LETTERS

Metamorphic enzyme assembly in polyketide diversification

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Natural product chemical diversity is fuelled by the emergence and ongoing evolution of biosynthetic pathways in secondary metabolism[1]. However, co-evolution of enzymes for metabolic diversification is not well understood, especially at the biochemical level. Here, two parallel assemblies with an extraordinarily high sequence identity from *Lyngbya majuscula* form a β-branched cyclopropane in the curacin A pathway (Cur), and a vinyl chloride group in the jamaicamide pathway (Jam). The components include a halogenase, a 3-hydroxy-3-methylglutaryl enzyme cassette for polyketide β-branching, and an enoyl reductase domain. The halogenase from CurA, and the dehydratases (ECH_1s), decarboxylases (ECH_2s) and enoyl reductase domains from both Cur and Jam, were assessed biochemically to determine the mechanisms of cyclopropane and vinyl chloride formation. Unexpectedly, the polyketide β-branching pathway was modified by introduction of a γ-chlorination step on (*S*)-3-hydroxy-3-methylglutaryl mediated by Cur halogenase, a non-haem Fe(II), α-ketoglutarate-dependent enzyme[2]. In a divergent scheme, Cur ECH_2 was found to catalyse formation of the α,β enoyl thioester, whereas Jam ECH_2 formed a vinyl chloride moiety by selectively generating the corresponding β,γ enoyl thioester of the 3-methyl-4-chloroglutaconyl decarboxylation product. Finally, the enoyl reductase domain of CurF specifically catalysed an unprecedented cyclopropanation on the chlorinated product of Cur ECH_2 instead of the canonical α,β C=C saturation reaction. Thus, the combination of chlorination and polyketide β-branching, coupled with mechanistic diversification of ECH_2 and enoyl reductase, leads to the formation of cyclopropane and vinyl chloride moieties. These results reveal a parallel interplay of evolutionary events in multienzyme systems leading to functional group diversity in secondary metabolites.

The biosynthesis of secondary metabolites is 'diversity oriented'[3,4], targeting the variable environment by producing a vast array of complex chemical structures. This productivity is largely fuelled by the rapid evolution of biosynthetic genes and functional alteration of the corresponding enzymes[1,4]. However, our current understanding of pathway diversification is largely based on genetic and bioinformatics approaches.

The curacin and jamaicamide metabolites from *Lyngbya majuscula* are mixed-polyketide nonribosomal-peptide natural products with potent anticancer and sodium channel blocking activities, respectively[5,6]. The corresponding biosynthetic pathways (Fig. 1a) provide an unusual opportunity to investigate the biosynthetic origin of chemical diversity. Within these dissimilar pathways, the parallel, highly conserved components catalyse formation of a cyclopropane ring for curacin and a vinyl chloride for jamaicamide[5,7]. Comparative biochemical studies on the variant functions and selectivities of these highly parallel biosynthetic components form the subject of this report.

Two parallel Cur and Jam enzyme assemblies are incorporated into the early polyketide synthase modules, and are predicted to catalyse polyketide β-branching reactions in the growing chain elongation intermediates[5,7]. These catalytic domains and discrete enzymes span from CurA to CurF and from JamE to JamJ, and are grouped into three subsets (Fig. 1a): (1) halogenases (Hals) embedded in CurA and JamE; (2) 3-hydroxy-3-methylglutaryl (HMG) enzyme cassettes containing a tandem acyl carrier protein (ACP) tridomain (ACP_3) including ACP_I, ACP_{II} and ACP_{III} embedded in CurA and JamE, discrete CurB and JamF ACP_{IV}s, CurC and JamG ketosynthase-like enzymes (KSs), CurD and JamH HMG-CoA synthase-like enzymes (HCSs), CurE and JamI ECH_1s, and ECH_2s embedded in CurF and JamJ; and (3) enoyl reductase domains (ERs) embedded in CurF and JamJ (Fig. 1a). The sequence identities of the Cur and Jam Hals, ACP_3s, ACP_{IV}s, KSs, HCSs and ECH_1s are extraordinarily high (~90%), whereas the ECH_2s and ERs are substantially lower (~60% identity) (Fig. 1a). Our bioinformatic analysis suggests that a 'di-acyltransferase (AT) domain replacement' might have occurred to insert the parallel AT-Hal-ACP_I-ACP_{II}-ACP_{III}-ACP_{IV}-KS-HCS-ECH_1-ECH_2-ER-KS-AT gene assembly into precursors of the modern Cur and Jam gene clusters by homologous recombination (Supplementary Figs 1 and 2).

