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Functional analysis of Cdk7-interacting proteins Mat1 and Hint in model organisms

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ABBREVIATIONS

aa	amino acids
$AMPNH_2/NH_2pA/APA$	adenosine-5'-monophosphoramidate
ßGP	ß-glycero-phosphate
BES	N,N-bis[2-Hydroxyethyl]-2-aminoethane-sulfonic acid
bp	base pairs
CAK	Cdk-activating kinase
Cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CKI	Cdk inhibitor
CTD	carboxy-terminal domain
C terminus	carboxy terminus
DTT	dithiothreitol
E	embryonic day
ECL	enhanced chemiluminescence
ER	estrogen receptor
ES cell	embryonic stem cell
GST	glutathione S-transferase
INK4	Inhibitors of Cdk4
kb	kilobase
kDa	kilodalton
MEF	mouse embryonic fibroblasts
mRNA	messenger ribonucleic acid
N terminus	amino terminus
NPAT	nuclear protein mapped to the AT locus
neo	neomycin
NER	nucleotide excision repair
P	post natal day
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PFA	paraformaldehyde
PMSF	phenylmethylsulfonyl fluoride
pol II	RNA polymerase II
RAR	retinoic acid receptor
Rb	retinoblastoma susceptibility protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMCC	SRB- and MED-containing cofactor complex
TFIIH	transcription factor IIH
TRAP	tyroid hormone receptor-associated protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications.

I Rossi, D.J., Londesborough, A.*, **Korsisaari, N.***, Pihlak, A., Lehtonen, E., Henkemeyer, M., Mäkelä, T.P.: Inability to enter S phase and defective RNA polymerase II CTD phosphorylation in mice lacking *Mat1*. *EMBO J* 20, 2844-2856, 2001.

II **Korsisaari, N.***, Rossi, D.J.*, Paetau, A., Charnay, P., Henkemeyer, M., Mäkelä, T.P.: Conditional ablation of the Mat1 subunit of TFIIH in Schwann cells provides evidence that Mat1 is not required for general transcription. *J Cell Sci* 115, 4275-4284, 2002.

III **Korsisaari, N.** and Mäkelä, T.P.: Interactions of Cdk7 and Kin28 with Hint/PKCI-1 and Hnt1 histidine triad proteins. *J Biol Chem* 275, 34837-40, 2000.

IV **Korsisaari, N.***, Rossi, D.J.*, Luukko, K., Huebner, K., Henkemeyer, M., Mäkelä, T.P.: The histidine triad protein Hint is not critically required for murine development or Cdk7 function. *Submitted*.

*) equal contribution

ABSTRACT

Cell division is a fundamental process of all uni- and multicellular organisms. The cell division cycle is intimately governed by a group of proteins called the cyclin-dependent kinases (Cdk) and their regulatory cyclin subunits. Originally purified as a biochemical activity, Cdk7 together with cyclin H was shown to phosphorylate and activate the major cell cycle Cdks *in vitro*. Soon thereafter Cdk7 and cyclin H, together with a third protein MAT1, were also identified to be part of a general transcription factor TFIIH. Consequently the physiological function of Cdk7-cyclin H-MAT1 became a subject of active research. We aimed at elucidating the functions of Cdk7 kinase by studying two proteins that interact with Cdk7: 1) MAT1, which had previously been found to interact with Cdk7, and 2) Hint, which we identified as a novel Cdk7-interacting protein.

In order to investigate the requirement for *Mat1* in murine development, we performed targeted mutagenesis in mice. We found that the *Mat1*-deficient mice remained viable only until the blastocyst stage of embryogenesis (day 3-4), and died before implantation concomitant with the depletion of maternal Mat1 protein. Interestingly, when *Mat1*-deficient blastocysts were taken into culture, they did not give rise to the mitotic inner cell mass cells, whereas the non-mitotic trophoblast cells remained viable. Further investigations of the *Mat1*-deficient trophoblast cells suggested that they were unable to enter endoreduplicative S-phase, a phenotype consistent with a defect in Cdk activation.

To investigate the role of Mat1 in adult lineages, we generated a conditional allele of *Mat1* which allowed us to study *Mat1* deficiency in the mitotic germ lineage cells and in the post-mitotic Schwann cells of adult mice. We noted that similar to the embryonic inner cell mass cells, the rapidly proliferating germ lineage was rendered non-viable upon *Mat1* ablation. While this result is again consistent with a defect in Cdk activation, failure in some aspect of transcription cannot be excluded. Interestingly, the *Mat1*-deficient post-mitotic myelinated Schwann cells remained viable for several weeks, and they were fully capable of attaining a mature myelinated phenotype. As the process of myelination and the maintenance of the myelin sheath require vast transcriptional activity, this data provides evidence that Mat1 is not essential for *general* transcription. The myelinated Schwann cells did however succumb to Mat1 loss at approximately 3 months of age resulting in a hypomyelinating phenotype. These observations suggested a role for Mat1 in the regulation of a subset of transcripts. Such a subset may include target genes activated by the nuclear hormone receptors (RARα, RARγ, and ERα) which are known to be regulated by TFIIH kinase activity. However, the involvement of Mat1 in nucleotide excision repair or in a yet unidentified function cannot be excluded.

Importantly our results suggest that Mat1 is not required for general RNA polymerase II-mediated transcription in mammalian cells. This is in stark contrast to the absolute necessity of the *Saccharomyces cerevisiae* Mat1 homologue Tfb3 for transcription and viability. Our results therefore provide important new information on the regulation of general transcription in mammals.

To further elucidate the functions of the Cdk7 kinase, we searched for novel Cdk7-interacting proteins. Through a yeast two-hybrid screening, we identified that Hint, a small homodimerizing nucleotide-binding protein that belongs to the HIT protein family, interacts physically with Cdk7. *In vitro* studies utilizing overexpressed human proteins in yeast cells revealed that the Cdk7-Hint interaction was independent of Cdk7 kinase activity and of cyclin H binding. Physiological relevance to the interaction was yielded by a demonstration of a genetic interaction between Cdk7 and Hint *S. cerevisiae* homologues *KIN28* and *HNT1*, respectively. A regulatory role for Hint was suggested by the preference of Hint-associated endogenous Cdk7 to phosphorylate RNA polymerase II CTD over Cdk2 *in vitro*.

In order to study the interaction between Cdk7 and Hint *in vivo* in a mammalian context, targeted disruption of *Hint* was performed in mice by homologous recombination in ES cells. In contrast to the paramount importance of the functions proposed for Cdk7, *Hint*-deficient mice were viable and lived a life span comparable to control mice. Moreover, no histological abnormalities were observed in the $Hint^{-/-}$ tissues where *Hint* transcripts were found to be abundantly expressed in wild-type mice. As the *in vivo* levels of Thr160-phosphorylated Cdk2 as well as total RNA polymerase II (pol II) and pol II phosphorylated on CTD were comparable in $Hint^{-/-}$ mouse embryonic fibroblasts to control cells, these data suggest that Cdk7 activity in mice may not be critically regulated by Hint.

REVIEW OF THE LITERATURE

1 The cell division cycle

The eukaryotic cell cycle is divided into four phases. During two of these phases, cells execute the two basic events in cell division: generation of a single and faithful copy of its genetic material (the synthesis or S phase) and partitioning of all cellular components between two identical daughter cells (mitosis or M phase). The two other phases of the cycle, G1 and G2, are important gap periods, during which cells carefully prepare themselves for the successful completion of the S and M phases. When cells cease dividing, they exit the cell cycle and enter a quiescent state known as G0.

Other, more unconventional types of cell cycles also exist in eukaryotic cells. For example, in certain types of specialized cells (Keighren and West, 1993) DNA synthesis occurs by a process known as endoreduplication, whereby successive rounds of G and S phases proceed without intervening mitosis leading to polyploid or polytene genomes (reviewed in Zybina and Zybina, 1996).

The current model of the cell cycle control holds that the transitions between the different cell cycle phases are regulated at checkpoints, governed by enzymes called cyclin-dependent kinases (Cdk) and their activator subunits, the cyclins.

1.1 Cyclin-dependent kinases (Cdks)

Progression through the cell cycle is driven by successive activities of Cdk-cyclin pairs (Figure 1). In mammalian cells nine catalytic subunits, Cdks (Cdk1-9), and at least 16 regulatory cyclins have been identified (reviewed in Roberts, 1999). Cdk4-cyclin D and Cdk6-cyclin D pairs govern the G1 phase. One of the key substrates of Cdk4-cyclin D is the retinoblastoma protein pRb (Ewen *et al*., 1993; Kato *et al*., 1993). Unphosphorylated, active pRb is bound to a transcription factor E2F, which is central in the promotion of cell proliferation. Upon pRb phosphorylation by Cdk4-cyclin D, E2F is freed to activate the transcription of its target genes (Chellappan *et al*., 1991), one of which is cyclin E. Cdk2-cyclin E activity propels the cell cycle progression through the G1-S border. During S phase, Cdk2 binds to another cyclin, cyclin A. The G2 and M phases involve the activities of Cdk1 (cdc2)-cyclin A, and later in mitosis the activity of Cdk1-cyclin B. Following mitosis and cytokinesis, a rapid degradation of cyclin B by ubiquitin targeted pathway signals the cell to exit from the cell cycle (Glotzer *et al*., 1991; Hershko *et al*., 1991).

The complete list of the substrates of the Cdk-cyclins in not known, and thus the downstream events of Cdk activation are less clear. In addition to pRb, phosphorylation of NPAT (nuclear protein mapped to the AT locus) by Cdk2-cyclin E has been shown to have a role in S phase entry (Zhao *et al*., 1998), and in activation of histone gene transcription (Ma *et al*., 2000; Zhao *et al*., 2000). Several studies have shown that histone H1 is phosphorylated significantly during the S and M phases of the cell cycle (reviewed in

Hohmann, 1983). In the starfish, Cdk1 was shown to be the M phase-specific histone H1 kinase (Arion *et al*., 1988; Labbe *et al*., 1989). Cdks have also been implicated in DNA replication. During G1/S transition, Cdks promote the conversion of the pre-initiation complex into an active replication form (reviewed in Dutta and Bell, 1997). The Cdks appear also to exert a negative effect on DNA replication by catalyzing a series of phosphorylation events that render replication factors unable to re-enter the pre-initiation state, thus importantly preventing re-replication of the genome (reviewed in Jallepalli and Kelly, 1997).

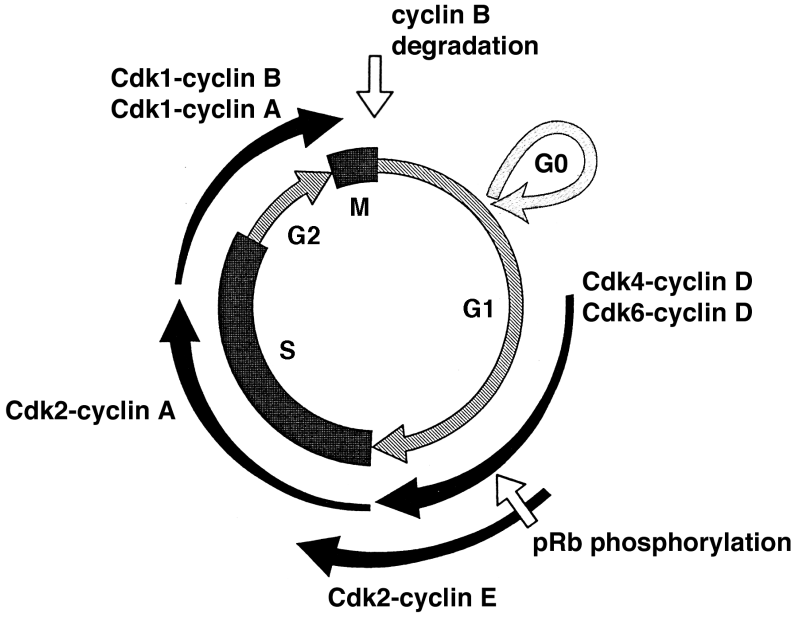

Figure 1. The mammalian cell division cycle.
The non-dividing phase (G0) and the division cycle phases G1, S, G2, and M with their governing Cdk-cyclin pairs. The point of pRb phosphorylation demarcating the decision to enter the S phase, and the point of cyclin D degradation demarcating the exit from mitosis, are marked with white arrows. Modified from (Sherr, 1993).

1.2 Regulation of Cdks

Cdks are amongst the most highly regulated enzymes known with their activities controlled through multiple mechanisms (reviewed in Morgan, 1995). The primary regulator of Cdk activity is the cyclin subunit. Cyclins were originally identified for their oscillating protein levels during the different phases of the cell cycle (Evans *et al*., 1983). Today they are recognized for their sequence homology and ability to bind and activate Cdks. A so-called cyclin box of approximately 100 amino acids harbors the homology and is also responsible for the Cdk binding and activation (Kobayashi *et al*., 1992; Lees and Harlow, 1993). Studies on Cdk2 and cyclin A (Jeffrey *et al*., 1995) have demonstrated that cyclin binding induces structural changes to the Cdk subunit, which lead to a repositioning of the ATP phosphates for the subsequent phospho-transfer reaction.

