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The microtubule-associated histone methyltransferase SET8, facilitated by transcription factor LSF, methylates α-tubulin

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Running title: *SET8-mediated methylation of α-tubulin on K311*

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ABSTRACT

Microtubules are cytoskeletal structures critical for mitosis, cell motility, and protein and organelle transport, and are a validated target for anticancer drugs. However, how tubulins are regulated and recruited to support these distinct cellular processes is incompletely understood. Post-translational modifications of tubulins are proposed to regulate microtubule functions and dynamics. Although many of these modifications have been investigated, only one prior study reports on tubulin methylation and an enzyme responsible for this methylation. Here, we used *in vitro* radiolabelling, mass spectrometry, and immunoblotting approaches to monitor protein methylation, and immunoprecipitation, immunofluorescence, and pull-down approaches to measure protein-protein interactions. We demonstrate that N-lysine methyltransferase 5A (KMT5A or SET8/PR-Set7), which methylates lysine 20 in histone H4 (H4K20), bound α-tubulin and methylated it at a specific lysine residue, Lys-311. Furthermore, LSF/CP2, a known transcription factor, bound both α-tubulin and SET8, and enhanced SET8-mediated α-tubulin methylation *in vitro*. In addition, we found that the ability of LSF to facilitate this methylation is countered by factor quinolinone inhibitor 1 (FQI1), a specific small-molecule inhibitor of LSF. These findings suggest the general model that microtubule-associated proteins, including transcription factors, recruit or stimulate protein-modifying enzymes to target tubulins. Moreover, our results point to dual functions for both SET8 and LSF, not only in chromatin regulation, but also in cytoskeletal modification.

Microtubules (MTs), the polymerized heterodimers of α-tubulin and β-tubulin, are major cytoskeletal components that play important roles in key cellular processes such as structural support, localization of organelles, and chromosome segregation (1,2). A number of post-translational modifications (PTMs) of tubulins have been reported, which contribute to the functional diversity of MTs and affect MT dynamics and

organization (3). This led to the hypothesis of a tubulin code (1), in which tubulin modifications specify biological outcomes through changes in higher-order microtubule structure by recruiting and interacting with effector proteins. As in the well-established, parallel histone code paradigm, each specific modification would be anticipated to either directly recruit or interrupt the interaction between the MTs and specific interactor(s). Most identified tubulin PTMs, including tyrosination, glutamylation, glycylation, map to the unstructured tubulin C-termini that regulate interaction with motors and other microtubule-associated proteins (MAPs) (3). The extensively studied acetylation on K40 of α-tubulin is unusual, in that it is located in the lumen of MTs (4); this modification marks stable MTs, and may be induced by transient breakage (5,6). Notably, tubulin methylation has been less studied than other types of tubulin modification.

SET8/PR-Set7 is a N-lysine methyltransferase responsible for the monomethylation of both histone and non-histone proteins in higher eukaryotes (7). It is functionally characterized as a histone H4 lysine 20-specific monomethyl-transferase (8); this modification is often a mark for transcriptional repression, although it can also be associated with active promoters. Both SET8 and H4K20me are specifically enriched during mitosis (9,10). SET8 is required for both DNA replication and mitosis during cell cycle progression, with deletion or RNAi-mediated depletion of the enzyme leading to impaired replication origin licensing and reduced chromosome compaction (11-18). Previous findings, in particular, suggested that SET8 and H4K20me1 are required for mitotic entry (19). In addition, enhanced expression or impaired cell cycle-specific degradation of SET8 can lead to premature chromosome condensation, mitotic delay, or impaired cytokinesis (20,21). SET8 also mediates monomethylation of other substrates, including p53, which results in repression of p53 target genes (22). However, how H4K20me1 is regulated and how it functions to promote cell cycle progression remains an open question, including the possibility that other non-histone substrates may be involved.

LSF (also named CP2), previously characterized widely as a transcription factor, is an oncogene in hepatocellular carcinoma that is signficantly overexpressed in hepatocellular carcinoma cell lines and patient samples (23-28), as well as in other cancer types (29). LSF is involved in cell cycle progression and cell survival (30-32). Initially, LSF was described as a regulator of G1/S progression (32) and essential for inducing expression of the gene encoding thymidylate synthase (*TYMS*) in late G1. Additional involvement of LSF in mitosis was initially demonstrated through characterization of the effects of Factor Quinolinone Inhibitor 1 (FQI1), a specific small molecule inhibitor of LSF (31). The biological specificity of FQI1 for LSF was confirmed by the parallel mitotic phenotypes between treatment with FQI1 and with siRNA targeting LSF (33). These include mitotic delay with condensed but unaligned chromosomes, incomplete cytokinesis, and multinucleation. FQI1 not only abrogates the DNA-binding and corresponding transcriptional activities of LSF (31), but also specific LSF-protein interactions (34). Finally, FQI1 inhibits growth of hepatocellular carcinoma tumors in multiple mouse models, and causes cell death via mitotic defects in hepatocellular carcinoma cell lines (31,35).

In this study, we demonstrate that these three regulators of mitosis, SET8, LSF, and α-tubulin, all interact with each other both *in vitro* and within cells. Furthermore, we demonstrate that SET8 is a microtubule-associated methyltransferase that specifically methylates K311 of α-tubulin *in vitro*. Finally, in parallel to how transcription factors stimulate histone modification by interacting both with the chromatin writers and the DNA, LSF stimulates *in vitro* methylation of α-tubulin by SET8. Overall, these results suggest that LSF and SET8 have biological implications beyond gene transcription and histone methylation, respectively.

Results

SET8 directly interacts with tubulin

Although SET8 in some studies has been reported to be solely a nuclear protein, consistent with its identified histone H4, PCNA and UHRF1

substrates, localization of SET8 also in the cytoplasm of human cells has been previously documented by others, in a cell type-specific manner (36,37). Furthermore, even in the same cells, SET8 localization was shown to switch between the cytoplasm and the nucleus during cell cycle progression (38). In order to investigate the localization of SET8 in the cytoplasm, GFP-SET8 expressed in a COS7 cell line, in which it is substantially localized in the cytoplasm, was analyzed in greater detail (Fig 1A). Upon screening for co-association specifically with various cytoplasmic structural features by staining with relevant fluorescence dyes or antibodies along with GFP-SET8 expression, GFP-SET8 significantly co-localized only with α-tubulin, indicating association with MTs. MT co-localization was observed at stages throughout the cell cycle (Fig 1A, Fig S1A). The most obvious association was in G1 phase, when SET8 exhibited the same pattern as the filamentous tubulin distributed throughout the cytoplasm, emphasized by the yellow in the merged image. In S phase, a larger percentage of GFP-SET8 was also nuclear (Fig 1A). In order to verify that this colocalization with α-tubulin was not an artefact of the overexpression of the fusion of SET8 with GFP or the use of monkey cells, immunofluorescence was used to image endogenous SET8 and α-tubulin in the HCT116 human colon cancer cell line. Once again, although some SET8 was nuclear, it was abundant in the cytoplasm, where it colocalized with α-tubulin (Fig 1B). In addition, we performed biochemical fractionation of human HEK293T cells (Fig 1C), which were used in the subsequent experiments. Whereas the nuclear fraction still contained some cytoplasmic markers (tubulins, likely due to attachment of cytoplasmic proteins to the nuclear membrane), the cytoplasmic fraction lacked any significant amount of nuclear marker. The endogenous SET8 was present in both the nuclear and the cytoplasmic fractions, although predominantly in the nucleus in these cells (Fig 1C).

