

A CK1 FRET biosensor reveals that DDX3X is an essential activator of CK1 ϵ

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Summary statement: A FRET biosensor reveals DDX3X as an essential activator of the CK1 kinase in living cells. It is recruited by CK1 through phosphorylation, but CK1 peptides can disrupt this activation.

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Abstract:

Casein kinase 1 (CK1) plays central roles in various signal transduction pathways and performs many cellular activities. For a long time CK1 was thought to act independent of modulatory subunits and in a constitutive manner. Recently, DEAD box RNA helicases, in particular DEAD box RNA helicase 3 X-linked (DDX3X), were found to stimulate CK1 activity *in vitro*. In order to observe CK1 activity in living cells and to study its interaction with DDX3X, we developed a CK1-specific FRET biosensor. This tool revealed that *DDX3X* is indeed required for full CK1 activity in living cells. Two counteracting mechanisms control the activity of these enzymes. Phosphorylation by CK1 impairs the ATPase activity of DDX3X and RNA destabilizes the DDX3X/CK1 complex. We identified possible sites of interaction between DDX3X and CK1. While mutations identified in the *DDX3X* genes of human medulloblastoma patients can enhance CK1 activity in living cells, the mechanism of CK1 activation by DDX3X points to a possible therapeutic approach in CK1-related diseases like tumors driven by aberrant Wnt/ β -catenin and Sonic hedgehog (SHH) activation. Indeed, CK1 peptides can reduce CK1 activity.

Introduction

CK1 kinases are serine/threonine (Ser/Thr) protein kinases, which were considered to act as monomers and independent of cofactors. Members of the CK1 family are involved in many different biological processes through phosphorylation of a myriad of substrate targets, including key components of signaling cascades with well-established roles in development, tumor initiation, and tumor progression (Knippschild et al., 2014). The involvement and requirement of CK1 family members in Wnt/ β -catenin signaling has been studied extensively (reviewed in Clevers and Nusse, 2012; Knippschild et al., 2005; Niehrs and Acebron, 2012). Moreover, an increasing number of reports link altered CK1 expression or activity to cancer (Knippschild et al., 2005)(Dolezal et al., 2010; Fuja et al., 2004; Lin et al., 2014; Modak and Chai, 2009; Rodriguez et al., 2012; Schitteck and Sinnberg, 2014), indicating that regulation and control of CK1 activity is of high importance for cells. Although CK1 isoforms were long considered to be constitutively active kinases (Tuazon and Traugh, 1991), subcellular localization and posttranslational modifications (especially phosphorylation) of CK1 isoforms may contribute to the regulation of their kinase activity. Another molecular control mechanism was recently suggested by the finding that DEAD box RNA helicases, in particular DDX3X, are able to modulate CK1 kinase activity *in vitro* by enhancing the velocity of the kinase reaction (Cruciat et al., 2013).

Several reports linked mutations in *DDX3X* to tumors that are caused by uncontrolled Wnt/ β -catenin or SHH signaling pathways (Jones et al., 2012; Kool et al., 2014; Pugh et al., 2012; Robinson et al., 2012). As CK1 activity plays important roles in these two signaling pathways, we started to investigate the cellular role of DDX3X in activating CK1 in living cells and the mechanisms by which the carcinogenic mutations in *DDX3X* could affect these two signaling pathways. Towards this end we developed a biosensor that is capable of specifically monitoring CK1 activity in the cell over time. The results showed that the DDX3X-CK1 interaction is essential for high levels of CK1 activity in living cells. We further present results that indicate that the CK1 activating function of the RNA helicase DDX3X is regulated by phosphorylation and by RNA binding. These studies demonstrate the importance of the role of DDX3X in activating CK1 in living cells and that this enhancement is not properly controlled anymore in mutant DDX3X that are associated with medulloblastoma.

Results

A CK1 biosensor specifically monitors CK1 activity in living cells

In order to analyze CK1 activity directly in living cells and to explore the effects of possible co-factors on CK1 activity, we generated a CK1 biosensor on the basis of a ratiometric FRET sensor (Förster Resonance Energy Transfer) (Fritz et al., 2013; Hukasova et al., 2012; Komatsu et al., 2011). The CK1 FRET biosensor was constructed by substituting the sensor domain of AKAR3EV, a Protein kinase A sensor (PKA sensor) (Komatsu et al., 2011) with the non-canonical CK1 recognition sequence RRKDLHDDEEDEAMTIAD and with RRKDLHDDEEDEAMAIAD for the non-phosphorylatable T/A control (Fig. 1A). The unimolecular sensor consists of two fluorophores (eCFP and YPet) separated by three different domains: the phosphorylation motif domain, containing a non-canonical phosphorylation motif for CK1 (D/En-X-X-T), where T517 is targeted for phosphorylation, the phospho-binding domain FHA1 (forkhead-associated domain 1; (Durocher and Jackson, 2002)) and a linker domain that separates the two neighboring domains (Fig. 1A). The phosphorylation motif domain is also called the CK1 sensor domain (CK1 SD). In its phosphorylated state (P-T517), CK1 SD binds to the ligand domain FHA1 and induces a conformational change that reduces the distance between the two fluorophores YPet and eCFP as well as their orientation towards one another, leading to an increase in FRET (Fig. 1B). The FRET/CFP ratio of the emission signal serves as a readout for the T517 phosphorylation levels as already described for biosensors for other kinases (Komatsu et al., 2011). A nuclear export signal (NES) directs this biosensor into the cytoplasm for monitoring of cytoplasmic T517 phosphorylation and CK1 activity.

Quantifications of the FRET/CFP ratio were performed in a microplate reader. eCFP was excited and the emission spectra of cells expressing the biosensor were measured. They displayed two peaks for the two individual fluorophores (Fig. 1C). Phosphorylation-independent FRET changes were monitored by using a mutated sensor in which the essential CK1 target site was changed from Thr to Ala (T517A, named T/A control). This led to an overall reduced FRET/CFP ratio (Fig. 1C,D). LC-MS/MS analysis of the purified sensor from either control or CK1 ϵ overexpressing HEK293T cells confirmed the expected phosphorylation changes at T517 whereas a phosphorylation site outside of the CK1 target sequence, S269, was unaffected (Fig. 1E). Importantly, the FRET/CFP ratio of the T/A control sensor was not significantly altered by the same experimental conditions (Fig. 1F), thus indicating that the observed changes are phosphorylation-dependent. Note that the FRET/CFP ratio is shown relative to the ratio measured when cells are over-expressing the respective sensor only (either CK1 sensor or T/A control sensor). Next, we demonstrated the ability of the sensor to respond to cellular CK1 activity in the presence of the small molecule CK1 inhibitor D4476 and other published CK1 inhibitor compounds (compound 17 (CPD 17, Peifer et al., 2009), CPD 5 (Bischof et al., 2012) and CPD 2 (Richter et al.,

2014)). The FRET/CFP ratio was reduced due to the inhibitor treatment while it was elevated when CK1 ϵ was overexpressed (Fig. 1F- I).

The general CK1 phosphorylation motif used in the sensor allowed us to detect the enhanced CK1 activation driven by overexpression of different CK1 isoforms. Not only overexpression of CK1 ϵ enhanced the FRET/CFP ratio of the CK1 sensor, but also overexpression of CK1 δ and in particular CK1 α 1, although all CK1 isoforms are expressed at comparable levels (Fig. 2A,A'). Indeed, CK1 α 1 is less expressed which points to the fact that its effect on the sensor is underestimated. The stimulation of the sensor by overexpression of different CK1 isoforms could also be observed microscopically in individual single cells (Fig. 2B,C). This analysis clearly shows that the sensor measures CK1 activity in living cells. The co-expression of the kinase-dead mutant of CK1 ϵ (CK1 ϵ^{K38R} ; Fig. 2D) did not enhance the FRET/CFP ratio of the sensor. This proofed that the structural protein of CK1 ϵ is not sufficient to induce a change in the FRET/CFP ratio of the sensor, which is also consistent with the notion that the FRET/CFP ratio monitors the CK1 kinase activity.

Next we tested the specificity of the sensor for this particular kinase subgroup. Various other cytoplasmic protein kinases did not affect the FRET/CFP ratio, namely CDK2 (cyclin-dependent kinase 2), CK2 α (Casein kinase 2 alpha subunit), NLK1 (nemo-like kinase, type 1) and GSK3 β (glycogen-synthase-kinase 3 β), as well as ERK1, ERK2 (extracellular-regulated kinases 1 and 2) and constitutively active RAF (Rapidly Accelerated Fibrosarcoma; Fig. 2D,E). As an additional control, the Protein kinase A sensor (PKA sensor), which provided the framework for the CK1 sensor, was analyzed side by side with the CK1 sensor. Co-expression of CK1 isoforms led to a specific increase of the FRET/CFP ratio from the CK1 sensor, but not from the original PKA sensor, thus corroborating the specificity of the CK1 sensor (Fig. 2F).

DDX3X is required for full cellular CK1 activity and synergizes specifically with CK1 ϵ

Previous *in vitro* experiments had suggested that DDX3X influences the activity of CK1 by direct protein-protein interaction (Cruciat et al., 2013). Analyzing the CK1 biosensor in *siDDX3X* treated cells revealed that the FRET/CFP ratio was reduced to approximately the same level as in cells treated with the CK1 inhibitor D4476, demonstrating that *DDX3X* is required for normal activity of CK1 in living human cells (Figs 3A, S1A,B). Previously, it was shown that DDX3X synergizes with CK1 ϵ to induce Dishevelled (Dvl) phosphorylation and thereby activates Wnt/ β -catenin signaling (Fig. S1C; Cruciat et al., 2013). This synergy was corroborated with the microplate assay as well as with single cell measurements. Expressing *CK1 ϵ* together with *DDX3X* led to a significantly enhanced FRET/CFP ratio

indicative of enhanced CK1 kinase activity (Fig. 3B-D). Moreover, *DDX3X* expression alone was not sufficient to significantly enhance endogenous CK1 activity, which is consistent with previous results that showed that *DDX3X* is required, but not sufficient for Wnt signaling and Dvl hyperphosphorylation (Cruciat et al., 2013). Under the co-expression conditions *DDX3X* predominantly synergized with limiting amounts of CK1 ϵ rather than with CK1 δ and CK1 α isoforms (Fig. 3E). This might point to CK1 ϵ being the predominant *DDX3X*-stimulated CK1 isoform in HEK293T cells. In summary, using the specific FRET based CK1 biosensor we were able to show that *DDX3X* is required for full CK1 activity in living cells.

ATPase and kinase-activating functions of DDX3X appear mutually exclusive

We next investigated the connection between the two biological activities of *DDX3X*: its enzymatic RNA helicase function and its kinase-activating function. In general, the addition of RNA to DEAD box RNA helicases stimulates their ATPase activity (Linder and Jankowsky, 2011). Interestingly however, if CK1 ϵ was added, the RNA-stimulated ATPase activity of full length *DDX3X* purified from HEK293T cells was reduced to background levels (Fig. 4A). As an additional control that the RNA helicase activity indeed derives from *DDX3X* and not from other helicases, we extensively washed the purified *DDX3X* and checked the recombinant proteins for co-purification of other RNA helicases known to bind to *DDX3X* like *DDX5* and *RIG-1*. These RNA helicases were not detectable in the purified *DDX3X* fraction, suggesting that *DDX3X* did not form unspecific large RNA helicase aggregates and that our assay indeed analyzed the helicase activity of *DDX3X* (Fig. S1D).