Negative regulation of Cdks during the G1 phase and the S phase entry is achieved by Cdk inhibitors (CKIs; Russo *et al*., 1996a). Two families of CKIs have been identified: the INK4 proteins (inhibitors of Cdk4), which include $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$, and the Cip/Kip family, which includes $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (reviewed in Sherr and Roberts, 1995). The INK4 family of inhibitors binds only to Cdk4 and Cdk6, whereas the Cip/Kip proteins inhibit cyclin E- and A-dependent kinases. A variation to the known activities of the Cip/Kip family CKIs came about when it was noted that their binding to cyclin D-dependent kinases *positively* regulates the Cdk-cyclin. This was demonstrated by the assistance of $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ in Cdk4-cyclin D assembly (LaBaer *et al*., 1997).

Phosphorylation of hydroxyl groups of serine, threonine or tyrosine residues is a widely used mechanism of post-translational regulation. Cdks can be negatively regulated by phosphorylation of residues within the ATP-binding pocket (Thr14 and Tyr15 in human Cdk2). While the mechanism of the inhibition is not fully known, Wee1, originally identified in *S. pombe* (Parker *et al*., 1992), has been described as the Tyr15 kinase. The identification of the Thr14 kinase has been more elusive; in *Xenopus* Myt1 has been identified as the responsible kinase (Mueller *et al*., 1995). Dephosphorylation of Thr14 and Tyr15 on Cdk2 and Cdk1 is performed by the Cdc25 family phosphatases Cdc25A, Cdc25B, and Cdc25C (reviewed in Nilsson and Hoffmann, 2000).

Similarly, a complete Cdk activation requires phosphorylation. This activating phosphorylation occurs at a conserved threonine residue (Thr160 in human Cdk2) on the so-called T-loop, which leads to a structural change facilitating the substrate binding (Russo *et al*., 1996b). The kinases that catalyze this phosphorylation are known as Cdk-activating kinases (CAKs).

2 Cdk-activating kinases

CAK was originally isolated as an activity in a cell extract capable of phosphorylating the conserved threonine residue within the T-loop of various Cdks (Desai *et al*., 1992; Solomon *et al*., 1992). The enzyme exercising CAK activity in metazoan species was surprisingly noticed to be structurally related to Cdks, and to have previously been isolated from *Xenopus* under the name $p40^{MO15}$ (Shuttleworth *et al*., 1990). Subsequently a novel cyclin was identified by biochemical purification methods and found to be associated with $p40^{MO15}$ by yeast two-hybrid method (Fisher and Morgan, 1994; Mäkelä *et al*., 1994). The cyclin was designated cyclin H and $p40^{MO15}$ was renamed CDK7. A third subunit, MAT1 (for ménage à trois), a 36 kDa RING finger protein has also been identified to be associated with the CDK7-cyclin H complex. MAT1 was originally identified as an assembly factor (Devault *et al*., 1995; Tassan *et al*., 1995) promoting a stable interaction between CDK7 and cyclin H and increasing the activity of CDK7-cyclin H complex (Fisher *et al*., 1995).

2.1 Ménage à trois (MAT1)

In contrast to all the other cyclin-dependent kinases, the CDK7-cyclin H dimer is associated with a third protein. Ménage à trois, MAT1, was first co-purified with cdk7 and cyclin H from starfish oocytes, where it was reported to function as a stabilizer of the dimeric complex (Devault *et al*., 1995). *In vitro*, MAT1 was subsequently shown to be required for the formation of an active human CDK7-cyclin H complex (Tassan *et al*., 1995), and its binding was suggested to be an alternative means to T-loop phosphorylation, to achieve an active CAK complex (Fisher *et al*., 1995). Interestingly, MAT1 has also been implicated in the regulation of substrate specificity of CDK7-cyclin H, as the MAT1-bound kinase was shown to prefer a RNA polymerase II C-terminal domain (CTD) substrate over a Cdk2 substrate (Inamoto *et al*., 1997; Rossignol *et al*., 1997; Yankulov and Bentley, 1997). Moreover, MAT1 was shown to be required for the efficient phosphorylation of p53 (Ko *et al*., 1997) and Oct-1 (Inamoto *et al*., 1997) by CDK7-cyclin H complex.

MAT1 is a 36 kDa RING finger protein. The N-terminal RING finger domain has been shown to be dispensable for the formation of the trimeric complex and for CDK7 activation (Tassan *et al*., 1995). RING finger proteins have emerged as a new and apparently widespread class of ubiquitin ligases (reviewed in Jackson *et al*., 2000). Whether MAT1 might function in ubiquitin-mediated degradation, is however unclear. Studies on deletion mutants of MAT1 have suggested that the hydrophobic C terminus of MAT1 is critically involved in binding cdk7-cyclin H and in activation of the kinase, whereas the RING finger domain appeared to be involved in transcription activation and CTD phosphorylation (Busso *et al*., 2000). The central coiled-coil motif was primarily suggested to mediate cdk7-cyclin H-MAT1 binding with the core TFIIH through interactions with XPD and XPB (Rossignol *et al*., 1997; Busso *et al*., 2000; Sandrock and Egly, 2001). A schematic of MAT1 and the proposed roles of the different domains are shown in Figure 2.

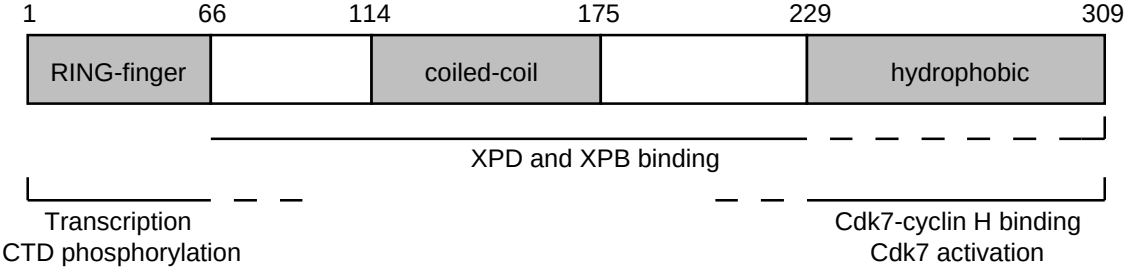

Figure 2. Schematic of MAT1.
The RING finger, coiled-coil, and C-terminal hydrophobic domains and their corresponding amino acid positions are shown. The proposed functional roles for the distinct domains are indicated. Modified from (Busso *et al*., 2000).

MAT1 homologues have been isolated from both *S. cerevisiae* and *S. pombe* (Pmh1; GenBank accession number AF191500). The budding yeast MAT1 homologue Tfb3/Rig2, associated with a homologous kinase-cyclin pair Kin28-Ccl1, has been shown to be essential for transcription in experiments utilizing a temperature sensitive allele of Tfb3

(Faye *et al*., 1997). It was shown that upon shifting to the restrictive temperature, transcription of the genes that were assayed was completely shut down within 30 minutes. This experimental setting thus recognized the *S. cerevisiae* Tfb3 critical for RNA polymerase II-mediated transcription.

2.2 The Cdk-activating kinase – from mammals to yeast

In vitro, the mammalian Cdk7-cyclin H-MAT1 trimer has been shown to phosphorylate and activate Cdk1, Cdk2, Cdk3, Cdk4, and Cdk6 (reviewed in Kaldis, 1999), thus identifying Cdk7-cyclin H-MAT1 as a potent candidate for a metazoan CAK. In support of this, in the fission yeast *Schizosaccharomyces pombe*, the Cdk7 orthologue Mcs6 kinase was found to possess CAK activity *in vivo* (Hermand *et al*., 1998; Hermand *et al*., 2001). Perhaps surprisingly, in *Saccharomyces cerevisiae*, the CAK activity was identified to be afforded by a monomeric protein Cak1/Civ1 *in vivo* (Espinoza *et al*., 1996; Kaldis *et al*., 1996; Thuret *et al*., 1996). To date, the best evidence for Cdk7-cyclin H-MAT1 functioning as a CAK in metazoan species comes from experiments with *Drosophila* Cdk7 orthologue, which demonstrated that DmCdk7 kinase activity was required for the proper activation of the mitotic Cdk-cyclins *in vivo* (Larochelle *et al*., 1998). Moreover, in *Xenopus* egg extracts, cdk7 was shown to be the physiological CAK (Fesquet *et al*., 1997).

3 Cdk activation meets basal transcription

RNA polymerase II (pol II) is responsible for mRNA synthesis in eukaryotes. In addition to the multi-subunit pol II, transcription initiation requires a number of additional proteins. The current model for activation of transcription involves the assembly of the preinitiation complex at the core elements of the promoter (TATA box and initiator). In addition to pol II, the preinitiation complex includes the general transcription factors (reviewed in Orphanides *et al*., 1996). General (or basal) transcription factors include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and TFIIJ. These factors were originally identified as proteins sufficient for promoter recognition and low levels of transcription from core promoter elements *in vitro*. The conventional model for ordered transcription initiation is characterized by a distinct series of events: 1) recognition of core promoter elements by TFIID, 2) recognition of the TFIID-promoter complex by TFIIB, 3) recruitment of a TFIIF-pol II complex, 4) binding of TFIIE and TFIIH to complete the preinitiation complex, 5) promoter melting and formation of an open initiation complex, 6) synthesis of the first phosphodiester bond of the nascent mRNA transcript, 7) release of pol II contacts with the promoter (promoter clearance), and elongation of the RNA transcript.

The preinitiation complex formation also involves pol II interaction with proteins of the Mediator complex (TRAP/SMCC in humans) to receive signals from the transcription activators, which are bound to the upstream activator sequences. Mediator complex (reviewed in Malik and Roeder, 2000) was originally identified as an interface between gene-specific regulatory proteins and the general transcription apparatus, and its existence

was revealed already more than a decade ago by functional studies in a yeast pol II transcription system (Kelleher *et al*., 1990). Figure 3 depicts the schematized relations of the proteins involved in activation of transcription.

An entirely new perspective on Cdk7 function was opened when Cdk7 along with cyclin H and MAT1 were identified to comprise the kinase subunit of the transcription factor IIH (TFIIH; Roy *et al*., 1994; Serizawa *et al*., 1995; Shiekhattar *et al*., 1995). The Cdk7 homologous kinase in *S. cerevisiae*, Kin28 (Simon *et al*., 1986), along with a cyclin H homologue Ccl1 (Valay *et al*., 1993) and a MAT1 homologue Tfb3/Rig2 (Faye *et al*., 1997; Feaver *et al*., 1997) were also identified within TFIIH (Feaver *et al*., 1994; Feaver *et al*., 1997). Subsequently, it became an intriguing question whether the *in vivo* functions of the mammalian Cdk7-cyclin H-MAT1 complex lie in cell cycle progression, basal transcription, or both, particularly since Kin28-Ccl1-Tfb3 was found not to possess CAK activity (Cismowski *et al*., 1995).

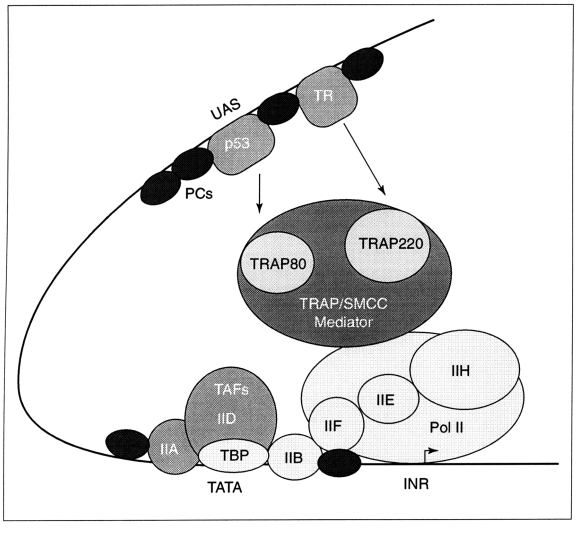

Figure 3. Activation of transcription through multiple protein-protein interactions.
A model including RNA polymerase II (pol II) and the general transcription factors (IIA, IIB, IID, IIF, IIE, and IIH) in addition to the Mediator complex and the gene specific activators upstream. INR, initiator; PCs, positive cofactors; SMCC, SRB- and MED-containing cofactor complex; TAFs, TBP-associated factors; TBP, TATA-box-binding protein; TR, thyroid-hormone receptor; TRAP, thyroid-hormone-receptor-associated-protein; UAS, upstream activator sequence. From (Malik and Roeder, 2000).

