from applying the power of reverse genetics to the study of cell-cycle regulation.

Acknowledgements
Work in our laboratory is supported by the National Institutes of Health and the W.M. Keck Foundation.

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0962-8924/03/$ - see front matter
doi:10.1016/S0962-8924(03)00148-X

Secretory granules: and the last shall be first…

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By tagging secretory granules with the fluorescent protein dsRed-E5, which changes its emission from green to red over time, Duncan *et al.* analysed the age-dependent distribution of secretory vesicles within chromaffin cells. This elegant study illustrates as never before how age is a critical factor that segregates granules with respect to their localization and mobility and the probability of them undergoing exocytosis in response to different stimuli.

The field of regulated exocytosis is always 'brighter'. So it appears, as the coupling of each new variant of green (GFP) and red (*Discosoma* sp. Red, dsRed) fluorescent proteins to cell cargoes is exploited to better track the biogenesis, transport and exocytosis of secretory granules in living neuroendocrine cells. Before the advent of GFP, investigators relied on loading secretory vesicles with fluorescent dyes such as FM1–43 [1] and acridine orange [2], which, however, can also be taken up by the endosomal–lysosomal system. It was first shown in 1997 that regulated secretory proteins fused to GFP variants were properly stored in the neurosecretory granules of rat phaeochromocytoma PC12 cells [3,4]. The use of such GFP chimaeras has thus improved the possibility of labelling granules specifically rather than other membranous compartments. Immature secretory granules (ISGs) in PC12 cells, for example, could be selectively viewed by confocal microscopy after conjugation of the

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cargo chromogranin B to a temperature-sensitive GFP mutant, which folds properly at 20°C but not at 37°C [5]. However, once properly folded at the lower temperature, this GFP variant remains stable and fluorescent even at 37°C. Upon the shift from 20°C (at which the budding of secretory vesicles from the *trans* Golgi network (TGN) is blocked) to 37°C, GFP-labelled ISGs moved within seconds in a microtubule-dependent fashion from the TGN to the F-actin-rich cell cortex (Fig. 1), where they remained spatially restricted while continuing their maturation. Insulinoma cell granules labelled with a fusion protein between dsRed and phogrin, an intrinsic protein of the granule membrane [6], were shown to require the motor protein ATP-dependent conventional kinesin for their anterograde transport along microtubules [7] (Fig. 2).

The behaviour of granules within the actin cortex has been further elucidated by total internal reflection fluorescence microscopy (TIRFM). This approach is uniquely suited to observing in real-time single organelles that are within ~300 nm of the cell surface [8,9]. The consensus emerging from these investigations is that the mobility of granules is slowed considerably as they approach the plasma membrane, because they either become entrapped ('caged') in the cortical actin meshwork or are anchored to a tethering apparatus [10–12] (Fig. 2). Assembly of the SNARE fusion complex between vesicles and the plasma membrane is unlikely to account entirely for the restricted motion of granules, for at least two reasons. First, granule mobility was not increased upon transfection with *Clostridium* toxins [12], which cleave

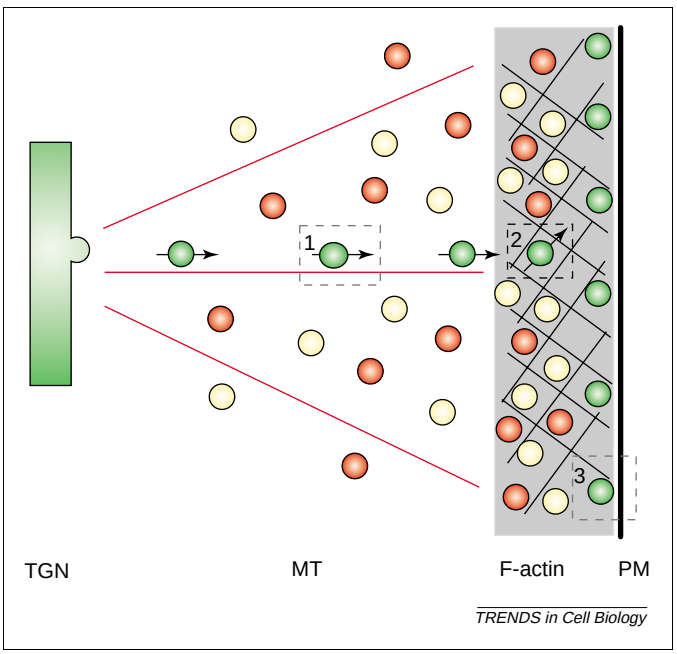

Fig. 1. Scheme illustrating the age-dependent distribution of secretory granules in bovine chromaffin cells. The colour-coded spheres depict secretory granules according to their age: green, youngest granules; yellow, older granules; red, oldest granules. Note that the youngest granules are found in close proximity to the plasma membrane, whereas the older ones are localized to the interior of the cell. The perforated boxes depict newly formed granules during microtubule-dependent (1) or actin-dependent transport (2), or docked beneath the plasma membrane (3). The black arrow indicates the transport of newly formed granules from the TGN to the plasma membrane. The grey box adjacent to the plasma membrane depicts the cell cortex enriched in F-actin (meshwork of black lines). Abbreviations: TGN, *trans* Golgi network; MT, microtubules; PM, plasma membrane.