The colocalization of SET8 in the cytoplasm with microtubules suggested that SET8 might be a microtubule-associated protein. Since purified tubulin preparations from mammalian tissues are known to contain microtubule-associated proteins that co-purify with the polymerized tubulin, we tested whether such a preparation (>97% tubulin) contained SET8. By immunoblotting, SET8 was detectable, although as a minor component (Fig S1B). To confirm that endogenous cellular SET8 associates with tubulins in HEK293T cells, we immunoprecipitated protein complexes from cell extracts. Using antibody against SET8, α-tubulin was also precipitated. In addition, some β-tubulin co-precipitated, although to a considerably lesser extent (Fig 1D). Conversely, upon expression of Flag-tagged α-tubulin or β-tubulin in the cells, endogenous SET8 co-immunoprecipitated with both, to roughly similar extents compared to the level of expression of the tagged tubulin (Fig 1E). As α- and β-tubulins stably heterodimerize in cells, *in vitro* experiments were required in order to determine whether either of these interactions between SET8 and tubulin was direct. To this end, purified recombinant proteins fusing maltose binding protein (MBP) to either α-tubulin (TUBA1A) or β-tubulin (TUBB) were individually tested for interactions with His-tagged SET8 purified from *E. coli*. SET8 directly interacted only with α-tubulin, but not with β-tubulin (Fig 1F). To map the region of SET8 that interacts, recombinant proteins fusing glutathione S-transferase (GST) to either full-length, or the N- or C-terminal overlapping portions of human SET8 were tested for interactions *in vitro* with purified mammalian tubulin. The purified heterodimeric tubulin interacted only with the full-length and N-terminal portion of SET8, even though th C-terminal SET8 fusion protein was present at a higher level than the others (Fig 1G), indicating specificity of this interaction. Taken together, these data demonstrate that α-tubulin and SET8 directly interact with each other, whereas β-tubulin only associates in a complex with SET8 in the presence of α-tubulin.

SET8 methylates α-tubulin

SET8 was characterized historically as a histone H4K20 specific methyltransferase, and subsequently as a regulator of the non-histone protein p53. However, since SET8 bound strongly

to α-tubulin, we tested whether tubulins could be a novel substrate of the enzyme. Purified porcine α/β-tubulin was incubated with the cofactor S-adenosyl-L-[methyl-^{3}H] methionine (AdoMet) and purified, recombinant GST-SET8. In the presence of both SET8 and AdoMet, radioactivity was incorporated into a protein band migrating at the position of α- and β-tubulins, in addition to a less pronounced automethylation of GST-SET8 (Fig 2A, lane 3), but no radioactive product was present at the position of α- and β-tubulins when either tubulin or SET8 was omitted from the reaction (Fig 2A, lanes 2 and 4). Interestingly, when histone H4 was also included in the reaction, the amount of tubulin modification was reduced (Fig 2A, lane 1), indicating that histone H4 strongly competed with tubulins for the methylation activity of SET8. Furthermore, histone H4 also competed with SET8 itself as a substrate, as shown by the significant reduction in SET8 automethylation in the presence of histone H4.

Since purified tubulin is composed of α-and β-tubulin heterodimers, we sought to determine which species is methylated by SET8. Recombinant fusion proteins of either α-tubulin or β-tubulin with MBP were purified and incubated with SET8 along with the radioactive methyl donor. Upon incubation of SET8 with α/β tubulin and AdoMet, both SET8 and tubulin(s) were labeled. However, only MBP-α-tubulin, but not MBP-β-tubulin, was methylated along with SET8 itself, when the individual recombinant proteins were tested (Fig 2B). These data indicate that α-tubulin is the target for SET8. Mass spectrometry was used to determine which lysine residue(s) of α-tubulin were methylated by SET8. In control samples lacking exogenous SET8, lysine methylation of α-tubulin on K304 (Fig S2A), and of β-tubulin on K19 and K297 (Fig S2B) were detected, none of which have previously been reported. As anticipated from the previous data (Fig 2B), incubation with exogenous SET8 did not induce detectable methylation at any other sites on β-tubulin. However, SET8 did induce methylation of three additional lysine residues of α-tubulin - K280, K311 and K352 - which were all monomethylated (Fig 2C, Fig S2A). Of these three lysines, only K311 is located on the outside surface of MTs, whereas K352 is at the interface between α-tubulin and the β-tubulin in the adjacent heterodimer, and K280 is on the inside surface of MTs (Fig 2D, Fig S2C,D). In addition, only the sequence surrounding K311 ($RHGK_{311}$) resembles those of other known SET8 target sequences: histone H4 ($RHRK_{20}$) and p53 ($RHKK_{382}$) (22). In contrast, the sequences of the other α-tubulin sites, $SAEK_{280}$ and $TGFK_{352}$, do not resemble other known physiological SET8 targets. Therefore, in order to determine the relative efficiency of methylation by SET8 *in vitro* at the identified sites, each was independently mutated in the context of the full-length MBP-α-tubulin, and purified proteins were tested for incorporation of radioactivity upon incubation with SET8. Each lysine was mutated to serine, maintaining a similar structure and hydrophilicity, but removing the charge. Consistent with the K311 surrounding sequence being the best match with other SET8 targets, mutation of K311 abolished modification by SET8. In contrast, mutation of K280, K304, or K352 did not appreciably affect the degree of methylation of the substrates (Fig 2E).

In order to test further the targeting of the various α-tubulin sites by SET8, peptides spanning these three sites (K280, K311, K352), as well as K40, reported to be methylated by SETD2 (39), and K304, modified in purified porcine tubulin (Fig S2A), were incubated with purified wild type SET8 *in vitro*. Only the K311-containing peptide was robustly methylated (Fig S3A,B, Table S1). In addition, radioactive incorporation into the K311-containing peptide was absent when incubated with catalytically inactive SET8 (D338A) *in vitro*, and methylation abolished if the K311 residue was either mutated (K311A, K311S) or already modified (K311Me, K311Ac) (Fig S3B, Table S1). Although the *in vitro* targeting of the α-tubulin K311-containing peptide by SET8 is robust, SET8 methylates histone H4 much more efficiently (Fig S3C), consistent with the ability of Histone H4 to strongly compete against tubulin for methylation by SET8 (Fig 2A).

As a comparison, we tested methylation by SETD2 of the α-tubulin K40-containing peptide. The original account describing methylation of α-

tubulin by SETD2 included the statement in the supplemental experimental procedures, as data not shown, that purified SETD2 did not methylate the target peptide *in vitro* (39). We confirmed this unexpected finding, that methylation of the K40-containing peptide was not detectable over background, despite the reported methylation of histone H3 by the purified SETD2 enzyme *in vitro* (Fig S3D). K40 is the only residue in α-tubulin previously reported to be targeted by an identified tubulin methyltransferase (39).

Taken together, these observations indicate that SET8 methyltransferase can directly, specifically, and effectively methylate α-tubulin at K311.

Transcription factor LSF associates with both SET8 and tubulin

DNA-binding proteins recruit chromatin writers to modify histones (40-43), suggesting the possibility that tubulin-binding proteins might similarly recruit SET8 to target sites on microtubules resulting in tubulin modification. Our previous studies showed that the transcription factor LSF interacts with DNMT1, and addition of an inhibitor of the LSF-DNMT1 interaction resulted in alterations in the genomic DNA methylation profile (34); this is consistent with recruitment of DNMT1 to DNA by LSF in order to facilitate DNA methylation at specific sites. Since DNMT1 complexes with SET8, and both SET8 and LSF (31) are required for mitotic progression, we proposed the novel hypothesis that the transcription factor LSF might also recruit SET8 to microtubules in order to facilitate α-tubulin methylation by SET8. In support of this notion, there is precedent for some DNA-binding transcription factors binding microtubules (44-49), although in all these instances for the purpose of sequestering the transcription factors in the cytoplasm and/or facilitating their transport into the nucleus.