3.1 Transcription factor IIH (TFIIH)

TFIIH has been shown to be crucial in transcription initiation and in promoter escape (reviewed in Dvir *et al*., 2001). Biochemical purification coupled with recombinant reconstitution experiments have indicated that the nine-subunit TFIIH can be purified in several subcomplexes; a core TFIIH complex comprised of five subunits [XPB (xeroderma pigmentosum complementation group B), p62, p52, p44, and p34], and the Cdk7-cyclin H-MAT1 kinase complex. The remaining XPD (xeroderma pigmentosum complementation

group D) subunit can be found associated with either the core TFIIH or with the kinase subunit, and appears to mediate the interactions between the kinase subunit and the core TFIIH (Busso *et al*., 2000; Sandrock and Egly, 2001). Structural studies have revealed that TFIIH is composed of a ring-shaped structure, which harbors many of the core proteins, and a bulge, where the immunoreactivity of Cdk7 and cyclin H has been detected (Chang and Kornberg, 2000; Schultz *et al*., 2000).

Of all the basal transcription factors, the nine-subunit TFIIH is among the few to possess enzymatic activities. In addition to the kinase activity afforded by the Cdk7-cyclin H-MAT1 subunit, ATPase/helicase activities are provided by both the XPB and the XPD subunits. XPB activity is essential for promoter opening and transcription initiation, whereas XPD activity is dispensable, however enhancing transcription (Coin *et al*., 1999; Tirode *et al*., 1999).

The XPB and XPD subunits also function critically in nucleotide excision repair (NER). Mutations in XPB or XPD can lead to the human DNA repair deficiency syndromes Xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy (reviewed in Egly, 2001; Lehmann, 2001). The role of the TFIIH *kinase* activity in NER is still unclear. Some studies have suggested that the kinase activity would negatively regulate NER (Araujo *et al*., 2000), while other studies have suggested that the TFIIH kinase activity plays no role in DNA repair (Svejstrup *et al*., 1995).

3.2 Substrates of the TFIIH kinase

The most widely studied substrate of the TFIIH kinase Cdk7-cyclin H-MAT1 is the C-terminal domain (CTD) of the large subunit of RNA polymerase II. Numerous studies have indicated the importance of CTD in transcription initiation, elongation, and in mRNA processing (reviewed in Oelgeschlager, 2002). CTD is composed of numerous repeats of a conserved heptapeptide sequence YSPTSPS. Cdk7 has been mostly implicated in phosphorylation of the serine in position 5 (Gebara *et al*., 1997; Hengartner *et al*., 1998; Sun *et al*., 1998; Trigon *et al*., 1998), although some evidence also exists for phosphorylation of the serine at position 2 (Watanabe *et al*., 2000). The role of CTD phosphorylation in transcription has been of a great interest over the past years. Studies using highly purified reconstituted transcription systems have given variable results. In one experimental setting, transcription required CTD phosphorylation by Cdk7 (Akoulitchev *et al*., 1995), yet in another system, transcription occured in the absence of the TFIIH kinase (and an active Cdk7; Mäkelä *et al*., 1995; Rossignol *et al*., 1997). Nonetheless, the presence of the TFIIH kinase strongly enhances transcriptional activity (Rossignol *et al*., 1997; Tirode *et al*., 1999). The fact that CTD phosphorylation by the TFIIH kinase does not require promoter opening (Tirode *et al*., 1999) suggests that its significance lies somewhere else than in transcription initiation.

The emerging consensus on the importance of CTD phosphorylation is in the enhancement of the processing of nascent transcripts (capping, splicing and polyadenylation of pre-

mRNA). Indeed, the recruitment of the capping enzyme guanylyltransferase appears to be facilitated by CTD phosphorylation (Cho *et al*., 1997; McCracken *et al*., 1997a; Yue *et al*., 1997). Furthermore, truncation of pol II CTD leads to inefficient splicing, and polyadenylation of mRNAs in addition to ineffective capping (McCracken *et al*., 1997b). Moreover, a hyperphosphorylated pol II CTD strongly activates splicing, whereas hypophosphorylated CTD can inhibit the reaction (Hirose *et al*., 1999). Interestingly, in *S. cerevisiae*, the CTD kinase responsible for the targeting of the capping enzyme is Kin28, in contrast to either of the orthologous CTD kinases Srb10-Srb11 (Cdk8-cyclin C) or CTDK-I (Cdk9-cyclin T) (Rodriguez *et al*., 2000).

In addition to being a pol II CTD kinase, the TFIIH kinase has also been reported to phosphorylate the basal transcription factors TFIID (Ohkuma and Roeder, 1994), TFIIE (Ohkuma and Roeder, 1994; Rossignol *et al*., 1997; Yankulov and Bentley, 1997), and TFIIF (Ohkuma and Roeder, 1994; Yankulov and Bentley, 1997). TFIIH kinase also phosphorylates p53 (Ko *et al*., 1997; Lu *et al*., 1997) and the octamer transcription factor Oct-1 (Inamoto *et al*., 1997). Cdk7 has also been implicated in binding of the HIV-1 transcriptional transactivator Tat (Cujec *et al*., 1997). Interestingly, mounting evidence suggests that the physiological function of the TFIIH kinase may lay in the regulation of the transcription factors of activated transcription, as Cdk7 has been shown to phosphorylate and activate retinoic acid receptor (RAR) α (Rochette-Egly *et al*., 1997; Keriel *et al*., 2002), RARγ (Bastien *et al*., 2000), and estrogen receptor α (ERα; Chen *et al*., 2000).

How these proposed functions of Cdk7 might be mediated, is still unclear. However, it would be reasonable to expect, that specific subcomplexes containing Cdk7 may mediate different functions. This notion is supported by the observation that the trimeric Cdk7-cyclin H-MAT1 functions efficiently as a CAK *in vitro*, whereas the CTD phosphorylation is mediated by Cdk7 within the basal transcription factor TFIIH (Rossignol *et al*., 1997; Yankulov and Bentley, 1997). The experimental evidence available at the time that Studies I-IV were initiated pertaining to the involvement of Cdk7, cyclin H, and MAT1 and their yeast homologues in Cdk activation, RNA pol II-mediated transcription, and NER is schematized in Figure 4.

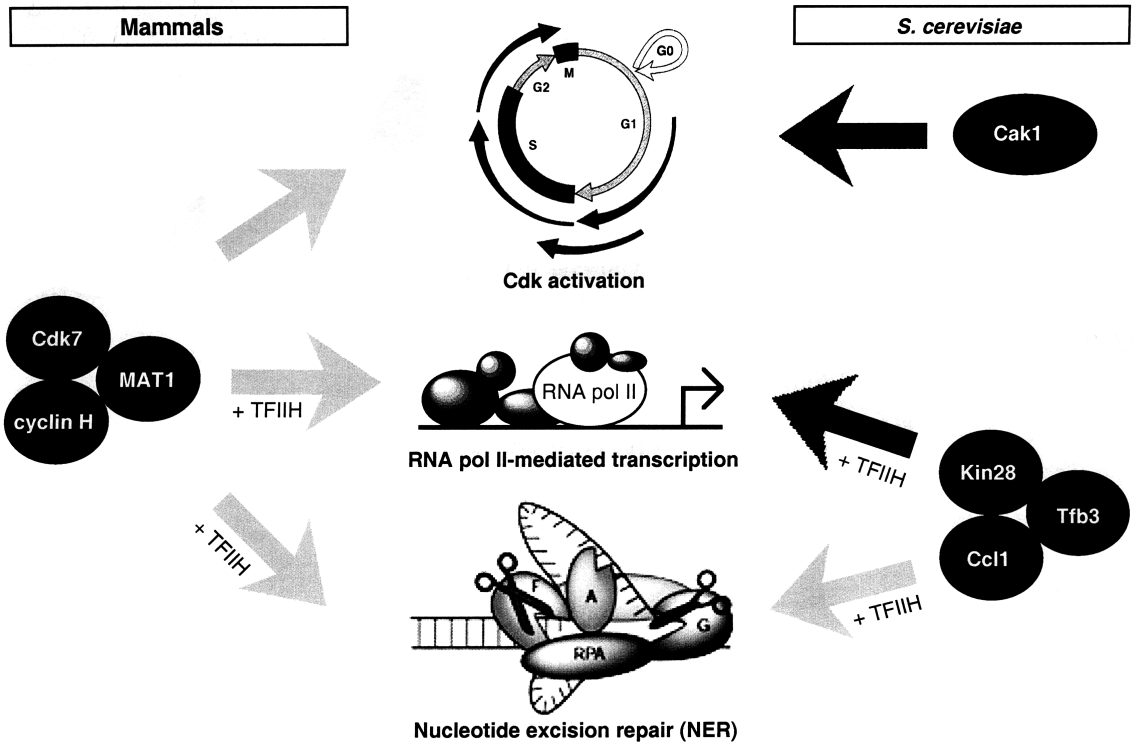

Figure 4. A comparison of Cdk activation, RNA pol II-mediated transcription, and NER functions in mammals and in *S. cerevisiae.*
Black arrows indicate a demonstrated *in vivo* involvement, while gray arrows indicate that the functional involvement of the trimeric complex (Cdk7-cyclin H-MAT1 or Kin28-Ccl1-Tfb3) *in vivo* is unclear [whereas the involvement of the core TFIIH (+ TFIIH) is unequivocal]. The image used for illustrating NER is from (Hoeijmakers, 2001).

4 Histidine triad (HIT) gene family

The HIT protein family was first introduced in 1992 (Seraphin, 1992), when Bertrand Séraphin noticed that the protein coded by a *Saccharomyces cerevisiae* open reading frame (Frohlich *et al*., 1991) upstream of CDC48 gene, resembled a previously purified dimeric protein from the bovine brain (Pearson *et al*., 1990). As significant sequence similarity was also shared with proteins published in GenBank from the cyanobacterium, nitrogen fixing bacterium, and mycoplasma, Séraphin named the group of proteins “the HIT protein family” for a characteristic histidine triad motif near their C terminus (HXHXHXX where X is a hydrophobic amino acid). Later, orthologous HIT proteins were reported form maize (*Zea mays*) and rice (*Oryza sativa*), which extended the HIT protein family to plants (reviewed in Robinson and Aitken, 1994). To date, homologous HIT proteins named after the mammalian protein Hint have been found from a wide diversity of organisms, indicating high conservation throughout evolution. The thus far identified HIT proteins from humans and budding yeast are listed in Table 1 and will be reviewed below.

Table 1. Human and *S. cerevisiae* HIT genes

H. sapiens			*S. cerevisiae*		
gene	aa *	map	gene	aa *	map
HINT	125	5q31.2	*HNT1*	158	YDL125C
HINT2	163	9p11.2	–	–	–
HINT3	182	6q22.33	–	–	–
APTX	168/342	9p13.3	*HNT3*	217?	YOR258W
FHIT	147	3p14.2	*HNT2/APH1*	217	YDR305C
GALT	379	9p13	*GAL7*	366	YBR018C

* number of amino acids

Modified from (Brenner, 2002)

4.1 The origin of Hint

In 1985, Hint was purified from the bovine brain as a Ca^{2+}-binding protein and a potent inhibitor of protein kinase C (PKC) with a mass of 17 kDa (McDonald and Walsh, 1985). In a later study, the amino acid sequence was determined and the protein was named PKCI-1 for protein kinase C inhibitor 1 (Pearson *et al*., 1990). However, subsequent work by the same group (Fraser and Walsh, 1991) coupled with an inability of other groups to reproduce PKC inhibition by PKCI-1 (Mozier *et al*., 1991; Robinson *et al*., 1995; Gilmour *et al*., 1997) cast some doubt on the physiological relevance of the inhibitory effect of PKCI-1 on PKC. Since that time, PKCI-1 has been renamed *HINT* for histidine triad nucleotide-binding protein (Brenner *et al*., 1997). Human *HINT* encodes a 125 amino acid protein, which has a 14 kDa predicted molecular mass.

4.2 FHIT

During the emergence of the Hint branch of the HIT proteins, diadenosine tri- and tetraphosphate hydrolase activities were isolated from budding (Brevet *et al*., 1991) and fission yeast (Robinson *et al*., 1993), followed by the identification of these proteins to represent a related branch of HIT proteins (Huang *et al*., 1995; Chen *et al*., 1998). It was not however until the cloning of the human orthologue spanning the chromosomal fragile site at *FRA3B*, that this second HIT protein branch was named *FHIT* for fragile histidine triad (Ohta *et al*., 1996). FHIT is perhaps the most widely known of the HIT proteins. It has repeatedly been implicated in tumor suppression in humans (reviewed in Huebner and Croce, 2001), and more recently also in mice (Fong *et al*., 2000; Zanesi *et al*., 2001).

4.3 GalT

The publication of the three-dimensional structures (discussed in detail below) of the human and rabbit Hint proteins (Lima *et al*., 1996; Brenner *et al*., 1997) led to an identification of a structural similarity with galactose-1-phosphate uridylyltransferase, GalT (Wedekind *et al*., 1995). GalT functions as a UDP transferase between galactose- and glucose-1-phosphate, and its deficiency in humans causes galactosemia (Tedesco *et al*., 1975). Despite the low amino acid sequence identity besides an imperfect histidine motif (HXHXQ), it was indeed noted that the structure of the dimeric Hint shared an extensive similarity with the structure of the monomeric core of GalT, therefore identifying GalT as a distant member of the HIT superfamily of proteins (Brenner *et al*., 1997; Holm and Sander, 1997).

