# A motif in HSP90 and P23 that links molecular chaperones to efficient estrogen receptor α methylation by the lysine methyltransferase SMYD2

Received for publication, April 20, 2018, and in revised form, August 31, 2018 Published, Papers in Press, September 6, 2018, DOI 10.1074/jbc.RA118.003578

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Edited by Norma M. Allewell

**Heat shock protein 90 (HSP90) is a molecular chaperone that supervises folding of cellular signaling proteins such as steroid receptors and many protein kinases. HSP90 relies on ATP hydrolysis for powering a conformational circuit that helps fold the client protein. To that end, HSP90 binds to co-chaperone proteins that regulate ATP hydrolysis rate or interaction with client proteins. Co-chaperones such as P23, cell division cycle 37 (CDC37), or activator of HSP90 ATPase activity 1 (AHA1) interact with the N-terminal or middle domain of HSP90, whereas others, such as HSP70/HSP90-organizing protein (HOP), use tetratricopeptide repeat (TPR) domains to bind the EEVD motif at the very C-terminal end of HSP90. Recently, the lysine methyltransferase SET and MYND domain–containing 2 (SMYD2) has been proposed as an HSP90-binding partner, and interaction analyses indicate that SMYD2 binding to HSP90 is independent of the EEVD motif. Using the amplified luminescence proximity homogeneous assay (Alpha) technique, I identified a new (M/I/L/V)P*X*L motif at the C termini of HSP90 and P23 that mediates an interaction with SMYD2, and synthetic peptides harboring this motif dissociated this complex. Of note, the HSP90- and P23-dependent client estrogen receptor α (ERα), was a major methylation target of SMYD2. In a reconstituted system in bacteria, I analyzed HSP90/P23–associated, SMYD2-mediated ERα methylation and found that when SMYD2 binds to the molecular chaperones, it considerably increases methylation of Lys-266 in ERα. Because methylation represses ERα activity, the observed complex formation between SMYD2 and HSP90/P23 may contribute to ERα regulation.**

HSP90 is a major molecular chaperone in the eukaryotic cytosol that oversees folding and degradation of a subset of client proteins and therefore contributes to cellular protein homeostasis. The HSP90 client spectrum ranges from steroid hormone receptors (*e.g.* glucocorticoid receptor, progesterone receptor, and estrogen receptor) and protein kinases (*e.g.* SRC, CDK4, and AKT) to transcription factors (*e.g.* OCT4 or P53 tumor suppressor) and others such as cystic fibrosis transmembrane conductance regulator or Tau protein (1). Many client proteins, when mutated or deregulated, are related to well-known diseases such as various cancer types, cystic fibrosis, and neurodegenerative disorders (1–3). HSP90 function depends on ATP hydrolysis that drives a conformational cycle during which the protein client either folds or is triaged for proteolytic degradation (4–6). To fulfill its tasks, HSP90 is assisted by a multitude of cochaperone proteins that modulate its ATP hydrolysis rate or mediate the interaction with client proteins. Some cochaperones, such as P23, CDC37, or AHA1, interact with the N-terminal domain or the middle domain of the molecular chaperone (6). Others, such as HOP, CHIP, DNAJC7, PP5 (protein phosphatase 5), and the immunophilins, use tetratricopeptide repeat (TPR)[2] domains to clamp the C-terminal EEVD motif of HSP90 for interaction (7–9).

SMYD2 was initially identified as a histone H3–specific lysine methyltransferase that interacted with HSP90 (10, 11). Histone methylation activity suggested a role for SMYD2 as regulator of gene expression. Shortly after, additional nonhistone methylation targets of SMYD2 were reported, including the transcription factors tumor suppressor P53 and estrogen receptor α (ERα) and the molecular chaperone HSP90 (12–14). SMYD2-catalyzed methylation reduces P53 and ERα transcriptional activity and therefore represses P53 and ERα target gene expression (12, 14). Crystal structures of SMYD2 in complex with histone, P53, and ERα target peptides were solved (15–18). Accordingly, SMYD2 consists of an N-terminal catalytic domain (N-lobe) and a C-terminal domain (C-lobe) with structural similarity to the TPR domains of cochaperone proteins that bind to the EEVD motif of HSP90. Therefore, it was proposed that the C-lobe of SMYD2 may bind to HSP90 in a manner similar to the TPR clamp mechanism of HSP90 cochaperones, such as HOP (15). However, this hypothesis was never tested experimentally. Moreover, the purpose of the SMYD2–HSP90 interaction remains elusive, although one may speculate that the molecular chaperone may affect SMYD2 target protein methylation.

In the present study, binding of SMYD2 to HSP90 and HOP to HSP90 was compared and found to be fundamentally differ-

This work was supported in part by Mukoviszidose Institut gGmbH (Bonn, Germany) Grant S03/10, the research and development arm of the German Cystic Fibrosis Association Mukoviszidose e.V. (to W. O.), and in part by Ruhr-University Bochum FoRUM Grant F829 (to W. O.). The author declares that he has no conflicts of interest with the contents of this article.
This article contains Tables S1–S3 and Figs. S1–S7.

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[2] The abbreviations used are: TPR, tetratricopeptide repeat; ERα, estrogen receptor α; ERαK266, ERα Lys-266; Alpha, amplified luminescence proximity homogeneous assay; SAM, *S*-adenosylmethionine; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

