Unlike stomatal development, cell rearrangements play an important part, so it is unclear whether polarity switching would provide a robust mechanism for patterning in these cases. Thus, although sharing many elements with animal systems, the fixed nature of plant cells may lead to distinctions in the way cells pattern themselves in dynamically changing tissues.

References and Notes
19. BASSL protein becomes localized in the cell periphery at a position that minimizes the inverse distance from all points along the new division boundaries. See equation in the modeling section of the supporting online material.

X-ROS Signaling: Rapid Mechano-Chemo Transduction in Heart
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We report that in heart cells, physiologic stretch rapidly activates reduced-form nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) to produce reactive oxygen species (ROS) in a process dependent on microtubules (X-ROS signaling). ROS production occurs in the sarcolemmal and t-tubule membranes where NOX2 is located and sensitizes nearby ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR). This triggers a burst of Ca2+ sparks, the elementary Ca2+ release events in heart. Although this stretch-dependent “tuning” of RyRs increases Ca2+ signaling sensitivity in healthy cardiomyocytes, in disease it enables Ca2+ sparks to trigger arrhythmogenic Ca2+ waves. In the mouse model of Duchenne muscular dystrophy, hyperactive X-ROS signaling contributes to cardiomyopathy through aberrant Ca2+ release from the SR. X-ROS signaling thus provides a mechanistic explanation for the mechanotransduction of Ca2+ release in the heart and offers new therapeutic possibilities.

In the heart, mechanical stretch during diastolic filling activates mechanotransduction signaling pathways that have broad implications for cardiac health and disease (1, 2). A small diastolic stretch (8%) (3) of a ventricular myocyte causes a burst of Ca2+ sparks (4), the elementary events corresponding to the release of free Ca2+ from intracellular stores (Fig. 1, A to C) (5, 6). The mechanism by which this occurs has remained elusive. To address this question, we created tools to improve investigation of single-cell function (Fig. 1A, fig. S2, and movies S1 and S2) (4, 7, 8). We firmly attached single myocytes to stiff glass micro-riods with a biological adhesive (MyoTak) (8). This allows precise control of cell length and the measurement of isometric force, thus permitting us to examine the details of stretch-dependent signal transduction. Such work reveals that stretch-activated Ca2+ sparks are triggered by a mechano-chemo signaling pathway that regulates local production of reactive oxygen species (ROS) in heart cells.

Stretching of myocytes initiated a burst of Ca2+ sparks that was rapid in onset (milliseconds) and large in magnitude (a nearly twofold increase) (Fig. 1C). Although diastolic stretch in normal myocytes produces the spark burst, under pathological conditions the identical stretch generates transient increases in the intracellular concentration of free Ca2+ ([Ca2+]i) that propagate as waves through the cell (Ca2+ waves). Such conditions include Ca2+ overload of the sarcoplasmic reticulum (SR) (Fig. 1D) and pharmacological sensitization of ryanodine receptors (RyR2s) (fig. S3). Stretch-activated sparks are not influenced by stretch-activated channels, Ca2+ influx, Na+ influx, nitric oxide signaling (9), nor acute stretch-induced increases in SR [Ca2+]i (9). To explore how stretch activates Ca2+ signals under normal and pathological conditions, we examined cardiomyocytes from the stretch-sensitive muscular disorder, Duchenne muscular dystrophy (DMD). The frequency of Ca2+ waves produced by cellular stretch was increased in cardiomyocytes from the mouse model of DMD, the mdx mouse, compared with wild-type (WT) cells (Fig. 1, E and F). Recent reports that ROS-dependent nitrosylation (10) and oxidation (11, 12) of RyR2s contribute to aberrant Ca2+ release from the SR and arrhythmia in mdx myocytes encouraged us to examine ROS signaling.

ROS and reactive nitrogen species (RNS) react with cysteine residues on RyR2 to rapidly and reversibly modulate RyR2 [Ca2+] sensitivity (10, 13–15). Application of the antioxidant N-acetylcysteine (NAC) to healthy myocytes blocked the stretch-induced burst of Ca2+ sparks (Fig. 2A), suggesting that stretch-dependent oxidation of RyR2s (11, 14, 15) may underlie the increase in spark frequency. To assay whether such a mechanism could account for the rapid changes in Ca2+ spark rate observed (Fig. 1C), we superfused cells with 200 μM H2O2 and assayed spark activity (fig. S4A). Superfusion of H2O2 reversibly increased Ca2+ spark rate, with kinetics and magnitude similar to that observed with physiologic stretch (fig. S4, A to C), confirming that an oxidative mechanism can account for rapid and reversible regulation of RyR2 [Ca2+] sensitivity during stretch.