The observed inhibition of the ATPase activity of *DDX3X* might either be caused by phosphorylation of *DDX3X* or by a structural change induced by the binding of CK1 ϵ . Two previous studies had already described that *DDX3X* becomes phosphorylated by kinases while stimulating their activity (Cruciat et al., 2013; Gu et al., 2013). In order to identify *DDX3X* residues that can be phosphorylated by CK1 ϵ , mass spectrometric analyses were performed using the *DDX3X* helicase core (aa 168-582) and the C-terminal helicase domain (aa 414-582). While phosphorylation of S429 and S543 could be identified for the *DDX3X* helicase core, residues T469, S470, and S543 were found to be in a phosphorylated state in the C-terminal helicase domain (Table 1). As can be seen in Fig. 4B, pre-incubating CK1 ϵ with the CK1 inhibitor D4476 prevented the inhibition of the ATPase activity of *DDX3X*, indicating that CK1 ϵ kinase activity is needed for this inhibitory effect. To further test this hypothesis, *DDX3X* was pre-incubated with the constitutively active CK1 $\epsilon\Delta C$ (CK1 ϵ lacking its regulatory C-terminal domain) to allow phosphorylation of *DDX3X* *in vitro* (Fig. 4B, last four lanes). The helicase activity of this phosphorylated *DDX3X* was much less stimulated by dsRNA. Pre-treating

DDX3X with the kinase-dead mutant CK1 ϵ Δ C^{K38R} as control still allowed dsRNA stimulation of the ATPase activity (“non-P-DDX3X” in Fig. 4C,C’). Overall, the general ATPase activity was diminished after this treatment. However, comparing the relative ATPase activity, DDX3X pre-incubated with the active form of the kinase showed reduced ATPase activity, implying that phosphorylation of DDX3X impairs its ATPase activity.

Next, we tested whether the enzymatic activity of the helicase influences the kinase activating function of DDX3X. For this we added RNA to the *in vitro* kinase assay. Double stranded RNA (dsRNA) or single stranded RNA (ssRNA) impaired the kinase activating potential of DDX3X without significantly affecting CK1 ϵ activity at the concentrations used (Figs 4D, S1E,F). Importantly, pre-incubation of DDX3X with RNA clearly reduced its binding to CK1 ϵ (Fig. 4E). This suggests that RNA interferes with the formation or stability of the DDX3X/CK1 ϵ complex.

Mapping and modeling the interaction between DDX3X and CK1

We set out to identify the minimal domain of CK1 that is required for interaction with DDX3X. Previous studies had already indicated that the C-terminal helicase domain of DDX3X is sufficient to enhance CK1 activity (Cruciat et al., 2013). We found that the DDX3X region containing only residues 414-582 was sufficient for the kinase stimulation function (Fig. S1G). In order to narrow down the regions within the CK1 protein that interact with DDX3X, we took advantage of a peptide library that was synthesized based on the sequences of CK1 δ and CK1 ϵ (Figs 5A, S2). Cruciat et al. found that *in vitro* all CK1 isoforms, including CK1 δ and CK1 ϵ , were activated by DDX3X. Because this activating effect was most pronounced for C-terminally truncated CK1 ϵ and because CK1 δ and CK1 ϵ display very high sequence similarity we focused on peptides derived from the kinase domain of CK1 δ and CK1 ϵ (Cruciat et al., 2013). The interaction between different CK1 peptides and the minimal CK1-activating fragment His-DDX3X⁴¹⁴⁻⁵⁸² was initially assessed by ELISA. Strongest binding was observed for peptides δ -31, ϵ -61, δ -91, δ -141, and ϵ -241, and to a lesser extent also for peptides δ -1, δ -41, ϵ -41, and δ -231 (the number indicates the position of the N-terminal amino acid of each 15mer peptide in the full-length sequences of CK1 δ or CK1 ϵ ; Figs 5B; S3A,B). Peptide δ -341, originating from the C-terminal domain of CK1 δ , served as negative control. Peptide δ -101 showed no binding and was therefore chosen as additional negative control for subsequent experiments. In order to validate these initial data, the CK1 δ -/CK1 ϵ -derived peptides that showed binding to His-DDX3X⁴¹⁴⁻⁵⁸² in the ELISA assay were also tested in fluorescence thermal shift (FTS) assays to determine whether they enhance the stability of His-DDX3X⁴¹⁴⁻⁵⁸². This would also be indicative of binding. Indeed, in the presence of peptides δ -1 and δ -41 the melting

temperature (T_m) of His-DDX3X⁴¹⁴⁻⁵⁸² increased by 3.5 and 5°C, respectively (Fig. 5C). For the δ -1 and δ -41 peptides, dissociation constants (K_d) of 15.61 and 5.49 μ M, respectively, could be determined for their interaction with the DDX3X fragment (Fig. S3C), establishing these as putative DDX3X binding sequences. Interestingly, also the peptides ϵ -1 and ϵ -41 lead to significant T_m changes of His-DDX3X⁴¹⁴⁻⁵⁸² (4.6 and 6.3°C, respectively; Fig. 5C). These peptides originate from the CK1 ϵ amino acid sequence and show only minor sequence differences to the corresponding CK1 δ peptides δ -1 and δ -41 (Fig. S2). The negative control peptides δ -101 and δ -341 did not cause T_m shifts (Fig. 5C).

If the interactions between the identified CK1 peptides and DDX3X reflect the interactions that are relevant for the activation of CK1 by DDX3X, these peptides may block access of DDX3X to the CK1 protein and this should then lead to impaired kinase activation. Indeed, *in vitro* incubation of CK1 peptides δ -1, δ -41, and ϵ -41 with recombinant DDX3X impaired the stimulation of CK1 ϵ kinase activity without affecting kinase activity itself (Fig. 5D,E). In contrast to these three peptides, ϵ -1 and the non-binding control peptide δ -101 did not interfere with the DDX3X stimulation. To test whether these peptides also affect endogenous CK1 activity in living cells we transfected the aforementioned peptides into cells expressing the CK1 biosensor and measured the resulting FRET/CFP ratio. As can be seen in Fig. 5F,G, the transfection of the three peptides δ -1, δ -41, and ϵ -41, but not ϵ -1 or the negative control peptide δ -341, reduced the FRET/CFP ratio, indicating that peptides δ -1, δ -41, and ϵ -41 interfere with endogenous CK1 activity in living cells.

Biolayer interferometer (BLItz) binding studies showed that the C-terminal helicase domain of DDX3X strongly interacted with δ -1 rather than with δ -41 or the control peptide δ -101 (Fig. 5H). Within the three-dimensional structure of CK1 δ (PDB: 4TWC; Bischof et al., 2012) peptides δ -1 and δ -41 are located in a well exposed position on the surface of the kinase' N-lobe. These structures as well as their CK1 ϵ counterparts therefore seem in a good position to interact with DDX3X (Fig. 5I). To better understand the interaction between the peptides and DDX3X we modeled the binding of the δ -1 and δ -41 peptides to DDX3X using the Schrödinger Suite “Maestro” as molecular modeling software. The results suggested that peptides δ -1 and δ -41 bind inside the cleft between the N-terminal and C-terminal domains of DDX3X (Fig. 5J; PDB: 2I4I; Högbom et al., 2007). For peptide δ -341, which we have used as a negative control, the molecular modeling revealed that effective binding to DDX3X would be sterically hindered (Fig. 5J). Based on this binding simulation, the binding peptides δ -1 and δ -41 might interfere with the access to the RNA- and ATP-binding motives of DDX3X. These include the adenine-binding motif Q, the P-loop in motif I that interacts with the phosphate moiety of ATP, and motif VI, possibly participating in ATP-hydrolysis (Cordin et al., 2006; Högbom et al., 2007).

DDX3X mutations associated with medulloblastoma hyperstimulate CK1 activity

Exon-sequencing of medulloblastoma patient samples revealed that the occurrence of mutations within *DDX3X* is significant, suggesting a causative connection (Jones et al., 2012; Kool et al., 2014; Pugh et al., 2012; Robinson et al., 2012). Medulloblastoma tumors are classified into four subgroups. Subgroup 1 and 2 are associated with aberrant Wnt/ β -catenin and sonic hedgehog signaling (SHH), respectively, and all identified *DDX3X* mutations belonged to tumor samples of the Wnt or SHH subgroups (Kool et al., 2014). Most mutations led to an amino acid change and were distributed along the *DDX3X* helicase core domain (Kool et al., 2014; Pugh et al., 2012). Pugh et al. demonstrated for seven of these mutations that they synergistically hyperactivate Wnt/ β -catenin signaling together with a mutated variant of β -catenin (S33Y) that prevents its degradation (Pugh et al., 2012). The fact that CK1 activity is needed in Wnt/ β -catenin and SHH signaling suggested that mutations in *DDX3X* might affect CK1 activity and that this could be the reason why they cluster in the Wnt and SHH medulloblastoma tumor groups. We therefore used the CK1 biosensor to test the hypothesis that mutant *DDX3X* versions identified by Pugh et al. affect CK1 activity. Transfected into HEK293T cells together with the sensor construct, the two *DDX3X* mutants *DDX3X*^{R376C} and *DDX3X*^{R528H}, which are located in the N-terminal and C-terminal helicase domain, respectively, enhanced the FRET/CFP ratio of the CK1 sensor significantly stronger than the wild-type *DDX3X* (Fig. 6A,B) even though wild-type *DDX3X* was expressed at least as strongly as the mutants (Fig. S4A). Enhancement of CK1 kinase activity by these mutant *DDX3X* forms could be confirmed by studying their effect on the phosphorylation of a CK1 target in the Wnt pathway, Dvl. Analyzing Dvl phosphorylation levels by a shift detectable on Western blots revealed that the mutant forms of *DDX3X* stimulated Dvl phosphorylation while wild-type alone could not (Fig. 6C). This indicates that both mutants enhance CK1 activity in living cells. *In vitro* kinase assays further confirmed these results as both mutants were able to significantly enhance CK1 ϵ activity (Fig. 6D).

At high dsRNA concentrations *DDX3X*^{R376C} and *DDX3X*^{R528H} proteins displayed reduced and no significant ATPase activity, respectively, as had been shown already for other *DDX3X* mutations associated with medulloblastoma (Fig. 6E; Epling et al., 2015). We considered the possibility that failure of the mutant *DDX3X* protein to bind RNA might cause the CK1 hyperactivation phenotype. However, *in vitro* RNA binding was still observed with the mutant forms (Fig. S4B). On the other hand, *DDX3X*^{R376C} seemed to bind more strongly to CK1 ϵ than any of the other *DDX3X* variants (Fig. S4C), suggesting that this mutation might enhance the binding affinity to CK1 ϵ . However, in co-localization experiments both

mutant forms of DDX3X showed the same distribution and co-localization pattern as wild-type DDX3X with CK1 ϵ (Fig. S4D).

While complex formation between the wild-type helicase and CK1 as well as subsequent activation of CK1 is generally impaired upon addition of RNA (see Fig. 4E), mutant DDX3X^{R528H} is still able to stimulate and to bind to CK1 ϵ even in the presence of RNA (Fig. 6F,G). DDX3X^{R376C}, on the other hand, behaves in this regard more like the wild-type protein because dsRNA still inhibits its CK1 activation potential and binding to CK1 (Fig. 6F,G).

The CK1 sensor revealed that wild-type DDX3X mainly cooperated with CK1 ϵ in living cells and not with CK1 α (Fig. 2A). Interestingly, in cultured cells both *DDX3X* mutations significantly cooperated not only with CK1 ϵ , but also with CK1 δ and even stronger with CK1 α (Fig. 6H). The finding that mutations in *DDX3X* cause the activation of other CK1 isoforms besides CK1 ϵ in living cells is striking and might contribute to the pathological effects of *DDX3X* mutations found in medulloblastoma.

Discussion

In this study we developed a unimolecular biosensor enabling us to monitor CK1 kinase activity in living cells. The sensor is specifically phosphorylated by CK1 and the treatment of cells with CK1 inhibitors or overexpression of specific constructs caused the expected changes in the FRET activity of the sensor. Thus, we established a specific and sensitive biosensor that can be used to study CK1 kinase regulation in signaling pathways. Employing this sensor we demonstrated that *DDX3X* is required for full activation of CK1 in living cells. Specifically, knock down of *DDX3X* reduces CK1 activity to the same level as the addition of CK1 inhibitors, indicating that most or all of the cellular CK1 activity towards the sensor depends on *DDX3X*. This is a surprising finding given that for decades CK1 family members were regarded as constitutively active enzymes. This earlier view was based on the fact that all CK1 isoforms, once purified from cells, displayed kinase activity (Tuazon and Traugh, 1991). However, the existence of a CK1 ϵ activation/inactivation cycle has already been demonstrated in the signal transduction processes following activation of group I metabotropic glutamate receptors. Within this cycle CK1 ϵ is activated by calcineurin-mediated dephosphorylation and again inactivated by intramolecular autophosphorylation (Liu et al., 2002). Similarly, activation of CK1 ϵ in Wnt signaling is mediated via dephosphorylation of CK1 ϵ by PP2A/P61 ϵ , which is located at the Frizzled-LRP5/6 receptor complex (Vinyoles et al., 2017). Especially in the context of Wnt signaling both mechanisms, dephosphorylation of CK1 ϵ and interaction of CK1 ϵ with *DDX3X*, may cooperate to fully activate CK1 ϵ . Focusing on the latter mechanism, our results led to a detailed characterization of the *DDX3X*-mediated activation of CK1 ϵ , advancing the knowledge on CK1 activity and regulation.