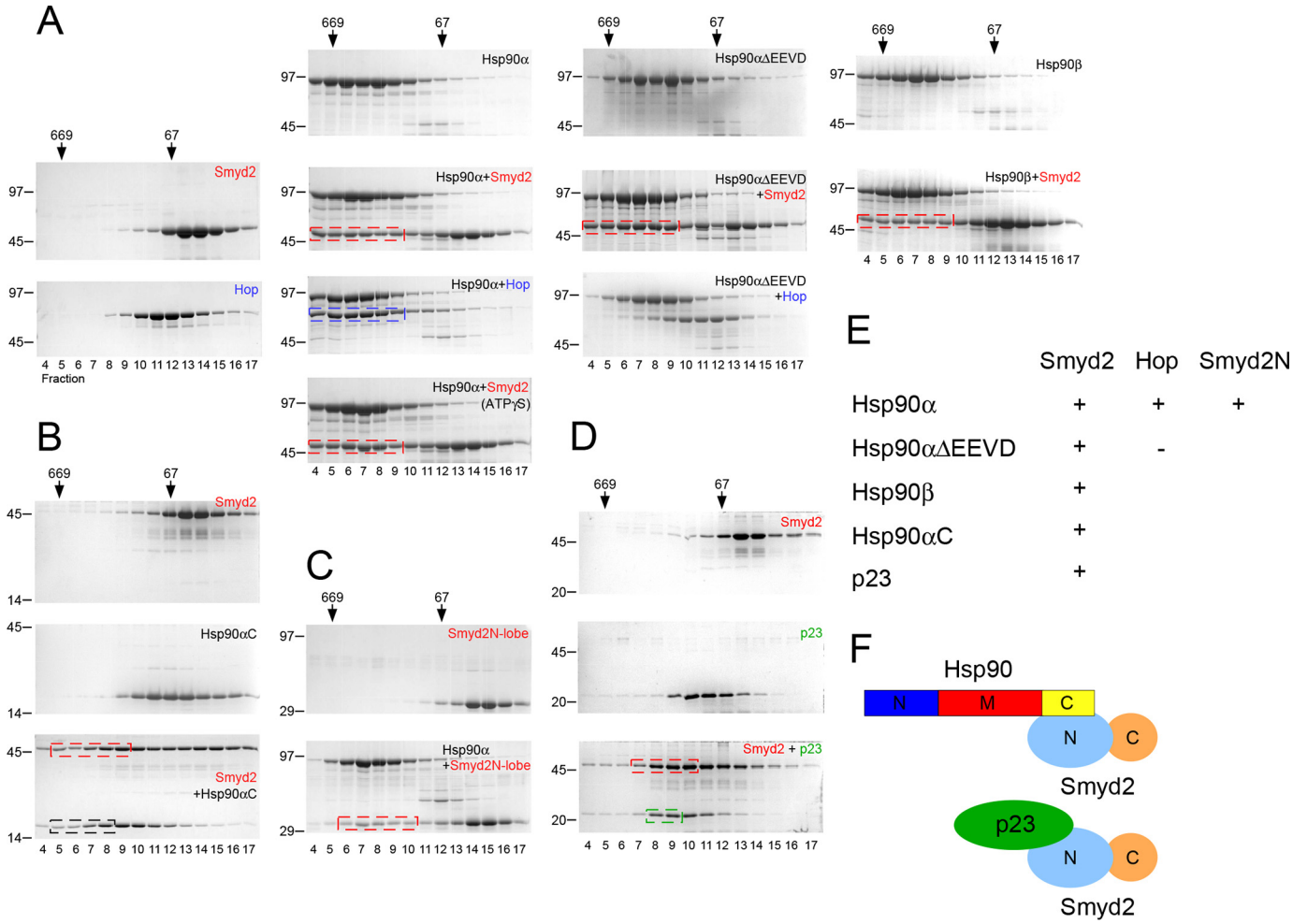


**Figure 1. SMYD2 binds to HSP90 and P23.** *A*, SMYD2 and HOP were incubated with HSP90α, HSP90αΔEEVD, or HSP90β and analyzed by gel filtration chromatography. A shift of SMYD2 and HOP together with HSP90α, HSP90αΔEEVD, or HSP90β toward higher molecular weight indicates the formation of robust protein complexes (indicated by *red* and *blue dashed boxes*). Marker proteins (thyroglobulin (669 kDa) and BSA (67 kDa)) are shown on *top*. SMYD2 binds to HSP90α and HSP90β, independent from the EEVD motif at the C terminus of the molecular chaperone, in contrast to the cochaperone HOP. The interaction between SMYD2 and HSP90α is insensitive to ATPγS. *B*, the C-terminal domain HSP90αC is sufficient for interaction to SMYD2. Shifts of SMYD2 and HSP90αC are indicated by *red* and *black dashed boxes*. *C*, SMYD2N-lobe, the catalytic domain of SMYD2, interacts with HSP90α, as indicated by a *dashed red box*. *D*, SMYD2 binds to the HSP90 cochaperone P23. Shifts of SMYD2 and P23 are indicated by *red* and *green dashed boxes*. *E*, compilation of the protein–protein interaction results. *F*, summary model of SMYD2–HSP90 and SMYD2–P23 binary protein complexes. Marker proteins are indicated in kDa.

ent. Whereas HOP interaction required the C-terminal EEVD motif of HSP90, this sequence was dispensable for SMYD2 binding. Using Alpha (amplified luminescence proximity homogeneous assay) for interaction analysis, an (M/I/L/V)P*X*L motif, present in HSP90 and in the cochaperone P23, was identified as the binding site for SMYD2. When SMYD2-catalyzed lysine methylation of P53, ERα, and HSP90 was compared, ERα turned out to be the major target. The HSP90/P23– dependent client protein ERα was used to investigate the functional role of HSP90/P23–SMYD2 complex formation. To that purpose, an HSP90/P23– chaperoned ERα expression system was reconstituted. Efficient lysine methylation of ERα depended on interaction between SMYD2 and the molecular chaperones HSP90/P23. This suggests that the molecular chaperone–SMYD2 alliance may keep ERα in an inactive methylated state during folding, before activation by estrogen. Accordingly, HSP90/P23 contribute to another layer of ERα regulation by SMYD2-dependent methylation.

## Results

### *SMYD2 binds to HSP90 independently of the C-terminal EEVD motif and to the cochaperone P23*

Previous studies reported that SMYD2 binds to the molecular chaperone HSP90 in human culture cells (11, 13). To test this observation, SMYD2 and HOP were mixed with HSP90α, and the interaction was analyzed by gel filtration chromatography (19–21). A shift of the SMYD2 elution profile and co-migration together with HSP90α indicated complex formation between the two proteins, independent of nucleotide (Fig. 1*A*). Likewise, SMYD2 bound to HSP90β indistinguishably from HSP90α (Fig. 1*A*).