Cur and Jam Hals were predicted to be α-ketoglutarate-dependent non-haem Hals (less than 20% sequence identity to characterized homologues)[8–10] that catalyse halogenation of unactivated carbon atoms[9–13] through a non-haem Fe(IV)=O intermediate[14]. HMG enzyme cassettes have been demonstrated to catalyse polyketide on-assembly-line β-branching to generate a pendant methyl or ethyl group from a polyketide β-carbonyl[15–17]. Cur and Jam ERs show ~50% sequence identity to other ERs in Cur and Jam polyketide synthase modules, and belong to the acyl-CoA reductase family that catalyses NADPH-dependent reduction of α,β enoyl thioester in acyl-CoAs or acyl-ACPs[18].

HMG β-branching includes a series of modifications on the β-carbonyl group of a polyketide intermediate typically tethered to the tandem ACPs[15–17]. As illustrated for CurA (Fig. 1b), CurB malonyl-ACP_{IV}, CurC KS and CurD HCS lead to formation of (*S*)-HMG-ACP_3 (**1**-ACP_3). As shown previously, ECH_1 catalyses dehydration of **1**-ACP_3 to 3-methylglutaconyl-ACP_3 (**2**-ACP_3), followed by ECH_2 decarboxylation to generate 3-methylcrotonyl-ACP_3 (**3**-ACP_3)[15,19], a presumed precursor for (1*R*,2*S*)-2-methylcyclopropane-1-carboxyl-ACP_3 (**5**-ACP_3) (Fig. 1b). This initial study raised three important questions regarding the role of the Cur and Jam Hals, HMG enzyme cassettes, and ERs in the formation of cyclopropane

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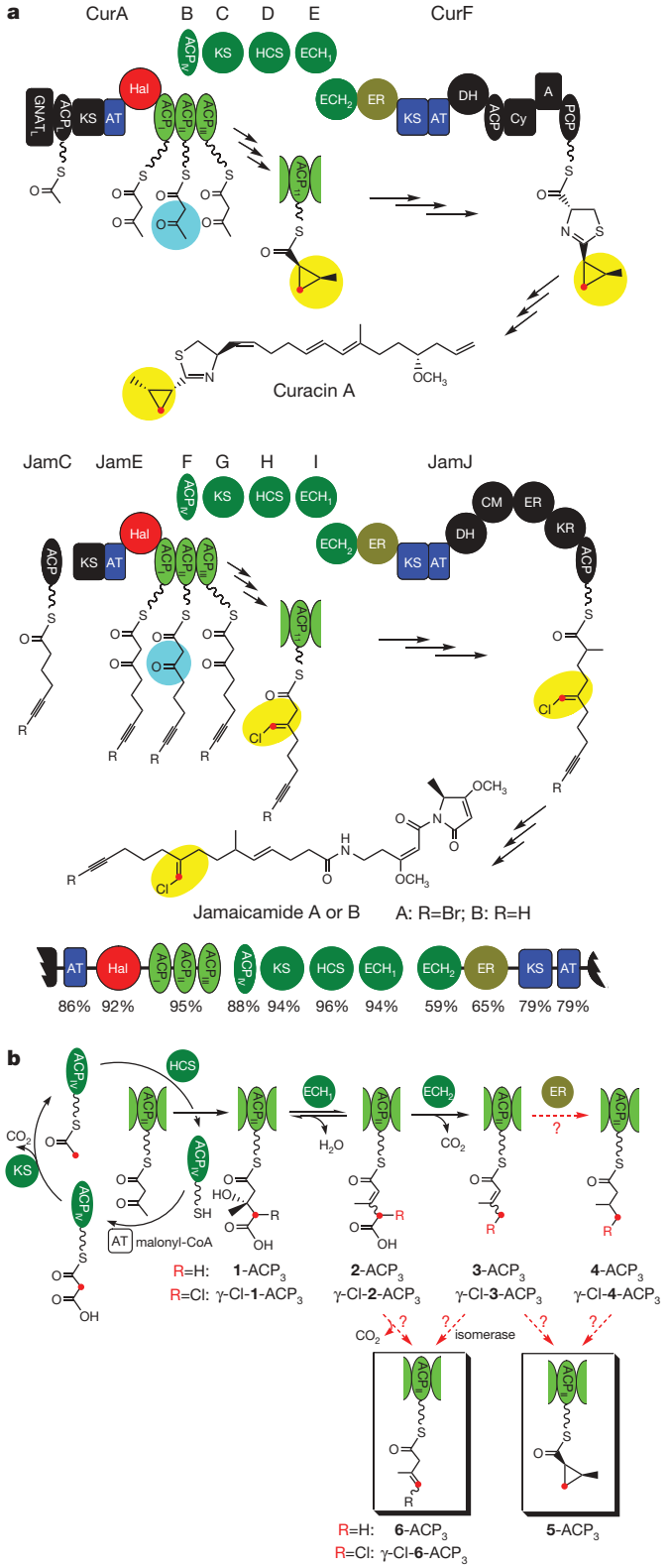