4.4 Novel members of the HIT family: aprataxin, Hint2, and Hint3

A novel member of the HIT protein superfamily was identified when two independent groups simultaneously described a protein named aprataxin (Date *et al*., 2001; Moreira *et al*., 2001), a frequent cause of ataxia-ocular apraxia (AOA) in humans. Aprataxin exists in two splice variants; a long 342-amino acid form, and a short 168-amino acid protein. The long form contains an additional N-terminal domain which shares distant homology with polynucleotide kinase 3'-phosphatase (PNKP) (Moreira *et al*., 2001). The 168-amino acid short splice form of aprataxin containing the HIT motif and a DNA-binding C2H2 zinc-finger motif, corresponds to a homologous sequence in *S. cerevisiae* named *HNT3* (Brenner, 2002). The physiological function of aprataxin, or how it causes AOA is unknown. The human gene encoding aprataxin was named *APTX*.

Recently two genes closely related to *HINT* have been identified and named *HINT2* (GenBank accession no AF356515) and *HINT3* (GenBank accession no NM_138571) in humans. Human *HINT2* encodes a 163-amino acid, 17 kDa protein that has also been identified from *Mus musculus* and *Bos taurus*. Human *HINT3* encodes a 182-amino acid protein. Neither Hint2, nor Hint3 has orthologues in the budding yeast.

Of the HIT proteins, the greatest amount of amino acid sequence identity is shared between Hint and Hint2 (60% over a 123-amino acid sequence alignment), while for example human Hint and Fhit only share a 19% sequence identity (over a 111-amino acid sequence alignment). An amino acid sequence alignment and a phylogenetic tree of the human HIT proteins Hint, Hint2, Hint3, aprataxin, and Fhit with *S. cerevisiae* HIT orthologues Hnt1, Hnt2, and Hnt3 are shown in Figure 5 (reviewed in Brenner, 2002).

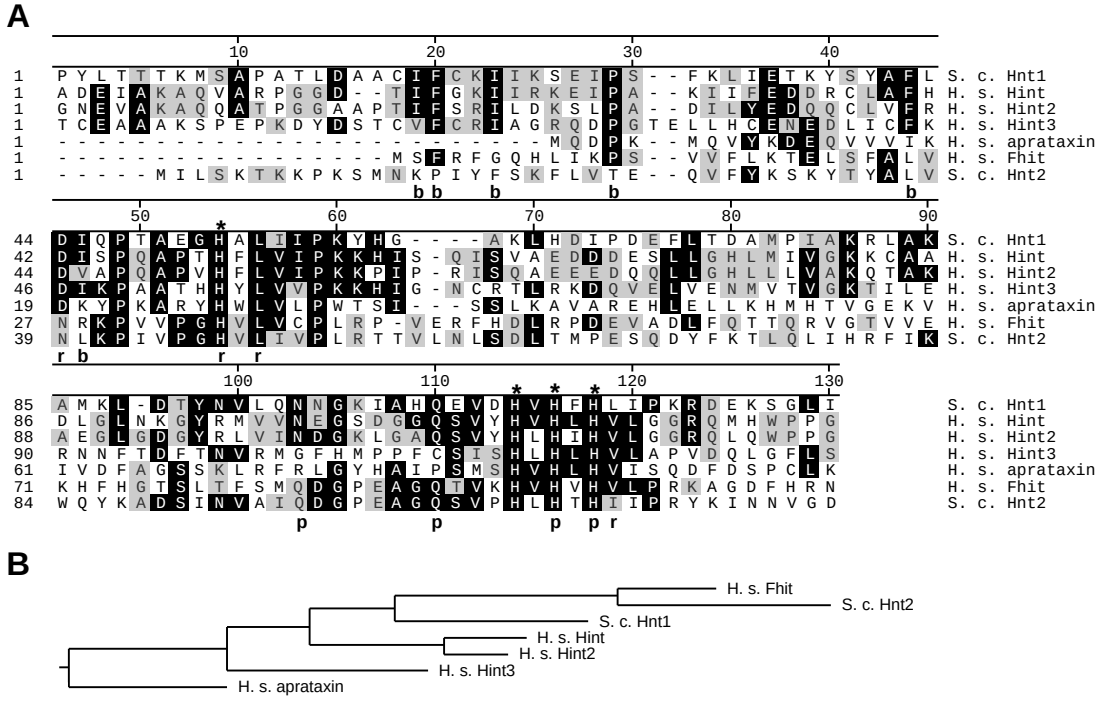

Figure 5. HIT family proteins.
(A) Amino acid sequence alignment of 110 amino acids of sequence alignment of *Saccharomyces cerevisiae* (*S. c.*) Hnt1 (amino acids 8-130 of the full length 158 aa-cDNA), *Homo sapiens* (*H. s.*) Hint (1-125/125), *H. s.* Hint2 (37-163/163), *H. s.* Hint3 (31-159/182), *H. s.* aprataxin short splice form (1-100/168), *H. s.* Fhit (1-110/147), and *S. c.* Hnt2 (1-123/217). Residues identical to the majority are boxed with black and residues with similarity to the majority are boxed in gray. The four conserved histidines (His51, His110, His112, and His114 in human Hint) are marked with an asterisk above the alignment. See the text below for more information on the base-binding residues (b), the ribose-binding residues (r), and the 5'-phosphate-binding residues (p) identified for rabbit Hint (Brenner *et al*., 1997). (B) Phylogenetic tree of the alignment in (A).

4.5 HIT proteins – from structure to function

Structural studies have had a central role in analyzing Hint and Fhit. X-ray crystallography of the three dimensional structure of the human Hint showed that Hint is a homodimer of two 13.7 kDa polypeptides, each consisting of two alpha-helical regions and five beta-strands (Lima *et al*., 1996). The two polypeptides come together to form extensive contacts between a helix and the C terminus, whereby analogous amino acids of both protomers meet. Crystal structure of the rabbit Hint (Brenner *et al*., 1997) revealed striking similarity to the structure of human Hint (Lima *et al*., 1996), suggesting a stable fold for the Hint branch of HIT proteins in mammals, and perhaps throughout nature.

Shortly after the cloning of the human *FHIT*, and parallel to the first structural studies of Hint, it was established that the human Fhit protein functions as a diadenosine 5',5'''-P^1,P^3-triphosphate (Ap_3A) hydrolase with a K_m value of 1.3 µM, while related substrates were shown to be hydrolyzed with lesser efficiency (Barnes *et al*., 1996). It was further demonstrated that the four histidines conserved in most HIT proteins; the N-terminal His35

and the C-terminal triad His94, His96, and His98 in human Fhit were all required for full activity, while His96 was absolutely essential (Barnes *et al*., 1996). This suggested that the non-FHIT-type HIT proteins might also interact with nucleotide phosphate compounds, and that the central histidine (His112 in human Hint) might be critical to function. Purification of Ap_3A hydrolase activity from *S. cerevisiae* (Brevet *et al*., 1991) led to the identification of the budding yeast Fhit orthologue Hnt2 (Aph1) as the *in vivo* Ap_3A hydrolase (Chen *et al*., 1998), while the fission yeast Fhit orthologue aph1 was found to be an *in vivo* diadenosine 5',5"'-P^1,P^4-tetraphosphate (Ap_4A) asymmetrical hydrolase (Robinson *et al*., 1993; Huang *et al*., 1995; Ingram and Barnes, 2000). Surprisingly, the Ap_3A hydrolase activity of FHIT does not correlate with its tumor suppressor function (Siprashvili *et al*., 1997; Werner *et al*., 2000).

Hint has also been found to possess nucleotide phosphate-binding activity (Gilmour *et al*., 1997), for which reason the rabbit Hint was crystallized in complex with several different small nucleotide phosphate compounds (Brenner *et al*., 1997). Subsequently the amino acids contributing to the binding of the substrates were identified. It was reported, that the binding pocket for the base is composed of four isoleucines (Ile18, Ile22, Ile27, and Ile44) and two phenylalanines (Phe19 and Phe41). The ribose is recognized by a mixture of polar and non-polar amino acid side chains (Asp43, His51, Leu53, and Val108), whereas the 5'-phosphate interacts with the side chains of Asn99, Gln106, His112, and His114 of the rabbit Hint (Brenner *et al*., 1997). The high conservation of these 14 residues (Figure 5) within the Hint and the Fhit branches of the HIT superfamily provides further evidence for also Hint to function in nucleotide binding.

The three dimensional structure of FHIT (Lima *et al*., 1997a) shows that the tertiary structures of the core domains of the human Hint (Lima *et al*., 1996) and FHIT are similar (Figure 6). *FHIT* encodes two alpha-helices and seven beta-strands. Most notably FHIT diverges from Hint on its C-terminal extension. A parallel investigation of the enzymatic functions of Hint and FHIT performed *in vitro* (Lima *et al*., 1997b), showed that Hint hydrolyzes adenosine 5'-diphosphate (ADP) into adenosine monophosphate (AMP) and inorganic phosphate (P_i) with a K_m value of 800 µM. (In this experimental setting Fhit hydrolyzed Ap_3A at a K_m value of 65 µM.) A more recent study by Bieganowski *et al*. (2002) identified adenosine-5'-monophosphoramidate ($AMPNH_2$, NH_2pA, or APA) as the preferred *in vitro* substrate of the rabbit Hint and its *S. cerevisiae* orthologue Hnt1. The central histidine (His116 in Hnt1) was indeed noted to be essential for the enzymatic activity. Although the *in vitro* kinetics of the hydrolysis of $AMPNH_2$ by the rabbit Hint were impressive (K_m = 0.068 µM), it remains to be seen whether $AMPNH_2$ is the *in vivo* substrate of Hint across kingdoms. However, as Bieganowski *et al*. (2002) conclude, the substrate of Hint is likely to consist of an AMP molecule with a perhaps yet unidentified protonated leaving group.

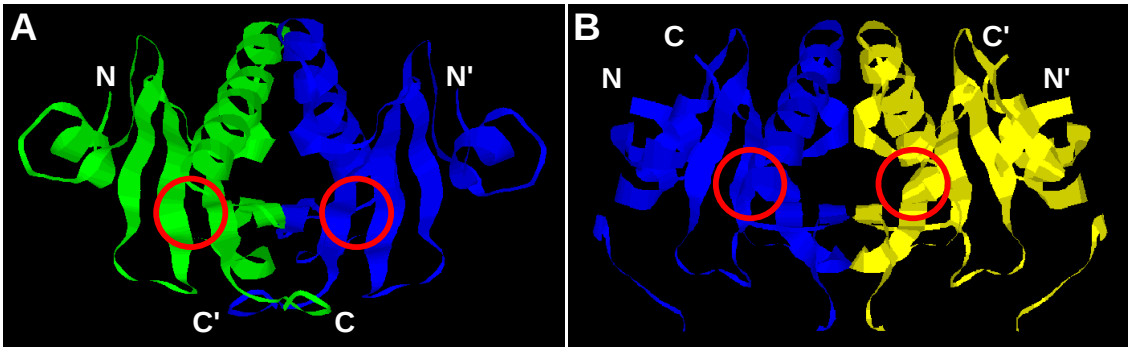

Figure 6. Three-dimensional structures of human Hint and Fhit.
Ribbon diagrams of human Hint (A) and Fhit (B) dimer structures. The N and C termini of each monomer have been marked with N/N' and C/C', respectively. The nucleotide phosphate-binding regions harboring the conserved histidine residues are circled in each monomer. Hint PDB accession code is 1kpb (Lima *et al*., 1996), and Fhit PDB accession code is 1fit (Lima *et al*., 1997a).

4.6 Other Hint-interacting cellular proteins

Hint has been implicated in interactions with a variety of proteins in yeast two-hybrid studies. *HINT* clones have been identified in screenings with PKC β regulatory domain (Lima *et al*., 1996) and with *ATDC*, an early erroneous candidate for ataxia-telangiectasia (AT) -complementing gene (Brzoska *et al*., 1995). The biological significance of these results was however weakened by the identification of only single and/or partial Hint clones. An experimentally more solid association was demonstrated in a study of microphthalmia (*mi*), which suggested that Hint was a negative regulator of the *mi* transcription factor (Razin *et al*., 1999). *Mi* plays a major role in the regulation of growth and function in mast cells and melanocytes. No follow-up on the observed Hint-microphthalmia interaction has however been published to date.

AIMS OF THE STUDY

This study was undertaken to investigate the *in vivo* function of the Cdk7 kinase. Cdk7-cyclin H-MAT1 trimer has been implicated in Cdk activation and in RNA polymerase II-mediated transcription. At the onset of these investigations, only biochemical evidence existed for the function of Cdk7-cyclin H-MAT1 kinase in mammals.

To elucidate Cdk7 function, we studied two Cdk7-interacting proteins: 1) Mat1, which had previously been identified to associate with Cdk7, and 2) Hint, which we identified as a novel Cdk7-interacting protein.