It was proposed that the C-lobe of SMYD2 is a TPR-like domain and binds to HSP90 dependent on the EEVD motif, similar to cochaperone proteins such as HOP or DNAJC7 (7, 8). However, alignment of the SMYD2 C-lobe sequence with *bona fide* TPR domains of HSP90 binding partners revealed that

amino acid residues of the dicarboxylate clamp responsible for EEVD interaction are not conserved in SMYD2 (Fig. S1). This suggests that the EEVD motif is dispensable for interaction with SMYD2. To test this assumption, the EEVD motif of HSP90α was cleaved off, and the truncated protein (HSP90αΔ EEVD) was analyzed for complex formation with SMYD2 and HOP (Fig. 1*A*). Whereas HSP90αΔEEVD showed no interaction with HOP as expected, SMYD2 binding to HSP90α did not require the EEVD motif (Fig. 1*A*), although SMYD2 binding was mediated by HSP90αC (Fig. 1*B*). This suggests that SMYD2, in contrast to HOP, does not use the TPR clamp mechanism for interaction with HSP90. Furthermore, the catalytic SMYD2N-lobe was sufficient for interaction with HSP90α (Fig. 1*C*). To determine whether SMYD2 forms complexes with yet unidentified partners, the methyltransferase was applied as bait in a yeast two-hybrid screen using a universal human library. Hits identified were fragments encompassing amino acids 697–732 in the C terminus of HSP90α, confirming the result shown in Fig. 1*B*, and amino acids 110–160, representing a C-terminal fragment of the HSP90 cochaperone P23 (Fig. S2). Interaction between SMYD2 and P23 was confirmed by gel filtration chromatography using the purified proteins (Fig. 1*D*). The results of the protein interaction analysis are compiled in Fig. 1*E*. In summary, SMYD2 forms complexes with both chaperone partners HSP90 and P23 (Fig. 1*F*).

### *A new (M/I/L/V)PXL motif at the C termini of HSP90 and P23 connects the molecular chaperones to SMYD2*

Based on the result of the yeast two-hybrid screen, the binding site between SMYD2 and HSP90α could be mapped to a C-terminal fragment of the molecular chaperone comprising amino acids 697–732, consistent with the biochemical data (Fig. 1*B*). Alpha technology (22) was used to further explore the interaction between SMYD2 and the HSP90 polypeptide (Fig. 2*A*). Candidate proteins are attached to GST donor beads or nickel-chelate acceptor beads and brought to close proximity upon protein interaction. Laser excitation leads to emittance of singlet oxygen from donor beads and triggers emission of luminescence by acceptor beads that can be quantified. Mutant proteins or the addition of inhibitors prevent interaction-dependent excitation of acceptor beads (Fig. 2*A*). A variety of GST-HSP90α fusion peptides and $His_6$-tagged SMYD2 or SMYD2N proteins were purified and analyzed for interaction (Fig. S3 and Table S1). Full-length HSP90, HSP90C, and HSP90(697–732) bound to SMYD2, whereas HSP90(1–696) did not (Fig. 2*B*). Subsequently, HSP90(697–732) was trimmed at both termini, and the GST-fused fragments were tested for binding to SMYD2. Eventually, the nonapeptide EMPPLEGDD (HSP90(715–723)) was identified to be sufficient for interaction with SMYD2 (Fig. 2*B*). In an effort to locate the peptide binding site on SMYD2, the smallest protein that could be stably expressed was SMYD2(1–240). This fragment bound to HSP90(715–723) equally well as full-length SMYD2 (Fig. 2*C*). Next, the sequence motif essential for HSP90α–SMYD2 complex formation should be identified. Therefore, peptide EMPPLEGDD was subjected to iterative rounds of amino acid substitutions, and the mutant peptides were purified and analyzed for interaction with SMYD2 by Alpha (Fig. S4 and Table S2). It turned out that the motif (M/I/L/V)P*X*L, where Met could be substituted with amino acid Ile, Leu, or Val and *X* could be any amino acid in HSP90α, was essential for binding to SMYD2 (Fig. 2*D*). The corresponding sequence in HSP90β is IPPL, consistent with this finding (Fig. 2*D*). To further corroborate the (M/I/L/V)P*X*L motif as the SMYD2-binding site, the essential proline was substituted by an alanine, leading to HSP90αP717A, and the full-length protein was purified. After the addition of SMYD2 or HOP, complex formation was analyzed by gel filtration chromatography (Fig. 2*E*). The P717A mutation starkly affected binding to SMYD2 but did not alter interaction with HOP, confirming the specificity of the (M/I/L/V)P*X*L motif for SMYD2.

Another binding partner of SMYD2 is the cochaperone P23, and the interaction of the fragment P23(110–160) with the methyltransferase could be verified by Alpha based on GST-tagged P23 proteins (Fig. S5*A* and Table S3). Sequence alignment using the HSP90α peptide revealed the presence of an (M/I/L/V)P*X*L motif at the very C terminus of P23, suggesting that this piece is responsible for P23–SMYD2 complex formation (Fig. 2*F*). Mutation of each of the amino acids Met-156, Pro-158, and Leu-159 in Pro-23 to alanine completely abolished binding of P23 to SMYD2, confirming that the cochaperone uses its very C-terminal end for interaction with the methyltransferase (Fig. S5*A* and Table S3). To test whether SMYD2 binds to other GST-tagged chaperone proteins, a C-terminal part of Hsc70 (Hsc70C), HOP, and AHA1 were purified and analyzed by Alpha (Fig. S5*B*). Neither Hsc70C nor any of the cochaperones bound to the methyltransferase (Table S3). Several cochaperone proteins compete with each other for binding to HSP90, although they employ different sites on the molecular chaperone for interaction (19, 23). Therefore, HSP90-HOP and HSP90-AHA1 were incubated with SMYD2, and complex dissociation was analyzed. Although HOP and AHA1 use a binding motif or interaction site different from SMYD2, the methyltransferase disturbs complex formation with HSP90, suggesting steric overlap between SMYD2 and the cochaperones HOP and AHA1 (Fig. 2*G*).

