Eight erythroid protein structures resolved in their native multiprotein complex. Sometimes for scientists ground-breaking papers are like buses, after waiting patiently for a breakthrough for a long time, they then arrive at the same time. In this issue of Nature Structural Biology we have not one but two manuscripts containing unprecedented structural detail regarding a key red blood cell (RBC) membrane multiprotein complex.1 Vallese et al.,2 and Xia et al.,3 describe the successful purification of partially intact native RBC Band 3 multiprotein membrane complexes that they then use to capture the structures and interactions of multiple erythrocyte proteins using high-resolution cryo-EM.

Strikingly, across both papers we are presented with 8 new erythroid protein structures (band 3, glycophorin A (GPA), RhAG, RhCE, Aquaporin 1, protein 4.2, ankyrin and glycophorin B (GPB)) in various associated native states2,3. Crucially, these new structures not only enhance our current understanding of RBC structure function in health and disease, but also, they provide a broader interest due to the occurrence of similar types of transporter-cytoskeleton associations that are replicated in other cells of the body by isoforms of the same protein families.

The RBC is the most abundant cell type in the body, and the anion exchanger band 3 (also known as Anion Exchanger 1 or AE1) is the highest expressed protein within its membrane. In the RBC many of the key membrane proteins such as band 3 are found in multiprotein complexes which are corralled and tethered via adaptor proteins to the underlying spectrin-based cytoskeleton4. This ensures that key proteins are more evenly distributed around the red cell surface for efficient gas exchange, and these interactions are essential for providing stability to the membrane. The two most abundant multiprotein complexes are the Band 3-ankyrin multiprotein complex1 and the 4.1R junctional multiprotein complex5. The correct delivery and assembly of these multiprotein complexes are crucial for imparting the recognisable biconcave structure of the RBC. Mutations in ankyrin or band 3 for example, can disrupt these associations and cause Hereditary spherocytosis6 or Southeast Asian Ovalocytosis7. Mutations of band 3 or the associated protein RhAG can cause Hereditary Stomatocytosis due to cation leaks8.
Over many years, scientific research has made considerable progress to improve our understanding of the molecular structure and assembly of these erythrocyte membrane complexes during erythropoiesis. There have been crystal structures e.g. of the band 3 N-terminal domain, the band 3 membrane domain, and also structures predicted through homology modelling e.g. of Rhesus proteins, protein 4.2 and even attempts at the whole complex. The interactions within these complexes have been elucidated from careful studies on red blood cells of individuals with genetic mutations that result in specific erythroid protein deficiencies, and from the painstaking biochemical studies from numerous research groups around the world. Although we have good information about how these proteins interact and associate, we can now finally visualise the individual associations and proteins found in the human erythrocyte band 3 ankyrin multiprotein complex in situ through these two landmark papers in unprecedented detail.

In both papers we are treated to the first full structures of native band 3. Xia et al., provide a 2.8 Å cryo-EM structure of band 3 in the outward binding facing state and this structure fills in the gaps for previously unresolved regions of band 3 in the reported crystal structure (Figure 1). These new structures of band 3 will be a boon for computational scientists and modellers to help further explore how the anion exchanger family proteins transport their substrates. Unexpectedly, cryo-EM structural studies show that the band 3 chaperone protein glycophorin A (GPA) associates with band 3 in the plasma membrane as a monomer rather than the expected dimer. The GPA monomer associates closely with a GPA monomer on a separate band 3 dimers, which may hint at how these complexes assemble or maintain integrity if connectivity is lost to the cytoskeleton.

Both papers describe the anticipated associations of dimeric band 3 with ankyrin but with some differences. Xia et al., describe a dimer of band 3-GPA associated with both protein 4.2 and ankyrin repeats region 6-13 of ankyrin and another dimer of band 3-GPA at ankyrin repeats 17-20 (Figure 1). Vallese et al., identify an additional associated dimer of band 3 and GPA at ankyrin repeat region 21-24, suggesting that each ankyrin can bind three separate dimers rather than the anticipated two dimers (Figure 2). The band 3 dimer associated at the N terminus of ankyrin repeat region is indirectly associated via protein 4.2 in the Vallese et al., complex structure. Whether these differences are due to inherent flexibility of binding associations at the N terminus of ankyrin facilitating multiple conformations and complexes, or produced as an artifact of the isolation method will have to be determined. Importantly though, in support of the former, Vallese et al complement cryo EM structures by providing sub-tomogram average of particles in vesicles generated by extrusion of native erythrocyte membrane.