To test our hypothesis, multiple assays were initially performed to evaluate whether LSF interacts with SET8 and tubulin(s). *In vitro*, direct interaction between recombinant, purified SET8 and purified LSF was evaluated by a GST pull-down assay (Fig 3A). Using fusion proteins between GST and either full length SET8, or its N- or C-terminal fragments, His-LSF bound specifically to the N-terminal region of SET8 (Fig 3A), the same domain that bound purified tubulins (Fig 1F). The binding of purified α/β-tubulin to purified GST-LSF was also evaluated and mapped to specific regions within LSF. Both α- and β-tubulins showed similar binding profiles to the panel of LSF fusion proteins, as expected given their stable heterodimeric structure (Fig 3B). Binding of tubulins to the full-length GST-LSF was greater than to the control GST, although quite weak compared to some of the other fusion proteins; this was ascribed to the sensitivity of the full-length GST-LSF fusion protein to cleavage in bacterial culture, resulting in a significant fraction of the purified preparation representing the GST domain alone. However, the tubulins interacted strongly with two specific domains of LSF whose GST fusion proteins were stable: the DNA binding domain (DBD), and to a lesser extent, the sterile alpha motif domain (SAM; Fig 3B). Further analysis suggests that it is the C-terminal portion of the DBD that contains the tubulin interaction surface in this domain, since the GST-LSF 2 protein also binds both tubulins to a high degree. Finally, purified His-LSF also interacted in parallel assays with purified recombinant full-length GST-α-tubulin (Fig 3C). These *in vitro* protein-protein interaction results indicate that all pairwise interactions among SET8, LSF, and α-tubulin occur through direct binding with each other.

To examine whether interactions of LSF with both SET8 and tubulin also take place in cells, multiple approaches were taken. First, upon co-expression of GFP-SET8 and 3xFlag-tagged LSF in transient transfection assays, the two proteins significantly co-localized, predominantly in the cytoplasm (Fig 3D). Although LSF, as a transcription factor, is localized in the nucleus, endogenous LSF has also been shown to localize in the cytoplasm in a cell type-specific manner (36), consistent with these immunoflouresence results. Second, the presence of complexes between endogenous cellular proteins was tested by co-immunoprecipitation experiments using HEK293T cell lysates. With antibodies against endogenous

LSF, but not control antibodies, both endogenous SET8 and endogenous α-tubulin co-immunoprecipitated with LSF (Fig 3E). Reciprocally, SET8 antibodies not only specifically co-immunoprecipitated its previously identified partner proteins, PCNA (50-52) and UHRF1 (37), but also endogenous LSF (Fig 3F). Finally, the possibility of relevant LSF-tubulin interactions was investigated by analyzing whether LSF was present in commercial, highly purified tubulin preparations. These preparations are obtained in part by multiple rounds of polymerization/depolymerization of the tubulin, and are more than 97-99% pure. They are well known to contain additional proteins that are defined as microtubule-associated proteins (MAPs). Immunoblots using a LSF monoclonal antibody did indeed detect a band comigrating with LSF, albeit at a very low level (Fig 3G). LSF was reproducibly detected in this manner in multiple commercially purified preparations of tubulin.

Taken together, these results demonstrate that LSF interacts directly with both SET8 and α-tubulin *in vitro*, and also associates with both of these proteins *in vivo*. Furthermore, LSF, although a transcription factor, appears to be a previously unidentified MAP.

LSF promotes tubulin methylation by SET8

The demonstration of pairwise, physical interactions between LSF, tubulin, and SET8 set the stage for directly testing the hypothesis that LSF could mediate the methylation of α-tubulin by SET8. Thus, recombinant GST-SET8 and the radioactive methyl donor were incubated with tubulin in the presence of increasing concentrations of purified His-LSF (Fig 4A). Tubulin methylation increased upon increasing LSF from a 1:4 to 2:1 molar ratio of LSF:GST-SET8, suggesting that LSF can mediate tubulin methylation by SET8. Note that in this experiment, there was more SET8 relative to tubulin than in other experiments (Fig 4A, bottom), resulting in a greater degree of automethylation of SET8 compared to tubulin methylation (Fig 4A, top), presumably due to substrate competition between SET8 itself and α-tubulin. A similar experiment was performed using recombinant MBP-α-tubulin as substrate for SET8, which also showed that increasing levels of LSF enhanced methylation of MBP-α-tubulin (Fig S4A).

The LSF small molecule inhibitor, FQI1, inhibits LSF binding to DNA (35), as well as binding of LSF to certain protein partners (34). To determine whether FQI1 would diminish the interaction between LSF and α-tubulin in cells, cell lysates from vehicle- versus FQI1-treated cells were analyzed by co-immunoprecipitation assays. These demonstrated a significant reduction in the LSF-α-tubulin interaction after FQI1 incubation for 24 hours (Fig 4B). Since FQI1 can inhibit the LSF-tubulin interaction *in vivo*, it was used to interrogate whether the interaction of LSF and tubulin was important for stimulating the SET8-mediated methylation of α-tubulin *in vitro*. Given that LSF is already present in the tubulin preparations, FQI1 was initially added to reactions containing only SET8, radioactive methyl donor, and purified tubulin. Tubulin methylation decreased with increasing concentrations of FQI1 (Fig 4C), consistent with the presence of LSF and its ability to enhance SET8-dependent tubulin methylation. Whether FQI1 specifically inhibits LSF in these assays was tested in two ways. First, it was demonstrated that the presence of FQI1 prevented any increase in tubulin methylation upon addition of purified His-LSF (Fig S4B, compare lanes 7 and 8). Second, the possibility that FQI1 directly inhibits SET8 catalytic activity was tested using Histone H4, instead of α-tubulin, as a substrate. Limiting amounts of histone H4 were added in this experiment to enhance the sensitivity of the assay. FQI1 did not inhibit methylation of histone H4 by SET8 (Fig 4D), in contrast to its effect on α-tubulin methylation (Fig 4C). In addition, when SET8 was incubated with histone H4 plus whole cell extract in the presence of the radioactive methyl donor, FQI1 did not appreciably diminish methylation of any other proteins in the extract either (Fig S4B, compare lanes 1 and 2), in contrast to its ability to inhibit methylation of tubulin (Fig S4B, compare lanes 3 and 4).

In order to verify that this methylation occurs *in vivo*, we generated an antibody against an α-tubulin peptide containing K311me. Upon immunblotting HEK293T whole cell lysates, only

two proteins were identified with the antibody, one of which co-migrated with tubulins (Fig 4E). Purified mammalian tubulin also was detected, when sufficiently large amounts were loaded onto the gel. Specificity of the interaction to the methylated lysine in both proteins detected was demonstrated by the ability to compete the signal with methylated, but not unmethylated peptide. The identity of the larger protein is not yet known, and the subject of ongoing investigation, but this experiment definitively demonstrates that α-tubulin is methylated on K311 *in vivo*. Upon treating cells with a SET8 inhibitor, the degree of methylation of α-tubulin, as detected with the K311me-specific antibody, was diminished (Fig 4F, bottom). Treatment with FQI1 to inhibit LSF reduced the level of LSF in these HEK293T cells (Fig 4F, top), as was previously noted (35). More importantly, FQI1 also partially diminished the degree of K311 methylation of α-tubulin, but to a lesser degree than did the SET8 inhibitor (Fig 4F, bottom).

