partial protein sequencing and degenerate-primer PCR [9], and that once identified its sequence showed no relationship to any other known prenyl transferase. Yet *dmaW* homologs are evident in numerous secondary metabolite clusters, including the newly identified terrequinone A cluster [1]. In the future, other previously unrecognized, but essential and conserved, gene families might be identified for other metabolite gene clusters. For example, the loline alkaloid (*LOL*) gene cluster of *Neotyphodium uncinatum* contains ten genes, none of which fall into the four aforementioned categories [10]. Even so, the relationships of *LOL* proteins to pyridoxal phosphate enzymes, monooxygenases, or nonheme-iron oxygenases strongly suggest that this is a secondary metabolism cluster.

Thus, two approaches appear promising for identifying novel secondary metabolism clusters in sequenced genomes: identification of signature protein families [2], and demonstration of coordinated regulation with other metabolite gene clusters [1]. Bok et al. [1] demonstrate that the latter approach holds considerable promise for *Aspergillus* species. The degree to which the technique can be applied to other fungal groups depends on whether global regulation is typical or atypical across the kingdom. A promising observation is that homologs of *laeA* are apparent in sequenced genomes of other fungi in the phylum Ascomycota. But, there is actually an embarrassment of riches. For example, a BLASTp search of LaeA sequence against the protein database for *Gibberella zeae* PH-1—also known for its secondary metabolites and toxins—brings up 37 hits with E values ranging from 1×10^{-47} to 7×10^{-21} (personal observation). Obviously, these cannot all be global regulators. Does the top of this list represent the functional homolog of LaeA, or does the next one (at 9.7×10^{-46}), or do none of them? Follow-up studies of other systems will have evolutionary implications as well. If global regulation appears to be typical of diverse fungal genera, what ecological conditions might they have in common that would have selected this behavior? If, on the other hand, other fungi tend not to exhibit global regulation of secondary metabolism, what are the ecological variables that have led to such divergent regulatory strategies?

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A Tighter RVxF Motif Makes a Finer Sift

Most partners of protein phosphatase 1 rely on an instance of the so-called RVxF motif for interaction with the enzyme. In this issue of *Chemistry & Biology*, a stringent definition of the motif targeting high-affinity instances enabled Meiselbach and colleagues to recognize novel binding partners with high specificity [1].

For a protein, a key to durable protection from mutation is the acquisition of one or more essential functions that involve interactions with multiple partners. Few proteins are better placed to illustrate the success of this strategy than protein phosphatase 1 (PP1), an enzyme that removes phosphoryl moieties from Ser or Thr residues in proteins [2–5]. Beginning in early eukaryotic evolution, this enzyme formed interactions with proteins that target and regulate its activity toward various specific substrates. Some of the earliest of these alliances, presumably including those with the mitotic regulator Sds22 [6] and the less well characterized Inhibitor-3 [7], became indispensable for eukaryotic survival. The ensuing need for conservation of the different interaction sites involved has severely constrained further mutation of the enzyme [2].

However, the structural rigidity of PP1 has not halted its functional evolution. To the contrary, to meet new regulatory demands in different eukaryotic lineages, its

stable structure has continued to accommodate novel interactions with inhibitors, substrate specifiers, which restrict the access to the catalytic site, and targeting proteins, which recruit PP1 to specific subcellular locations or substrates. For instance, some time before the divergence of fungi and animals, PP1 and a coopted glycogen-targeting protein engaged in the controlled dephosphorylation and reactivation of glycogen synthase [2]. Bound to other regulatory subunits, PP1 functions in the regulation of various aspects of cell cycle progression and arrest, the metabolism of small molecules and macromolecules, actin and actomyosin reorganization, and signaling through receptors, ion channels, and ion pumps [3–5].