Although Cruciat and colleagues (Cruciat et al., 2013) previously showed that *DDX3X* is able to stimulate CK1 isoforms α , δ , and ϵ to the same extent *in vitro*, our biosensor data showed that in living cells only the activities of CK1 δ and ϵ , but not CK1 α , are stimulated by co-expressed *DDX3X*. This finding might indicate that other interaction partners of *DDX3X* exist within the cell. By acting as scaffolding proteins these partners might recruit *DDX3X* specifically to CK1 ϵ and its highly related isoform CK1 δ via their C-terminal tails outside of the kinase domain, but they would not stimulate the interaction with the shorter isoform CK1 α . This work also provides new mechanistic insights into how the RNA helicase *DDX3X* performs its multiple functions in the cell (Fig. S4E). First, RNA-bound *DDX3X* displays ATPase activity and fails to stimulate CK1 ϵ kinase presumably due to reduced binding between the two proteins. The modeling (Fig. 5I,J) suggests that CK1 interacts with the open conformation of *DDX3X* and RNA binding could therefore interfere with the CK1-*DDX3X* interaction by inducing the closed form of *DDX3X* and/or by masking the CK1 binding site. Second, CK1 counteracts the ATPase activity of the RNA helicase *DDX3X* by direct phosphorylation. This mechanism explains, how the

moonlighting RNA helicase DDX3X is able to perform different and apparently alternative biological activities. Its helicase function does not seem to be required for its kinase activating function and it is even reduced by phosphorylation when it interacts with the kinase. Indeed, only a rather small structural part of DDX3X is required for the kinase activation function as the C-terminal helicase part alone, which does not possess helicase activity, is sufficient to stimulate the kinase. Interestingly, a kinase activating function of DDX3X has been suggested for another kinase, too. Gu et al. could show that DDX3X directly interacts with the kinase IKK ϵ to promote its autophosphorylation and activation (Cruciat et al., 2013; Gu et al., 2013). However, it remains to be determined whether the underlying mechanisms are comparable to the activation of CK1 isoforms.

CK1 in turn is also able to phosphorylate DDX3X. However, the exact targets of phosphorylation have not been determined so far. By using mass spectrometry we determined several sites within DDX3X that are phosphorylated by CK1 ϵ . However, the functional consequence of CK1 ϵ -mediated site-specific phosphorylation remains to be determined in future studies. While general phosphorylation of DDX3X by CK1 has already been shown to reduce the enzymatic activity of the RNA helicase (Fig. 4; Cruciat et al., 2013; Gu et al., 2013) the modeling simulation suggests a second mechanism that may contribute to the reduction of this enzymatic activity. The CK1 peptides that interfere with the stimulation of DDX3X were modeled to bind in the vicinity of the RNA and ATP binding sites of DDX3X, suggesting that the kinase may block these sites upon interaction. The interaction with the kinase may therefore interfere with binding of the helicase to the RNA, thereby ensuring that DDX3X remains a kinase activator.

The fine-mapping of CK1 δ and CK1 ϵ domains that interact with DDX3X was performed using a CK1 δ -/CK1 ϵ -derived peptide library and this approach demonstrated the power of peptides as biological tools. Especially peptides δ -1 and δ -41, as well as their CK1 ϵ homologues ϵ -1 and ϵ -41, displayed strong binding to DDX3X *in vitro*. Apart from ELISA, peptide-protein interactions were validated by FTS and BLItz assays. There, however, no binding of peptide δ -41 could be observed, possibly because the binding domain for δ -41 in DDX3X might be masked by the binding of DDX3X to the BLItz sensor surface. This hypothesis is supported by the fact that δ -41 also only shows moderate binding in ELISA. This limitation of the sensor-based methods highlights the advantage of the FTS assay, which can be used to measure a wide range of affinities with no surface-fixed components. All components are measured in aqueous solution as free phase. Finally, binding of peptides δ -1, ϵ -1, δ -41, and ϵ -41 could be clearly demonstrated by FTS assay and with the exception of peptide ϵ -41 all of these peptides also showed remarkable effects in subsequent analyses.

Peptides δ -1 and δ -41/ ϵ -41 reduced the interaction between DDX3X and CK1 ϵ in the binding assays, further supporting the view that the sequences covered by these peptides might reflect actual

binding sites for DDX3X on CK1 δ and CK1 ϵ proteins (Fig. 5B,C). In cell culture, peptides δ -1 and δ -41/ ϵ -41 reduced the CK1 activity in our CK1 biosensor studies (Fig. 5F,G). While this reduction was evident, it was less pronounced than what was observed with the CK1-specific small molecule inhibitors or siRNA directed against DDX3X. This difference could for instance be due to limited uptake of the peptides into the cells or degradation of peptides within the cells. For a more efficient use of peptides as CK1 inhibitors for cultured cells, cell-penetrating peptide sequences or nanoparticle-mediated peptide delivery could be used instead. We also noted that even a conservative single amino acid change from R to K had a strong effect in this assay. The CK1 peptides δ -1 (R) and ϵ -1 (K) differ only in this position in the middle of the peptide (Fig. S2) and peptide δ -1 was able to reduce the FRET/CFP ratio, while ϵ -1 did not (Fig. 5F,G). Further studies are needed to find out what the basis of this different behavior is.

The CK1 FRET biosensor monitors CK1 activity in living cells, providing a tool to analyze the importance of *in vivo* stimulation of CK1 by DDX3X and whether this activity needs to be regulated. This allowed us to start studying human malignancies that involve changes in DDX3X. Several reports had linked mutations in DDX3X to tumors that are caused by overactive Wnt/ β -catenin or Shh signaling pathways. Medulloblastoma, the most common form of brain cancer in children, is one of these (Jones et al., 2012; Kool et al., 2014; Pugh et al., 2012; Robinson et al., 2012). Analyzing DDX3X mutants discovered by Pugh et al. (Pugh et al., 2012) revealed that both mutants enhanced CK1 activity *in vitro* and in cell culture (Fig. 6). Furthermore, as opposed to the wild-type DDX3X, both mutant forms synergized not only with CK1 ϵ , but also with other CK1 isoforms. The basis of their altered interaction with CK1 and their hyperstimulation of CK1 activity might be a stronger or more stable physical interaction with CK1 independent of simultaneous binding of RNA. Surprisingly however, this enhanced interaction became only apparent in two different experiments. While DDX3X^{R376C} showed enhanced binding to CK1 ϵ in comparison to the wild-type protein (Fig. S4C), DDX3X^{R528H} binding to CK1 was more resistant to regulation by dsRNA (Fig. 6F,G).

The findings presented here shed new light on DDX3X and its functions beyond its helicase activity. In the context of medulloblastoma formation and other tumors involving Wnt/ β -catenin and SHH signaling mechanisms, such elevated CK1 kinase activity and the loss of its control can lead to hyperstimulation of these signaling pathways thereby supporting tumor formation. It will be similarly interesting to find out whether DDX3X or a related DEAD box RNA helicase contribute to these signaling mechanisms during normal development. The results presented here therefore warrant further investigations into the role of DEAD box RNA helicases in the activation of the CK1 isoforms. The fluorescent CK1 sensor described in this study could prove useful again to explore CK1 activity in living cells and even in tumor models being used to study the impact of CK1 activity on various signaling

pathways. Furthermore, our data also showed that not only CK1-specific small molecule inhibitors, but also peptides specifically blocking the interaction between CK1 and DDX3X (or other reagents mimicking this effect) could be considered as a therapeutic option for medulloblastoma or other tumors sharing this mutational signature.

Materials and Methods

Expression constructs

Human *DDX3X* (uniprot entry O00571) and its published mutants were kindly provided by T.J. Pugh (Pugh et al., 2012) and human *CK1ε* (uniprot entry P49674) by C. Niehrs (Institute of Molecular Biology, Mainz, Germany). The kinase dead mutant of *CK1ε*^{K38R} was ordered via Addgene (deposited by David Virshup; (Rivers et al., 1998). Kinases for control experiments were a gift from V. Heussler and O. Pertz (ERK1, ERK2, and c.a. RAF, Institute of Cell Biology, Bern). Tagged constructs were generated by inserting full length or deleted forms of the respective gene either into a pCS-based vector containing a N-terminal Flag or HA-tag for human cell culture work, or into the bacterial expression vector pET28a (Novagen). The *CK1εΔC* (wild type and mutant) deletion constructs lacks the C-terminal 112 amino acids. AKAR3EV (PKA FRET biosensor) has been kindly provided by Prof. Kazuhiro Aoki (Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University). The CK1 FRET biosensor was constructed by substituting the sensor domain of AKAR3EV, a Protein kinase A sensor (PKA sensor; Komatsu et al., 2011) with the non-canonical CK1 recognition sequence RRKDLHDDEEDEAMTIAD and with RRKDLHDDEEDEAMAIAD for the T/A control.

Cell culture and siRNAs

HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium. Note that the cell culture medium was substituted by a phenol red-free medium (FluoroBrite DMEM, Gibco) prior to microplate reader-based fluorometric analysis. Metafectene (Biontex) or PEI (Polyethylenimine, Polysciences) were used for DNA transfections. siRNAs were purchased from GE Healthcare Dharmacon and transfected either as a pool consisting of all four siRNAs or single siRNAs using Dharmafect: siDDX3X-1:GCAAAUACUUGGUGUUAGA, siDDX3X-2:ACAUUGAGCUUACUCGUUA, siDDX3X-3:CUAUAUCCUCCUCAUUUA, siDDX3X-4:GGUAUUAGCACCAACGAGA.

Purification of recombinant DDX3X and CK1

Purification of flag-DDX3X from HEK293T cells was performed as described previously (Cruciat et al., 2013). Importantly, during the last washes before elution, the beads were washed with a high salt buffer (150 mM NaCl) to prevent co-purification of endogenous CK1 protein. Oligomerization control experiments with DDX3X¹⁶⁸⁻⁵⁸² revealed that only very low levels of endogenous DDX3X were co-purified by oligomerizing with flag-DDX3X. For bacterial expression the pET28a expression vectors were transformed into Rosetta2 cells (DDX3X variants, Novagen) or Lemo21(DE3) (*CK1εΔC*, NEB). Gene expression was induced by the addition of 0.5mM IPTG at OD₆₀₀=0.5 and incubation continued for 16h at 20°C. Cells were lysed by sonication in a lysis buffer consisting of 50mM Tris, 200mM NaCl,

5mM imidazole, 1mM TECEP, pH 8.0. The cell lysate was cleared by high-speed centrifugation and subjected to TALON affinity chromatography resin (Clontech) according to the manufacturer's instructions. Proteins for ELISA and FTS were dialyzed in 50mM HEPES, pH 8 containing protease inhibitor (25µg/ml aprotinin).

Biolayer Interferometer experiments (BLItz)

Biolayer interferometry was measured on the BLItz system (Pall ForteBIO LLC). High Precision Streptavidin biosensor chips were loaded three times with 100µM biotin-coupled CK1 peptides in 50mM Tris (pH 7.5), 10mM NaCl for 30sec, followed by a step to block free biotin binding sites with 10µM biocytin. Association and dissociation times of DDX3X⁴¹⁴⁻⁵⁸² (250µM) were fixed at 60 seconds. Binding and dissociation curves were recorded three times.

Generation of CK1δ/CK1ε-derived peptides

According to the amino acid sequences of the N-terminal and the kinase domain of human CK1δ (uniprot entry P48730), 30 CK1δ-derived peptides were generated. In order to investigate the effects of sequence-specific differences in the sequence of CK1ε (uniprot entry P49674) compared to CK1δ, seven additional CK1ε-derived peptides were generated (Fig. S3). Each peptide consists of 15 amino acids with the first and last five amino acids always overlapping the previous and the following peptide, respectively. At the amino terminus the peptides were coupled to biotin via a four amino acid spacer sequence (SGSG). For peptide position and sequence information see Fig. S3. All peptides were pre-dissolved in DMSO (final concentration of 12.5% DMSO in the peptide stock).

