Role of DEAD box 1 in retinoblastoma and neuroblastoma

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Analysis of hereditary and nonhereditary retinoblastoma led to the formulation of the two-hit hypothesis of cancer in the early 1970s. The two-hit hypothesis was validated in the 1980s when both copies of the *RB1* gene were shown to be mutated in hereditary and nonhereditary retinoblastoma. However, consistent genetic abnormalities other than *RB1* mutations suggest that additional events may be required for the formation of these malignant tumors. For example, *MYCN* amplification has long been known to occur in both retinoblastoma and neuroblastoma tumors and is strongly associated with poor prognosis in neuroblastoma. The DEAD box gene, DEAD box 1 (*DDX1*), is often coamplified with *MYCN* in both these childhood tumors. Here, we examine possible roles for *DDX1* overexpression in retinoblastoma and neuroblastoma.

Keywords: DEAD box 1, DEAD box protein, gene amplification, MYCN, neuroblastoma, retinoblastoma, RNA helicase

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DEAD box proteins

DEAD box genes encode a family of putative RNA helicases (ATP-dependent RNA unwinding proteins) whose primary role is to alter the secondary structure of RNA molecules. DEAD box proteins share nine conserved domains (Q, I, Ia, Ib, II–VI) [1,2] with roles in ATP binding/hydrolysis, RNA binding and RNA unwinding. The signature domain (motif II) of DEAD box proteins, D(asp)-E(glu)-A(ala)-D(asp), is involved in ATP binding/hydrolysis and the coupling of ATPase and RNA unwinding activities (**Figure 1**). Motifs I and VI contain ATP-binding sites, whereas motif III may link ATP hydrolysis with helicase activity, and motif Q has been postulated to regulate ATP binding and hydrolysis. Motifs Ia, Ib, IV and V are believed to play roles in RNA binding and unwinding [3,4]

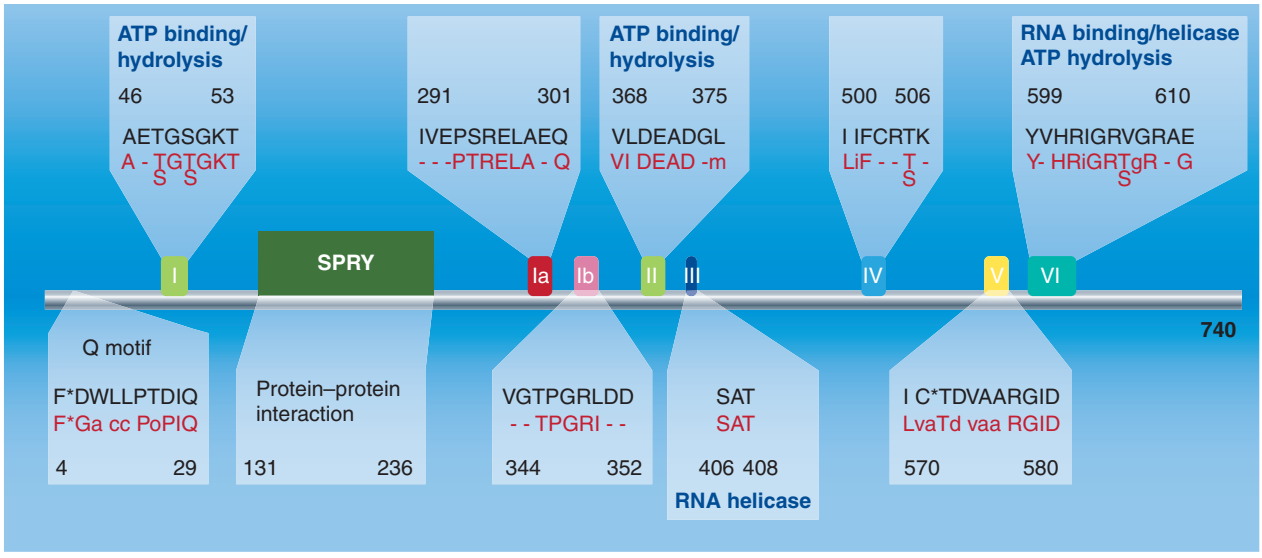
DEAD box proteins are found in most organisms, from complex multicellular plants and animals to bacteria and viruses. A total of 36 DEAD box proteins have been identified in the human genome [5]. Related to the DEAD box proteins are the DEAH proteins, which appear to be primarily involved in RNA splicing, and the DExH proteins. DEAD/DEAH/DExH proteins are all part of the helicase superfamily 2 [6], and are generally thought to have little processive activity, acting more like clamps to prevent RNAs from reassociating [7]. The only member of this family shown to have processive activity is the DExH protein NPH-II from *Vaccinia virus* [8]. NPH-II can unwind up to 83 bp of dsRNA at a rate of 6 bp per kinetic step.

One of the best-characterized eukaryotic DEAD box proteins is the translation initiation factor eIF-4A (DDX2). eIF-4A binds nucleic acids and unwinds RNA–RNA and RNA–DNA duplexes in an ATP-dependent manner, and has RNA-dependent ATP hydrolysis activity [9,10]. eIF-4A is believed to be a nonprocessive or slightly processive RNA helicase whose activity is enhanced in the presence of other proteins, such as eIF-4B and eIF-4G, especially in the presence of extensive RNA secondary structure [11–14]. Proposed biochemical functions for eIF-4A include:

- Unwinding the 5′ ends of mRNAs to facilitate translation;
- Motor activity, whereby the eIF-4A/ribosome is used to translocate mRNA as ATP is hydrolysed;
- Alteration of RNA structure to either facilitate or inhibit the binding of translational factors and other factors [10].