### *The HSP90/P23–dependent client ERα is a major methylation target of SMYD2*

SMYD2 was originally described as a histone H3 lysine 36–specific methyltransferase (10). Shortly after, several non-histone target proteins were reported, among them the tumor suppressor P53 (12), the molecular chaperone HSP90 (13), and ERα (14). To obtain insight into the target-specific methylation activity of SMYD2, 36-mer peptides of P53, HSP90α, and ERα containing the proposed methylation sequences were produced as GST fusions. For comparability, target lysines were at the same position within each sequence, using the P53(358–393) peptide as a blueprint (17) (Fig. 3*A*). GST alone and $His_6$-HSP90(544–732) served as controls. After incubation with SMYD2, lysine methylation was detected with two different specific antibodies. Relative signal intensity was strongest with ERα(254–289), considerably weaker with P53(358–393), and invisible with HSP90α(603–638) or $His_6$-HSP90(544–732), suggesting that ERα is a major SMYD2 methylation target (Fig. 3*B*). To ensure that Lys-266 is indeed the SMYD2-dependent

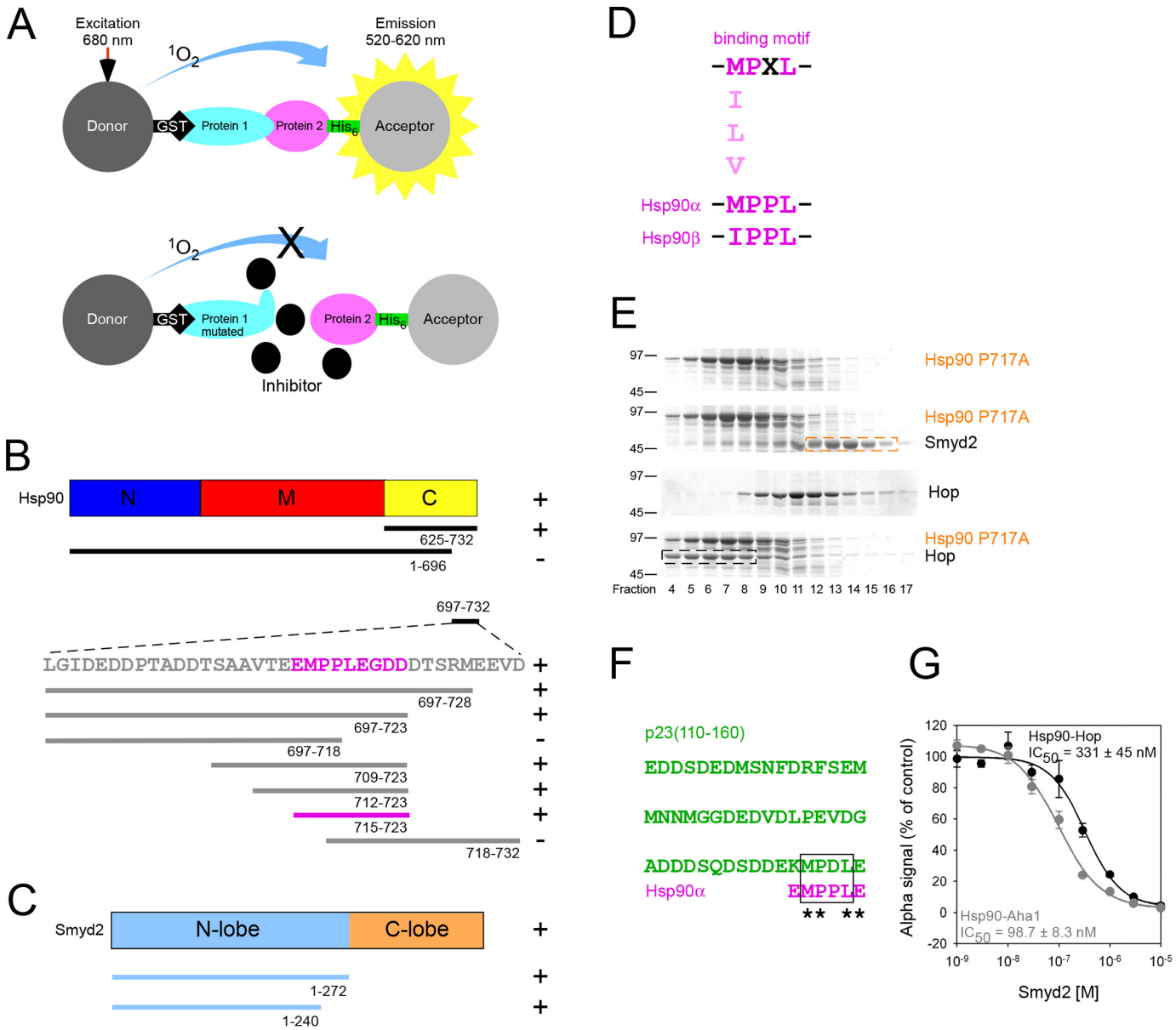


**Figure 2. Identification of the (M/I/L/V)PXL motif responsible for binding to SMYD2 by Alpha assay.** *A*, illustration of the Alpha assay principle. Proteins 1 and 2 are attached to donor and acceptor beads via their GST or $His_6$ fusion tags. Interaction of the proteins brings donor and acceptor beads to close proximity. Accordingly, short-lived singlet oxygen generated from donor beads by laser excitation can reach acceptor beads and triggers emission of luminescence light. Mutant proteins or inhibitors prevent protein interaction and luminescence light emission. *B*, HSP90α GST fusion proteins tested for interaction with $His_6$-SMYD2 by Alpha assay. The peptide sequence EMPPLEGDD in the C-terminal domain of HSP90α interacted with SMYD2 (Table S1). *C*, SMYD2N-lobe, the catalytic domain of SMYD2, is sufficient for interaction with HSP90 (Table S1). *D*, mutation of the EMPPLEGDD peptide sequence identified (M/I/L/V)PXL as the SMYD2 interaction motif in HSP90α. Binding between mutant GST fusion peptides and SMYD2 was measured by Alpha assay (Table S2). Alignment of respective HSP90α and HSP90β sequences shows the conservative exchange of Met to Ile in HSP90β. *E*, the HSP90 mutant P717A interacts with HOP but not with SMYD2, consistent with the presence (*black*) or absence (*orange*) of a protein shift together with HSP90, as revealed by gel filtration analysis. *F*, the EMPPLE sequence of HSP90α matches with the very C-terminal amino acids in P23, disclosing the (M/I/L/V)PXL motif in the cochaperone indicated by a *black box*. Mutational analysis confirms P23 as a SMYD2-binding protein (Table S3). *G*, SMYD2 competes with HOP and AHA1 for complex formation with HSP90. Although SMYD2, HOP, and AHA1 use different motifs or sites for interaction, HSP90 allows only binary complexes with each of the three proteins, suggesting that steric requirements hinder binding of more than one protein to the molecular chaperone. Marker proteins are indicated in kDa. *Error bars*, S.D.

methylation target in ERα(254–289), mutants K266A and K266R were tested, and both abolished the lysine methylation signal (Fig. 3*C*). SMYD2 catalytic activity toward P53 can be inhibited by LLY-507, a chemical compound that is specific for this methyltransferase (24). Thus, we tested methylation of the target peptide ERα(254–289) in the presence of LLY-507 (Fig. 3*D*). As a result, SMYD2-catalyzed ERα(254–289) methylation was disturbed by LLY-507 in a concentration-dependent manner.

