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**Journal Club**

Michalski K, Syrjanen JL, Henze E, Kumpf J, Furukawa H, Kawate T. (2020). The Cryo-EM structure of pannexin 1 reveals unique motifs for ion selection and inhibition. Elife. 9:e54670. doi: 10.7554/eLife.54670.

Deng Z, He Z, Maksaev G, Bitter RM, Rau M, Fitzpatrick JAJ, Yuan P. (2020). Cryo-EM structures of the ATP release channel pannexin 1. Nat Struct Mol Biol. 27(4):373-381. doi: 10.1038/s41594-020-0401-0.

Ruan Z, Orozco IJ, Du J, Lü W. (2020). Structures of human pannexin 1 reveal ion pathways and mechanism of gating. Nature. doi: 10.1038/s41586-020-2357-y. Online ahead of print.

**Article Summary**

Pannexins are anion-selective channels which can mediate release of ATP from the cell, making them important modulators of purinergic signalling, particularly with respect to P2X7 receptor activation. Michalski *et al.* (2020), Deng *et al.* (2020) and Ruan *et al.* (2020) report the 3D-structures of *Xenopus tropicalis* and/or human pannexin-1 (PanX1) using single particle cryo-electron microscopy (cryoEM). PanX1 is shown to form a heptameric channel resembling a truncated cone, with a central pore of minimum diameter approx. 9Å in the extracellular domain, large enough to permit the passage of ATP. The extracellular constriction presents a ring of tryptophan residues forming π-cation interactions with arginine residues in adjacent subunits, which has an important role in governing anion selectivity. Each subunit possesses 4 transmembrane domains (TMs), with an intracellular C-terminal domain, the final 50 amino-acids of which form an unstructured C-terminal tail (CTT). Ruan *et al.* (2020) provide direct structural evidence that in the resting state, the CTT blocks the channel pore, and that seven ‘side channels’ in the intracellular domain permit the conduction of small anions (but not ATP), further demonstrating that CTT cleavage removes the pore blockage, rendering the channel constitutively open to ATP. While Michalski *et al.* (2020) used mutagenesis and functional assay to propose an allosteric site for the antagonist

carbenoxolone (CBX) in the extracellular domain, Ruan *et al.* (2020) solved a structure of human PanX1 bound to CBX which showed additional density in the pore at the site of the extracellular constriction, strongly suggesting pore blockade as the mechanism of CBX antagonism. These structures represent a transformative advance in our understanding of pannexin structure, ion selectivity, gating and antagonism, and further demonstrate the power and applicability of cryoEM as a structural tool for ion channel proteins.

**Commentary**

Pannexins are a family of three non-selective anion channels in humans (PanX1, PanX2 and PanX3), proposed to play roles in nucleotide release (particularly during apoptosis), the regulation of blood pressure, neuropathic pain, cancer progression and oocyte development (see references in Michalski *et al.* (2020), Deng *et al.* (2020) and Ruan *et al.* (2020)). Pannexins can be activated by multiple stimuli including changes in membrane potential, shear stress, elevated extracellular potassium, and interactions with other proteins (e.g. caspases) (Dahl G (2018) FEBS Lett. 592:3201-9). The physiological importance of pannexins makes them key drug targets, but the fundamental understanding of their structure-function relationship has been hampered by a lack of high-resolution structural information, compounded by a lack of substantial amino-acid sequence homology to other proteins thought to be in a similar structural family (e.g. connexins).

Single particle cryoEM has revolutionised structural biology, in particularly the study of membrane protein structure, often permitting the study of wild-type proteins in a lipid-containing environment, and not requiring either the production of tens of milligrams of protein or the formation of 3D-crystals (Cheng Y (2018) Science 361:876-80). Michalski *et al.* (2020), Deng *et al.* (2020) and Ruan *et al.* (2020) have each used this technique to determine the 3D-structure of the 428 amino-acid PanX1 channel, either from *Xenopus*