Nearly a decade ago, a peptide display study described a short peptide motif that is sufficient for interaction with PP1 [8]. Interestingly, this motif, which consists of a few basic residues followed by Val, a poorly defined residue, and Phe or Trp, occurs in most PP1 binding proteins. At about the same time, the cocrystallization of PP1 with a fragment of a glycogen-targeting subunit revealed the binding of this very motif in atomic detail; the Val and aromatic side chains nest in a hydrophobic channel at some distance from the catalytic site of the enzyme, while the basic residues adhere to the electrostatically favorable surface near the entrance of the channel [9]. Although functional on their own, instances of the RVxF motif, as it is most often referred to, often join forces with additional PP1 binding sites. The recently published crystal structure of a myosin-targeting subunit in complex with PP1 nicely illustrates this observation [10]. On the other hand, some well-studied PP1 partners, like Sds22 [11], have been shown to lack an RVxF sequence altogether, which leaves the RVxF binding channel free to accommodate additional protein ligands.

The prospect for the recognition of novel binding partners of PP1 inspired approaches to a more rigorous description of the motif. Wakula et al. proposed a definition based on known and experimentally validated RVxF instances and an exhaustive exploration of the residues allowed at the penultimate position of the motif [12]. The compatibility of a residue was derived from the inhibitory potency of mutated versions of a fragment of the Nuclear Inhibitor of PP1 that depends on a functional RVxF sequence for binding. The resulting formal definition of the motif read [KR]-$X_{(0,1)}$-[VI]-{P}-[FW] (Val or Ile separated by any residue but Pro from Phe or Trp, with Arg or Lys at at least one of the two preceding positions). This definition recognizes virtually all established RVxF instances, but with its predicted and observed random occurrence of roughly one instance per thousand residues (or per three average-length protein sequences), it lacks specificity. Given a generous estimate of about 200 genuine RVxF instances in the mammalian proteome [3], the specificity of the motif as defined by Wakula et al [12] would approach 2.5%. Hence, compatibility with this motif is an inadequate screening criterion for candidate regulators of PP1, even when combined with context filters that exclude poorly conserved occurrences or instances in putative extracellular and transmembrane regions.

In this issue, Meiselbach et al. suggest a more restrictive definition of the motif, based on the coprecipitation of PP1 with mutated versions of a PP1 binding fragment of the mGluR7b receptor with an RVxF sequence [1]. A complete loss of interaction in these pull-down assays [1] roughly corresponded to a reduction of the inhibitory potency by a factor of two in the inhibition assays [12]. The choice of a less sensitive binding assay resulted in the exclusion of Ile from position 3 of their 5 residue motif and of about half of the naturally occurring residues from positions 2 and 4. The sensitivity of the new definition appears somewhat disappointing, as it only recovers about 40% of the established RVxF instances. However, at roughly one instance per ten thousand residues, the predicted and the observed random occurrence of this stricter motif are an order of magnitude lower than that of the earlier, more sensitive definition. Under the assumption that the overall sensitivity approaches the 40% sensitivity among established instances, this lower random occurrence should translate to an improved specificity of about 10%. The authors furthermore report that subsequent context filtering can eliminate more than half of the candidate instances based on a predicted segregation from the intracellular PP1, which results in a combined expected specificity of roughly 25%. Strikingly, on a small test set of proteins not known to be binding partners, the proposed procedure performed even better than expected. Pull-down assays validated seven out of eight tested candidates, and for each of those a competition assay demonstrated the involvement of an RVxF sequence. Even when an allowance is made for a substantial margin of error in view of the small size of the test population, the efficiency of the proposed screening procedure is very impressive. Hence, a further exploration of the untested candidates holds the promise of the identification of several dozens of novel PP1 partners.