Direct binding ELISA

His-DDX3X⁴¹⁴⁻⁵⁸² protein fragment was coated onto 96-well Nunc MaxiSorp plates (NUNC, Denmark) at a final concentration of 0.01µg/µl in 100mM sodium carbonate buffer (pH 9.6) at 4°C overnight. Unspecific binding sites were blocked with 5% FCS in PBS. Biotinylated CK1δ-/CK1ε-derived peptides (1µg/well) were added and incubated for 2.5h at room temperature. DMSO (0.35%) and water were used as negative controls. Detection of the bound biotinylated peptides was performed by incubation with HRP-streptavidin (1:8,000 in 0.5% FCS in PBS). Finally, plates were incubated with ABTS detection reagent (50mM potassium phosphate buffer (pH 5.7), 5% ABTS solution (1mg/ml), 0.05% H₂O₂). Absorption was measured at 405nm after 40min incubation.

Fluorescence thermal shift (FTS) assay

FTS reactions were performed in a total volume of 20 μ l in 50mM HEPES (pH 8) and contained 10 μ M His-DDX3X⁴¹⁴⁻⁵⁸², a final concentration of 20 μ M of each indicated CK1 δ -/CK1 ϵ -derived peptide, as well as 10xSYPRO-Orange (Invitrogen). DMSO (0.35%) and water were used as controls. Measurements were performed in a LightCycler® 480 device for real-time PCR (Roche) according to the manufacturer's instructions. Respective protein melting temperatures were calculated using the protein melting evaluation software supplied by Roche.

Determination of peptide dissociation constants (K_d)

Dissociation constants were determined using the FTS assay described above and varying concentrations of the CK1 δ -derived peptides. Following preliminary assays, the peptide concentration range was adjusted and centered with higher density of data points around the previously estimated K_d value. DMSO was used as control. Protein melting temperatures were calculated using the protein melting evaluation software supplied by Roche and first fitted to a model for single site ligand binding using Prism 6 (GraphPad) as described in (Vivoli et al., 2014). Since cooperative binding could not be excluded, data were also fitted to a model for simple cooperative ligand binding. Fit of data to the different models was compared using extra sum-of-squares F test and data of the preferred model was chosen.

RNA synthesis

The RNA substrate used in RNA binding and ATPase assays was generated with pGEM3 and pGEM MO1/2 vectors as described (Méthot et al., 1994). Transcriptions were carried out as recommended by the supplier (Ambion). RNA was purified and annealed as described in order to get double stranded RNA with a short sequence of annealed nucleotides and overhangs on both sides (Li et al., 2010). For kinase assay we either used single stranded RNA derived from the pGEM3 construct or the annealed double stranded RNA from both vectors.

Kinase assays

Kinase assays were carried out essentially as described (Cruciat et al., 2013). For kinase assays full length DDX3X protein was expressed and purified from HEK293T cells as described for the ATPase assay. Deletion constructs were purified from *E. coli*. The eluted proteins were visualized using Coomassie Blue and the intensity of the protein bands was measured using ImageJ. The amounts used for kinase assays were estimated based on this staining. Where indicated, dsRNA was premixed with proteins at RT for 15min before subjecting sample to radioactive kinase assays. The CK1 substrate peptide RRKDLHDDEEDEAMSITA for the kinase assay and samples was analyzed using p81 phosphocellulose

(Biomol) as described (Zhai et al., 1992). 0.3 μ M CK1 δ/ϵ -derived and control peptides were used (Schacherl et al., 2015). For mass spectrometric analyses DDX3X helicase core (His-DDX3X¹⁶⁸⁻⁵⁸²) as well as the C-terminal helicase domain (His-DDX3X⁴¹⁴⁻⁵⁸²) were *in vitro* phosphorylated by CK1 ϵ . Also reactions without addition of kinase were performed and analyzed in order to correct for false positive results. Mass spectrometry data acquisition and analysis of trypsin-digested samples were performed at the Core Unit Mass Spectrometry and Proteomics (Ulm University) essentially as described in (Meng et al., 2016).

ATPase assays

For the ATPase assay flag-tagged DDX3X full-length constructs were expressed in HEK293T cells. After purifying and eluting DDX3X from the beads, an aliquot of the eluates was loaded on an SDS PAGE gel in order to monitor the different DDX3X levels via Western blotting and Image J. Based on this comparison similar amounts of full length DDX3X protein were used. Approx. 100ng flag-DDX3X protein and 10ng His-CK1 ϵ were used in a 60 μ l reaction mix (reaction buffer: 20mM Tris pH 7.4, 200mM NaCl, 0.5mM DTT, 1mM MgCl₂, 1mM ATP) with dsRNA (17nM, 34nM, 68nM); the reaction was carried out at 37°C for 40min. To phosphorylate DDX3X, 500ng His-CK1 $\epsilon\Delta$ C or His-CK1 $\epsilon\Delta$ C^{K38R} were added in kinase buffer before eluting the DDX3X protein from the sepharose magnetic flag-beads (Sigma). Incubation was performed at 37°C for 30min. Then, beads were washed with 50mM Tris pH 7.5 and 500mM NaCl before elution with flag-peptide. Termination of the helicase reaction was done with 10 μ l of 50% TCA solution added to each reaction. Centrifugation at 3,000g for 5min at 4°C followed. Reaction mix was mixed with coloring solution (35mM ammonium molybdate, 10mM zinc acetate, 10% ascorbic acid, pH 5) in a 96-well plate before analysis using a spectrometer (wavelength 700nm).

Microplate reader-based fluorometric analysis

HEK293T cells were seeded at a density of 20,000 cells/well on 96-well black microplates (PS, μ Clear, clear bottom, chimney well, Greiner No.655090). Transfection was performed as described above. Unless indicated otherwise, the respective cell populations were transfected with 50-100ng CK1 FRET biosensor, 100ng regulator (i.e. N-flag DDX3) and 1-10ng kinase (i.e. N-flag CK1 ϵ). Either empty pCS2 N-flag vector or luciferase were used to transfect the same amount of DNA in every well. Generally, the different samples were run in triplicates for each individual experiment. Due to the varying values for the FRET/CFP ratios based on transfection efficiency, cell density and the time point of analysis, we always set the FRET/CFP ratio of cells only transfected with the CK1 sensor to 1 in order to compare individual experiments. Importantly, the individual figures with the CK1 sensor represent only experiments performed on the same 96-well plate with the same cell density, transfection efficiency and time point.

Where applicable, the small-molecule CK1 inhibitor D4476 (BioVision) was added to the cells 2-4h prior to the analysis. For each triplicate the respective volume of D4476 (in DMSO) was added together with 1µl Fugene HD (Promega) to 60µl serum-free medium. The solution was mixed, incubated for 15min and added to the cells. The addition of Fugene HD is necessary due to the poor cell-permeability of D4476 (Rena et al., 2004). End concentrations of D4476 before transfection are indicated in figure panels and do not necessarily reflect the uptake by the cells. In the corresponding controls an equal amount of DMSO instead of the inhibitor was added to the medium. Analysis was performed 48-60h after transfection on an Infinite M1000 PRO microplate reader (Tecan) by exciting CFP with a wavelength of 433nm and measuring the resulting emission from 460-600nm in 2nm increments. Fluorometric spectra were background subtracted with spectra recorded from wells with non-transfected cells. Resulting FRET/CFP ratios were calculated by dividing the intensity values at the respective peaks (478nm for CFP, 528nm for YFP) and analyzed essentially as described (Fritz et al., 2013). Where indicated, CK1δ/ε-derived peptides were transfected into cells using Metafectene (Biontex) one hour before measurements.

Live-cell ratiometric FRET imaging

Cells were seeded at a density of 50,000 cells/compartiment on CELLview cell culture dishes with glass-bottom and four compartments (Greiner) one day prior to transfection. Alternatively, 35mm glass-bottom petri dishes (Mat Tek, No.P35G-1.5-10-C) were used. Dishes had been coated previously with 1% PEI in PBS (10min) or Poly-L-Lysine (30min) to facilitate cell adherence. Transfection was performed as described above using 100ng CK1 FRET biosensor, 200ng N-flag DDX3 and 10ng N-flag CK1ε for the respective compartments. Cells were imaged 48h later on a Leica TCS SP8 confocal microscope (63x objective, 457nm Argon laser line) with a heating chamber set to 37°C. Ratiometric analysis was performed in ImageJ by calculating the FRET/CFP ratio for each pixel and quantifying the mean ratios within the cytoplasm of selected cells. Since the use of hybrid detectors (HyD) resulted in negligible background fluorescence, no background subtraction was performed during image analysis.

Immunoprecipitation of CK1 sensor and phosphopeptide analysis

1,000ng CK1 FRET biosensor, 750ng flag-DDX3X, and 100ng CK1ε were transfected into HEK293T cells seeded on 6-well plates. Extracts were added to 50µl magnetic beads (XS Protein G Magnetic Sepharose Xtra Beads, GE Healthcare) previously incubated with anti-GFP (3E6-2, mouse, 14µg/mL). IPs were separated by SDS-PAGE and gel bands sent for mass spectrometry. Mass spectrometry data acquisition and analysis (Programm SpC PepShaker) was performed at the Mass Spectrometry and

Proteomics Laboratory (Department of Clinical Research, University of Bern) essentially as described in (Stettler et al., 2014).

Molecular Modeling

Molecular Modeling was performed on a Lenovo ThinkPad T440p (Lenovo) using Schrödinger Suite Maestro (Maestro, version 10.2, Schrödinger, LLC, 2015) and Schrödinger Biologics Suite (BioLuminate, version 1.9, Schrödinger, LLC, 2015). A human DDX3X model was generated based on the high resolution structure PDB 2I4I (resolution = 2.2 Å) using the protein preparation wizard (standard settings; Schrödinger Suite 2015-2 Protein Preparation Wizard; Epik version 3.2, Schrödinger, LLC, 2015; Impact version 6.7, Schrödinger, LLC, 2015; Prime version 4.0, Schrödinger, LLC, 2015). Peptide models were generated using the build fragments tool and molecular dynamics calculation (MacroModel, version 10.8, Schrödinger, LLC, 2015). All systems were minimized using OPLS3 force field by default settings implemented on the Schrödinger software package. Subsequently, peptides were docked to human DDX3X and CK1 δ/ϵ , respectively, performing protein-protein docking.

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Table 1: DDX3X is phosphorylated by CK1ε *in vitro*.

analysed protein	site of phosphorylation (probability of phosphorylation in %*)			
	S429	T469	S470	S543
His-DDX3X ¹⁶⁸⁻⁵⁸²	100	-	-	88.7
His-DDX3X ⁴¹⁴⁻⁵⁸²	-	100	100	92.7

*phosphorylated serine or threonine residues were detected by mass spectrometry analysis following tryptic digestion during sample preparation routine. His: 6x histidine tag; S: serine; T: threonine; -: no phosphorylation detected

