KDM6A mutations promote acute cytoplasmic DNA release, DNA damage response and mitosis defects

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Abstract

Background

KDM6A, encoding a histone demethylase, is one of the top ten mutated epigenetic cancer genes. The effect of mutations on its structure and function are however poorly characterized.

Methods

Database search identified nonsense and missense mutations in the N-terminal TPR motifs and the C-terminal, catalytic JmjC domain, but also in the intrinsically disordered region connecting both well-structured domains. KDM6A variants with cancer-derived mutations were generated using site directed mutagenesis and fused to eGFP, which served as an all-in-one affinity and fluorescence tag to study demethylase activity by an ELISA based assay in vitro, complex assembly by Co-immunoprecipitation and localization by microscopy in cellulo.

Results

Independent of the mutation and demethylase activity, all KDM6A variants were detectable in the nucleus. KDM6A truncations displayed changes in complex assemblies: affecting (1) known interactions with the COMPASS complex component RBBP5 and (2) KDM6A-DNA associated assemblies with the nucleolar protein Nucleophosmin. Furthermore, we observed a severe cellular phenotype characterized by multiple acute effects on nuclear integrity, namely, release of nuclear DNA into the cytoplasm, increased level of DNA damage indicators RAD51 and p-γH2A.X, and hence mitosis defects.

Conclusion

These observations reveal novel effects of pathogenic variants pointing at new specific functions of KDM6A as well as at a dominant negative effect of KDM6A truncation variants. The underlying mechanisms and affected pathways have to be investigated in future research to understand how tumor cells cope with and benefit from KDM6A truncations.

Background

In the past decade, deep sequencing approaches have revealed mutations in many genes encoding epigenetic modifiers, prominently KDM6A (1). KDM6A is frequently mutated in several cancer types, especially in urothelial cancer (2–4), and also in the hereditary Kabuki syndrome (5). The human protein H3K27-demethylase KDM6A, also referred to as Ubiquitously transcribed Tetratricopeptide repeat, X chromosome (UTX), specifically demethylates the ε-di/trimethylation on lysine 27 of histone H3, a repressive histone modification (6). KDM6A interacts with other chromatin-modifying protein complexes.
In particular, interactions with KMT2C/D-complexes (COMPASS) with the core components WDR5, RBBP5, ASH2L and DPY30 (7) appear to be demethylase-dependent at bivalent H3K27me3/H3K4 domains (8). KDM6A binding at promoter sequences of actively transcribed genes (9) is mainly demethylase-independent and involves interactions with the SWI/SNF chromatin remodeling complex (10), coactivators CBP/p300 (11), but also components of the transcription elongation machinery, like the SUPT6H-RNA Polymerase II complex (12).

The KDM6A protein has three functionally and structurally relevant regions conserved among several vertebrates. These include the eight N-terminal tetratricopeptide repeats (TPRs, 92–385 aa in human), which represent a common protein interaction motif (13). Interestingly, the human KDM6A TPR3 and TPR5 conform poorly to the consensus (1–4%) as highlighted in Figure S1 and Table S1, but the helix-turn-helix motif is still preserved. The well-characterized C-terminal and highly conserved, catalytically active Jumonji C domain (JmjC) is important for histone H3K27me2/3-recognition, binding and demethylation (14). In addition to the JmjC core (approx. aa 1095–1260), flanking regions termed pre- and post-JmjC are essential for its catalytic activity (approx. aa 880–1395). The two well-structured domains, TPR and JmjC, are connected by an intrinsically disordered region (IDR) (15). Interestingly, a high number of other chromatin-associated proteins, such as Nucleophosmin, p300 and KMT2, possess such flexible sequences, which are common and crucial motifs for protein and DNA/RNA interactions (16).

In cancer, KDM6A mutations can be found throughout the whole coding region, most prevalently within the functionally relevant domains. Knowing the precise effect of mutations on the functionality of KDM6A is key to fully understand the pathogenicities resulting from impairments of this protein and to develop strategies for targeted therapies. Therefore, we developed a workflow to systematically and simultaneously follow changes of the intrinsic properties of KDM6A substitution and truncation variants, using eGFP as an all-in-one fluorescence marker and affinity tag. We characterized the impact of KDM6A mutations on demethylase activity by an ELISA-based demethylase assay, protein stability by western blotting, intracellular localization by immunofluorescence and interactions by co-immunoprecipitation (Co-IP). This analytical approach revealed in particular that especially truncating mutations of KDM6A dramatically affect nuclear integrity.

