and its mutation results only in retinopathy (Aherne et al., 2004). IMPDH2 is expressed in all tissues and its depletion is lethal during early embryogenesis (Gu et al., 2003). IMPDH inhibitors are actively explored in anticancer and antimicrobial drug design due to its absolute requirement for cell proliferation (Shu and Nair, 2008).

Several reports established that IMPDH might have moonlighting functions as a polynucleotide binding protein (Hedstrom, 2009; Mortimer et al., 2008; Xu et al., 2008). Its CBS domain is sufficient for binding RNA and DNA *in vitro* and *in vivo* (Gan et al., 2002; McLean et al., 2004). Mutations in the CBS domain of IMPDH1 are associated with the retinal degeneration disease *Retinitis pigmentosa* and exhibit decreased affinity to polynucleotides (Mortimer and Hedstrom, 2005). Mortimer and colleagues demonstrated that IMPDH binds rhodopsin mRNA and associated ribosomes and

proposed that *Retinits pigmentosa* might be explained by reduced binding of mutant IMPDH to rhodopsin mRNA (Mortimer et al., 2008).

Drosophila melanogaster as a model system

For nearly a century, the fruit fly *Drosophila melanogaster* has been used as a model organism to address numerous biological questions. Being complex enough to study major conserved developmental processes and simple enough to breed and maintain, *Drosophila* is even more attractive now, when multiple genetic and molecular tools have been developed by generations of *Drosophila* scientists.

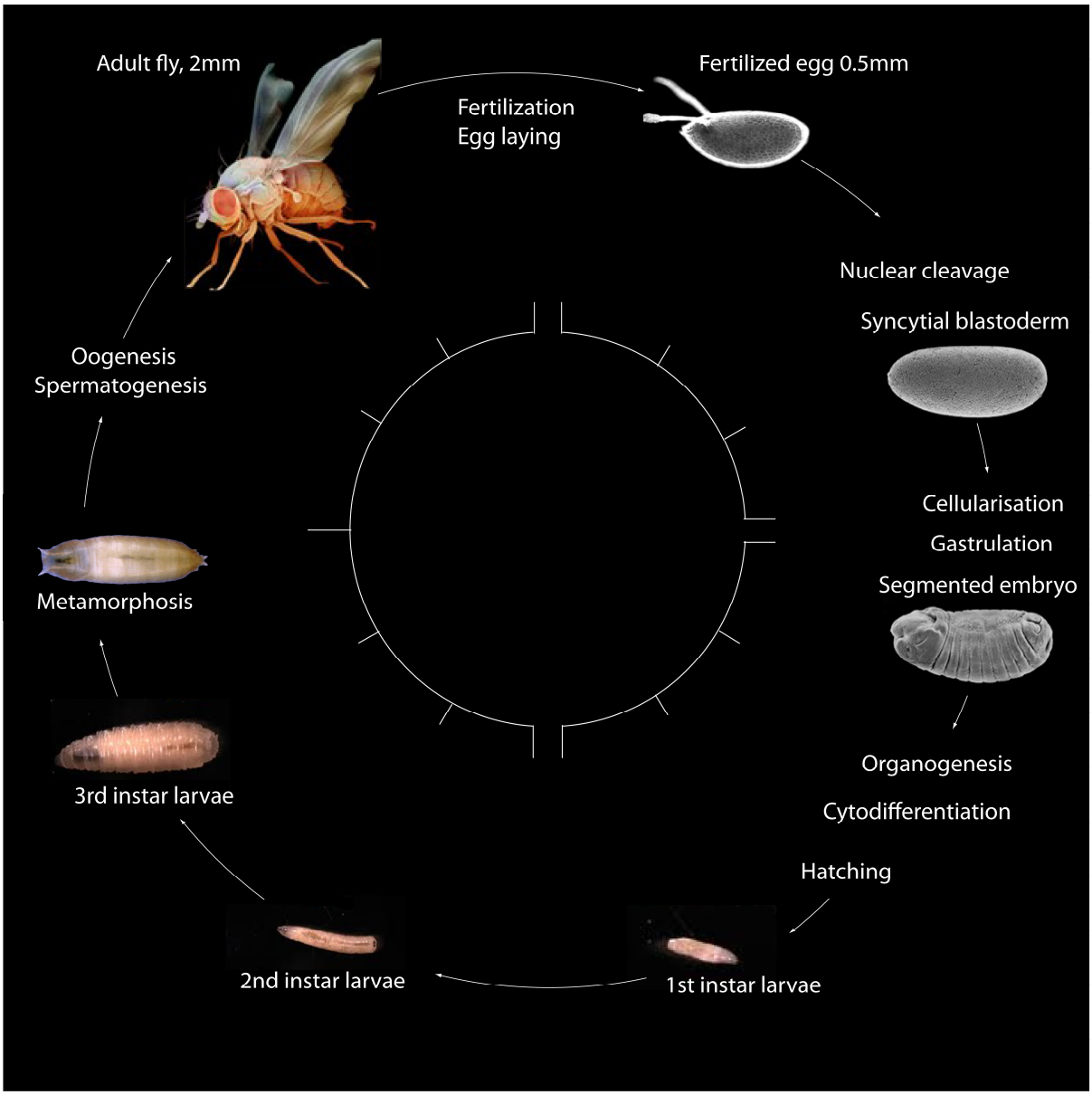

Figure 4 Life cycle of *Drosophila melanogaster*.

Essentially, the fruit fly life cycle consists of embryogenesis, three larval stages, a pupal stage and imago. Transitions between these stages are promoted by the steroid hormone ecdysone. Embryonic development lasts approx. 15 hours when the ventral cord and primordial organs called imaginal discs, that will later form the CNS and adult body parts, develop. Larval stages involve voracious feeding, growth promoted by ecdysone and proliferation of primordial organs. After four days, the third instar larva encapsulates itself inside a hard and dark-colored puparium. In 4 days, during this pupal stage, *Drosophila* larvae undergo metamorphosis and develops into imago (Figure 4).

Up to date, colossal collections of mutant fly strains and gene manipulation systems have been generated. The yeast UAS-GAL4 system was adopted to be used in fly genetics: a gene of interest placed downstream of a UAS sequence can easily be activated by co-expressing GAL4 (Brand and Perrimon, 1993). Regulatory elements of tissue-specific genes or the *heat shock 70* gene are used to drive GAL4 expression, thus, allowing activation of the gene of interest in a single tissue. Recently, a library of UAS-RNAi lines was generated comprising more than 22 thousand fly strains covering more than 12 thousand *Drosophila* genes (approx. 88% of the *Drosophila* genome) (Dietzl et al., 2007). Together with GAL4 drivers, these tools allow the depletion of virtually any protein in a tissue-specific manner at any stage of development (Duffy, 2002).

Another powerful tool offered by *Drosophila* is the third instar larval salivary glands that contain giant polytene chromosomes. These chromosomes form through several endocycles – chromosome duplications without cell divisions and separation of homologues - and contain about 1000 DNA strands. Immunofluorecsent analysis of the protein distribution along these chromosomes allows the mapping of a protein binding loci with about 10 kb accuracy. Co-immunostainings with two or more antibodies help to investigate protein co-localization. RNAi knock-down with a salivary-gland specific driver can be used to test whether the distribution of two proteins is interdependent.

Many more sophisticated tools and techniques including memory and behavior test systems are now available to fly geneticists enabling relatively easy and cost effective ways to address virtually any biological question.

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Scope of the Thesis

Transcriptional regulation of gene expression is one of the major mechanisms that controls cell proliferation, differentiation, and development of a living organism. During more than two decades molecular biology research has been focused on transcription factors and co-regulatory complexes that comprise the gene regulation machinery. However, cell physiology and general metabolism have not been incorporated into the general model of cell regulatory mechanisms. In recent years, metabolic enzymes and intracellular metabolites emerge as transcriptional co-factors that significantly affect gene expression. This thesis is aimed to identify metabolic enzymes with a potential role in transcription and to elucidate their impact on gene expression and cell function. We used *Drosophila* as a model system to study the role of two enzymes of *de novo* GMP biosynthesis, IMPDH and GMPS, in regulation of transcription.

Chapter 2 describes a new activity of the metabolic enzyme IMPDH in gene transcription. It demonstrates genome-wide analysis of IMPDH target genes and its regulation during cell cycle. In this chapter we demonstrate an unusual mode of DNA recognition by IMPDH. GMPS is one of the metabolic enzymes that moonlight in regulation of gene expression. The aim of Chapter 2 is to discover functional relationships between GMPS and its partner USP7 in the course of *Drosophila* development. We explore the cooperation of GMPS and USP7 using mutant and overexpression fly lines and we test the effect of catalytic mutations in USP7 and GMPS on the transcriptional function of these proteins. We attempt to identify developmental processes that are regulated by the USP7/GMPS complex. This question is further developed in Chapter 4 that describes genome-wide transcriptional changes and developmental abnormalities caused by depletion of GMPS and USP7. This chapter also contains an overview of mass spectrometry analysis of USP7 and GMPS binding partners.

CHAPTER 2

Metabolic enzyme IMPDH couples gene expression to cellular state by acting as a transcription factor

(*Under revision*)

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Summary

Cells need to adapt their gene expression program to metabolic state, but how they do this remains poorly understood. Inosine monophosphate dehydrogenase (IMPDH) controls the guanine nucleotide pool and, thereby, cell proliferation. We found that *Drosophila* IMPDH is also a DNA-binding transcriptional repressor. IMPDH regulates the S phase-specific *histone* genes and *E2f*, a key driver of cell proliferation. The nuclear localization of IMPDH and, consequently, its transcriptional activity is controlled by the cell cycle. IMPDH only accumulates in the nucleus during G2, or following metabolic- or oxidative stress. Thus, IMPDH creates a feedback loop that couples expression of histones and E2F to cellular state. IMPDH has an unusual mode of DNA recognition. Genome-wide profiling and in vitro binding assays established that IMPDH binds sequence-specifically to single-stranded, CT-rich DNA elements. Through its dual functions of nucleotide biosynthetic enzyme and transcription factor, IMPDH both enables and restricts cell proliferation.

Introduction

Functional compartmentalization and division of labor are central tenets of cell biology. Consequently, gene regulation and metabolism are conducted by separate classes of proteins. However, cells need to coordinate gene expression and metabolic state. Numerous transcription factors govern cellular metabolism by regulating levels of metabolic enzymes. For example, E2F and MYC transcription factors regulate the expression of nucleotide biosynthesis genes and, this way control the proliferative capacity of the cell (Dang and Lewis, 1997). Conversely, there is little knowledge on how the physiological state of the cell influences gene transcription programs (McKnight, 2010). Intriguingly, there are examples of metabolic enzymes performing double-duty as transcriptional coregulators (McKnight, 2003). For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an essential part of the OCA-S coactivator complex that is recruited by the sequence-specific transcription factor Oct-1 to activate *histone H2B* transcription (Zheng et al., 2003; Dai et al., 2008). Nicotinamide adenine dinucleotide (NAD^+) metabolism also impinges in multiple ways on chromatin and transcription (Rutter et al., 2001; Zhang and Kraus, 2010). Previously, we reported that the nucleotide biosynthetic enzyme guanosine monophosphate synthase (GMPS) can regulate target genes through stimulation of USP7-mediated histone H2B deubiquitylation (Frappier and Verrijzer, 2011; van der Knaap et al., 2010; van der Knaap et al., 2005). GMPS and USP7 form a corepressor complex that is recruited to- and regulates specific genes.

The enzyme directly upstream of GMPS in the guanine nucleotide biosynthesis pathway is IMPDH. IMPDH is conserved from prokaryotes to humans and catalyzes the NAD^+-dependent oxidation of IMP to xanthosine monophosphate (Hedstrom and Gan, 2006; Hedstrom, 2009). This is the pivotal step in the GMP biosynthesis pathway, which controls the guanine nucleotide pool size. Thus, IMPDH is one of the key enzymes that allow genome replication and cell proliferation. Because of this crucial function, IMPDH is a target for antiviral, anticancer and immunosuppressive drugs (Hedstom, 2009; Jackson et al., 1975; Shu and Nair, 2008). In addition to its role as a classic biosynthetic enzyme, there is evidence that IMPDH might engage in additional cellular activities (Hedstrom and Gan, 2006; Hedstrom, 2009). IMPDH can bind nucleic acids and associates with polyribosomes independently from its catalytic activity, which might suggest a role in translation regulation (Mortimer et al., 2008; Xu et al., 2008). Inspired

by our identification of GMPS as a transcriptional corepressor, however, we wondered if IMPDH could be involved in transcription control.

Here, we show that IMPDH also functions as a sequence-specific DNA-binding transcription factor. Although IMPDH is largely cytoplasmic, it accumulates in the nucleus and binds DNA after completion of DNA replication. In addition, cellular stress can induce nuclear IMPDH. When nuclear, IMPDH binds and represses *E2f*, the master driver of the G1/S transition, and the S phase-specific *histone* genes. Thus, as a transcription factor, IMPDH creates a negative feedback loop that couples expression of proliferation genes to cellular state. Genome-wide profiling and biochemical experiments established that IMPDH binds CT-rich unwound DNA elements. Our finding provides a molecular explanation for the enigmatic function of these DNA elements, which have long been implicated in gene control. We discuss the implications of our results for understanding how cellular state can influence gene expression.

Results

Nuclear IMPDH is cell cycle regulated

We investigated the subcellular localization of IMPDH in *Drosophila* S2 cells. Immunofluorescence using anti-IMPDH antibodies (green), in parallel with DAPI staining of DNA (blue), revealed a bimodal distribution. In ~60% of the cells IMPDH was excluded from the nucleus, whereas in the remaining ~40% IMPDH was present in both cytoplasm and nucleus (Figure 1A). Nuclear IMPDH coincided with high cellular levels of cyclin A and cyclin B, suggesting it accumulates in the nucleus during late S- and G2 phase (Figure 1B-D). Next, we tested if nuclear IMPDH was associated with chromatin. Immunofluorescence on third instar larval salivary gland polytene chromosomes revealed that IMPDH (green) displayed a specific pattern of binding to mainly the densely packed chromosome bands (Figure 1E, upper part). A set of chromosomes originating from another nucleus that were marked by PCNA, indicative of ongoing DNA replication, were not bound by IMPDH (lower part). Collectively, these results indicate that nuclear IMPDH is cell cycle regulated and mainly restricted to the G2 phase (Figure 1F).

