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P68/Ddx5 RNA Helicase Interacts and Co-Localizes *In vivo* with the *De Novo* DNA Methyltransferases Dnmt3a1 and Dnmt3a2

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Abstract

The 5-methyl cytosine (5meC) genomic methylation patterns play crucial roles in mammalian development and are altered in cancer. The enzymes that create, maintain and modify the DNA methylation patterns are the DNA methyltransferases (Dnmts) which are all encoded by essential genes. The *de novo* Dnmts -Dnmt3a and Dnmt3b- establish the DNA methylation patterns early in mammalian development by introducing DNA methylation marks where no previous methylation exists. These enzymes do not exhibit affinity for specific DNA sequences, thus their recruitment to specific DNA loci and their activities must be tightly regulated. In particular, Dnmt3a2 –one of the two protein isoforms produced by the Dnmt3a locus- is the most abundant DNA methyltransferase in mouse Embryonic Stem Cells. To identify Dnmt3a (and DNA methylation) regulators we have searched for Dnmt3a2 interacting proteins in mESCs by pull down and Mass Spectrometry. The *DEAD box* p68/Ddx5 RNA helicase was identified to directly interact with Dnmt3a1 and Dnmt3a2 *in vitro* and *in vivo*. We have created a mutant Ddx5 ($Ddx5^{MUT}$) protein exhibiting an altered nuclear localization pattern compared to the wt protein. Both wt and mutant Ddx5 interact directly *in vitro* and co localize *in vivo* with Dnmt3a proteins. Our data suggest that the Dnmt3a/Ddx5 interaction might be significant for modulating the DNA methylation/demethylation dynamics *in vivo*.

Introduction

DNA methylation in mammals is associated with important developmental phenomena such as genomic Imprinting and X-inactivation and is inversely correlated with transcription initiation [1]. The DNA methylation patterns, thought to characterize each cell type as a molecular fingerprint, are established early in mammalian development and are altered in cancer [2-5]. The mouse DNA methyltransferases (Dnmts), the enzymes that create, maintain and modify the DNA methylation patterns are all encoded by essential genes. These enzymes are categorized according to their ability to introduce new DNA methylation marks into genomic regions where no previous DNA methylation exists (*de novo* Dnmts) or to copy pre-existing methylation into newly synthesized DNA strands in the context of the genomic CpG nucleotides (maintenance enzyme, Dnmt1) [6-9]. The *de novo* Dnmts belong to the Dnmt3 family which contains three members, namely Dnmt3a, Dnmt3b and Dnmt3L. Dnmt3a and Dnmt3b are active enzymes while Dnmt3L is an auxiliary molecule enhancing the enzymatic activity of Dnmt3a/3b proteins [10]. The Dnmt3a locus (together with Dnmt3L) is responsible for establishing the differential DNA methylation marks associated with genomic Imprinting, which results into the "parent of origin"- specific monoallelic expression of ~100 autosomal genes [11]. Additionally, DNMT3A is associated with cancer pathogenesis because it is found mutated in 20% of acute myeloid leukemia patients [12].

The Dnmt3a genomic locus produces two proteins differing in that a 219aa unique N-terminal "tail" lacking from the Dnmt3a2 is present in the longer isoform Dnmt3a1. Dnmt3a1 and Dnmt3a2 proteins are expressed in mouse Embryonic Stem Cells (mESCs) and in early stages of mouse development which are thought to be enriched in Stem and precursor cells. Dnmt3a1 is expressed in low levels in adult somatic cells. In contrast, the Dnmt3a2 mRNA and protein expression ceases to undetectable levels in later stages of development, in adult somatic cells and upon mESCs differentiation [6].

P68/Ddx5 is a prototypical member of the DEAD box family of RNA helicases exhibiting ATPase and RNA unwinding activities. The DEAD box family is named after the amino acid sequence of its conserved Motif II (also known as the Walker B motif) containing the amino acids asp-glu-ala-asp (D-E-A-D). Ddx5 is a multifunctional protein involved in transcription regulation, mRNA splicing, miRNA biogenesis and in DNA damage repair pathways [13]. In particular, in DNA repair, Ddx5 is associated with Thymidine DNA glycosylase (TDG), a protein which is essential for active DNA demethylation [14]. However, the Ddx5 roles in this context are not well understood.

New, exciting findings point towards the notion that the DNA methylation patterns, thought to characterize each cell type as a molecular fingerprint are dynamically built up and erased during development and in adult tissues [15-17]. Currently, our understanding of the DNA methylation/demethylation dynamics involves the actions of several groups of enzymes such as **a)** the DNA methyltransferases (Dnmts) which introduce and maintain the 5-methylcytosine (5meC), **b)** the ten-eleven translocation (TET) family of enzymes that modifies

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5meC by successive hydroxylation and oxidation towards 5-hydroxymethyl cytosine (5hmC), **c)** the activation induced cytidine deaminases (AID and Apobec 1-3) which are thought to deaminate (5meC or 5hmC) and **d)** the DNA glycosylases of the Base Excision Repair (BER) pathway initiating the replacement of modified cytosines (5meC or 5hmC) with unmodified ones thus, effecting DNA demethylation.

