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Regulation of the human Suv3 helicase on DNA by inorganic cofactors

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Abstract

Mitochondria are essential organelles and consequently proper expression and maintenance of the mitochondrial genome are indispensable for proper cell function. The mitochondrial Suv3 (SUPV3L1) helicase is known to have a central role in mitochondrial RNA metabolism and to be essential for maintenance of mitochondrial DNA stability. Here we have performed biochemical investigations to determine the potential regulation of the human Suv3 (hSuv3) helicase function by inorganic cofactors. We find that hSuv3 helicase and ATPase activity *in vitro* is strictly dependent on the presence of specific divalent cations. Interestingly, we show that divalent cations and nucleotide concentration have a direct effect on helicase substrate stability. Also, hSuv3 helicase is able to utilize several different nucleotide cofactors including both NTPs and dNTPs. Intriguingly, the potency of the individual nucleotide as energy source for hSuv3 unwinding differed depending on the included divalent cation and nucleotide concentration. At low concentrations, all four NTPs could support helicase activity with varying effectiveness depending on the included divalent cation. However, at higher nucleotide concentrations, only ATP was able to elicit the helicase activity of hSuv3. Consequently, we speculate that the capacity of hSuv3 DNA unwinding activity might be sensitive to the local availability of specific inorganic cofactors.

Keywords

Helicase; Cofactor; Nucleotide; Divalent cations; DNA binding protein

1. Introduction

Mitochondria are essential organelles serving multiple roles, which are indispensable for normal cell function [1]. According to the Warburg hypothesis, a driving force in

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Conflict of interest

None.

tumourigenesis is a change in cell energy metabolism due to insufficient mitochondrial function. Recently, maintenance of the mitochondrial DNA (mtDNA) was causatively linked to cancer suppression in the mouse model of the mitochondrial helicase Suv3 [2]. Heterozygous (*mSuv3*$^{+/-}$) mice were found to be predisposed to cancer, a phenotype which was aggravated through each generation. The pathologies were passed maternally to both offspring with heterozygous (*mSuv3*$^{+/-}$), but also homozygous (*mSuv3*$^{+/+}$) *SUV3* gene status. In addition, cohort studies of human breast cancer patients found the expression of human Suv3 (hSuv3) to be reduced in the malignant cells compared to the normal cells. Accordingly, hSuv3 was classified as a tumor suppressor [2].

Originally, the Suv3 protein was described to play a role in mitochondrial RNA metabolism, where it forms the degradosome complex in association with a nuclease [3–5]. In *Saccharomyces cerevisiae* and *Trypanosoma brucei* the nuclease is believed to be Dss1p, while in humans it is believed to be PNPase [4–8].

In addition, a potential role for Suv3 in DNA metabolism has been speculated. Loss of Suv3 is associated with a decrease in mtDNA copy-number in yeast, mice and human cells [2,9,10]. Inactivation of Suv3 in mice results in an increase in mtDNA mutations [2]. *In vitro*, hSuv3 has a preference for dsDNA over RNA substrates [11] and in *S. cerevisiae* Suv3 was found to associate with active origins of replication, without the Dss1p nuclease, suggesting a potential direct role in mtDNA replication [10]. In addition to the major mitochondrial function, a small fraction of hSuv3 localizes to the nucleus [12–14]. In the nucleus, knockdown of hSuv3 results in an elevated incidence of sister-chromatid exchange [14] and hSuv3 has been found to have several nuclear interaction partners involved in nuclear genome maintenance including BLM, WRN, RPA and FEN1 in human cells [12,14] and Sgs1, Ddc1 and Mec3 in yeast [15].

The Suv3 protein belongs to the SF2 superfamily of helicases [16]. It catalyzes the ATP-dependent separation of double stranded DNA and RNA substrates [11,12]. The hSuv3 unwinding activity is known to be strictly dependent on the presence of a divalent cation [11], however, the specific efficiency of different divalent cations is unknown. Stabilization of nucleotide binding by (RecA)-like helicase domains by way of coordination with a divalent cation is a common feature among most SF2 helicase family members [17]. Previously, various protein activities were shown to be differentially affected by different divalent cations. Interestingly, the mitochondrial polymerase gamma (Polγ) requires either Mg^{2+} or Mn^{2+} for *in vitro* activity, and while the biological role is currently unclear, Mn^{2+} supports reverse transcriptase activity of Polγ at the cost of fidelity [18,19]. Also, the helicase activity of the WRN helicase can be supported by Mn^{2+} or Ni^{2+} in addition to Mg^{2+}, while its exonuclease activity is stimulated in the presence of Zn^{2+} [20].

Suv3 is highly conserved across species but shares less than 25% sequence similarity to any known protein structure [16,21]. Based on sequence analysis and inspection of the crystal structure it was found that of the classical nine DEAD and DExD/H box helicase motifs (Q, I, Ia, Ib, II–VI), Suv3 lacks the Q-motif involved in ATP binding [21,22]. Normally, residues within the Q-motif interact specifically with atoms of the adenine group of ATP [22]. Instead, the hSuv3 protein was found to interact with the nucleotide via base-stacking

between aromatic amino acids within a RecA domain with no specific interactions between protein and the adenine base atoms [21]. This may suggest that other nucleotides can be utilized by hSuv3 as well.

ATP is a major energy cofactor within the cell and is used by many different proteins in various cellular pathways [17]. Some helicases are restricted to the use of ATP, while others are more permissive allowing the use of other classic nucleotides e.g. UTP, GTP and CTP [23]. The mitochondrial helicase Twinkle is able to utilize all four ribonucleotides, with UTP resulting in the most efficient dsDNA unwinding activity [24,25]. In *T. brucei*, the mitochondrial degradosome activity was found to be stimulated *in organello* by increasing local UTP concentration. The ability of Suv3 to utilize energy cofactors other than ATP has not been addressed previously, although it may be important in regulating activity according to local or global changes in energy metabolism and nucleotide pools.

Here we examined the effect of inorganic cofactors on hSuv3 activity. Using an *in vitro* biochemical assay, we have analyzed the ATPase and helicase unwinding activity of hSuv3 in the presence of different divalent cations. Having identified the divalent cations that are able to support hSuv3 function, we next compared the efficiency of hSuv3 in the presence of the four major ribonucleotides, ATP, UTP, GTP and CTP, finding a divalent cation dependent difference in hSuv3 DNA unwinding efficiency between the ribonucleotides analyzed.