Next, we wondered if metabolic state could influence the subcellular localization of IMPDH (Figure 1G,H). Following oxidative stress, caused by hydrogen peroxide (H_2O_2), virtually all cells have nuclear IMPDH. Depletion of the deoxynucleotide pool by

Kozhevnikova *et al.*, Fig. 1

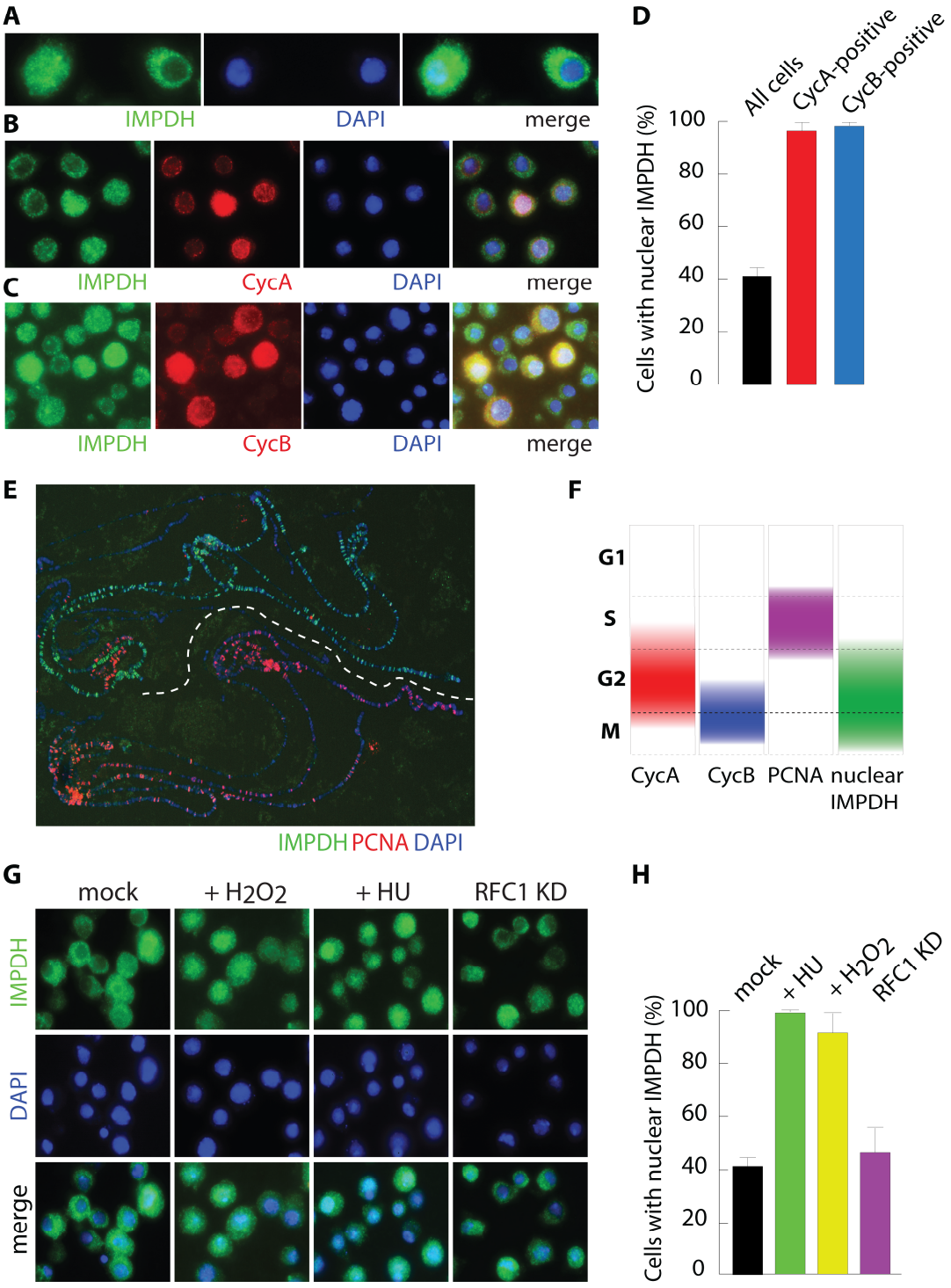

Figure 1. IMPDH nuclear localization is regulated by cell cycle and stress
(**A**) IMPDH shows variable subcellular distribution. In ~60% of the cells IMPDH is excluded from the nucleus, whereas in ~40% of the cells IMPDH is both cytoplasmic and nuclear. S2 cells were fixed and stained with antibodies against IMPDH (green) and nuclei were visualized by DAPI staining of DNA

(blue). (**B**, **C**) IMPDH nuclear localization is largely restricted to (**B**) CycA- and (C) CycB-positive cells. CycA (red), CycB (red) and IMPDH (green) were detected by immunofluorescence. (**D**) Quantification of IMPDH subcellular distribution. The number of S2 cells with either only cytoplasmic IMPDH or both nuclear and cytoplasmic IMPDH were counted and expressed as percentage. The quantification was based on three independnet immunostaining experiments, averaged and shown as a bar graph. (**E**) IMPDH binds specific chromosomal loci but dissociates during S phase. 3rd instar larval salivary gland polytene chromosomes were fixed, squashed and stained with anti-IMPDH (green) and anti-PCNA (red) antibodies. The DNA is visualized by DAPI staining (blue). The merged image is from chromosomal spreads originating from two different nuclei, separated by the dotted white line. (**F**) Nuclear accumulation of IMPDH is cell cycle regulated and mainly restricted to the G2 phase of the cell cycle. The use of CycA, CycB and PCNA as markers of cell cycle stage revealed that IMPDH accumulates in the nucleus from late S through G2 phase and early mitosis. (**G**) IMPDH subcellular localization responds to metabolic stress. Whereas only 40% of mock-treated cells have nuclear IMPDH, after treatment with either H_2O_2 or hydroxyurea (HU), virtually all cells have nuclear IMPDH. In contrast, defective replication due to depletion of replication factor C (RFC) does not significantly alter the IMPDH distribution. The corresponding cell cycle profiles are shown in Figure S1.(**H**) Quantification of IMPDH subcellular distribution.

hydroxyurea (HU), which leads to replicative- and metabolic stress, induced nuclear accumulation of IMPDH. We used hydroxyurea (HU), a ribonucleotide reductase inhibitor, to deplete the deoxynucleotide pool and induce replicative- and metabolic stress. Incubation of cells with HU caused a pronounced nuclear accumulation of IMPDH. In contrast, RNAi-mediated depletion of replication factor C (RFC) did not cause nuclear accumulation of IMPDH. Whereas RFC depletion caused an accumulation of cells in G2/M as well as S phase, HU did cause an accumulation of cells in G1- and S phase of the cell cycle (Figure S1). These results suggest that the effect of HU upon IMPDH localization is more likely due to the cell sensing deoxynucleotide depletion than strictly the consequence of an indirect cell cycle effect. We conclude that nuclear accumulation of IMPDH is controlled by the cell cycle and mainly restricted to the G2 phase. In addition, metabolic stress can also induce nuclear IMPDH.

IMPDH binds and represses *E2f* and the *histone* genes

Because IMPDH binds selective DNA loci, one potential nuclear function of IMPDH is the regulation of gene transcription. Therefore, we compared the genomic distribution of IMPDH and RNA polymerase II (RNA pol II) on polytene chromosomes (Figure 2A, S2A). IMPDH (green) and RNA polymerase II (RNA pol II, red) bind predominantly non-overlapping genomic loci, consistent with a repressive- rather than an activating role for IMPDH. We also noted that IMPDH was associated with chromosomal region 39D-E, which harbors the *histone* gene cluster. In *Drosophila*, the genes encoding histone 1 (*His1*) and the core histones (*His2A, His2B, His3 and His4)* form a unit that is repeated

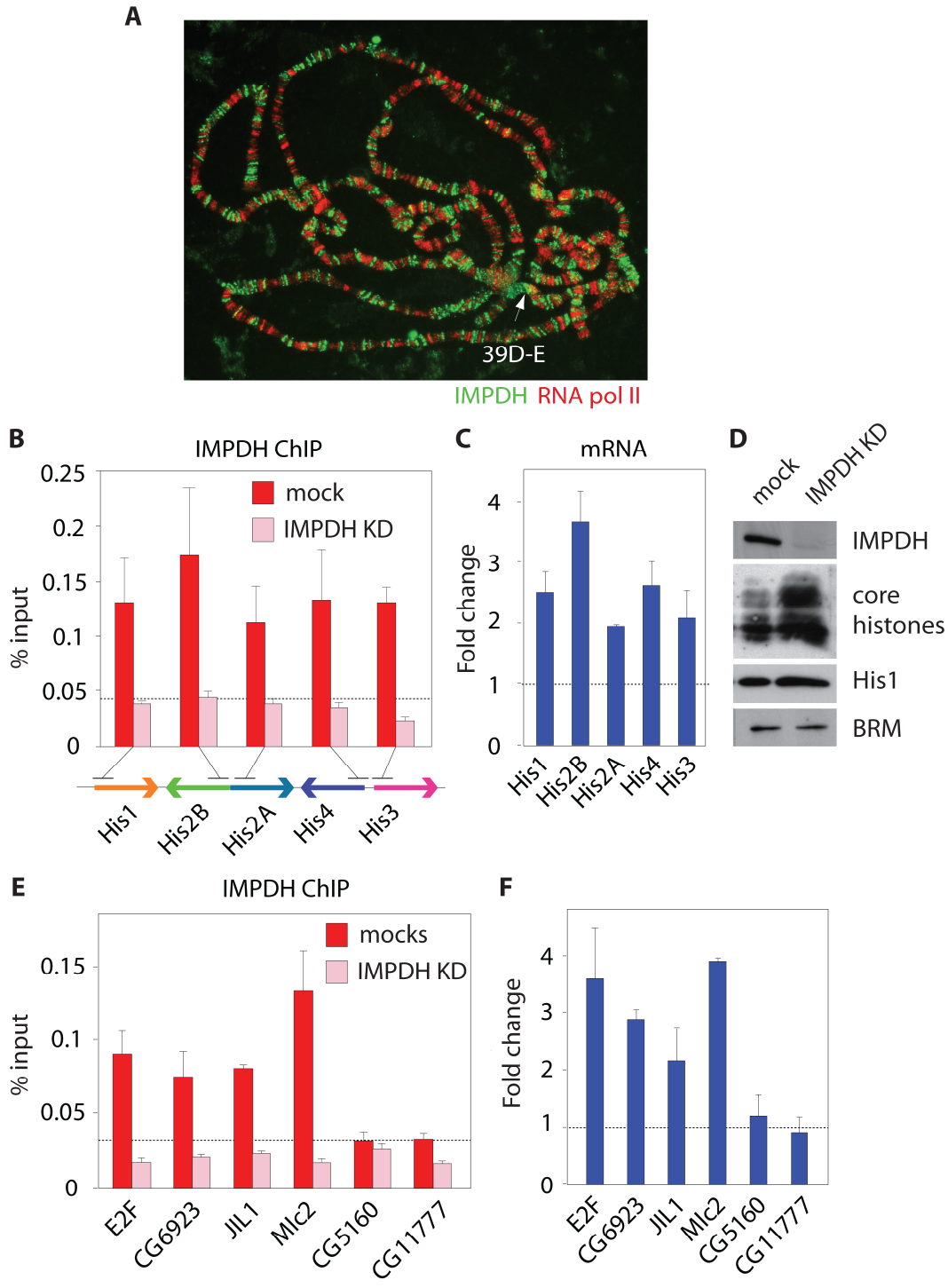

Figure 2. IMPDH binds chromatin and represses its target genes

(**A**) IMPDH and RNA Pol II binding sites do not overlap significantly. Polytene chromosomes were stained with anti-IMPDH (green) and anti-RNA Pol II (red) antibodies. IMPDH binding to chromosomal region 39D-E, harboring the *histone* gene cluster is indicated. Images from different color channels are shown in Figure S2A.

(**B**) IMPDH binding to histone genes in mock- (red) or IMPDH-depleted cells (IMPDH KD, pink), was determined by ChIP-qPCR. ChIP signals are shown as percentage of input. (**C**) The expression of histone genes upon IMPDH knock down was determined by RT-qPCR. *Tub84B* and *brahma* mRNA levels were averaged and used for normalization. Derepression is represented as fold change relative to mock-treated cells. The corresponding cell cycle profiles are shown in Figure S2B. (**D**) Western blotting analysis of mock- or IMPDH-depleted cells using antibodies directed against the core histones or His1.

Brahma (BRM) serves as loading control. (**E**) To validate the genome-wide ChIP-chip results, we used ChIP-qPCR on 4 IMPDH target genes (*E2F*, *Mlc2*, *CG6923*, *JIL1*) and 2 genes that were not bound by IMPDH (*CG5160* and *CG11777*). IMPDH was ChIPed from mock- (red) or IMPDH-depleted (pink) cells. (**F**) IMPDH represses transcription. The effect of IMPDH depletion on the expression of 3 target genes and 2 negative controls was determined by RT-qPCR, as described above.

23 times. Expression of the *histone* genes is tightly coupled to DNA replication and is limited to S phase (Osley, 1991). Therefore, it is an attractive possibility that after IMPDH accumulates in the nucleus at the completion of replication, it will repress *histone* gene transcription. To corroborate IMPDH binding to the *histone* genes, we used chromatin immunoprecipitation in S2 cells followed by quantitative PCR (ChIP-qPCR). In agreement with the polytene localization, ChIP-qPCR showed that IMPDH was associated with all five *histone* genes (Figure 2B). Confirming the specificity of the ChIP, signals were significantly reduced after RNAi-mediated IMPDH depletion. Importantly, loss of IMPDH caused a robust increase in *histone* mRNA- and protein levels (Figure 2C,D). The induction of *histone* genes was not an indirect consequence of an S phase arrest. IMPDH depletion does leads to a drop of cells in G1 and an accumulation of cells at the G2/M phase of the cell cycle (Figure S2B). In conclusion, IMPDH binds and represses the *histone* genes. Following genome duplication and re-packaging into chromatin, it is crucial to shut down histone expression because excess histones are toxic to cells (Groth et al., 2007). Our results suggest that at the end of S phase IMPDH accumulates in the nucleus, binds the *histone* genes and represses their expression.

To obtain a high-resolution genome-wide view of IMPDH distribution, we mapped the genomic IMPDH binding sites in S2 cells using ChIP followed by hybridization to *Drosophila* tiling arrays (ChIP-chip). After intersecting the IMPDH binding peaks from three independent biological replicates at a false discovery rate (FDR) of <0.05, we derived 2910 IMPDH binding loci. The genome-wide IMPDH binding site identification was supported by ChIP-qPCR of selected loci (Figure 2E). We noted that one of the IMPDH targets is the *E2f* gene, encoding a transcription factor that is crucial for the G1/S transition and DNA replication in animal cells (Blais and Dynlacht, 2004; van den Heuvel and Dyson, 2008; Dimova and Dyson, 2005). Consistent with a repressive role in transcription for IMPDH, its target genes, including *E2f*, were up-regulated following its depletion (Figure 2F). In contrast, the expression of control genes that were not bound by IMPDH, remained unaffected. One of the ways E2F drives the G1/S transition is by up-regulation of nucleotide biosynthetic enzymes (Blais and Dynlacht, 2004; van den Heuvel and Dyson, 2008; Dimova and Dyson, 2005). Our

results suggest that once its biosynthetic function is completed at the end of S phase IMPDH creates a negative feedback loop. After completion of DNA replication, or upon cellular stress, IMPDH enters the nucleus and attenuates histone and E2F expression.

