Studying Ras Nanocluster Formation on the Cell Membrane with Correlative Superresolution and Electron Microscopies

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It has now been well accepted that the biological membrane is far from a mosaic of proteins in a fluid lipid bilayer, but instead is laterally segregated into heterogeneous compartments with distinct compositions, structures, and functions [1]. This physical and functional partitioning of the membrane could have profound biological consequences. For example, different molecules may localize to the same membrane compartments and interact with each other with high efficiency and specificity, even if their apparent affinities are weak, because the molecules are locally co-enriched in the same space [2]. Aside from promoting heterotypic interactions, local enrichment in nanoscopic membrane domains would also facilitate homotypic interactions to yield higher order structures such as protein nanoclusters [3], which in many cases have been implicated in regulating the biological activities of the protein.

Among others, the Ras small GTPases are prototypical examples of membrane proteins that form nanoclusters [4]. All three major Ras isoforms, namely H-, N-, and K-Ras, have been shown to exhibit membrane nanoclusters when over-expressed based on immuno electron microscopy (immuno-EM) studies, and the nanoclusters have been linked to Ras effector pathway activation. There is also evidence that the nanoclusters of different Ras isoforms reside in different membrane nanodomains, in a large part due to the differences in the isoform-specific membrane targeting motifs. This spatial mechanism explains why the Ras isoforms are highly homologous in their functional (globular) domains but have non-redundant biological functions and distinct mutational spectra in human cancers [5]. Thus, understanding the mechanisms through which Ras forms nanoclusters is critical to understanding how Ras functions in cells and targeting mutant Ras in human cancers.

Using quantitative superresolution microscopy (SRM), we have recently shown that mutant K-Ras forms dimers when expressed at near-endogenous expression levels and activates Raf-MAPK [6]. Formation of higher order K-Ras nanoclusters occurs at over-expression conditions, but it is not necessary for Raf-MAPK activation. H-Ras also forms dimers and higher order nanoclusters at physiologically relevant expression levels. To further elucidate how Ras forms dimers and higher clusters on the cell membrane, we have combined quantitative SRM with scanning electron microscopy (SEM). In this approach, SRM provides the precise localizations and stoichiometry of fluorescently tagged Ras, and SEM allows nanoscopic visualization of the immediate membrane context of various Ras species (monomers, dimers, and higher order structures) [7]. With optimized imaging workflows and image analysis strategies, we were able to demonstrate correlative SRM – SEM imaging of Ras on prepared membrane sheets with ~20 nm combined spatial resolution. In the correlative images, Ras monomers, dimers, and higher order nanoclusters could be detected and precisely localized along with

their associated membrane ultrastructures such as the cortical cytoskeleton and various membrane vesicles, including clathrin-coated pits (Fig. 1).

With this approach, we systematically analyzed and compared the membrane structures associated with H-Ras or K-Ras monomers, dimers, or higher order nanoclusters. We found that H-Ras and K-Ras both are associated with multiple membrane structures, but each preferentially target to distinct subsets of ultrastructures to form dimers and/or higher order nanoclusters. The two Ras isoforms also form different sizes of clusters depending on the associated membrane structures. Lastly, the membrane clustering properties were found to be dominated by the C-terminal, membrane targeting motifs for both Ras isoforms. Together, these results provide a more complete picture of Ras nanocluster formation on the cell membrane and offer useful guidance to future studies aiming at defining each of the Ras-associated membrane structures. Our approach should also be generally applicable to study the nanoclusters of other membrane proteins.

References:

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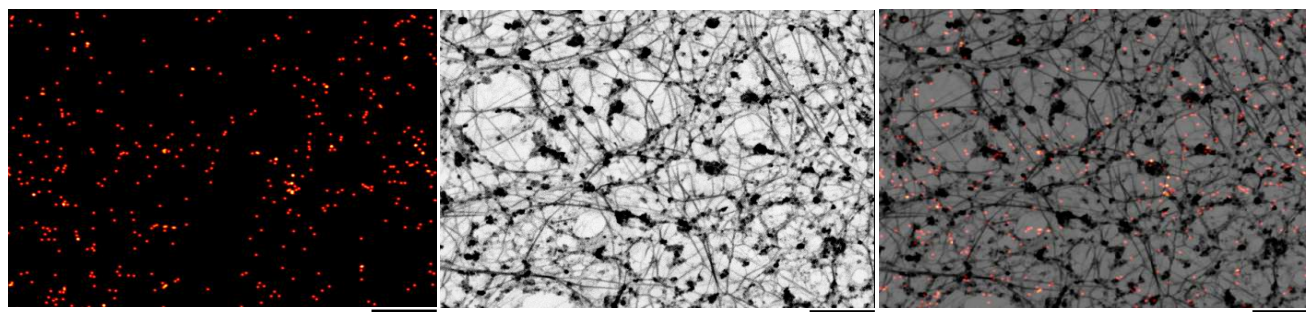[7] Y Zhang et al., PLoS One **12(5)** (2017), p. e0176839.

Figure 1. Correlative SRM and SEM for visualizing Ras proteins and their associated membrane ultrastructures. Membrane sheets prepared from U2OS cells stably expressing PAmCherry1-tagged tK (C-terminal tail of K-Ras) were fixed and imaged first with SRM (left), followed by SEM sample preparation and imaging (middle). The SRM and SEM images of the same field-of-view were then registered using gold nanoparticles as fiduciaries. Scale bars, 500 nm.