To get insights on how the DNA methylation/demethylation dynamics are regulated we sought to identify Dnmt3a2 interacting proteins in Mouse Embryonic Stem Cells (mESCs). We have identified that the p68/Ddx5 *DEAD box* RNA helicase is specifically pulled down from mESCs nuclear extract preparations by a GST-tagged Dnmt3a2 bait protein. We show that the interaction between the two proteins is direct. To explore the functional significance of this interaction *in vivo*, we have created a Ddx5 mutation that is expected to be an active ATPase with a compromised Helicase activity. We have expressed fluorescently tagged (GFP and RFP) versions of Dnmt3a1/3a2 and wt and mutant Ddx5 and examined their sub-cellular localization. We find that unlike wt Ddx5, the mutant protein does not form nuclear speckles. Both wt and mutant Ddx5 proteins co-localize better with Dnmt3a2 and to a lesser extent with Dnmt3a1 in the cell nucleus.

Materials and Methods

Cell lines, plasmids and transfections

HEK 293T were maintained in ES medium [DMEM supplemented with 10% Fetal Bovine Serum (*PAN Biotech*), 50U/ml Penicillin/Streptomycin, 2mM L-Glutamine and 0.1 mM β-Merkaptoethanol. The cells were grown on round cover slips placed in tissue culture dishes and coated with 0.1% gelatin. Transfections were performed using equal molar amounts of GFP/RFP constructs, with the $CaCl_2$ method as described previously [18]. *Dnmt3a1* and *Dnmt3a2* cloning is reported in [18]. The Gal4 DNA binding domain (DBD) bacterial expression vector was constructed by introducing the Gal4-DBD encoding sequences and polylinker from *pBXG1* vector into a pRSET vector background. Dnmt3a2 cDNA was subsequently cloned into the resulting vector (*pR-Gal4*) in frame with the Gal4-DBD sequences. Ddx5 cDNA was obtained via RT-PCR from total RNA of mouse Embryonic Stem Cells (129/3). For reverse transcription and PCR, Superscript III (*Invitrogen*) and Expand Long Template kit (*Roche*) were respectively used. The *Ddx5* cDNAs were first cloned into TOPO vector (*Invitrogen*). The S279A and T281A mutations which alter the SAT motif to AAA were introduced by standard overlapping PCR. The introduction of the double mutation was verified by the presence of a PstI site and by sequencing. Ddx5 Nter and Cter cDNAs were cloned by PCR using the Expand Long Template kit (*Roche*). The wt Ddx5 was cloned into pRSETB vector to acquire the 6xHIS tag. The wt and mutant Ddx5 as well as the Nter and Cter cDNAs were sub-cloned into pEGFPC1 (*Clontech*) and pRFPC1 to acquire the EGFP and RFP tags N-terminally.The wt and mutant Ddx5 cDNAs were also sub-cloned into the pEGFPN3 to obtain the EGFP tag C-terminally.Primer sequences are available upon request.

Gal4 recruitment assay

For the Gal4 recruitment assay, 100ng of a biotinylated DNA fragment bearing five tandem Gal4 binding sites was immobilized on Dynabeads M-280 Streptavidin (*Dynal/Invitrogen*) and loaded with purified Gal4-DBD or Gal4-DBD tagged Dnmt3a2. The coupled beads were incubated with three different amounts of purified recombinant His-Ddx5 or with His-Ddx5^{MUT} proteins. The beads were washed twice with PBS+0.01% Tween and five times with PBS+0.01% Tween plus 0.3 M NaCl. The beads were subsequently boiled in SDS PAGE buffer and analyzed in Western blots.

Fluorescence and confocal microscopy

After 24h the cover slips bearing the transfected cells were washed twice with PBS and the cells were fixed in 4% PFA for 10 min at RT. The cells were washed twice for 5min with PBS. Excess fluid was removed and the cover slips were placed "face down" on a drop of ProLong Gold anti-fade reagent with DAPI (*Invitrogen*) on objective slides, so as the fixed cells to be immersed into the DAPI containing solution. Excess fluid was removed and the preparations were left to dry for 30 min at RT in the dark. Direct fluorescence (figure 3 of this manuscript) was monitored on the Observer D1 fluorescence microscope (*Zeiss*). Areas of interest were scanned with x63 objective lens and images were captured by the AxioVision Rel. 4.8.2 program (*Zeiss*). The co-localization studies of the co-transfected protein constructs were conducted with the use of a Leica TCS SP5 with the dual (Tandem) Scanner (*Leica*). Green and Red direct fluorescence were excited by laser beams at 488nm and 555nm respectively. Emissions were then sequentially acquired. Areas of interest were scanned with a x40 oil immersion objective lens.

Silver staining

The following modified silver staining protocol which is compatible with the LC-MS/MS analysis, was used for the SDS PAGE gel treatment. Briefly, the gels were immersed in a solution of 50% Methanol, 5% Acetic Acid for 20 min, followed by a 10 min wash in 50% methanol and a 2h wash in milli-Q water. The gel was subsequently immersed for 1 min in 0.02% $Na_2S_2O_3$ and was washed briefly twice with milli-Q water. The gel was placed into a 0.1% $AgNO_3$ for 20 min, followed by two brief washes with milli-Q water. The gel was immersed into a solution of 2% Na_2CO_3, 0.04% Formaldehyde until the appearance of the protein bands. The gel was washed twice for 10 min, in 5% acetic acid and stored in 1% acetic acid until the bands were excised for further analysis.