The approaches we took involved generation of genetically engineered mutations of *Mat1* in mice and of *Hint* in mice and yeast. Using these genetic models combined with biochemical analysis, we more specifically aimed at answering the following questions:

1. The requirement of Mat1 in murine development.

2. The involvement of Mat1 in the mediation of cell cycle progression.

3. The role of Mat1 in RNA polymerase II CTD phosphorylation.

4. The requirement of Mat1 in general transcription.

5. The physiological relevance of the newly identified interaction between Cdk7 and Hint in mammals and in yeast.

6. The requirement of Hnt1/Hint in *S. cerevisiae* and in murine development.

MATERIALS AND METHODS

Generation of genetically engineered *Mat1* (I, II) and *Hint* mice (IV)

Genomic sequences of the *Mat1* and *Hint* loci were cloned and subjected to restriction mapping and sequencing. For *Mat1*, one exon encoding the 3' half of the RING finger domain corresponding to nucleotides 242-394 of the murine *Mat1* cDNA (GenBank accession no. U35249) was targeted for ablation utilizing a loxP conditional targeting strategy. This yielded the target construct for generation of the conditional (*flox*) allele (Study II). To generate the null allele of *Mat1* (Study I), the loxP conditional target construct was transformed into a bacterial strain expressing the Cre recombinase (Buchholz *et al*., 1996) before introduction into ES cells. For *Hint*, exons 2 and 3 corresponding to nucleotides 163-267 and 268-575 of the murine *Hint* cDNA (GenBank accession no. AK002965) were targeted by insertion of flanking loxP sites to the genomic locus. All target constructs were linearized and electroporated into ES cells. Individual ES cell clones (2900 for *Mat1* null, 800 for *Mat1 flox*, and 700 for *Hint*) were isolated, expanded, frozen and DNAs extracted from these cells were screened for homologous recombination by Southern blotting. ES cell clones were confirmed to be correctly targeted (10 for *Mat1* null, 3 for *Mat1 flox*, and 7 for *Hint*), and cells from individual lines were subsequently injected into blastocysts from C57/BL6 mice, and the blastocysts implanted into pseudopregnant females. Germline transmission of the targeted allele from several coat color positive animals was confirmed by Southern blotting and PCR genotyping. Experimental and control mice were maintained on several heterogeneous genetic backgrounds. The animal welfare committees of the Haartman Institute, University of Helsinki and the State Provincial Office of Southern Finland have approved the generation of the mice and the experimental procedures reported in this study.

Generation and analysis of *Hint*$^{-/-}$;*Fhit*$^{-/-}$ mice (IV)

A cross was set up to produce mice deficient for both *Hint* and *Fhit* (Fong *et al*., 2000). Subsequently cohorts of *Hint*$^{+/-}$;*Fhit*$^{-/-}$ and *Hint*$^{-/-}$;*Fhit*$^{-/-}$ littermates were generated and aged. Careful necropsy and histological analysis of the stomach, small intestine, colon, ovary/testis, liver, kidney, spleen, pancreas, brain, and blood was performed at approximately 18 months of age. The skin was palpated for possible detection of sebaceous tumors. The histological analysis was performed as described below.

Generation of *S. cerevisiae HNT1* disruptant (III)

To disrupt the *HNT1* locus oligos hnt-his 5' (5' *ATG GAG CCA TTG ATA TCG GCA CCG TAC CTA ACA ACA ACA A*CC ATA ATT CCG TTT TAA GAG 3', *HNT1* homologous sequence in italics) and hnt-his 3' (5' *CTA ATC GGA GCC TTC TAG TTT GGC AAG CAA TTC CTT GTG T*CA TAT GAT CCG TCG AGT TCA 3', *HNT1* homologous sequence in italics) were used to amplify a *HIS3* selection cassette. PCR reactions were performed in a

thermocycler by heating the reaction at 95°C for 30 seconds followed by 25 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute. The purified PCR products were subsequently transformed into a haploid and a diploid *S. cerevisiae* strain and correct integration to *HNT1* locus was confirmed by PCR genotyping. The viable haploid *HNT1* disruptant strain *hnt1::HIS3* was used in subsequent studies. *kin28-ts3* strain was a kind gift from Dr. Gérard Faye (Valay *et al*., 1993). Mating, sporulation, and random spore analysis were performed according to standard procedures to produce *hnt1::HIS3*; *kin28-ts3* double mutant haploid strain, identified by auxotrophy analysis, PCR genotyping, and temperature sensitivity.

PCR genotyping (I, II, III, IV)

Tail clips from ear-marked mice (3-4 weeks old) were incubated over night at 58°C in 200 µl of lysis buffer (50 mM KCl, 0.1 mM Tris-HCl pH 8.3, 0.2 mM $MgCl_2$, 0.1% gelatin, 0.45% IGEPAL CA-630, 0.45% Tween 20 and 1 mg/ml proteinase K). The following day proteinase K was inactivated by heating the tubes at 100°C for 15 minutes followed by PCR using 1 µl of tail prep as a template. Genotyping was achieved with the following primers. For *Mat1*: M10; 5'- GCC CTA TTT CAG GAG CCA GTC C, M12; 5'- TGA CCA AGC ATT TGT ATC TAT GAG CC, N4; 5'- GTC AGT TTC ATA GCC TGA AGA ACG. M10 and M12 amplify 385 bp and 477 bp fragments corresponding to the wild-type and flox alleles, respectively, while M10 and N4 amplify a 310 bp fragment corresponding to the null allele. For *Hint*: H7; 5'- GCA GGG AGC ACG CGG GAA GAG TCT GC, H10; 5'- CTG AAT ACA CAA GAA TGG GAA GAC C, N4; see above. H7 and H10 amplify 240 bp and 330 bp fragments corresponding to the wild-type and flox alleles, respectively, whereas N4 and H10 amplify a 310 bp fragment corresponding to the null allele. PCR genotyping of the *Fhit* mice was achieved with the following primers: FHIT for; 5'- CTT GAA TCT AGG CTG CAT TCT AGC GAG, FHIT rev; 5'- GAT TCC TTG CTT ACC TTT TGG GGA TGG, and FHIT neo; 5'- TGG GCT CTA TGG CTT CTG AGG C (personal communication with Dr. Kay Huebner). FHIT for and FHIT rev amplify a 450 bp fragment corresponding to the wild-type allele, whereas FHIT rev and FHIT neo amplify a 280 bp fragment corresponding to the null allele. PCR reactions were performed in a thermocycler by heating the reaction at 95°C for 5 minutes followed by 35 cycles of 95°C for 50 seconds, 58°C for 50 seconds, 72°C for 50 seconds. Reaction products were analyzed on 1.5% agarose gels.

For PCR genotyping the *hnt1::HIS3* disruptant yeast, chromosomal DNA was extracted. Washed cells were resuspended in breaking buffer (2% v/v Triton X-100, 1% v/v SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), where 300 µl of glass beads, and 200 µl phenol/chloroform/isoamyl alcohol were added. Samples were vortexed for 3 minutes after which 200 µl TE buffer was added. Aqueous layer was mixed with ethanol and precipitated nucleic acids were resuspended in 400 µl TE buffer. 30 µl RNAse A (1 mg/ml) was added and incubated at 37°C for 5 minutes, 10 µl of 4 M ammonium acetate and 1 ml of ethanol were added to precipitate the DNA. One microliter (of 100 µl total

volume) of resuspended DNA was used for PCR reaction with the following external *HNT1* primers: 5' hnt ext.; 5'- GTG CGA ATC GTT ACA GAA TA and 3' hnt ext.; 5'- CGT CCA AAA GTG GTT TAT GTT which produce a 1845 bp *HNT1* disrupted fragment and a 1230 bp *HNT1* wild-type fragment. Reaction products were analyzed on 1% agarose gels.

Protein analysis (I, II, III, IV)

To prepare total protein lysates from cultured mammalian cells, cells were lysed on ice in ELB lysis buffer (150 mM NaCl/300 mM KCl, 50 mM HEPES, pH 7.4, 5 mM EDTA, 0.1% IGEPAL CA-630) with 10 mM ß-glycero-phosphate (ßGP), 1 µg/ml leupeptin, 12.5 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) added. For western blot analysis with RNA pol II antibodies H5 and H14 cells were lysed in boiling LSB followed by a brief sonication. Yeast cell lysates were prepared in ELB lysis buffer with 1% IGEPAL CA-630, where 300 µl of Glass Beads (Sigma) was added prior to mechanical lysis with a tissue disrupter. Protein concentrations were analyzed by a spectrophotometer utilizing Bio-Rad protein assay reagent. Immunoprecipitations from yeast lysates were performed from 500 µg of total protein lysate according to standard procedures. Protein lysates from snap-frozen or fresh sciatic nerves were achieved by sonication of the minced tissue in LSB. For western blotting analysis, boiled samples were separated on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes after electrophoresis, and immunoblotted followed by detection with ECL reagents.

Immunofluorescence and immunohistochemistry (I, II, III)

Protocol for immunofluorescence detection was varying depending on the antibodies used. Blastocyst outgrowths and tissue culture cells were fixed on coverslips with 3.5% paraformaldehyde (PFA) for 20 minutes and permeabilized with 0.1% Triton X-100 or with methanol. Cryosections of sciatic nerves were fixed in 4% PFA or methanol and permeabilized with 0.2% Triton X-100. Blocking of the samples was done in 5 or 10% fetal bovine serum or in 5% goat serum in PBS. Primary antibodies were left on cells for 1 to 3 hours, and secondary antibodies generally for 1 h. DNA was stained with Hoechst 33342. Samples were viewed with a Zeiss Axiophot or Axioplan microscope and documented with Sensicam (Cooke Corp. Mich.) or Zeiss Axiocam. Merged images were made with Zeiss AxioVision multichannel imaging software. DNA content was quantified from the digital images with NIH Image software.

BrdU labeling (I, II)

For BrdU labeling of blastocysts and outgrowths *in vitro*, cells were cultured in the presence of 10 µM BrdU for 16 and 46 hours, respectively. Cells were then fixed in 4% PFA, washed with PBS and treated with 0.5 N HCl for 30 minutes followed by indirect immunofluorescence analysis. For *in vivo* labeling of sciatic nerves, experimental and

control animals were injected intraperitoneally once a day for 10 days with 50 µg/g body weight of BrdU in 0.9% NaCl, 7 mM NaOH solution. On day 11 sciatic nerves were dissected, embedded in Tissue-Tek O.C.T. compound (Sakura) and cryosectioned. Sectioned tissues were fixed in methanol, pretreated with 0.5 N HCl, and immunostained for BrdU.

Blastocyst outgrowths (I)

Mat1 heterozygous animals were intercrossed and plugged females were sacrificed 2.5 days post-coital. Morula stage embryos (16-32 cell) were then flushed from the oviducts and maintained in culture for 48 hours. Developed blastocysts were then transferred onto coverslips in micro-well plates, and maintained in culture for up to 7 days.

Microinjection (I)

Trophoblast giant cells from blastocyst outgrowths were microinjected with 25 ng/µl of pEGFP-N2 (Clontech) and 0.1 mg/ml Texas Red dextran tracer dye. Cells were injected for 0.5 seconds under 120 hPa pressure using an Eppendorf microinjector and transjector and a Zeiss Axiovert microscope. After injection, cells were washed twice with media, and analyzed 24 hours later by fluorescence microscopy.

Histology (II, IV)

For standard histological preparations, tissues were fixed over night in 4% PFA at 4°C, dehydrated through increasing ethanol series to xylene, and embedded in paraffin. Sections were cut at 7 µm and mounted on glass slides. After rehydration, hematoxylin/eosin or hematoxylin only staining was performed according to standard procedures and analyzed by light microscopy. For semithin sections (1 µm) of sciatic nerves, tissues were fixed in 1% PFA, 0.5% glutaraldehyde for 2 hours, treated with 1% osmium tetroxide (OsO_4), dehydrated in ethanol, and embedded in epoxy resin. Sections were stained in Toluidine blue and boric acid and analyzed by light microscopy.

Transmission electron microscopy (II)

Ultrathin sections (60-90 nm) of plastic embedded sciatic nerves were cut on grid and stained with uranyl acetate and lead citrate before observing with JEOL 1200EX transmission electron microscopy. Micrographs were captured on negatives.

ß-galactosidase and alkaline phosphatase staining (II)

Sciatic nerve cryosections were fixed on ice with 0.2% glutaraldehyde in PBS with 50 mM EGTA, pH 7.3, and 100 mM $MgCl_2$. For ß-galactosidase staining, slides were washed with *lacZ* wash buffer (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% IGEPAL CA-630 in

PBS) and stained with *lacZ* stain solution (0.5 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM ferrocyanide in *lacZ* wash buffer) at 37°C. For alkaline phosphatase (AP) staining, endogenous alkaline phosphatases were heat inactivated at 70°C for 30 min, samples were rinsed with PBS, and washed with AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$). AP staining was performed with BM Purple (Roche Molecular Biochemicals) substrate solution at 4°C. After washing, glasses were dehydrated to xylene and mounted for analysis on light microscope.