To probe whether LSF recruits SET8 to tubulin, we used FQI1 to disrupt the LSF-tubulin interactions (Fig 4B). FQI1 did diminish co-immunoprecipitation of endogenous tubulin with SET8 antibodies (Fig S4C), supporting the recruitment model. However, a caveat to this straightforward interpretation was that SET8 immunoprecipitation was also somewhat diminished, although to a lesser degree, after incubation of the cells with FQI1.

Taken together, these findings indicate that SET8 methylates α-tubulin at K311. Furthermore, they suggest that LSF enhances this ability of SET8 to modify α-tubulin, and conversely that FQI1 therefore impedes methylation of α-tubulin by SET8. The data support a model in which LSF recruits SET8 to tubulin, and/or in which LSF binding as a ternary complex with both SET8 and tubulin activates the methylase activity of SET8 already associated with tubulin (Fig 4G).

Discussion

Tubulin PTMs are generally thought to regulate the binding of proteins to the microtubule cytoskeleton, thereby regulating microtubule function, the cell cycle, and signaling events in the cell. To date, a large variety of microtubule associated proteins (MAPs) have been characterized, which stabilize or destabilize microtubules, are associated with the coupling of molecular motors and microtubules, and play critical roles in spindle formation (53). Here, we identify additional sites of methylation on tubulins, in particular methylation of K311 on α-tubulin, two additional unanticipated MAPs: the N-lysine methyltransferase SET8 that methylates α-tubulin K311, and the transcription factor LSF, and provide support for a novel mechanism for facilitating tubulin modifications.

Tubulin methylases and sites of methylation

A number of post-translational modifications of microtubules are well established, although limited insights have been obtained regarding their biological roles, as tubulin PTMs have generally remained less amenable to straightforward functional studies. Identified posttranslational modifications of tubulins mediated by specific modifying enzymes include acetylation of lysine 40 in α-tubulin by αTAT1 (MEC-17 in *C. elegans*), deacetylation of the same residue by Sirt2 (Sirtuin type2), HDAC5, and HDAC6, and polyglutamylation of both α- and β-tubulins at multiple C-terminal glutamate residues by TTLL4, 5, and 7 (54,55). Despite the extensive research on these various modified sites and their relevant enzymes, only one study previously identified lysine methylation of a tubulin (39), which is the focus of this report. Walker's group reported that SETD2, known as a histone methyltransferase for a chromatin activation mark, H3K36me3, also methylates α-tubulin at K40. Surprisingly, however, although purified SETD2 (1392-2564 aa) apparently methylated both purified bovine tubulin and recombinant TUBA1A-myc *in vitro*, it was noted as data not shown in the report that the enzyme was not able to methylate either a K40-containing peptide or purified GST-TUBA4A [see supplemental experimental procedures of (39)]. This is despite the fact that all six TUBA1 and TUBA3 isoforms have identical amino acid sequences betweem residues 1-74, and TUBA4A differs at only 4 residues in that stretch, with the

nearest nonidentical residue 10 away from K40 (residues 7, 16, 50, and 54). We have now confirmed this unexpected finding that purified SETD2 (1392-2564 aa) did not detectably methylate a α-tubulin K40-containing peptide *in vitro*. It will be interesting to understand the basis of this discrepancy.

Herein, we describe a distinct, novel lysine methylation of α-tubulin at K311 and identify an enzyme responsible for its modification both *in vitro* and *in vivo* as SET8, which is fully capable of methylating the target peptide, as well as both intact recombinant human protein and purified porcine tubulin. Given that the SET8 inhibitor did not entirely eliminate the K311me modification in cells, it is possible that another as yet unidentified methyltransferase also contributes to modification of this lysine. The $RHGK_{311}$ motif is highly conserved in α-tubulins, being present in eight human TUBA isotypes (TUBA1A-C, TUBA3C-E, TUBA4A, TUBA8).

In addition, we identify methylation of β-tubulin purified from mammalian brain at K19 and K297. K19 is conserved in all human β-tubulin isotypes, and the surrounding sequence in 6 of the 9 isotypes. K297 is conserved in 7 of 9 β-tubulin isotypes, whereas 2 isotypes instead have R297. We are pursuing identification of the enzymes responsible.

Biological consequences of tubulin methylation

SETD2 and SET8 not only target different sites in α-tubulin, but lead to differing methylation states (tri- *versus* mono-methylation, respectively). Thus, it is anticipated that each would lead to distinct biological consequences, including binding of different proteins. This is especially the case since these sites are on completely different locations on the microtubules – K40 in the lumen and K311 on the outer surface. Phenotypically, disruption of SETD2 or SET8 both result in mitotic defects and subsequent genomic instability, however with distinct features. SETD2-null mouse embryo fibroblasts exhibited a mitotic delay with delayed congression, multipolar spindles, lagging chromosomes and cytokinesis failure, resulting in polyploidy and polynucleation (39). In contrast, loss of SET8 results in premature chromosome condensation leading to delayed mitotic progression, in addition to defects in S phase (11-19). Conversely, lack of timely SET8 degradation in mitosis also delayed progression between metaphase and anaphase (20,21).

Precise modulation of SET8 levels is required for proper mammalian cell cycle progression. Previous reports therefore suggested that SET8 and its modification of histone H4, H4K20me1, functioned as novel regulators of cell cycle progression, with the focus on regulation of S phase (56). With our demonstration that SET8 can also methylate α-tubulin, the roles of non-histone substrates must also be considered as causes for SET8-mediated regulation of the cell cycle, and in particular of mitosis when SET8 is most abundant (9). Similarly to mammals, SET8 is an essential protein for *D. melanogaster*, as SET8 mutants die during larval development. However, flies in which all histone H4 genes were replaced by multiple copies of the mutant histone H4K20A unexpectedly survived to adulthood without apparent phenotypic defects, although they did exhibit a significant delay in development (57). Thus, contrary to the prevailing view, histone H4 was not the most critical biological target for SET8. Given the minimal biological effects in *Drosophila* of mutating histone H4K20, we propose that α-tubulin methylation is a strong candidate for mediating critical SET8 consequences. Notably, *D. melanogaster* and human α-tubulins are 98% identical with all the lysines throughout the sequence being conserved.

Targeting SET8 to tubulin by transcription factor LSF: a general model

Beyond identification of the tubulin PTMs and the enzymes that catalyze these modifications is the question of what drives the spatiotemporal access of such enzymes to MTs. Relevant to this process, we also demonstrate here that a transcription factor, LSF, apparently moonlights as a microtubule-associated protein, and that LSF has the ability to recruit SET8 to tubulin and/or enhance SET8's enzymatic modification of α-tubulin. Such a recruitment mechanism would mirror mechanisms

of targeting histone writers to chromatin, expanding the model of the parallel nature between the generation of the histone and tubulin codes. Furthermore, these data suggest that transcription factors more generally may be able to regulate tubulin modifications, and thereby microtubule dynamics. Although several transcription factors have previously been reported to bind microtubules, including c-myc (44,45), MIZ-1 (46), p53 (47,49), and Smads (48), in all these cases the biological relevance proposed or demonstrated was to sequester the transcription factors in the cytoplasm, and/or to help transport the transcription factor into the nucleus. Thus, all previous transcription factor-microtubule interactions were proposed to regulate transcription activity, not microtubule function. The *in vitro* results regarding LSF, although requiring further validation *in vivo*, provide the first instance in which binding of a transcription factor directly to microtubules affects tubulin modification, and presumably therefore to altered microtubule function. We propose that this may represent a new paradigm that reflect functions of other transcription factors, as well.