Trypsin digestion and LC-MS/MS analysis

Trypsin digestion and Mass Spectrometry was performed as described in [19].

SEQUEST analysis of MS-results

Tandem mass spectra of the peptides were analyzed by SEQUEST (Bioworks Browser 3.3, Thermo Electron). For this analysis, the trypsin cleavage rule was applied and the peptide mass tolerance was 2.0, and the fragment ion tolerance was 0.5 amu. The search was carried out against the IPI mouse-specific database version 3.55. The peptides were filtered based on the Xcorr scores versus charge state (>1.5 for +1, >2.0 for +2) and the DeltaCN >0.1 [20].

Results

Dnmt3a1 and Dnmt3a2 directly interact with Ddx5 RNA helicase

Dnmt3a1 and Dnmt3a2 are nuclear proteins expressed in mESCs. The bulk Dnmt3a1 and Dnmt3a2 remain chromatin associated upon nuclei extraction with 0.5M NaCl [18].Dnmt3a1 and 3a2 solubilization requires chromatin degradation in addition to 0.65M chromatin extraction. The reduction of the salt concentration of the soluble

Dnmt3a protein extract to physiological levels (0.1-0.15M) by step dialysis, results in excessive Dnmt3a protein precipitation. Thus, this extract is not suitable for use in co-immunoprecipitation experiments.

To identify Dnmt3a2-interacting proteins in mESC nuclear extracts, we used bacterially-produced glutathione S-transferase (GST)-tagged Dnmt3a proteins (Figure 1A). The recombinant GST-tagged Dnmt3a1 and Dnmt3a2 are active DNA methyltransferases *in vitro* [*unpublished data*]. Sepharose-glutathione beads with immobilized GST-Dnmt3a2 protein or control GST, were then incubated with 500 μg soluble nuclear extracts from wild type mouse Embryonic Stem Cells. The beads were washed under high stringency conditions and analyzed by SDS PAGE. Silver staining identified five differentially-appearing bands specifically in the GST-Dnmt3a2 pull-down reaction (Figure 1B, *compare lane GST-3a2 with lane GST-3a2+N.E*). The bands were excised from the gel and underwent trypsin digestion followed by LC-MS/MS Analysis. Four peptides were identified with the set criteria and additional two with lower scores (Table 1). Two of the acceptable peptides were unique for Ddx5. One of them is the tryptic peptide NFYQEHPDLAR. Figure 2 shows the table (**A**) and the spectrum (**B**) of the observed y and b-ions identified by MS/MS analysis, which

IPI:IPI00420363.2	SWISS-PROT:Q61656	Orivavke ATP-dependent RNA helicase RNA helicase DDX5		Score	Coverage
Peptide	MH+	DeltaM	z	XC	DeltaCn
				56.18	10.40
K.APILIATDVASR.G	1226.71033	-0.54730	2	3.54	0.60
K.NFYQEHPDLAR.R	1389.65454	-0.39329	2	2.71	0.49
K.FVINYD YPNSSEDYIHR.I	2131.97192	0.38712	2	2.32	0.47
K.LLQLVEDR.G	985.56769	0.00848	1	1.58	021
R.GLDVEDVK.F	874.45166	0.09570	1	1.17	0.09
R.DWVLNEFK.H	1050.52551	-0.1670	1	0.95	0.09

Table 1: Detailed information of the Ddx5 tryptic peptides identified by SEQUEST. Peptide sequence, precursor mass, DeltaMass, charge (z), Xcorr (XC) and, DeltaCn are displayed.