Other DEAD box proteins with RNA unwinding activity, RNA-dependent ATPase activity, and the ability to unwind/destabilize RNA, include p68 (DDX5), *Drosophila* vasa (DDX4), *Xenopus* An3 (DDX3), human p54 (DDX6) and yeast Dbp5p (DDX19) [15–20]. Despite considerable interest in this protein family, the biological role of the majority of DEAD box proteins remains poorly understood. DEAD box proteins have been proposed to serve as multifunctional RNA chaperones that promote the formation of particular RNA structures and transport RNAs from one compartment of the cell to another [21]. For example, Dbp5p, required for mRNA export out of the nucleus, has recently been shown to be activated by the nuclear pore protein Gle1 and phosphoinositide IP6, suggesting that Dbp5p RNA binding/ATPase activity at

Figure 1. DEAD box motifs and SPRY domain in DDX1.



DDX1 has the nine conserved motifs found in all DEAD box proteins (Q, I, Ia, Ib, II, III, IV, V and VI), as well as a SPRY domain. The amino acids spanning each conserved motif are indicated, with 740 referring to the total number of amino acids in DDX1. Activities associated with motifs I, II, III and VI are shown in blue. Capital letters represent amino acids that are conserved in the majority (>80%) of DEAD box proteins; small case letters represent amino acids that are less well-conserved (50–79%) [7]. For the Q motif, the asterisk represents 16 amino acids; a = F,W,Y; c = D,E,H,K,R; I = I,L,V; o = S,T [90].

the nuclear pore is necessary for mRNA export [22–24]. Some DEAD box proteins are believed to function as RNPases involved in RNA-protein association and dissociation [25,26]. A recent analysis of p68 function in *Drosophila* has revealed a possible connection between DEAD box proteins, transcript clearance and gene repression [27]. Flies carrying *p68* mutations do not release completed mRNAs from their transcription sites and have problems in shutting off active genes. These authors postulate that p68 may be required to remove transcripts from a gene before the chromatin can be reset to an inactive state.

DEAD box proteins & cancer

A number of DEAD box proteins have been implicated in cancer. For example, the *p54* and *DDX10* genes are found at sites of chromosome translocations [28–30] and p54 has been shown to be overexpressed in a number of cancer types [31–33]. As p54 can unwind *c-Myc* RNA *in vitro*, it has been suggested that p54 may contribute to cancer by restructuring mRNAs, thus affecting their translation efficiency. In this regard, it is noteworthy that Myc–Max heterodimers have been implicated in the transcriptional activity of two DEAD box genes: *MrDd* (*DDX18*) and *Drosophila* pitchoune [34,35]. These results suggest a cancer-related feedback loop between members of the DEAD box protein family and Myc, with Myc activating DEAD box protein transcription and DEAD box proteins promoting Myc production.

Two related DEAD box proteins, p68 and p72 (DDX17), can function either as transcriptional coactivators for estrogen receptor-α (ERα) and tumor suppressor p53, or as co-repressors, depending on the promoter context [36–38]. p68 is recruited to the promoter of the ERα target gene, *pS2*, in the presence of estrogen and stimulates transcription from p53-responsive promoters, suggesting a direct role in transcriptional regulation [36,37]. Phosphorylation of p68 activates transcription of the cyclin D and c-Myc genes resulting in an increase in PDGF-induced cell proliferation [39]. p68 is overexpressed in colorectal cancer and overexpression of this DEAD box protein in normal cells results in their transformation [40,41]. p68 is phosphorylated in cancer cell lines but not in normal cells, suggesting that p68 phosphorylation regulates the activity of genes important for tumor formation [42]. Recently, phosphorylated p68 has been shown to be required for epithelial-mesenchymal transition, a process believed to be critical in enabling epithelial cells to become invasive, by promoting cytoplasmic β-catenin nuclear translocation [43].

DDX3, also known as PL10, An3, CAP-Rf and DBX, has been postulated to play a role in nuclear–cytoplasmic shuttling of RNAs, pre-mRNA splicing, mRNA transport and transcriptional activation. DDX3 upregulates the promoter activity of *CDKN1A* ($p21^{WAF1/CIP1}$) and has an inhibitory effect on cell growth [44]. DDX3 is reduced in hepatoma tumors and is translocated from the nucleus to the cytoplasm in cutaneous squamous cell carcinomas [44]. These data support a role for DDX3 in tumor suppression.

DEAD box 1 identification, structure & expression

DEAD box 1 (*DDX1*) was identified by differential screening of a subtracted retinoblastoma cDNA library prepared from two retinoblastoma cell lines, Y79 and RB522A [45]. Subsequent Northern and Southern blot analyses revealed amplified copies of the *DDX1* gene and overexpression of the *DDX1* transcript in a subset of retinoblastoma cell lines with *MYCN* amplification. Like the *MYCN* gene, *DDX1* is located on chromosome 2p24 [45].

In addition to the nine motifs characteristic of DEAD box proteins, DDX1 has a SPRY domain of around 130 amino acids. Although the function of SPRY domains remains enigmatic, they are believed to play a role in either protein–protein or protein–RNA interactions (**Figure 1**) [46–49]. SPRY domains have been identified in more than 70 proteins encoded by the human genome, including heterogeneous nuclear ribonucleoprotein (hnRNP) U and RanBPM. In hnRNP U, the region encoding the SPRY domain mediates binding of TFIIH to the RNA polymerase II holoenzyme [50]. RanBPM binds to the neurotrophin receptor TrkA through its SPRY domain [51].

Table 1. Comparison of the predicted amino acid sequences of DDX1 proteins from different species.