Figures

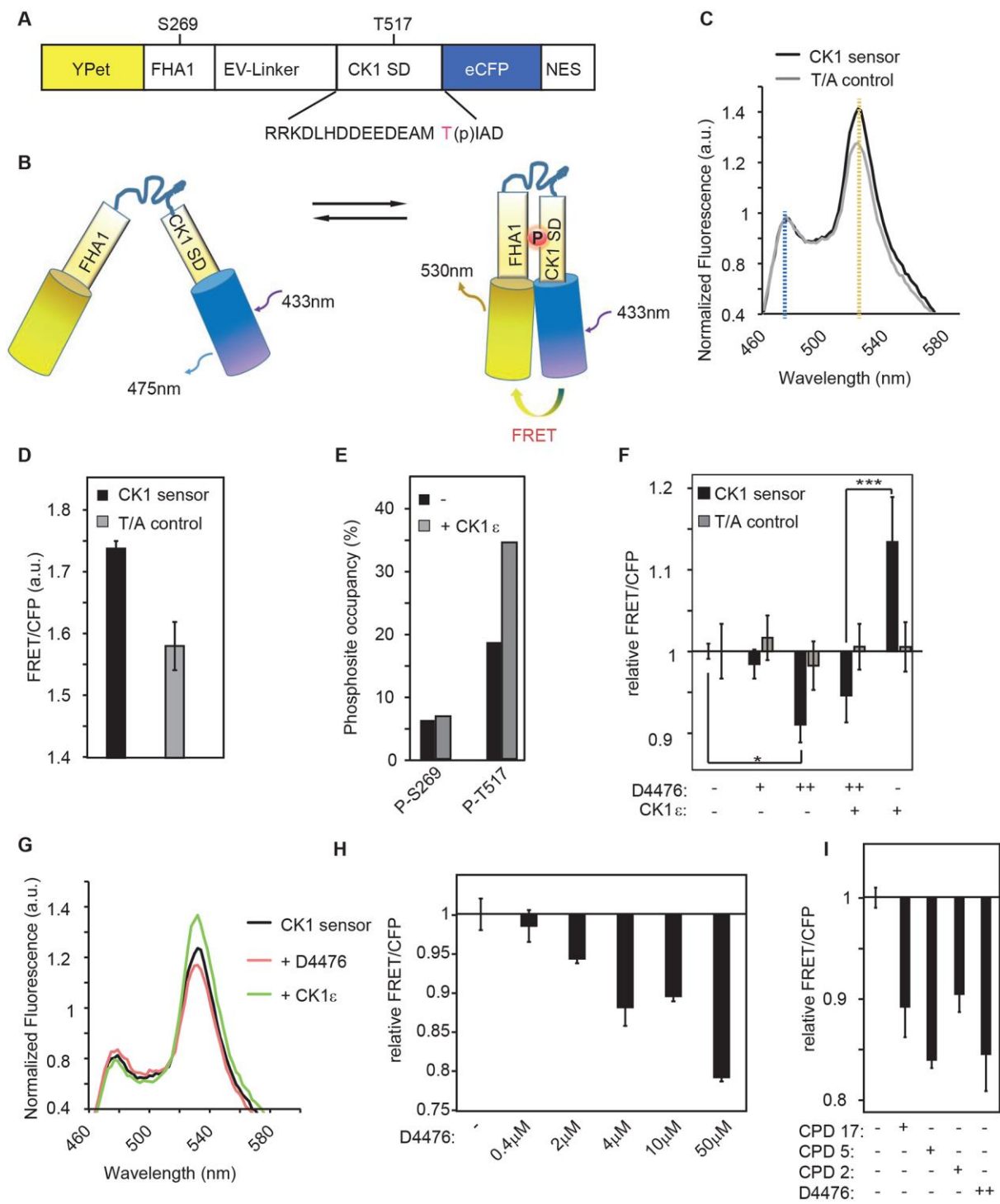


Fig. 1: The CK1 biosensor specifically responds to CK1 activity.

(A-B) Domains of FRET-based CK1 biosensor (A) and its schematic activity (B). YPet: Yellow-Fluorescent-Protein Variant; FHA1: Forkhead-associated domain 1; EV-Linker: Eevee linker; CK1 SD: CK1 sensor domain; eCFP: enhanced cyan fluorescent protein; NES: nuclear export signal. (C) Emission spectra in HEK293T cells transiently expressing CK1 sensor (black) or T/A control (grey). Excitation wavelength: 433nm; a.u.: arbitrary units. (D) FRET/CFP ratio is distinct for CK1 sensor and T/A control. (E) Phosphopeptide analysis of immunopurified CK1 sensor. The phosphosites in the biosensor are shown in (A). (F-H) CK1 sensor reacts to overexpression of CK1 and to the CK1-specific inhibitor D4476. HEK293T cells expressing CK1 sensor or T/A control and, if indicated, CK1 epsilon. CK1 inhibitor application (D4476) is also indicated. CK1 sensor and T/A control were normalized separately and set to 1. + (0.4μM), ++ (2μM) D4476. (G) Emission spectra measured from HEK293T cells transiently expressing the CK1 sensor alone (black), together with CK1ε (green) or in the presence of the CK1 inhibitor D4476 (red, 2μM). Excitation wavelength: 433nm; a.u. arbitrary units. (H) Dose dependence of CK1 inhibition by D4476 is monitored in HEK293T cells transiently transfected with CK1 sensor construct. (I) Other CK1 inhibitors affect the CK1 sensor as well. HEK293T cells expressing CK1 sensor are treated with different compounds (CPD#). Error bars indicate s.d.s, n=3. Asterisk reflects student's t-test: * p<0.05, ***p<0.001.

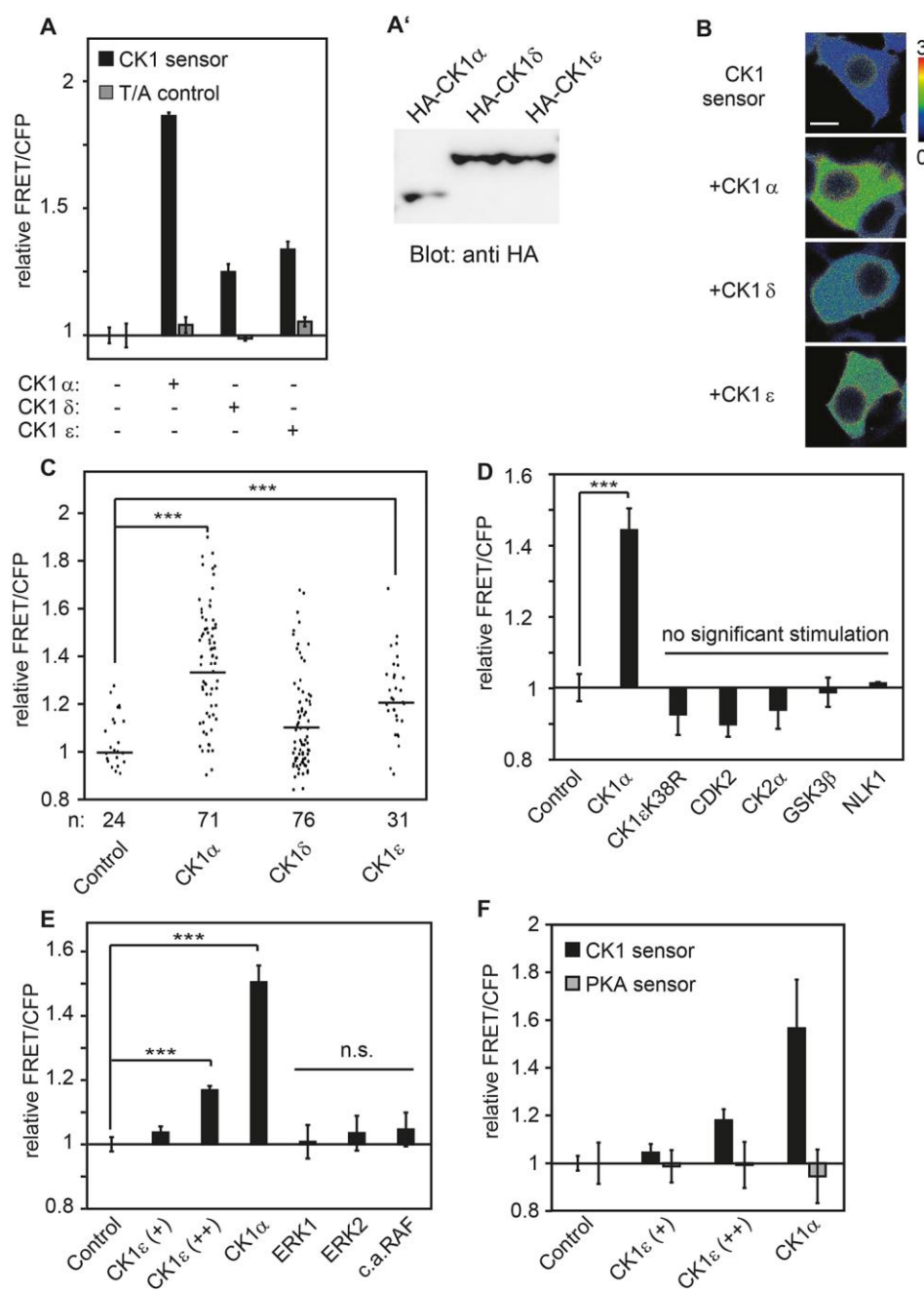


Fig. 2: CK1 isoforms stimulate the CK1 sensor and not other tested kinases.

(A) All tested CK1 isoforms are able to stimulate the CK1 sensor. CK1 sensor only and T/A control only were normalized separately and set to 1. (A') Expression of CK1 isoforms in transfected HEK293T cells analyzed by Western blotting. (B) Live imaging of cells expressing CK1 sensor and different CK1 isoforms. Heatmap displays FRET/CFP ratio. Scale bar: 10 μ m. (C) Live imaging of cells transfected with CK1 sensor and CK1 isoforms. Single cell analyses displaying FRET/CFP ratios. (D-E) CK1 sensor is

stimulated by CK1 isoforms and not by other cytoplasmic kinases. HEK293T cells transiently transfected with CK1 sensor and indicated constructs. c.a. RAF: constitutive active RAF (F) CK1 sensor reacts to higher CK1 levels while PKA sensor does not. Error bars indicate s.d.s, n=3. Asterisk reflects student's t-test: ***p<0.001.

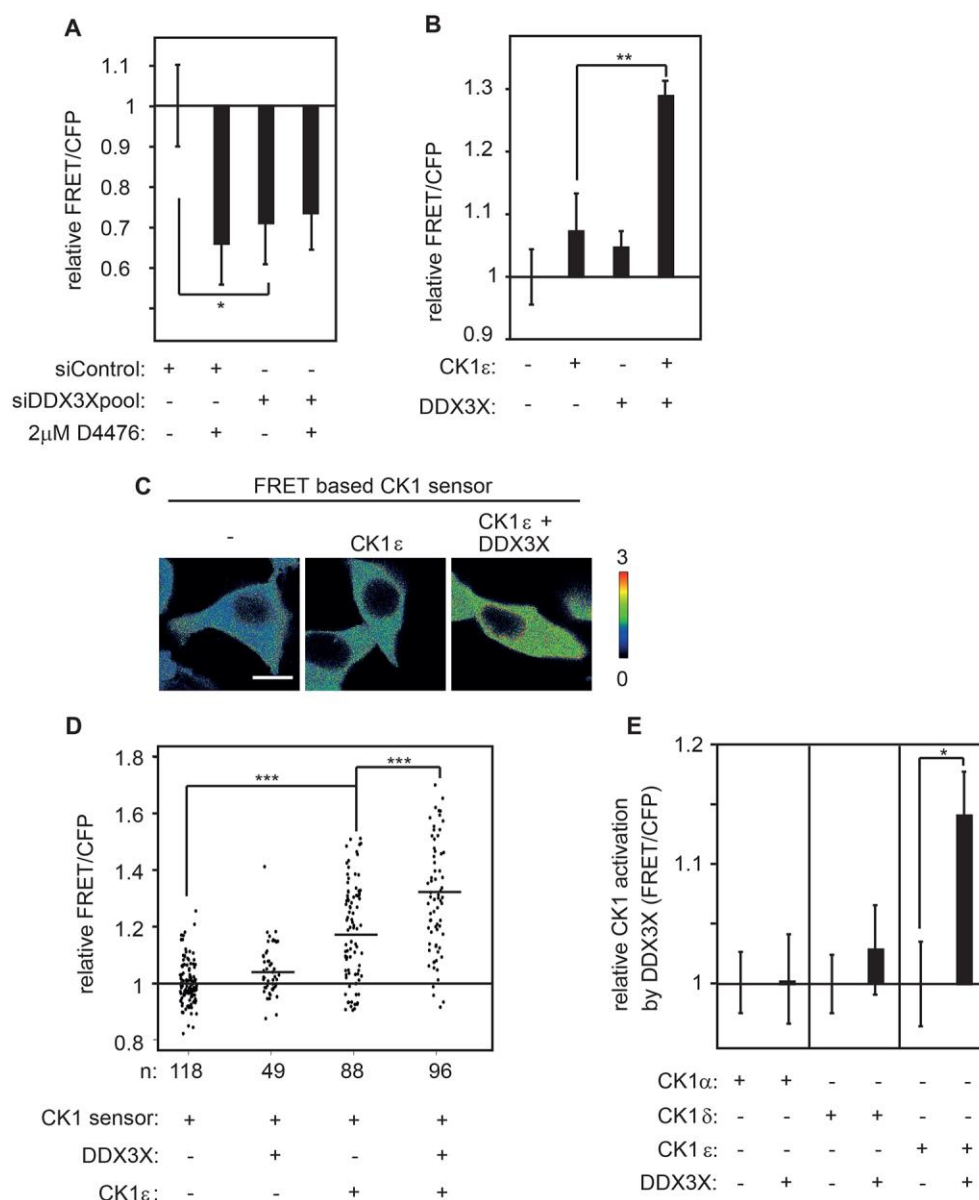


Fig. 3: DDX3X is required for full CK1 activity in human cells.