### *SMYD2 recruitment by the (M/I/L/V)PXL motif in HSP90 and P23 results in efficient methylation of ERα at Lys-266*

The next aim was to examine the effect of (M/I/L/V)PXL-mediated SMYD2– chaperone association on the methylation status of ERα. Identification of this motif allowed synthesis of short peptides derived from HSP90α and P23 that contain the SMYD2-binding sequence. Accordingly, peptides HSP90(707–723) spanning the amino acid sequence DTSAAVTEEMPPLEGDD and P23(147–160) spanning the sequence DSQDSD-

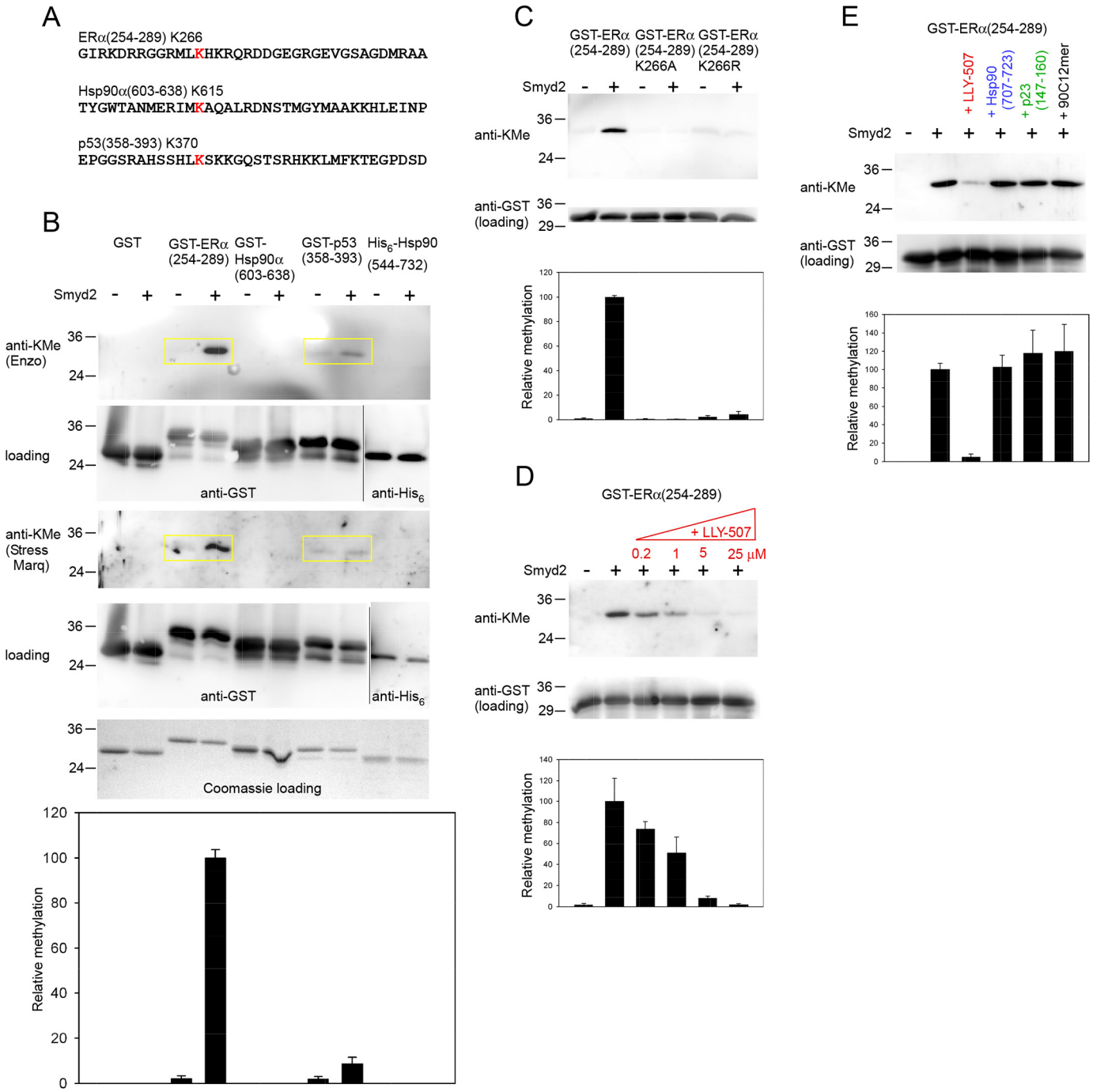


**Figure 3. ERα is a major methylation target of SMYD2.** *A*, 36-mer GST fusion sequences of ERα, HSP90α, and P53 used for methylation by SMYD2. The lysine residue supposed to be the methylation target is indicated in *red*. *B*, relative methylation of ERα, HSP90α, and P53 by SMYD2. ERα is a major and P53 is a minor methylation target of SMYD2, as detected by independent blotting with lysine methylation–specific antibodies ADI-KAP-TF121 (Enzo) and SPC-158F (Stress-Marq). GST-HSP90α(603–638) and $His_6$-HSP90α(544–732) showed no detectable methylation signal when tested together with ERα and P53. GST served as a negative control, and protein loading was monitored by subsequent blotting with anti-GST or anti-$His_6$ antibody and by Coomassie staining of an identical gel. *C*, Lys-266 in ERα is the methylation target of SMYD2. Mutants K266A and K266R are no longer methylated by SMYD2. A GST loading control is shown *below*. *D*, methylation of GST-ERα(254–289) is prevented by the SMYD2 inhibitor LLY-507 in a concentration-dependent manner. A GST loading control is shown *below*. *E*, peptides HSP90(707–723), P23(147–160), and 90C12mer do not affect SMYD2-dependent methylation of ERα(254–289) when used at 200 μM, in contrast to the SMYD2 inhibitor LLY-507. A GST loading control is shown *below*. Relative methylation levels of ERα(254–289) are indicated, with S.D. indicated by *error bars*. Marker proteins are indicated in kDa.

