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FOCUS ARTICLE

Arabidopsis FRIGIDA stimulates EFS histone H3 Lys36 methyltransferase activity

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Flowering time in plants is coordinately regulated by environmental cues and endogenous signals. FRIGIDA (FRI) regulates flowering time by upregulating the transcription of FLOWERING LOCUS C (FLC). EARLY FLOWERING IN SHORT DAYS (EFS) is a histone methyltransferase implicated in FRI-mediated transcriptional activation (Kim et al. 2005; Ko et al. 2010; Zhao et al. 2005). We recently showed that FRI works with the EFS histone methyltransferase (HMTase) (Ko et al. 2010), but the molecular mechanism by which FRI and EFS function to upregulate FLC transcription remains unclear. To clarify this mechanism, we systematically examined the effect of FRI on EFS activity. First, we measured the effect of the FRI core domain (FRI_{core}, a.a. 131–432) on EFS HMTase activity using the EFS SET domain ($EFS\_{}_{SET850}$, a.a. 850–1166), a nucleosomal array as a substrate, and ^{3}H-*S*-adenosyl-methionine (^{3}H-SAM) as a cofactor (Fig. 1a). Figure 1a clearly shows FRI stimulates EFS activity in a dose-dependent manner.

Next, we performed a kinetic analysis on FRI-mediated EFS activation by increasing the amount of nucleosomal substrate for each concentration of FRI_{core} (Fig. 1b). We observed that the EFS V_{max} increases as the concentration of FRI increases, and that the EFS K_m drops in the presence of FRI (Fig. 1b; Supplemental Figure 1). To determine whether FRI specifically activates the HMTase activity of EFS, we performed an HMTase assay using human homologs of EFS, ASH1L, and the methyltransferase G9a. FRI does not activate the histone methylation activity of G9a, but marginally increases the activity of hASH1L, which shares the conserved SET domain with EFS (Supplemental Figure 2 and Supplemental Figure 3). These data indicate that FRI specifically increases the catalytic activity of EFS protein family members.

We then performed a His-tag pull-down assay using His-tagged FRI_{core} to determine whether FRI_{core} physically interacts with EFS. For the assay, we used $EFS\_{}_{SET917}$ (a.a. 917–1263), which has higher purity than $EFS\_{}_{SET850}$. We found clear evidence that $EFS\_{}_{SET917}$ physically interacts with FRI_{core} (Fig. 1c). Because FRI is a highly basic protein composed of 14 stacked helices (Hyun et al. 2016), we speculated that FRI may interact with nucleosomes and that this interaction may be an important contributor to FRI's stimulation of EFS activity. To test this hypothesis, we incubated mononucleosomes with increasing amounts of FRI_{core} and measured their interactions via a gel-retardation assay. Figure 1d shows that the FRI_{core} does indeed interact with nucleosomes, but $EFS\_{}_{SET917}$ does not. This suggests that FRI may recruit EFS to the nucleosomes for histone methylation.

The lesions in EFS reduce H3K4 methylation at the *FLC* locus (Kim et al. 2005) and *efs* mutants show dramatically reduced H3K36 methylation, but not H3K4 methylation at *FLC* (Zhao et al. 2005). We, therefore, decided to clarify the substrate specificity of EFS in the absence and presence of FRI. We first measured the histone methyltransferase activity of $EFS\_{}_{SET850}$ with a G5E4 nucleosomal array and