Results

Generation of amino acid substitution and truncated variants of KDM6A based on the mutational landscape of KDM6A in tumor tissues and cell lines. Based on 64668 tested samples from 44 tissue types, 2496 unique mutations are found in COSMIC v92 (GRCh 38, November 2020) for KDM6A. Among the cancer tissues, meninges and the urinary tract exhibit the highest mutation frequency in each more than 30% of the tested samples (411/1336 cases for UC). The three most frequent point mutations across all tissues are the synonymous mutation Q1037= (c.3111G > A) in the JmjC domain, the missense mutation T726K (c.2177C > A) and the truncating Q555* (c.1663C > T) both in the intrinsically disordered region. We selected T726K as a hotspot substitution variant and substitution mutations, typically found
in urothelial cancer cell lines and tissues: E315Q (located in TPR6) and D336G (TPR7), P966R (loop in a beta sheet cavity containing the active center), V1338F (zinc-binding domain) and C1361Y (zinc-binding). To compare the impact of these substitution mutations, we additionally selected the variants Q1133A (H3-tail interaction) and H1329A (hydrophobic patch near active center) that have been shown to be catalytically inactive (14). Positions P966, Q1133, V1338 and C1361 are conserved in the KDM6A zebrafish orthologue and its closest functional homologs KDM6B and KDM6C. We created all substitution variants by site-directed mutagenesis using eGFP-KDM6A<sub>origin</sub> as a template (Fig. 1A). As nonsense mutations make up nearly one quarter of all point mutations and generate truncated variants with partial losses of the IDR and JmjC, we established a set of truncated variants with an eGFP-tag fused to their N-terminus: δTPR, δIDR, ΔJmjC, TPR (= δIDR/ΔJmjC) and JmjC (= ΔTPR/ΔIDR) (Fig. 1C). These variants were transiently transfected into urothelial cancer cell lines with wildtype (SW-1710) or mutated (T-24) KDM6A/KMT2C/D proteins. All KDM6A substitution and deletion variant proteins were detectable at the expected sizes (Fig. 1B/D), except ΔIDR, which was predicted to have the lowest solubility of all truncated variants (Table S2). In a second step, we analysed all variants for their demethylation activity. KDM6A JmjC and the flanking Zinc-binding domain are known to recognize and bind several amino acids between H3R17-H3T32 of the H3K27 di- and tri-methylated N-terminal histone tail to ensure substrate specificity (14). Consequently, mutations in the JmjC and flanking domains have a high potential to impede or even abolish the catalytic activity. However, it is unknown to what extent mutations in the TPR and IDR might contribute to KDM6A demethylase activity.

**KDM6A demethylase activity is strongly affected by substitutions and deletions within the JmjC domain.** To assess the demethylase activity of KDM6A variants, we established an ELISA-based H3K27me3 demethylation assay to screen for the catalytic activity of all truncated and substitution variants. The assay was carried out with the purified protein attached to GFP-dynabeads. We used fluorescence emission of the eGFP tag to determine and normalize the amount of protein before and after binding to dynabeads (Figure S2A). The activity of the variants was measured by colorimetric readout of the demethylated products and subsequent fitting via 4PL-regression (Figure S3A/B). The eGFP-KDM6A WT species served as a reference point for demethylase activity (Figure S3C). The activity of substitution variants was assessed in a quantitative manner, yielding specific activities summarized in Table 1. As shown in Fig. 1A, eGFP-KDM6A variant T726V featured a demethylase activity in the range of WT. Note that TPR substitutions E315Q and D336G had a slightly higher activity. Interestingly, activity in T726K decreased time-dependently within two days after transfection (Figure S4). H1329A and V1338F had strongly reduced activity, while P966R, Q1133A and C1361Y were considered as non-active.
Table 1
Specific activity of substitution variants.

| No. | KDM6A variant, affected domain | Specific activity [10⁻³ µmol min⁻¹ mg⁻¹] |
|-----|--------------------------------|------------------------------------------|
| 1   | WT                             | 2.15 ± 0.24                              |
| 2   | E315Q, TPR                     | 3.96 ± 0.58                              |
| 3   | D336G, TPR                     | 3.76 ± 0.89                              |
| 4   | T726K, IDR                     | 0.69 ± 0.10                              |
| 5   | T726V, IDR                     | 1.54 ± 0.11                              |
| 6   | P966R, JmjC                    | <0.07 ± 0.03                             |
| 7   | Q1133A, JmjC                   | <0.09 ± 0.02                             |
| 8   | H1329A, JmjC                   | 0.28 ± 0.05                              |
| 9   | V1338F, JmjC                   | 0.29 ± 0.03                              |
| 10  | C1361Y, JmjC                   | <0.12 ± 0.03                             |

Specific activity of KDM6A variants highly depends on the mutation site. Mutations affecting JmjC domain impair catalytic activity, whereas TPR substitution mutation enhanced activity. IDR mutation T726K showed time-dependent reduction of activity. Specific activity was calculated as described in additional method 1.

The demethylase activity of the truncated KDM6A variants was assessed in a qualitative manner since changes in the fluorescence emission spectra of the truncated variants did not allow proper quantification of the amount of protein used in the assay (Figure S2C). As expected, experiments with truncated (Δ- and ΔΔ-) variants exclusively yielded activity when the JmjC domain was preserved (Figure S2D).

