Yeast cell wall integrity (CWI) signaling serves as a model of the regulation of fungal cell wall synthesis and provides the basis for the development of antifungal drugs. A set of five membrane-spanning sensors (Wsc1 to Wsc3, Mid2, and Mtl1) detect cell surface stress and commence the signaling pathway upon perturbations of either the cell wall structure or the plasma membrane. We here summarize the latest advances in the structure/function relationship primarily of the Wsc1 sensor and critically review the evidence that it acts as a mechanosensor. The relevance and physiological significance of the information obtained for the function of the other CWI sensors, as well as expected future developments, are discussed.

**SENSOR STRUCTURES AND MECHANICAL PROPERTIES**

The five CWI sensors of *S. cerevisiae* can be divided into two small protein families, namely, (i) Wsc1 to Wsc3 and (ii) Mid2 and Mtl1 (Fig. 2). It should be noted that a similar protein, Wsc4, does not reside at the plasma membrane and thus is not a CWI sensor. The two families differ in the amino-terminal head group, which is presumed to physically connect with cell wall polysaccharides and/or proteins (13). In Wsc-type sensors, the head group is composed of a region comprising eight cysteine residues (CRD for cysteine-rich domain, also referred to as a WSC domain) that has features reminiscent of a lectin binding domain and is presumed to be in contact with the cell wall glucans (14, 15). The two Mid-type sensors each carry an N-glycosylated asparagine residue in a similar position, which is required for sensor function (16), and can be imagined to be intertwined with the glucan network or the mannoproteins in the outer layer (Fig. 2). The rest of the overall structures of all five sensors appear similar, in that a serine/threonine-rich (STR) region forms a spring-like structure that extends into the cell wall and a single transmembrane domain provides a second anchor for the sensors besides the head groups described above. Cytoplasmic tails of various lengths provide the link to the downstream components of the CWI signaling pathway, first by interacting with the GDP/GTP exchange factor Rom2 (17, 18). The cytoplasmic tails also determine the primary distribution of the sensors within the plasma membrane, as shown by fluorescence microscopy of hybrid Wsc1/Mid2 sensors (19).

A major breakthrough in the determination of sensor functions came with the investigation of single Wsc1 sensors by atomic-force microscopy (AFM) (20), a technique that is increasingly used in microbiology (21). In brief, the CWI sensors are too short to reach the cell surface to be detected by AFM (22). Therefore, the Wsc1 sensor was elongated by adding the STR sequence of Mid2,
of yeast bud scars, where the cell wall is considerably thinner (20).

In formational activate the CWI pathway. Although such a scenario seems logical, it has yet to be supported by direct experimental evidence of mechanoreception.

**SENSOR DISTRIBUTION AND FUNCTION**

Another interesting feature deduced from the single-molecule AFM data was that the modified Wsc1 sensor was not distributed evenly at the yeast cell surface but rather was gathered in patches with an approximate diameter of 200 nm (Fig. 3C) (26). Both sensor density and patch sizes increased substantially upon heat and osmotic stress, indicating that sensor clustering may be related to CWI signaling capacity. Support for such a correlation between sensor abundance and cell wall synthesis also came from studies of mutants in which the rapid turnover of Wsc1 sensors by endocytosis was blocked. Thus, mutants with defects in clathrin-mediated endocytosis displayed a drastically increased Wsc1 concentration in the lateral plasma membrane, which led to a thicker cell wall, as judged from images generated by transmission electron microscopy (27). This was also confirmed by *in vivo* AFM studies of sensors lacking the endocytosis signal (22). The Wsc1 sensor accumulates at sites of polar cell growth, such as the bud tip and at the bud neck prior to cell division, which has been observed in fluorescence microscopy studies (19, 28). A similar distribution was also observed for the Wsc2 sensor, the lack of which, contrary to previous reports on its minor role in CWI signaling, reduced the fitness of yeast cells in growth competition experiments when they were mixed with wild-type cells (29). More importantly, green fluorescent protein (GFP)-tagged versions of the two Wsc-type sensors displayed a patchy distribution in the lateral plasma membrane in live-cell fluorescence microscopy, reminiscent of the patches observed in the AFM experiments described above. Although the size and stability of the Wsc1 patches are also reminiscent of yeast eisosomes (30), they do not colocalize (our unpublished results).

In the context of sensor clustering, single-molecule AFM studies on Wsc1 yielded another interesting observation. Mutants with cysteine-to-alanine changes in the CRD head group completely lost the ability to cluster at the cell surface (26). This phenotype could be copied by adding dithiothreitol as a reducing agent to cells carrying the modified wild-type sensor (31). Thus, the structure of the CRD, which presumably is conferred by disulfide bridges, is a prerequisite for its ability to mediate clustering. Regarding its *in vivo* performance, the cysteine-to-alanine mutants displayed growth phenotypes similar to those caused by complete wsc1 deletions, indicating that sensor function is lost concomitantly with the ability to form clusters. In fact, it was previously observed that truncated Wsc1 proteins lacking the entire CRD region also lacked their signaling capacity, which could be restored by overproduction of the truncated sensors (32). We suggest that this could be due to the increased sensor density in the membrane, which may mimic clustering.