Species	Identity (%)*
Homosapiens	100
Rattus norvegicus	97.7
Mus musculus	97.5
Gallus gallus	93.2
Danio rario	84.1
Drosophila melanogaster	58.7
Caenorhabditis elegans	46.5

**Percent identity is shown relative to human DDX1. The* Danio rerio *(zebrafish) sequence was compiled from CAH68924 and CAI20707.*

Sequence comparison analysis reveals little about the function of DDX1 as the predicted amino acid sequence of DDX1 is approximately 30% identical (over the core 350 amino acid region containing the nine conserved motifs) to other DEAD box proteins, regardless of species. The evolutionary relationship of DDX1 to other members of the human DEAD box protein family is illustrated in **Figure 2**. While DDX1 is not closely related to any of the other human DEAD box proteins, its closest relatives appear to be DDX2 (eIF-4A), DDX6 (p54), DDX19, DDX20, DDX25, DDX39 and DDX48.

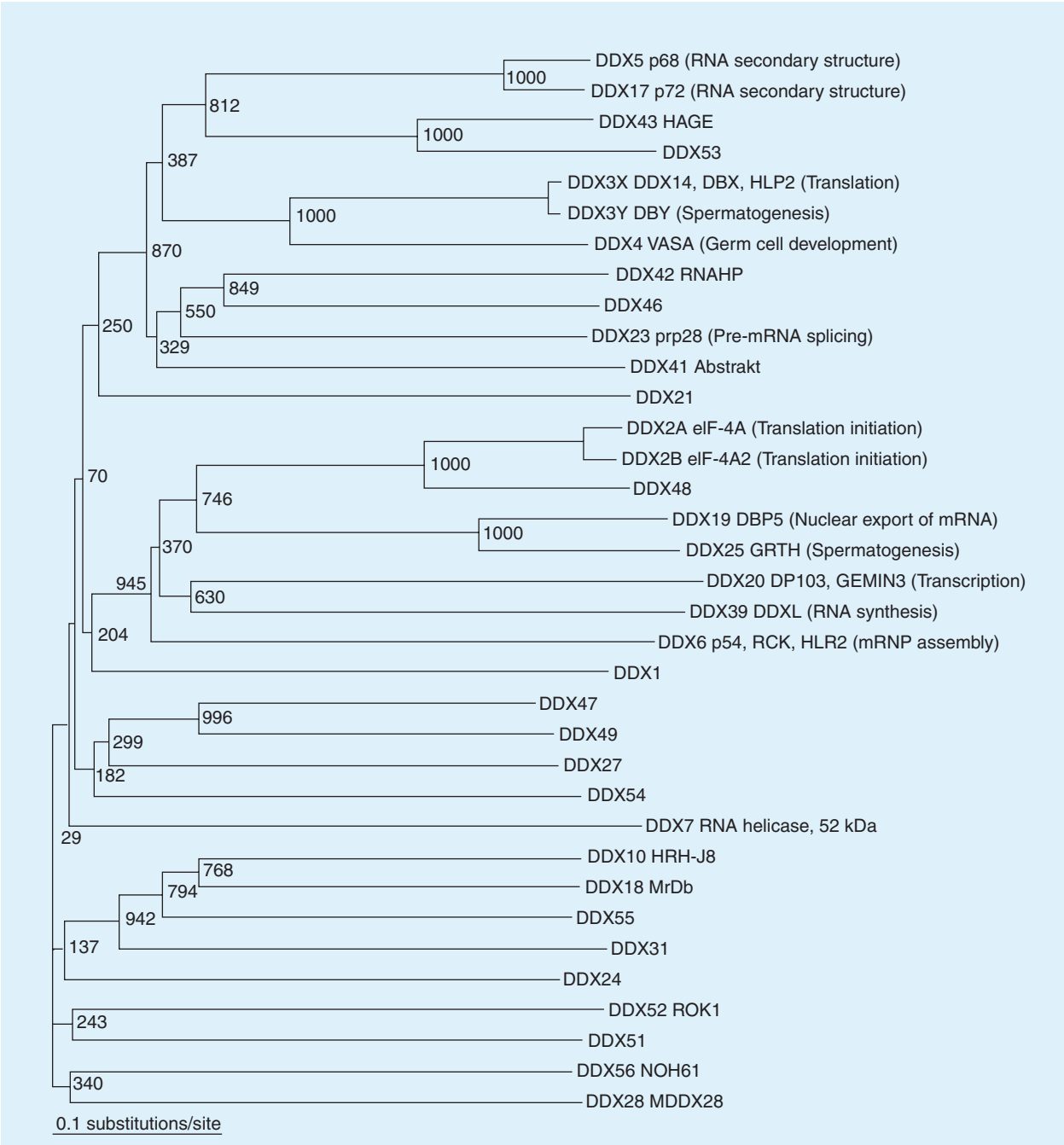
DDX1 is expressed in all tissues and cell lines tested to date, with the highest levels found in proliferating cells, cancer cells and cells of neuroectodermal origin [45,52]. *In silico* analysis of DDX1 tissue distribution reveals the highest levels of *DDX1* RNA in adult eye and brain compared with other adult tissues (**Figure 3**). With the exception of retinoblastoma and nonglioma brain tumors, which have significantly higher numbers of *DDX1*-expressed sequence tags in tumor compared with normal tissue, *DDX1* RNA levels appear similar in normal and cancer cells. DDX1 orthologues are found in rodents (*Mus musculus*, *Rattus norvegicus*), chicken (*Gallus gallus)*, zebrafish (*Danio rerio*), fruit fly (*Drosophila melanogaster)*, and nematode (*Caenorhabditis elegans*; the putative Y55F3BR.1 protein) (**Table 1**).