Yeast strains and culture conditions (III)

S. cerevisiae yeast strains used were EGY48 (MATa, *ura3-52, trp1-Δ63, his3-Δ200, leu2-Δ1, 6LexAop-LEU2*) (Gyuris *et al*., 1993), YPH499 (MATa, *ade2-101, lys2-801, ura3-52, trp1-Δ63, his3-Δ200, leu2-Δ1*), YPH499-500 (MATα/a, *ade2-101, lys2-801, ura3-52, trp1-Δ63, his3-Δ200, leu2-Δ1)* (a kind from Dr. Vincent Van Mullem), JGV4 (MATα, *ura3-52, trp1-Δ63, his3-Δ200, leu2-Δ1, kin28-ts3*) (a kind gift from Dr. Gérard Faye; Valay *et al*., 1993), NKK1 (YPH499 *hnt1::HIS3*; see above), and NKK2 (NKK1 + JGV4). As non-selective growth medium YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose, to plates 2.5% Bacto-agar was added) was used, and for selection purposes Synthetic complete with drop-out mix; 0.17% Bacto yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% dextrose (here SD), 2% galactose (SG) or 2% galactose with 1% raffinose (SGR), (2.5% Bacto-agar), 1x drop-out mix lacking histidine (-h), tryptophane (-t), uracil (-u), or combinations of them (-hut, -ht) was used. For selection of colonies expressing *lacZ* gene and leucine (l) X-Gal indicator plates were used. The medium was made of 1:1:2 volume ratios of Solution I, (8% galactose, 4% raffinose, 4x drop-out mix; -hutl or -htl, pH 7.0), Solution II, (0.4 M KH_2PO_4, pH 7.0), and X-agar (0.34% Bacto yeast nitrogen base without amino acids, 1% ammonium sulfate, 5% Bacto-agar), respectively. X-Gal was added at 0.08 mg/ml concentration.

Yeast transformation (III)

Logarithmically growing cells were washed with PBS and resuspended in 1 ml of 100 mM LiAc, centrifuged and resuspended in 400 µl of 100 mM LiAc. For one transformation 50 µl of the resuspension was used. 240 µl of 50% polyethylene glycol, 36 µl 1.0 M LiAc and 25 µl ssDNA (single-stranded DNA from salmon sperm 2.0 mg/ml) were added on centrifuged cells. Plasmid DNA (1 µg of each plasmid) was added in 50 µl volume. After vortexing, the mixture was incubated for 30 minutes at 30°C and heat shocked at 42°C for 15 minutes. Cells were then plated at two different densities on suitable selection plates.

Production of GST proteins and solution binding assay (III)

Production of GST, GST-Hint, GST-Cdk2, and GST-CTD were done in bacterial AD202 or DH5 alpha strain. Induction with isopropyl-ß-D-thiogalactopyranoside (0.126 mM, Fluka) was started at approximately OD 595 nm of 1.000 followed by incubation of the cultures

for a further 15 hours at 25°C. Lysis of bacteria was achieved by resuspending the pellets in 1% Triton X-100, 0.01% lysozyme (in PBS, 10 mM ßGP, 1 µg/ml leupeptin, l2.5 µg/ml aprotinin, 0.5 mM PMSF, 1 mM DTT), and incubating on ice for 25 minutes prior to sonication twice for 1 minute. Filtered supernatants were run twice through columns containing 1.2 ml of prewashed 75% slurry of Glutathione-Sepharose (Amersham Pharmacia Biotech). GST fusion proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 7.7, 200 mM NaCl. Protein concentrations and purity of the collected fractions were measured with Bio-Rad protein assay reagent and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Selected fractions were pooled, glycerol was added to 10% (final), and fractions were stored at -70°C. For a subsequent solution binding assay, 1.5 mg of U2OS human osteosarcoma cell extract in ELB lysis buffer was incubated with 5 µg of GST fusion protein for 1 hour at 4°C, incubated with Glutathione-Sepharose for 2 hours, and washed with ELB. Bound proteins were subjected to SDS-PAGE, western blotting, and immunodetection with the appropriate antibodies.

Transfection (III)

Mammalian cells were routinely cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum, supplemented with penicillin/streptomycin and glutamine. U2OS osteosarcoma cells were transfected according to the standard calcium-phosphate precipitate protocol (Sambrook *et al*., 1989). In brief, $CaCl_2$ was added to DNA solution at 250 mM final concentration, followed by addition of an equal volume of 2 x BES (N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid), and incubated at room temperature for 40 minutes. The precipitated solution was added on cells in a dropwise manner and washed 24 hours later. Cells were then incubated for further 24 to 48 hours prior to analysis.

***In vitro* kinase assay (III, IV)**

Immunoprecipitation was performed from freshly prepared mouse embryonic fibroblast or yeast ELB lysates, and kinase activity of immunoprecipitates was measured against known *in vitro* substrates (GST-CTD and GST-Cdk2 for Cdk7) in the presence of 20 µCi [γ-^{32}P]ATP in 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$ with 2.5 mM $MnCl_2$ and 1 mM DTT freshly added. Kinase reaction samples were run on SDS-polyacrylamide gels and detected with autoradiography from the gels.

***In situ* hybridization (IV)**

Hybridization was carried out using sense and anti-sense probes made by *in vitro* transcription of the open reading frames in the presence of ^{35}S-UTP. *In situ* hybridizations were performed according to standard protocols (Wilkinson, 1990) with modifications (Luukko *et al*., 1996).

Modeling of three dimensional structure (IV)

Modeling of human Hint2 structure was performed with the SWISS-MODEL protein structure server at http://www.expasy.ch/swissmod/SWISS-MODEL.html by using previously published crystal structures of human Hint (1KPBB; Lima *et al*., 1996) and rabbit Hint (6RHN; Brenner *et al*., 1997) as related structures. Images were obtained using SwissPdbViewer and Adobe Photoshop software.

RESULTS AND DISCUSSION

Targeted disruption of *Mat1* leads to embryonic lethality (I)

To study the function of Mat1 within the mammalian Cdk7-cyclin H-Mat1 kinase *in vivo*, a targeted disruption of *Mat1* was generated in mice. Sequences encoding most of the RING finger domain were targeted to generate a null (-) allele. Homologous recombination of a *Mat1* null construct in ES cells, injection of the targeted ES cells to blastocysts, and subsequent generation of chimeric animals lead to germline transmission of the *Mat1* null allele.

Analysis of $Mat1^{+/-}$ intercrosses revealed that no homozygous null progeny was recovered at weaning age. This suggested that the disruption of *Mat1* was embryonic lethal. Following examination of embryos recovered at different stages of development indicated that $Mat1^{-/-}$ embryos die shortly after implantation. Taken that the *Saccharomyces cerevisiae* homologous gene *TFB3*/*RIG2* is essential (Faye *et al*., 1997), it was surprising that the *Mat1*-deficient embryos survived to the implantation stage (day 3-4). We therefore immunostained for the possible existence of the Mat1 protein of maternal origin at 8-cell, 16-cell, and blastocyst stage, and observed decreasing signal in the $Mat1^{-/-}$ embryos such that by the blastocyst stage Mat1 immunoreactivity was barely observable.

These data indicate that the disruption of *Mat1* leads to embryonic lethality shortly after implantation, which is caused by the diminished amount of maternal Mat1 to a level no longer able to sustain essential function and to support viability.

Mat1 is required for the survival of mitotic but not post-mitotic embryonic lineages (I)

To further examine the role of *Mat1* in cell proliferation and differentiation, we took blastocyst stage embryos from $Mat1^{+/-}$ intercrosses into culture. It was soon observed that while the control embryos outgrew into a cluster of proliferative inner cell mass (ICM) cells on a layer of trophectoderm-derived trophoblast giant cells, the *Mat1*-deficient embryos never gave rise to the ICM cells. The viability of the post-mitotic trophoblast giant cells was however comparable to the control cells.

This indicated that Mat1 was required for the viability of mitotic embryonic lineages, but not for post-mitotic embryonic lineages. Additionally, this result enabled us to study the function of *Mat1* further by using the blastocyst outgrowth system to generate a population of viable $Mat1^{-/-}$ cells.

Mat1 specifically regulates the cellular protein levels of Cdk7 and cyclin H (I)

Since Mat1 has been identified as an assembly factor in the Cdk7-cyclin H-Mat1 trimeric kinase (Devault *et al*., 1995; Fisher *et al*., 1995; Tassan *et al*., 1995), we wanted to investigate whether the expression of Cdk7 or cyclin H was affected in the *Mat1*-deficient

trophoblast cells. Indirect immunofluorescence detection revealed a dimished signal in Cdk7 and cyclin H staining in the *Mat1*-deficient cells compared to the robust nuclear staining of the control cells. As a temperature sensitive disruption of the *S. cerevisiae Mat1* homologue *TFB3*/*RIG2* leads to a cessation of all transcription within 30 minutes after shifting to the restrictive temperature, we wanted to investigate whether the diminished levels of Cdk7 and cyclin H could be a result of a low general level of transcription. To this end, expression levels of several proteins were assayed for. These included proliferation cell nuclear antigen (PCNA), p53, Cdk2, cyclin E, cyclin D1, Cdk6, and the large subunit of RNA polymerase II. We found that the expression levels of all of these proteins were comparable in *Mat1*$^{-/-}$ and control cells.

These data indicate that the loss of Mat1 resulted in a specific downregulation of the protein levels of Cdk7 and cyclin H and suggested that the loss of Mat1 does not deregulate transcription or translation in general.

Mat1 modulates RNA polymerase II CTD phosphorylation (I)

As the most widely studied substrate of Cdk7-cyclin H-Mat1 kinase is the C-terminal domain (CTD) of the large subunit of RNA polymerase II, we wanted to investigate whether the phosphorylation status of the CTD was affected in the *Mat1*-deficient trophoblast cells. This was achieved by utilizing antibodies specific for phosphorylated and unphosphorylated serine 2 or 5 residues of the conserved heptapeptide repeat, (Thompson *et al.*, 1989; Patturajan *et al.*, 1998) in an indirect immunofluorescent staining on trophoblast outgrowths. The stainings indicated that while the total level of pol II (non-phospho-Ser-2 antibody 8WG16) was unchanged in *Mat1*$^{-/-}$ cells, signals from both phosphorylated Ser-2 (H5), and Ser-5 (H14) were dimished.

These data suggest that Mat1 regulates the CTD phosphorylation levels *in vivo* either by affecting the activity of Cdk7-cyclin H kinase directly, or by modifying the activities of the other CTD kinases.

Transcription and translation of an exogenous cDNA in Mat1$^{-/-}$ cells (I)

To achieve a more direct assessment of whether or not the *Mat1*$^{-/-}$ cells had an ability to transcribe and translate *de novo*, an exogenous reporter construct was introduced to the *Mat1*-deficient cells. Microinjection of a CMV-promoter driven EGFP (enhanced green fluorescent protein) construct to the trophoblast giant cells and the subsequent analysis of the cells with a fluorescent microscope on the following day showed that *Mat1*$^{-/-}$ cells were able to engage in *de novo* transcription and translation like the control cells were.

Mat1 is required for S phase entry in trophoblast giant cells (I)

DNA synthesis in trophoblast giant cells occurs by endoreduplication whereby successive rounds of G and S phases proceed without an intervening mitosis (Barlow *et al.*, 1972;

Gardner, 1983). During the course of investigations of the outgrown cells, we observed that while the viability of the *Mat1*$^{-/-}$ trophoblast giant cells was comparable to that of the control cells, their nuclei appeared smaller. To investigate the possibility that *Mat1*$^{-/-}$ cells would have less DNA in their nuclei, we measured the intensity of a large number of Hoechst 33342 stained nuclei of *Mat1*-deficient and control cells as well as control mouse embryonic fibroblasts. This analysis revealed that the DNA content of the *Mat1*$^{-/-}$ cells was dramatically lower than that of control cells. Cells of both genotypes had heterogeneous amounts of DNA, indicating that also the *Mat1*-deficient cells had engaged in endoreduplication to some degree. With a further demonstration of an inability to incorporate BrdU, we concluded that the *Mat1*-deficient trophoblast cells were unable to enter S phase likely upon the depletion of the maternal Mat1 protein. This data coupled with the earlier observations of an intact transcriptional ability, suggests that the endocycle arrest of *Mat1*$^{-/-}$ cells may be due to a defect in Cdk activation.