DEKMPDLE were made. To test the potency of the peptides to interfere with binding, HSP90α–SMYD2 and P23–SMYD2 protein complexes were incubated with various concentrations of the peptides, and dissociation was measured by Alpha (Fig. 4*A*). 90C12mer (GDDDTSRMEEVD), a peptide that disturbs binding of TPR proteins to HSP90 (7, 8) and methylation cofactor *S*-adenosylmethionine (SAM) served as controls. HSP90(707–723) and P23(147–160) readily abrogated formation of homologous HSP90α–SMYD2 and P23–SMYD2 complexes with an $IC_{50}$ in the low micromolar range (Fig. 4*A*). In the

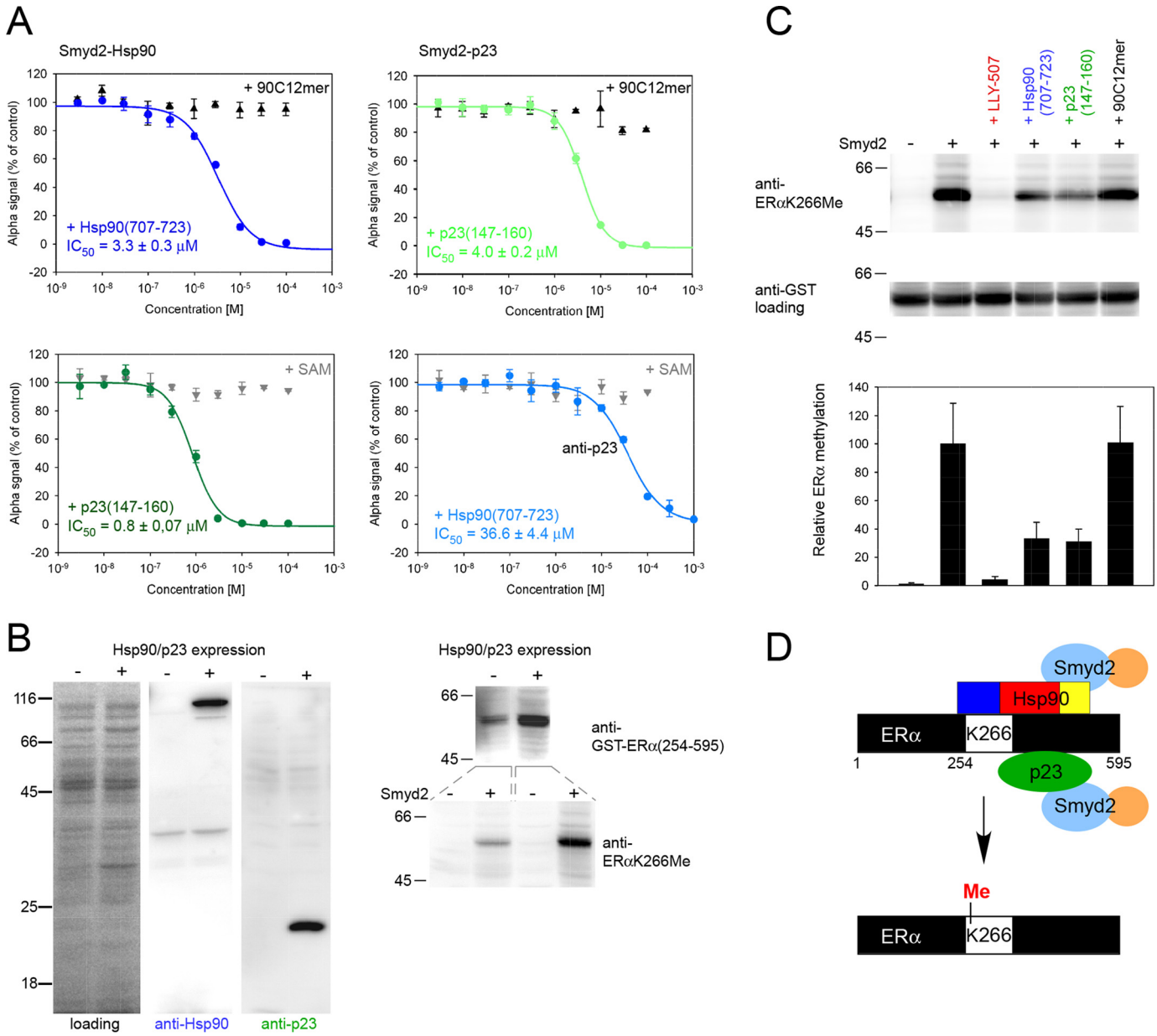


**Figure 4. Efficient methylation of ERα depends on recruitment of SMYD2 by the molecular chaperones HSP90/P23.** *A*, peptide HSP90(707–723) or P23(147–160) dissociates SMYD2–HSP90 or SMYD2–P23 complex and vice versa, as analyzed by Alpha assay. $IC_{50}$ values are indicated. Peptide 90C12mer and SAM had no effect on complex formation. Measurements were done in triplicate, and S.D. values are shown by *error bars*. *B*, GST-ERα(254–595) was expressed alone (−) or together with HSP90/P23 (+) in bacterial cytosol. Equal protein loading is shown by Ponceau staining and HSP90 and P23 expression confirmed by Western blotting (*left*). HSP90/P23 expression boosts GST-ERα(254–595) levels and increases SMYD2-dependent methylation of ERα (*right*). *C*, dissociation of SMYD2–chaperone complexes by peptides HSP90(707–723) and P23(147–160) decreases ERαK266 methylation to about one-third of untreated control. The SMYD2 inhibitor LLY-507 inhibits methylation completely, whereas peptide 90C12mer has no effect on ERαK266 methylation. All compounds were added at 200 µM. *D*, illustration of SMYD2 dependent methylation of Lys-266 in the hinge region of ERα in the presence of molecular chaperones HSP90 and P23. It is not intended to propose stoichiometry of the protein components. Marker proteins are indicated in kDa.

heterologous situation, P23(147–160) was even more efficient toward HSP90α-SMYD2 than HSP90(707–723), but toward the P23–SMYD2 complex, HSP90(707–723) was ~10-fold less effective compared with P23(147–160). Given that the (M/I/L/V)P*X*L motif sits at the very end of P23 but is flanked by C-terminal amino acids in HSP90, P23 might be accommodated more readily by SMYD2, resulting in higher binding affinity. To confirm the results obtained by Alpha, HSP90α and SMYD2 were mixed; incubated with peptide HSP90(707–723), P23(147–160), 90C12mer, or LLY-507; and analyzed for interaction by gel filtration chromatography (Fig. S6). HSP90(707–723) and P23(147–160) but neither 90C12mer nor LLY-507 dissociated HSP90–SMYD2 complexes.