(A) DDX3X is required for CK1 activity. Transiently transfected HEK293T cells expressing the CK1 sensor and indicated constructs. Small interfering RNAs (siDDX3X), a mock target (siControl) and CK1 inhibitor (D4476) were applied. (B) DDX3X synergizes with CK1 ϵ to enhance FRET/CFP ratio of CK1 sensor. (C-D) Live imaging of cells transfected with CK1 sensor and indicated constructs. (C) Heatmap of transfected HEK293T cells displaying FRET/CFP ratio in a color look-up table. Scale bar: 10 μ m. (D) Single cell analysis of FRET/CFP ratio in living cells reveals cooperation of CK1 ϵ and DDX3X. (E) At lower CK1 expression DDX3X mainly cooperates with CK1 ϵ in living cells. Error bars indicate s.d.s, n=3. Asterisk reflects student's t-test: * p<0.05, **p<0.01, ***p<0.001.

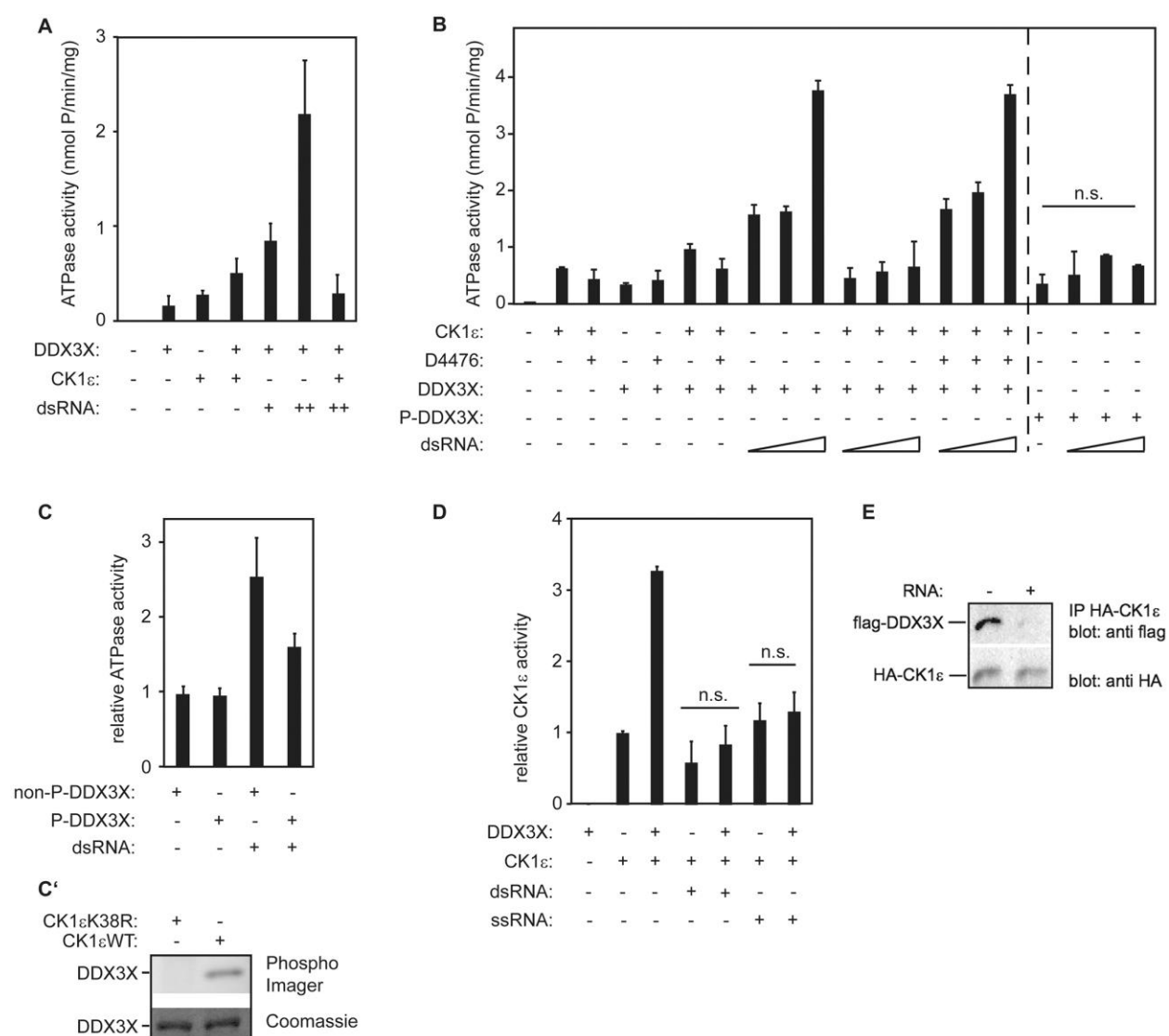


Fig. 4: CK1 counteracts ATPase activity of DDX3X by direct phosphorylation and RNA neutralizes the kinase stimulatory function of DDX3X.

(A-B) ATPase assay with recombinant proteins and dsRNA titration (+: 17nM and ++: 34nM RNA). (A) RNA-induced ATPase activity of DDX3X is impaired by CK1ε. (B) Phosphorylated DDX3X displays impaired ATPase activity. P-DDX3X: DDX3X pre-treated with CK1ε to allow phosphorylation. Note: because the pretreatment of the helicase might affect the enzyme activity in additional ways, it is not clear whether the last 4 columns can be quantitatively compared to the rest (indicated by a dashed line). The controls in Fig. 4C, however, show that at least a large fraction of the effect is due to the kinase activity of CK1ε. dsRNA titration: 17, 34, 68nM, respectively. (C) CK1ε-induced phosphorylation of DDX3X

impairs ATPase activity of the RNA helicase in vitro. ATPase assay with recombinant proteins. Recombinant DDX3X was pre-treated either with CK1 ϵ to allow phosphorylation (P-DDX3X) or with the kinase dead mutant CK1 ϵ^{K38R} (non-P-DDX3X). PhosphoImager blot shown in **C'**. **(D)** RNA inhibits the DDX3X induced CK1 activation. Radioactive kinase assay with recombinant proteins and CK1 peptide substrate. Where indicated, recombinant DDX3X was pre-treated with either dsRNA or ssRNA. **(E)** RNA impairs complex formation or stability of CK1 ϵ and DDX3X. Purified flag-DDX3X protein was incubated with total RNA where indicated and subjected to bead-coupled HA-CK1 ϵ . n.s.: not significant. Error bars indicate s.d.s, n=3.

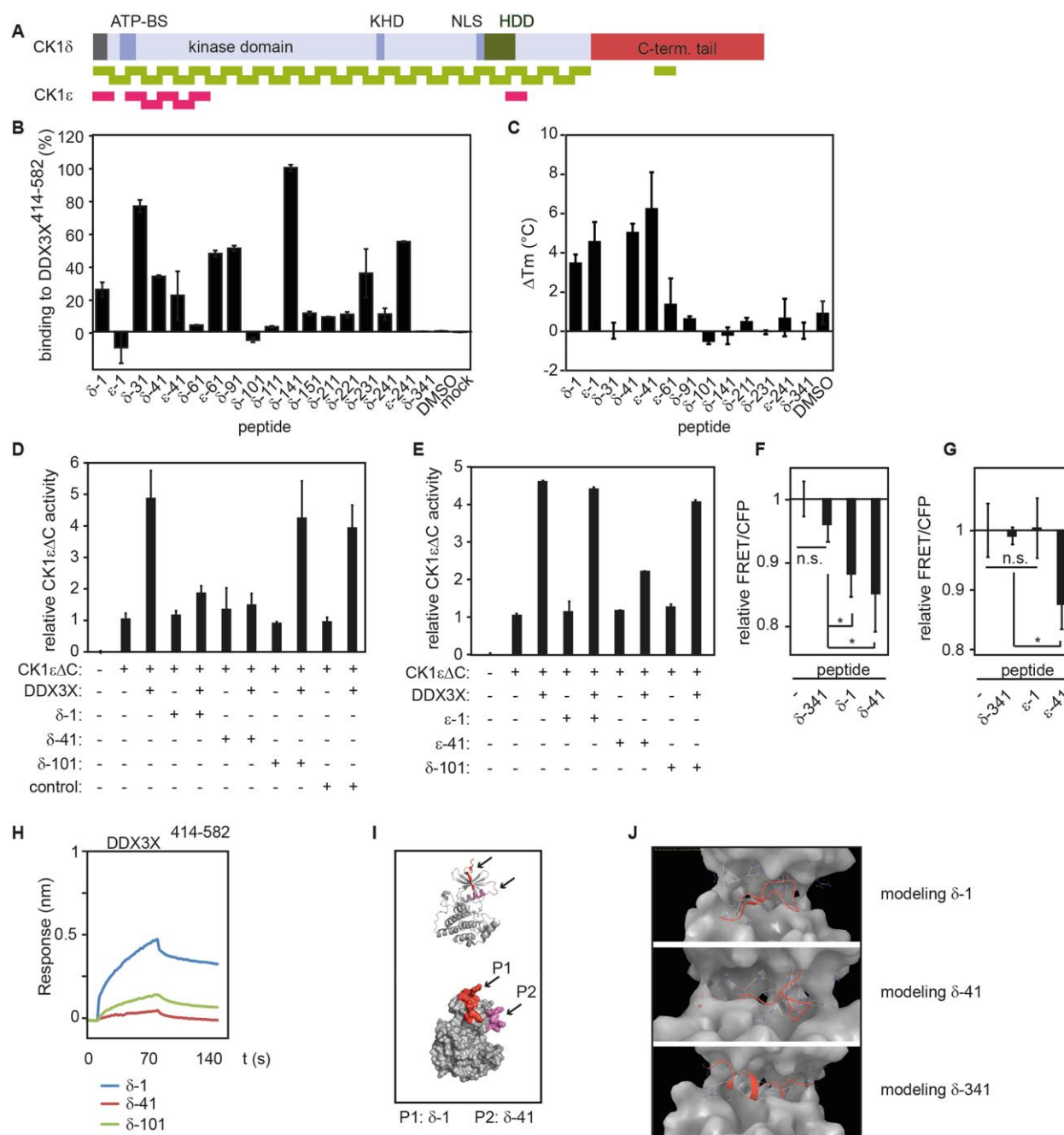


Fig. 5: Fine-mapping of CK1 interaction domain with DDX3X.

(A) CK1 δ - and ϵ -derived peptides were used to fine-map CK1 domains interacting with DDX3X. Schematic representation of the CK1 peptide library illustrating the peptide differences of CK1 δ versus CK1 ϵ . Binding of peptides to His-DDX3X⁴¹⁴⁻⁵⁸² were analyzed using (B) ELISA and (C) fluorescence thermal shift (FTS) assays. (B) Data were normalized to peptide δ -141, which was set to 100%. (C) CK1 δ -derived peptides were able to stabilize His-DDX3X⁴¹⁴⁻⁵⁸² in FTS assays. (D-E) Peptides δ -1 and

δ/ϵ -41 but not ϵ -1 and δ -101 inhibit DDX3X-stimulated CK1 activity in vitro. Radioactive kinase assay with indicated recombinant proteins and peptides. CK1 $\epsilon\Delta$ C activity alone was set to 1 for each experiment. **(F-G)** Peptides δ -1 and δ/ϵ -41 reduce endogenous CK1 activity in cultured cells. HEK293T cells transfected with CK1 sensor and indicated peptides were analyzed 1.5h after peptide transfection. Peptide δ -341 served as negative control. CFP/FRET ratio from cells only transfected with biosensor were set to 1 for each individual experiment. **(H)** Peptides δ -1 bind to the C-terminal DDX3X helicase domain, and peptides δ -41 display a slight binding, too. BLItz analysis of Biotin-peptides with the C-terminal helicase domain of DDX3X. **(I)** Crystal structure of CK1 δ with indicated peptides δ -1 (P1, red) and δ -41 (P2, purple). **(J)** Molecular modeling suggests peptide δ -1 and δ -41 (red) binding inside the cleft between N-terminal domain (blue) and C-terminal domain (yellow) of DDX3X. For control peptide δ -341 binding is sterically hindered and only lipophilic interactions could be observed. Error bars indicate s.d.s, n=3.