2. Material and methods

2.1. Recombinant proteins

Recombinant hSuv3 protein, hSuv3-(47–722)-WT and hSuv3-(47–722)-K213A helicase-dead mutant, were expressed in *E. coli* and purified to apparent homogeneity as described previously (Fig. S1) [12].

2.2. Oligonucleotide substrate

PAGE-purified oligonucleotides were used for preparation of substrates: D49, 5′-TTT GTT TGT TTG TTT GTT TGT TTG CCG ACG TGC CAG GCC GAC GCG TCC C-3′; D50-2, 5′-GGG ACG CGT CGG CCT GGC ACG TCG GCT TTG TTT GTT TGT TTG TTT GTT TT-3′. ^{32}P 5′-end labeling of the D50-2 oligonucleotide was performed as described previously [12]. In brief, D50-2 was incubated with [γ-^{32}P] ATP and T4 polynucleotide kinase at 37 °C. Labeling was terminated by adding EDTA to a final concentration of 10 mM. Unincorporated [γ-^{32}P] ATP was removed using a G-25 spin column. KCl was added to the eluate to a final concentration of 50 mM together with 1.5-fold excess of the D49 oligonucleotide and the two were annealed by initial incubation at 70 °C for 10 min, followed by slow-cooling of the reaction to room temperature.

2.3. Helicase activity assay

Helicase unwinding assessment was performed as described previously with some modifications [12]. Briefly, the recombinant hSuv3(47–722) protein (50 nM) was added to the reaction buffer containing 20 mM Tris–HCl, pH 7.4, 1 mM ATP if not otherwise indicated, 3 mM divalent cation if not otherwise specified, 1 mM DTT, 5% glycerol and 0.1

mg/ml BSA. The reactions were initiated by addition of the ^{32}P 5′-end-labeled substrate (0.5 nM), incubated at 37 °C for 30 min, and terminated by addition of helicase stop buffer to a final concentration of 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 10% glycerol, 0.3% SDS, 0.01% Bromophenol Blue and 5 nM unlabeled oligonucleotide. The products were resolved on a native 12% polyacrylamide gel and detected using a PhosphorImager followed by analysis using QuantityOne software.

2.4. ATPase activity assay

ATPase activity assay was conducted as described before, but with some modifications [26]. In short, reactions were initiated by adding recombinant hSuv3(47–722) protein (50 nM) to the reaction buffer containing 20 mM Tris–HCl, pH 7.4, 990 μM ATP, 10 μM γ-P^{32} ATP, 3 mM divalent cation, 1 mM DTT, 5% glycerol and 0.1 mg/ml BSA with or without 0.5 nM unlabeled DNA. Reactions were incubated at 37 °C for 30 min and stopped by adding EDTA to a final concentration of 50 mM. Samples were analyzed by thin layer chromatography in 0.75 M KH_2PO_4 and the signal was detected using a PhosphorImager followed by analysis using QuantityOne software.

2.5. DNA filter binding

DNA filter binding was performed as described by others with modifications [27]. Recombinant hSuv3(47–722) protein (25, 50 and 100 nM) was added to the reaction buffer containing 20 mM Tris–HCl, pH 7.4, 1–5 mM AMP-PCP, 3 mM divalent cation (as indicated), 1 mM DTT, 5% glycerol and 0.1 mg/ml BSA. The reactions were initiated by addition of the ^{32}P 5′-end-labelled single stranded D50-2 substrate (0.5 nM) and incubated at 37 °C for 30 min. Reactions were moved to ice and loaded directly on a double membrane platform with a nitrocellulose membrane on top of a nylon membrane binding the flow through DNA not bound by protein. The membranes were equilibrated in and washed with the binding-buffer: 50 mM Tris pH 7.5, 5% glycerol and 1 mM EDTA. The result was visualized using a PhosphorImager followed by analysis using QuantityOne software.

2.6. DNA melting temperature

For assessment of DNA melting temperature (Tm) reactions contained 250 nM annealed, unlabeled D50-2/D49 DNA fork, 20 mM Tris–HCl, pH 7.4, 1 mM ATP, 3 mM divalent cation (as indicated), 1 mM DTT, 5% glycerol, 1X SyBR green (Invitrogen) and 0.1 mg/ml BSA. Reactions were analyzed using a Stratagene Mx3000P qPCR analyzer determining a dissociation curve from 37 °C to 98 °C.

3. Results

Recently, a crystal structure of the hSuv3 helicase was published describing both typical helicase motifs and a unique nucleotide-binding pocket [18]. Therefore, the combination between divalent cation and nucleotide energy molecules may be important for hSuv3 function. In order to analyze the potential implications of these unique features on hSuv3 activity, we investigated the effect of inorganic cofactors on hSuv3 activity *in vitro*.

3.1. The hSuv3 helicase and ATPase activity is affected by divalent cation in vitro

To determine the specificity of divalent cation requirement on hSuv3 unwinding activity, we tested a variety of divalent cations by substituting the commonly used Mg^{2+} with either Mn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} or Zn^{2+} (Fig. 1A and B). In addition to Mg^{2+}, Mn^{2+} was also able to promote hSuv3 unwinding activity, whereas none of the other tested cations supported hSuv3 unwinding activity. The ATPase mutant, hSuv3-K213A, was unable to produce any significant unwinding for either of the cations, supporting that the product observed was indeed due to hSuv3 helicase activity. Preliminary testing also included Co^{2+}, however, our specific experimental conditions resulted in a DNA-protein complex resistant to high SDS and protease treatment, rendering it impossible to determine helicase activity. Consequently, this cation was omitted.

