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Current Opinion in Chemical Biology

# Profiling and inhibiting reversible palmitoylation

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Protein palmitoylation describes the posttranslational modification of cysteines by a thioester-linked long-chain fatty acid. This modification is critical for membrane association, spatial organization, and the proper activity of hundreds of membrane-associated proteins. Palmitoylation is continuously remodeled, both by spontaneous hydrolysis and enzyme-mediated de-palmitoylation. Bioorthogonal pulse-chase labeling approaches have highlighted the role of protein thioesterases as key regulators of palmitoylation dynamics. Importantly, thioesterases are critical for regulating the spatial organization of key oncogenic proteins, such as Ras GTPases. New inhibitors, probes, and proteomics methods have put a spotlight on this emerging posttranslational modification. These tools promise to advance our understanding the enzymatic regulation of dynamic palmitoylation, and present new opportunities for drug development.

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**Current Opinion in Chemical Biology** 2013, **17**:20–26

This review comes from a themed issue on **Omics**

Edited by **Matthew Bogyo** and **Pauline M Rudd**

For a complete overview see the Issue and the Editorial

Available online 1st January 2013



http://dx.doi.org/10.1016/j.cbpa.2012.11.023

## Introduction

Protein palmitoylation was first reported just a few months before the classic discovery of tyrosine phosphorylation [1,2], yet more than 30 years later, the importance of palmitoylation is only now gaining significant attention as a widespread, dynamic posttranslational modification. This is likely due to a historical lack of robust methods for sensitive analysis of this nonpolar, nonantigenic modification. Until recently, the only method to study palmitoylation involved metabolic labeling with [$^3$H]-palmitate, followed by lengthy exposure times ranging from days to weeks. Given the lack of straightforward methods, the dynamics and regulation of protein palmitoylation is largely unexplored.

Protein palmitoylation is clearly important in establishing the spatial localization of many well-studied signaling complexes. Cellular transformation by oncogenic v-Hras (H-Ras$^{G12V}$) requires membrane anchoring [3,4], and mutation of a single palmitoylation site eliminates the protein's oncogenic potential [3]. The rate of palmitate turnover on inactive GDP-bound H-Ras is accelerated >15 times upon activation [5]. Similarly, activation of G-alpha-s accelerates palmitate turnover nearly 50-fold [6]. Similar findings have been observed for the synaptic scaffolding protein PSD-95, which is rapidly depalmitoylated following glutamate stimulation [7]. On the basis of these observations, dynamic palmitoylation may be a general regulatory mechanism controlling signal-dependent spatial localization.

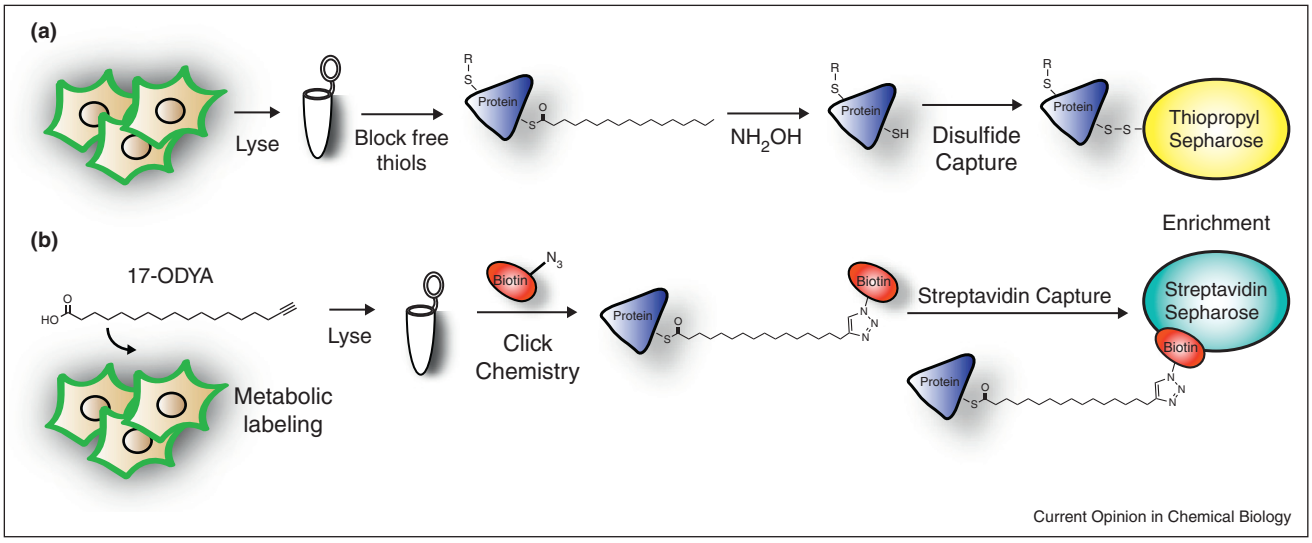
The goal of this review is to present recent advances for the detection, annotation, and quantification of dynamic palmitoylation, as well as a discussion of the potential for thioesterase inhibitors to modulate key signaling pathways.