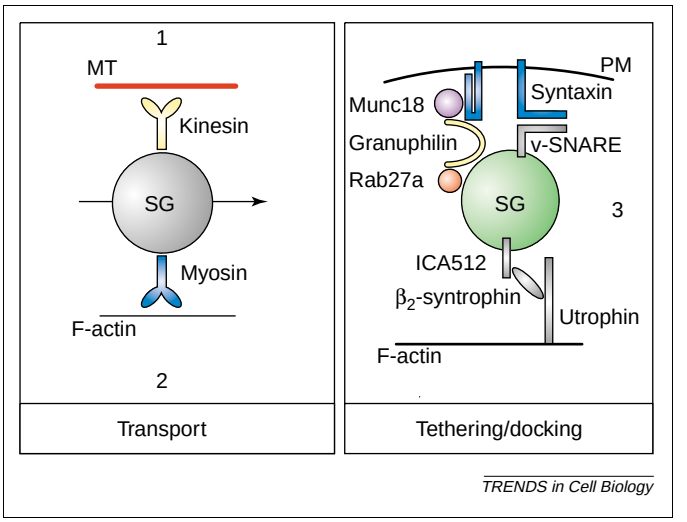

Fig. 2. Models of transport and docking of secretory granules. (Left) Granules depend on kinesin for transport along microtubules (MT) and on myosin for transport along F-actin. No colour code is given to the granule (SG) because these types of transport can apply to all three of the age-dependent pools indicated in Fig. 1. (Right) Granules are docked at the plasma membrane (PM) via the v/t-SNARE complex. For clarity, the plasma membrane t-SNARE, SNAP25, has been omitted. Granules are tethered to the plasma membrane through Rab 27a, granuphilin, Munc18 and 'backfolded' syntaxin. Granules are tethered to the cortical actin via the transmembrane protein ICA512/IA-2, β_2-syntrophin and utrophin. The numbers 1–3 refer to the steps indicated in Fig. 1 (perforated boxes).

SNARE proteins. Second, the plasma membrane SNARE protein syntaxin should diffuse in the lipid bilayer, unless it is itself attached to the cytoskeleton. Cytoskeletal elements might also bind effector proteins of the Rab GTPases (e.g. Rab3a), which are associated with the cytoplasmic face of the granule membrane. The Rab3a effector rabphilin, for example, has been reported to bind α-actinin, which cross-links actin filaments into a bundle [13]. Accordingly, rabphilin might link Rab3a to the cortical cytoskeleton. Another candidate molecule for tethering granules to the actin cortex is the receptor tyrosine phosphatase-like protein ICA512/IA-2, a transmembrane protein of secretory granules that interacts with the PDZ domain of β_2-syntrophin in insulinoma cells [14] (Fig. 2). β_2-Syntrophin, in turn, binds utrophin, a spectrin-related protein of the dystrophin family that is associated with cortical actin microfilaments (Fig. 2). It remains to be established whether such complexes are related to the filaments beneath the plasma membrane and in close proximity to granules that have been observed in chromaffin cells by electron microscopy [15].

Colour codes for ageing granules

The work of Duncan *et al.* has refined our understanding of neuropeptide secretion by demonstrating that granules are spatially and functionally segregated according to their age [16]. These authors specifically examined the granules of bovine chromaffin cells, which store both the peptide hormone atrial natriuretic factor (ANF) and catecholamines. They initially looked at the secretion from chromaffin cells that were transfected for 48 h with a fusion protein of ANF and enhanced GFP (ANF–EGFP) [4]. Maximal stimulation of the cells with nicotine triggered the release of ~60% of the ANF–EGFP compared with 10–15% secretion of the total catecholamine content. Since the

ANF–EGFP-labelled granules could not have been older than 2 days, while vesicles have an average life-time of 18 days, the authors concluded that newer secretory granules were preferentially recruited for exocytosis. They also noted, using confocal microscopy, that ANF–EGFP-labelled granules at the cell periphery were disproportionally depleted compared with those in the central region. This observation leads on to the most original aspect of their article: the temporospatial separation of newly synthesized granules using a chimaera between ANF and the 'timer' protein dsRed-E5, which progressively shifts its fluorescence emission from green to yellow and finally to red within 16 h [17]. Of the vesicles less than 3 days old, 67% appeared yellow, whereas the youngest (green) and the oldest (red) comprised 22% and 11% of the total ANF–dsRed-E5-labelled vesicles, respectively. The green granules were closer to the plasma membrane than the yellow and the red granules (Fig. 1), and 99% of them were released upon stimulation with nicotine compared with only 69% of the yellow and none of the red.