The helicase assay provides an estimation of the ability of the divalent cations to support active unwinding of the helicase alone. The unwinding rate depends on both the translocation velocity and the activation energy barrier of separating the base-pairs [23,28]. The classifying enzymatic activity of helicases is their ability to lower the activation energy barrier, which may vary depending on conditions e.g. temperature. Consequently, it is possible that certain divalent cations can support hSuv3 translocation while not enabling sufficient unwinding. In order to estimate the ability of the different divalent cations to support hSuv3 translocation, the *in vitro* ATPase activity of hSuv3 was determined. Recombinant hSuv3 had a low but similar level of ATPase activity in presence of Mg^{2+} compared to Mn^{2+} (Fig. 1C), in contrast to what was observed for helicase unwinding activity where Mn^{2+}-dependent activity was higher compared to Mg^{2+} (Fig. 1A–B). Surprisingly, Ni^{2+} and Zn^{2+} supported an even higher ATPase activity (Fig. 1C), suggesting that Ni^{2+} and Zn^{2+} are able to efficiently support helicase translocation but not double stranded DNA separation. While the ATPase activity in the presence of Mg^{2+} or Mn^{2+} was not affected significantly by the absence or the presence of DNA (data not shown), the helicase activity was substantially higher in the presence of Mn^{2+} compared to Mg^{2+}. Thus, rate of helicase product formation was higher in the presence of Mn^{2+} than in presence of Mg^{2+} at all time-points tested between 1 and 30 min (Fig. 2A, S2A).

3.2. The ratio between cation and nucleotide cofactor affects hSuv3 helicase activity in vitro

One possible contribution to the observed difference may be a variation in the binding of ATP by the two divalent cations. The energy co-factor ATP is considered to be biologically active only when it is in complex with a positively charged divalent cation, which stabilizes the negative charge of ATP (ATP^{4-}). Based on preliminary titrations, we found that the potency of ATP in supporting hSuv3 unwinding activity is highly dependent on Mg^{2+} concentration, with the optimum being 1 mM ATP and 3 mM $MgCl_2$ (unpublished work). Based on calculations by Storer and Cornish-Bowden, approximately 90% of ATP should be in complex with Mg^{2+} at the ratio used in our experiments [29], resulting in the true concentrations of these ions of approximately Mg_2:ATP (0.9 mM), Mg^{2+} (1.2 mM) and ATP^{4-} (0.1 mM). Importantly, these numbers may be different for Mn^{2+} depending on the binding strength of the Mn_2:ATP complex. Thus, it is possible that the optimal ratio between ATP and the divalent cations in relation to hSuv3 activity differs between Mg^{2+} and Mn^{2+},

explaining the difference in product formation between the two. Consequently, we tested a variety of different concentrations of ATP and Mg^{2+} (Fig. 2B, blue bars) or Mn^{2+} (Fig. 2B, orange bars). Low (0.5 mM) and high (10 mM) concentrations of divalent cation inhibited helicase activity. However, at none of the combinations tested, did reactions containing Mg^{2+} result in higher unwinding activity than reactions containing Mn^{2+} (Fig. 2B, S2B).

3.3. The divalent cations affect substrate stability

An alternative contribution to the observed difference between Mg^{2+} and Mn^{2+} may relate to interactions between the divalent cation and the DNA substrate. During the systematic investigation a slight difference was observed in the level dsDNA destabilization for the negative control without protein (Fig. 1A, lane 1), which was around 1–3% product formation for Mg^{2+} and 2–4% for Mn^{2+}. This raised the question of whether differences in cation-DNA interactions contributed to the observed difference. In addition, divalent cations also serve an important role in DNA stability because they act as enzyme cofactors. As such, duplex DNA has been shown to be stabilized against thermal or acid denaturation by alkaline earth metals such as Mg^{2+} and Ca^{2+}, while transition metals (Mn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}) caused destabilization [30]. A difference between DNA melting temperatures would entail different energy requirement for separation of base pairs in the duplex region. To address this issue, the DNA melting temperature (Tm) of the D50-2/D49 substrate in presence of the various divalent cations was assessed by generating a DNA dissociation curve. The T_m varied quite significantly depending on the choice of divalent cation (Table 1). Mn^{2+} lowered the T_m 4.5 °C compared to Mg^{2+} (Table 1). This may explain the difference in observed efficiency of helicase unwinding, as less energy was required to unwind the DNA fork duplex in presence of Mn^{2+} compared to Mg^{2+} due to lowered activation barrier for base-pair separation. Meanwhile, several of the other cations, which did not elicit any DNA unwinding, had an even lower T_m than Mn^{2+}. Therefore, the product formation in the presence of Mn^{2+} should be considered the result of protein activity of hSuv3. However, based on these results it cannot be concluded whether the higher product formation is due to improved hSuv3 catalytic activity, to a lowered T_m, or to a combination of both.

3.4. Comparable substrate T_m determined by titrating nucleotide concentration against cation concentration

In order to establish reaction conditions where the T_m is comparable between Mn^{2+} and Mg^{2+}, a titration of ATP was performed. Divalent cations in complex with ATP^{4-} are unable to interact with DNA and thereby affect its stability. Considering the chemical equation: $2Mx^{2+} + ATP^{4-} \leftrightarrow Mx_2ATP$ where Mx is the divalent cation, it can be speculated that the concentration of free divalent cations can be reduced by increasing the concentration of ATP, effectively decreasing the proportion of the divalent cation interacting with DNA. Therefore, we determined the T_m of the helicase substrate D50-2/D49 at increasing ATP concentration from 0 to 8 mM while keeping the divalent cation concentration at 3 mM (Fig. 3). The stabilizing effect of Mg^{2+} could indeed be affected resulting in a reduction in T_m by app. 2 °C increasing the ATP concentration from 0 to 5 mM. Meanwhile, increasing the ATP concentration in Mn^{2+} based buffers caused an increase in substrate T_m of almost 6 °C from 0 to 5 mM ATP. From the point of 5 mM ATP, the T_m of the substrate was 79 °C for both

Mn^{2+} and Mg^{2+} and further increasing the ATP concentration did not change the T_m significantly. Consequently, with regard to the helicase substrate, the reaction conditions for Mn^{2+} and Mg^{2+} can be considered comparable using 5 mM ATP in combination with 3 mM divalent cation. However, as these settings clearly constitute suboptimal conditions for unwinding activity (Fig. 2B), no strong conclusion can be made in regard to the more potent cofactor of the two divalent cations. Collectively, these findings advise caution in interpretation of data based on comparison of cation-dependent helicase activities, as divalent cations directly affect the substrate as well as the protein.