Relevance of LSF and SET8 to cancer

LSF, like SET8, is required for mitotic progression, as evidenced by mitotic defects upon reduction of LSF by siRNA or by inhibition of LSF activity by the small molecule inhibitor FQI1 (31,33). Consistent with LSF's role in the cell cycle progression (30,31,33), LSF has been implicated as an oncogene in multiple cancer types (29). In particular, LSF enhances tumorigenesis in hepatocellular carcinoma (23,26). Hepatocellular carcinoma is the sixth most common cancer worldwide and the second highest cause of cancer-related death globally (58). LSF is overexpressed in human hepatocellular carcinoma cell lines, and over 90% of human hepatocellular carcinoma patient samples, showing significant correlation with stages and grades of the disease (23), and elevated levels of LSF in patient tumors correlate with decreased survival (28). SET8 levels are also elevated and contribute by multiple mechanisms to cancer progression (56,59), including in hepatocellular carcinoma (60). Furthermore, elevated expression of specific α-tubulins (e.g. TUBA1B) have also been associated with this disease (61).

Mitosis, in which both LSF and SET8 are involved, is viewed as a vulnerable target for inhibition in cancer (62). The lead LSF inhibitor, FQI1, induces apoptosis in hepatocellular carcinoma cell lines *in vitro* as a consequence of mitotic defects and significantly inhibits tumor growth in multiple mouse hepatocellular carcinoma models, with no observable toxicity to normal tissues (31,35). Our new findings that LSF interacts with α-tubulin and SET8, and that FQI1 hinders the LSF-α-tubulin interaction, may relate to the impact of the LSF inhibitors in hepatocellular carcinoma cells and tumors. Given the current large unmet medical need, further investigation into the relevance of the LSF-α-tubulin-SET8 pathway for hepatocellular carcinoma and other cancer types in which LSF is oncogenic may aid in novel targeted and effective treatments.

Experimental procedures

Cell Culture, immunoprecipitation, and immunofluorescence

HEK293T and COS7 cells were cultured in DMEM media supplemented with 10% fetal bovine serum at 37°C. HCT116 were cultured in McCoy's media with 10% fetal bovine serum according to ATCC recommendations. For treatment with LSF and SET8 specific inhibitors, HEK293T cells were incubated with 2.5 μM FQI1 (Millipore/Sigma, 438210) or 10 μM UNC0379 (Selleckchem, S7570), respectively. FQI1 treatment was for 24 hours, or as indicated.

Immunoprecipitation and immunofluorescence experiments were carried out as described previously (63,64). For the immunoprecipitation, 1 mg of total HEK293T cellular extract was incubated with 5 μg of anti-SET8 antibody (Active Motif, 61009), anti-SET8 antibody (Millipore, 06-1304), anti-LSF antibody (Millipore, 17-10252), or mouse anti-FLAG antibody (Sigma-Aldrich, F1804,). The immunoprecipitates were blotted with anti-α-tubulin (Sigma, T9026), anti-β-tubulin (Sigma, T8328), anti-SET8 (Abcam, ab3798), anti-Pr-Set7 (D11) (Santa Cruz, sc-377034), anti-LSF

(BD Biosciences, 610818), anti-PCNA (Cell Signaling Technology, 2586), anti-UHRF1 (anti-ICBP90; BD Biosciences, 612264), or anti-FLAG (Cell Signaling Technology, 14793) antibodies, as per the manufacturer's dilution recommendations. Cellular extracts were also immunoprecipitated with normal IgG (Cell Signaling Technology) as a negative control for all immunoprecipitation experiments.

For immunofluorescence to detect α-tubulin and SET8 co-localization, COS7 cells were grown on coverslips and transfected with a GFP-SET8 expression plasmid. After cells were fixed with paraformaldehyde, the cells were incubated with anti-α-tubulin and the microtubules visualized with an anti-mouse IgG coupled with Alexa Fluor 488 (Molecular Probes) using a confocal microscope (Zeiss LSM510). For the detection of endogenous α-tubulin and SET8 co-localization, HCT116 cells were fixed with formaldehyde followed by methanol and blocked for 1 hr with 5% BSA-T-PBS. Cells were incubated with anti-SET8 antibody (Millipore), followed by anti-rabbit IgG coupled with Alexa Fluor 488, and then with anti-α tubulin (Sigma, T9026), followed by anti-mouse IgG coupled with Alexa Fluor 594 (Molecular Probes). Colocalization was detected using LSM880 confocal microscope (Zeiss). For the detection of SET8 and LSF co-localization, COS7 cells were co-transfected with GFP-SET8 and 3XFlag-LSF expression plasmids; the epitope tagged LSF was detected by mouse anti-FLAG antibody (F3165, Sigma-Aldrich) and visualized with an anti-mouse IgG coupled with Alexa Fluor 488 (Molecular Probes). DAPI was used to stain nuclear DNA.

GST and MBP pull down assays

LSF, SET8 and α-tubulin cDNAs were cloned into the pGEX-5X-1 (GE Healthcare) or pMalC4X (New England Biolabs) vector and GST-tagged or MBP-tagged proteins were captured using Glutathione Sepharose beads (GE Healthcare) or amylose resin (New England Biolabs), respectively. Sepharose beads containing approximately 10 μg of fusion protein were incubated for 2 hours at 4°C with purified tubulin (MP Biomedicals), recombinant His-tagged LSF, or recombinant His-tagged SET8, the latter two being purified from *E. coli*. Proteins bound to the beads were resolved by 10-20% SDS-PAGE. LSF, SET8 and α-tubulin were visualized by immunoblotting by using anti-LSF (BD Biosciences), anti-SET8 (Active Motif) or anti-α-tubulin (Sigma-Aldrich), respectively.

In vitro methylation assays

Approximately 1 μg of recombinant GST-SET8 (in 50% glycerol) and 2 μg of the purified tubulin (MP Biomedicals, 08771151), recombinant MBP-α-tubulin, or recombinant MBP-β-tubulin were incubated with 6 μM radioactively labeled [^{3}H] AdoMet (Perkin Elmer, NET155V001MC) in 1X HMT buffer containing 5 mM Tris pH 8.0, 0.5 mM DTT at room temperature for overnight. As indicated, histone H4 (New England Biolabs, M2504S), recombinant His-LSF protein, or FQI1 inhibitor were added to the reaction. Samples were separated by electrophoresis through a 10% Tricine Gel (Invitrogen) and the gel was stained with Coomassie Brilliant Blue (shown in grayscale in the figures) and incubated with EN3HANCE (Perkin Elmer) solution. The gel was dried and exposed to autoradiography film for 1 week. For the peptide assays, the specific peptides of α-tubulin were synthesized from AnaSpec Inc. Sequences are listed in Table S1. Two μg of each peptide and 2 μg of purified wild type or mutant SET8 or SETD2 (aa 1392-2564, Active Motif) were incubated with radioactively labeled [^{3}H] AdoMet at room temperature overnight. Samples were spotted onto P81 filters (Whatman 3698325) and the filters were washed 3 times with 0.3 M ammonium bicarbonate. The level of incorporated [^{3}H]CH_3 was determined using liquid scintillation counting.