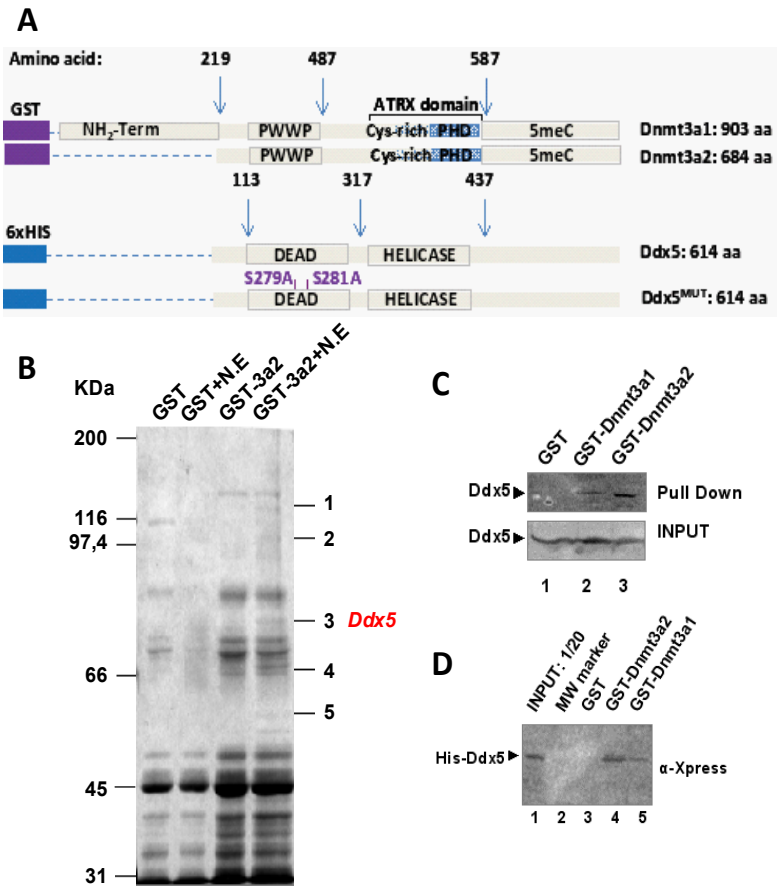

Figure 1: *Dnmt3a* directly interacts with the *DEAD box* p68/*Ddx5* RNA helicase. **A.** Schematic representation of *GST*-tagged *Dnmt3a1* (*Top*), *Dnmt3a2* (*Middle*) and His-tagged Ddx5 and Ddx5^{MUT} (*Bottom*) proteins. Numbers indicate amino acid positions. **B.** Silver staining of an SDS-PAGE gel of pull-down reactions performed with GST-Dnmt3a2 (GST-3a2) and controls from mouse embryonic stem cell nuclear extracts (N.E). **C.** *Western blot of* pull-down reactions performed with GST, GST-Dnmt3a1 and GST- Dnmt3a2 and mESC nuclear extracts. Detection was performed with Pab204 antibody. **D.** *Western blot of* pull-down reactions performed with GST, GST-Dnmt3a1 and GST- Dnmt3a2 and recombinant His-Ddx5 protein. Detection was with α-Xpress antibody.

result from fragmentation of this peptide. Thus, the processed band of approximately 70 kDa was identified as the *DEAD box* Ddx5 RNA helicase, (Figure 1B**,** *band 3,* uniprot accession number: Q61656.as).

To verify this interaction between Dnmt3a2 and Ddx5, sepharose-glutathione beads with immobilized GST-Dnmt3a1, GST-Dnmt3a2 or control GST proteins were incubated with wt mESC nuclear extracts in pull-down reactions. The reactions were washed under high stringency conditions and analyzed in Western blots with anti-Pab 204 monoclonal antibody (*Upstate*, 20), to detect nuclear Ddx5. This antibody recognizes an epitope between aa 483-503 of mouse and human Ddx5. We found that both GST-Dnmt3a1 and GST-Dnmt3a2 proteins pulled down endogenous Ddx5 from the mESC nuclear extract, with a more prominent interaction between GST-Dnmt3a2 and Ddx5 (Figure 1C, *compare lanes 2* and *3*).

To discriminate between a direct or indirect interaction between Dnmt3a and Ddx5, we expressed and purified recombinant His-tagged Ddx5 protein from bacteria under non-denaturing conditions. His-Ddx5 protein migrated at ~70 kDa in SDS PAGE and showed basal ATPase activity *in vitro* [*unpublished data*].

Sepharose-Glutathione beads with immobilized GST-Dnmt3a1, GST-Dnmt3a2 or GST control proteins were used in pull-down reactions with recombinant purified His-Ddx5 protein. His-Ddx5 was detected with anti-Xpress monoclonal antibody in Western blots for both GST-Dnmt3a2 and GST-Dnmt3a1 pull-downs, but not in the control GST reaction (Figure 1D), confirming that GST-tagged Dnmt3a1 and Dnmt3a2 proteins directly interacted with recombinant His-Ddx5 protein *in vitro*.

Dnmt3a1 and Dnmt3a2 co-localize with Ddx5 and Ddx5^{MUT} *in vivo*

To further investigate the interaction between Dnmt3a and Ddx5 *in vivo* we have created a Ddx5 protein with a mutated SAT motif (Ddx5^{AAA} referred to as Ddx5^{MUT}) by introducing a double mutation at amino acid positions S279A and T281A of Ddx5 protein. This results in a SAT motif (or motif III) mutation (SAT to AAA) of Ddx5 [21]. Thus this mutation is expected to preserve the ATPase but to compromise the helicase activity of the protein presumably by disrupting the communication between the two domains [21].

We tested whether wt and mutant Ddx5 proteins interact with Dnmt3a2, in an *in vitro* recruitment assay involving a biotinylated DNA fragment containing five yeast Gal4 binding sites immobilized on streptavidin-coated magnetic beads. Gal4 DNA binding domain (DBD) and Gal4 DBD tagged-Dnmt3a2 (Gal4-Dnmt3a2, shown in Figure 3A) were expressed and purified from bacteria (Figure 4A, bottom) and where left to interact with the immobilized biotinylated DNA fragment. The streptavidine beads where subsequently washed and the unbound protein was removed. The streptavidine beads containing the Gal-DBD and the *Gal4-Dnmt3a2* bound DNA fragments were then incubated with three different amounts of *His-Ddx5* and *His-Ddx5^{MUT}* proteins. The reactions were washed under high strigency conditions, the beads where re-suspended in SDS-loading buffer, were boiled and analysed in western blots. The Gal4-Dnmt3a2 but not the control Gal4-DBD bound DNA fragments, recruited both His-Ddx5 and His-Ddx5^{MUT} proteins in this assay (Figure 4A), suggesting that the Ddx5 mutation did not affect its binding to Dnmt3a2.We conclude that both Ddx5 and Ddx5^{MUT} interact with GAL4-Dnmt3a2 protein *in vitro*.