Figure 1 | Comparison of enzyme assemblies in the Cur and Jam pathways. **a**, Formation of cyclopropane and vinyl chloride functional groups. Comparative amino acid sequence identities of the Cur and Jam enzymes are shown. A, adenylation domain; CM, *C*-methyltransferase domain; Cy, condensation/cyclization domain; DH, dehydratase domain; $GNAT_L$, GCN5-related *N*-acetyltransferase-like domain with bifunctional decarboxylase/*S*-acetyltransferase activity; PCP, peptidyl carrier protein domain. **b**, Formation of **3**-ACP_3 in the Cur pathway, and hypothesized reactions for **4**-ACP_3, **5**-ACP_3 and **6**-ACP_3. The hypothetical chlorinated intermediates are shown along with the non-chlorinated ones. The β-branching carbon atoms are highlighted in red.

and vinyl chloride moieties: it is not clear (1) whether the CurF ER is involved in cyclopropyl ring formation, and based on its canonical function whether it can reduce **3**-ACP_3 to isovaleryl-ACP_3 (**4**-ACP_3) (Fig. 1b); (2) whether a chlorination step is involved in formation of the **5**-ACP_3 cyclopropane ring (Fig. 1b); and (3) how the unusual β,γ C=C of the pendant vinyl chloride group is formed in the Jam pathway. As previously proposed, 3-methyl-3-butenoyl-ACP_3 (**6**-ACP_3) might be generated from **3**-ACP_3 by isomerization[2], or by differential regiochemical control of double-bond formation during ECH_2 decarboxylation[19] (Fig. 1b).

First, we tested whether CurF ER can saturate **3**-ACP_3, the previously established product of Cur ECH_2[15]. The function of excised Cur ER was assessed by coupling it with the Cur ECH_1 and ECH_2 reactions. The substrates **1**-ACP_{II} and **1**-ACP_3 were prepared with HMG covalently linked to the phosphopantetheine arm[20] of the ACP (Supplementary Figs 3 and 4), and mass changes were detected by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) and infrared multiphoton dissociation (IRMPD) methods. As reported[15], Cur ECH_1 catalysed the reversible dehydration of **1**-ACP_{II} to generate **2**-ACP_{II}, and Cur ECH_2 catalysed decarboxylation of **2**-ACP_{II} to generate **3**-ACP_{II} (Fig. 2b, c). Cur ECH_1 showed substrate preference for (*S*)-HMG-ACP_{II} over (*R*)-HMG-ACP_{II} (Supplementary Fig. 5a–c), consistent with our previous results using the CoA-linked substrates[15]. With Cur ER and