Coamplification of *DDX1* & *MYCN*

The *DDX1* gene is coamplified with the *MYCN* gene in four childhood tumors: retinoblastoma (of 30 retinoblastoma cell lines tested, four were *MYCN*- and *DDX1*-amplified; none of the lines with normal *MYCN* copy numbers were amplified for *DDX1*) [45, Our Unpublished Data], neuroblastoma (1/2 to 2/3 of *MYCN*-amplified cell lines and tumors) [53–56], alveolar rhabdomyosarcoma (6/6 *MYCN*-amplified tumors) [57] and medulloblastoma (1/1 *MYCN*-amplified tumor) [58]. There is a strong correlation between *DDX1* gene copy number, *DDX1* mRNA levels and DDX1 protein levels in *DDX1*-amplified retinoblastoma and neuroblastoma tumor cell lines and tissues [56,59,60].

The *MYCN* amplification unit usually ranges from 300,000 to more than 1 million base pairs. A core amplification domain of approximately 130 kb was identified by analyzing the commonly amplified DNA regions in

Figure 2. Evolutionary relationship of human *DDX1* with other human DEAD box proteins.



Amino acid sequences of 35 human DEAD box proteins were obtained from the NCBI website [101]. Alternative names are shown after the official DDX designation with the roles of the better characterized DEAD box proteins indicated in parentheses. The bootstrap neighbour-joining tree was constructed with CLUSTALX [91]. Positions with gaps were excluded for the analysis. The bootstrap values (based on number/1000 replicate trials) are indicated on each node.

33 *MYCN*-amplified neuroblastoma tumors. The core domain amplified in 32 out of 33 tumors consists of the *MYCN* gene, 5 kb of DNA telomeric to *MYCN* and 125 kb of centromeric DNA [61]. *DDX1* is located outside the core amplification domain, approximately 310 kb telomeric of *MYCN*. As DNA mutations and rearrangements are common in amplified DNA regions, it is generally believed that only those amplified genes that provide a

growth advantage to a cell or tumor will be overexpressed. *DDX1* overexpression in *DDX1*-amplified cells suggests that elevated levels of this protein play an important role in either the development or maintenance of retinoblastoma and neuroblastoma tumors.

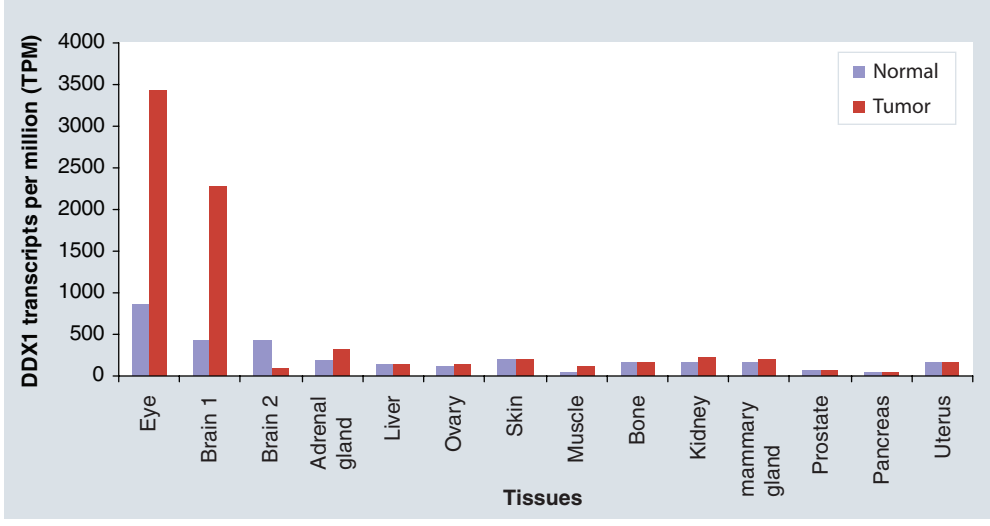
A number of genes other than *DDX1* have been reported to be amplified with *MYCN* in neuroblastoma. These include *NAG* (neuroblastoma-amplified gene), DNA repair protein *NSE1*, *LPIN1* (lipin 1), *SMC6* (structural maintenance of chromosome 6) and *SDC1* (syndecan 1). Of the *MYCN*-coamplified genes, *DDX1* is most consistently amplified and overexpressed in neuroblastoma tumors, followed by *NAG*, which is 380 kb telomeric to *MYCN* (**Figure 4**) [60,62].

There is evidence that the DDX1 protein itself may be tumorigenic. Transfection of NIH3T3 cells with a DDX1 expression vector results in the formation of colonies that demonstrate many hallmarks of cell transformation, including increased growth rates, increased anchorage independence and the ability to form tumors in nude mice [63].

Prognostic significance of *DDX1* amplification

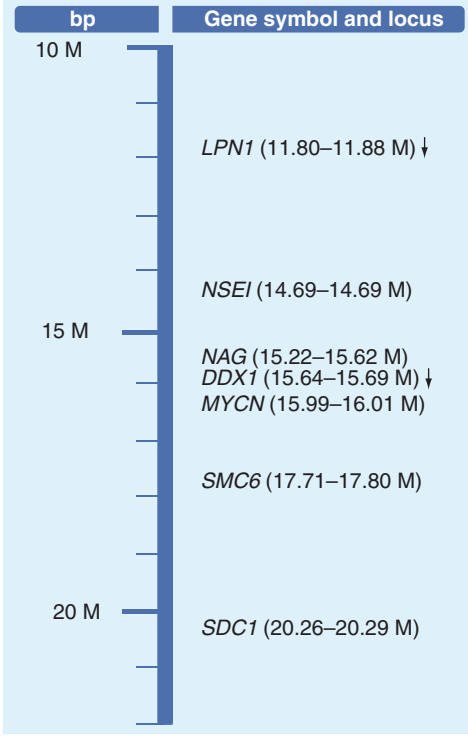
Retinoblastoma is a relatively rare tumor of the eye with a worldwide incidence of approximately 1 in 20,000 live births. An estimated 40% of retinoblastomas are a direct consequence of germline mutations in the *RB1* gene on chromosome 13q14, with tumors arising when the remaining normal copy of the *RB1* gene is inactivated. Children with the heritable form of retinoblastoma have multiple tumor foci distributed in one eye (unilateral) or both eyes (bilateral). Nonhereditary retinoblastoma is unilateral and unifocal since the likelihood of randomly inactivating both copies of the *RB1* gene in more than one target cell in the same individual is exceedingly small. Based on age at diagnosis of bilateral versus unilateral retinoblastoma, Knudson estimated that only two mutations are required for retinoblastoma onset [64]. These data have led to the hypothesis that complete inactivation of the *RB1* gene is sufficient for the development of retinoblastoma. However, in addition to the loss of *RB1* gene function, retinoblastoma tumors harbor a number of common cytogenetic