We have constructed mammalian expression plasmids encoding RFP and EGFP tagged full length Ddx5 and Ddx5^{MUT} proteins, EGFP tagged Ddx5 amino acids 1-437 (termed EGFP-Ddx5 Nter) and EGFP tagged Ddx5 amino acids 438-614 (termed EGFP-Ddx5 Cter). Additionally, we have constructed expression plasmids encoding RFP tagged Dnmt3a1 and Dnmt3a2 mammalian *de novo* DNA methyltransferases. The entire panel of the produced GFP/RFP tagged proteins is shown in Figure 3A.

A

AA	B-ions	Y-ions
N	115.0502039	-
F	262.1186178	1275.611662
Y	425.1819463	1128.543248
Q	553.2405238	965.4799192
E	682.2831169	837.4213417
H	819.3420288	708.3787486
P	916.3947926	571.3198368
D	1031.421736	474.2670729
L	1144.505800	359.2401299
A	1215.542913	246.1560659
R	-	175.1189522

B

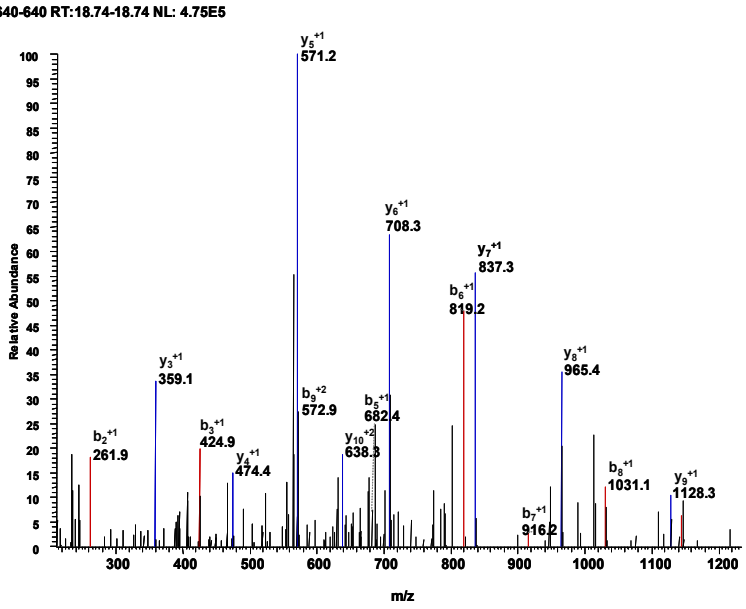

Figure 2: ***MS/MS identification of Ddx5.*** **A.** Table of the observed y- and b-ions identified by the MS/MS analysis resulting from the fragmentation of the doubly charged DDX5 tryptic peptide NFYQEHPDLAR. The parent peptide has a mass of 1389.65 Da. **B.** Spectrum of the observed y- and b-ions identified by the MS/MS analysis resulting from the fragmentation of the doubly charged DDX5 tryptic peptide NFYQEHPDLAR.

First we have tested the sub-cellular localization of EGFP tagged Ddx5 and Ddx5^{MUT} proteins by fluorescent microscopy. Both wt and mutant Ddx5 are located in the nucleus (as defined by the DAPI staining in Figure 3B). EGFP-Ddx5 exhibited a granular staining pattern known as nuclear speckles (Figure 3B, *upper panel, white arrows of inset image A*). In sharp contrast, the EGFP-Ddx5^{MUT} staining is more diffused in the nucleoplasm but it is excluded from the nucleolus; the mutant protein is not forming speckles (Figure 3B, *lower panel, inset image B*).

Next, we investigated the sub-cellular localization of the EGFP-Ddx5Nter and EGFP-Ddx5Cter domains by confocal microscopy. The EGFP-Ddx5Cter protein appears to be exclusively in the nucleus (Figure 3C, *lower panel*). However, the EGFP-Ddx5Nter is predominantly cytoplasmic (Figure 3C, *upper panel*). From the above experiments we conclude that in contrast to Ddx5^{MUT}, the wt Ddx5 forms nuclear speckles and that a strong nuclear localization signal is presumably located at the Ddx5 C-terminus, between amino acids 437-614.

In a second set of experiments, we have examined whether EGFP tagged Dnmt3a1 and Dnmt3a2 co-localize with RFP tagged Ddx5 and Ddx5^{MUT} proteins in the cell nucleus. To this end the mammalian expression plasmids encoding the corresponding tagged proteins were used to co-transfect human embryonic kidney HEK 293T cells. The protein co-localizations were assessed by confocal microscopy. RFP-Ddx5 and RFP-Ddx5^{MUT} co-localize well with EGFP-Dnmt3a2 protein in this assay (Figure 4, *second and fourth panels*).

We have noted that RFP-Ddx5 but not the RFP-Ddx5^{MUT} also forms nuclear speckles in this assay (Figure 4, *compare RFP-Ddx5 and RFP-Ddx5^{MUT} panels*). Additionally, EGFP-Dnmt3a2 appears to form intense nuclear speckles in many cells. Interestingly, these speckles correlate with the Dnmt3a2 enzymatic activity as the Dnmt3a2 C706A mutant which is inactive as a DNA methyltransferase appears to diffusely stain the nucleus [*unpublished data*]. The co-localization of RFP-Ddx5 and RFP-Ddx5^{MUT} with EGFP-Dnmt3a1 is partial (Figure 4, *first and third panels*); however, RFP-Ddx5 and RFP-Ddx5^{MUT} proteins co-localize with EGFP-Dnmt3a1 in ~40% of cells expressing both proteins. We conclude that RFP tagged Ddx5 and Ddx5^{MUT} proteins co-localize with both Dnmt3a proteins albeit their co-localization with Dnmt3a2 is better.