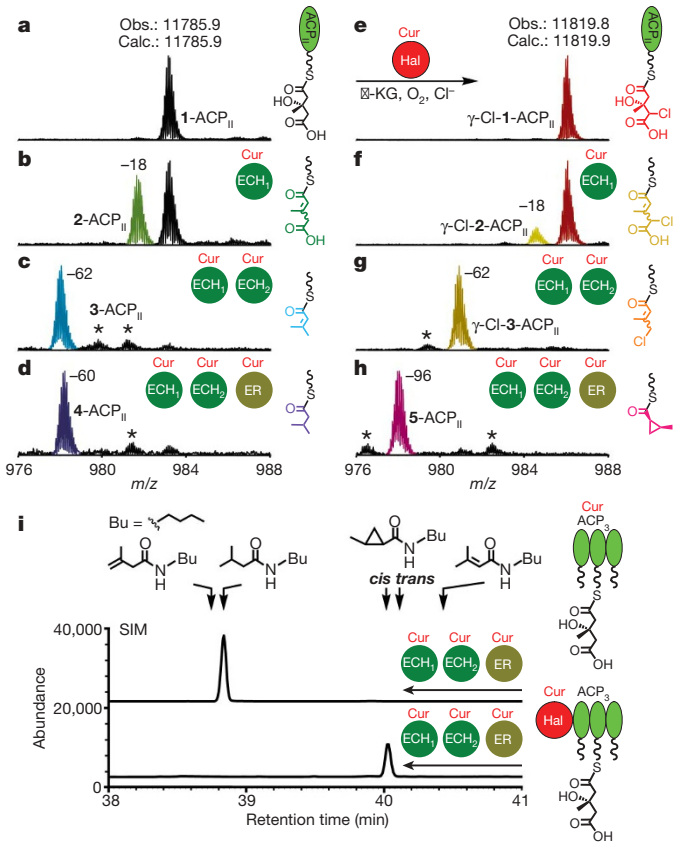

Figure 2 | Halogenation and cyclopropanation in the Cur pathway. **a**–**h**, Partial FTICR mass spectra (12+ charge state of ACP_{II}) for Cur ECH_1, ECH_2 and ER reactions excluding (**a**–**d**) or including (**e**–**h**) the Cur Hal chlorination step. **1**-ACP_{II} was incubated with Cur Hal for 2 h to generate the γ-Cl-**1**-ACP_{II} substrate. Reactions were incubated at 30 °C for 2 h for the **1**-ACP_{II} substrate and 30 min for the γ-Cl-**1**-ACP_{II} substrate. Asterisks denote unidentified species. α-KG, α-ketoglutarate. **i**, GC-MS analysis of the enzyme products after butylamine cleavage, and comparison with authentic standards. For optimal sensitivity, the chromatograms were recorded at selective ion mode (SIM) by monitoring 55, 57, 83, 115, 155 and 157 atomic mass units (a.m.u.). Retention times of the products were confirmed by co-injection with the authentic standards.

NADPH, a 2-Da mass addition was observed for **3**-ACP_{II} (Fig. 2d and Supplementary Fig. 6a), corresponding to saturation of the α,β enoyl thioester to generate **4**-ACP_{II}. To confirm the structure of the Cur ER reaction product tethered to ACP_3, we cleaved it from the phosphopantetheine arm with butylamine and analysed the corresponding butylamide derivative by gas chromatography (GC)-MS[21] (Fig. 2i). We used **1**-ACP_{II} and **1**-ACP_3 as substrates for Cur ECH_1, ECH_2 and ER reactions, and their products were confirmed as **4** by correlation with authentic standards (Fig. 2i, upper trace). However, the relatively poor efficiency of Cur ER-catalysed reduction of **3**-ACP_{II} (see below) suggested that its native substrate remained to be identified.