Kozhevnikova *et al.*, Figure 3

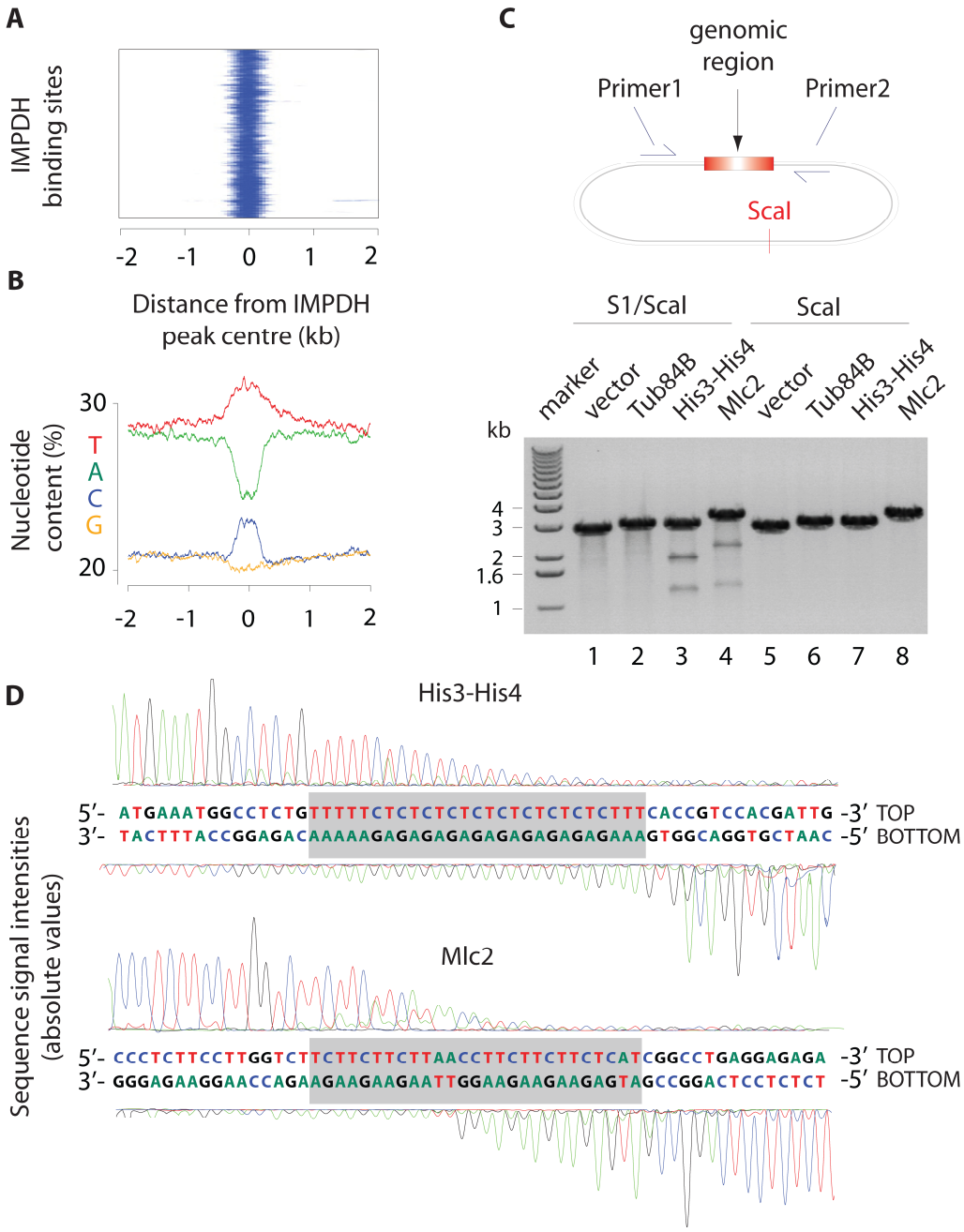

Figure 3. IMPDH binding sites have the tendency to unwind and form ssDNA
(**A**) Heatmap displaying the ChIP-chip enrichment of IMPDH at 2910 aligned binding sites. Four kb regions were aligned at the centre of the IMPDH binding peaks. (**B**) IMPDH loci are CT-rich. The average nucleotide content of aligned IMPDH binding sites is displayed as percentages. Note that the overrepresentation of Cs and Ts is inconsistent with Watson-Crick base pairing, suggesting coprecipitation of ssDNA with IMPDH. (**C**) IMPDH binding sites have the tendency to unwind. The genomic IMPDH-bound DNA sequences within the *His3-His4* promoter and within the *Mlc2* gene, as well

as a region from the *Tub84B* that was not bound by IMPDH, were cloned in pGEM-T. Supercoiled plasmids were subjected to S1 nuclease treatment (lanes 1-4), or incubated with a buffer control (lanes 5-8), and then digested by ScaI. Products were resolved by agarose gel electrophoresis. (**D**) Sequencing of the S1/ScaI generated fragments from the *His3-His4* promoter region or *Mlc2* gene. Four-color plots represent intensities of the four nucleotides detected in the sequencing reactions. The loss of signal occurs precisely within the CT-rich IMPDH target sequences, reflecting S1 nuclease digestion at these sites (grey boxes).

IMPDH binds CT-rich single-stranded DNA elements

To gain insight in IMPDH targeting, we aligned the 2910 identified genomic IMPDH loci, centered on the peak of IMPDH binding. The ChIP enrichment is presented as a heatmap, illustrates the well-defined binding of IMPDH (Figure 3A). Next, we plotted the averaged nucleotide composition of the aligned IMPDH loci (Figure 3B). Strikingly, IMPDH binding sequences were significantly enriched for Cs and Ts. This result violates Chargaff's rules, which stipulate that in DNA the amount of G is equal to C and the amount of A is equal to T. We note that, as expected, our ChIP-chip analyses of other chromatin factors always yielded equal ratios of A to T and G to C. One possible explanation of the CT bias of IMPDH loci could be the presence of single stranded DNA (ssDNA). This is a particularly attractive possibility, because CT-rich genomic sequences have been reported to adopt non-canonical, unwound DNA structures (Gilmour et al., 1989; Htun and Dahlberg, 1988; Johnston, 1988; Mace et al., 1983). These CT-rich DNA elements have long been associated with gene control, but the underlying molecular mechanism has remained unclear. To test the propensity of IMPDH targets to unwind, we subcloned the *His3*/*His4* bidirectional promoter and *Mlc2* IMPDH binding element into the pGEM-T-Easy vector. We probed supercoiled plasmids harboring these genomic fragments for local DNA unwinding using S1 nuclease, which preferentially cuts ssDNA. Supercoiling is believed to mimic packaging tension in chromatin, providing the energy to promote unwinding of DNA sequences that have the tendency to do so (Htun and Dahlberg, 1988; Larsen and Weintraub, 1982; Mace et al., 1983). Following S1 nuclease digestion, plasmids were digested with ScaI and analyzed by gel electrophoresis (Figure 3C). S1 nuclease digestion, reflected by the appearance of 2 extra bands, was only observed in the plasmids harboring the *Mlc2* or *His3-His4* promoter sequences, but not within the vector or *Tub84B* control sequences. The location of S1 nuclease digestion was mapped at high resolution by direct DNA sequencing of the isolated DNA fragments (Figure 3D). The loss of sequence signal intensities, due to S1 nuclease digestion, occurred precisely within the CT-rich genomic

IMPDH binding sites. These results show that the CT-rich IMPDH target loci have an intrinsic propensity to unwind.

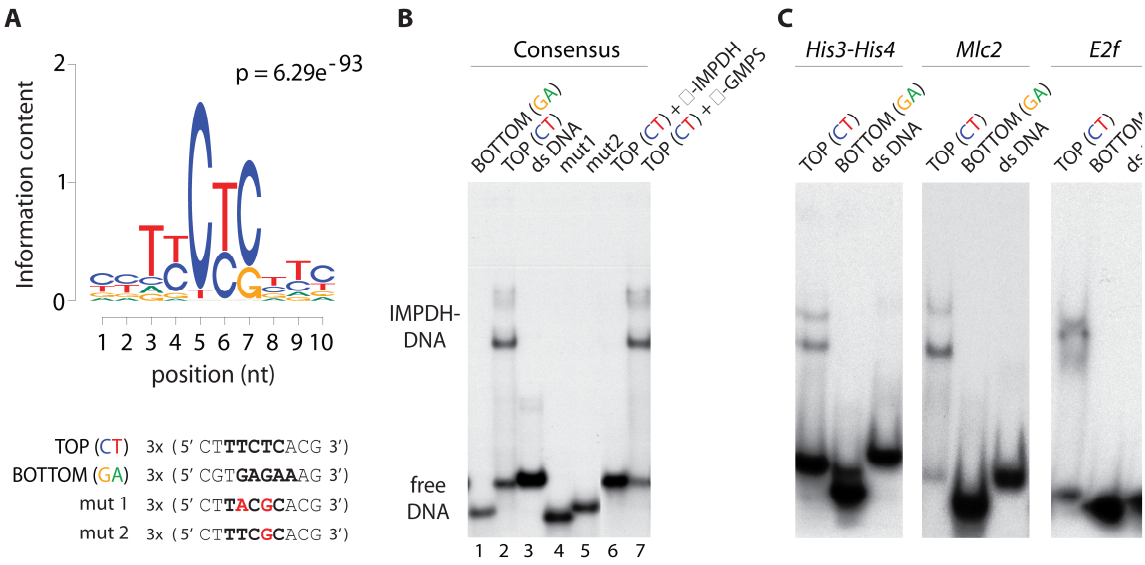

Figure 4. IMPDH is a sequence-specific ssDNA-binding protein
(**A**) Position weight matrix illustrating the overrepresentation of a T(C/T)C(T/C)C consensus motif ($p = 6.29e^{-93}$). We synthesized ssDNA oligonucleotides harboring 3 copies of the CT-rich consensus motif (TOP), the complementary GA-rich bottom strand, and hybridized the latter two to generate a dsDNA probe. We also synthesized two ssDNA probes harboring mutations (red) in the consensus. Oligonucleotides were radiolabeled and used in bandshift assays. (**B**) Bandshift testing the binding of recombinant, purified IMPDH to the indicated probes. IMPDH binds the CT-rich ssDNA consensus probe (lane 2), but not to its GA-rich complementary strand (lane 1). Mutations in the consensus causes a loss of IMPDH binding to the ssDNA probe (lanes 4 and 5). The addition of affinity purified anti-IMPDH antibodies abrogates DNA binding (lane 6), whereas irrelevant (anti-GMPS) antibodies do not affect IMPDH binding (lane 7). Free DNA and IMPDH-DNA complexes are indicated. (**C**) IMPDH also binds in vitro to its natural genomic sites, identified by ChIP. IMPDH binding to CT-rich (TOP), GA-rich (BOTTOM) and dsDNA probes derived from its genomic loci within the *His3-His4* promoter, *Mlc2* and *E2f* was tested by bandshift. The probes used for *His3-His4* and *Mlc2* correspond to the boxed sequences in Figure 3D. Note that IMPDH only binds the CT-rich ssDNA probes.

A more detailed analysis of the genomic IMPDH sites revealed the significant overrepresentation of the T(C/T)C(T/C)C consensus motif ($p = 6.29e^{-93}$; Figure 4A). To test if IMPDH could recognize this DNA sequence directly, we used recombinant, purified IMPDH and radiolabeled oligonucleotides harboring 3 copies of the consensus motif in bandshift assays. We compared binding to the CT-rich strand (TOP), the complementary GA-rich strand (BOTTOM) and double stranded DNA (dsDNA), in the presence of excess nonspecific competitor DNA. We found that IMPDH bound efficiently to the CT-rich top strand, but recognized neither the complementary strand, nor the

dsDNA probe (Figure 4B, lanes 1-3). IMPDH binding to the ssDNA probe was sequence-specific because mutations in the consensus sequence abrogated DNA binding (compare lanes 2 and 4-5). The addition of affinity purified antibodies against IMPDH blocked DNA binding, confirming the specificity of the bandshift (lane 6). In contrast, irrelevant antibodies did not affect IMPDH binding (lane 7). Finally, we tested IMPDH binding to its natural sites within the *His3-His4* promoter, *E2f* and *Mlc2* genes. Again, we detected efficient binding to the CT-rich ssDNA probes, but not to their complementary strands or to the dsDNA probes (Figure 4C). We conclude that IMPDH is a sequence-specific ssDNA binding transcription factor. Its genomic binding sites have the propensity to unwind, which might be promoted by DNA transactions such as nucleosome formation or transcription, which can create tension in the DNA that facilitates unwinding of susceptible DNA sequences.

Discussion

Our results demonstrate that the classic nucleotide biosynthetic enzyme IMPDH does double duty as a cell cycle-regulated transcription repressor. As a key metabolic enzyme that is rate-limiting for guanine nucleotide synthesis, IMPDH prepares the cell for DNA replication (Hedstrom, 2009; Hedstrom and Gan, 2006). However, IMPDH also acts as a cell cycle regulated transcription factor. At the end of S phase, IMPDH accumulates in the nucleus and represses proliferative genes such as the histones and *E2f*, the master driver of the G1/S transition. Furthermore, we found that metabolic stress, such as deoxynucleotide depletion caused by HU, triggers the accumulation of nuclear IMPDH. Through its dual functions, IMPDH balances metabolic state and cell cycle progression. This coupling is critical in animal cells because deficient nucleotide synthesis can drive genomic instability during early oncogenesis (Bester et al., 2011). As IMPDH undergoes an array of conformational changes during catalysis (Hedstrom, 2009; Hedstrom and Gan, 2006), its role in transcription might be modulated by cellular nucleotide levels. Noticeably, through GAPDH, mammalian *His2B* activation by the OCA-S coactivator is stimulated by NAD^+, but inhibited by NADH (Zheng et al., 2003; Dai et al., 2008). Suggestively, IMPDH converts NAD^+ to NADH, thus potentially blocking OCA-S-mediated activation of *histone* gene expression.

As a transcription factor, IMPDH has an uncommon mode of DNA binding. Genome-wide ChIP-chip analysis and in vitro binding assays established that IMPDH binds sequence-specifically to CT-rich, ssDNA elements. Interestingly, these pyrimidine-

rich DNA elements have long been associated with gene control, and have been proposed to assume non-canonical DNA structures harboring regions of ssDNA (Gilmour et al., 1989; Htun and Dahlberg, 1988; Johnston, 1988; Mace et al., 1983; Pestov et al., 1991; Takimoto et al., 1993). However, how these elements might influence transcription has remained enigmatic. Our results now suggest that IMPDH, or similar proteins, bind these elements and modulate transcriptional output similar to a classic DNA-binding transcription factor. We propose that the long known association of IMPDH activity and cancer (Hedstom, 2009; Jackson et al., 1975; Shu and Nair, 2008) involves both its role as a nucleotide biosynthetic enzyme and its regulation of proliferative genes as a transcription factor. It has been found that *retinitis pigmentosa*, a condition that leads to incurable blindness, is associated with mutations in *IMPDH1* that do not affect its catalytic activity (Bowne et al., 2002; Hedstrom and Gan, 2006). One suggested explanation has been that IMPDH is involved in control of rhodopsin mRNA translation (Mortimer et al., 2008). Our findings raise the possibility that IMPDH's role in human diseases also involves its activity as a sequence-specific transcription factor. We hypothesize that direct transcription control by IMPDH might be the tip of a larger gene regulatory network governed by nucleotide metabolic enzymes.

Experimental procedures

Immunological procedures

IMPDH amino acids 1 to 295 was expressed as GST fusion protein, purified, and used for immunizations as previously described (Chalkley and Verrijzer, 2004). Anti-histone antibodies were raised against purified endogenous core histones (Katsani et al., 2001), Anti-Brahma and anti-H1 have been described (Mohd-Sarip et al., 2006; Moshkin et al., 2007; van der Knaap et al., 2005) 2005. PCNA was detected using PC10 (Dako) and RNA polymerase II was detected using a mixture of 8WG16, H5, and H14 (Covance).