Figure 3. *DDX1*-expressed sequence tags in cDNA libraries generated from human normal and cancerous tissues.



Expressed sequence tag (EST) counts for each tissue, standardized as number of transcripts per million (TPM), were obtained from UniGene [102]. The number of DDX ESTs per total number of ESTs analyzed for each tissue is as follows: normal eye tissue (171/199770), retinoblastoma (158/46540), normal brain (396/940132), nonglioma brain tumor (294/131694), glioma brain tumor (9/107372), normal adrenal gland (6/33090), adrenal tumor (4/12709), normal liver (30/206292), liver tumor (13/96035), normal ovary (13/101514), ovarian tumor (13/76350), normal skin (41/197072), skin tumor (25/124904), normal muscle (6/107044), soft tissue/muscle tissue tumor (10/125489), normal bone (12/71669), bone tumor (15/99678), normal kidney (35/211066), kidney tumor (16/69067), normal mammary gland (22/152723), breast cancer (18/93685), normal prostate (12/191102), prostate tumor (6/111058), normal pancreas (11/214133), pancreatic tumor (5/104993), normal uterus (36/233118) and uterine tumor (13/90467).

Figure 4. Map of *MYCN* and neighboring genes on chromosome 2p24.



Gene symbols are shown in italics with location on chromosome 2 indicated in parentheses. Transcription direction is indicated by the arrows. M: Million base pairs.

abnormalities, including the acquisition of genetic material at chromosome 6p (usually in the form of an isochromosome 6p) and chromosome 1q [65], suggesting that additional events may be necessary for retinoblastoma tumor formation. In support of this idea, retinomas, benign retinal tumors, are often found in retinoblastoma patients [66], suggesting that initiating events may not be sufficient for malignant cell transformation. Preliminary analysis indicates that these lesions have mutations in both copies of the *RB1* gene [67].

MYCN amplification has been reported in approximately 5–15% of retinoblastomas [68], with *MYCN*-amplified tumors exhibiting increased levels of *MYCN* RNA and protein [69]. Although the *MYCN* amplicon is not as well characterized in retinoblastoma as it is in neuroblastoma, it is noteworthy that *DDX1* is coamplified with *MYCN* in all four *MYCN*-amplified retinoblastoma cell lines identified to date. As the cure rate for retinoblastoma is generally very high, with enucleation being the most common treatment, it may be difficult to address the clinical significance of *MYCN* or *MYCN/DDX1* amplification in retinoblastoma.