Given the above results, we reasoned that a chlorination step might be essential for cyclopropane ring formation. An important clue about the timing of chlorination at the β-branching carbon came from previous precursor-incorporation studies in curacin A biosynthesis (Supplementary Fig. 7). To identify the function of Cur Hal, it was purified anaerobically[9,10] as an excised domain (Supplementary Fig. 3), treated with a mixture of metals and α-ketoglutarate, and shown by inductively coupled plasma mass spectrometry (ICP-MS) to bind Fe^{2+} preferentially (90%). Seven acyl-ACP substrates bearing the target pendant β-branching carbon were tested to establish the substrate identity for Cur Hal, including malonyl-ACP_{IV}, acetyl-ACP_{IV}, **1**-ACP_{II}, **2**-ACP_{II}, **3**-ACP_{II}, **4**-ACP_{II} and **6**-ACP_{II} (Fig. 1b). Consistent with the precursor-incorporation experiment noted above, we observed formation of the mono-chlorinated species exclusively on **1**-ACP_{II} to generate γ-Cl-**1**-ACP_{II}. The chlorinated product was confirmed by FTICR-MS and IRMPD analysis (Fig. 2e and Supplementary Fig. 6a), and corroborated by GC-MS (see below, Fig. 3g). As expected, Cur Hal showed the same selectivity for (*S*)-HMG-ACP_{II} (**1**-ACP_{II}) as did Cur ECH_1, and O_2 and α-ketoglutarate dependence (Supplementary Fig. 5). Chlorination on a carboxylated γ-carbon of HMG is unique for α-ketoglutarate-dependent non-haem Hals, with previous reports limited to unactivated substrates[9–13].

Next, we sought to investigate how the chlorination of **1**-ACP_{II} affects efficiency of the downstream reaction sequence with the HMG cassette enzymes. **1**-ACP_{II} was converted to γ-Cl-**1**-ACP_{II} by Cur Hal (Fig. 2e), and reacted sequentially with Cur ECH_1, ECH_2 and ER. ECH_1 dehydrated γ-Cl-**1**-ACP_{II} and the γ-Cl-**2**-ACP_{II} product was decarboxylated by ECH_2 to generate γ-Cl-**3**-ACP_{II} (Fig. 2f, g). The ECH_1/ECH_2-coupled dehydration and decarboxylation with γ-Cl-**1**-ACP_{II} was ~4-fold faster than with **1**-ACP_{II} (Supplementary Fig. 8), which might be due to the electron-withdrawing effect of the γ-chlorine or to more effective binding of the halogenated substrate in the enzyme active sites. Unexpectedly, when Cur ER was added with Cur ECH_1 and ECH_2 in the presence of γ-Cl-**1**-ACP_{II}, no saturation product was obtained. Instead, we observed a 34-Da mass reduction from γ-Cl-**3**-ACP_{II} (Fig. 2h and Supplementary Fig. 6a), demonstrating elimination of chlorine to yield **3**-ACP_{II}, **5**-ACP_{II} or **6**-ACP_{II} (Fig. 1b) as the putative product(s). To establish definitively the identity of the final product in the presence of both Hal and HMG-cassette enzymes, a one-pot reaction using Cur Hal-ACP_3, ECH_1, ECH_2 and ER was performed, and the product was cleaved and analysed by GC-MS (Supplementary Methods). A single species was identified as the *cis*-2-methylcyclopropane-1-carboxyl compound (Fig. 2i, lower trace), demonstrating the formation of **5**-ACP by an unprecedented ER-catalysed cyclopropanation reaction, presumably via an intramolecular nucleophilic substitution.

We next assessed whether Jam ER can catalyse cyclopropanation when presented with γ-Cl-**3**-ACP_{II}, owing to the similarity of Cur and Jam ERs (~65% sequence identity, Fig. 1a). Unexpectedly, Jam ER yielded only the saturated product, γ-Cl-**4**-ACP_{II} (Fig. 3c and Supplementary Fig. 6b), indicating that it shows the activity of a canonical polyketide synthase ER domain. The distinct functions of Cur and Jam ERs motivated us to compare the catalytic efficiencies of Cur ER cyclopropanation against Jam ER saturation for the chlorinated substrate, and the efficiencies of cyclopropanation for the chlorinated substrate against saturation for the non-chlorinated substrate by