As stated above, we also suggest that the double-anchored polypeptide chain of Wsc1 forms a crucial part of a sensor by which mechanical stress at the cell surface is detected by stretching of the nanospring. This leads to conformational changes in the CRD, stabilized by disulfide bridges, and triggers both sensor clustering and conformational changes in the cytoplasmic domain, which then interacts with the downstream CWI components. The assembly of sensors in plasma membrane microdomains that recruit intracellular signaling components thus leads to the formation of a Wsc1 sensosome and enhances CWI signaling capacity (33).
The similarity of the overall structures of the five CWI sensors described above suggests that the data obtained for Wsc1 also apply to the other sensors. Indeed, AFM revealed nanospring properties similar to those of Wsc1 in a modified Mid2 sensor (34). However, one major difference between Wsc- and Mid-type sensors is the nature of their head group, i.e., CRD in the former and an N-glycosylated asparagine in the latter. Indeed, using high-resolution AFM imaging, clustering could not be observed for an elongated, His-tagged Mid2 sensor, either under normal growth conditions or under heat or osmotic stress, indicating that, at least during vegetative growth, Mid2 does not form sensosomes (Fig. 3D) (34). This observation is consistent with live-cell fluorescence imaging, where Mid2-GFP shows a more uniform distribution in a dense, network-like structure, rather than forming patches (35). In high-resolution AFM images, Mid2 appeared to be three times as abundant in bud scars as on the lateral cell surface, probably reflecting a greater chance of detection because of the thinner cell wall in this region (34).

Because of the severity of the growth defects in different sensor deletion mutants, Wsc1 and Mid2 have been suggested to apply to the bulk of CWI signaling, with partially overlapping functions (13,14). However, the differential responses of wsc1- and mid2-null mutants to different stress agents in serial-dilution assays and in transcriptome analyses suggest that the sensors evolved to specialize in different responses (19, 36). This may not be closely correlated with their membrane distribution, since switching of the Wsc1 endocytosis signal to a Mid2 sensor resulted in its untypical accumulation at sites of polar growth (similar to that of Wsc1) but could not suppress the growth defects of a wsc1 deletion (29). Alternatively, with regard to the mechanosensor hypothesis, the differential responses of the sensors to different stress agents could be mediated by probing different points in the lateral cell wall. Thus, the STR domains of the five sensors vary in length, so that their head groups would be anchored at corresponding distances from the plasma membrane (Fig. 2)(12). Different stress agents could affect the different layers of the cell wall to various degrees and thereby act primarily on a specific sensor. This would then trigger the appropriate adaptive physiological responses.

Whether the distribution of the sensors in specific microdomains within the plasma membrane (see above) also contributes to the differential response remains to be elucidated. It should be mentioned that Mtl1 has been proposed to serve a function in the oxidative-stress response (18, 37). Recently, it has been suggested to work in conjunction with Wsc1 or Mid2 to regulate the nuclear/cytoplasmic distribution of cyclin C and thereby programmed cell death in yeast (38). If and how these functions are related to the presumed mechanosensing properties of the sensors remain to be determined. In this context, comparative studies with other yeast species may be of value. Thus, in K.
lactis, a closely related yeast that has not undergone a whole-genome duplication (11), only three CWI sensors have been identified, excluding an Mtl1 homolog (39). Since CWI signaling functions seem to be similar in K. lactis and S. cerevisiae (8), it would be interesting to see which of the remaining sensors is involved in the oxidative-stress response of the milk yeast. In the more distantly related yeast Schizosaccharomyces pombe, Wsc1 and Mid2 homologs have also been characterized (with the latter designated SpMtl2), but they did not trigger the CWI pathway (40). It has also been suggested that Wsc1 has a role in biofilm formation in a certain S. cerevisiae strain independent from the downstream CWI signaling pathway (41). Thus, mechanosensing may trigger not-yet-anticipated cellular responses in addition to cell wall remodeling.

**QUO VADIS?**

Despite the progress made in recent years regarding the functional characterization of CWI sensors as summarized above, the most pertinent question to be addressed is if they indeed react to mechanical cues at the yeast cell surface. The AFM tools developed so far to pull on specific sensor molecules could be exploited to this end. In combination with live-cell fluorescence microscopy and superresolution imaging techniques (42), one can design the appropriate experimental setup. A broad AFM tip modified with Ni\(^{2+}\)-NTA could be employed to probe the surface of a yeast cell carrying the elongated, His-tagged Wsc1 sensor. Pulling on several sensor molecules at a time and following the distribution of GFP-tagged downstream components of the CWI pathway would provide conclusive evidence of the formation of sensosomes. Alternatively, the AFM tip could be modified with an antibody and used in conjunction with a sensor tagged accordingly, in order to enhance the strength of the interaction.

It should also be noted that similar AFM approaches could be applied to study any cell surface proteins that can be elongated to penetrate the yeast cell wall (e.g., the pheromone receptors) and also for the investigation of basically any cell surface protein in other organisms (33).

Finally, apart from the interesting questions of exactly how the sensors function and if there is mechanosensing in yeast cells, there are also two applied aspects to this research. (i) The data available so far suggest that the nanospring properties of the sensors are essential for their signaling function. These mechanical features are conferred by the highly mannosylated STR region and probably also by the anchoring of the head group in the cell wall. Thus, drugs interfering with either protein mannosylation or specific inhibitors of the CRD interactions within the Wsc-type sensors would be expected to impair cellular integrity, leading to cell lysis. In fact, mutants defective in O-mannosylation have been shown to have cell lysis defects (43). Expressing CWI sensors of pathogenic fungi, preferably in a deletion mutant of S. cerevisiae lacking its endogenous sensor genes, should allow screening for such specific drugs. (ii) We already elongated the Wsc1 and Mid2 sensors to reach through the cell wall in order to be detectable by...
single-molecule AFM (23, 34). Instead of displaying a His tag, similar constructs could be used to display specific antigens on the yeast cell surface, e.g., to trigger the immune system to attack and destroy tumor cells, as already reported for other yeast surface display systems (44, 45). The use of the CWI sensors for surface display would have the advantage that the antigens remain tightly attached to the yeast cells since they form one polypeptide chain with the downstream transmembrane domain.

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