*tropicalis* (Michalski *et al.* (2020), Deng *et al.* (2020)) or human (Deng *et al.* (2020) and Ruan *et al.* (2020)). It is an indication of the competitive nature of the research field that three additional papers reporting cryoEM structures of human PanX1 were recently presented back-to-back in the May edition of Cell Research (Qu *et al* (2020) Cell Res. 30: 446-448, Jin *et al* (2020) Cell Res. 30: 449-451 and Mou *et al* (2020) Cell Res. 30: 452-454), and so a wealth of structural information has been provided in a relatively short time. The methodology used in each of the six papers was subtly different; constructs, expression systems, purification procedures and data collection/analysis methods vary, and the final resolution of each structure is different, but all report the same key findings.

Each PanX1 monomer is composed of a bundle of 4 TM helices, with TM1 lining the pore, two extracellular loops (ECL1 and 2; composed of a mixture of α-helix and β-strands) and largely helical intracellular loop (ICL) and C-terminal domains. The overall protomer has structural homology with other large pore forming channels such as connexins and innexins. The biological unit is an inverted cone-shaped heptamer narrower in the extracellular domain than the intracellular domain. This is contrary to previous low-resolution studies which suggested a hexameric arrangement similar to connexins. The channel pore is relatively wide, with its narrowest point (approx. 9Å diameter) positioned in the extracellular region and lined by tryptophan and arginine residues (Trp-74 and Arg-75). The channel pore widens to a diameter of >30Å in the intracellular domain, and the intracellular CTT region is unstructured.

Michalski *et al.* (2020) solved the structure of a truncated *Xenopus tropicalis* PanX1 (XtPanX1) at a resolution of 3.0Å. XtPanX1 (which is 66% identical to human PanX1 at the amino-acid level) was selected in an orthologue screen according to its enhanced expression and stability, and both the CTT and part of the ICL between TM2 and TM3 were removed, which gave rise to a receptor with enhanced channel currents compared to the wild-type. The

structure lacked information for regions in ECL1 (residues 88-100) and the ICL (157-194), and the first 10 amino-acids of the N-terminal domain. A region of the N-terminal domain (the N-terminal loop) was shown to contribute to a constriction of the channel pore (approx. 16Å diameter) in the intracellular domain. The authors demonstrated that mutation of the Trp-74 and Arg-75 residues in ECL1 (the residues that form the most constricted part of the channel pore) substantially affected ion selectivity, and concluded that maintenance of a π-cation interaction between the two residues in adjacent subunits was the key determinant. Michalski *et al.* (2020) hypothesised that the mechanism of action of CBX was allosteric, and proposed a putative binding site in the hydrophobic groove between the first and second extracellular loops (ECL1/2), demonstrating that mutations within this region affected the ability of CBX to antagonise the channel.

Deng *et al.* (2020) solved the structures of full-length XtPanX1 (resolution 3.4Å) and human PanX1 (resolution 3.7Å). XtPanX1 was selected as most stable in an orthologue screen, and although the wild-type construct was used, no density for the N-terminal domain or CTT was observed in the structure. Additionally, no density was observed in ECL1 (88-102) and the ICL (181-195), similar to Michalski *et al.* (2020). Deng *et al.* (2020) also demonstrated the π-cation interaction between Trp-74 and Arg-75 in adjacent subunits, additionally showing a salt bridge between Arg-75 and Asp-81 in adjacent subunits which they proposed would stabilise the configuration of the tryptophan ring. Within the transmembrane domains, extensive interactions with lipids were preserved, which may play a role in enhancing subunit-subunit contact in the membrane region. Compared to the XtPanX1 structure, the human PanX1 structure had the same overall heptameric architecture, but the C-terminal region was disordered and could not be resolved.