3.5. High nucleotide concentration results in lowered DNA binding by hSuv3 in vitro

To gain further insight into the molecular mechanisms underlying the observed differences in helicase activity between divalent cations and ATP concentrations, we analyzed the DNA binding activity of hSuv3. The ability of hSuv3 to retain radioactively labeled DNA on a protein binding membrane was assessed in the presence of a non-hydrolysable ATP analog, AMP-PCP, to avoid substrate release as a result of ATP hydrolysis (Fig. 4, lane 1–8, S3). A DNA binding nylon membrane was used to collect the flow through DNA to verify equal amount of labeled DNA between samples (Fig. 4, lane 1*–8*). Testing the DNA binding of increasing amounts of recombinant hSuv3 protein revealed a trend toward slightly more efficient binding of hSuv3 in the presence of Mn^{2+} compared to Mg^{2+} (Fig. 4, compare row 2–4 and 6–8 in the top panel with row 2–4 and 6–8 in bottom panel, respectively). Binding by the K213A ATP-binding mutant was considerably reduced compared to the WT protein (Fig. 4, compare A and C with B and D, respectively), suggesting that ATP binding stabilizes the interaction between DNA and hSuv3. Furthermore, increasing the ATP concentration to 5 mM resulted in a significantly lowered substrate binding compared to experiments with low ATP (1 mM) for both divalent cations (Fig. 4, compare lane 2–4 with 6–8). Consequently, less DNA binding and higher substrate T_m are likely to contribute to the lower Mg^{2+}-dependent helicase activity of hSuv3 compared to Mn^{2+} at low ATP concentrations (1 mM) in spite of comparable translocating activity.

3.6. The hSuv3 helicase activity can be supported by different nucleotides and the potency of the individual nucleotide depends on the individual divalent cation

As described above, the crystal structure of hSuv3 has revealed that hSuv3 lacks the Q-motif sequence, which normally contains amino acids that interact specifically with the adenine ring of ATP through hydrogen bonds [21]. Instead aromatic amino acids perform base stacking with the adenine ring through π–π interactions. This binding entails a much less stringent recognition of the energy cofactor, suggesting that the ribonucleotide requirements of hSuv3 could be met by ribonucleotides other than ATP. When testing hSuv3 unwinding activity at optimal conditions (1 mM nucleotide), we found the activity to be comparable with either one of ATP, UTP, CTP and GTP alone when the reaction contained Mg^{2+} (Fig. 5A and B). In addition, NTPs were tested to determine if the presence of all four ribonucleotides together would affect hSuv3 unwinding activity. As the concentration of the individual ribonucleotides was 1 mM, the same concentration was used for mixed NTPs. However, 1 mM NTP would contain four times as many negative charges as 1 mM of a single nucleotide, e.g. ATP. Therefore, an additional sample of 0.25 mM NTP was included, which contained a sum of charges corresponding to the 1 mM ATP. Again, it seems that the

ratio between charges and divalent cation had a substantial impact on activity, as 0.25 mM mixed NTPs resulted in an activity corresponding to 1 mM of each ribonucleotide alone (Fig. 5A and B). Based on these findings we conclude that there is no inhibitory or augmented effect of a mixed ribonucleotide pool on Mg^{2+}-dependent activity of hSuv3.

When substituting Mg^{2+} with Mn^{2+} the outcome changed. The presence of UTP resulted in a much lower hSuv3 unwinding activity in comparison to the other three ribonucleotides (Fig. 5C and D). The value for NTP corresponds to the average of the four ribonucleotides alone, which suggests that UTP does not cause competitive inhibition of hSuv3 activity by ATP, CTP and GTP. Based on this it seems likely that the binding of UTP in the active site is much lower compared to the others, since no negative competition is observed. The T_m of the D50-2/D49 substrate did not vary significantly between the different ribonucleotides (Data not shown).

Collectively, hSuv3 is able to utilize four different ribonucleotides, ATP, UTP, GTP and CTP, with the efficiency depending on the identity of the divalent cation included in the reaction.

Interestingly, we found that dNTP could also serve as an energy source for hSuv3 helicase activity, although resulting in lower activity than NTP (Fig. 5A–D). Based on this finding, we further examined the efficiency of the individual dNTPs as an energy source for hSuv3 unwinding activity in presence of Mg^{2+} or Mn^{2+} (Fig. 6A and B, S4). In either case dTTP resulted in approximately 20% activity compared to dATP. In the case of Mg^{2+}, reactions with dGTP and dCTP also resulted in slightly lower activity than dATP. Activity for the mixed sample dNTP corresponded to the average activity for the individual dNTPs, again indicating that the pool of nucleotides did not have a synergistic effect, nor did the less efficient energy source dTTP cause any significant competitive inhibition of the reaction. In reactions using Mn^{2+} as the divalent cation, dGTP and dCTP dependent helicase activity was also slightly lower compared to dATP, while the mixed dNTPs showed close to additive effect on hSuv3 helicase activity.

3.7. The hSuv3 helicase activity can only be supported by ATP at high nucleotide concentration

The experiments described above were conducted at the optimal ATP concentration (1 mM) for hSuv3. To address whether the difference in T_m between Mg^{2+} and Mn^{2+} at these conditions could be contributing to the observed difference in nucleotide-dependent efficiency between the two, comparison of the individual ribonucleotides was conducted in the presence of 5 mM nucleotide (Fig. 7A and B, S5). Surprisingly, for both of the divalent cations, at high nucleotide concentrations only ATP was able to support helicase activity, while UTP, GTP and CTP were unable to do so. Helicase activity in the presence of dNTP was barely detectable and hence it was not possible to reliably discriminate between the individual dNTPs as in Fig. 6, as the low values would be highly affected by the general background variation. As the T_m is lowered for Mg^{2+} while increased for Mn^{2+}, it seems unlikely that the uniform change is caused by T_m alteration. On a technical note, initially when we tested the DNA binding of hSuv3 we used the ATP-analog ATPγS, however, variations between experiments and samples within the same experiment was high. This

could be due to the fact that this analog, though often described as non-hydrolysable, actually should be considered slowly hydrolysable [31]. Hence, slow substrate release may occur, defeating the intent of the binding assay. While decreased substrate binding may explain the general decrease in overall activity, it does not provide an explanation for the discrepancy between nucleotide efficiency. This leaves open the possibility of hSuv3 discrimination between nucleotides being sensitive to nucleotide levels.

Altogether, these result further support a flexibility of the hSuv3 nucleotide binding pocket permitting the use of several different energy cofactors, thereby establishing the potential for an accurate helicase activity regulation based on inorganic cofactor binding.