Mass-spectrometric analysis

For identification of tubulin modifications, purified tubulin (MP Biomedicals, 08771151) was incubated with nonradioactive AdoMet, with or without recombinant GST-SET8, overnight at room temperature and the samples were separated by electrophoresis through a 10% Tris-glycine gel. Excised gel bands were digested with either subtilisin or trypsin in 0.01% ProteaseMax in 50

mM NH_4HCO_3 for 1 hr at 50°C. Digestion was quenched with trifluoroacetic acid and samples were dried. Each digest was individually reconstituted and analyzed by direct injection onto an analytical column 25 cm 100 µm ID Aqua 3 µm with Easy n1000 nLC-QExactive at 300 nL/min. Acquired HCD spectra were searched using Proteome Discoverer 2.0.0.802 with Sequest using the SWISSPROT June 2015 database (416061 sequences) and Cys = 57.02146 static modification. Dynamic modifications were set for Met = 15.99492. Two missed and/or nonspecific cleavages were permitted. Searches were semi-specific (trypsin semi-specific R,K) with K = 14.016 dynamic modification. The mass tolerance for precursor ions was 10 ppm, and for fragment ions was 0.02 Da. Results were filtered with Percolator (q-value < 0.01) for high confidence spectrum matches. The target strict false discovery rate was 0.01, as determined by Percolator.

Biochemical fractionation of HEK293T cells

Subcellular fractions from cultured HEK293T cells were obtained using the Cell Fractionation Kit (Cell Signaling Technology, 9038) according to the manufacturer's protocol. Cytoplasm, nuclear, and whole cell extracts were separated by electrophoresis through a 10-20% Tris-Glycine and the resulting membrane immunoblotted with anti-MEK1/2 (Cell Signaling Technology, 8727), anti-histone H3 (Cell Signaling Technology, 9715), anti-α-tubulin (Sigma, T9026), anti-β-tubulin (Abcam, ab15568), and anti-SET8 (Active Motif, 61009).

K311me-α-tubulin antibody

The custom rabbit polyclonal antibody was generated and purified by Eurogentec using the peptide CDPRHK(me)YMA. Specific antibodies were purified using a methylated peptide-conjugated resin followed by depletion of unmethylated peptide reactivity. Quality control ELISA analysis indicated specificity to the methylated peptide. Immunoblotting with the antibody was performed with 2 µg purified antibody; for the indicated blots, the antibody was preincubated overnight with 100 µg of either methylated or unmethylated peptide prior to incubation with the membranes.

Data Availability

The tubulin methylation mass spectrometry data from this publication, entitled Microtubules methylation LC-MSMS have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository [https://www.ebi.ac.uk/pride/archive/] and assigned the dataset identifier PXD014257.

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Figures

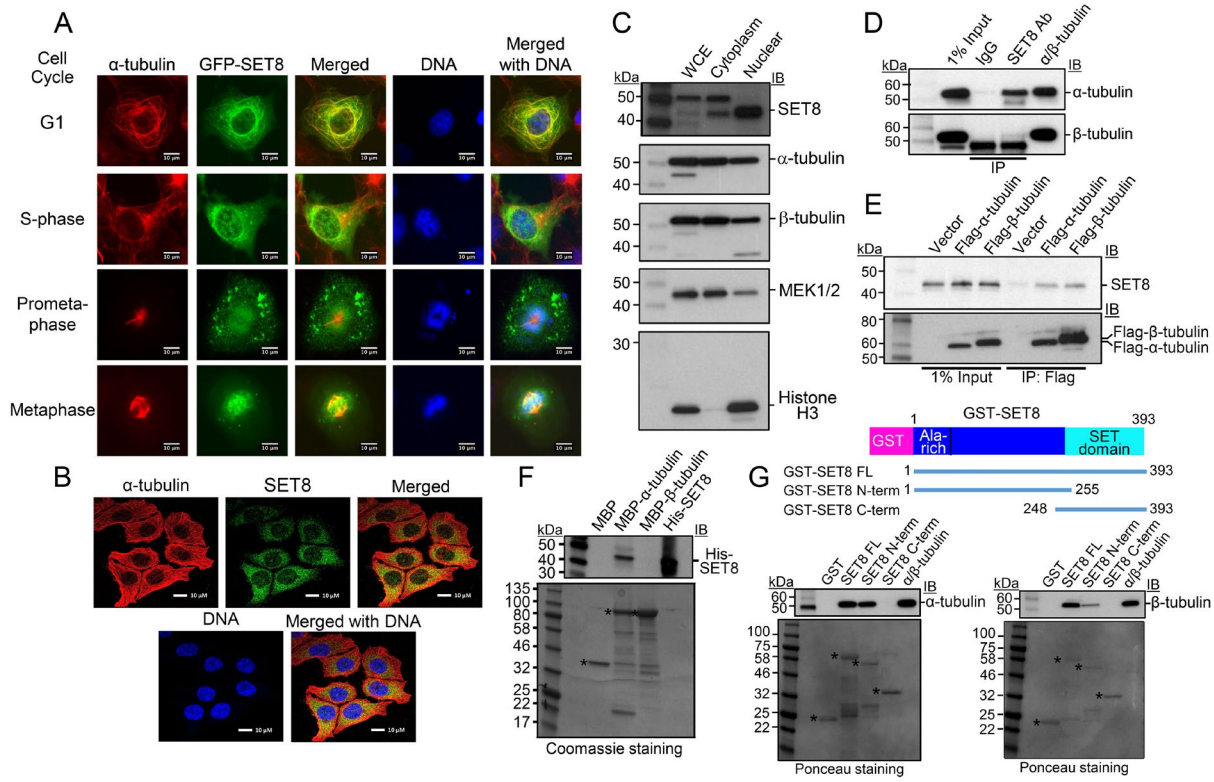

Figure 1. SET8 associates with tubulin in cells and directly interacts with α-tubulin *in vitro*. *A,* Colocalization of SET8 and α-tubulin in COS7 cells. GFP-SET8 (green) was expressed in asynchronous cells, tubulin was detected with anti-α-tubulin antibody (red) and DNA with DAPI (blue). Yellow in the merged image indicates colocalization of SET8 and α-tubulin. Images are from cells identified as being in the indicated stages of cell cycle progression. Scale bars: 10 μm. *B,* Colocalization of endogenous SET8 and α-tubulin in human HCT116 cells. *C,* Endogenous SET8 is localized in both nucleus and cytoplasm in HEK293T cells. Ten μg each of whole cell extract (WCE), cytoplasmic, and nuclear fractions were analyzed for the presence of SET8, α- and β-tubulins (cytoplasmic marker), MEK1/2 (predominantly cytoplasmic marker), and histone H3 (nuclear marker). *D,* Co-immunoprecipitation from HEK293T cells of endogenous tubulins with endogenous SET8, using SET8 antibody (Ab). Right lane: >99% pure tubulin (MP Biomedicals, 08771121) as a positive control. Immunoprecipitates were analyzed using antibodies against the indicated proteins by immunoblotting (IB). *E,* Co-immunoprecipitation from HEK293T cells of endogenous SET8 with transiently expressed Flag-tagged tubulins, as detected by immunoprecipitation with antibody against Flag. *F,* MBP-pull down analysis of purified His-SET8 with MBP-α-tubulin, but not MBP-β-tubulin. Top: Immunoblot (IB) in which biotinylated molecular weight markers are visualized; bottom: Coomassie staining of the same gel (shown in grayscale) in which standard molecular weight markers are visualized. *Expected positions of migration of the MBP-proteins. *G,* GST-pull down analysis of purified porcine brain tubulin to full-length or the indicated overlapping segments of SET8 fused to GST. Top: immunoblot (IB) in which biotinylated molecular weight markers are visualized; bottom: Ponceau staining of the same gels (shown in grayscale) in which standard molecular weight markers are visualized. *Expected positions of migration of the GST-proteins.