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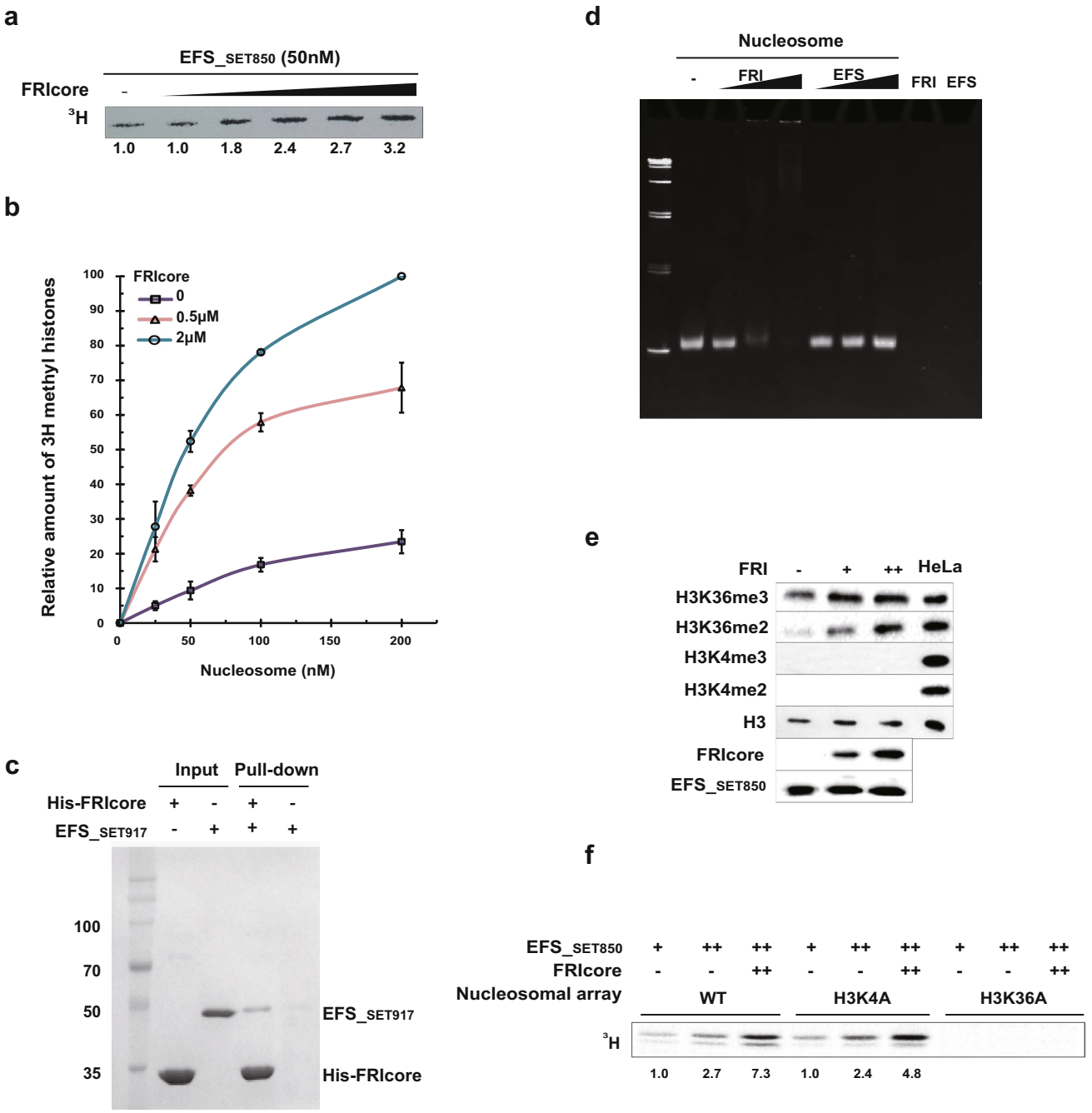

Fig. 1 FRI stimulates the histone H3 Lys36 methyltransferase activity of EFS. **a** FRI stimulates the histone methyltransferase activity of EFS in a dose-dependent manner. The incorporation of methyl groups by histones was measured using ^{3}H-SAM and visualized by autoradiography. The *numbers* under the gel indicate relative band intensities. EFS activities were observed with increasing concentrations of FRI_{core} (0, 0.125, 0.25, 0.5, 1, 2 μM). **b** Kinetic analysis of FRI-mediated activation of the EFS histone methyltransferase activity. EFS activities were measured with three different concentrations of FRI_{core} (0, 0.5, 2 μM). **c** FRI_{core} interacts with EFS_{SET917}. His-tagged FRI_{core} was used for the Ni-NTA pull-down assay. His-tagged FRI_{core} was incubated with EFS_{SET917} and pulled down with Ni-NTA resin. **d** FRI_{core} binds nucleosomes, but EFS_{SET917} does not. Binding in a 4% native TBE gel was visualized with EtBr. **e** The methylation status of histones methylated by EFS_{SET850} analyzed by western blot using H3K36me3-, H3K36me2-, H3K4me3-, and H3K4me2-specific antibodies. Hela histones were used as positive controls for the antibodies and for the amounts of each histone. FRI and EFS were monitored with histone H3, His, and GST-specific antibodies. **f** The substrate specificity of EFS was analyzed using wild-type G5E4 nucleosomal arrays (WT), or nucleosomal arrays containing either histone H3 K4A or H3 K36A mutants. The numbers under the blot indicate relative band intensities