**KDM6A single substitutions do not alter nucleoplasmic localization.** In SW-1710 cells, wildtype eGFP-KDM6A was located in the nucleoplasm and in the cytoplasm, sometimes with a tendency to cytoplasmic speckle formation (dependent on the protein dose of the transient overexpression). All substitution variants showed cytoplasmic and predominantly nucleoplasmic localization with different degrees of cytoplasmic speckle formation, as well as weak accumulation around the nucleoli (Fig. 2A). KDM6A transport to the nucleus depends largely on the KMT2C/D COMPASS complex (17). Therefore, the observation that KDM6A WT and all substitution variants localized in the nucleus suggests functional nuclear import, as well as interaction with the COMPASS complex. Notably, a recent study [17] found that KDM6A TPR mutations, among them the D336G variant, predominantly localized in the cytoplasm in stably transfected HeLa cells (which have no detectable endogenous KDM6A expression). We performed
a pull-down experiment of KDM6A WT, D336G and T726K variants with RBBP5 to test for impaired association with the KMT2C/D-complex. RBBP5 is a core component of KMT2 complexes that directly interacts with ASH2L, WDR5 and DPY30 within the WRAD complex to bind specific chromatin sequences, stabilize and activate the methyltransferase activity of KMT2 proteins. RBBP5 was pulled down with all three KDM6A variants to similar extents, but not with the eGFP control (Fig. 2B), suggesting that they are present in the same complex. Furthermore, perinuclear KDM6A speckles also stained DAPI positive, indicated newly formed micronuclei (Figure S5). Micronucleus formation, an indicator of genomic instability, is common in urothelial cancer cell lines and was slightly enhanced in KDM6A transfected cells. An increased tendency for DNA release was identified with some KDM6A substitution variants, which colocalized at the released DNA sequences (Fig. 2A, catalytically impaired variants). This phenomenon was much stronger in KDM6A truncation variants (see below).

**KDM6A truncated variants cause severe nuclear damage.** Compared to the correctly localized KDM6A control variant, deletion of any functional domain resulted in either one of two phenotypes: (1) a “mild” phenotype with weak cytoplasmic and nucleoplasmic KDM6A-Δ-variant expression as shown in Figure S7 and (2) a “severe” phenotype with a speckled, perinuclear distribution of the transfected protein. JmjC and ΔJmjC exhibited a more wildtype-like localization pattern with normally shaped nuclei, whereas following transfection of TPR and ΔIDR, nuclei were malformed or cells displayed multiple non-segregated nuclei and nuclear defects (Fig. 3, Figure S7). Nuclear DNA, which was excessively released into the cytoplasm, colocalized with KDM6A truncation variants. These results indicate the intrinsic ability of all variants to bind to chromatin, either directly through the JmjC or indirectly via other protein-protein interactions by TPR-containing variants. The presence or absence of the intrinsically disordered region (IDR) strongly affects stability and solubility of KDM6A, but not its enzymatic activity. All variants principally localized to the nucleus, but (to various extents) showed anomalies like partial nuclear redistribution to nucleoli or accumulation in perinuclear DNA-associated speckles. These observations raise two further questions, namely (1) how deletions of one or two KDM6A domains impair functional interactions with known proteins such as RBBP5, the KMT2C/D (COMPASS) complex and (2) whether Nucleophosmin (NPM1), a prominent shuttling and chaperone protein found in nucleoli, may be involved in KDM6A interactions with these organelles. To address these questions, we performed protein immunoprecipitation (Co-IP) and co-staining of transiently transfected KDM6A variants with NPM1 and RBBP5.

**Full-length KDM6A is needed for maximal binding of the COMPASS-complex core component RBBP5.** First, we tested for RBBP5 interaction with KDM6A by Co-IP experiments 48 h post transfection of selected KDM6A truncation variants (Fig. 4A). We had previously shown that RBBP5 was enriched in KDM6A-tagGFP2 Co-IPs in different urothelial cancer cell lines (18). Interestingly, we observed that for maximum interaction with RBBP5 all KDM6A domains are needed (Fig. 4B). Deletion of any domain impaired RBBP5 binding. Specifically, TPR-containing variants (ΔJmjC and TPR, Figure S8) and to some extent IDR-containing variants (ΔTPR) bound RBBP5 to some degree, but JmjC alone did not at all.
Intriguingly, MS data analysis from two KDM6A-tagGFP2 stably transduced urothelial cancer cell lines showed a significant enrichment of nucleolar proteins, such as Nucleolin, NPM1 and ribosomal subunits, in addition to histone variants (Figure S9, Table S4). However, in Co-IP experiments NPM1 was only significantly enriched with the KDM6A T726K mutant (Fig. 4C) whereas other selected KDM6A variants failed or gave very weak bands (ΔTPR and ΔJmjC, Fig. 4C white stars). Therefore, we searched for a complementary approach to detect associations of KDM6A with NPM1.

Scatterplots are widely used to detect colocalization. Typically, the intensities of two channels are plotted in a xy-diagram and the resulting populations elucidate on colocalization, which is displayed in Pearson (P between −1 and 1) or channel-wise Manders values (M1, M2 between 0–1). However, one drawback of simple P, M1, or M2 values is that the connection between scatterplot populations and the spatial cellular information is lost. For this reason, we generated scatterplots to identify populations of interest, mapped them back to the respective cellular compartment using the freely available Fiji plugin ScatterJ (19) and compared the outcome with line profiles through the corresponding cellular compartments. By this approach, we identified localization and interaction changes among the KDM6A variants.

**KDM6A WT and NPM1 form nucleoplasmic populations.** As shown in the scatterplot for KDM6A WT and NPM1 (Fig. 5A, left panel), two unique fractions appeared at the y- or x-axis, representing signals in only one of the two channels. The corresponding eGFP-KDM6A WT-only fraction is shown in green and the NPM1-only fraction in red. A third fraction with green and red signals of different intensities is shown in orange and named from here on “intermediate” fraction. Cellular back mapping of the selected populations (Fig. 5A, middle panel), clearly showed KDM6A WT-only signal predominantly in the cytoplasm and little in the nucleoplasm. As expected, the NPM1-only signal was present in nucleoli and in nucleoplasm. The intermediate fraction was always associated with NPM1-only fraction at the nucleoli rim and within the nucleoplasm, indicating a dynamic exchange of NPM1-only, mixed complexes and KDM6A WT-only fractions at specific sites within the nucleus. The line profile through the nucleus with DAPI as a DNA indicator (Fig. 5A right panel) corroborates these findings: High NPM1 (red) signal intensities were exclusively found in nucleoli, whereas green-red overlapping signals represent the KDM6A WT-NPM1 complexes.