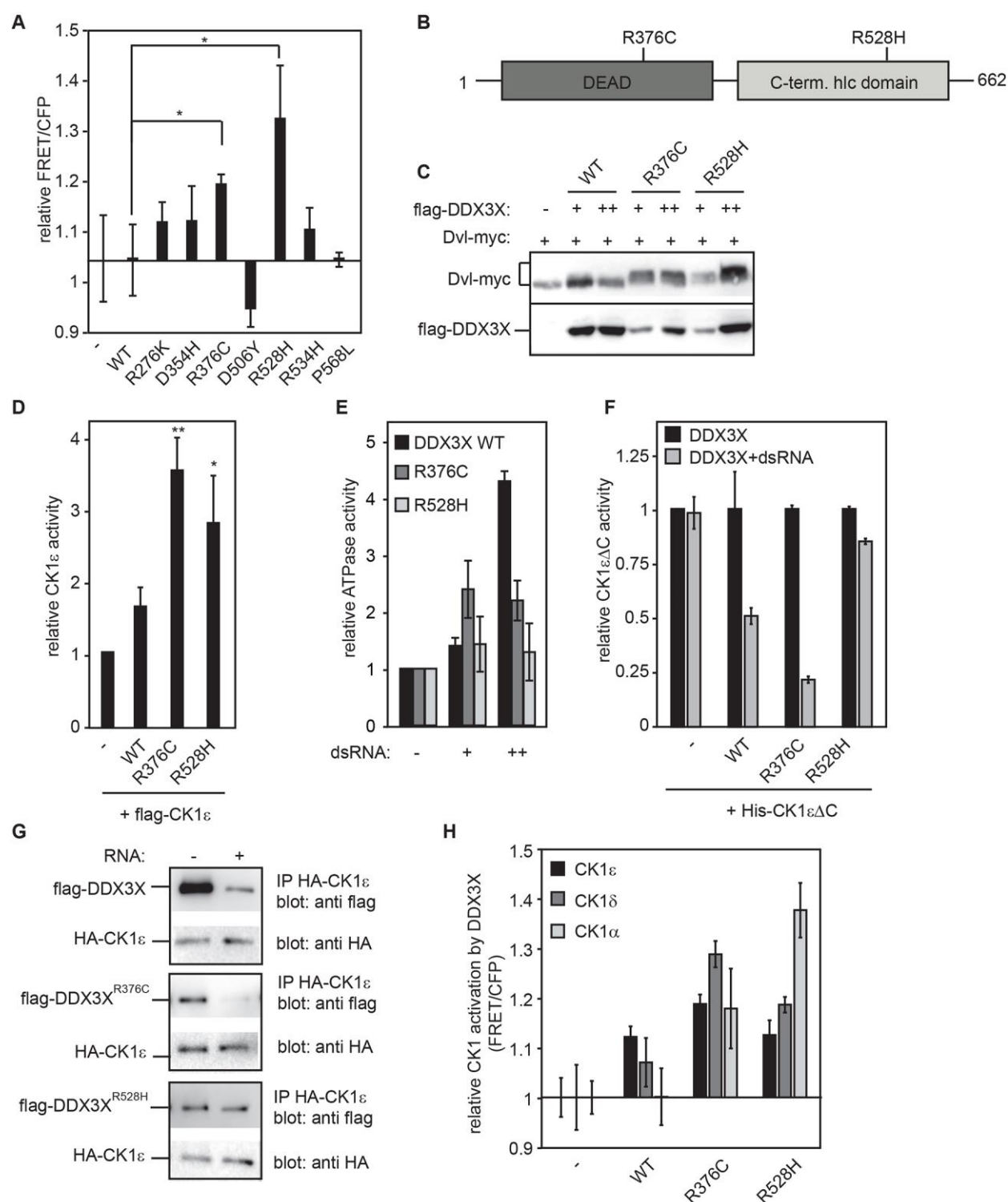


Fig. 6: Mutant DDX3X associated with medulloblastoma display enhanced CK1 activation.

(A) Expression of CK1 sensor and mutated DDX3X enhances CK1 activity. Microplate reader analysis of CK1 sensor and indicated constructs. (B) Schematic representation of DDX3X domains with strongest enhancing point mutations. Dark grey: N-terminal helicase domain, light grey: C-terminal helicase domain. (C) DDX3X^{R376C} and DDX3X^{R528H} stimulate Dvl phosphorylation. Western blot of lysate from transfected HEK293T cells; + (50ng), ++ (100ng) transfected flag-DDX3X DNA. (D) DDX3X mutant proteins display enhanced CK1 activation potential in vitro. Radioactive kinase assay with CK1 peptide substrate and recombinant DDX3X proteins. (E) DDX3X mutant proteins show impaired ATPase activity. ATPase assay with recombinant flag-DDX3X mutant proteins. ATPase activity was stimulated by two different doses of double stranded RNA (dsRNA; +: 17nM and ++: 34nM). (F-G) RNA interferes with CK1 activation and binding to DDX3X WT and mutant R376C. This is much less pronounced with mutant R528H. (F) Radioactive kinase assay with CK1 peptide substrate and recombinant DDX3X proteins. Each condition with DDX3X or kinase alone was set to 1. RNA was added where indicated. (G) Western blot with purified flag-DDX3X proteins. These were incubated with total RNA where indicated and subjected to bead-coupled HA-CK1ε. (H) DDX3X mutant proteins synergize with all tested CK1 isoforms. Microplate reader analysis of CK1 sensor and indicated constructs. FRET/CFP signal was set to 1 for each CK1 isoform expressed alone. Error bars indicate s.d.s. n= 3. Asterisk reflects student's t-test: * p<0.05, ** p<0.01.

Figure S1

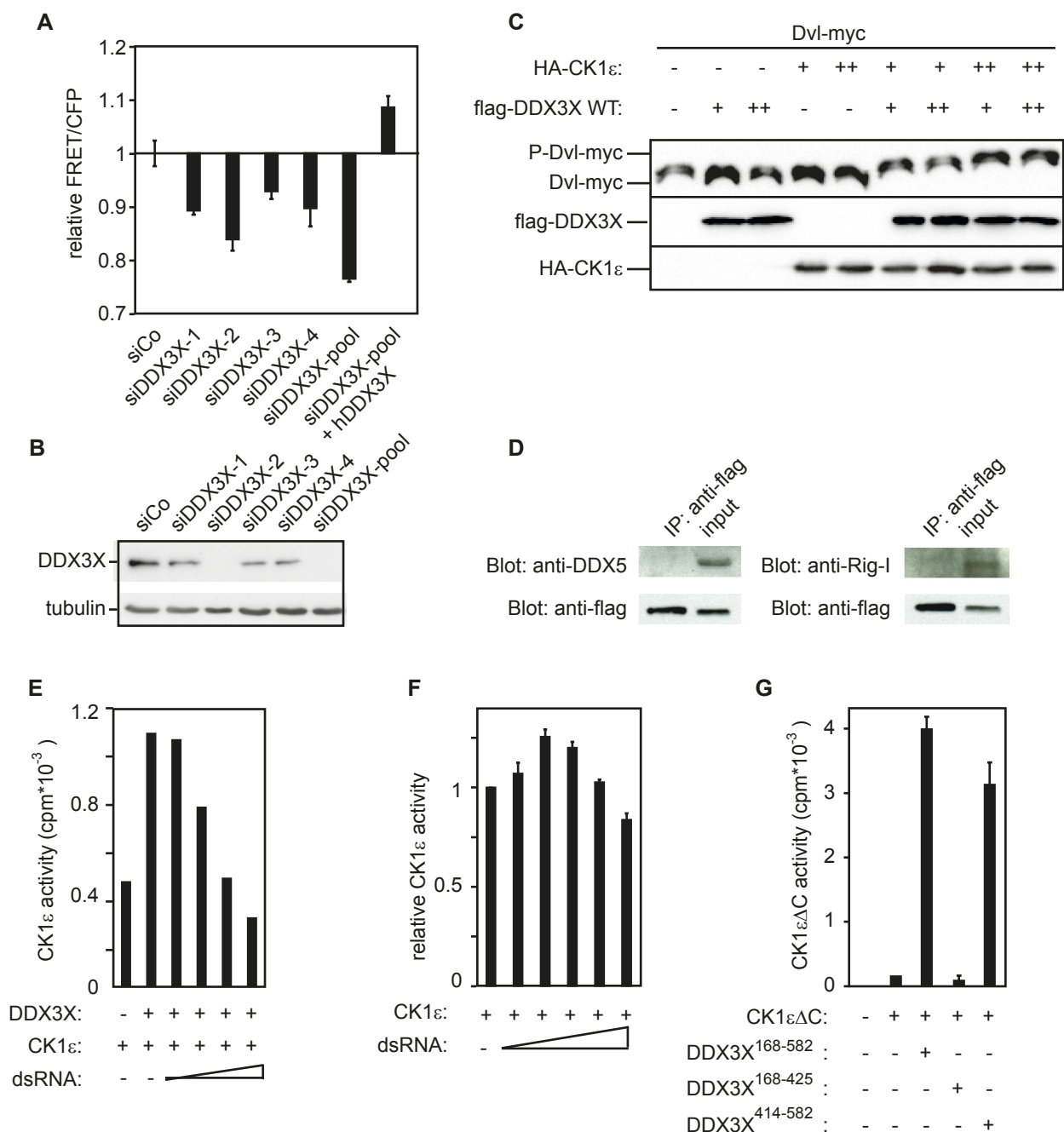


Fig. S1: Effects of siRNAs targeting DDX3X, phosphorylation and RNA on DDX3X protein.

(A) Four individual siRNAs targeting DDX3X inhibit FRET/CFP ratio of CK1 sensor. HEK293T cells transfected with indicated siRNAs and CK1 sensor. (B) siRNAs specifically reduce DDX3X protein levels. Western blot of cell lysate from treated cells. (C) Western blots of lysates derived from HEK293T cells transfected with the indicated constructs. HA-CK1ε: + (1ng) and ++ (5ng) of transfected DNA; flag-DDX3X: + (50ng) and ++ (100ng) of transfected DNA. (D) No other tested RNA helicase was co-purified with DDX3X. Western blot of co-IPs from transfected cell lysate. (E-F) RNA impairs kinase stimulatory function of DDX3X. Radioactive kinase assay with indicated recombinant proteins and titration of double stranded RNA. Used dsRNA titrations: 17, 34, 51, 68nM and in addition in (F) 85nM. (F) Only high amounts of dsRNA reduced CK1ε activity. (G) C-terminal helicase domain of DDX3X, His-DDX3X⁴¹⁴⁻⁵⁸², is sufficient to stimulate CK1εΔC activity. Error bars indicate s.d.s, n=3. cpm, counts per minute.