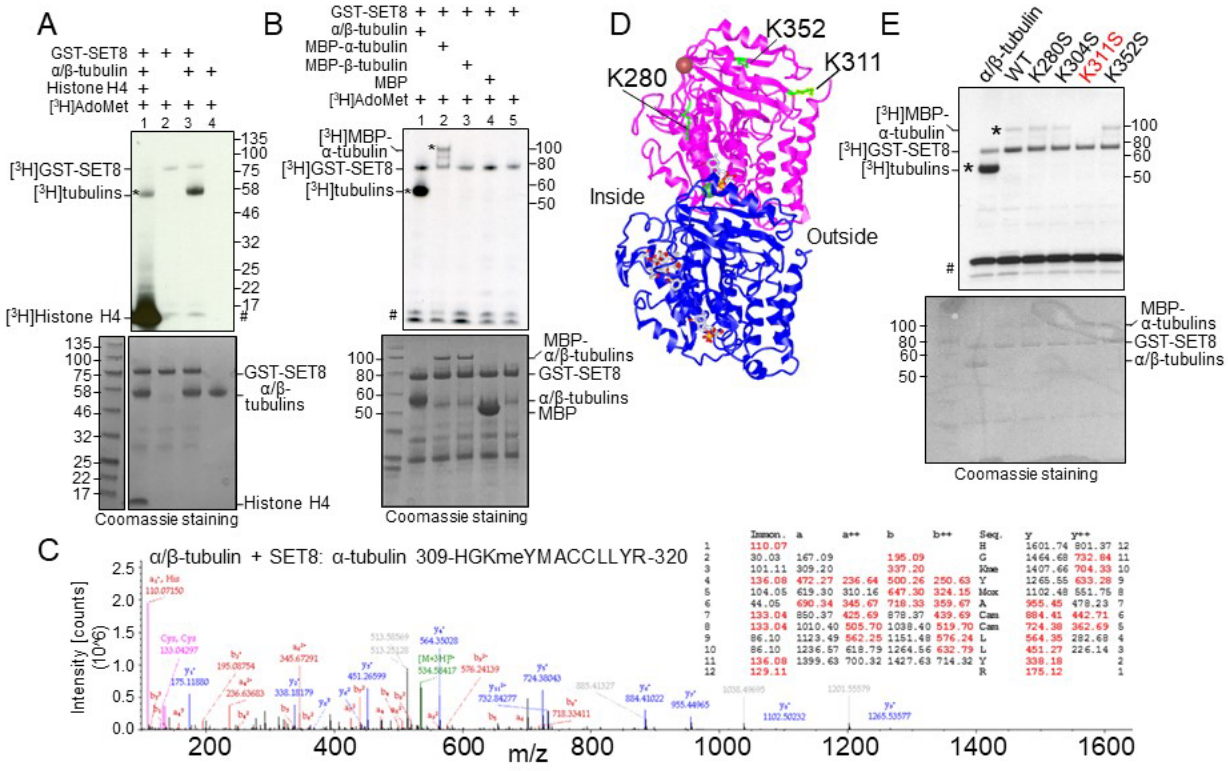

Figure 2. Histone methytransferase SET8 methylates α-tubulin at K311. *A,* Purified porcine tubulin (rPeptide, T-1201-1) is methylated by SET8. Lane 1: histone H4 (1 µg) was added in addition to tubulin as substrates. Top: autoradiogram of methyltransferase assays, showing methylation of tubulin (*), histone H4, and automethylation of GST-SET8. # indicates the migration of [^{3}H]-labeled impurities, which migrated at a similar position to that of Histone H4. Bottom: Coomassie-staining of the same gel (shown in grayscale) indicating relative levels of the components in the reaction. *B,* Recombinant human MBP-α-tubulin (*), but not MBP-β-tubulin, is methylated by SET8. Autoradiogram (top) and Coomassie-staining (bottom) are as described in *A*. Protein bands <50 kDa are from the purified GST-SET8 preparation, and are more evident in this experiment than in other reactions. *C,* Mass spectrum and table of expected m/z of the peptide fragments (with observed fragments in red) confirming methylation on K311 of α-tubulin after incubation of purified tubulin with SET8. *D,* The 3-dimensional structure of the α/β-tubulin heterodimer (PDB ID 1JFF; purple: α-tubulin; blue: β-tubulin), indicating positions of lysines in α-tubulin targeted by SET8 *in vitro* (green). Inside and outside surfaces of the MT structure are indicated. *E,* Mutation solely of K311 in recombinant MBP-α-tubulin (K311S) substantially reduced methylation by GST-SET8 *in vitro*. Autoradiogram (top) and Coomassie-staining (bottom) are as described in *A*.