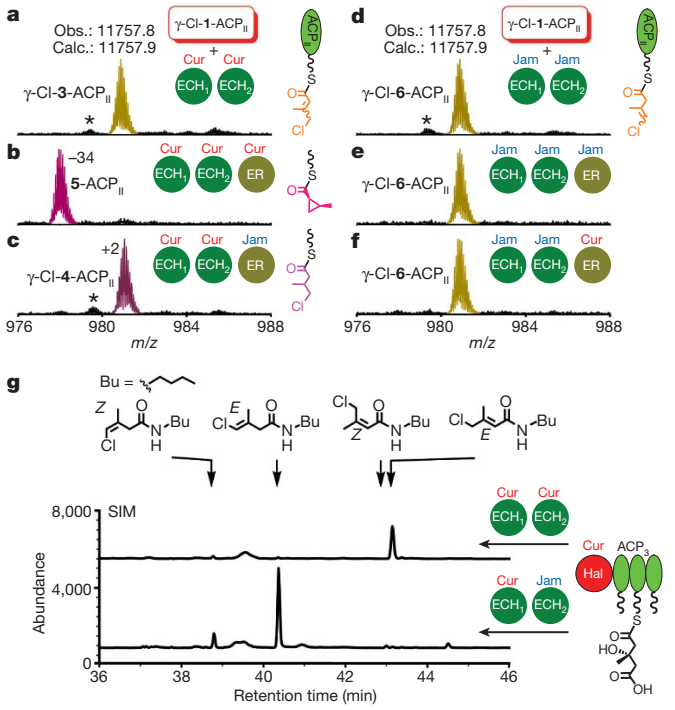

Figure 3 | Comparison of ECH_2s and ERs in Cur and Jam pathways. **a–f**, Partial FTICR mass spectra (12+ charge state of ACP_{II}) for Cur and Jam ECH_1, ECH_2 and ER reactions with the γ-Cl-**1**-ACP_{II} substrate. The reactions were incubated at 30 °C for 30 min. **g**, GC-MS analysis to identify the structures of Cur and Jam ECH_2 products. The chromatograms were recorded at SIM by monitoring 57, 117, 154 and 189 a.m.u. The retention times of products were confirmed by co-injection with the authentic standards.

Cur ER (Supplementary Fig. 9–11). This comparison confirmed that Jam ER has retained the canonical function as an α,β enoyl reductase, in contrast to the Cur ER as a cyclopropanase.

The basis for vinyl chloride group formation in the Jam pathway remained to be established. Given the extraordinarily high similarity between Cur and Jam Hals (92% sequence identity), we surmised that the two pathways diverge after the halogenation step. In the two assemblies, the Cur and Jam ECH_2 domains have lowest sequence identities (59%) (Fig. 1a), and probably function as a key branch-point determinant. The functions of Jam ECH_1 and ECH_2 were investigated with Cur substrates starting from **1**-ACP or γ-Cl-**1**-ACP_{II} to establish what controls introduction of the β,γ vinyl chloride group. For both **1**-ACP_{II} and γ-Cl-**1**-ACP_{II} substrates, Jam ECH_1 and ECH_2 catalysed successive dehydration and decarboxylation steps as expected (Fig. 3d and Supplementary Fig. 12b, c, f, g). However, when Jam ER was included, only ~20% of the saturated product was detected for the non-chlorinated substrate (derived from **1**-ACP_{II}, Supplementary Fig. 12i). No product was observed for the corresponding chlorinated substrate (derived from γ-Cl-**1**-ACP_{II}, Supplementary Fig. 12j), indicating that the Jam ECH_2 product is not a substrate for Jam ER. Thus, the Jam ECH_2-catalysed decarboxylation product of γ-Cl-**2**-ACP_{II} was predicted to be γ-Cl-**6**-ACP_{II} (β,γ C=C; Fig. 3d) with a vinyl chloride group, instead of γ-Cl-**3**-ACP_{II} (α,β C=C; Fig. 3a). Consistent with this prediction, Cur and Jam ECH_2 decarboxylation products showed different ultraviolet absorption patterns between 250 and 280 nm (Supplementary Fig. 13b), which distinguishes the isomeric α,β and β,γ enoyl thioester functionality.

To determine the structures of the decarboxylation products, one-pot reactions using Cur Hal-ACP_3, Cur ECH_1 and Cur or Jam ECH_2s were performed as described above. The main product of Cur ECH_2 contained primarily an α,β C=C in the *E* configuration, with trace