Figure S2

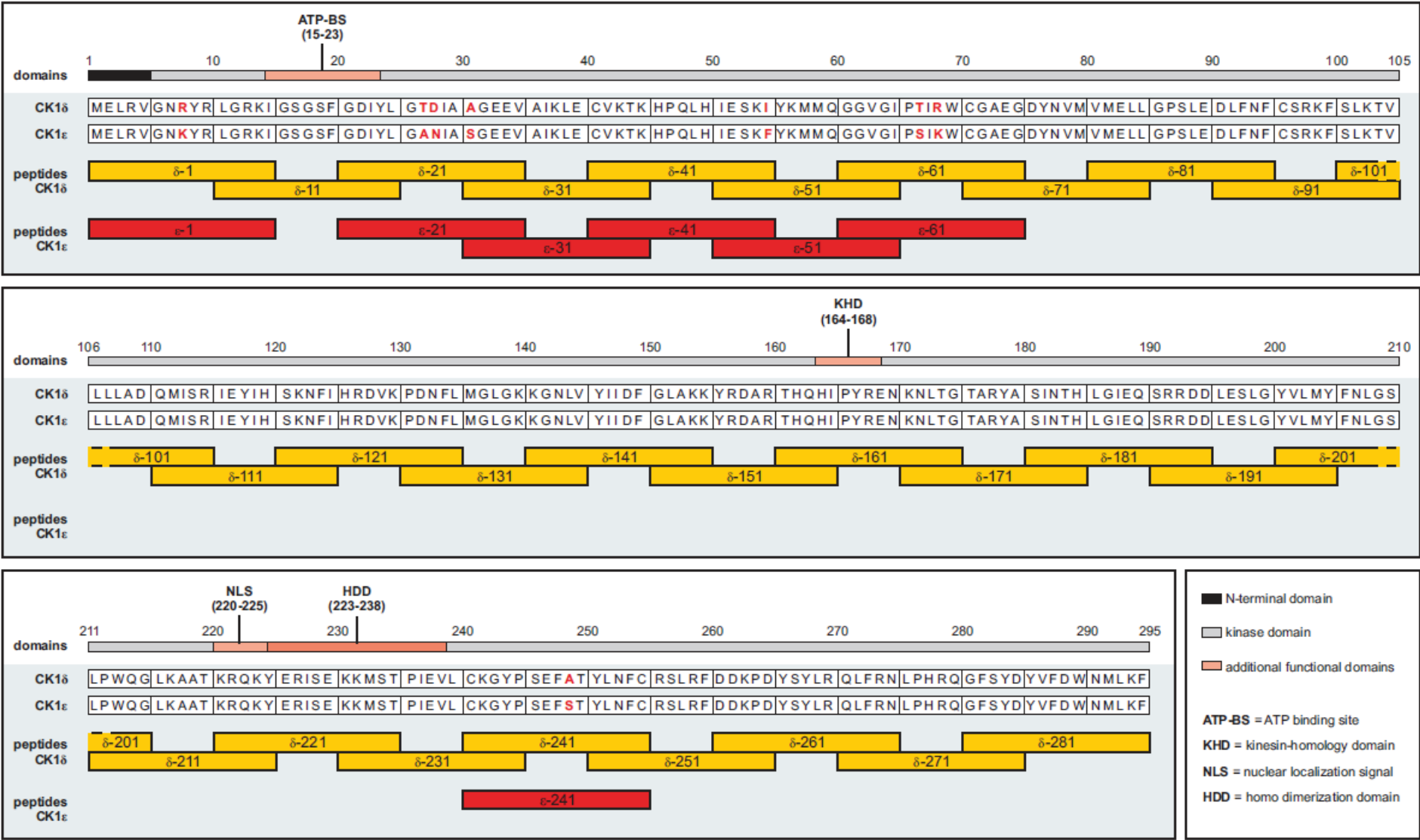


Fig. S2: Schematic illustration of CK1δ- and CK1ε-derived peptide library with indicated peptide sequences.

In ELISA and FTS assays peptide δ-341 was used as non-binding control peptide. Peptide δ-341 originates from the C-terminal domain of CK1δ and its position is outside of this figure. The full sequence of peptide δ-341 is biotin-SGSG-APPTPLTPTSHTANT.

Figure S3

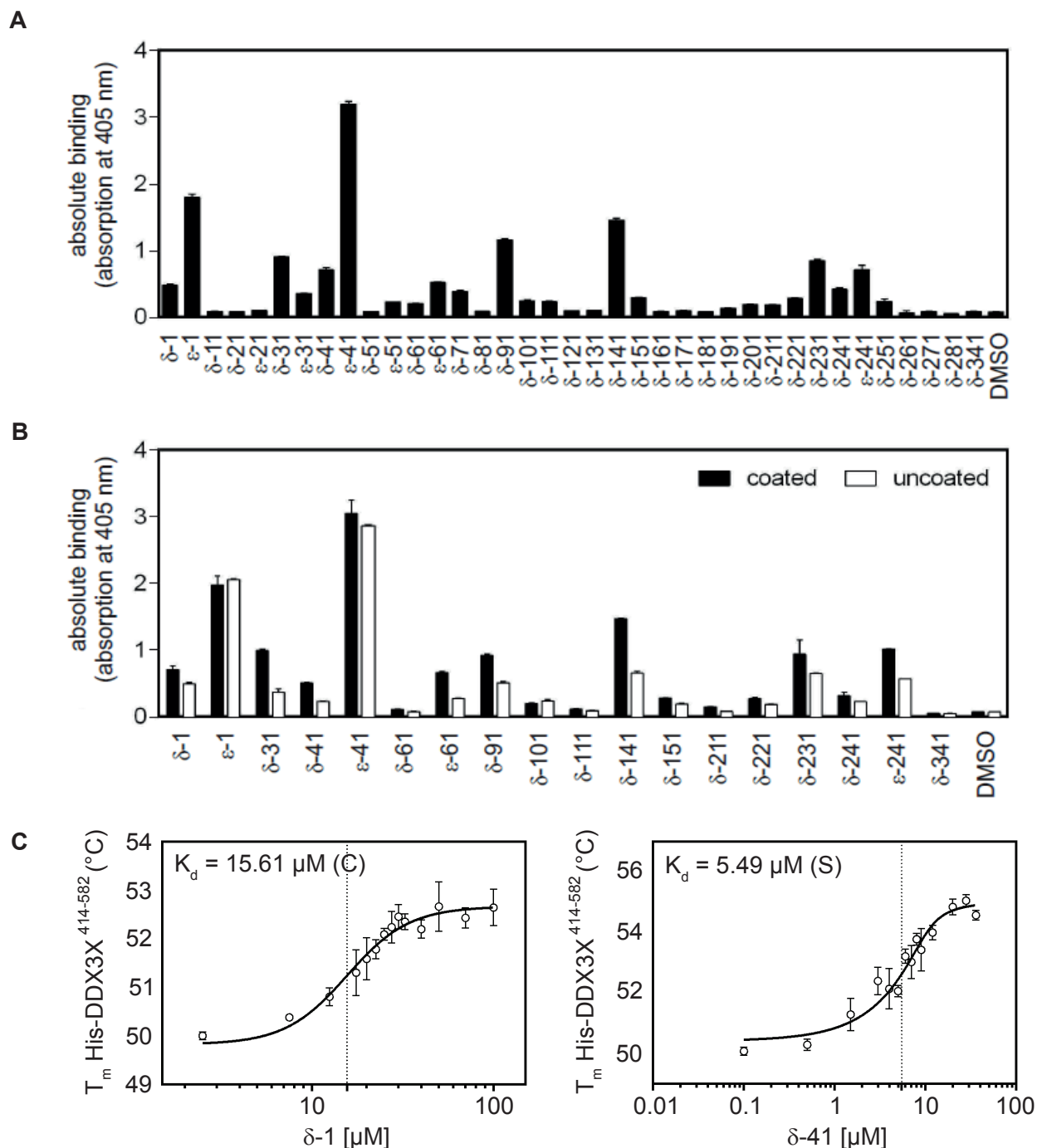


Fig. S3: Binding of CK1 δ -derived peptides to His-DDX3X⁴¹⁴⁻⁵⁸².

(A-B) Absolute binding of CK1 δ -derived peptides to coated His-DDX3X⁴¹⁴⁻⁵⁸². For ELISA 1 μg of His-DDX3X⁴¹⁴⁻⁵⁸² was coated onto the surface of each well of a 96-well ELISA plate. One μg of CK1 δ -derived peptides was used for binding analysis. DMSO was used as a control. (B) Positive peptides were reanalyzed in order to compare coated and uncoated wells, corroborating specificity of the binding seen in (A). Measurements were performed with three biological replicates each. (C) K_d values were determined using an FTS assay approach. Fifteen peptide dilutions were distributed around the estimated K_d value. Melting temperature data were fitted to models for single site ligand binding (S) or simple cooperative binding (C). In each case the best fitting model was chosen according to extra sum-of-squares F test. Each data point was measured with three biological replicates. K_d : dissociation constant; T_m : melting temperature.

Figure S4

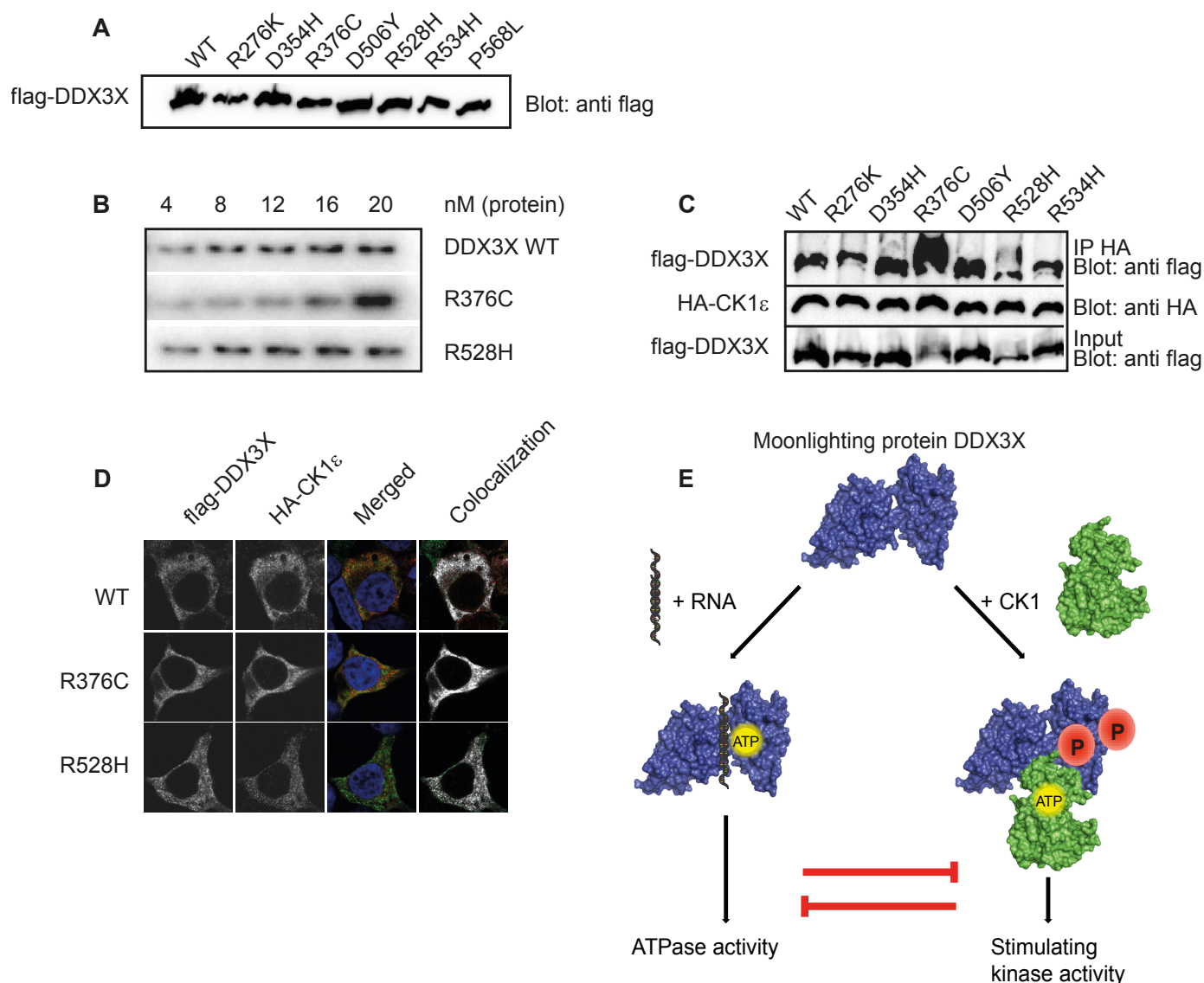


Fig. S4: Analysis of DDX3X mutant proteins associated with medulloblastoma.

(A) All flag-tagged DDX3X expression constructs are similarly expressed. Western blot with lysate from transfected HEK293T cells. (B) All recombinant DDX3X proteins can bind a generic single stranded RNA. Radioactive single stranded RNA was dose dependently UV-crosslinked with DDX3X recombinant proteins and separated on SDS-PAGE. (C) DDX3X^{R376C} shows enhanced binding to CK1ε. Binding of purified DDX3X mutant protein to bead-coupled HA-CK1ε analyzed via Western blot. (D) Wild-type and mutant DDX3X protein co-localize with CK1ε. Immunofluorescence experiment with transfected flag-tagged DDX3X constructs and HA-CK1ε in HEK293T cells. (E) Suggested model: DDX3X is regulated by RNA and phosphorylation, which control its different functions in the cell.