**The T726K hotspot mutant is absent from nucleolamina and additionally forms cytoplasmic complexes.** By comparing the scatterplot presentations from T726K or WT KDM6A and NPM1, we observed a much broader intermediate fraction with similar KDM6A-T726K signal intensities but higher NPM1 intensities (Fig. 5B), which was also visible in the line profile. By cellular back mapping, we identified the intermediate population in the nucleoplasm and in the cytoplasm, where KDM6A T726K (green population) was also detectable. In contrast, the nucleoplasmic NPM1-only fraction was completely absent with this mutant. In the original image (Figure S10A), DAPI staining clearly indicated DNA release into the cytoplasm. Scatterplot analysis of KDM6A T726K with DAPI (Figure S10B) showed KDM6A T726K associated to DNA in the nucleus and at the released DNA (population 3). Furthermore, we identified a DAPI-only fraction (population 4), which was also negative for NPM1 at the nuclear lamina.
Truncated KDM6A variants likewise form complexes with NPM1 at extranuclear DNA segments. As described above, truncated KDM6A variants elicited more severe nuclear DNA release and nuclear damage (Fig. 3). We exemplary selected KDM6A JmjC (Fig. 5C) and KDM6A TPR (Figure S10C) for further analysis. Scatterplot analysis and line profiling of the truncated variants with NPM1 clearly indicated an enriched, colocalizing intermediate fraction at extranuclear DNA segments. As observed before, KDM6A JmjC and TPR were also localized to the cytoplasm. In contrast to KDM6A WT, these variants were always associated with DNA, as shown by DAPI-KDM6A scatterplot analysis and cellular back mapping approaches (Fig. 5, Figure S10 B/C). By performing scatterplot analysis combined with the cellular back mapping approach, we identified KDM6A-NPM1 compositions of variable stoichiometry and different localization, which were hardly detectable by Co-IP/WB analysis (Fig. 4). Importantly, scatterplot analysis and the cellular back mapping approach highlighted differences among the wildtype, substitution and truncated KDM6A variants. The T726K variant represents a non-wildtypic phenotype with reduced enzymatic activity in a time-dependent manner and sometimes even DNA release. The severe phenotype of the truncated variants is characterized by a massive DNA release and forms nearly equal complexes with NPM1, pointing to a role of NPM1 different or in addition to the wildtype.

The severe phenotype of KDM6A truncation variants is characterized by mitotic defects and DNA release. As DNA release and micronuclei formation have been observed, we analysed whether the typical indicators for a DNA damage response are activated by KDM6A truncation variants, namely phospho-γH2A.X and RAD51. As shown in Figure S11, we detected elevated levels of phospho-γH2A.X with truncated KDM6A variants, whereas overall phospho-γH2A.X levels were unchanged in KDM6A WT transfected cells, even in cells with cytoplasmic aggregates. Visual inspection of phospho-γH2A.X in cells with truncated variants revealed defects in mitosis (Fig. 6A). These defects occurred (1) in anaphase as lagging chromosomes and multiple fragmentation events and (2) in telophase and cytokinesis by persisting chromosome bridges and accumulation of DNA damage sites at chromosome bridges (see also Figure S7C). Line profiles through the chromatin bridges with stained endogenous DNA damage response markers RAD51 and phospho-γH2A.X together with KDM6A JmjC or ΔTPR and additionally NPM1 indicated colocalization of all these proteins at DNA (Fig. 6C). Cell counting by features of DNA release, mitotic defects and micronuclei formation in untransfected cells or cells transfected with eGFP, KDM6A WT and truncated variants showed no statistically significant changes in the extent of micronuclei formation, except for ΔJmjC, but strongly increased mitotic defects and DNA release (Fig. 6B).

Discussion

In this study, we developed and utilized a comprehensive analysis tool kit to understand the relationship and interplay of known and predicted regulatory features of the multi-domain protein KDM6A. We demonstrate how single substitution mutations and deletions of the main three functional and regulatory domains, TPR, IDR and JmjC, affect the intrinsic properties of the target protein and its interactions with the cellular environment. We could show that mutations within the JmjC domain affected the catalytic activity. Although nuclear localization changed little, interactions with RBBP5 and NPM1 were affected,
especially in truncated variants, where we observed a cellular mechanism to dispose of harmful KDM6A variants, namely by newly formed micronuclei and cytoplasmically released DNA-KDM6A complexes. Harmful KDM6A variants cause mitosis defects and DNA damage.