amounts (~3%) of the β,γ C=C isomer (Fig. 3g, upper trace). In contrast, Jam ECH_2 showed high regiochemical control to generate exclusively the β,γ C=C product, with ~85% in the *E* configuration and ~15% of the *Z* isomer (Fig. 3g, lower trace). The minor amount of *Z* conformation is probably due to the use of the Cur substrate, which is less bulky than the Jam substrate (Fig. 1a). Notably, Jam ECH_2 decarboxylation had lower regiochemical control using the non-chlorinated substrate, and generated ~80% β,γ C=C and ~20% α,β C=C products, which further explains the partial enoyl reduced product observed after Jam ECH_1, ECH_2 and ER reactions with this substrate (Supplementary Fig. 12i). Given the canonical function of ER to catalyse reduction of α,β enoyl thioesters, the selective formation of β,γ C=C product by Jam ECH_2 renders Jam ER superfluous in the biosynthesis of jamaicamides. In general, α,β C=C ECH_2 products are energetically preferred and frequently identified or predicted in other pathways[16,17,22–25], with the notable exception of pederin and its structural analogues[26,27] (Supplementary Fig. 14).

We sought to understand further the regiochemical control of ECH_2-catalysed decarboxylation. The Cur ECH_2 crystal structure[19] was modelled with the chlorinated substrate and site-directed mutagenesis experiments were performed to identify key residues for regiochemical control (Supplementary Fig. 15). We compared the catalytic efficiencies of the wild type (WT) and mutants of Cur ECH_2, and measured the ratios of the two possible decarboxylation products γ-Cl-**3**-ACP_{II} and γ-Cl-**6**-ACP_{II} (Supplementary Methods). The data revealed that positioning of a non-conserved Tyr 82 residue in a hypervariable region of the enzyme active site is crucial for regioselective protonation after collapse of the presumed enolate intermediate (Fig. 4c and Supplementary Fig. 15a). This result suggests that ECH_2 regiochemical control might be readily affected by mutations in this hypervariable region to provide functional group diversification.

The Cur and Jam pathways enable us to witness the remarkable process of evolutionary diversification in secondary metabolism. Both Cur and Jam contain Hal domains that were recruited and embedded in a modular polyketide synthase to impart new chemical diversity (Fig. 4a). Recent studies on this class of α-ketoglutarate-dependent non-haem Hals have reported them as being discrete enzymes in secondary metabolite pathways[2,9–12], but this integrated domain represents an unprecedented example of pathway diversification. Cur and Jam are further diversified by the amino acid sequence variation in downstream enzymes to yield different catalytic activities (Fig. 4a). Specifically, the Cur ER domain was shown to be a cyclopropanase catalysing nucleophilic displacement of the chlorine atom leading to a highly strained and unusual functional group (Fig. 4b). In contrast, the Jam ER domain was found to retain reductase function for the Cur α,β enoylthioester substrate, but it is inactive against the corresponding β,γ enoylthioester isomer. Thus, in addition to the cyclopropanation strategies of Zn^{2+}-dependent CmaC[9,28] and the recently reported FAD-dependent dehydrogenase KtzA[29] where chloride also serves as a leaving group, the NADPH-dependent Cur ER-catalysed cyclopropanation represents a new strategy for generating a thioester enolate and subsequent ring formation. Structural insights to reveal Cur ER sequence variations that stabilize the α-carbanion while supporting closure of the highly strained cyclopropane are key to understanding its functional evolution. Further pathway diversification is reflected in select amino acid sequence changes that direct alternative double-bond regiochemistry in the Jam products (Fig. 4c). These parallel yet distinct systems demonstrate the mutability of enzymes within complex metabolic pathways, and