Chromatin immunoprecipitation

ChIP experiments were performed as described (Mohd-Sarip et al., 2006) with minor modifications. In brief, S2 culture cells were fixed with 2% formaldehyde for 15 minutes at room temperature. Crosslinking was blocked by the addition of glycine at a final concentration of 150 mM for 5 minutes. Next, cells were washed with PBS and lysed. Chromatin was sonicated into fragments of ~500-700 bp. For ChIP, 100 µg of sonicated chromatin was incubated with 40 µl of α-IMPDH antibodies and 20 µl of pre-blocked protein A Agarose (Upstate) overnight, followed by extensive washes, de-crosslinking and DNA elution with QIAquick PCR purification kit (Qiagen).

ChIP-chip data analysis

Raw ChIP-chip hybridization intensities were analyzed using R and R/Bioconductor packages. Log2-scaled genomic DNA (5 replicas) hybridization intensities were quantile normalized, averaged and served as input. Genome-wide ChIP-chip enrichment (score) was calculated by ChIP.score = average ($\log_2$(ChIP/Input) norm) – average (log2(Mock/Input) norm). Three independent biological replicates were used. Peaks were selected at FDR < 0.05 based on random permutation. Binding sites were determined by intersecting sets of significant ChIP-chip peaks. Data was deposited to Gene Expression Omnibus. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hfwpjacemcwmklg&acc=GSE29202)Average nucleotide content was calculated for aligned IMPDH loci. Sequences were retrieved using R/Bioconductor and BSgenome.Dmelanogaster.UCSC.dm3 package. De novo sequence motif discovery was performed by applying feature model motif approach described in and available from (Sharon et al., 2008).

S1 nuclease digestion

PCR products obtained from qPCRs were cloned into pGEM-T-Easy vector (Promega), transformed into bacterial cells and purified as supercoiled plasmids. Plasmids (5 µg) were digested with 300 units of S1 nuclease (Promega) for 10 minutes at 25°C, purified using the QIAquick PCR purification kit (Qiagen) and digested with ScaI (Roche). For control digestion, supercoiled plasmids were only digested with ScaI. Digested material was resolved by agarose gel electrophoresis. The fragments generated by S1 nuclease and ScaI digestion were isolated and purified using the QIAquick gel extraction kit (Qiagen). Recovered DNA fragments were sequenced using flanking primers.

Protein purification and band shift assays

IMPDH was cloned as his-tag fusion within pDEST26 (Invitrogen) vector and introduced into 293-T cells using polyethyleneimine-mediated transfection. The protein was purified on Ni-NTA agarose (Qiagen) according to the manufacturer's instructions and dialyzed against buffer containing 20mM HEPES-KOH, 10% glycerol and 100mM KCl. Single-stranded probes were end-labelled using polynucleotide kinase (New England Biolabs) and when appropriate annealed. For bandshift assays, 20µl binding reactions containing 20 mM HEPES-KOH, pH 7.6, 10% glycerol, 5 mM $MgCl_2$, 1 mM $ZnCl_2$, 1µg poly-dIdC, 1µg BSA, 75 mM KCl, 0.01% NP40 and recombinant IMPDH were incubated at room temperature for 20 minutes. Next, labelled probes were added to the reaction mixtures at a final concentration of 0.4pM and incubated for an additional 20 minutes at room temperature. Binding reactions were then resolved in non-denaturing polyacrylamide gels, dried and exposed to X-ray film.

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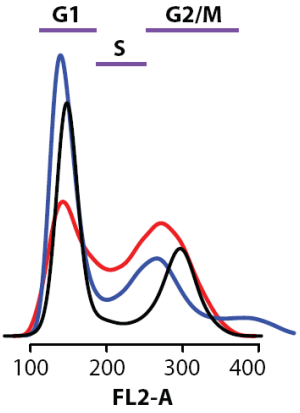
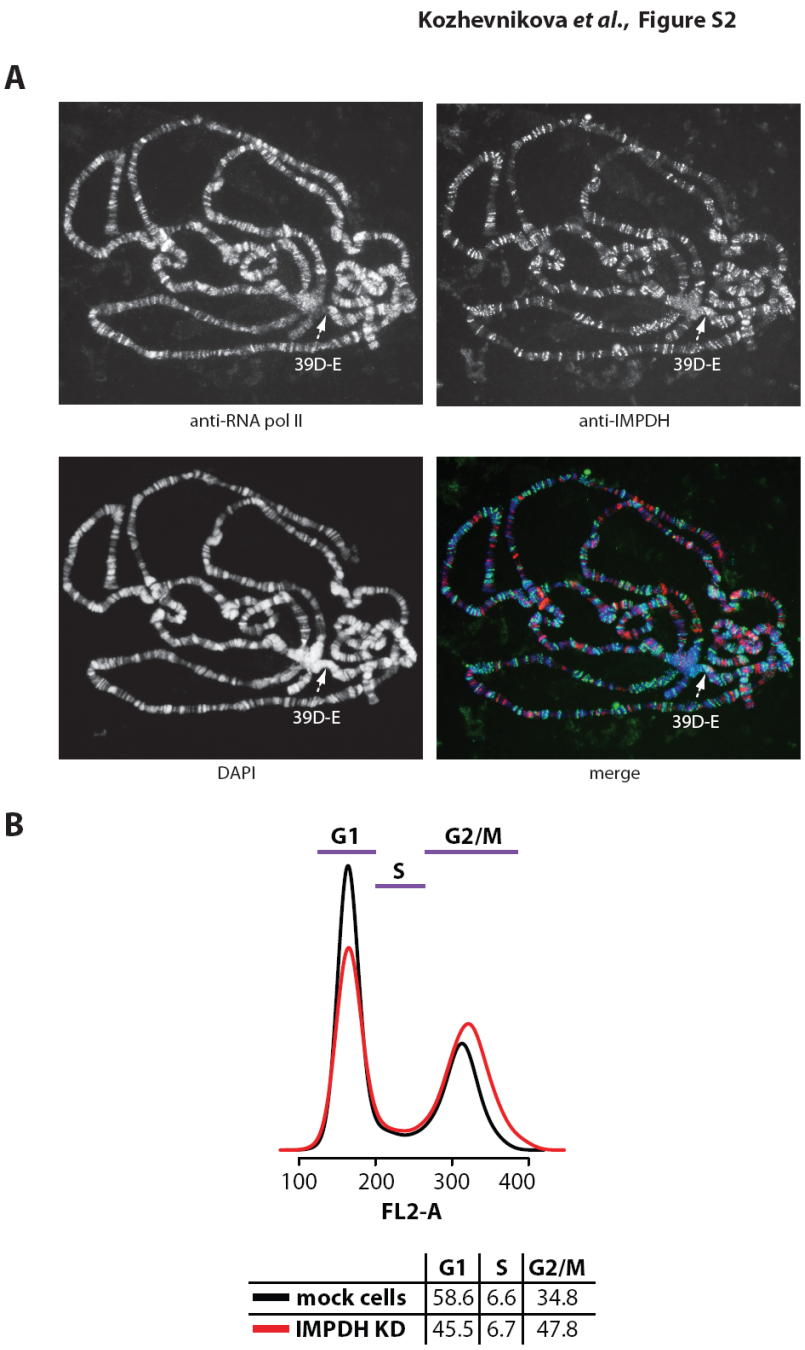


	G1	S	G2/M
mock cells	58.6	6.6	34.8
HU	60.11	11.36	28.54
RFC1 KD	37.46	19.15	43.4

Supplementary figure S1
Cell cycle profiles after either mock treatment, incubation with hydroxyurea (HU) or RNAi-mediated depletion of RFC (see **Figure 1G, H**), were determined by FACS analysis. Quantification is based on gated cells. The percentage of cells in G1, S or G2/M phases and corresponding peaks are indicated.



Supplementary figure S2

(**A**) IMPDH and RNA Pol II binding sites do not overlap significantly.3rd instar larval salivary gland polytene chromosomes were fixed, squashed and stained with anti-IMPDH (green) and anti-RNA Pol II (red) antibodies. The DNA is visualized by DAPI staining (blue). IMPDH binding to chromosomal region 39D-E, harboring the *histone* gene cluster is indicated. The images from different color channels and a merged image are shown. (**B**) Effect of IMPDH depletion on the S2 cell cycle distribution. IMPDH depletion leads to a modest accumulation of cells in G2/M. S2 cells were either mock-treated (black) or treated with dsRNA directed against IMPDH (IMPDH KD, red) and analyzed by FACS.

CHAPTER 3

Biosynthetic Enzyme GMP Synthetase Cooperates with Ubiquitin-Specific Protease 7 in Transcriptional Regulation of Ecdysteroid Target Genes

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Biosynthetic Enzyme GMP Synthetase Cooperates with Ubiquitin-Specific Protease 7 in Transcriptional Regulation of Ecdysteroid Target Genes[▽]

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Drosophila **GMP synthetase binds ubiquitin-specific protease 7 (USP7) and is required for its ability to deubiquitylate histone H2B. Previously, we showed that the GMPS/USP7 complex cooperates with the Polycomb silencing system through removal of the active ubiquitin mark from histone H2B (H2Bub). Here, we explored the interplay between GMPS and USP7 further and assessed their role in hormone-regulated gene expression. Genetic analysis established a strong cooperation between GMPS and USP7, which is counteracted by the histone H2B ubiquitin ligase BRE1. Loss of either GMPS or USP7 led to increased levels of histone H2Bub in mutant animals. These *in vivo* analyses complement our earlier biochemical results, establishing that GMPS/USP7 mediates histone H2B deubiquitylation. We found that GMPS/USP7 binds ecdysone-regulated loci and that mutants display severe misregulation of ecdysone target genes. Ecdysone receptor (EcR) interacts biochemically and genetically with GMPS/USP7. Genetic and gene expression analyses suggested that GMPS/USP7 acts as a transcriptional corepressor. These results revealed the cooperation between a biosynthetic enzyme and a ubiquitin protease in developmental gene control by hormone receptors.**

Proper development requires the coordination of growth and differentiation. Hormones perform essential signaling functions in metazoan organisms, controlling a plethora of processes ranging from homeostasis to key developmental transitions. The steroid hormone 20-hydroxyecdysone (ecdysone) provides critical temporal triggers that direct major developmental transitions in *Drosophila* (14, 22). A high ecdysone pulse at the end of the third-instar larval stage starts the larval-to-prepupal transition (pupariation). About 10 h later, another ecdysone pulse sets off the prepupal-pupal transition (pupation). The primary mediator of ecdysone signaling is a heterodimer of the Ecdysone Receptor (EcR) and Ultraspiracle (USP), the fly RXR homolog. The EcR/USP heterodimer belongs to the class of nuclear receptors (NRs) that bind their cognate regulatory DNA elements both in the absence and in the presence of hormone (14, 22). Gene regulation by NRs involves the antagonistic activities of transcriptional corepressors and coactivators. Nonliganded NRs recruit transcriptional corepressors to their target regulatory elements, thus directing gene silencing. Upon hormone binding, there is a conformational transition that causes the replacement of corepressors by coactivators, leading to activation. Typically, coregulators modulate the structure of chromatin and include histone acetyltransferases, deacetylases, methyltransferases, demethylases, and ATP-dependent chromatin-remodeling factors (3, 4, 18, 23).

We previously implicated the biosynthetic enzyme GMP synthetase (GMPS) in transcription regulation via modulation of histone H2B deubiquitylation by ubiquitin-specific protease 7 (USP7) (29). USP7 is an evolutionarily conserved protein which was originally isolated as a binding partner of the herpes simplex virus protein Vmw110/ICP0, hence its alternate name, HAUSP (herpesvirus-associated ubiquitin-specific protease) (11). Several other associated factors and substrates of USP7 have been identified, including p53 (16), Epstein-Barr nuclear antigen 1 (EBNA1) (12), MDM2 (15), DAXX (26), FOXO4 (28), and PTEN (25).

An intriguing feature of GMPS is that it strongly stimulates the activity of USP7 (29). We found that a portion of cellular GMPS was tightly associated with USP7 and was required for H2B deubiquitylation. Histone H2B monoubiquitylation at lysine 120 (H2Bub) by the E3 ligase BRE1 is an active mark linked to transcriptional elongation, whereas H2Aub is associated with silencing (10, 30). In addition, we showed that GMPS and USP7 both act as enhancers of Polycomb-mediated silencing of homeotic genes *in vivo* (29). These findings suggested that GMPS does double duty: it is required for de novo GMP synthesis but is also involved in transcription control, at least in part, through cooperation with USP7. However, the extent of the involvement of GMPS and USP7 in developmental gene expression control was unclear. Here, we used a combination of genetics, *in vivo* gene expression analysis, and biochemistry to establish essential roles for GMPS/USP7 in ecdysone signaling. Our results revealed that GMPS/USP7 binds and regulates ecdysone target loci, implicating a complex of a biosyn-

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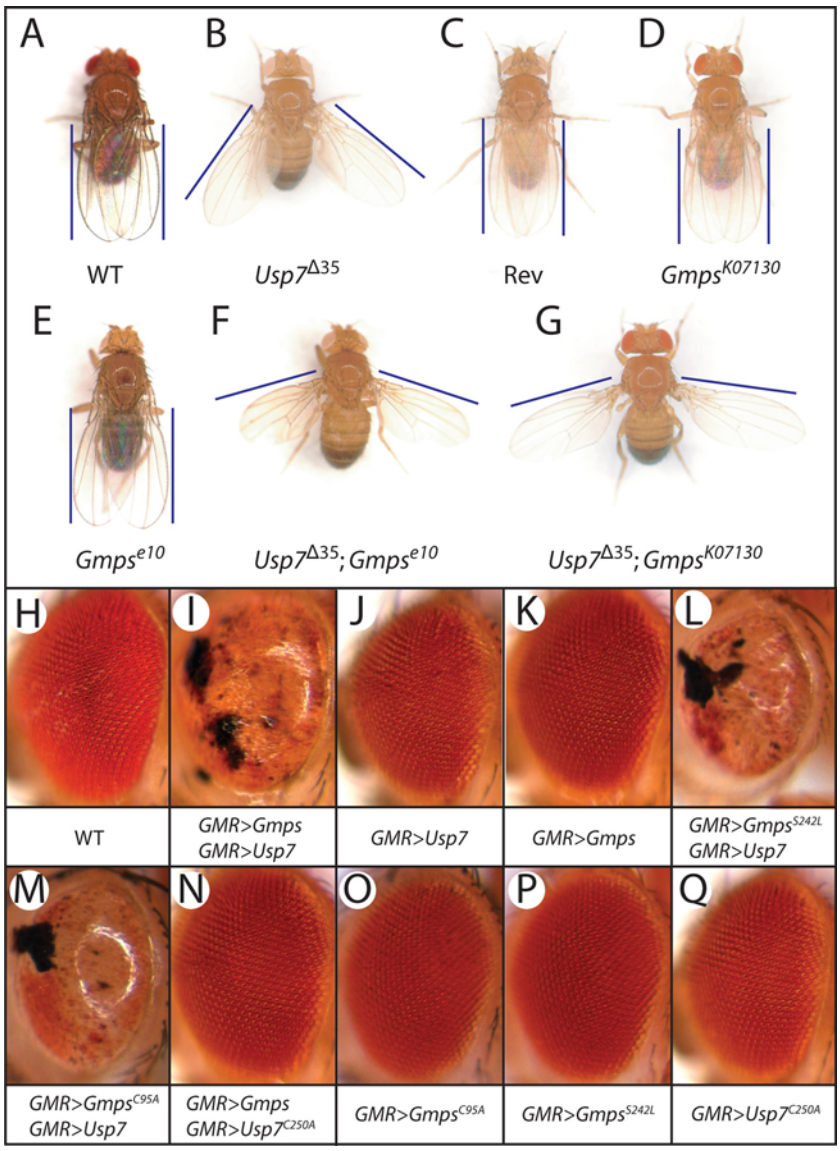

FIG. 1. *Usp7* and *Gmps* interact genetically. (A to G) Gmps loss-of-function mutations enhance the $Usp7^{\Delta 35}$ held-out wing phenotype. The hypomorphic allele $Usp7^{\Delta 35}$ was generated by imprecise excision of the *P* element in $Usp7^{KG06814}$, whereas the revertant (Rev) resulted from precise excision of the *P* element. As indicated, the male flies are WT, hemizygous for $Usp7^{\Delta 35}$, and/or heterozygous for the indicated *Gmps* alleles. (H to Q) Ectopic expression of both *Usp7* and *Gmps* driven by the GMR enhancer (*GMR>Gmps*/*GMR>Usp7*) causes defective eye development characterized by disorganized or missing ommatidia, loss of bristles, and black necrotic patches. Overexpression of either *Usp7* or *Gmps* by itself did not disrupt eye development. Mutations that abrogate either glutamine hydrolysis ($Gmps^{C95A}$) or ATP hydrolysis ($Gmps^{S242L}$) do not affect GMPS cooperation with USP7. However, overexpression of the $Usp7^{C250A}$ catalytic mutant together with GMPS no longer gave an eye phenotype. Single overexpression of $Gmps^{C95A}$, $Gmps^{S242A}$, or $Usp7^{C250A}$ has no effect on eye development.

thetic enzyme and a ubiquitin protease in gene control by hormone receptors.