Discussion

Dnmt3a1 and Dnmt3a2 are co-expressed -along with Dnmt3b- in mouse Embryonic Stem Cells (mESCs) and in early stages of mammalian development [6,7]. Previously, we have shown that Dnmt3a1 up-regulates transcription of specific genes at the promoter level and epigenetically silences chromosomal gene clusters in mESCs [18]. Dnmt3a2 is the most abundant *de novo* Dnmt in mESCs [22]. To dissect the Dnmt3a2 specific functions *in vivo*, we aimed to identify and analyze Dnmt3a2 interacting partners. Dnmt3a proteins are difficult material for protein co-immuno precipitation experiments. For example, Tachibana et al. [23] failed to reproducibly detect the G9a and Dnmt3a1 association in ES cells by protein co-immunoprecipitation, although the two proteins interact *in vivo* [18,24]. To circumvent this problem and identify Dnmt3a2 interacting proteins we have added purified and immobilized recombinant glutathione S-transferase (GST)-tagged Dnmt3a2 protein in mESCs soluble nuclear extracts.

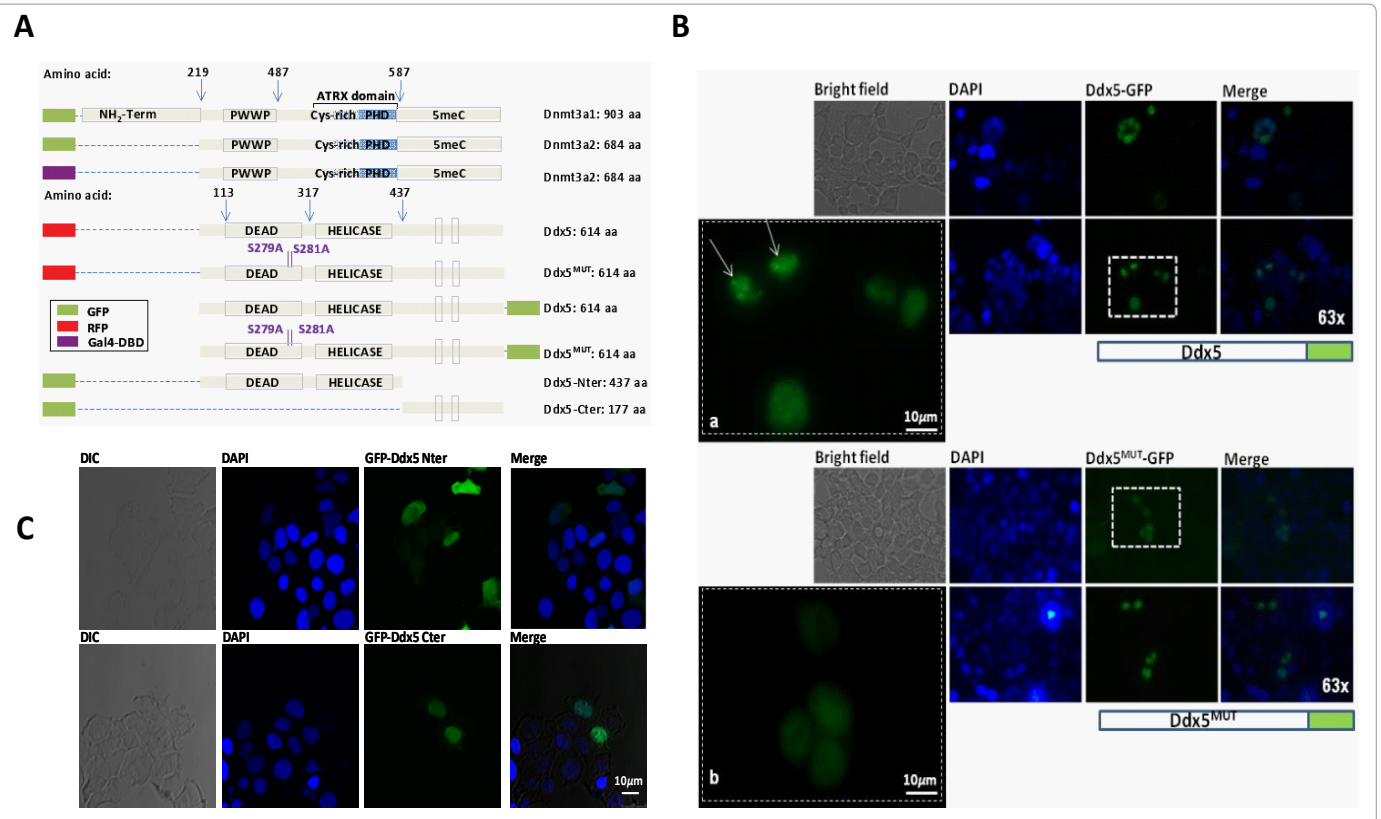

Figure 3: ***Subnuclear localization of GFP-tagged Ddx5 and Ddx5^{MUT} proteins.*** Schematic representation of GFP-tagged Dnmt3a1, Dnmt3a2, Ddx5-Nter, Ddx5-Cter, RFP-tagged Ddx5 and Ddx5^{MUT} and Gal4(DBD)-Dnmt3a2 proteins. Numbers indicate amino acid positions. **B.** Fluorescence microscopy of EGFP-Ddx5 (*Top*) and Ddx5^{MUT} (*Bottom*) proteins. White arrows indicate EGFP-Ddx5 nuclear speckles. **C.** Confocal microscopy of EGFP-Ddx5Nter and EGFP-Ddx5Cter proteins.