4. Discussion

The Suv3 helicase is essential for maintenance of the mitochondrial genome and its haploinsufficiency have been causatively linked to cancer [2]. Therefore, elucidating the underlying molecular mechanisms of hSuv3 activity regulation by cofactors *in vitro* may aid in dissecting its regulation *in vivo* in response to local energy metabolism. Even though biochemical experiments are simplifications of a cellular enzymatic process, they still provide valuable information about the absolute capacities of the protein. Protein activities can be affected by divalent cation binding, which may affect binding of energy cofactors. Here, we demonstrate that both Mg^{2+} and Mn^{2+} were able to support hSuv3 unwinding activity, while other cations, including Ca^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+} and Zn^{2+}, failed to do so (Fig. 1A and B). Mg^{2+}-dependent product formation did not exceed Mn^{2+}-dependent activity at any of the tested time-points or ratios to ATP (Fig. 2B). The ATPase activity of hSuv3 did not differ significantly between Mg^{2+} and Mn^{2+}, suggesting translocation was equally efficient with either of the two (Fig. 1C). Instead the difference in DNA unwinding could at least partly be ascribed to a difference in T_m of the DNA fork substrate. Thus, a difference in energy requirement for separating the DNA double stranded region was lowered in presence of Mn^{2+} compared to Mg^{2+}, therefore requiring a lower energy input to unwind the DNA fork (Table 1). As other transition metals had even lower T_m without resulting in DNA destabilization, the product formation in the presence of Mn^{2+} should be considered true enzymatic activity. Indeed, the unwinding activity of hSuv3 with either Mg^{2+} or Mn^{2+} was similar at conditions resulting in a comparable T_m (Figs. 2B and 3). Both Ni^{2+} and Zn^{2+} supported ATPase activity of the WT hSuv3 protein, but not the K213A ATP-binding mutant, indicating that the observed effects were indeed protein generated. The ability to support translocation, but not unwinding, may have a biological role for hSuv3 when in complex with other proteins, which do not require unwinding function of hSuv3, or it may be sequence specific. Dependence of hSuv3 ATPase activity on either Mg^{2+} or Mn^{2+} has been reported by others [11]. Also, Shu et al. conducted a systematic comparison of Suv3 ATPase activity finding all tested cations, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and Ca^{2+} to be able to support activity to some extent [32]. However, as previously discussed [12], the recombinant Suv3 protein used by Shu et al. displayed diverging results on directionality. In any case, this is the first report of systematic analysis of hSuv3 unwinding activity depending on divalent cation, and further comparison to ATP hydrolysis activity.

Here, we show that the concentration of divalent cation has a significant effect on substrate stability *in vitro* (Fig. 3). Hence, we argue that keeping the concentration constant between the cations may provide the better ground for comparison of protein capacities in these artificial settings, although this may not reflect the physiological conditions. The estimated concentration of freely available Mn^{2+} and Mg^{2+} in the mitochondrial matrix has been estimated to be around 10–50 nM and 0.8–1.5 mM, respectively [33,34]. Still, these measures may not reflect the bioavailable concentration. Information on the regulation of metal ion levels within the mitochondrial matrix is currently scarce [35]. Transport of cations into the matrix is believed to be highly regulated requiring either channels or translocases. In the case of Fenton active ions, such as Fe^{2+} and Mn^{2+}, co-metallochaperones are expected to bind cations until usage, thereby ensuring correct bioavailability and specific delivery. The following binding of the cation to a mitochondrial metallo-protein is believed to occur upon protein import. The protein is unfolded to become imported and once inside the matrix metal ions are inserted as the protein is being re-folded in conjuction with the co-metallochaperones [35]. Regulation of the specific divalent cation to be bound can therefore be expected to be much more complex than simply by free ion concentration alone. An example is the mitochondrial polymerase gamma, Polγ, is able to switch between polymerase and reverse transcriptase activity depending on divalent cation binding [18,19]. While the biological role of the switch is currently unclear, it may contribute to the frequent misincorporation of single ribonucleotides in the mitochondrial genome [36,37]. The hSuv3 helicase has been reported to unwind both double stranded RNA and DNA substrates *in vitro* [11,12], and is essential for mtRNA decay and mtDNA stability. Consequently, it may be tempting to speculate that binding of a specific divalent cation might be one way to regulate discrete activities in RNA and DNA metabolism. Mitochondrial function is strictly dependent on maintenance of the mitochondrial genome ensuring timely expression and replication to sustain the energy balance. One reason for maintaining a small genome within the mitochondria, in spite of the inherent risks and costs associated with it, might be to enable local regulation of proteins involved in oxidative phosphorylation in accordance to energy requirement. Helicases are the energy requiring components of both transcription and replication [38], and as such a possible point for regulation of the reactions by energy levels. The crystal structure of hSuv3 indicates that ribonucleotides other than ATP may fit as energy cofactors for the enzyme. To our knowledge, we here show for the first time, hSuv3 activity on nucleotide energy co-factors besides the classic ATP [11,32]. Mg^{2+}-dependent helicase activity of hSuv3 resulted in similar activity between the four NTPs. In contrast, a significant difference was observed when the reaction included Mn^{2+} as the divalent cation. We found that Mn^{2+}-dependent hSuv3 activity using UTP was far lower than with any of the other three NTPs. A UTP-dependent mode of regulation in mitochondria has previously been demonstrated for the *T. brucei* degradosome. Increasing UTP concentration stimulated RNA decay facilitated by the degradosome dependent on polyU tail formation [8,39]. In human cells, decreased hSuv3 protein levels result in perturbed RNA decay and the appearance of 3′ poly-uridylated RNAs, normally not detected [9,40]. The ATPase activity of hSuv3 *in vitro* is stimulated by the presence of oligonucleotides with poly(U)-ribonucleotides [11] which may suggest efficient overall degradosome activity on 3′-poly(U) tailed RNA species. Interestingly, another major mitochondrial helicase, Twinkle, is also able to utilize all four

ribonucleotides, but displays the highest activity with UTP [24,25]. Curiously, the *de novo* synthesis of uridine is coupled to the inner mitochondrial membrane and is dependent on an active electron transport chain (ETC) [41]. Consequently, rho^0 cells who lack functional ETC, need uridine supplementation [42]. Altogether, it is tempting to speculate that UTP levels might have a role in local regulation of mitochondrial genome expression in response to energy requirement and OXPHOS efficiency. In any case, the fact that the two major mitochondrial helicases have opposite efficiencies with a specific energy cofactor is indeed interesting.