MATERIALS AND METHODS

Fly strain, genetics, and DNA constructs. Fly stock maintenance and crosses were performed using standard procedures. $Usp^{\Delta 35}$ was generated by imprecise excision of the *P* element in $P[SUPor\text{-}P]Usp7^{KG06814}$ (29). Genomic sequence analysis revealed 24 bp of the *P* element remaining and loss of a part of the 5′ untranslated region (UTR) and the coding sequence for its first 23 amino acids. $P[EP]Bre1^{kim1}$ and $Bre1^{RNAi}$ were purchased from GenExel (strain GE22117) and VDRC (strain 15620) (9), respectively. $Gmps^{RNAi}$ was obtained from the National Institute of Genetics Fly Stock Center (strain 9242R-3). To generate *UAS-Usp7* and $UAS\text{-}Usp7^{C250A}$ transgenic lines, cDNAs encoding full-length USP7, $USP7^{C250A}$, $GMPS^{S242L}$, and $GMPS^{C95A}$ were cloned into pUAST and verified by sequencing. *P* element-mediated germ line transformation was performed according to standard procedures. The $Gmps^{e10}$ and *UAS-Gmps* lines were a kind gift of Yong Rao (19). $Gmps^{K07130}$ has been described previously (29). EcR^{V559fs} and EcR^{A483T} were acquired from the Bloomington stock center. *UAS-Usp7#J2* and $UAS\text{-}Usp7^{C250A\#C4}$, both integrated on the second chromosome, were each combined with *UAS-Gmps#2* (*UAS-bur#2* in reference 19) and crossed with a *GMR-Gal4* driver line. A stable *GMR>Usp7*/*GMR>Gmps* line (#2091) was created by recombination of *UAS-Usp7* (line J1, on the third chromosome) and *UAS-Gmps* (UAS-bur#1 in reference 19) and crossed with a *GMR-Gal4* driver line (on the second chromosome). Crosses were performed at 25°C. Details will be provided upon request.

Protein-protein interaction assays. Recombinant glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21-Codon-

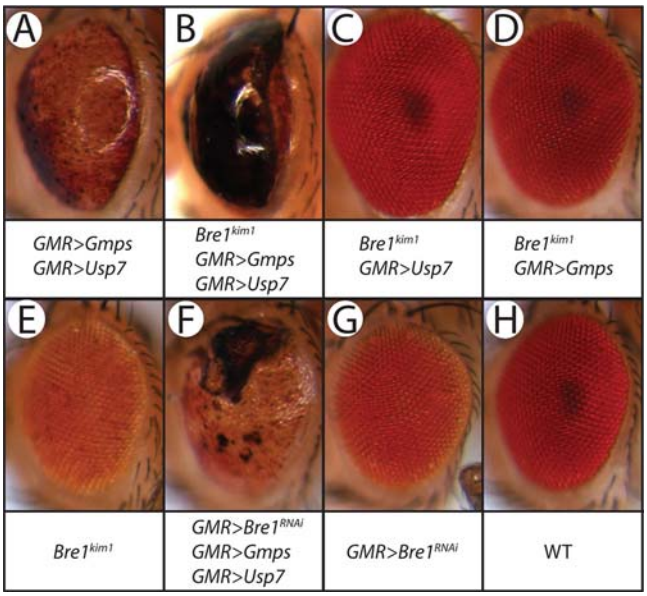

FIG. 2. *Usp7*, *Gmps*, and *Bre1* interact genetically. Concomitant reduction of histone H2B ubiquitin ligase BRE1 levels with GMPS and USP7 overexpression strongly enhances the eye phenotype. (A to E) Heterozygous $Bre1^{kim1}$ males that overexpress GMPS and USP7 by the GMR-GAL4 driver have a 50% reduction in viability, whereas escapers display an enhanced eye phenotype. Heterozygous $Bre1^{kim1}$ males which overexpress only USP7, only GMPS, or neither factor have normal eyes. (F) When GMR-GAL4 drives the expression of an RNAi targeting the *Bre1* mRNA, as well as overexpression of USP7 and GMPS, we also observed a clear enhancement of the eye phenotype. (G) $GMR>Bre1^{RNAi}$ by itself does not display any eye defect. WT eyes are shown as a reference.

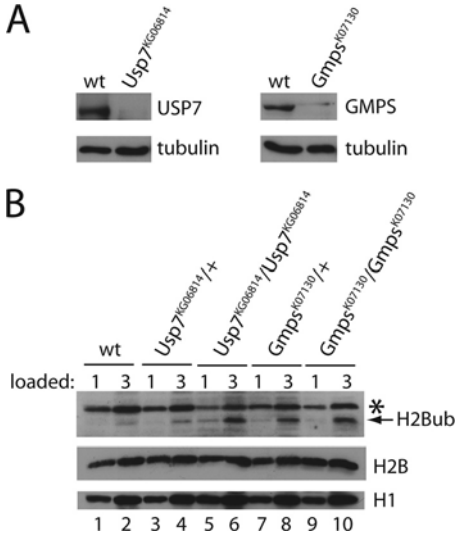

FIG. 3. USP7/GMPS targets H2Bub *in vivo*. (A) Homozygous $Usp7^{KG06814}$ and $Gmps^{K07130}$ mutants have reduced levels of USP7 and GMPS, respectively. Protein extracts of third-instar larvae were analyzed by immunoblotting using the indicated antibodies. (B) Homozygous $Usp7^{KG06814}$ and $Gmps^{K07130}$ mutants contain increased levels of histone H2Bub. Immunoblotting on protein extracts of third-instar larvae with the indicated genotypes is shown. A threefold different protein amount of each extract was loaded to ensure that blotting analysis was within a quantitative range. The asterisk indicates a background band that served as an additional loading control. Antisera were directed against the indicated proteins.

Plus(DE3)-RIL and purified by glutathione Sepharose 4 fast flow (GE Healthcare) chromatography according to standard procedures (8). ^{35}S-labeled proteins were expressed using TNT coupled rabbit reticulocyte lysates (Promega). Prior to binding reactions, beads were blocked with 3% fetal calf serum in HEMG buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol) containing 1 mM dithiothreitol, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 150 mM KCl (making HEMG/150), and 0.1% NP-40. Binding reactions were in the same buffer but lacking serum. Following three washes with HEMG/300/0.1% NP-40 and two with HEMG/150/0.01% NP-40, bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

Immunological procedures. For antibody production, GMPS (amino acids 310 to 563 and 310 to 623) and BRE1 (amino acids 231 to 718) were expressed as GST fusion proteins, purified, and used for immunizations as previously described (8). Anti-USP7, anti-H1, and anti-H2B (PV57/58, raised against purified core histones and mainly recognizing H2B) have been previously published (13, 20, 29). To detect RNA polymerase (Pol) II, we used a mixture of monoclonal antibodies 8WG16, H5, and H14 (Covance). Anti-ubiquityl-histone H2B (clone 56) was from Millipore. Anti-EcR (DDA2.7 and Ag10.2), developed by C. Thummel and D. Hogness, was obtained from the Developmental Studies Hybridoma Bank (Department of Biological Sciences, National Institute of Child Health and Human Development, University of Iowa, Iowa City). Analysis of polytene chromosomes was done as described previously (21). For analysis of polytene chromosomes upon knockdown of GMPS, $Gmps^{RNAi}$ was crossed with an actin driver line. To analyze H2Bub levels, 10 third-instar larvae were lysed in SDS-gel loading buffer using an Eppendorf tube pestle.

Gene expression analyses. For prepupal analysis, the $Usp7^{kim1}$, $Usp7^{KG06814}$, and $Gmps^{K07130}$ mutant lines were rebalanced with green fluorescent protein (GFP)-marked balancer chromosomes. GFP-negative mutant prepupae and wild-type (WT) prepupae were collected at 2-h intervals from the moment of pupariation ($t = 0$) for 12 h. RNA was extracted using TRIzol. S2 cells were cultured and treated with double-stranded RNA (dsRNA) as described previously (33). After 3 days, dsRNA was reapplied, and after 5 days, cells were harvested and RNA was extracted using TRIzol. Knockdowns were performed in biological triplicates. Knockdown efficiency was monitored in parallel by immunoblotting. RNA levels in prepupae or S2 cells were analyzed by first-strand cDNA synthesis with Superscript II reverse transcriptase (Invitrogen) and subsequent quantitative PCR (qPCR) with SYBR green I using a MyiQ single-color real-time PCR detection system (Bio-Rad). Analysis of the reverse transcription (RT)-qPCR data was performed using the $2^{-\Delta\Delta C_T}$ method (17). CG11874 was used as an internal control mRNA.

The RT-qPCR primers used were as follows: CG11874, 5′-AGTGTTGCTCTG CCTAAGTGG-3′ and 5′-CGGATGATGGTGCGGATTGG-3′; GAPDH, 5′-TG CTGGAGCCGAGTATGTGG-3′ and 5′-GCCGAGATGATGACCTTCTTGG-3′; ImpL3, 5′-ATCGGCAGCGGCACCAAC-3′ and 5′-CGGCAATGTTCACTC CAGACC-3′; ImpE2, 5′-ACTTCCTGGGCGGCAATCG-3′ and 5′-CTACGGCT GGCTTCTCTGTGG-3′; Ftz-F1, 5′-ATGGAAGGCGAACGAAGGATAC-3′ and 5′-AGTTGGTGGTAGTAGTGATGATGC-3′; E74A, 5′-GATGGTCGTCTT GTTGGAGGTC-3′ and 5′-TGCGGGTTGTTCGGATTGC-3′; E75A, 5′-CCTTT CATTGACTAACTGCCACTC-3′ and 5′-CGAAACGAAACGAACGGAACG-3′; E75C, 5′-CGGCTCGGAAGTTTGTGGTTAG-3′ and 5′-GCTGATGCTGCT GCTGATGC-3′; CG1381, 5′-ACACCCGAACAGGCGAGAATCC-3′ and 5′-TC ATCATCGTCGTCGTTGTCATCC-3′.

RESULTS

GMPS and USP7 interact genetically. Previously, we presented biochemical evidence revealing that parts of GMPS and USP7 associate in a highly stable complex, mediating epigenetic gene silencing (29). Here, we set out to explore their cooperation *in vivo* during developmental gene control. To facilitate the genetic analysis of USP7, which is encoded by an essential X-chromosomal gene, we created new alleles by mobilizing the *P* element in the $Usp7^{KG06814}$ mutant strain. One of the derived alleles, $Usp7^{\Delta 35}$, turned out to be a hemizygous viable hypomorph. $Usp7^{\Delta 35}$ lacks part of the *USP7* 5′ UTR and

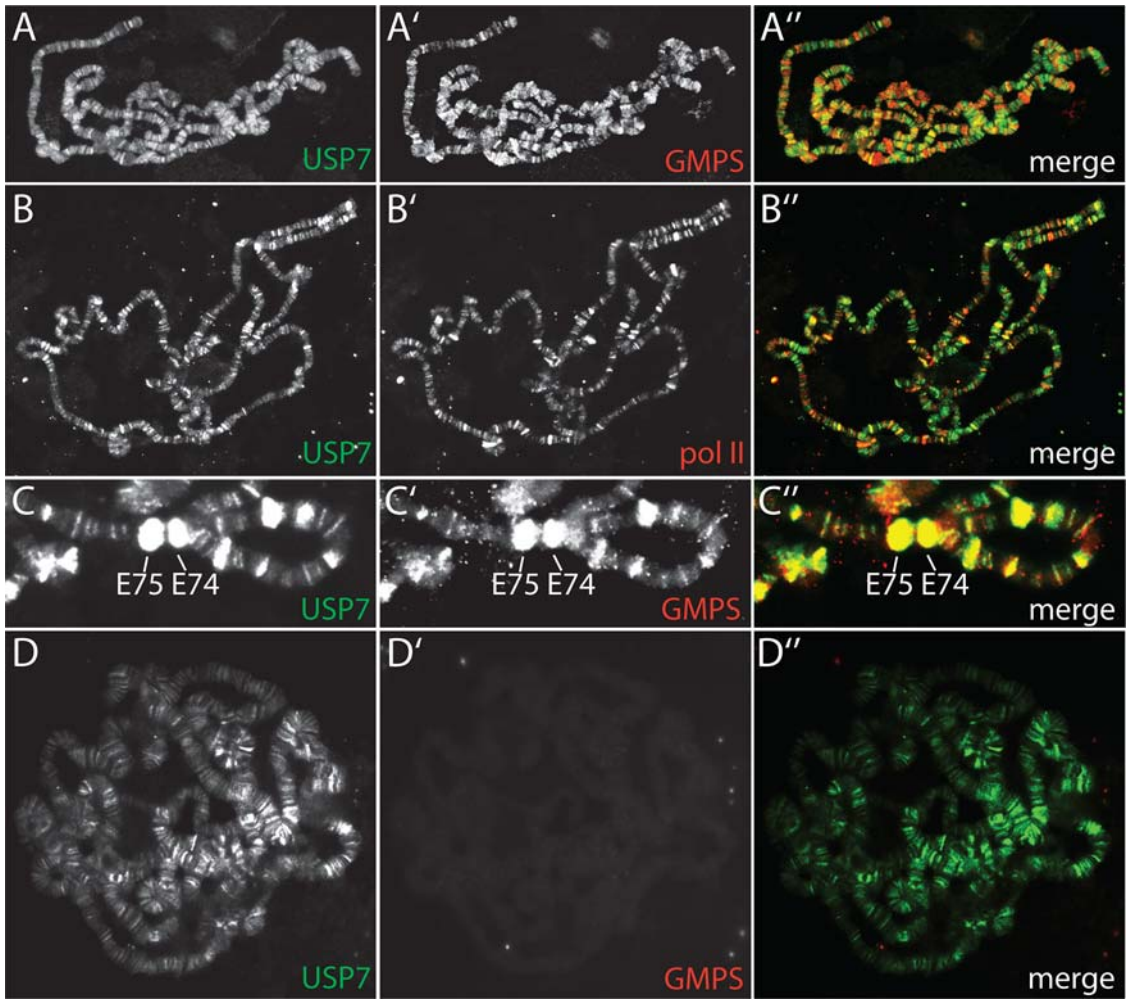

FIG. 4. GMPS and USP7 bind ecdysone-regulated loci. (A to E) The distribution of USP7, GMPS, and RNA Pol II on *Drosophila* salivary gland polytene chromosomes was determined by indirect immunofluorescence using the appropriate antibodies. (A) USP7 and GMPS colocalize at many sites but also bind unique loci. (B) USP7 and RNA Pol II distribution is mostly mutually exclusive. (C) Higher magnification of a polytene chromosome showing the binding of USP7 and GMPS at ecdysone-regulated loci *E74* and *E75* prior to puffing. (D) USP7 remains associated with polytene chromosomes upon RNAi-mediated knockdown of GMPS.

the coding sequence for its first 23 amino acids and expresses USP7 at a reduced level. Hemizygous $Usp7^{\Delta 35}$ males, but not revertants, generated by precise *P* element excision, displayed a "held-out wing" phenotype (Fig. 1A to C). This phenotype is convenient for genetic interaction assays. Indeed, we found that the "held-out wing" phenotype was strongly enhanced when $Usp7^{\Delta 35}$ was combined with either a heterozygous $Gmps^{e10}$ or $Gmps^{K07130}$ allele, which by itself did not affect wing positioning (Fig. 1D to G). These results showed that USP7 and GMPS interact not only biochemically but also genetically.