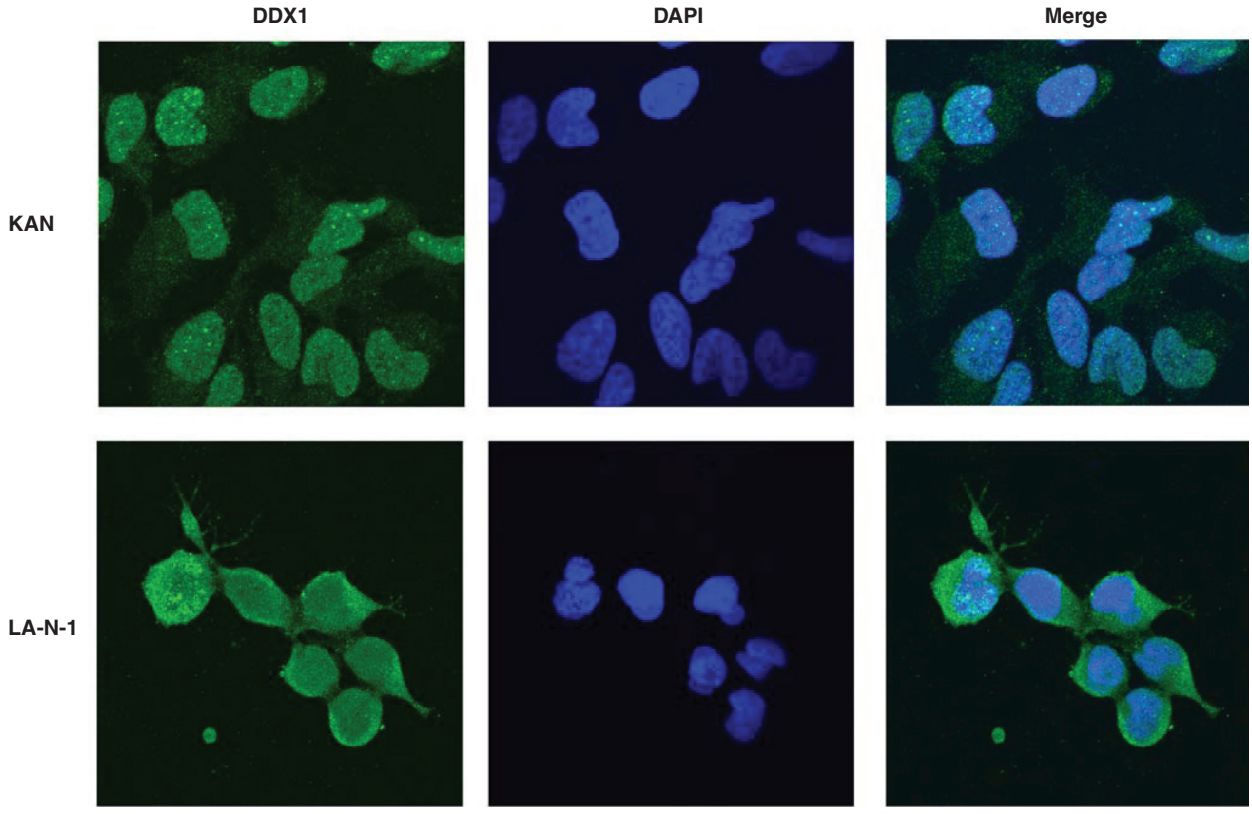
MYCN amplification is observed in approximately 25% of neuroblastoma tumors and is strongly associated with a poor prognosis. The 6-year survival probability for all patients with neuroblastoma is approximately 60%, compared to less than 30% for neuroblastoma patients with *MYCN* amplification. The fact that some patients with *MYCN* amplification are long-term survivors suggests that factors other than *MYCN* determine patient outcome. Two early reports that included relatively small numbers of *MYCN*-amplified neuroblastoma tumors (13 cases in the first study, 38 cases in the second study) showed a trend towards a worse clinical prognosis in *MYCN/DDX1*-amplified tumors in comparison with *MYCN*-amplified tumors [54,70]. However, more recent studies including larger cohorts suggest a better prognosis if *DDX1* is coamplified with *MYCN*. For example, analysis of 98 *MYCN*-amplified primary neuroblastomas revealed a better clinical outcome when *DDX1* was coamplified with *MYCN* ($p = 0.027$) [56]. There was no correlation with outcome for the other *MYCN*-coamplified genes tested, such as *NAG*, *NSE1*, *LPIN1*, *EST-AA581763*, *SMC6* and *SDC1*. Importantly, Weber *et al.* noted that 35% of patients with *DDX1/MYCN* coamplification diagnosed before 1996 (with a minimum follow-up time of 6 years) were long-term survivors in contrast with patients without *DDX1* amplification, all of whom died within 28 months of initial diagnosis ($p = 0.01$) [56]. Although these data were contested by a second group who found no adverse or beneficial prognostic value associated with *DDX1* amplification [71], the difference in results between the two groups could be accounted for by the lower survival probability of the patient cohort in the De Preter *et al.* study (10–15%) compared with the Weber *et al.* study (20–25%). In yet another report, 113 primary neuroblastoma tumors were examined for *MYCN*, *DDX1* and *NAG* amplification and expression. Patients aged 18 months or older were found to have a significantly better outcome if *DDX1*, but not *NAG*, was coamplified with *MYCN* [60]. These data have led to the hypothesis that overexpression of DDX1 may make the tumor more susceptible to therapy. Further insight into the role of *DDX1* amplification and overexpression in clinical prognosis will require analysis of additional cohorts of uniformly treated patients.

Subcellular localization of DDX1

DDX1 is primarily located in the nucleus of non-*DDX1*-amplified cells (**Figure 5**) [59]. However, in *DDX1*-amplified cells, DDX1 is abundant in both the nucleus and cytoplasm, with a staining intensity in the nucleus similar to that of nonamplified cells (**Figure 5**). The fact that nuclear DDX1 does not appreciably increase in *DDX1*-amplified cells suggests that DDX1 levels in the nucleus may be regulated. DDX1 has a punctate pattern in the nucleus and is generally excluded from the nucleoli. In most normal and cancer cells, DDX1 can also be found in dense foci of approximately 0.5 µm (usually 2–3 per cell), known as DDX1 nuclear bodies [72,73].

Different types of nuclear bodies, including cleavage bodies, Cajal bodies and gems, have been identified in the nucleus. Cleavage bodies, Cajal bodies and gems all contain proteins associated with RNA metabolism and have been proposed to serve as storage or assembly sites for factors involved in RNA synthesis, splicing, processing and degradation. Protein markers specific for each nuclear body (cleavage stimulation factor [CstF]-64 for cleavage bodies, p80 coilin or the splicing-associated factor Sm for Cajal bodies and SMN for gems) have been used to examine the spatial relationship of DDX1 and other nuclear bodies as a function of the cell cycle. DDX1 nuclear bodies were found to colocalize with cleavage bodies and to reside adjacent to Cajal bodies and gems during the S phase of the cell cycle [73]. Fluorescence resonance energy transfer and co-immunoprecipitation indicate that DDX1 is not only in close proximity to CstF-64 but also exists in a complex with this protein [72].

Figure 5. Distribution of DDX1 protein in KAN and LA-N-1 neuroblastoma cell lines.



DDX1 is primarily found in the nucleus of non-DDX1-amplified cells (exemplified by the neuroblastoma cell line KAN). Levels of DDX1 in the nucleus of DDX1-amplified LA-N-1 cells are similar to that of KAN; however, DDX1 in the cytoplasm of LA-N-1 is much more abundant than in the cytoplasm of KAN. Method: cells growing on coverslips were fixed in 1% paraformaldehyde in PBS for 10 min and permeabilized for 5 min in 0.5% Triton-X-100/PBS. DDX1 was immunolabelled with anti-DDX1 polyclonal antibody (batch 2923) followed by Alexa 488-conjugated goat antirabbit secondary antibody. Coverslips were mounted onto slides with 1 mg/ml ρ-phenylenediamine containing 1 µg/ml DAPI, which binds to DNA and delineates the nucleus. The signal was detected by immunofluorescence microscopy.