Furthermore, at high concentrations of ribonucleotides (5 mM) only ATP was able to support helicase unwinding activity for both Mg^{2+} and Mn^{2+} (Fig. 7). The difference in nucleotide usage cannot be ascribed simply to a change in T_m of the substrate, as T_m did not change significantly for reactions including Mg^{2+}. Consequently, it is tempting to speculate that high ATP levels may overrule any regulation dependent upon the use of other nucleotides by hSuv3. Under normal circumstances, the concentration of ATP exceeds that of the other nucleotides by up to 10-fold [43,44]. The concentrations of NTPs used in this and comparable studies [24,25] are for the most above the estimated physiological conditions ranging from 0.1 to 2 mM (CTP < UTP < GTP ≪ ATP) [43,44]. Again, in order for values to be comparable, and to produce values sufficiently above background levels, lower concentrations than 1 mM could not be analysed (Fig. 2B) As such, it may be extrapolated that ATP serves as the primary energy source for hSuv3 when energy supply is sufficient, still low energy levels allow for the use of other nucleotides. GTP, CTP, UTP and dNTPs can in this way serve as backup, and furthermore enable fine-tuned regulation of local energy requiring activities within the mitochondrion. Also, we suggest that the, somewhat counterintuitive, lowered helicase activity *in vitro* at high ATP concentrations (5 mM) at least in part can be explained by diminished DNA binding by hSuv3. According to calculations by Storer and Cornish-Bowden, at this ratio of Mg^{2+} (3 mM) and ATP (5 mM) basically all Mg^{2+} will be chelated by ATP [29], leaving no divalent cation to neutralize the negative charge of DNA. We speculate that this may contribute to the reduced binding between DNA and hSuv3 (Fig. 4).

Finally, we found that hSuv3 was able to utilize dNTPs as energy cofactors as well, but with a lower efficiency than NTPs. Examination of the crystal structure of the hSuv3 nucleotide binding pocket shows that no specific interactions are taking place between the 2′-OH group of the ribose of energy cofactor and amino acids of hSuv3. Therefore, the use of dNTP as an energy cofactor is compatible with the crystal structure. Several mammalian helicases have been found to utilize dNTPs [25,45]. The WRN helicase displays unwinding activity in presence of dATP and dCTP, however, with lower efficiency than ATP and CTP [45]. The physiological role of this finding is currently not understood [45]. While mitochondrial replication is not coupled to the cell cycle, formation of the abortive mitochondrial replication product 7S was recently found to be sensitive to dNTP concentration and to increase markedly during the S-phase of the cell cycle [46]. A role of Suv3 in relation to mitochondrial replication has been speculated [10,12], but it still remains to be established.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.11.003.

Abbreviations

bp	basepair
BSA	bovine serum albumin
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded DNA
Dss1p	deletion of Suv3 suppressor 1
FEN1	flap endonuclease 1
mtDNA	mitochondrial DNA
mtRNA	mitochondrial RNA
NTP	nucleoside triphosphate
OXPHOS	oxidative phosphorylation
PNPase	polynucleotide phosphorylase
Polγ	polymerase gamma
ssDNA	single stranded DNA
Suv3	suppressor of Var1 – 3
T_m	DNA melting temperature
WRN	Werner protein

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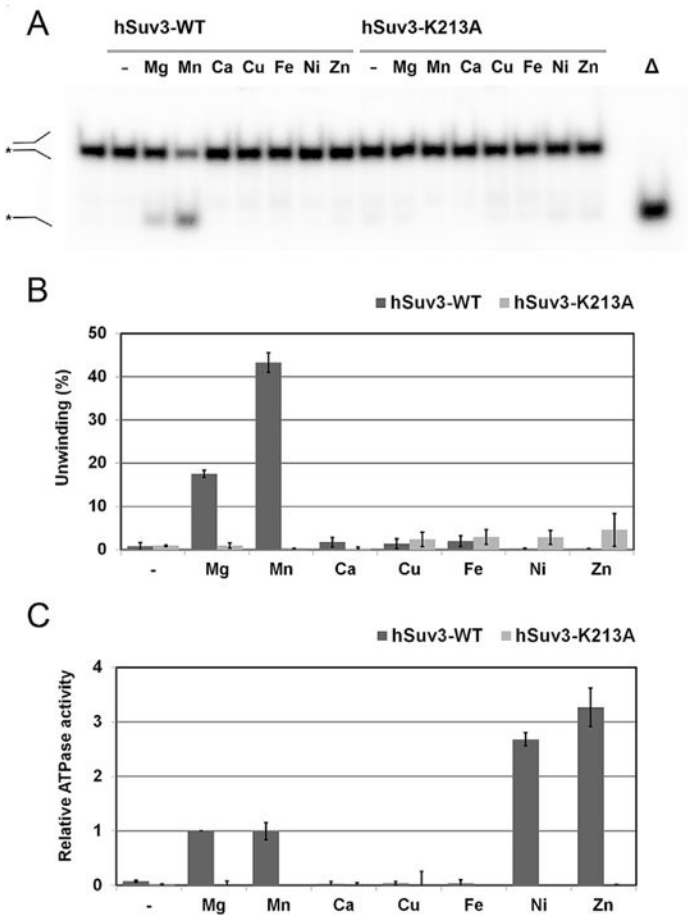

Fig. 1.

Human Suv3 activity is affected differentially in the presence of different divalent cations. A) hSuv3 helicase activity in presence of different divalent cations. Representative experiment showing hSuv3 helicase activity on a forked substrate in the presence of divalent cations. Purified recombinant hSuv3 protein (50 nM) wildtype (WT) or ATPase deficient (K213A) was incubated for 30 min at 37 °C with a forked DNA substrate in the presence of 3 mM of the indicated cation. B) Quantification of A. Error bars represent S.D. (mean value for three independent experiments). C) Quantification of hSuv3 ATPase activity in the presence of different divalent cations. Purified recombinant hSuv3 protein (50 nM) wildtype or ATPase deficient (K213A) was incubated in the presence of 32P-γATP for 30 min at 37 °C with an unlabeled forked DNA substrate in the presence of 3 mM of the indicated cation. Error bars represent S.D. (mean value for three independent experiments).