## Nonradioactive detection of palmitoylation

Two complementary methods have been developed in recent years for the nonradioactive detection, enrichment, and mass spectrometry-based annotation of palmitoylated proteins. The first method, termed acyl-biotin exchange, is useful for the static analysis of palmitoylated proteins in native tissues or cells [8–10]. In this method, lysates are first treated with methyl methanethiosulfonate (MMTS) or maleimide to block free thiols. Next, thioesters are hydrolyzed with hydroxylamine, which releases the acyl chain and exposes new free thiols for disulfide capture [11]. One drawback to this approach is the enrichment of proteins with native thioesters, such as ubiquitin ligases and lipoamide-linked dehydrogenases. New modifications of this approach employ activated thiol resins for more simplified enrichment [12••] (Figure 1a).

The second method uses metabolic labeling with the bioorthogonal fatty acid analog 17-octadecynoic acid. The alkynyl fatty acid analog is incorporated by the endogenous palmitoylation machinery into native sites palmitoylation. After lysis, labeled proteins are ligated to azide-linked reporter tags by click chemistry [13,14] (Figure 1b). Importantly, all reagents are commercially available and relatively inexpensive. The key advantages are a simplified workflow, high sensitivity, reduced nonspecific labeling, and the ability to examine palmitoylation turnover dynamics by classic pulse-chase methods. Unlike ABE, this method only enriches native sites of long-chain fatty acid modification, and not other endogenous thioesters [9,10].

**Figure 1**




Methods for palmitoylated protein enrichment. **(a)** Resin-assisted capture of hydroxylamine-sensitive cellular thioesters for static analysis of palmitoylation. After reduction and alkylation, lysates are treated with hydroxylamine to hydrolyze thioesters. Free thiols are captured by disulfide formation using activated thiol sepharose resin. **(b)** Bioorthogonal enrichment of 17-ODYA metabolically labeled sites of palmitoylation. Biotin-azide is conjugated by click chemistry to 17-ODYA labeled proteins for streptavidin enrichment.

Both enrichment methods have been used to globally annotate palmitoylated proteins by mass spectrometry in a variety of organisms, tissues, and cell lines [9,10,13,15,16,17•]. Altogether, more than 500 palmitoylated proteins have been annotated in mammalian cells. This list contains both integral and membrane-associated proteins, including channels, receptors, and scaffolding proteins. On the basis of these results, there are likely thousands of palmitoylated cysteine residues in the proteome [15], solidifying protein palmitoylation as pervasive as other widely studied polar posttranslational modifications.

## Quantitative analysis of palmitoylation

Ras is the prototypical palmitoylated protein, and has been used as a model to study the spatial organization, dynamics, and turnover of protein palmitoylation. Upon microinjection of fluorescent, palmitoylated N-Ras, the semi-synthetic protein rapidly distributes to all membranes, and enters a pathway of dynamic palmitoylation and de-palmitoylation [18•,19]. N-Ras is quickly de-palmitoylated in the periphery, but re-palmitoylated at the Golgi and recycled back to the plasma membrane through the secretory pathway. Complementary live-cell fluorescence imaging with transfected photo-convertible fluorescent protein fusions confirmed these observations. On the basis of these experiments, palmitoylation is hypothesized to stabilize the membrane attachment and increase the residency time of N-Ras at the plasma membrane [20]. This specific example demonstrates how dynamic palmitoylation can promote spatial organization and function of a key oncogenic signaling protein. Despite these promising observations, there is little evidence studying the dynamics of native proteins to support these findings.

Bioorthogonal metabolic pulse-chase labeling provides a direct approach to profile the global dynamics of palmitoylation on native proteins. Preliminary studies in Jurkat T-cells demonstrated an increase in the rate of Lck de-palmitoylation after pervanadate treatment [21••]. Importantly, the rate of palmitoylation turnover was attenuated by treatment with the generic lipase inhibitor methyl arachidonyl fluorophosphonate (MAFP) [22]. This was the first demonstration on native proteins that unspecified serine hydrolases regulate the turnover of palmitoylation on native proteins.

To measure fractional changes in palmitoylation, SILAC quantitative proteomics methods [23] were applied in conjunction with metabolic 17-ODYA and enrichment. BW5147-derived mouse T-cell hybridoma cells were first passaged in isotopic media according to standard SILAC protocols [24••]. First, a list of high confidence palmitoylated proteins was established by performing two parallel experiments. In the first experiment, light cells were treated with 17-ODYA for eight hours, and the heavy cells with palmitic acid, or *vice versa* (Figure 2). In a second experimental group, heavy and light cells were both treated with 17-ODYA for eight hours, and one cell lysate was incubated with hydroxylamine to cleave thioesters and release 17-ODYA. In both experimental groups,