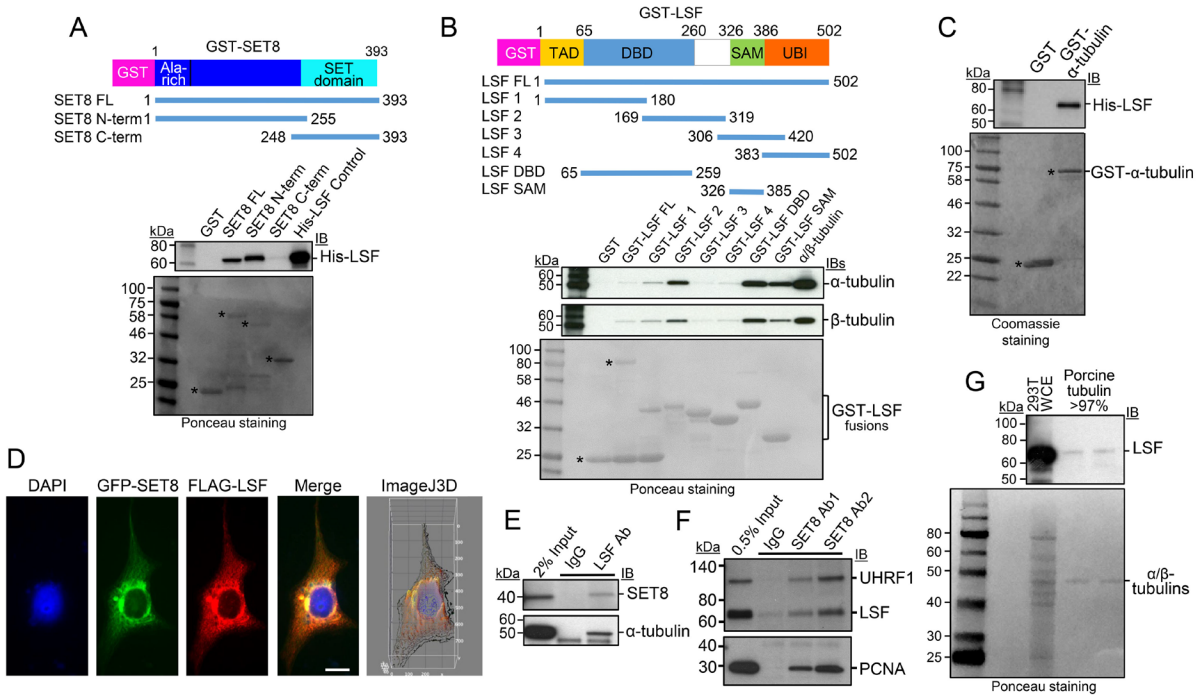

Figure 3. LSF interacts directly with SET8 and tubulin. *A,* GST-pull down analysis of purified His-LSF to full-length or the indicated overlapping segments of SET8 fused to GST. Top: immunoblot (IB) in which biotinylated molecular weight markers are visualized; bottom: Ponceau staining of the same gels (shown in grayscale) in which standard molecular weight markers are visualized. *Expected positions of migration of the GST-proteins. *B,* GST-pull down analysis of purified porcine tubulin to purified full-length or the indicated overlapping segments of LSF fused to GST. Gels are as described in *A*. * or bracket: Expected positions of migration of the GST-proteins. *C,* GST-pull down analysis of recombinant, purified His-tagged LSF to purified α-tubulin fused to GST. Gels are as described in *A*, except that the protein gel was stained with Coomassie. *D,* Plasmids expressing 3xFLAG-LSF and GFP-SET8 were transfected into COS7 cells. Anti-FLAG antibody was visualized with a red fluorescing secondary antibody, and DNA was visualized with DAPI. The merged image indicates colocalization of GFP-SET8 with FLAG-LSF (yellow), concentrated largely near the nuclear membrane (Manders correlation coefficient of LSF and SET8 colocalization is 0.9, as determined via the Image J 3D analysis). The majority of overexpressed 3XFlagLSF was cytoplasmic with only a minority detected in the nucleus. *E,* Specific co-immunoprecipitation of endogenous SET8 (top) and endogenous α-tubulin (bottom) from HEK293 cellular extracts, using antibodies to LSF as compared to control IgG. *F,* Specific co-immunoprecipitation of endogenous LSF from HEK293 cellular extracts, using antibodies to SET8 (Ab1: Active Motif; Ab2: Millipore) as compared to control IgG. Co-immunoprecipitation of PCNA and UHRF1 are also shown as positive controls. *G,* Immunoblotting of purified porcine brain tubulin (rPeptide, >97%) shows the presence of LSF, using a LSF monoclonal antibody. Representative also of results obtained using a separate source of purified tubulin: MP Biomedicals >99%. Positive control for LSF migration: 293T WCE (HEK293T cell whole cell extract). Top: immunoblot; bottom: Ponceau staining using standard molecular weight markers.

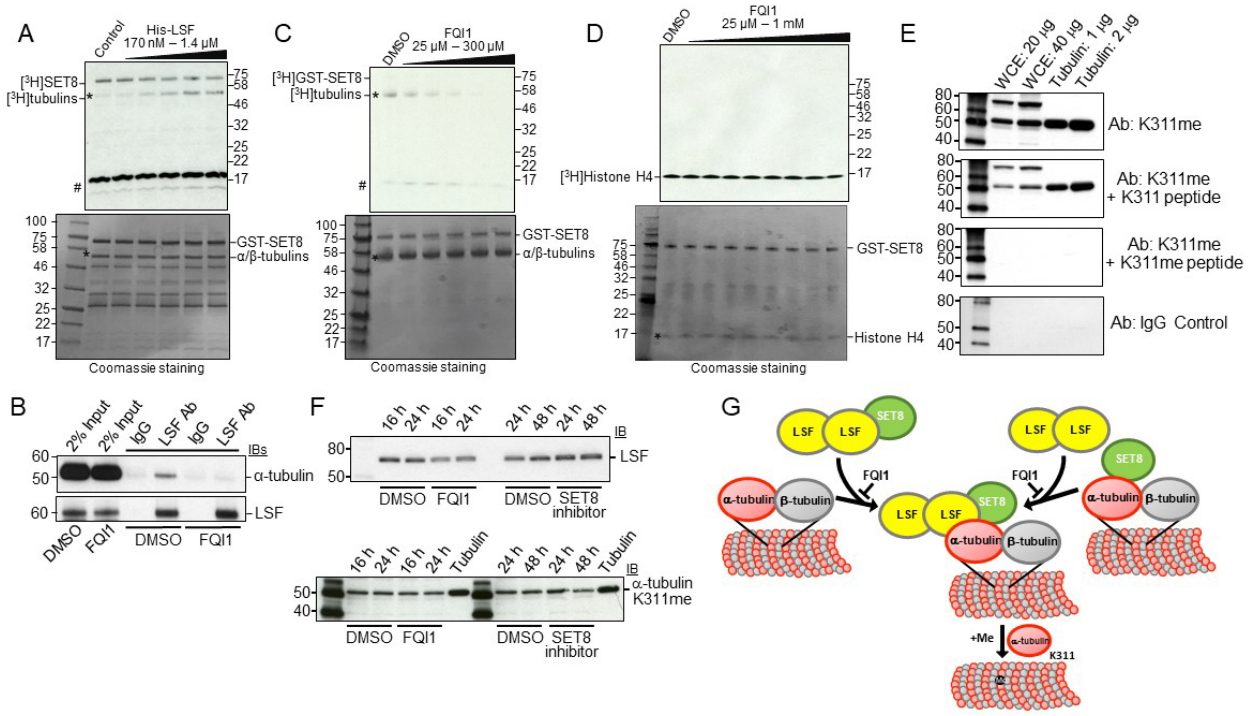

Figure 4. LSF and FQI1 oppositely affect methylation of tubulin by SET8. *A,* Tubulin (>99%, MP Biomedicals) methylation reactions were performed with addition of the indicated, increasing range of concentrations of LSF. Top: autoradiogram of methyltransferase assays, showing methylation of tubulin (*) and automethylation of GST-SET8. The higher relative levels of GST-SET8 to α/β-tubulin in this experiment led to greater initial automethylation of SET8 relative to tubulin methylation. # indicates the migration of [^{3}H]-labeled impurities. Bottom: Coomassie-staining of the same gel (shown in grayscale) indicating relative levels of the components in the reaction. As in Fig 2B, protein bands <50 kDa are from the purified GST-SET8 preparation, and are more evident in this experiment. *B,* Co-immunoprecipitation of endogenous α-tubulin with endogenous LSF from HEK293T cell lysates was disrupted upon treatment of the cells with 2.5 μM FQI1 for 24 hr. *C,* Tubulin (>99%, MP Biomedicals) methylation reactions were performed with addition of the indicated, increasing range of concentrations of FQI1. At 100 μM FQI1 (lane 4), methylation is decreased ~3-fold. Gels are labeled as in A. *D,* Histone H4 methylation reactions at limiting amounts of histone H4 (200 ng) were performed with addition of the indicated, increasing range of concentrations of FQI1. Gels are labeled as in *A*. *E,* Specific methylation of tubulin on K311. Immunoblots of HEK293T cell lysates and purified tubulin, at the indicated concentrations, with α-tubulin K311me or non-specific IgG antibodies. Specificity to methylated K311 was demonstrated by preincubation of the antibody with methylated, versus non-methylated α-tubulin K311 peptides. *F,* Treatment of HEK293T cells with either LSF or SET8 inhibitors somewhat reduces the level of methylated K311 on α-tubulin. *G,* Model for the recruitment and/or activation of SET8 at microtubules by LSF, and the subsequent methylation of α-tubulin by SET8.

The microtubule-associated histone methyltransferase SET8, facilitated by transcription factor LSF, methylates α-tubulin

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