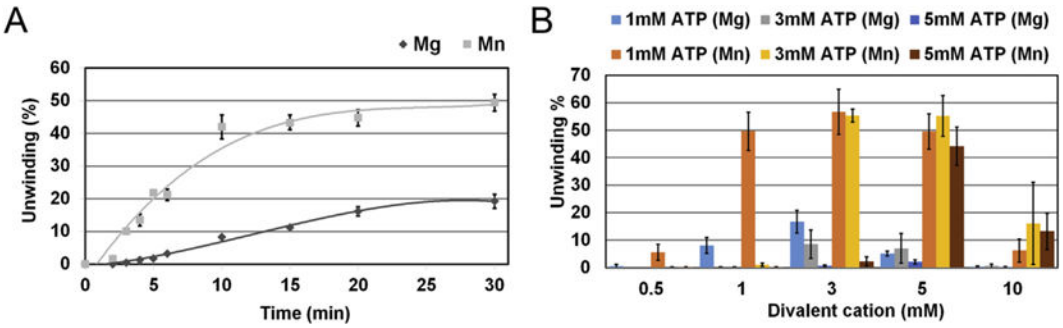

Fig. 2.

Divalent cation affects product formation and substrate stability. A) Quantifications of time-course experiments of hSuv3 helicase activity in the presence of either Mg^{2+} or Mn^{2+}. Purified recombinant hSuv3 (50 nM) WT protein was incubated at 37 °C with a forked DNA substrate in the presence of 1 mM ATP and 3 mM Mg^{2+} (◆) or Mn^{2+} (■) and incubated for the indicated time interval. B) Effect of ratio between ATP and divalent cation on helicase activity. Purified recombinant wildtype hSuv3 protein (50 nM) was incubated at 37 °C with a forked DNA substrate for 30 min in the presence of 0.5–10 mM Mg^{2+} (orange) or Mn^{2+} (blue) and 1–5 mM ATP. Error bars represent S.D. (mean value for three independent experiments).

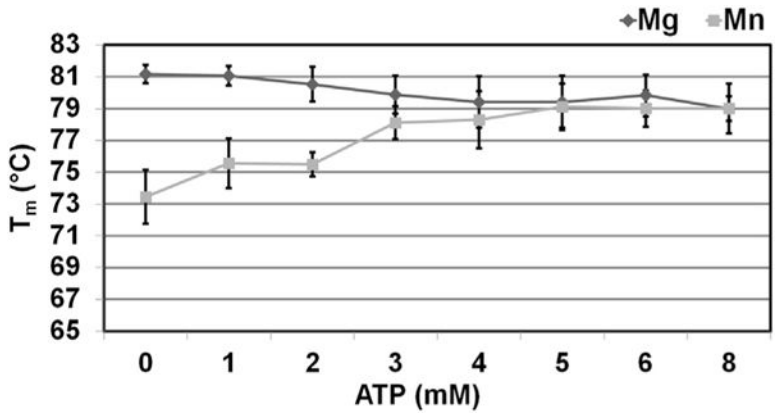

Fig. 3.

ATP concentration affects DNA melting temperature (T_m). Determination of T_m values for the D50-2/D49 substrate at 0 to 8 mM ATP in the presence of 3 mM Mg^{2+} (◆) or Mn^{2+} (■). Error bars represent S.D. (mean value for three independent experiments).

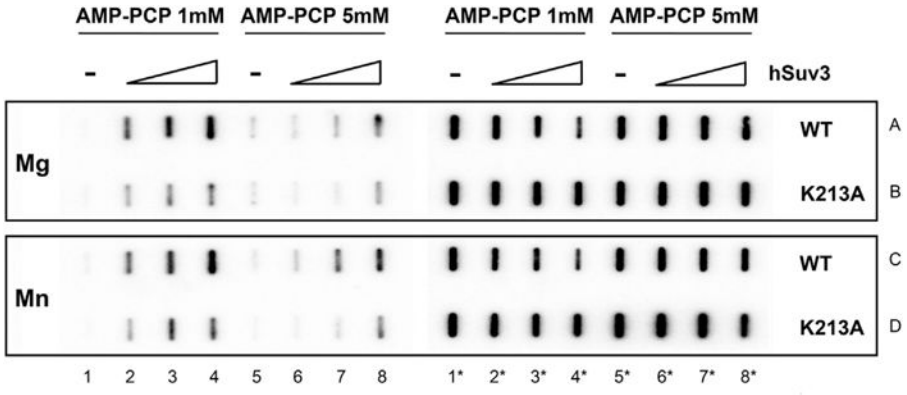

Fig. 4.

DNA binding by hSuv3 changes with nucleotide concentration. DNA binding by hSuv3 was estimated by DNA nitrocellulose filter-binding. Purified recombinant hSuv3 protein (25, 50 and 100 nM, respectively), WT or K213A, was incubated with radioactively labeled DNA in presence of the indicated concentration of non-hydrolysable ATP analogue AMP-PCP and 3 mM Mg^{2+} (row 1–2) or Mn^{2+} (row 3–4) at 37 °C for 10 min. The sample was passed through a nitrocellulose membrane to bind protein (lane 1–8), on top of a nylon membrane to bind flow through DNA (lane 1*–8*).