CstF is a heterotrimeric complex (CstF-64, CstF-77 and CstF-50) involved in the 3′ end processing of pre-mRNAs. The cleavage and polyadenylation steps associated with 3′ end processing require the AAUAAA hexanucleotide, which is located 10–30 bases upstream of the cleavage site, and a GU- or U-rich element located 20–40 bases downstream of the cleavage site. The AAUAAA sequence is bound by the cleavage and polyadenylation specificity factor complex, whereas the GU element is bound by the CstF complex, with CstF-64 shown to bind directly to the RNA [74]. In support of a role for DDX1 in 3′ end processing, Sunden *et al.* [75] demonstrated an interaction between DDX1 and CstF-77 using the yeast-two-hybrid system. DDX1 also binds hnRNP K, a multifunctional protein associated with the regulation of transcription, mRNA export, splicing and translation initiation, as well as to poly(A) *in vitro* [76]. The association of DDX1 with CstF-64, CstF-77 and hnRNP K links DDX1 to RNA processing and/or events associated with RNA processing, such as translation initiation and nuclear export.

DDX1 & RNA transport/translation

Kanai *et al.* identified 42 proteins in RNA-transporting granules of neurons that were isolated as binding partners of kinesin KIF5, including DDX1, DDX3 and elongation factor-1 α (eEF1A) [77]. Through coimmunoprecipitation experiments, DDX1 was found in the same complex as eEF1A, a protein involved in translation. These authors postulate that DEAD box proteins located in RNA-transporting granules may play a role in translation initiation similar to that of eIF4A. As an alternative, they propose that DDX1 could play a role in RNA transport, perhaps through interaction with kinesin along microtubules.

DDX1 has been shown to associate with proteins found in pseudopodial protrusions, along with DDX3 and DDX13, and proteins involved in cell signaling such as RACK1, IQGAP1, RhoGD1 and ROCK. The discovery of DEAD box proteins in these structures has led to the hypothesis that pseudopodial proteins may be involved in the recruitment of RNAs for local translation [78]. DDX1 also associates with 14–3-3 proteins [79,80]. 14–3-3 proteins have been implicated in the regulation of a wide range of cellular processes, including cellular trafficking, transcription, RNA processing, protein synthesis and cell cycle.

DDX1 & viral infection

DDX1 plays an important role in the function of Rev, an early gene product expressed during the HIV-1 replication cycle [81]. All HIV-1 proteins are made from a single transcript in its spliced and unspliced forms. Rev binds to the Rev-responsive element, a structured RNA sequence present in the viral transcripts. Rev, with CRM1 as a cofactor, allows nuclear export of transcripts containing introns. DDX1, as well as DDX3, has been shown to interact with Rev and to play critical roles in the export of incompletely spliced HIV-1 RNAs [81,82]. As these results suggest that DDX1 may facilitate HIV-1 replication, this has lead to speculation that DEAD box proteins could be used in novel anti-HIV-1 strategies and therapeutics. In agreement with this, endogenous DDX1 alters the distribution of Rev protein in astrocytes, resulting in an unfavorable microenvironment for Rev function [83].

DDX1 enhances production of the JC virus, a human polyomavirus, by transactivating the JC virus promoter when overexpressed in HEK293 cells [84]. DDX1 has also been shown to bind to the 3′(+) untranslated region (UTR) and reverse complementarity 5′(-) UTR of HCV [85]. As the 3′ UTR is believed to play a role in the initiation and regulation of viral RNA replication and protein translation, binding of DDX1 to this region may be important for viral replication.

Conclusion

Retinoblastoma appears to be a genetically simple and well-characterized tumor, with inactivation of both copies of the *RB1* gene apparently sufficient to at least initiate tumor formation. Yet, a number of recurrent chromosome abnormalities, including *MYCN* and *DDX1* amplification, and the presence of *RB1*$^{-/-}$ benign retinomas in retinoblastoma patients, suggest that additional events may be required to produce the highly malignant phenotype characteristic of these tumors. Furthermore, in spite of extensive mutational analysis, *RB1* mutations in the blood and tumor tissue of patients with retinoblastoma are not always found [86], leaving the door open to the possibility that not all retinoblastomas are initiated as a consequence of loss of *RB1* function.

Although neuroblastoma is not, in genetic terms, as well characterized as retinoblastoma, a number of chromosome abnormalities have been associated with neuroblastoma, including deletions of the short arm of chromosome 1

(25–35% of tumors) and loss of 11q (35–45% of primary tumors) [87]. *MYCN* amplification/overexpression (~25% of primary tumors) is most consistently associated with a poor clinical outcome in neuroblastoma. The effectors of MYCN underlying the aggressive nature of *MYCN*-amplified neuroblastomas have not been conclusively identified. The coamplification of *DDX1* commonly observed in *MYCN*-amplified neuroblastoma suggests a possible role for DDX1 in these tumors.

Based on the proposed roles for DEAD box proteins, overexpression of DDX1 in retinoblastoma, neuroblastoma and other tumor cells could have profound effects on transcript levels, RNA transport and protein production. DDX1 has been shown to associate with factors involved in the processing of pre-mRNAs (CstF-64, CstF-77 and 14–3-3), RNA transport and transcription (hnRNP K, Rev and 14–3-3) and translation (eEF1A). As these processes are interrelated, and DDX1 is found in both the cytoplasm and nucleus, we propose that DDX1 may be linked to mRNAs from the time that they are transcribed in the nucleus to the time they are translated in the cytoplasm. Since DDX1 is specifically enriched in the cytoplasm of *DDX1*-amplified cells, we further propose that the end result of *DDX1* overexpression may be spatial and/or quantitative abnormalities in protein distribution and/or production.