To set up a test system to explore the effect of (M/I/L/V)P*X*L-mediated SMYD2–chaperone association on the methylation status of ERα, the hormone receptor should be expressed alone or together with chaperones HSP90α and P23 and methylated by SMYD2. Therefore, ERα(254–595) was cloned as a GST fusion protein. This fragment contains the hinge region of ERα with the SMYD2 methylation target Lys-266 and the C-terminal part of the receptor that harbors the ligand binding domain and associates with HSP90 and P23 (25–28) (Fig. 4*D*). HSP90

and P23 were cloned into a bicistronic vector for joint expression (see "Experimental procedures"). Accordingly, GST-ERα(254–595) was expressed together with HSP90 and P23 or in their absence (Fig. 4*B*). HSP90/P23 markedly increased the expression level of GST-ERα(254–595). Gel filtration analysis showed that GST-ERα(254–595) is retained when expressed together with HSP90 and P23, suggesting that the molecular chaperones associate with the steroid receptor and prevent its aggregation (Fig. S7). Moreover, SMYD2-dependent methylation of GST-ERα(254–595) was higher in the presence of HSP90 and P23 (Fig. 4*B*). This indicates that the chaperones hold GST-ERα(254–595) competent for methylation and/or recruit SMYD2. To analyze this further, peptides HSP90(707–723) and P23(147–160) should be used to block the interaction between the chaperones and SMYD2 to measure methylation of GST-ERα(254–595) in the absence of HSP90α–SMYD2 and P23–SMYD2 complexes. To rule out any adverse effect of HSP90(707–723) and P23(147–160) on the catalytic activity of SMYD2, methylation of GST-ERα(254–289) was assayed in the presence of the peptides using LLY-507 and 90C12mer as controls (Fig. 3*E*). HSP90(707–723) and P23(147–160) did not affect the catalytic activity of SMYD2 at 200 μM, the concentration used for the following assay (Fig. 3*E*). To test the effect of HSP90–SMYD2 and P23–SMYD2 complex formation on ERα Lys-266 (ERαK266) methylation, the assay was performed in the presence of HSP90(707–723) and P23(147–160) to disturb SMYD2 recruitment by the chaperones using LLY-507 and 90C12mer as controls. Complex dissociation by HSP90(707–723) and P23(147–160) peptides resulted in a decrease of GST-ERα(254–289) Lys-266 methylation to about one-third of control (Fig. 4*C*). This provides evidence that the (M/I/L/V)P*X*L motif recruits SMYD2 to HSP90/P23 for efficient methylation of the chaperone-associated client protein ERα at Lys-266 (Fig. 4*D*).

## Discussion

The present study shows that the lysine methyltransferase SMYD2 binds to the C-terminal domain of HSP90, yet independent of the EEVD motif that is used by TPR domain cochaperones such as HOP for interaction (7, 8). In lieu thereof, a new (M/I/L/V)P*X*L binding motif present at the C termini of the molecular chaperones HSP90 and P23 mediates complex formation with SMYD2. Although SMYD2 uses this unique sequence for HSP90 binding, it competes with HOP and the HSP90 ATPase activator AHA1 for interaction with the molecular chaperone. This suggests that SMYD2 may add another layer of regulation to HSP90-dependent client protein activation, apart from the established functions that are performed by cochaperones such as AHA1 and HOP. To further dissect the role of HSP90–SMYD2 complex formation, relative lysine methylation of nonhistone SMYD2 target proteins was measured. This analysis revealed Lys-266 in the hinge region of ERα as a major methylation target of SMYD2, when compared with previously reported proteins such as P53 or HSP90. ERα belongs to the steroid receptor family and is a *bona fide* HSP90/P23–dependent client protein. Therefore, an HSP90/P23–chaperoned ERα expression system was reconstituted to decipher the role of SMYD2–chaperone complexes toward estrogen receptor α methylation. This expression system indicated that the amount of soluble ERα protein produced was contingent on HSP90/P23, approving the significance of the molecular chaperones for client protein folding and prevention of aggregation.

Furthermore, SMYD2-catalyzed methylation of ERα was considerably higher in the presence of the molecular chaperones HSP90 and P23. In contrast, dissociation of SMYD2 from the molecular chaperones HSP90/P23 by interfering with synthetic peptides considerably reduced ERαK266 methylation, suggesting that SMYD2–chaperone complexes are required for efficient methylation of ERα. This finding raises the question of the purpose of SMYD2-associated methylation of the HSP90/P23–dependent client protein ERα. SMYD2-catalyzed methylation puts an inhibitory mark on ERαK266 and P53 Lys-370, prevents binding of these transcription factors to their respective promoters on the DNA, and thereby prevents their gene expression activity (12, 14). Because P53 promotes apoptosis of cancer cells, lysine methyltransferase SMYD2 that inactivates this tumor suppressor may be considered a cancer-promoting oncogene (12). Following the standard model of steroid receptor activation, HSP90 interacts with ERα to keep the receptor in an inactive folding-competent state (29, 30). Accordingly, in the absence of activating hormone estrogen, methylation of the hinge region at Lys-266 by SMYD2 in the presence of HSP90/P23 may prevent premature dimerization and coactivator binding of ERα and allow proper chaperone-assisted folding of this steroid receptor. Upon estrogen binding, however, the chaperones HSP90/P23 are released from ERα together with SMYD2, resulting in lower Lys-266 methylation and receptor activation. The methylation mark on ERαK266 and P53 Lys-370 is a reversible modification and can be removed by LSD1, a lysine-specific demethylase (14, 16, 31), which is associated with ERα and P53 activation. As a consequence, demethylated ERαK266 gains transcriptional activity.

In an alternative model that has been proposed recently, ERα is engaged in coregulator complexes together with HSP90, where the molecular chaperone may act as a scaffold (32). In such an ensemble, it is conceivable that HSP90-dependent SMYD2 recruitment leads to methylation and deactivation of ERα. In turn, LSD1-mediated demethylation would activate ERα. This balance of methylation/demethylation events may add a layer of regulation to ERα activity that is, in part, brought about by the molecular chaperones HSP90 and P23.