Ddx5 was detected specifically in the GST-Dnmt3a2 protein pull down reaction and the two proteins were shown to interact directly *in vitro*.

The *DEAD box* p68/Ddx5 RNA helicase shares an antigenic epitope with the polyoma virus SV-40 large T antigen, a DNA dependent helicase, capable of transforming a variety of cell types. Furthermore, Ddx5 shares with SV-40 large T the ability to interact with p53 tumor suppressor gene and the transcription co-activators CBP/p300 [13]. Ddx5 is a multi-functional protein participating in an astonishing array of biochemical pathways *in vivo*. This variety of Ddx5 *in vivo* functions are difficult to be dissected out by merely knocking the protein out or down, especially because Ddx5 shares some functions with the paralogous protein p72/p82, with which it shares a 77.9% identity. The two proteins are often co-expressed in many cell types [25].To address the unique Ddx5 functions we have created a mutant Ddx5 protein (Ddx5^{MUT}) which is expected to be an active ATPase but to have compromised the coordination between the ATPase and Helicase domains. Here, we show that wt but not the mutant Ddx5 forms nuclear speckles in HEK 293T cells. The formation of nuclear speckles could not be an artifact attributed to protein over-expression because both Ddx5 and Ddx5^{MUT} expression is derived from transcripts emanating from isogenic constructs. Furthermore, our findings agree with immunohistochemistry experiments performed with Pab 204 antibody recognizing Ddx5 which reveal, a granular staining pattern excluded from the nucleoli [26]. We suggest that the ability of Ddx5 protein to form nuclear speckles correlates with its ability to coordinate its ATPase and Helicase activities. What is the nature of the Ddx5 formed nuclear speckles?The metazoan nucleus comprises a dynamic compartmentalized environment containing more than ten membrane-less compartments such as, the nucleoli, the Cajal bodies, the PML bodies, the histone locus, the nuclear stress bodies, the paraspeckles, and the nuclear speckles [27]. The nuclear speckles or Interchromatin Granule Clusters (IGCs) are 30-50 specialized nuclear entities which are enriched in splicing factors and often organize around them actively transcribed genes. For example, the *ERα* regulated genes reposition in the vicinity of the nuclear speckles upon ligand activation, to be coordinately regulated [28]. Furthermore, several types of coding and noncoding RNAs can function as structural elements and as "nucleators" driving the formation of nuclear bodies [29]. Ddx5 functions in splicing [30,31] and is a transcriptional co-activator for nuclear receptor *ERα* [32-34]. Moreover, human DDX5 is associated with the steroid receptor RNA activator (SRA), a non-coding RNA; DDX5 bound to SRA is required for the CTCF insulator function [35]. Taken together our data suggest that Ddx5 participates in the "nuclear speckles". It could thus be the case that the SRA RNA orchestrates the formation of the Ddx5 nuclear speckles. However, whether or not the Ddx5^{MUT} protein interacts with SRA RNA is not yet known.

Finally, the identified interaction between Dnmt3a2 and Ddx5 is a novel and intriguing finding for two reasons. Firstly, Ddx5 is associated with a highly purified ribonucleoprotein (RNP) complex