Generation of *Mat1* loxP conditional mice (II)

In order to study the consequences of the loss of Mat1 *in vivo* without the restrains of embryonal lethality, we generated a loxP conditional allele of *Mat1* (*flox*) to achieve a tissue-specific disruption in adult mice. A vector designed to conditionally disrupt *Mat1* in a Cre/loxP-mediated manner was constructed and introduced into ES cells. Homozygous mice for the conditional allele (*Mat1*$^{flox/flox}$), generated by intercrossing *Mat1*$^{flox/+}$ mice were found to be healthy and fertile, indicating that the conditional allele had no overt hypomorphic effects. The functionality of the conditional allele was tested *in vivo* by crossing the *Mat1*$^{flox/flox}$ mice to a phosphoglycerate kinase 1 (PGK-1) promoter-driven Cre-deleter mouse (a kind gift from Dr. Peter Lonai; Lallemand *et al*., 1998). Subsequently the null allele (-) was observed in all offspring. To generate both mitotic and post-mitotic *Mat1*$^{-/-}$ cell populations in a spatio-temporal manner, *Mat1*$^{flox/-}$ animals were crossed to mice in which the *Cre* recombinase was knocked-in to the transcription factor *Krox-20* locus (*KCN*). This enabled us to study the loss of *Mat1* in the mitotic germ cells (Voiculescu *et al*., 2000) and in the post-mitotic myelinated Schwann cells (Topilko *et al*., 1994). (A detailed characterization of the Krox-20-Cre expression pattern in Voiculescu *et al*., 2000).

Mat1 is required for the viability of the mitotic germ cells (II)

As *Krox-20* is known to be expressed in the germ lineage of sexually mature mice (Voiculescu *et al*., 2000), we histologically examined the seminiferous tubules of experimental *Mat1*$^{flox/-}$*;KCN* and control male mice to study the function of Mat1. While before sexual maturation, the developing germ lineage of the mutant animals was indistinguishable from the control, at six weeks of age, pycnotic germ cells and the loss of spermatogonia and spermatocytes from a subset of the tubules were apparent. Death of the germ lineage was further exacerbated in later adulthood, as at 14 weeks the seminiferous tubules were noted to be totally devoid of all germ cells. These data indicate that *Mat1* is

essential for the viability of the mitotic germ cells, and thus extend the requirement of *Mat1* from the embryonic mitotic lineages to the adult proliferating lineages.

Targeted disruption of *Mat1* in the myelinated Schwann cells (II)

Myelination of the peripheral axons in mice starts approximately at the time of birth and takes the first four weeks of postnatal life (P0-P30) to be completed. (See Figure 7 for Schwann cell differentiation.) Myelination is controlled by the successive expression of several transcription factors. *Krox-20* has been shown to be elemental for the onset of myelination (Topilko *et al*., 1994), as it controls the expression of numerous genes involved in the myelin sheath synthesis. Upon committing to the myelinated Schwann cell identity, a Schwann cells exits the cell cycle and becomes post-mitotic. A great amount of transcriptional and translational activity is required to generate the structural components of myelin, and to maintain the myelin sheath integrity (Stahl *et al*., 1990). By using the *Krox-20-Cre* mice we were able to study the role of Mat1 in transcription, unencumbered by the proposed essential functions of Cdk7-cyclin H-MAT1 in Cdk activation.

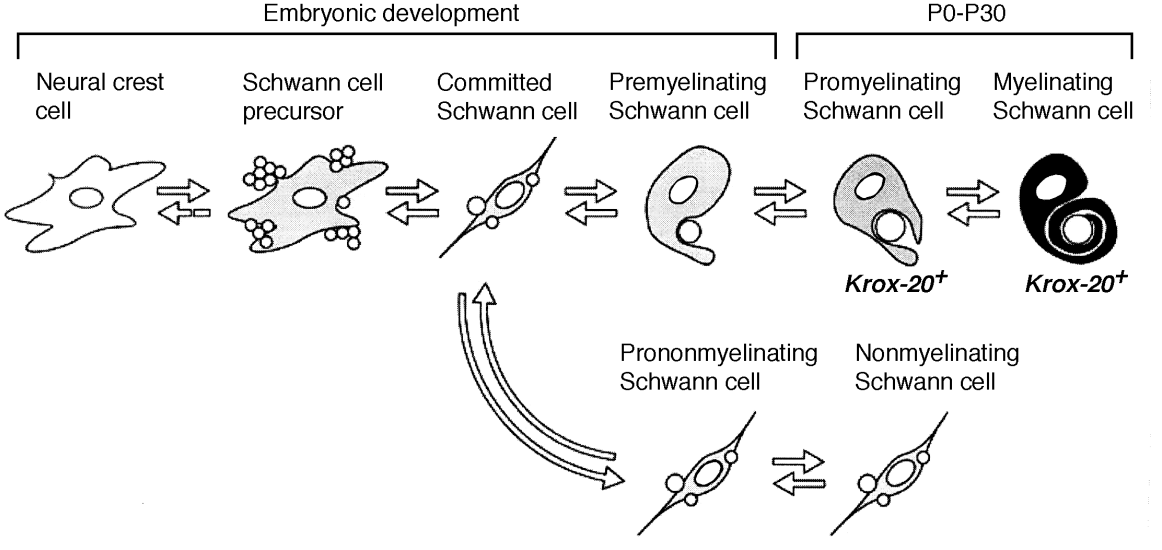

Figure 7. Differentiation of Schwann cells.
The maturation of the myelinated and the non-myelinated Schwann cells. Cells expressing *Krox-20* have been marked (*Krox-20*$^{+}$). [In the myelinated Schwann cell lineage, *Krox-20* expression continues throughout life (Topilko *et al*., 1994).] The nuclei of the Schwann cells are drawn in oval shape, while the axons are drawn in round shape with their number ranging from many to one for every Schwann cell. Modified from (Zorick and Lemke, 1996).

Histological examination of the sciatic nerves at one month of age revealed that the Mat1-deficient myelinated Schwann cells were fully capable to achieve a mature myelinated phenotype. Furthermore, normal myelin thickness was maintained at two months of age, which was also indicated by the expression of comparable levels of myelin structural proteins.

At approximately three months of age however, the *Mat1*$^{flox/-}$*;KCN* mice began to exhibit symptoms of neuropathy and subsequent muscular atrophy, including gait abnormalities and a loss of hind limb body mass. Histological analysis of the sciatic nerves at three months of age revealed apparent signs of hypomyelination. Denuded axons and abnormally thin myelin sheaths were frequently found, and at five months of age, virtually all myelinated Schwann cells were affected.

These data indicate that Mat1 is not essential for the transcription underlying myelination, but is nonetheless required for the sustained viability of the myelinated Schwann cells. This suggests an essential role for Mat1 in the regulation of transcription of a *subset* of genes required for maintaining Schwann cell viability, rather than a role in *general* transcription.

Remyelination by the non-myelinated Schwann cells (II)

The histological and ultrastructural analysis of the sciatic nerves from the experimental animals indicated that the Mat1-deficient myelinated Schwann cells were rendered non-viable, and that a frequent remyelination of the denuded axons was occurring. This apparent turnover in the Schwann cell pool was examined by an *in vivo* BrdU labeling and a subsequent Krox-20/BrdU double staining. The results showed increased proliferation of the Schwann cell lineage in the experimental *Mat1*$^{flox/-}$*;KCN* mice.

Many morphological observations in the literature are compatible with the hypothesis that upon nerve injury and the subsequent axon withdrawal, the myelinated Schwann cells extrude their myelin sheath, lose their myelin gene expression, dedifferentiate and proliferate (Wallerian degeneration, reviewed in Stoll and Muller, 1999). To unravel the subject of reversible differentiation of the myelinated Schwann cells, a few studies have distinctly investigated the two lineages of Schwann cells, the myelinated and the non-myelinated, and have indicated that non-myelinated Schwann cells contribute to the proliferative response *in vivo* (Griffin *et al*., 1990; Messing *et al*., 1992).

Interestingly, granted by our system where targeted recombination of *Mat1* is restricted to the *Krox-20*-positive myelinated Schwann cells, we suggest that the non-myelinated Schwann cells (see Figure 7) serve as the stem cell pool capable of remyelination of the large caliber denuded axons.

Cdk7 and Kin28 associate with Hint and Hnt1 (III)

We were interested in identifying novel proteins interacting with the Cdk7 kinase. To this end, a yeast two-hybrid screening with a LexA-Cdk7 bait was performed (Mäkelä *et al*., 1994) prior to the onset of Study III. Initially ignored, characteristically small and intensely blue colonies were identified as cDNA clones of a gene named *PKCI-1* (protein kinase C-interacting protein 1; Pearson *et al*., 1990) or *HINT* (histidine triad nucleotide-binding protein; Brenner *et al*., 1997). As all the identified *HINT* clones contained the entire open reading frame, Cdk7-Hint interaction was likely to require a full-length Hint.

Identification of a yeast two-hybrid interaction between the Cdk7 and Hint *S. cerevisiae* homologues Kin28 and Hnt1, respectively, indicated that the association was conserved from man to yeast. Cdk7-Hint and Kin28-Hnt1 interactions were further verified in a complex formation assay by immunoprecipitation from yeast lysates, which showed that both Hint and Hnt1 co-immunoprecipitated with Cdk7 and Kin28, respectively. Homodimerization of Hint and Hnt1 was also demonstrated in this assay. Moreover, with 34% amino acid sequence identity between the human and the yeast HIT orthologues, Hint and Hnt1 were able to form a complex with each other (unpublished data), indicating structural conservation.

An association between Hint and the endogenous mammalian Cdk7 was also confirmed by co-purification of endogenous Cdk7 with a GST-Hint from mammalian cell lysates.

These data indicate that the physical association between Cdk7 (an *in vitro* CAK and a CTD kinase) and the HIT protein Hint, is conserved between the *S. cerevisiae* Kin28 (exclusively a CTD kinase) and Hnt1 proteins.

Cdk7-Hint interaction is independent of Cdk7 kinase activity and cyclin H binding (III)

To elucidate the possible functional significance of Cdk7-Hint interaction, we characterized the ability of Hint to associate with distinct Cdk7 mutants using the two-hybrid assay. A Cdk7 mutant, which is deficient in kinase activity (Cdk7-K41M) interacted with Hint. In contrast to cyclin H (Mäkelä *et al*., 1994), Hint was also able to bind Cdk7, in which the T-loop threonine had been mutated to mimic a constitutive activating phosphorylation (Cdk7-T170E). No interaction was noted between the N-terminally deleted Cdk7 (Cdk7Δ1-132) and Hint.

These data suggest that Hint binding is independent of Cdk7 kinase activity and of cyclin H binding. Nevertheless, Hint-associated Cdk7 retained its kinase activity, indicating that Cdk7-Hint interaction does not exclude cyclin H binding, which is required for Cdk7 activity. In conclusion, Hint apparently does not represent a Cdk inhibitor – rather it may be involved in modifying the Cdk7 substrate specificity as was suggested by the preferential phosphorylation of an *in vitro* RNA polymerase II CTD substrate by the Hint-associated Cdk7.

Genetic interaction between *KIN28* and *HNT1* (III)

The extension of the Cdk7-Hint association to the *S. cerevisiae* Kin28 and Hnt1, together with the availability of temperature-sensitive alleles of *KIN28* (Valay *et al*., 1993) prompted us to investigate whether *KIN28* and *HNT1* display genetic interactions. This was initiated by generating a disruption of *HNT1* in which a *HIS3* selection cassette replaced amino acids 14-146 (of 158) of *HNT1*. The haploid disruptant strain proved to be viable, and exhibited no apparent phenotype, indicating that *HNT1* is not an essential gene.

Subsequently a double mutant haploid strain was generated harboring the disruption of *HNT1* together with a *KIN28* temperature-sensitive allele *kin28-ts3* (JGV4, a kind gift from Dr. Gérard Faye; Valay *et al.*, 1993).

Analysis of the double mutant haploid indicated decreased colony formation compared to either parental strain and elongated, filament-like cell morphology at permissive temperature, most prominent on galactose media. The mutant morphology was suppressed with ectopic expression of Hnt1 or Kin28, as well as with expression of the human Hint. These data provide genetic evidence supporting the physiological relevance of the observed two-hybrid interaction between Kin28 and Hnt1.

The results have recently been verified and taken further by Charles Brenner and co-workers (Bieganowski *et al.*, 2002). Bieganowski *et al.* (2002) additionally showed that *hnt1* deficiency leads to a synthetic loss of viability with a thermo-sensitive allele of not only *kin28*, but also with *S. cerevisiae* cyclin H and MAT1 homologues *ccl1* and *tfb3*, respectively, which was shown to be suppressed by the ectopic expression of Hnt1. Moreover, a synthetic phenotype was shown to result from combining several different *kin28* alleles to *hnt1* disruption, and that the enzymatic activity of Hnt1 was essential for it. This was demonstrated by utilizing a Hnt1 mutant *hnt1-H116A*, which is inactive in $AMPNH_2$ hydrolysis (Bieganowski *et al.*, 2002). Alternative models for the regulation of Kin28 function by Hnt1 have been schematized in a recent review (Brenner, 2002). In brief; 1) Kin28-AMP is suggested to be a substrate of Hnt1 either alone or in addition to a yet unspecified AMP-conjugated molecule (AMP-x) and 2) small molecules (AMP-x) are proposed to be important Hnt1 substrates, whose insufficient hydrolysis could lead to Kin28 inhibition. The elucidation of the physiological significance of Hint/Hnt1 on the function of Cdk7/Kin28 is still however incomplete.