To complement these loss-of-function analyses with an *in vivo* ectopic expression assay, we employed the GAL4/UAS system (6). We used the glass multimer reporter (GMR) to drive GAL4 expression in the developing eye, resulting in overexpression of USP7 or GMPS under UAS control. Concomitant ectopic expression of USP7 and GMPS (*GMR>Usp7*/*GMR>Gmps*) caused clear defects in eye development characterized by disorganized or missing ommatidia, loss of bristles, and black necrotic patches (Fig. 1I). When either only USP7 (Fig. 1J) or only GMPS (Fig. 1K) was expressed ectopically, we observed no eye defects. Thus, the phenotype was strictly dependent on the overexpression of both GMPS and USP7. Next we wondered whether the enzymatic activities of USP7 or GMPS were required for the distorted eye phenotype. The phenotype of ectopic coexpression of USP7 with either a GMPS mutant defective in ATP hydrolysis ($GMPS^{S242L}$) or a GMPS mutant defective in glutamine hydrolysis ($GMPS^{C95A}$) was similar to that resulting from the coexpression of WT GMPS (Fig. 1L and M). Thus, in agreement with our earlier *in vitro* results (29), GMPS's enzymatic activity is not required for stimulation of USP7. In contrast, concomitant overexpression of GMPS and $USP7^{C250A}$, a deubiquitylation-defective substitution mutant, no longer affected eye development (Fig. 1N). As is the case for the WT proteins, ectopic overexpression of only $GMPS^{C95A}$, $GMPS^{S242L}$, or $USP7^{C250A}$ had no effect on eye development. (Fig. 1O, P, and Q). We conclude that GMPS and USP7 cooperation is critically dependent on USP7's

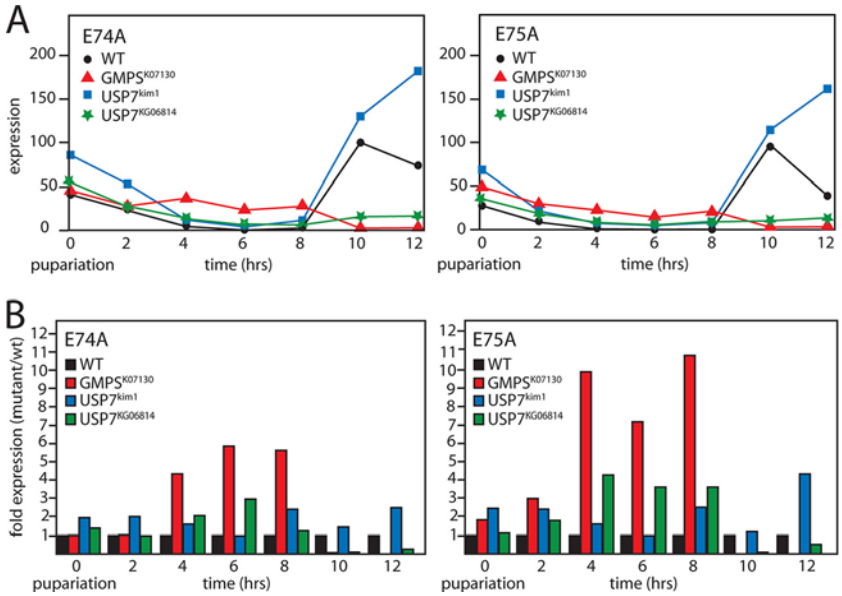

FIG. 5. *Usp7* and *Gmps* mutants misexpress EcR target genes. (A) WT and homozygous $Usp7^{kim1}$, $Usp7^{KG06814}$, or $Gmps^{K07130}$ mutant prepupae were isolated at 2-h intervals from pupariation ($t = 0$) for 12 h. RNA was extracted, and relative expression levels of E74A (left panel) and E75A (right panel) were determined by RT-qPCR. The highest level of expression in WT prepupae was set at 100. Note that $Gmps^{k07130}$ and $Usp7^{KG06814}$ mutants die during pupation. We omitted the error bars for clarity, but the variation was always less than 5%. (B) E74A and E75A expression in homozygous $Usp7^{kim1}$, $Usp7^{KG06814}$, and $Gmps^{k07130}$ mutant prepupae plotted relative to that in WT animals.

deubiquitylating activity but not on de novo GMP synthesis by GMPS.

GMPS/USP7 counteracts BRE1 by deubiquitylation of histone H2B. Our earlier *in vitro* reconstitution experiments suggested that histone H2Bub is a target for deubiquitylation by GMPS/USP7 (29). Because BRE1 is the E3 ubiquitin ligase that catalyzes H2B ubiquitylation, we wondered whether reduced BRE1 levels would modulate the GMPS/USP7 overexpression phenotype. To test this hypothesis, we combined a heterozygous $Bre1^{kim1}$ mutant allele with GMR-driven ectopic expression of GMPS and USP7. Note that the recombined *GMR>Gmps/GMR>Usp7* line used in these assays has a milder eye phenotype (Fig. 2A) than the one used above (Fig. 1I; see Materials and Methods for details). The heterozygous $Bre1^{kim1}$ allele, when combined with GMPS/USP7 overexpression, led to strongly (~50%) reduced viability, whereas the eye phenotype in surviving adults is strongly enhanced (Fig. 2B). The combination of $Bre1^{kim1}$ with overexpression of only USP7 or only GMPS had no effect on eye development, again emphasizing the dependence on cooperation between GMPS and USP7 (Fig. 2C and D). Development of $Bre1^{kim1}$ heterozygotes was normal (Fig. 2E). When we used the GMR-GAL4 driver for ectopic GMPS and USP7 expression and to express an interfering RNA targeting the *Bre1* mRNA, we also observed an enhanced phenotype (Fig. 2F). Importantly, the $GMR>Bre1^{RNAi}$ line by itself displayed no defective eye development (Fig. 2G). When $GMR>Bre1^{RNAi}$ was combined with either *GMR>Usp7* or *GMR>Gmps* alone, we observed no phenotype (data not shown). Collectively, these results strongly support the notion that GMPS and USP7 cooperate *in vivo* to counteract the activity of ubiquitin ligase BRE1.

The ability of GMPS/USP7 to deubiquitylate H2Bub *in vitro* (29) and the strong genetic interaction between GMPS/USP7 and BRE1 *in vivo* strongly support the relevance of histone H2Bub as a substrate. To provide further evidence for this notion, we determined H2Bub levels in $Gmps^{K07130}$ and $Usp7^{KG06814}$ mutants by immunoblot analysis of protein extracts made from third-instar larvae. Homozygous $Usp7^{KG06814}$ and $Gmps^{K07130}$ larvae have strongly reduced levels of USP7 and GMPS, respectively (Fig. 3A). We observed a clear increase in bulk H2Bub levels in homozygous $Usp7^{KG06814}$ larvae compared to WT or heterozygous larvae (Fig. 3B, compare lanes 5 and 6 with lanes 1 to 4). Note that to show that we are within a quantitative blotting range, we loaded two amounts of protein for each set. In support of the regulatory role of GMPS in the catalytic activity of USP7, we also observed an increase in H2Bub levels in homozygous $Gmps^{K07130}$ larvae (Fig. 3B, compare lanes 9 and 10 with lanes 1 and 2 and lanes 7 and 8). Collectively, our earlier results (29) and those presented here demonstrate that GMPS/USP7 deubiquitylates histone H2Bub.

GMPS and USP7 are required for ecdysteroid signaling *in vivo*. To compare their genome-wide distribution, we used indirect immunofluorescence to determine the binding of GMPS and USP7 on third-instar larval salivary gland polytene chromosomes (Fig. 4A). USP7 and GMPS colocalize on many of their chromosomal binding sites, but each also occupies unique loci. Thus, although USP7 and GMPS can form a tight complex, they can also bind DNA independently. In agreement with this notion, immunodepletion experiments revealed that portions of USP7 and GMPS are not associated with each other (data not shown). Overall, USP7 does not colocalize with RNA Pol II, indicative of a role in gene silencing rather than activation (Fig. 4B). This is consistent with its genetic function as an enhancer of Polycomb silencing (29) and its biochemical activity: removal of the active H2Bub mark. Analysis of their binding pattern revealed that both GMPS and USP7 associate

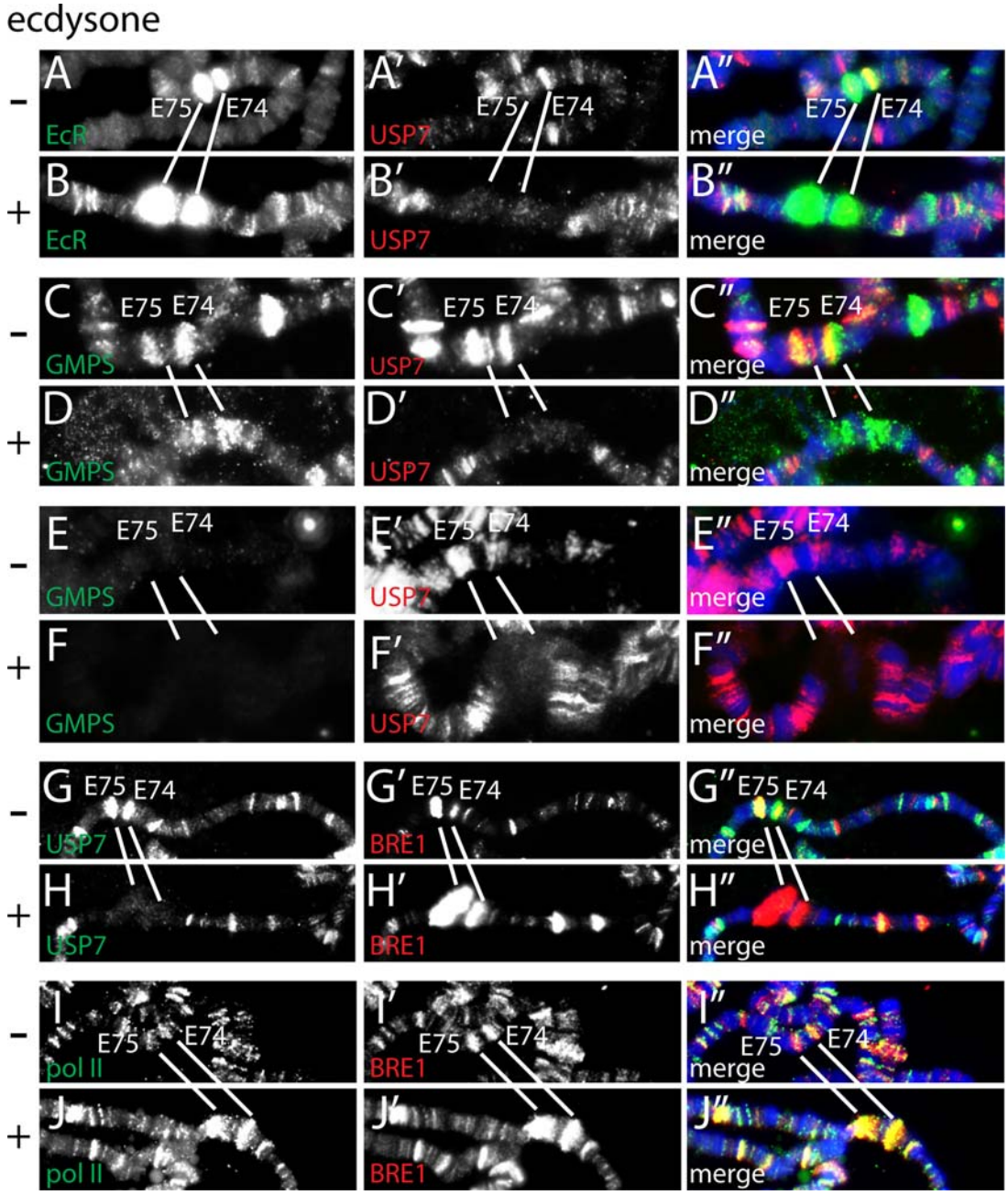

FIG. 6. Upon induction, USP7 leaves the *E74* and *E75* loci, whereas GMPS remains and BRE1 is recruited. Indirect immunofluorescence on third-instar larval polytene chromosomes before (−) and after (+) activation by ecdysone using the indicated antibodies is shown. DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining (blue). The *E74* and *E75* loci are indicated. (E and F) RNAi-mediated knockdown of GMPS does not affect the dynamics of USP7 association with *E74* and *E75*.

with the *E74* and *E75* loci harboring ecdysone early-response genes (7, 24) (Fig. 4C). We note that we only observed GMPS and USP7 colocalization in these regions prior to their induction (see below). RNA interference (RNAi)-mediated knockdown of GMPS does not affect USP7 binding to polytene chromosomes (Fig. 4D). These results suggest that the recruitment of USP7 to chromatin is independent of GMPS.