First, we developed an ELISA-based demethylase assay suited for use with eGFP tagged KDM6A variants. Our experiments revealed, that substitution variants within JmjC (catalytic domain) possessed reduced or abolished demethylase activity, especially if peptide recognition, peptide binding or stabilizing amino acids were changed. Among the selected cancer-associated point mutations, the hotspot mutation T726K is the third most frequently listed mutation in Cosmic v92 across all tissues and was the only substitution variant yielding post-expression time-dependent lowered demethylase activity. Since the demethylase activity is reduced for T726K, but not T726V, we expect a unique functionality for the amino acid position K726 (Table 1, Figure S4). We predicted and tested K726 as a possible methylation site, but mass spectrometry analysis did not show a PTM at K726 (data not shown). The activity of truncated KDM6A variants was in principle determined by the presence or absence of the JmjC domain. As expected, KDM6A JmjC, ΔTPR and ΔIDR exhibited demethylase activity, whereas TPR and ΔJmjC did not (Figure S3). However, removal of other domains negatively affected protein stability and solubility, especially in KDM6AΔIDR (Table S2). While variants with N-terminal or central truncations are mostly artificial, C-terminally truncated variants caused by nonsense mutations make up almost a quarter of all listed KDM6A mutations (COSMIC v92). In cancers, nonsense mutations would abolish demethylase activity by truncation or deletion of JmjC, e.g. in the urothelial cancer cell line T-24 with heterozygous mutations at E895* and E902* (18). A prevalent nonsense mutation is the Q555* with additional partial deletion of the IDR. This mutation site has been identified as an APOBEC3 hotspot (20). Other variants, like the moderately frequent Q333*, lack both IDR and JmjC and could potentially impair TPR8 functionality. Apart from lost demethylase activity, these variants have impaired interactions with other proteins. We have recently shown that KDM6A associates with RBBP5 in urothelial cancer cell lines dependent on the mutation status of KDM6A and KMT2C/D proteins (18) suggesting vital interactions of KDM6A with the COMPASS complex. Here we showed that substitution variants did not affect RBBP5 binding. However, especially the KDM6A mutation D336G has been shown to be predominantly cytoplasmic in HeLa cells caused by impaired binding to ASH2L in a pull-down experiment and consequential reduced nuclear import by the KMT2C/D complex (17). In a previous study we observed that KDM6A nuclear import was strongly decreased after double, but not single, knock down of KMT2C and KMT2D proteins (18). Systematic deletion of KDM6A domains clearly indicated that all domains, including TPR and IDR, are necessary for proper binding of RBBP5 (Fig. 4) independent of demethylase activity. Although JmjC alone does not bind RBBP5, the presence of this domain enhanced binding in KDM6A WT compared to KDM6AΔJmjC and KDM6A TPR. This might be due to facilitated association via chromatin binding. A recently published study indicated that RBBP5, WDR5 and the KDM6A JmjC domain share similar recognition and binding motifs at the Histone 3 tail (aa 1–57) (21). Furthermore, RBBP5, but not WDR5 of the WRAD sub-complex bound to H3K27me3 modified peptides and K27F peptides. All KDM6A variants were located in the nucleoplasm, albeit to different extents, independent of their mutation status. Truncations but not substitutions are characterized by (1) cytoplasmic DNA release, (2) additional
micronuclei formation, (3) enhanced levels of RAD51 and phospho-γH2AX as indicators of DNA damage, and (4) defects of mitosis caused by missegregated chromosomes at anaphase and persisting chromatin bridges at telophase and cytokinesis (Figs. 3, 6). All observed effects occurred on a short time scale within 36 h, both at relatively higher and lower expression levels. While transient and stable overexpression of the KDM6A WT reduces long-term cell growth and colony formation (18), we never observed effects of this kind, neither short-term nor long-term. In general, aneuploidy, replication stress and mitosis errors are common in cancers (22). Accordingly, all cancer cell lines used in this study exhibit these features, but they are profoundly enhanced after induction of KDM6A truncation variants (Fig. 6).

Among the severe phenotypes, cytoplasmic DNA release was most commonly observed. All KDM6A truncation variants were associated (directly or indirectly) with the DNA released from the nucleus as indicated by localization analysis and the cellular back mapping approach. Moreover, introduction of KDM6A variants, especially truncated variants, elicited elevated phospho-γH2A.X levels (Figure S11).

Phospho-γH2A.X is activated during the DNA damage stress response (23). Enrichment of proteins involved in DNA repair and stress response (DDR) appeared in our MS-data analysis from three different urothelial cancer cell lines with stably or transiently transfected KDM6A WT (Figure S9). Notably, KDM6A activity in differentiating embryonal stem cells has been linked to DNA damage response pathways by colocalization with γH2A.X positive foci (23). In addition, as an oxygen-dependent enzyme KDM6A serves as a sensor to control chromatin and cell fate (24). Thus, overexpressed (with a high dose-effect) and impaired KDM6A variants as well as oxygen-related stress have the tendency to increase DNA damage. This phenomenon was also observed in diabetic kidney disease (25). An additionally prominent feature of truncated KDM6A variants was a high degree of co-localization with NPM1 at extranuclear DNA and to appear, like all variants, as a mixed population of variable stoichiometry in the nucleoplasm (Fig. 5). As NPM1 is involved in rRNA processing, ribosome maturation and shuttling of ribosomal subunits between nucleoli, nucleoplasm and cytoplasm (26), KDM6A may be involved in these processes, too (see also respective GO terms in MS analysis in Table S4). However, the co-occurrence of NPM1 and KDM6A truncation variants might also result from the role of NPM1 as a chaperone (27). At this stage, we cannot completely rule out activation of the unfolded protein response pathway (UPR) or ER proteostasis (28) by truncated KDM6A, but consider it rather unlikely for the following reasons. First, we observed correct nuclear localization of all truncated variants (Figure S7). Second, perinuclear aggregation was observable in all KDM6A WT, substitution, truncation and control (eGFP) variants. Third, with truncated variants with a severe phenotype, KDM6A protein was always associated with DNA and never freely distributed throughout the cytoplasm. It is however possible that UPR stress sensors contribute to activation of the nuclear DNA damage response (28), which is indicated by enhanced phospho-γH2A.X levels. As KDM6A itself might act as a critical stress sensor, it is difficult to ascertain at this stage which signaling cascade might explain our observations best.