## Experimental procedures

### *Antibodies, peptides, and chemicals*

Mouse monoclonal antibodies specific for GST tag and $His_6$ tag were from Abcam; P23 mouse mAb JJ3 was from Thermo Fisher Scientific; rabbit polyclonal anti-methylated lysine antibodies ADI-KAP-TF121 and SPC-158F were from Enzo Life Sciences and StressMarq Biosciences, respectively; and rabbit polyclonal anti-ERαK266Me antibody was a kind gift from Xiaobing Shi. Synthetic peptides HSP90(707–723), P23(147–160), and 90C12mer were from Metabion International, and SMYD2 inhibitor LLY-507 was from Sigma.

### Expression constructs and recombinant protein purification

SMYD2 constructs were amplified from a human skeletal muscle first-strand cDNA preparation (Clontech) and cloned into $His_6$-tagged pProExHta expression vector. P53(358–393) was amplified from a human placenta first-strand cDNA preparation (Clontech) and cloned into GST fusion expression vector pGEX-4T1. ER$\alpha$(254–289) and ER$\alpha$(254–595) were amplified from IMAGE cDNA clone IRCMp5012F0638D (Source Bioscience) and cloned into GST fusion expression vector pGEX-4T1. HSP90, HOP, P23, and AHA1 expression vectors were described earlier (4, 21, 22). HSP90 and P23 fragments were expressed as GST fusion proteins from vector pGEX-4T1. Point mutations in expression constructs were generated with the QuikChange site-directed mutagenesis kit (Stratagene) using missense oligonucleotides. For expression of GST-ER$\alpha$(254–595) from pGEX-4T1 in the presence of chaperones, a dicistronic pET28b expression vector was constructed containing P23 and $His_6$-HSP90$\alpha$ in series following the EMBL dicistronic cloning protocol (https://www.embl.de/pepcore/pepcore_services/cloning/cloning_methods/dicistronic_cloning/index.html).[3]

All clones were verified by DNA sequencing. ER$\alpha$ and HSP90/P23 vectors were co-transformed into *Escherichia coli* following selection on ampicillin- and kanamycin-containing medium, with empty pET28b vector serving as control. Recombinant proteins were expressed in *E. coli* BL21(DE3)pLysS as GST and $His_6$ fusions and purified using GSH-Sepharose 4B (GE Healthcare) or nickel-chelate agarose (Qiagen) affinity beads. Further purification was performed by gel filtration chromatography on a Superose 12 HR10/30 column or by ion-exchange chromatography on a ResourceQ column using an ÄktaPurifier system (GE Healthcare).

### Protein interaction analysis by gel filtration chromatography

Each 5 $\mu$M concentration of purified proteins was mixed and incubated at room temperature for 10 min and on ice for another 10 min to allow complex formation. 500-$\mu$l samples were separated on a Superose 12 HR10/30 column in 40 mM HEPES/KOH buffer (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$ operated by an ÄktaPurifier system (GE Healthcare). 500-$\mu$l fractions were collected after a 6-ml elution volume and analyzed by SDS-PAGE. When bacterial cell lysates were examined by chromatography, proteins were visualized by Western blotting with specific antibodies after SDS-PAGE.

### Alpha assay

Purified protein interaction partners with N-terminal GST or $His_6$ tags were incubated in 40 mM HEPES, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 0.5% BSA for 10 min at 30 °C, followed by 10 min at 4 °C. Control reactions contained GST only together with the $His_6$ tag partner protein. Donor and acceptor beads (4 $\mu$g/ml) were added to protein complexes at a final concentration of 0.3 $\mu$M and further incubated at room temperature for 40 min in an AlphaPlate-384 (PerkinElmer Life Sciences). Luminescence signals were measured in an EnSpire Multimode Plate Reader (PerkinElmer Life Sciences). For competition studies, interaction partners were incubated in the presence of competing synthetic peptides or competing proteins without tags at various concentrations as indicated. Experiments were performed in triplicate, and $IC_{50}$ values were determined after data fitting to a four-parameter logistic curve equation using SigmaPlot software.

### Yeast two-hybrid screen

Yeast two-hybrid screening was essentially done as described previously (23). Human SMYD2 was cloned into vector pGBKT7 and transformed into the Y2HGold reporter strain (Clontech). Cells were mated with strain Y187 pretransformed with a Universal Human (Normalized) Mate & Plate Library in vector pACT2 (Clontech). After selection for protein–protein interactions on SD/−Leu/−Trp/−His plates by growth and blue staining on SD/−Leu/−Trp + X-$\alpha$-Gal medium, DNA was isolated from yeast cells and transformed into *E. coli* XL-1 blue, and pACT2 plasmids were isolated for sequencing of the prey inserts.

### Methylation assay

Fusion protein GST-ER$\alpha$(254–289), GST-HSP90$\alpha$(603–638), GST-P53(358–393), or $His_6$-HSP90$\alpha$(544–732) (3 $\mu$M) was incubated with 2 $\mu$M SMYD2 and 200 $\mu$M SAM for 1 h at 30 °C in 50 mM Tris, pH 9.0, 2 mM $MgCl_2$. Methylation was analyzed by blotting with anti-methylated lysine antibodies ADI-KAP-TF121 and SPC-158F. Subsequent blotting with anti-GST– or anti-$His_6$–specific antibodies was used for loading controls. When indicated, LLY-507 or peptides HSP90(707–723), P23(147–160), and 90C12mer were added at concentrations up to 200 $\mu$M to GST-ER$\alpha$(254–289) methylation assays. For analysis of HSP90/P23–associated SMYD2 methylation, GST-ER$\alpha$(254–595) was expressed alone or together with the chaperones in *E. coli* and the cell lysate used in the assay as described above in the presence or absence of methylation inhibitor LLY-507, peptides HSP90(707–723), P23(147–160), or 90C12mer as a control at 200 $\mu$M. Sequence-specific methylation of ER$\alpha$(254–595) was assayed with anti-ER$\alpha$K266Me antibody. Experiments were repeated at least three times, and quantification of methylation signals was done with ImageJ software.

*Author contributions*—W. M. J. O. conceptualization; W. M. J. O. data curation; W. M. J. O. formal analysis; W. M. J. O. funding acquisition; W. M. J. O. validation; W. M. J. O. investigation; W. M. J. O. methodology; W. M. J. O. writing-original draft; W. M. J. O. project administration.

*Acknowledgments—I thank Stefanie Darnauer, Svenja Schäfers, and Julia Hofmann for expert technical assistance. Polyclonal anti-ERαK266Me1 antibody was kindly provided by Xiaobing Shi (University of Texas M.D. Anderson Cancer Center, Houston, TX).*

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