The binding of USP7 and GMPS to the *E74* and *E75* loci suggested a role in the transcriptional regulation of these gene clusters. Consistent with this notion, strong USP7 and GMPS mutants die at the prepupal or pupal stage, when ecdysone signaling is critical. Furthermore, *Gmps* and *Usp7* mutant pupae frequently lack anterior spiracles, one of the hallmarks of defective ecdysteroid signaling during pupariation (data not shown). To test the role of USP7 and GMPS in ecdysone signaling, we compared the expression profiles of *E74A* and *E75A* in homozygous $Usp7^{KG06814}$, $Usp7^{kim1}$, and $Gmps^{K07130}$ prepupae to that in WT animals (Fig. 5A). Prepupae were collected at pupariation ($t = 0$), and RNA was extracted at 2-h intervals for 12 h and monitored by RT-qPCR. To facilitate

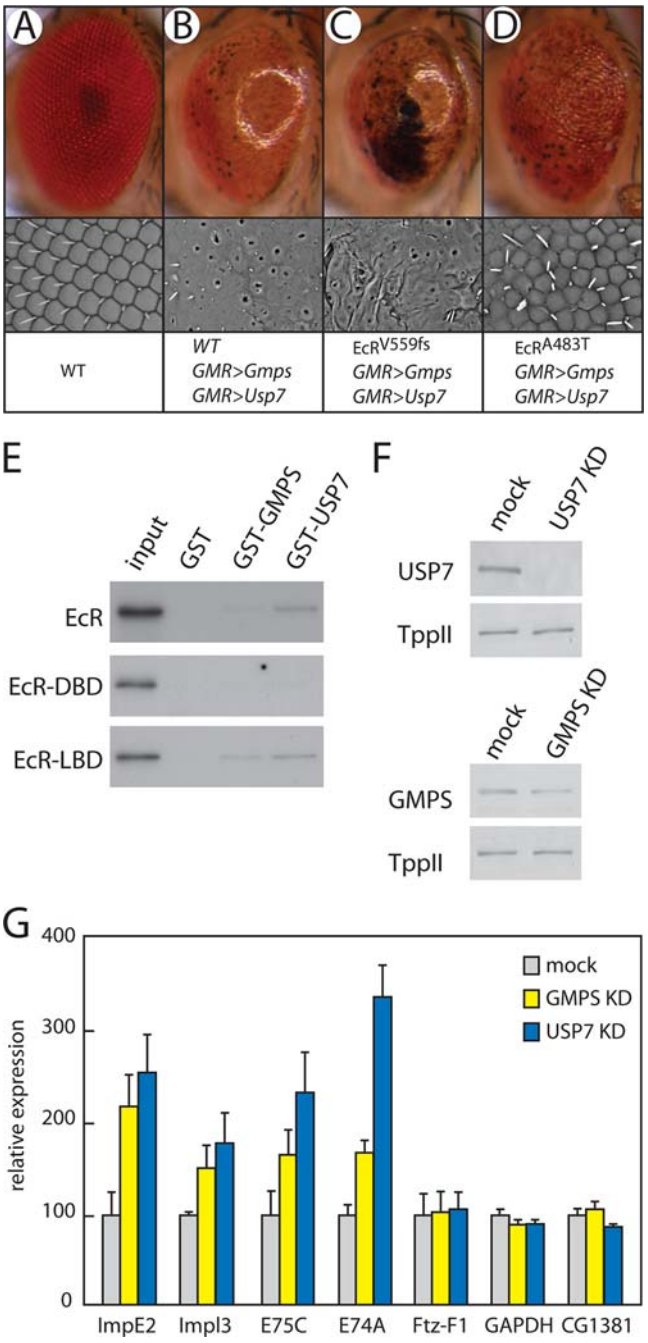

FIG. 7. GMPS/USP7 is an EcR corepressor. (A to D) Genetic analysis suggested that GMPS/USP acts as an EcR corepressor. The effect of ectopic expression of both *Usp7* and *Gmps* (*GMR>Gmps*/*GMR>Usp7*) during eye development is enhanced by EcR^{V559fs}, which is activation defective, and suppressed by EcR^{A483T}, which is defective for repression. The upper panels are regular photographs, and the lower panels are scanning electron micrographs. (E) USP7 binds the EcR *in vitro*. GST, GST-GMPS, or GST-USP7 immobilized on glutathione-Sepharose beads was incubated with ^{35}S-labeled full-length EcR, DBD, or LBD, and after extensive washes, bound proteins were resolved by SDS-PAGE and detected by autoradiography. Eight percent of the input was loaded. (F) RNAi-mediated knockdown of USP7 or GMPS was monitored by immunoblot analysis of whole-cell extracts. Tripeptidyl peptidase II (TppII) served as a loading control. (G) GMPS and USP7 depletion leads to upregulation of ecdysone-regulated genes. RT-qPCR analysis of mock-treated S2 cells or cells treated with dsRNA directed against either USP7 or GMPS. Relative expression levels of the indicated genes were determined by RT-qPCR using the $2^{-\Delta\Delta C_T}$ method. For each gene, the relative level in mock-treated cells was set at 100. Experiments were performed in three independent biological replicates. Error bars represent the standard error of the mean.

comparison, *E74A* and *E75A* expression levels in *Gmps* or *Usp7* mutants were also plotted relative to those in WT animals (Fig. 5B). In WT prepupae, *E74A* and *E75A* expression reflects the level of ecdysone signaling. Following a peak at pupariation, transcription ceases until, after ~10 h, another ecdysone pulse strongly activates *E74A* and *E75A* transcription. Compared to WT animals, $Usp7^{kim1}$ prepupae overexpress *E74A* and *E75A* at early and late time points. In $Gmps^{K07130}$ and $Usp7^{KG06814}$ mutants, there is a striking derepression from t = 4 to 8 h. In contrast to $Usp7^{kim1}$ mutants, which harbor a weaker allele than $Usp7^{KG06814}$ and are homozygous viable, the development of $Gmps^{K07130}$ and $Usp7^{KG06814}$ mutants arrests prior to pupation and *E74A* and *E75A* expression ceases after t = 10 h. In summary, GMPS and USP7 bind ecdysone target loci and are required for their normal regulation, most likely as transcriptional corepressors.

Binding dynamics at ecdysone target loci. We used indirect immunofluorescence of polytene chromosomes to monitor GMPS and USP7 binding to ecdysone target loci prior to or after hormone signaling (Fig. 6). An ecdysone pulse at the end of the third-instar larval stage causes activation of the *E74* and *E75* gene clusters, hallmarked by chromosome "puffing." EcR and USP7 both bind *E74* and *E75* prior to induction (Fig. 6A). However, following the ecdysone pulse, additional EcR is recruited but USP7 disappears, as expected of a corepressor (Fig. 6B). GMPS was present at the uninduced *E74* and *E75* loci (Fig. 6C) but, in contrast to USP7, persisted following activation (Fig. 6D). The dynamics of USP7 binding is independent of GMPS, because it is unaffected by RNAi-mediated depletion of GMPS (Fig. 6E and F). Our genetic analysis indicated that GMPS/USP7 acts antagonistically to BRE1 *in vivo* (Fig. 2). Therefore, we compared the binding dynamics of BRE1 with those of USP7 and RNA Pol II. In agreement with a low level of transcription prior to ecdysone induction, we detected both BRE1 and RNA Pol II at *E74* and *E75* (Fig. 6G and I). However, following activation, BRE1 and RNA Pol II levels were dramatically increased, whereas USP7 was removed (Fig. 6H and J). In conclusion, our analysis of binding to ecdysone target loci supports a role for GMPS/USP7 as a transcriptional corepressor. Moreover, in accordance with their genetic antagonism, and opposing biochemical activities, there is an exchange of the ubiquitin protease USP7 for the ubiquitin ligase BRE1 concomitant with RNA Pol II recruitment.

GMPS/USP7 acts as an EcR corepressor. To obtain additional evidence for the role of GMPS/USP7 as an EcR corepressor, we performed genetic interaction assays utilizing two distinct EcR mutants. EcR^{V559fs} is a loss-of-function mutation that has an activation defect (2). In contrast, EcR^{A483T} can still respond to hormone induction but its interaction with the corepressor SMRTER is abrogated, causing enhanced transcription in the absence of hormone (27). When EcR^{V559fs} was

combined with GMPS/USP7 ectopic expression in the eye, we observed a clear enhancement of the developmental defect (Fig. 7A to C). In striking contrast, EcR^{A483T} suppressed the GMPS/USP7 overexpression eye phenotype (Fig. 7D). By themselves, the EcR^{V559fs} and EcR^{A483T} mutations result in normal eyes (not shown). Thus, defective activation by EcR enhances the GMPS/USP7 ectopic expression phenotype, whereas loss of repression causes suppression. A plausible molecular explanation is that the failure of EcR^{A483T} to bind the SMRTER corepressor compensates for the overexpression of GMPS/USP7 by lowering the overall EcR-directed transcriptional repression. Alternatively, EcR^{A483T} might be defective for GMPS/USP7 recruitment, possibly through SMRTER. Suggestively, SMRTER was detected by mass spectrometry in our USP7 purifications from *Drosophila* embryos (J. A. van der Knaap, unpublished results). Whatever the detailed molecular mechanism, these genetic interactions provide further *in vivo* support for the notion that GMPS/USP7 acts as an EcR corepressor.

To test for direct binding, we expressed and purified GST-tagged GMPS or USP7, which was immobilized on glutathione-Sepharose beads and incubated with radiolabeled EcR. We found that EcR bound efficiently to USP7 but not to GMPS (Fig. 7E). Additional binding studies revealed that USP7 bound the EcR ligand binding domain (LBD) but not the DNA binding domain (DBD). We observed very weak binding of GMPS to the LBD, but considering the lack of binding to full-length EcR, this was most likely background. We conclude that GMPS/USP7 interacts not only genetically with the EcR but also physically, through binding of USP7 to the LBD.

Finally, we decided to test the effect of RNAi-mediated depletion of USP7 or GMPS on a selection of ecdysone-regulated genes in S2 tissue culture cells. Whereas USP7 was efficiently depleted when targeted by RNAi, only a modest reduction in GMPS levels could be achieved (Fig. 7F). Nevertheless, RT-qPCR revealed clear derepression of the ecdysone-regulated genes *ImpE2*, *ImpL3*, *E75C*, and *E74A* (1, 32) following USP7 or GMPS depletion. In contrast, reduced USP7 or GMPS levels did not affect the transcription of β*Ftz-F1*, *gapdh*, or *CG1381*. We conclude that GMPS/USP7 acts as a gene-selective transcriptional corepressor of ecdysone-inducible genes.

DISCUSSION

Our results demonstrated that a classic biosynthetic enzyme, GMPS, does double duty as a transcription factor involved in gene-specific repression by NRs. Genetic analysis revealed strong cooperation between GMPS and USP7, which was critically dependent on the deubiquitylating activity of USP7 but independent of GMP synthesis by GMPS. A series of observations established that GMPS/USP7 acts as a transcriptional corepressor of the EcR. (i) GMPS and USP7 mutants die at a developmental stage when ecdysone signaling is critical, and they display severe misregulation of endogenous ecdysone target genes. (ii) GMPS and USP7 bind ecdysone target genes when these are repressed, but USP7 dissociates upon activation and RNA Pol II recruitment. (iii) USP7 directly binds the EcR. (iv) GMPS/USP7 and the EcR interact genetically. EcR mutants defective for transcriptional activation enhance the GMPS/USP7 ectopic expression phenotype, whereas loss of EcR repression causes suppression. (v) Loss of USP7 or GMPS causes upregulation of ecdysone target genes, both *in vivo* and in S2 cells. We conclude that GMPS/USP7 acts as a corepressor during developmental gene control by hormone receptors. Combined with their role in Polycomb-mediated gene silencing (29), these observations revealed surprisingly broad functions in transcription control for the biosynthetic enzyme GMPS and the ubiquitin protease USP7.

Our genetic analysis supports antagonistic roles for GMPS/USP7 and the histone H2B ubiquitin ligase BRE1. These findings dovetail well with our *in vitro* analysis that showed that GMPS is required for H2B deubiquitylation by USP7 (29). Recently, addition of the repressive histone H2Aub mark has been implicated in silencing of mammalian NR targets. The H2A ubiquitin ligase 2A-HUB associates with the NR corepressor N-CoR and negatively regulates transcription (34). Conversely, the H2Aub ubiquitin protease 2A-DUB interacts with the transcriptional coactivator p/CAF and stimulates transcriptional activation by the androgen receptor (35). Thus, histone ubiquitylation/deubiquitylation seems to play a key role in gene control by NRs.

Finally, our finding that a key enzyme in the guanine nucleotide biosynthesis pathway is involved in NR target silencing suggests that GMPS might provide a relay between metabolic state and developmental gene switching. Consistent with this notion, GMPS expression is upregulated in proliferating and tumorigenic cells (5, 31). It will be of interest to take a closer look at other nucleotide biosynthetic enzymes and investigate whether more of them have a second function as transcription factors. They might help provide the critical coupling of cell growth, division, and differentiation that is required for development.

ACKNOWLEDGMENTS

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CHAPTER 4

A role of GMP Synthetase and Ubiquitin-Specific Protease 7 in axon guidance

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(*Work in progress*)

CHAPTER 5

General Discussion

Discussion

During the two past decades the main interest of molecular biologist studying regulatory events in the cell have been concentrated on mechanisms of gene transcription and chromatin structure. This field has advanced in understanding major regulatory processes occurring on chromatin and later through the lifetime of RNA and protein, and elaborated sophisticated approaches to study gene expression. At the same time, another, equally important aspect of cell biology, cellular metabolism, has been largely neglected with respect to its regulatory functions in the cell (McKnight, 2010). The intricate balance of cellular physiology, its metabolic status and oxidative state are essential for proper cellular fate commitment and function, however, how these metabolic signatures are established and maintained is largely unknown. If one side of this equation includes transcription factors, the regulators of genes encoding metabolic enzymes, and, logically the other should therefore include feedback mechanisms from these metabolic enzymes to gene activity. This might not only involve the regulation of metabolic genes themselves similar to the classic lac operon/lactose interactions (Mobbs et al., 2007), but also the regulation of many other genes.

Some important examples of such regulation are NAD^+/NADH dependent transcription factors including sirtuins, CtBP and NPAS/CLOCK (Fjeld et al., 2003; Rutter et al., 2001; Shi, 2004). All these proteins regulate different genes and processes but are driven by a common stimulus – the oxidative state of the cell manifested by NAD balance. Another example of NAD-dependent regulation of transcription shows that the GAPDH/LDH complex is involved in histone locus activation as part of the OCA-S co-activator complex (Dai et al., 2008; Zheng et al., 2003). Intriguingly, GAPDH and LDH are housekeeping metabolic enzymes serving the breakdown of glucose and energy metabolism. Earlier studies demonstrated the peculiar ability of these enzymes to bind single-stranded DNA-conjugated substrates, but these findings have not been developed further (Grosse et al., 1986; Morgenegg et al., 1986; Nicholls et al., 2011). Acetyl coenzyme A, which catalyses the transport of acetyl groups to the citric acid cycle, provides another striking case of a metabolic compound governing transcription. It is essential for histone acetylation in single-cell eukaryotes *in vivo* (Takahashi et al., 2006), but also activates RNA polymerase II-dependent transcription *in vitro* (Galasinski et al., 2000). Moreover, adenosine triphosphate (ATP)-citrate lyase is

required for histone acetylation in higher eukaryotes upon growth factor stimulation (Wellen et al., 2009), raising the possibility that the glucose levels can affect histone acetylation and gene transcription.

The importance of the metabolic state with respect to cell cycle, differentiation and cell type specificity has been clearly shown by Wang and colleagues. The authors demonstrated that embryonic stem cells have a unique metabolic profile and depend on threonine metabolism with copious expression of the threonine dehydrogenase gene (Wang et al., 2011; Wang et al., 2009). Moreover, this discovery allowed the authors to develop a threonine dehydrogenase inhibitor and arrest rapid cells division in embryonic stem cells, but not in other cell types (Alexander et al., 2011). These elegant studies suggest that the metabolic signatures of specific cell types might help to advance drug design to eventually target cancer cells.

Here we show that two nucleotide biosynthetic enzymes, IMPDH and GMPS, act as transcription regulators and affect major cellular and developmental pathways. This notion places the two enzymes in a position of potential feedback effectors between regulatory and metabolic sate of the cell. What are the possible relations between nucleotide metabolism and gene expression? What features of IMPDH and GMPS would allow them to mediate these relations? Attempting to address the first question, we disturbed nucleotide metabolism by treating cells with hydroxyurea and tested IMPDH's subcellular distribution, suspecting that nuclear shuttling might serve an excellent mechanism of IMPDH induction. Indeed, we found that IMPDH localizes to the nucleus upon hydroxyurea treatment, but not upon replication disruption, suggesting that IMPDH is a nucleotide-sensitive transcription factor (this thesis). Noteworthy, *histone* genes, the targets for IMPDH repression, are known to be down-regulated in hydroxyurea-treated fungal and mammalian cells (Carrino et al., 1987; Sittman et al., 1983), but not in cells arrested by other means. Taking into account that IMPDH is also a transcriptional repressor for cell cycle-promoting genes, it might therefore be responsible for hydroxyurea-mediated cell cycle arrest.