Our combined approach of mutagenesis, activity assay, localization and interaction analysis hints at possible new functions for KDM6A in the cell cycle. Specifically, the following questions are raised: At first, what is the role of KDM6A during mitosis and to which extent is any such function dependent on its catalytic activity and its interplay with RBBP5 and further components of the KMT2C/D-COMPASS
complex? Notably, many lysine demethylases (KDM) have cell-cycle specific roles (29, 30). KDM4C, KDM1A and KDM7B have already been linked to mitosis by regulation of chromosome segregation, transcriptional activation of mitotic checkpoint complex components (see refs in (29)). Moreover, WDR5 and KMT proteins, likely KDM6A interaction partners, have also been shown to be involved in mitosis (31, 32): WDR5 is part of the midbody in the spindle apparatus (30). Intriguingly, we found endogenous KDM6A, too, located along the midbody (Figure S11). At second, the multifaceted functions of NPM1 in chromatin remodeling, DNA repair, cell cycle control, apoptosis, mitotic spindle, centromere and cytoskeleton binding (27, 33) and its prominent enrichment in MS analysis suggests an important link between both proteins. Thus, under which conditions and how do both proteins directly or indirectly interact?

Conclusion

Overall, we have unraveled the structure-function relationship of several KDM6A variants concerning H3K27me3-demethylase activity, interaction with a COMPASS core component and localization upon transient transfection. In addition, we observed acute effects on nuclear integrity hinting at new functions of KDM6A in interphase and mitosis, which will be addressed in future research.

Methods

Cell lines and cell culture

Parental T-24 and SW-1710 urothelial carcinoma cell lines were obtained from the DSMZ (Braunschweig, Germany). Cells were cultured and treated in DMEM GlutaMAX-I (Gibco, Darmstadt, Germany) with 10% fetal bovine serum (FBS; Gibco™, Thermo Fisher Scientific) and 100 U/ml Penicillin/100 µg/ml Streptomycin (Sigma-Aldrich, Darmstadt, Germany). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. STR (short tandem repeat) profiling via DNA fingerprint analysis was performed for all cell lines in this study and is available upon request.

Generation of eGFP-KDM6A substitution and deletion variants

eGFP-KDM6A wildtype (WT) was synthesized by BioCat (Heidelberg, Germany) by cloning a codon-optimized eGFP-KDM6A (both sequences full-length, KDM6A main isoform 1 (Uniprot ID O15550) without additional linker between eGFP and KDM6A) into the pcDNA 3.1(+) vector, using NheI and NotI as flanking restriction sites. Generation of eGFP-KDM6A substitution variants was done using site-directed mutagenesis (SDM) using 10 ng eGFP-KDM6A WT plasmid and mutagenesis primers (listed in additional method 2, Table S5). 1.25 U PrimeSTAR GXL DNA Polymerase (Takara Bio, Kusatsu, Shiga, Japan) was used in PCR reactions. Successful PCR amplification and product length was checked by gel electrophoresis. After DpnI (NEB, Ipswich, MA, USA) digestion (20 U for 1 h at 37 °C), SDM amplicons were transformed into Escherichia Coli XL10-Gold® (Stratagene, Santa Clara, CA, USA) and spread on LB
amp plates. Colonies were picked, grown and DNA was isolated using a QIAPrep Spin Miniprep Kit (QIAGEN, Hilden, Germany). After sequencing, DNA from positive KDM6A substitution clones was re-transformed into E. Coli XL10-Gold® (Stratagene) and purified at a large scale using NucleoBond Xtra Maxi Plus EF kit (Macherey-Nagel, Dueren, Germany). Substitutions were then confirmed a second time by sequencing. Generation of eGFP-KDM6A deletion variants was done by “modularized” cloning of three inserts: TPR, res. 1-390, IDR, res. 391–885 and JmjC, res. 886–1401 from the original wildtype eGFP-KDM6A pcDNA3.1(+) into the pEGFP-C1 vector for the desired combinations. Each restriction enzyme (RE) site produces a two amino acids long linker. Constructs with one insert (eGFP-TPR, eGFP-IDR) have BspEI and HindIII as flanking RE sites. Constructs with two inserts (eGFP-KDM6AΔTPR, ΔIDR, ΔJmjC) have BspEI and EcoRI as flanking RE sites and HindIII as middle RE site. The control construct eGFP-KDM6Acloning has BspEI and KpnI as flanking RE sites and HindIII and EcoRI as mid RE sites. Amplification primers (as shown in Table S5) were designed according to the desired combination with appropriate overhangs and synthesized by Eurofins Genomics (Ebersberg, Germany). PrimeSTAR GXL DNA Polymerase (Takara Bio) was used for amplification according to the manufacturer’s instructions. T4 DNA ligase (NEB) was used for insert ligation (10 min at RT, 3.1 insert:vector ratio). All restriction enzymes were purchased from NEB. NucleoSpin Gel and PCR Clean-Up (Macherey-Nagel) was used to extract and clean up DNA. Cloning products were transformed into Escherichia Coli XL10-Gold® (Stratagene). An appropriate number of colonies were picked, grown and the DNA was isolated using a QIAPrep Spin Miniprep Kit (QIAGEN). After sequencing, positively cloned DNA was re-transformed into E. Coli XL10-Gold® (Stratagene) and purified in a large scale using a NucleoBond Xtra Maxi Plus EF kit (Macherey-Nagel).