Hint expression during murine development and adulthood (IV)

In order to study Hint in a mammalian *in vivo* context, we have investigated the expression of *Hint* in mouse development and in adulthood by *in situ* hybridization analysis. During the early and mid-gestational embryonic stages (E7-E13), the expression of *Hint* was ubiquitous and largely correlated with cell density. However, at E15 *Hint* expression was more pronounced in the peripheral sensory trigeminal, vestibulo-cochlear and dorsal root ganglia, suggesting expression in neuronal cells. Prominent expression was also noted in the heart, the mucosal epithelium of the intestine, and in the germ cells of the developing testis. As a comparison, the expression of *Cdk7* was ubiquitous and evenly distributed at E7-E15 (unpublished data).

In the adult, a low signal was noted in all analyzed tissues, while prominent signal of *Hint* was again more restricted. The coronal section of an adult mouse brain of the rostral level revealed a spotted pattern of pyramid cells expressing high levels of *Hint* mRNA within the caudate-putamen. Likewise, a high *Hint* expression was noted in the neurons of the myoenteric plexuses along the gastrointestinal tract. *Hint* was also expressed at a higher

level in the differentiated keratinized cells of the stratified epithelium of the stomach, and in the proximal tubules of the kidney. These data are in line with previous reports on the expression of Hint protein (McDonald *et al*., 1987; Klein *et al*., 1998). Hint expression in the adult testis continued to be most prominent in the germ cells where the highest expression was found in the pachytene spermatocytes, whereas abundant ovarian expression was located in the maturing follicles and the egg cytosol (unpublished data).

The particular and high expression of *Hint* in the myoenteric nerves along with the high expression in the peripheral sensory trigeminal, vestibulo-cochlear and dorsal root ganglia late in the embryonic development identifies specific subsets of neurons, where *Hint* expression is abundant. Also, as several epithelial cell populations seem to harbor prominent *Hint* expression, these data suggest that Hint may have a prominent role in at least these tissues.

Generation of the *Hint* conditional (*flox*) mice (IV)

To investigate the function of *Hint* in murine development further, we generated targeted mutagenesis of *Hint* in ES cells. A vector designed to conditionally disrupt *Hint* in a Cre/loxP-mediated manner was constructed and introduced into ES cells. Several ES cell clones were confirmed to be correctly targeted, and mice bearing the conditionally targeted allele ($Hint^{flox}$) were generated.

Intercrossing $Hint^{flox/+}$ mice yielded F1 offspring bearing combinations of all the expected alleles. The $Hint^{flox/flox}$ mice were healthy and fertile, indicating that the conditional allele had no overt hypomorphic effects. To test the functionality of the *flox* allele *in vivo*, the $Hint^{flox/flox}$ conditional mice were crossed to a phosphoglycerate kinase 1 (*PGK-1*) promoter driven Cre-deleter mouse (a kind gift from Dr. Peter Lonai; Lallemand *et al*., 1998) after which the recombined *Hint* null allele (-) was observed in all offspring ($Hint^{+/-}$). Subsequent $Hint^{+/-}$ intercrosses produced $Hint^{-/-}$ offspring in Mendelian ratios, indicating disruption of *Hint* developmentally viable.

Hint$^{-/-}$ tissues are histologically normal (IV)

The results obtained from the expression study of the embryonic and adult tissues, and the fact that Hint disruption was viable prompted us to analyze the tissues where high *Hint* expression was noted for pathological changes.

The histological analysis of the sympathetic nerves of the stomach, small intestine and colon in the adult $Hint^{-/-}$ animals revealed histological integrity. Moreover, the histology of the $Hint^{-/-}$ brain at the caudate-putamen was also normal, which was supported by the fact that the $Hint^{-/-}$ animals did not display any symptoms that would indicate autonomic nerve dysfunction. Histology of the gastrointestinal tract as well as kidney and liver (unpublished data) was also normal in the adult $Hint^{-/-}$ mice. Fertility of the $Hint^{-/-}$ males and females was

comparable to wild-type animals, indicating no defects in reproductive functions, or the production of the germ cells.

The normal development and the lack of histological abnormalities in the adult tissues of the *Hint*$^{-/-}$ animals corroborate with the natural life span observed for the *Hint*$^{-/-}$ animals. These data indicate that *Hint* is not an essential gene for long-term survival in the mouse, and suggest redundancy through overlapping functions with a related gene product.

Functional redundancy within the HIT family? (IV)

The possibility of functional redundancy within the HIT family was first investigated in the budding yeast *Saccharomyces cerevisiae*. We (Korsisaari and Mäkelä, 2000) and others (Bieganowski *et al*., 2002) have noted that the Hint homologue *HNT1* is not essential for viability in normal culture conditions. Likewise, the disruption of the budding yeast Fhit homologue *HNT2* is not lethal, albeit Hnt2 substrate accumulating in these cells (Chen *et al*., 1998). To address the possibility of functional overlap within the thus far identified HIT proteins, we generated a double disruptant of the *S. cerevisiae hnt1* (Korsisaari and Mäkelä, 2000) and *hnt2* (YPALST, a kind gift from Dr. Pierre Plateau; Chen *et al*., 1998), and noted that the viability of the *hnt1*Δ*hnt2*Δ was not compromised (unpublished data), indicating no functional redundancy between Hnt1 and Hnt2.

In order to address the possibility of overlapping functions between *Hint* and *Fhit* in mice, we generated a double mutant by crossing the *Hint*$^{-/-}$ mice to the *Fhit*-deficient mice (a kind gift from Dr. Kay Huebner; Fong *et al*., 2000). The viability of the *Hint*$^{-/-}$*;Fhit*$^{-/-}$ mice was noted to be comparable to wild-type animals. The analysis of the double deficient mice for the phenotypes reported for the *Fhit*$^{-/-}$ mice (Zanesi *et al*., 2001), revealed increased gastric polyposis, whereas the incidence of the other neoplasias and tumors reported for the *Fhit*-deficient mice was more modest in both the *Hint*$^{+/-}$*;Fhit*$^{-/-}$ and the *Hint*$^{-/-}$*;Fhit*$^{-/-}$ cohort. In conclusion, this data does not support functional redundancy between the murine *Hint* and *Fhit*.

Interestingly, a new member of the HIT protein family has been recently identified and named *Hint2* in mammals. The human Hint is more closely related with Hint2 [60% amino acid (aa) sequence identity] than with Fhit (19% aa sequence identity), which prompted us to gauge the likelihood of functional redundancy between the mammalian Hint and Hint2.

To this end, we modeled the 3-dimensional structure of Hint2 based on the known Hint structures (see Materials and Methods). The tertiary structure of Hint (6RHN; Brenner *et al*., 1997) and the modeled structure of Hint2 share significant resemblance with each other, whereas the crystal structure of Fhit (1FIT; Lima *et al*., 1997a) is distinct from these. Moreover, Hint2 exhibits very high conservation of the 14 amino acids (86%; 12/14 aa conserved) implicated in nucleotide phosphate-binding of rabbit Hint (Brenner *et al*., 1997) (see also Figure 5 in the Review of the literature). A small number of amino acid side chains of the modeled Hint2, notably Phe56, His88, and Gln143 are positioned differently in space compared to the corresponding side chains of Hint residues (Phe19, His51,

Gln106). The high conservation of these residues between Hint and Hint2 suggests that Hint2 possess a nucleotide phosphate compound-hydrolyzing activity similar to that of Hint. A conclusive answer to this however requires biochemical and genetic experimentation.

The TFIIH kinase subunit is stable and active in Hint$^{-/-}$ cells (IV)

The generation of *Hint*$^{-/-}$ mice allowed us to explore whether the lack of Hint in mammalian cells had any impact on the functions ascribed to the Cdk7 kinase and its associated proteins. Normal Cdk7 function was implicated by wild-type protein levels of Cdk7, cyclin H, and Mat1 in *Hint*$^{-/-}$ MEFs. When kinase activity of Cdk7 was analyzed following immunoprecipitation of the kinase complex from both wild-type and *Hint*$^{-/-}$ MEFs, no change was observed in the ability of Cdk7 to phosphorylate either a GST-CTD or a GST-Cdk2 substrate. Moreover, we saw no change in the total *in vivo* level of pol II, nor in the levels of pol II phosphorylated on serine 2 or serine 5 of the CTD when comparing *Hint* wild-type, heterozygous, and *Hint*$^{-/-}$ MEFs. Furthermore, we found that the phosphorylation of Cdk2 on T-loop Thr160 was unaltered in the Hint-deficient cells, indicating that loss of Hint did not alter Cdk7's ability to phosphorylate Cdk*2* in mouse embryonic fibroblasts under normal culture conditions

In contrast to the effect of mammalian Mat1 disruption on Cdk7 (Study I; Rossi *et al*., 2001), these results indicate that the loss of Hint causes no detectable changes on Cdk7 functions suggesting that Hint is not a critical regulator of Cdk7.

CONCLUDING REMARKS

During the past ten years, the field of molecular biology has witnessed great achievements. Reflecting the global advances in cell cycle research, the 2001 Nobel Prize in Physiology or Medicine was awarded to Leland Hartwell, Tim Hunt and Paul Nurse for their discoveries of key regulators of the cell cycle. Using genetic and biochemical methods, they identified Cdks and cyclins, and demonstrated that these proteins control the cell cycle in yeasts, insects, plants, animals and humans alike.

Cdk7 with its cyclin partner cyclin H and a third subunit MAT1 has been implicated in positive regulation of the other Cdk-cyclins *in vitro* (reviewed in Kaldis, 1999). After Cdk7, cyclin H, and MAT1 were also discovered to comprise the kinase subunit of the nine-subunit basal transcription factor TFIIH (Roy *et al*., 1994; Serizawa *et al*., 1995; Shiekhattar *et al*., 1995), the physiological function of the mammalian Cdk7-cyclin H-MAT1 has been a subject of active research. A different evolutionary solution has been indicated in *S. cerevisiae* where the homologous Kin28-Ccl1-Tfb3 complex only functions as a TFIIH kinase and not as a Cdk-activating kinase (Cismowski *et al*., 1995). However, in *Schizosaccharomyces pombe*, the Cdk7-related Mcs6 kinase complex activates Cdks *in vivo* (Hermand *et al*., 1998; Hermand *et al*., 2001). With the studies included in this dissertation, we have aimed at elucidating the function of the mammalian Cdk7 by utilizing genetic murine and yeast models to study previously reported and novel Cdk7-interacting proteins.

Our results on genetic mouse mutations of *Mat1* revealed an absolute requirement for Mat1 in mitotic embryonic inner cell mass cells and adult germ cells alike. As Cdk7-cyclin H-MAT1 trimer has been implicated in Cdk activation (reviewed in Kaldis, 1999), it is plausible that the cellular defects in these mitotic cell populations are due to deficits in CAK activity. These data suggest that Mat1, and by extension the trimeric Cdk7-cyclin H-Mat1 kinase, has an *in vivo* role in cell cycle progression. Such results for the homologous protein complex have to date only been obtained in *Drosophila* (Larochelle *et al*., 1998).

We also found unexpectedly that the *Mat1*-deficient post-mitotic trophoblast cells and the myelinated Schwann cells remained viable for extended periods of time. As transcription is a fundamental function of all cells, the viability of these *Mat1*$^{-/-}$ cell populations proposes that in the mouse, Mat1 (and by extension the TFIIH kinase) would not be *absolutely* required for RNA polymerase II-mediated transcription. Rather, these observations suggested a role for Mat1 in the regulation of a subset of transcripts, which may include target genes transactivated by the nuclear hormone receptors RARα, RARγ, or ERα, which have been suggested to be regulated by TFIIH kinase activity (Rochette-Egly *et al*., 1997; Bastien *et al*., 2000; Chen *et al*., 2000; Keriel *et al*., 2002). This is a very surprising result in the light of earlier experiments in budding yeast using temperature sensitive alleles of Cdk7, cyclin H, and MAT1 homologues, which resulted in a very rapid and near total cessation of transcription and inviability of cells upon shifting to the restricted temperature (Valay *et al*., 1993; Valay *et al*., 1995; Valay *et al*., 1996; Faye *et al*., 1997; Holstege *et al*.,

1998). These results could perhaps reflect profound differences in the regulation of transcription in mammals and in yeast *S. cerevisiae*.

In order to elucidate the functions of Cdk7 further, we searched for novel interacting proteins. We identified a novel Cdk7-interacting protein Hint. While the biochemical evidence indicated a regulatory role for Hint on Cdk7 activity, the *in vivo* studies of mammalian *Hint* did not support these observations. The finding that *Hint*-deficient mice were healthy, together with the fact that the activities of Cdk7 were unaltered in *Hint*-deficient mouse embryonic fibroblasts, suggested that Hint may not have a critical function in the regulation of Cdk7 in mice. Which pathways the proposed nucleotide phosphate hydrolase activity of Hint will impinge upon, remains a subject of future research.

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