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Uterine Leiomyoma-Linked MED12 Mutations Disrupt Mediator-Associated CDK Activity

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SUMMARY

Somatic mutations in exon 2 of the RNA polymerase II transcriptional Mediator subunit *MED12* occur at very high frequency (~70%) in uterine leiomyomas. However, the influence of these mutations on Mediator function and the molecular basis for their tumorigenic potential remain unknown. To clarify the impact of these mutations, we used affinity-purification mass spectrometry to establish the global protein-protein interaction profiles for both wild-type and mutant MED12. We found that uterine leiomyoma-linked mutations in MED12 led to a highly specific decrease in its association with Cyclin C-CDK8/CDK19 and loss of Mediator-associated CDK activity. Mechanistically, this occurs through disruption of a MED12-Cyclin C binding interface that we also show is required for MED12-mediated stimulation of Cyclin C-dependent CDK8 kinase activity. These findings indicate that uterine leiomyoma-linked mutations in MED12 uncouple Cyclin C-CDK8/19 from core Mediator and further identify the MED12/Cyclin C interface as a prospective therapeutic target in CDK8-driven cancers.

INTRODUCTION

Uterine leiomyomas (fibroids) are monoclonal neoplasms of the myometrium and represent the most common pelvic tumor in reproductive-age women (Stewart, 2001). Although benign, they are nonetheless associated with significant morbidity. They are the primary indicator for hysterectomy and a major cause of gynecologic and reproductive dysfunction, ranging from profuse menstrual bleeding and pelvic discomfort to infertility, recurrent miscarriage, and preterm labor (Stewart, 2001). Recently, we discovered that mutations in exon 2 of the *Xq13* gene encoding the transcriptional Mediator subunit *MED12* occur at very high frequency (~70%) in uterine leiomyomas (Mäkinen et al., 2011). Along with their high-frequency occurrence, two additional genetic findings suggest that MED12 mutations likely contribute to the genesis of uterine leiomyomas. First, all observed MED12 exon 2 mutations affect highly evolutionarily conserved regions of the MED12 protein, including three principal hot spot mutations in codons 36, 43, and 44 (Mäkinen et al., 2011). Second, localization of the missense mutations to a small number of amino acids suggests that the MED12 mutations are dominant and that MED12 acts as an oncogene (Vogelstein et al., 2013), providing a likely etiological basis previously lacking for the majority of these clinically significant tumors. Compatible with the key role of MED12 in controlling gene expression, we have also shown that the RNA expression patterns of MED12 mutant leiomyomas cluster tightly together and form a clearly separate branch distinct from all other leiomyomas (Mehine et al., 2013).

Mediator is a conserved multisubunit signal processor through which regulatory information conveyed by gene-specific transcription factors is transduced to RNA polymerase II (pol II). Structurally, Mediator is assembled from a set of core subunits into three distinct modules, termed "head," "middle," and "tail," that bind tightly to pol II in the so-called holoenzyme (Conaway and Conaway, 2011; Kornberg, 2005; Larivière et al., 2012; Malik and Roeder, 2010; Spaeth et al., 2011; Taatjes, 2010). MED12, along with MED13, Cyclin C, and CDK8 or CDK19, comprise a fourth "kinase" module that exists in variable association with core Mediator. The kinase module was originally implicated in negative regulation of pol II-dependent transcription

(Akoulitchev et al., 2000; Knuesel et al., 2009a; van de Peppel et al., 2005). Several more recent studies, however, have also characterized a positive role for CDK8 activity in transcription (Donner et al., 2010; Firestein et al., 2008; Morris et al., 2008).

MED12 links Cyclin C-CDK8 with core Mediator and also stimulates Cyclin C-dependent CDK8 kinase activity (Ding et al., 2008; Knuesel et al., 2009b). Although the mechanism by which MED12 activates CDK8 is unknown, MED12-dependent CDK8 activation is nonetheless required for nuclear transduction of signals propagated by several different oncogenic pathways with which MED12 is biochemically and genetically linked (Firestein et al., 2008; Kim et al., 2006; Spaeth et al., 2011; Zhou et al., 2006, 2012). Furthermore, MED12 itself is a target of oncogenic mutation, including exon 2 mutations linked to uterine leiomyomas (Barbieri et al., 2012; Je et al., 2012; Kämpjärvi et al., 2012; Mäkinen et al., 2011). However, the impact of these mutations on MED12 function and the molecular basis for their tumorigenic potential remain unknown.