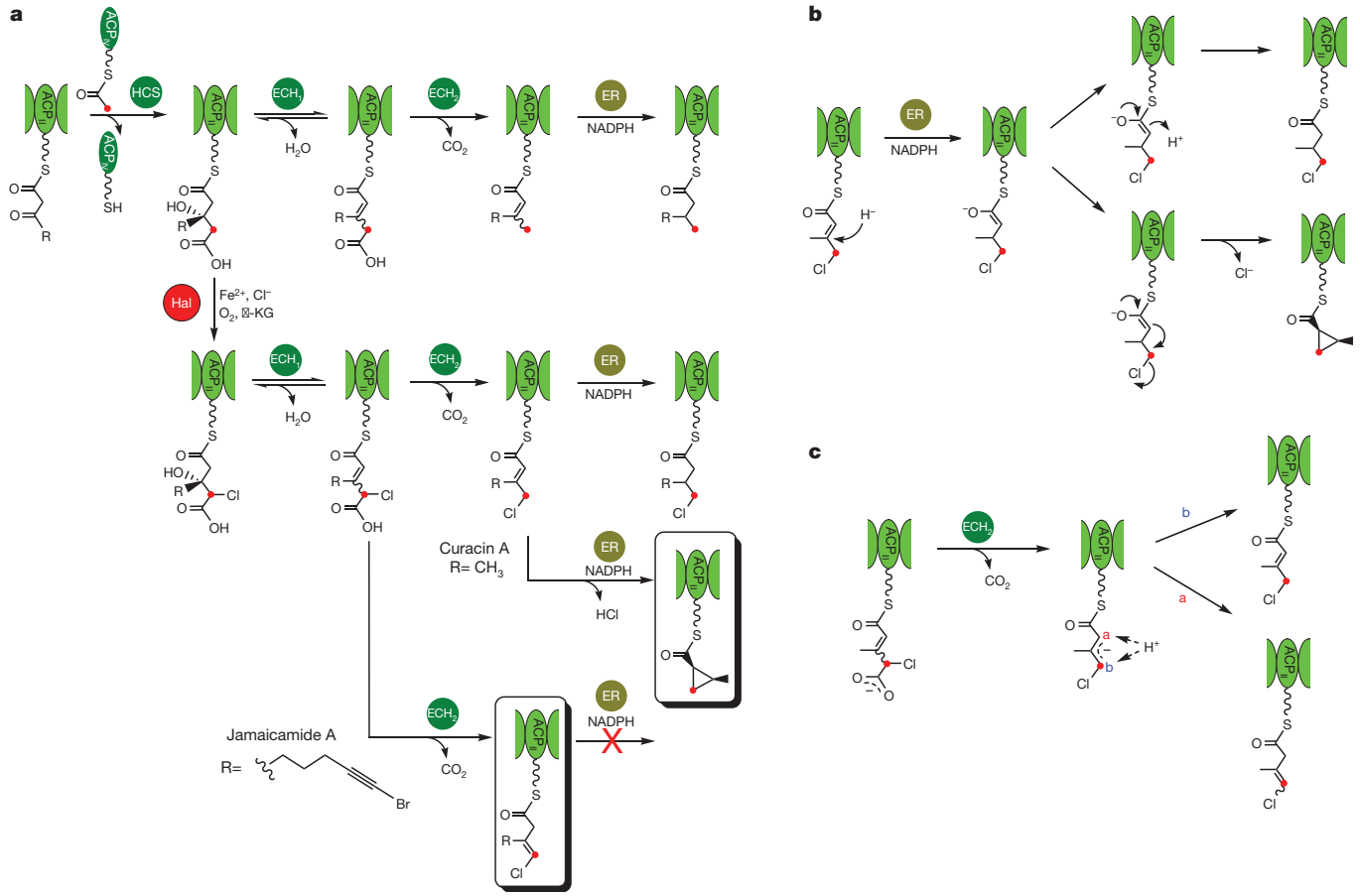

Figure 4 | Impact of enzyme assembly evolution on β-branching chemical diversity. **a**, Proposed ancestral forms of the enzyme assemblies in Cur and Jam pathways. α-KG, α-ketoglutarate. **b**, The functional diversification of ERs. **c**, Differential regiochemical control by ECH_2s. The β-branching carbon atoms are highlighted in red.

reveal their metamorphic properties for creating chemical diversity in biologically active natural products.

METHODS SUMMARY

All proteins used in the work were overexpressed in *Escherichia coli*, and purified by Ni-affinity chromatography. Specifically, the Cur Hal constructs were purified anaerobically and reconstituted with Fe^{2+} and α-ketoglutarate (refs 9, 10). FTICR-MS and IRMPD were used to detect mass changes on acyl-ACPs. The acyl groups tethered to ACP phosphopantetheine arms were cleaved by butylamine aminolysis, and corresponding butylamide derivatives were subsequently analysed by GC-MS and correlated with authentic standards for structure determination. IRMPD-based quantification was developed to measure yields of the ER-mediated saturation and cyclopropanation reactions. Detailed experimental procedures are described in the Supplementary Methods.

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