Ruan *et al.* (2020) solved a number of human PanX1 structures in different conditions, including the wild-type *apo*-state (2.8Å resolution), with the CTT cleaved (3.0Å

resolution), with CBX bound (4.4Å resolution) and with the N255A mutation which permits 'gap junction-like structure' formation (2.8Å resolution). The authors were able to model most of the structure at high resolution except for the ICL region (163–190) and the CTT (residues 374–426). At low resolution Ruan *et al.* (2020) were able to observe asymmetric density occluding the channel. This additional density (along with the asymmetry) was absent in their CTT-cleaved construct, suggesting that the CTT plugs the pore in the resting state, rendering the channel unable to transport ATP. In agreement with Deng *et al.* (2020), the π-cation interaction between Trp-74 and Arg-75 in adjacent subunits and the salt bridge between Arg-75 and Asp-81 were observed, along with extensive lipid interactions, including at the transmembrane subunit interfaces. PanX1 is thought to be unable to form gap junctions (head-to-head interactions) due to N-linked glycosylation at Asn-255. By mutating Asn-255 to Ala, Ruan *et al.* (2020) were able to prepare and resolve human PanX1 gap junction-like structures, demonstrating that a head-to-head interaction can form in the absence of glycosylation (although this may not be of immediate physiological relevance) and that these interactions are mediated by residues in ECL2. In the CBX-bound structure (with the caveat of the substantially lower resolution of 4.4Å), additional density was observed in the extracellular region of the pore adjacent to Trp-74, which was consistent with the presence of CBX blocking the pore (although the authors were unable to confirm this unambiguously). From this Ruan *et al.* (2020) propose that CBX may antagonise PanX1 by channel block, contrary to the allosteric mechanism proposed by Michalski *et al.* (2020). Additional density in the channel pore of human PanX1 (attributed to CBX binding and blocking the pore) was also observed by Jin *et al* in a structure solved at 4.6 Å resolution, although it was again not possible to attribute this unambiguously to CBX (Jin *et al.* (2020) Cell Res. 30: 449-451).

At the protomer level, the structure of hPanX1 solved by Ruan *et al.* (2020) exhibits a substantial difference in that the N-terminal domain forms an additional helix which lines the

channel pore, extending towards the extracellular face of the transmembrane domain. This region is either not observed or located in the cytoplasmic domain in the structures solved by Michalski *et al.* (2020), Deng *et al.* (2020) or Qu *et al* (3.2Å resolution; Qu *et al* (2020) Cell Res. 30: 446-448). Ruan *et al.* (2020) did not find any evidence to suggest that the N-terminal helix was involved in channel function, but suggested that its role was structural, helping to maintain a rigid transmembrane domain and large pore. Strikingly, the linker between the N-terminal helix and TM1 was shown to form small 'side channels', which were demonstrated to be accessible to water in molecular modelling studies. Modifying the size of the channels by mutagenesis greatly affected channel function; when the channels were constricted, current density in whole-cell recordings was markedly reduced, but when the channels were expanded (or the linker region was removed) current density significantly increased above wild-type levels. This suggested that the side channels could serve as conduction pathways for small ions (e.g. chloride) when the large pore is blocked with the CTT, perhaps explaining why PanX1 is chloride-permeable under certain conditions (e.g. following stimulation with positive membrane potential).

Taken together these studies convincingly demonstrate the heptameric architecture of PanX1, show that ion selectivity is largely governed by Trp-74, Arg-75 and Asp-81 in the extracellular pore region, and that (in the absence of the CTT) the pore is wide enough to permit the passage of ATP. They also provide a mechanism whereby cleavage of the CTT renders the channel constitutively open. Several open questions remain, however. Why is it that in most PanX1 structures the N-terminal helix is not positioned inside the channel pore? Could this be due to the resolution of the structures, or might there be more than one possible conformation of the N-terminal domain? What is the mechanism of CBX antagonism – is it allosteric antagonism or pore block? Higher resolution CBX-bound structures will be required to demonstrate conclusively that the additional density in the pore is indeed CBX,

and it may be that CBX is capable of binding both at an allosteric site and within the pore itself. Finally, how do other activation mechanisms (e.g. shear stress or elevated extracellular potassium) alter the conformation of the CTT to permit ATP release? Nonetheless, a substantial array of PanX1 3D-structures is now available, and these will act as templates for molecular modelling, docking and interpretation of structure-function experiments, greatly stimulating research on this fascinating and enigmatic ion channel family.