Interestingly, histones are regulated by three dehydrogenases, IMPDH, GAPDH and LDH; moreover, all these dehydrogenases bind single-stranded DNA, although GAPDH and LDH have not been shown to bind *histone* DNA directly. All dehydrogenases bind and utilize NAD^+ as a co-factor in its oxidized from, suggesting that the transcription of *histones* might be regulated by the NAD^+/NADH balance, and that IMPDH, GAPDH and LDH might serve as sensors of cellular oxidative state. Yeast cells

under dietary restrictions are know to adjust the cell cycle to their respiratory cycle: cell growth occurs during respiratory stage and consequently, oxidative phase, whereas DNA replication occurs during reductive phase, presumably, to minimize its exposure to reactive oxygen species generated during respiration (Tu et al., 2005; Tu and McKnight, 2006, 2007; Tu et al., 2007). Similar mechanisms might operate in multicellular organisms and IMPDH might be a good candidate to coordinate replication and oxidative processes as it binds nucleotides, NAD^+ and regulates transcription.

Next, we investigated the transcriptional function of another metabolic enzyme, GMPS that in complex with USP7 acts as transcriptional co-repressor for inducible genes. Importantly, USP7 and GMPS repress ecdysteroid-regulated genes that are essential for fly development (this thesis), because all stage transitions during the fly life cycle are regulated by ecdysone. Next, we show that the USP7/GMPS complex is also essential to a number of other developmental processes including nervous system development and axon guidance. GMPS catalyses GMP biosynthesis from the XMP precursor and utilizes L-glutamine and water as co-factors and ATP as an energy source. Therefore, GMPS might be capable of not only sensing nucleotide levels, but also the ratio of L-glutamine to L-glutamate. At the same time, ecdysone is a signalling effector that regulates expression of glutamate receptors (Downey et al., 2005). It would be interesting to test, whether GMPS is involved in transcriptional regulation of glutamate receptor genes and if glutamate levels might affect its transcriptional function. L-glutamate is a major neurotransmitter in the *Drosophila* visual system. At the same time, the mouse glutamate transporter gene *GLT-1* is under the control of steroid hormones (Dunlop et al., 1999) and steroid treatment of mouse brains induces expression of this gene. Given that GMPS regulates development of the *Drosophila* visual system, it might serve in a feedback loop from intracellular glutamate levels and expression of the glutamate transporter.

Thus, it would be of a great interest to study whether GMPS transcriptional regulation of steroid-inducible target genes is conserved from fly to human and if glutamate levels might alter this activity of GMPS. Another potent direction of future studies might involve elucidation of the exact mechanism of GMPS/USP7-mediated regulation of axon guidance including ChIP analysis of USP7 and GMPS binding and genetic analysis of potential target genes. Moreover, it would be important to elucidate whether the GMPS/USP7 complex might serve as a co-activator and understand the mechanisms of this alternative activity.

Investigation of the role of metabolic enzymes and intermediary metabolites in cell homeostasis and gene regulation does not only contribute to the general understanding of cell function but is also essential for applied medicine. For instance, IMPDH and GMPS inhibitors are actively used in cancer therapies due to their involvement in GMP biosynthesis. However, these treatments are yet unspecific because inhibition of GMP production affects all actively dividing cells. Now, given their role in gene regulation and, specifically, the dependence of IMPDH nuclear localization on the cellular state, it seems logical to investigate the specific metabolic state of cancer cells and the IMPDH repressor function under these conditions. Thus, by interfering with the cancer cell-specific metabolic state, it might be possible to trigger IMPDH-dependant repression of proliferation genes like E2F and to achieve tumour-specific cell cycle arrest. Recent studies of Wang and colleagues demonstrate that the metabolic state of the cell can indeed be used for specific inhibition of proliferation of stem cells (Wang et al., 2011; Wang et al., 2009). The involvement of metabolic enzymes and intracellular metabolites in gene regulation might be used in some cases as an alternative for gene therapy. If the physiological state of the cell regulates transcription then the addition of a metabolic drug might alter expression of some genes. This approach would have a significant advantage to gene therapies because the intracellular delivery of small compounds is generally more practical and effective. Understanding of the cross talk between metabolism and gene regulation as well as the tissue-specific metabolic signatures might serve as a basis for the development of specific drugs that might potentially allow a relatively simple way of targeted treatment of cancer cells.

In conclusion, we demonstrated that GMPS and IMPDH appear to be functionally more complex molecules than previously believed. We show that in addition to nucleotide biosynthesis they directly regulate gene activity, and IMPDH binds DNA as a canonical transcription factor. Therefore, we propose that they might represent a missing link bridging cellular metabolism and gene regulation. However, we have not yet explained the exact mechanisms that allow GMPS and IMPDH to integrate metabolic and regulatory states of the cell. Given that development of this problem is still in a rudimentary stage, we believe that our findings might provide a starting point for the future studies in this yet mysterious “quantum gravity” field.

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Summary

Nederlandse samenvatting

Summary

Metabolism has long been only seen as a source of energy and cellular building blocks and largely neglected with respect to gene regulation and development. However, the metabolic state should feed back to the regulatory machinery in order to adjust it to the cellular physiological needs. In this thesis we demonstrate that the GMP biosynthetic enzymes IMPDH and GMPS are capable of transcriptional regulation of multiple cellular and developmental processes. We also discuss how this regulation might bridge metabolic and regulatory states of the cell.

In Chapter 2 we show that *Drosophila* metabolic enzyme IMPDH is also a DNA-binding transcriptional repressor. IMPDH regulates the S phase-specific *histone* genes and *E2f*, a key driver of cell proliferation. The nuclear localization of IMPDH and, consequently, its transcriptional activity is controlled by the cell cycle. IMPDH only accumulates in the nucleus during G2, or following metabolic- or oxidative stress. Thus, IMPDH creates a feedback loop that couples expression of histones and E2F to cellular state. IMPDH has an unusual mode of DNA recognition. Genome-wide profiling and in vitro binding assays established that IMPDH binds sequence-specifically to single-stranded, CT-rich DNA elements. Through its dual functions of nucleotide biosynthetic enzyme and transcription factor, IMPDH both enables and restricts cell proliferation.

The Drosophila enzyme GMPS binds USP7 and is required for its ability to deubiquitinate histone H2B. The GMPS/USP7 complex cooperates with the Polycomb silencing system through removal of the active ubiquitin mark from histone H2B (H2Bub). In Chapter 3, we explored the interplay between GMPS and USP7 further and assessed their role in hormone-regulated gene expression. Genetic analysis established a strong cooperation between GMPS and USP7, which is counteracted by the histone H2B ubiquitin ligase BRE1. Loss of either GMPS or USP7 led to increased levels of histone H2Bub in mutant animals. These in vivo analyses complement our earlier biochemical results, establishing that GMPS/USP7 mediates histone H2B deubiquitination. We found that GMPS/USP7 binds ecdysone-regulated loci and that mutants display severe deregulation of ecdysone target genes. The ecdysone receptor interacts biochemically and genetically with GMPS/USP7. Genetic and gene expression analyses suggested that GMPS/USP7 acts as a transcriptional co-repressor. These results revealed the cooperation between a biosynthetic enzyme and a ubiquitin protease in developmental gene control by hormone receptors.

We attempted to find developmental processes that are regulated by GMPS and USP7. In Chapter 4 we show that USP7 and GMPS synergistically regulate axon guidance in the *Drosophila* visual system. Moreover, identification of USP7 and GMPS target genes using microarray analysis revealed that they affect expression of axon guidance genes. RT-qPCR analysis demonstrated down-regulation of transcription factors essential to axon pathfinding upon depletion of USP7 and GMPS in the *Drosophila* brain. Mass spectrometry of the USP7 and GMPS protein complexes revealed that they interact with numerous transcription factors and protein modifying enzymes. Thus, we uncovered a new developmental process regulated by USP7, demonstrated synergism between USP7 and GMPS, and identified their target genes. Finally, the protein interaction network presented in this study revealed possible transcription factors responsible for the axon guidance regulation by USP7 and GMPS. We believe that these results will contribute to understanding the role of ubiquitination in development. Moreover, it will help to elucidate the alternative functions of metabolic enzymes in a cell.

The mechanisms of reciprocal interactions between metabolic and regulatory states of the cell are yet poorly understood. Here we show that metabolic enzymes are directly involved in both processes and therefore might mediate a crosstalk between metabolism and transcription.

Nederlandse samenvatting

Metabolisme is heel lang alleen maar beschouwd als een bron van energie en bouwstenen voor de cel en haar rol in genregulatie en ontwikkeling is voor een groot deel genegeerd. De metabolische toestand moet echter terugkoppeling geven aan het regulatiesysteem, zodat er, afhankelijk van de cellulaire fysiologische behoeften, aanpassingen plaats kunnen vinden. In dit proefschrift tonen we aan, dat de enzymen IMPDH en GMPS, die betrokken zijn bij de *de novo* biosynthese van GMP, in staat zijn tot transcriptieregulatie van verscheidene cellulaire- en ontwikkelingsprocessen. We bediscussiëren ook hoe deze regulatie een brug slaat tussen de metabolische toestand en de reguleringstoestand van de cel.

In hoofdstuk 2 tonen we aan dat het metabolische enzym IMPDH van *Drosophila* een DNA-bindende transcriptionele repressor is. IMPDH reguleert zowel de S-fase specifieke *histon* genen, als *E2f*, wat een sleutelfactor van cellulaire proliferatie is. De nucleaire lokalisatie van IMPDH, en als gevolg daarvan haar transcriptionele activiteit, wordt gecontrolleerd door de celcyclus. IMPDH accumuleert zich alleen in de celkern gedurende G2, of als gevolg van metabolische of oxidatieve stress. Zo creëert IMPDH een terugkoppeling tussen de expressie van histonen en E2F, en de cellulaire toestand. IMPDH heeft een ongewone manier van DNA herkenning. Genoom-brede associatiestudies en *in vitro* bindingsproeven toonden aan dat IMPDH sequentiespecifiek bindt aan enkelstrengs, CT-rijke DNA elementen. Via haar dubbelrol als enzym in de nucleotide biosynthese en als transcriptiefactor maakt IMPDH zowel celproliferatie mogelijk en beperkt zij deze.

Het *Drosophila* enzym GMPS bindt aan USP7 en is nodig voor diens vermogen om histon H2B te deubiquitineren. Het GMPS/USP7 complex werkt samen met het Polycomb repressiesysteem door middel van het verwijderen van het actieve ubiquitine signaal van histon H2B. In hoofdstuk 3 hebben we de interactie tussen GMPS en USP7 verder onderzocht en hun rol in hormoon-gereguleerde genexpressie vastgesteld. Genetische analyse bewees een sterke samenwerking tussen GMPS en USP7, die wordt tegengegaan door de histon H2B ubiquitine ligase BRE1. Verlies van, of GMPS, of USP7 leidt to verhoogde niveaus van histon H2Bub in mutant dieren. Deze *in vivo* analyses complementeren onze eerdere biochemische resultaten, die vaststelden dat GMPS/USP7 histon H2B deubiquitinering tot stand brengt. Wij vonden dat GMPS/USP7

ecdyson-gereguleerde loci bindt en dat mutanten ernstige deregulatie van ecdyson-gereguleerde genen vertonen. De ecdyson receptor interacteert biochemisch en genetisch met GMPS/USP7. Genetische en genexpressie analyses suggereren dat GMPS/USP7 zich gedraagt als een transcriptionele corepressor. Deze resultaten onthullen de samenwerking tussen een biosynthetisch enzym en een ubiquitine protease in de ontwikkelingsspecifieke genregulatie door hormoonreceptoren.

We hebben geprobeerd om verdere ontwikkelingsprocessen te vinden, die gereguleerd worden door GMPS en USP7. In hoofdstuk 4 tonen we aan dat USP7 en GMPS synergistisch axon guidance in het visuele systeem van *Drosophila* reguleren. Bovendien wees de identificatie van genen, die gereguleerd worden door USP7 en GMPS, door middel van microarray analyse uit, dat beide de expressie van axon guidance genen beïnvloeden. RT-qPCR analyse toonde aan dat transcriptiefactoren, die essentieel zijn voor axon pathfinding, omlaag gereguleerd worden na depletie van USP7 en GMPS in het *Drosophila* brein. Massaspectrometrische analyses van USP7- en GMPS-eiwitcomplexen onthulde dat ze interacteren met talrijke transcriptiefactoren en eiwitmodificerende enzymen. Op deze wijze ontdekten we nieuwe ontwikkelingsprocessen, die gereguleerd worden door USP7, toonden we synergisme aan tussen USP7 en GMPS, en identificeerden we hun targetgenen. Tot slot onthulde het eiwitinteractie-netwerk in deze studie mogelijke transcriptiefactoren, die verantwoordelijk zijn voor axon guidance regulering door USP7 en GMPS. Wij zijn van mening dat deze resultaten zullen bijdragen aan het begrip voor de rol van ubiquitinering in ontwikkeling. Bovendien zal het helpen om de alternatieve functies van metabolische enzymen in de cel op te helderen.

De mechanismes van reciproke interacties tussen metabolische en regulerende toestanden van de cel worden nog steeds slecht begrepen. Hier tonen we aan dat metabolische enzymen direct betrokken zijn bij beide processen en dus een crosstalk to stand brengen tussen metabolisme en transcriptie.

List of Publications

1) Pindyurin A.V., Boldyreva L.V., Shloma V.V., Kolesnikova T.D., Pokholkova G.V., Andreyeva E.N., **Kozhevnikova E.N**., Ivanoschuk I.G., Zarutskaya E.A., Demakov S.A., Gorchakov A.A., Belyaeva E.S., Zhimulev I.F. Interaction between the Drosophila heterochromatin proteins SUUR and HP1. Journal of Cell Science, 2008 May 15; 121(Pt 10):1693-703.

2) van der Knaap J.A.[1], **Kozhevnikova E**.[1], Langenberg K., Moshkin Y.M., Verrijzer P. Biosynthetic enzyme GMP synthetase cooperates with ubiquitin-specific protease 7 in transcriptional regulation of ecdysteroid target genes. Mol Cell Biol. 2010 Feb; 30(3):736-44

[1] Equal contribution

3) Reddy B.A., Bajpe P.K., Bassett A., Moshkin Y.M., **Kozhevnikova E**., Bezstarosti K., Demmers J.A., Travers A.A., Verrijzer C.P. Drosophila transcription factor Tramtrack69 binds MEP1 to recruit the chromatin remodeler NuRD. Mol Cell Biol. 2010 Nov; 30(21):5234-44

4) **Elena N. Kozhevnikova**, Yuri M. Moshkin, Jan A. van der Knaap, Alexey V. Pindyurin**,** Zeliha Ozgur, Wilfred F.J. van Ijcken, and C. Peter Verrijzer. Metabolic enzyme IMPDH couples gene expression to cellular state by acting as a transcription factor. Under review with Mol Cell.

Curriculum Vitae

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