Notably, the huge gap between the expected and observed specificity may indicate that the overall sensitivity is considerably higher than the 40% coverage of known RVxF instances. This hypothesis implies that known binding partners bear more divergent RVxF instances with a lower affinity for PP1. The distinction between primary and secondary partners of PP1 [2] may offer an explanation. While the interaction of secondary partners with PP1 is subordinate to a distinct principal function, the recruitment, substrate specification, and regulation of PP1 together constitute the core function of primary PP1 partners. Primary partners, which include the majority of the known regulators, often rely on multiple interaction sites for the various aspects of their function. Cooperative binding of these sites may alleviate the affinity requirements for an involved RVxF instance. The PP1-related function of secondary PP1 partners, on the other hand, is typically limited to the direct recruitment of the phosphatase, which tends to be involved in the regulation of the distinct core function of that partner. An isolated PP1 binding site, typically an easily evolvable RVxF sequence, may suffice for recruitment if its affinity for the enzyme is high enough.

Most known examples of secondary PP1 partners are receptors, ion channels, ion pumps, or scaffold proteins [3]. Strikingly, five out of the seven candidates validated by Meiselbach and coworkers and the mGluR7b paradigm are also receptors and/or ion channels and therefore presumably secondary partners. If this subset

proves representative, the proposed approach may efficiently target secondary PP1 partners with high-affinity RVxF instances. Interestingly, given that the number of primary PP1 partners has stagnated in recent years, secondary interactors show the highest potential for growth via novel discovery approaches like the one proposed by Meiselbach and coworkers. Other recently described PP1 binding motifs [12–14] may inspire additional strategies.

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Specific Probes for Chemokine Receptors

Chemokine receptors have attracted a good deal of public attention as important therapeutic targets for many diseases and disorders. In this issue of *Chemistry & Biology*, Kumar and colleagues propose a new concept of synthetic modular modifications to generate unnatural chemokines, which exhibit high receptor selectivity [1].

In a postgenome and proteome era, selective agonists and antagonists can be highly useful for studies of receptor biology and for clinical applications. Chemokines belong to a chemotactic cytokine family that attracts and induces migration of leukocytes. Chemokines and their receptors play fundamental roles in physiological phenomena. Since these actions are relevant to many pathological disorders such as cancer and AIDS, chemokine receptors are thought to be critical drug targets.

Chemokine receptors are members of the seven-transmembrane G protein-coupled receptor (GPCR) family, which transduce signals of corresponding chemokines. The relationships between chemokines and their receptors are highly interconnected and complicated: a single chemokine recognizes a plurality of receptors, while one chemokine receptor recognizes several chemokines. Numerous chemokines lack receptor selectivity. Unnatural chemokines that have high receptor selectivity would be practically useful, not only as specific molecular probes for biological studies, but also as drug leads for clinical application. Furthermore, the development of systematic strategies to synthesize such unnatural ligands would be desirable.

In this issue of *Chemistry & Biology*, Kumar and colleagues report unnatural synthetic molecules as chemical probes of chemokine receptors. They present the concept of modular modifications to generate unnatural chemokines that possess receptor selectivity [1]. They created synthetically and modularly modified (SMM)-chemokines based on a combination of total chemical synthesis and modular modification. They chose CXCR4 [2] and CCR5 [3–7] as target receptors with the aim of potentially developing anti-AIDS drugs, since these are the two principal coreceptors that are required for HIV-1 entry. In addition to HIV infection/AIDS, CXCR4 has also been shown to be involved in several problematic diseases, such as cancer metastasis [8, 9], leukemia [10, 11], and rheumatoid arthritis [12, 13]. As such, CXCR4 represents one of the greatest therapeutic targets for the above diseases. Although natural chemokines for CXCR4 and CCR5 can inhibit HIV infection by blocking gp120 binding regions on CXCR4 and CCR5, respectively, serious problems remain concerning selectivity, side effects, and toxicity profiles. vMIP-II, which recognizes various chemokine receptors, was chosen among chemokine ligands as the parent molecule for modification [14]. For a detailed discussion of CXCR4/CCR5 antagonists, readers are referred to recent reviews [15, 16].