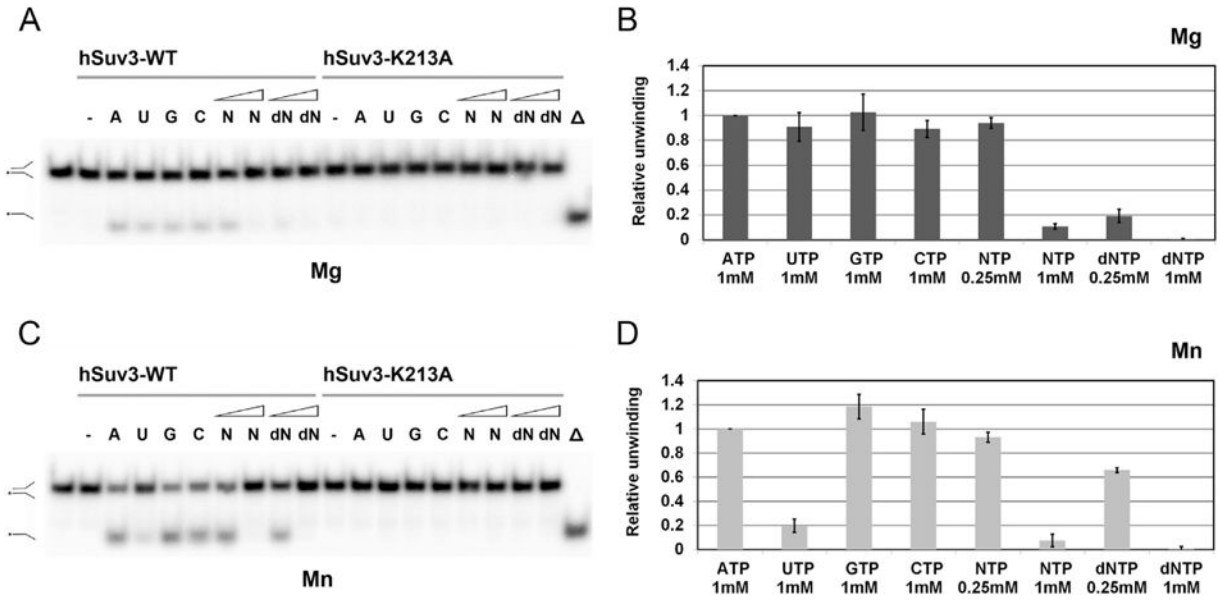

Fig. 5.

Effect of ribonucleotide and divalent cation combinations. A) Mg^{2+}-dependent hSuv3 helicase activity with different ribonucleotide energy cofactors. Purified recombinant hSuv3 protein (50 nM), wildtype or ATPase deficient (K213A) was incubated at 37 °C with a forked DNA substrate for 30 min in the presence of 3 mM Mg^{2+} or Mn^{2+} and 1 mM (or 0.25 mM) of the indicated nucleotide. B) Quantification of A. C) Mn^{2+}-dependent hSuv3 helicase activity with different energy cofactors. Reactions performed as in A. D) Quantification of C. Error bars represent S.D. (mean value for three independent experiments).

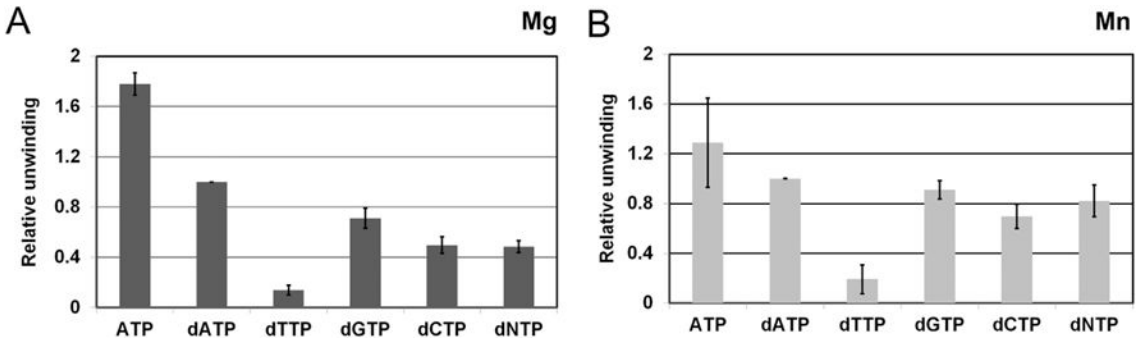

Fig. 6.

Effect of deoxyribonucleotide and divalent cation combinations. A) Quantification of Mg^{2+}-dependent hSuv3 helicase activity with different deoxyribonucleotide energy cofactors. Purified recombinant hSuv3 wildtype protein (50 nM) was incubated at 37 °C with a forked DNA substrate for 30 min in the presence of 3 mM Mg^{2+} or Mn^{2+} and 1 mM (or 0.25 mM) of the indicated nucleotide. B) Quantification of Mn^{2+}-dependent hSuv3 helicase activity with different deoxyribonucleotide energy cofactors. Error bars represent S.D. (mean value for three independent experiments).

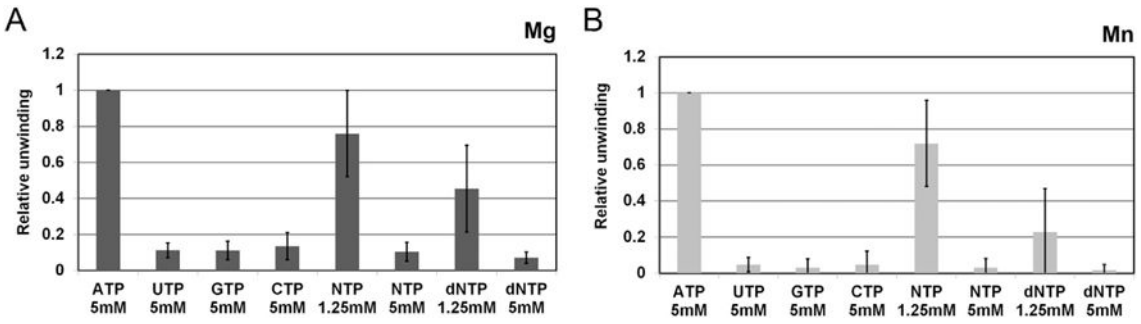

Fig. 7.

High nucleotide concentration affects nucleotide preference by hSuv3. A) Quantification of Mg^{2+}-dependent hSuv3 helicase activity with different nucleotide energy co-factors. Purified recombinant hSuv3 wildtype protein (50 nM) was incubated at 37 °C with a forked DNA substrate for 30 min in the presence of 3 mM Mg^{2+} or Mn^{2+} and 5 mM (or 1.25 mM) of the indicated nucleotide. B) Quantification of Mn^{2+}-dependent hSuv3 helicase activity with different nucleotide energy cofactors as in A). Error bars represent S.D. (mean value for three independent experiments).

Table 1

DNA melting temperature, Tm, of the substrate D50-2/D49 is affected by divalent cation.

Cation	T_m
None	80.4 ± 1.1
Mg	81.5 ± 0.8
Mn	77.5 ± 1.0
Ca	80.6 ± 0.5
Cu	NA
Fe	72.6 ± 4.8
Ni	68.9 ± 0.3
Zn	61.2 ± 7.5

The T_m value is determined from the negative first derivative of the dissociation curve. The Cu^{2+} ion interfered with the fluorophore resulting in an undetectable signal. Mean value and S.D. for three independent experiments.