Insight into the function of DDX1 in normal and cancer cells will require a better understanding of the natural DDX1 substrates. Substrates synthesized *in vitro* typically used in RNA helicase/unwinding assays consist of 10–14 bp of dsRNA flanked by relatively long ssRNA ends. Chen *et al.* found that immunoprecipitated DDX1, but not recombinant DDX1, was able to unwind an RNA substrate containing 10 bp, but not 14 bp, of dsRNA, leading to the suggestion that cofactors are required for RNA helicase activity [76]. However, until we identify the endogenous substrates of DDX1 and the proteins with which DDX1 interacts, only limited information can be derived from these experiments. DDX1–RNA and DDX1–protein coimmunoprecipitation experiments may shed light on the types of molecules that are preferentially bound by DDX1. Alternatively, a genomic or proteomic approach revealing which transcripts or proteins are altered as a consequence of *DDX1* overexpression may provide information on the types of molecules targeted by DDX1.

DDX1 amplification is consistently accompanied by a significant increase in *DDX1* RNA and protein, suggesting that increased *DDX1* expression in *MYCN*-amplified tumors may provide either a growth or survival advantage. In this regard, it is interesting that other DEAD box proteins (p54, p68, MrDr and pitchoune) have been associated with Myc [32,34,35,39]. An intriguing possibility is that cross-talk occurs between DDX1 and MYCN. Thus, elevated levels of DDX1 may enhance, balance or even counteract the oncogenic properties of *MYCN*.

In spite of extensive research, DEAD box proteins remain enigmatic molecules. Some of these proteins may be critically involved in a range of fundamental RNA metabolic processes, making it difficult to pinpoint their exact roles in the cell. Others may play specialized roles when associated with their natural substrates and cofactors, conditions that have yet to be reproduced in the laboratory. Future insight into the role of DDX1 in normal and cancer cells will come from the identification of its natural substrates and the cofactors with which it interacts.

Future perspective

Endogenous substrates of DDX1, as well as DDX1 interactors and cofactors, need to be identified if significant progress is to be made in understanding the biological consequence of *DDX1* overexpression in tumors. Identification of the molecules that DDX1 interacts with will provide a more relevant context for examining its biochemical properties and biological role in the cell.

With the exception of one report indicating that *DDX1* overexpression results in transformation of NIH3T3 cells, the consequences of experimentally manipulating DDX1 levels have not been addressed in higher eukaryotes. In *Drosophila*, *DDX1* mutations are associated with an embryonic recessive lethal phenotype [103]. We are currently attempting to generate *DDX1*$^{-/-}$ mice to address the role of DDX1 during development. Our attempts to overexpress *DDX1* in transgenic mice have been unsuccessful even though the *DDX1* gene was inserted in the genome in multiple copies, suggesting that levels of DDX1 may be regulated in nontumorigenic cells. Models need to be developed that allow manipulation of intracellular levels of DDX1 in both normal and tumor cells if we are to make progress in understanding its function.

Finally, *MYCN*-amplified and *MYCN/DDX1*-amplified neuroblastoma tumors provide a natural model system to investigate the consequence

of *DDX1* overexpression on tumor growth properties. Analysis of sufficient numbers of *DDX1/MYCN*-amplified versus *MYCN*-amplified neuroblastoma patients should resolve the issue of whether or not *DDX1* amplification affects patient prognosis. Should *DDX1* amplification and overexpression turn out to be associated with a worse prognosis, there may be ways of targeting *DDX1*-overexpressing tumor cells as part of the therapeutic regimen. For example, a small molecule inhibitor of translation initiation, pateamine, has recently been shown to function through uncontrolled stimulation of eIF4A-associated activities [88]. Furthermore, the tumor suppressor Pdcd4 can bind eIF4A, thus inhibiting its RNA helicase activity and cap-dependent translation of proteins [89]. Identification of molecules and proteins that interact with DDX1 may provide reagents that can be used to inhibit or limit its activity in tumor cells. On the other hand, should *DDX1* amplification and overexpression provide a clinical advantage to patients with neuroblastoma, this may lead to a more individualized approach to the treatment of *MYCN*-amplified versus *MYCN/DDX1*-amplified neuroblastoma tumors.

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Executive summary

- DEAD box proteins are ubiquitously expressed proteins implicated in all aspects of RNA metabolism.
- DDX1 is expressed in every cell type tested to date and enriched in cells of neuroectodermal origin.
- *DDX1* is amplified with *MYCN* and overexpressed in a subset of retinoblastoma and neuroblastoma tumors.
- The clinical significance of *DDX1* amplification in *MYCN*-amplified neuroblastoma remains controversial, although recent studies suggest improved survival when *DDX1* is coamplified with *MYCN*.
- DDX1 is expressed in both the cytoplasm and nucleus. In the nucleus, DDX1 has a punctate distribution pattern and is also found in nuclear bodies.
- During the S phase of the cell cycle, DDX1 nuclear bodies colocalize with cleavage bodies, with DDX1 bodies and cleavage bodies residing adjacent to Cajal bodies and gems.
- Cleavage stimulation factor-64 and -77, heterogeneous nuclear ribonucleoprotein K and elongation factor-1 alpha have been shown to exist in the same complex as DDX1, suggesting roles in processing 3′ ends of pre-mRNAs, as well as in RNA transport and transcription.
- DDX1 is found in RNA-transporting granules of neurons.
- We propose that DDX1 is a multifunctional RNA transport protein involved in facilitating the processing and translation of transcripts.

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