**Transient transfection**

For transient transfection, cells were seeded into 6-well plates with (imaging) or without (activity, western blot) glass cover slips. 24 h later, cells were transfected at ~ 70% confluence with XtremeGENE™ 9 or HP (Roche, Basel, Switzerland; application dependent use) in a 2:1 ratio (v/w) of transfection reagent to DNA. Total DNA transfected per well (9.6 cm²) did not exceed 2 µg. Transfection was carried out 24–48 h for activity assay and Western Blot applications and 36 h for localization analysis.

**Co-IP and Western blot analysis**

An appropriate amount of cells were lysed by suspension in SDS-free RIPA like buffer (RLB) consisting of 50 mM Tris-HCl (pH 7.5), 0.3% CHAPS, 150 mM sodium chloride, 1 mM sodium vanadate (Na₂VO₄), 10 mM sodium fluoride (NaF), 1 mM ethylene diaminetetraacetate (EDTA), 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′′,N′′′-tetraacetate (EGTA), 2.5 mM tetrasodium pyrophosphate Na₄O₇P₂. 1 µm Dithiothreitol and 1x HALT™ protease inhibitor cocktail (Sigma-Aldrich) were freshly added. Lysis was followed by immediate freezing in liquid nitrogen, thawing on ice for 30 min and repeated mixing by pipetting for 30 s each. After centrifuging the pellet, the lysate was either directly separated by SDS-PAGE on a 4–20% gradient gel or used in the following Co-IP steps. For Co-IP, the GFP containing lysate was incubated with GFP-trap dynabeads (Chromotek, Planegg-Martinsried, Germany) for 1 h at 4 C with constantly mild agitation to pull-down eGFP-KDM6A variants and complexed proteins. Dynabeads were
magnetically separated and washed three times with Co-IP buffer, resuspended in SDS-PAGE loading buffer, cooked and separated on a 4–20% gradient gel. After running, the gel was transferred onto an activated PVDF-membrane. The membrane was blocked in TBS/0.1% Tween (TBS-T) and 5% BSA for 1 h at RT and subsequently incubated with the respective primary antibody (Table S6) in TBS-T, 1% BSA overnight at 4 °C. The membrane was washed three times in TBS-T at RT. The secondary antibody (Table S6) was applied for 1 h at RT in TBS-T, 1% BSA. The membrane was washed again for three times, Clarity™ ECL (Bio-Rad, Hercules, CA, USA) was used to develop the signal.

**ELISA-based demethylase activity assay**

Preparation and lysis of the cells was done the same way as described for Co-IP. However, before the GFP-trap dynabeads were added, the fluorescent emission signal of the lysate was measured (470 nm ex., 485–650 nm em.) in a microliter cuvette. The dynabeads were then mixed in the lysate for 1 h at 4 °C under constant, mild agitation. Beads were then magnetically separated, the fluorescent emission of the remaining lysate was measured again with the same specifications as above. The delta in the emission spectra before and after bead incubation in the range of 500–530 nm was used to calculate the amount of eGFP-KDM6A pulled out of the lysate in each run. To eliminate residual RLB buffer, beads were washed two times with the activity assay (AA) buffer (50 mM TRIS-HCl (pH 7.45), 0.02% Triton X-100, 100 µM α-ketoglutarate, 50 µM Fe(NH₄)₂(SO₄)₂·6 H₂O, 100 µM ascorbic acid, 1 mM TCEP. Cofactors and TCEP being added freshly to avoid oxidation). H3K27me3 (ProteoGenix, Schiltigheim, France) and H3K27me2 (BioCat) peptides (Table S7) were dissolved in AA buffer and mixed with the loaded beads. The beads were incubated with the peptides for 4 h at 30 °C while maintaining constant suspension. Afterwards, the beads were magnetically separated and the supernatant containing the biotin-labeled peptide was loaded onto a streptavidin-coated 96-well plate (triplicate per variant/control, 50 µl per well). After 1 h of biotin binding at RT and removal of the solution, the wells were loaded with 50 µl of the a-H3K27me2 antibody in 0.1% TBS-T and incubated for 1 h at RT while shaking gently. The wells were then washed three times with 150 µl TBS-T. Subsequently, 50 µl of a 1:1000 alkaline phosphatase (ALP)-conjugated secondary antibody was added and incubated for 30 min at RT. The wells were washed four times for 5 min. For detection, 100 µl of p-nitrophenyl phosphate (pNPP, Sigma-Aldrich) was added into each well and incubated for 10 min at RT in the dark, mixing thoroughly. The reaction was then quenched by 100 µl 1 M NaOH. The signal was measured in a plate reader at 405 nm absorption, quantified, normalized and fitted. To fit the ELISA readout, we used a 4-PL-regression normalized to the standard curve (additional method 1, Activity assay analysis). The WT fit overlaid with the standard curve was used to directly calculate the relation between the amount of fluorescence signal and demethylated product. WT 4PL-regression fit was applied to calculate c₅₀ as a reference point to compare the WT value with the variants. Additionally, eGFP reference measurements were used to calculate the absolute amount of protein input and calculate a specific activity. Initial validation for the assay was done with recombinant full-length KDM6A (#31460, Active Motif).