Consistent with previous results, TIRFM showed that nicotine stimulation triggered the exocytosis of immobile granules that were located in close proximity to the plasma membrane; these are referred to as the readily releasable pool. This was not the case, however, for the granules that underwent exocytosis in response to Ba^{2+}, which is known to act on the reserve pool rather than on the readily releasable pool of granules. Analysis by confocal microscopy revealed that green granules were indeed insensitive to Ba^{2+}, whereas 83% of the yellow granules were released concomitantly with the secretion of 59% of the total catecholamine content. In addition, TIRFM showed that Ba^{2+}, unlike nicotine, did not induce the release of immobile granules near the plasma membrane that were labelled with acridine orange. The implication of these results is that Ba^{2+} preferentially promotes the exocytosis of older granules, including a large fraction of those not labelled with ANF–dsRed-E5 as they are older than 3 days. Nicotine and Ba^{2+} had an additive effect on secretion by releasing 82% of stored catecholamine, consistent with their activation of different granule pools.

Finally, Duncan *et al*. compared the mobility of the different ANF–dsRed-E5-positive vesicles using confocal microscopy. Green vesicles displayed the lowest mobility, being almost immobile near the plasma membrane. Yellow vesicles showed a varied mobility in terms of both speed and direction, and red vesicles were either immobile or moved rapidly in a saltatory manner along straight lines, suggesting their transport along tracks. Previous studies presented apparently contrasting results regarding the mobility of the granules that preferentially undergo exocytosis. In chromaffin cells, the readily releasable pool included mostly immobile granules docked at the plasma membrane (2,10), whereas rapidly moving granules were primarily responsible for the sustained release in neuronal growth cones [4]. Notably, sustained release from neuronal growth cones was induced with Ba^{2+} [16]. These results can be reconciled by assuming that Ba^{2+} elicits the exocytosis of granules that are part of the reserve pool (i.e. older) and, being fairly distant from exocytotic sites, require motor-protein-mediated transport. This scenario is consistent with the evidence that the second phase of insulin secretion from β-cells depends on kinesin-mediated transport [7]. If so, it might be possible that Ba^{2+} somehow promotes kinesin activity and/or the ATP-dependent myosin transport system (see below) by affecting ATP levels or through ATP-dependent pathways. However, it is possible that Ba^{2+} may act on mechanisms other than granule transport.

Concluding remarks

Evidence that newly synthesized endocrine hormones are the first to be secreted already existed [18], but the age-distinct features of granules have never before been revealed in this detail. The preferential exocytosis of newer vesicles might allow cells to adjust their secretory response to changing physiological needs quickly by modifying the granule composition. It is not known, however, how the segregation of granules according to age is accomplished at the molecular level. For example, what mechanism drives the continuous displacement of new granules from the plasma membrane if they have not undergone exocytosis shortly after docking? Several possibilities could be envisioned. The continuous cycling of β_2-syntrophin between conformations that differ in their ability to bind ICA512, even in resting conditions [14], could reversibly modulate the tethering of granules to the cortical cytoskeleton. Cleavage of tethering proteins might also play a role. Vesicular and plasma membrane SNARE proteins, as well as ICA512, can be substrates of μ-calpain, a Ca^{2+}-activated protease that is enriched at the plasma membrane [14,19,20]. The progressive shaving of proteins on the granule cytoplasmic side of the granule membrane might be part of the granule ageing process. The involvement of molecular switches such as small GTPases is also likely. First, because Rab3a and Rab27a are both enriched on granules and interact in their GTP-bound form with granuphilin/Slp4a, which in turn binds syntaxin 1a and its interactor Munc18 [21,22]. In such a way Rab3a and Rab27a might modulate the docking and fusion of granules through SNARE proteins (Fig. 2; see also Ref. [23]). Second, Rab proteins play a role in the targeting of secretory vesicles to membrane fusion sites through the exocyst complex [24].

Rab27a, on the other hand, could also participate in the actin-mediated transport of granules within the cell cortex by recruiting the myosin Va motor protein via granuphilin. A similar transport mechanism has been demonstrated for pigment-containing melanosomes, where surface-bound Rab27a-GTP recruits myosin Va through the granuphilin paralogue melanophilin [25]. There is direct evidence for a myosin Va-dependent transport of granules in PC12 cells, where association of this motor protein to a subpopulation of newly formed granules facilitates their homogeneous distribution in the actin cortex [26].

In conclusion, the use of fluorescent reporter molecules in the last few years has provided us with an unprecedented view of the dynamic complexity of the regulated secretory machinery in neuroendocrine cells. It is predictable that the future of this approach will be even brighter once combined with the down-regulation of individual granule proteins by techniques such as RNA interference.

Acknowledgements
We are grateful to Pietro De Camilli and Christiane Walch-Solimena for the critical reading of this manuscript.

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0962-8924/03/$ - see front matter
doi:10.1016/S0962-8924(03)00150-8