RESULTS AND DISCUSSION

Uterine Leiomyoma-Linked Mutations in MED12 Disrupt its Association with Cyclin C-CDK8/CDK19

To identify proteins that bind differentially to wild-type (WT) and oncogenic MED12, we engineered stable, tetracycline-inducible Flp-In 293 T-REx cell lines expressing C-terminally Twin-Strep-tag-modified WT MED12 or its most common leiomyoma mutant derivative (G44D) (see Experimental Procedures for details) (Glatter et al., 2009; Varjosalo et al., 2013). Quantitative immunoblot analysis revealed that tagged WT and mutant MED12 proteins attained induced levels of expression (~0.8–1.6 × 10^5 molecules per cell) comparable to that of endogenous 293 cell MED12 (~0.4–0.8 × 10^5 molecules per cell) (Figure S1A). Affinity-purification mass spectrometry (MS) (Figure 1A; Figure S1B) revealed a specific and reproducible (n = 3) reduction in the binding of Cyclin C, CDK8, and CDK19 to mutant versus WT MED12 (Figure 1B; Table S1).

Relative quantification of MED12-associated Mediator subunits confirmed a statistically significant loss of kinase module, as opposed to core Mediator subunits, in mutant versus WT MED12 affinity purifications (Figure 1C; Table S2). We confirmed the reduced association of CDK8 and CDK19 with MED12 G44D by immunoprecipitation (IP)-western blot (Figure 1D) and further established that this defect extends to other uterine leiomyoma-linked exon 2 mutations in MED12, including L36R, Q43P, and G44S. Thus, FLAG-specific immunoprecipitates from HEK293 cells expressing FLAG-tagged MED12 mutant derivatives bore significantly reduced levels of Cyclin C, CDK8, and CDK19, but not core Mediator subunits, as well as diminished pol II C-terminal domain (CTD)-directed kinase activity compared to those from WT MED12-expressing cells (Figure 1E).

Uterine Leiomyoma-Linked Mutations in MED12 Disrupt Its Direct Interaction with Cyclin C-CDK8

To determine whether leiomyoma-linked mutations in MED12 disrupt its direct interaction with Cyclin C-CDK8, we analyzed recombinant kinase module variants reconstituted from baculovirus-expressed subunits. CDK8 immunoprecipitates from insect cells coexpressing CDK8, Cyclin C, and either WT or mutant MED12 derivatives (L36R, Q43P, or G44S) were monitored for the presence of MED12 and the level of CDK8 kinase activity. Note that these reconstitution assays were performed in the absence of MED13, because the latter does not appreciably impact the integrity or activity of a trimeric MED12/Cyclin C/CDK8 submodule assembly (Figure S1C). Compared to WT MED12, all three of the MED12 leiomyoma mutants were severely compromised for both Cyclin C-CDK8 binding and activation (Figure 1F). We mapped the Cyclin C-CDK8 binding domain on MED12 to within its N-terminal 100 amino acids encoded largely by exons 1 and 2 (Figures 2A and 2B) and further confirmed that MED12 (1–100) binds to and activates Cyclin C-CDK8 (Figure 2C). This suggests that exon 2 mutations in MED12 likely disrupt its Cyclin C-CDK8 binding interface as opposed to triggering conformational masking of a distant interaction site elsewhere in the protein. Together, these findings identify a common functional defect associated with uterine leiomyoma-linked mutations in MED12 and further suggest that disruption of its Cyclin C-CDK binding activity contributes to leiomyoma formation.

MED12 Activates CDK8 through Direct Interaction with Cyclin C

To clarify the molecular basis by which exon 2 mutations in MED12 disrupt its direct interaction with Cyclin C-CDK8, we first resolved kinase module subunit interactions using recombinant baculovirus-expressed proteins. Immunopurification of the kinase module from insect cells expressing all possible combinations of its four constituent subunits permitted resolution of its hierarchical subunit organization. This analysis revealed that MED12 binds to Cyclin C, which in turn binds to CDK8 (Figure 3A; Figure S2A). MED12 also binds to MED13, which does not bind to either Cyclin C or CDK8 (Figure 3A; Figures S2A and S2B). Importantly, we did not detect an interaction between MED12 and CDK8 in the absence of Cyclin C (Figure 3A; Figures S2A and S2C), indicating that Cyclin C bridges MED12 and CDK8. These findings confirm those recently described for subunit assembly in *S. cerevisiae* and support a conserved molecular organization between the yeast and human kinase modules (Tsai et al., 2013).

To understand how MED12 binds to Cyclin C, we exploited information derived from prior structural resolution of both *S. pombe* and *H. sapiens* Cyclin C proteins (Hoeppner et al., 2005; Schneider et al., 2011). These structures reveal the presence of a unique surface groove that is phylogenetically conserved among Cyclin C family members but absent from cell-cycle-type cyclins (Figure 3B). We hypothesized that this surface groove could represent a binding interface through which MED12 stimulates Cyclin C-dependent CDK8 kinase activity. To test this hypothesis, we introduced substitution mutations at residues both within (W177A, N181A, D182A, and Y238A) and outside of (W6A and E98A) the structurally defined Cyclin C surface groove (Figure 3B) and examined their impact on MED12 binding and CDK8 activation. Accordingly, CDK8-specific immunoprecipitates from insect cells coexpressing CDK8, MED12, and either WT or mutant Cyclin C derivatives were examined for both the presence of MED12 and the