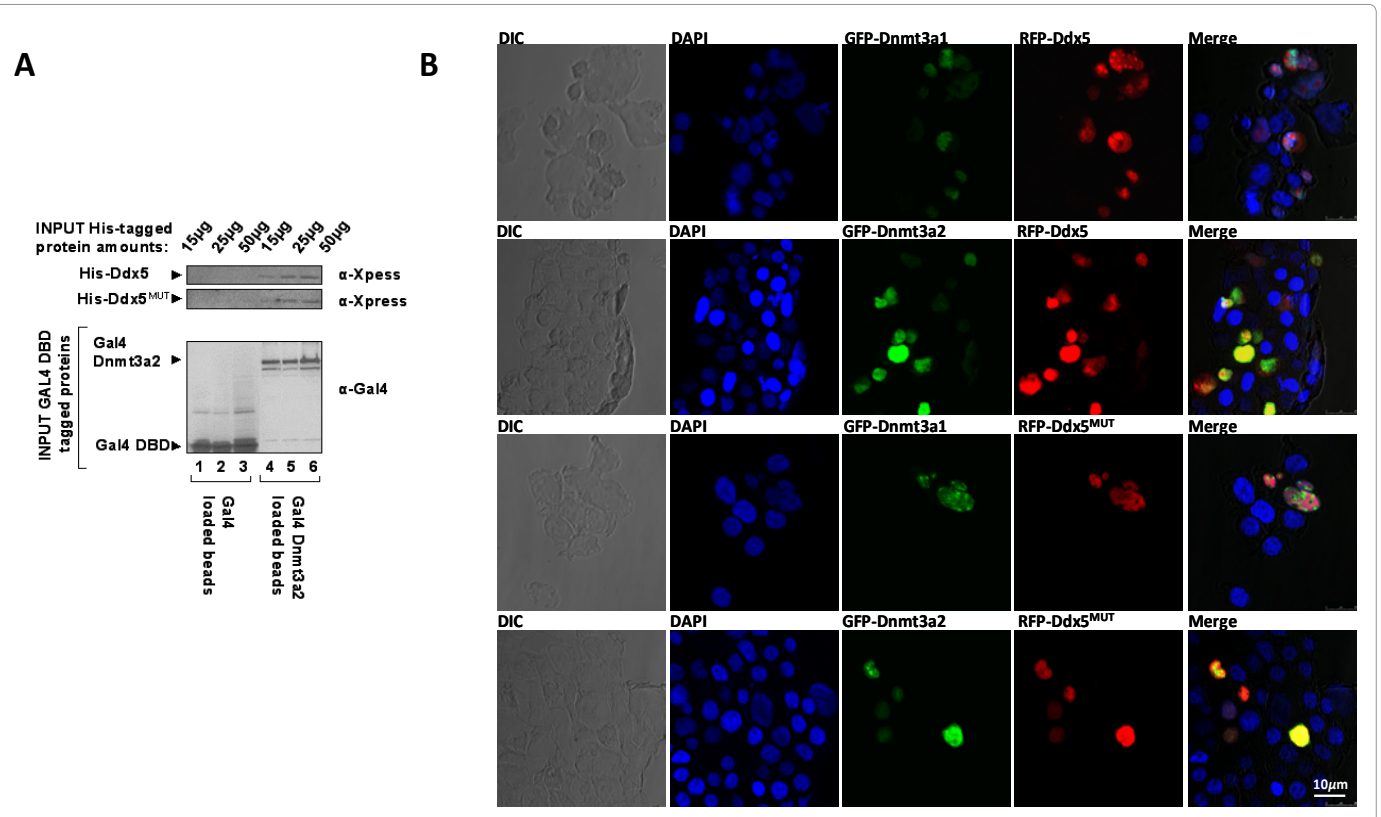

Figure 4: A. ***Ddx5 and Ddx5^{MUT} both interact with Dnmt3a2 in vitro.*** Recruitment assay performed with the yeast Gal4 (DBD) and Gal4-Dnmt3a2 recombinant proteins bound on a biotinylated DNA fragment containing five tandemly repeated Gal4 binding sites, which was immobilized on streptavidine coated magnetic beads. His-Ddx5 (lanes 4-6 *first panel*) and His-Ddx5^{MUT} (lanes 4-6 *second panel*) proteins are recruited to GAL4-DBD-tagged Dnmt3a2 protein-loaded beads but not to the control GAL4-DBD loaded beads (lanes 1-3 *first and second panel respectively)*. His-tagged protein recruitment was detected with the *α*-Xpress antibody. *α*-Gal4 antibody indicates the input amounts of Gal4-DBD (lanes 1-3, *third panel*) and Gal4- Dnmt3a2 proteins (lanes 4-6, *third panel*) in the first and second panel samples. **B.** ***RFP-tagged Ddx5 and Ddx5^{MUT} colocalize with GFP-tagged Dnmt3a1 and Dnmt3a2 proteins.*** *First and third panels*: Confocal microscopy of EGFP-Dnmt3a1 together with RFP-Ddx5 and RFP-Ddx5^{MUT} proteins. *Second and fourth panels*: Confocal microscopy of EGFP-Dnmt3a2 together with RFP-Ddx5 and RFP-Ddx5^{MUT} proteins.

combining 5meC DNA methyltransferase and glycosylase activity in chicken embryos [36]. Secondly, Ddx5 along with TDG and Dnmt3s are recruited via ligand-activated nuclear receptors, to the *Vtn* and p*S2* gene promoters in mouse and human cell lines respectively [34,37]. These promoters undergo active DNA demethylation prior to the transcription activation of the corresponding genes and the *de novo* Dnmts are switching activity and instead of functioning as DNA methyltransferases, they perform deamination of 5meCs. The deamination reaction produces thymines opposite to guanines; then, the Base Excision Repair (BER) pathway intervenes to excise the mistaken base. Thymidine DNA Glycosylase (TDG) is critical in the removal of the mistaken base. However, Ddx5 which is also recruited in demethylated promoters plays a yet unidentified role in the context of BER. In the light of our finding that Ddx5 and Dnmt3a proteins directly interact, we suggest that Ddx5 could modulate the Dnmt3a methyltransferase activity and play a fundamental role in the regulation of DNA methylation/demethylation dynamics on activated promoters. This complex could combine the Dnmt3a DNA methylation activity and the TDG demethylation action in one molecular entity. One would anticipate that a methyl-cytidine deaminase would also participate in this complex. Alternatively, because Dnmt3a in association with TDG is suggested to deaminate 5meC, Ddx5 could play a regulatory role in the switching of the complex activity from DNA methylation to DNA demethylation. Which is the case, will be revealed in future experiments, where the $Ddx5^{MUT}$ that we have created will be a useful tool in the investigation of this hypothesis.

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