**Immunocytochemistry**
Depending on the antibody requirements two protocols were used. For ICC with primary antibody incubation overnight, cells were seeded on coverslips, transiently transfected and fixed at > 80% confluence with 1% (v/v) para-formaldehyde, 0.02% (v/v) Triton X-100 for 20 min at RT. Blocking and permeabilization was done with 1% (w/v) BSA, 0.1% (w/v) saponin in PBS for 30 min at RT. After overnight incubation at 4 °C, coverslips were washed with PBS and incubated with the secondary antibody for 1 h at RT. Slides were washed with PBS, stained with DAPI (1:2000 for 2 min), washed again frequently with PBS and mounted. For ICC with primary antibody incubation for 1 h RT, cells were prepared as before, but fixed with 4% FA (v/v) for 10 min at RT. Permeabilization was done with 0.5% (v/v) Triton X-100 for 3 min at RT and blocking with 1% (w/v) BSA in PBS for 30 min at RT. The primary antibody was incubated shaking for 1 h at RT, coverslips were washed with PBS and incubated shaking with the secondary antibody for 1 h at RT. Slides were washed with PBS, stained with DAPI (1:2000 for 2 min), washed again several times with PBS and mounted. Primary and secondary antibodies are listed in Table S6.

Microscopy and image processing

Confocal imaging with live or fixed cells was performed on a confocal laser scanning microscope FV1000 IX81 inverted microscope (Olympus, Shinjuku, Japan) using a 60x water immersion UPLSAPO NA 1.2 objective. DAPI, eGFP and Star Red were excited at 405 nm, 488 nm and 635 nm, respectively, with the internal FV10-MARAD-2 main laser unit. Star Orange was excited at 559 nm with an external Opti λ 559 diode laser (NTT Electronics, Yokohama, Japan). Internal PMT detectors (Olympus) were used for detection. Confocal laser scanning microscope (LSM) processing routine was carried out with with the freely accessible Fiji and Huygens Pro 20.10 (SVI, Netherlands) for deconvolution. For deconvolution of images fulfilling the Nyquist criterion, we used an automatically computed theoretical point spread function based on our known microscopic parameters and a model of the Olympus IX81 provided by SVI and performed 30 iterative steps of classic maximum likelihood estimation (CMLE) on our images. The signal-to-noise ratio was determined for each channel and then kept constant for during image processing.

Scatterplot generation and cellular back mapping

We used the freely available, open source Fiji plugin ScatterJ (17). Processed images were converted into 8-bit grey scale tagged image file formats (.tiff) and opened in ScatterJ. 256 × 256 pixel scatterplots were saved as xy-lists (.dat) for processing in OriginPro. Within scatterplot, regions were selected using Fiji free-hand-tool and back mapped to the original image. The back-mapped image, as well as channel-wise images, were saved as text sequences (.dat) or portable networks graphics (.png) for further image and matrix analysis in OriginPro. The resulting cellular back-mapping-images representing the pixel-wise analysis of defined scatterplot populations are shown in Fig. 5 and the corresponding Figure S10.

Abbreviations

4-PL
Four parameter logistic

**aa**
Amino acid

**AA**
Activity assay

**CMLE**
Classic maximum likelihood estimation

**COMPASS**
Complex proteins associated with Set1

**DDR**
DNA repair and stress response

**ELISA**
Enzyme-linked immunosorbent assay

**GO**
Gene ontology

**IDR**
Intrinsically disordered protein

**JmjC**
Jumonji C domain

**KDM**
Lysine demethylase

**KMT**
Lysine methyltransferase

**M**
Manders coefficient

**MS**
Mass spectrometry

**NPM**
Nucleophosmin

**P**
Pearson coefficient

**PTM**
Post-translational modification

**RBBP**
Retinoblastoma-binding protein

**RLB**
RIPA-like buffer

**SDM**
Site-directed mutagenesis

**SWI/SNF**
SWitch/Sucrose non-fermentable
TPR
Tetra-tricopeptide repeat
UTX
Ubiquitously transcribed tetratricopeptide repeat, X chromosome
WDR
WD-repeat containing protein
WRAD
WDR5-RBBP5-ASH2L-DPY30 complex
WT
Wildtype

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
All data generated or analysed during this study are included in this published article and in supplementary information files or are available from the corresponding authors on reasonable request.

Competing interests
The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Contributions
AG, JK, WAS conceived the project and designed the experiments. AG and JK conducted the experiments, collected, interpreted the data and wrote the manuscript. AL and AP helped with experiments and
resources. MJH, WAS reviewed and edited the manuscript. All authors read and approved the final Manuscript.

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