We are also provided the first full length native structures for the associated adaptor proteins ankyrin and protein 4.2, solidifying pre-existing assumptions based on the partial structures or homology models for these proteins. Vallese et al., go a step
further, providing not only the band 3, ankyrin and protein 4.2 structures in their study, but providing full structures for an associated heterotrimer RhAG and RhCE (consisting of a ration of 2:1 RhAG to RhCE), glycophorin B and also aquaporin 1. With the direct native structural information for RhCE, the authors tantalisingly observe evidence of a channel, suggesting it may be a membrane transporter. These structures also show the interactions between RhAG, GPB and band 3, RhCE and aquaporin 1, and with both ankyrin and protein 4.2. Using these Vallese et al. suggest that the preassembly of the RhAG/CE, band 3 and GPB subcomplex could facilitate recruitment of ankyrin to the overall multiprotein complex by displacement of the ankyrin autoinhibition motif which would otherwise preclude complex assembly.

Intriguingly, no heterotrimer or density was detected for RhD, despite RhD positive blood being used. This may just be explained by heterogeneity within the RhAG-Rh complex and only RhCE containing multiprotein complexes being resolved for unknown reasons. However, deficiency of RhCE in humans results in structural abnormalities in the red blood cell membrane that are not replicated in mice. This is because in mice a proportion of Rh protein is associated with the 4.1 junctional multiprotein complex. Therefore, another explanation is that RhD is either simply not associated with the band 3 ankyrin complex, or alternatively it is associated with the 4.1R junctional complex as observed for mice. Exploration of prospective sub-complexes of RhAG with RhCE and RhD should be explored further in future studies on the human junctional complex.

Although these new structures provided represent a real quantum leap in our depth of understanding of the proteins and interactions within this important complex, there is however more work to be done. There are still proteins that are known to associate but are absent, perhaps because they are more loosely associated or found in other complexes that were not isolated. These missing proteins, CD47, and LW (ICAM4), comprise the remaining proteins of the Rhesus subcomplex. Structural information regarding the native complex environment of the marker of self CD47 with established dependencies upon protein 4.2 and RhCE proteins resolved by Vallese et al. remains elusive.

In summary, the abundant erythroid protein structures reported by Vallese et al. and Xia et al. combined provide an unprecedented structural understanding of a vital erythrocyte multiprotein complex important for gas exchange and which imparts membrane flexibility in humans. These papers provide confirmation to previous studies, fill in the gaps and provide finer details about the individual protein structures enhancing the current depth of understanding of how these important proteins likely interact with one another in the RBC membrane.

These data will eventually lead to an improved mechanistic understanding of how the specialised membrane of the RBC is built and also how the red cell adapts in the different environments to which it is exposed as it traverses the circulation. The possibility of application of the approaches used here to purify erythroid membrane complexes can be used in future studies to study rare patients' RBC known to lack
specific protein components due to genetic mutations is exciting. This would provide further understanding to how the complexes are assembled and elucidate how the cell adapts to specific protein deficiencies in human disease. Finally, we know that RBCs change the associations within the complexes to alter the cell shape or to enable the cell to deform and that phosphorylation can impart some of these changes. Therefore, an exploration of the structures obtained from RBCs exposed to different experimental conditions such as during oxygen/deoxygenation or after inducing phosphorylation will be enormously informative.

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Competing interests
The author declares no competing interests.

Figures and Legends

Figure 1: A cryo-EM map of domain organisations of band 3 dimer, protein 4.2 and ankyrin. Figure adapted from Xia et al.,

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Figure 2. Cryo-EM density map of a B3 ankyrin complex A) containing three band 3 dimers (band 3 I, band 3 II, and band 3 III), glycophorin A, glycophorin B, a heterotrimer of RhAG and RhCe, aquaporin 1, protein 4.2 and ankyrin. B) showing Band 3I, Glycophorin A, Rh Complex, ankyrin and protein 4.2). Figure adapted from Vallese et al.,2.

References