visualized the methylation status using methylation-specific antibodies. Figure 1e indicates EFS methylates H3K36, but not H3K4. It also shows FRI activates the H3K36-specific methylation activity of EFS. This suggests that $EFS_{\_SET}$ has an intrinsic H3K36-specific methyltransferase activity. We next generated nucleosomal arrays with a histone H3 Lys4Ala mutation (H3K4A) or a Lys36Ala mutation (H3K36A) and measured the resulting EFS methylation activities using the mutant nucleosomal arrays as substrates (Fig. 1f). While the H3K4A nucleosomal array shows methylation levels comparable to the wild-type (WT) nucleosomal array, the H3K36A nucleosomal array shows no measurable methylation. This unambiguously indicates that $EFS_{\_SET}$ harbors an H3K36-specific methyltransferase activity in vitro.

The crystal structure of the catalytic domain of hASH1L, a human ortholog of EFS, shows an auto-inhibitory loop in the Post-SET domain that occupies the substrate-binding pocket; this loop must be released for hASH1L to reach full activity (An et al. 2011). The exact mechanism of this inhibitory loop release, however, is unclear. The SET catalytic domains of EFS and hASH1L are highly similar in sequence. EFS and hASH1L are also unique in that their SET domains are located in the middle of the protein rather than at the C-terminus like other histone methyltransferases that contain SET domains. These similarities suggest that EFS activity might be also regulated by an auto-inhibitory loop. Because our data show that FRI physically interacts with EFS and enhances its activity, we speculate that the binding of FRI to EFS might release this auto-inhibitory loop, increasing the intrinsic enzymatic activity of EFS. Consistent with this model, the EFS V_{max} increases in the presence of FRI. Still, clarification of the exact molecular mechanism of FRI-mediated EFS activation awaits an atomic resolution structure of the FRI–EFS complex. FRI is a highly basic protein ($pI > 9$), meaning it is positively charged. Consistent with this, we show that FRI interacts with nucleosomes (Fig. 1d). Because our data indicate EFS lacks significant affinity for nucleosomes, it is possible that FRI recruits EFS to nucleosomes, increasing substrate accessibility sufficiently to increase EFS activity. In our kinetic analysis, however, we found that FRI increased the substrate affinity of EFS by lowering its K_m and also increasing its V_{max}. Therefore, FRI binding to EFS may cause multiple events; it may release an auto-inhibitory loop, increasing V_{max}, and increase nucleosome binding, reducing K_m. The exact mechanism will require further investigation.

The substrate specificity of EFS has been controversial, a problem hASH1L and Ash1 shared until recently. We showed hASH1L has histone H3 Lys36-specific, but not Lys4-specific activity in vitro. In this study, too, we show that EFS methylates histone H3 Lys36 but not Lys4. We cannot exclude the possibility, however, that EFS exhibits dual specificity toward histone H3 Lys4 and Lys36 in vivo upon interacting with an unknown binding partner. In summary, this work identified FRI as an activator of EFS and showed that EFS was a histone H3 Lys36-specific methyltransferase and biochemically links two key epigenetic regulators for flowering time.

Materials and methods

The materials and methods are described in detail in the Supplementary materials and methods.

Author contribution statement KH, YN, and JS developed the concepts, KH did all the experiments, and all authors examined the data and wrote the manuscript.

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Compliance with ethical standards

Ethical standards All authors complied with all ethical standards.

Conflict of interest The authors declare that there are no conflicts of interest.

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