Because NAC indiscriminately scavenges all ROS and reactive nitrogen species (RNS), we sought to identify the specific source of RyR2 oxidation. The reduced-form nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family of enzymes represents a major source of ROS in the cardiovascular system (16) and has a central role in the pathology of DMD (12, 17) and heart disease (18). Unlike other ROS sources (for example, xanthine oxidases,
the mitochondrial electron transport chain, or uncoupled nitric oxide synthases), NOX generates superoxide (O$_2^-$) in a highly regulated manner, which is ideal for a role in cell-signaling cascades (16). Furthermore, NOX-dependent ROS activates RyR1 in skeletal muscle in vitro (19). Therefore, we tested for a potential role of NOX by applying the inhibitor diphenyleneiodonium (DPI). DPI blocked the stretch-dependent burst of Ca$^{2+}$ sparks (Fig. 2B), which is consistent with the hypothesis that physiologic stretch triggers rapid production of ROS by NOX.

To test this hypothesis, we measured ROS generation during stretch of ventricular myocytes with the fluorescent ROS sensor 2',7'-dichlorofluorescein diacetate (DCF). The rate of change (slope) of the DCF fluorescence signal reports the rate of ROS generation within the cell (Fig. S5). An 8% stretch produces an immediate increase in DCF slope. Upon return to the original cell length, the DCF slope rapidly returns to the prestretch level (Fig. 2C, black trace). The derivative (Fig. 2C, red trace) of a polynomial fit [Fig. 2C, green trace; correlation coefficient ($r^2 = 0.96$)] to the DCF data reveals that ROS production is increased immediately upon stretch, gradually decreases during the prolonged stretch duration, and returns to its prestretch level upon relaxation (Fig. 2C). This correlates with the time course of stretch-dependent Ca$^{2+}$ sparks depicted in Fig. 2B and Fig. S5C. Treatment of cells with DPI fully blocked the stretch-induced increase in ROS production (Fig. 2, D and E), suggesting that NOX activation underlies stretch-dependent ROS production. However, DPI also inhibits mitochondrial ROS production (20). Mitochondrial oxidative metabolism results in constant ROS production, which is consistent with the mitochondrial-like pattern of DCF fluorescence in a resting cell (Fig. S6). We therefore uncoupled mitochondrial metabolism and subsequent ROS production by applying the proton ionophore carbonyl cyanide 3-chlorophenylhydrazine (CCCP). The strong mitochondrial fluorescence pattern with DCF was lost after CCCP application, but the stretch-dependent ROS burst and stretch-dependent Ca$^{2+}$ sparks were maintained (Fig. S6, C to E). Thus, mitochondria appear not to contribute to stretch-induced ROS production and resulting Ca$^{2+}$ sparks.

Along with NOX, microtubules represent a second component required for the stretch-dependent signaling pathway (4). We confirmed that disrupting microtubule integrity blocks the

Fig. 1. Stretch-activated Ca$^{2+}$ release in healthy and dystrophic cardiac myocytes. (A) Single rat ventricular myocyte attached to stiff glass micro- 
rods coated with MyoTak (8). (B) Fluorescent surface plot of stretch-activated Ca$^{2+}$ sparks in myocyte subject to 8% axial stretch. (C) (Top) Average 
positional output from piezo-electric length controller upon 8% diastolic stretch, which imposes a sarcomere length change of 1.84 ± 0.02 to 1.99 ± 0.03 μm. (Bottom) Ca$^{2+}$ spark histogram (500ms bins) before (black), during (red), and after (blue) stretch (n = 52 rat cells). (D) Frequency of 
Ca$^{2+}$ waves before and during stretch, under control conditions (1.8 mM [Ca$^{2+}$]o, n = 52 rat cells) and under conditions of Ca$^{2+}$ overload (5 mM [Ca$^{2+}$]o, n = 10 rat cells). (E) Fluorescent surface plot demonstrating stretch-induced Ca$^{2+}$ wave in dystrophic (mdx) myocyte. (F) Frequency of 
Ca$^{2+}$ waves in mdx myocytes (n = 38 cells) compared with WT myocytes (n = 25 cells) before and during stretch. *P < 0.05 compared with rest value. 
#P < 0.05 compared with control conditions value (D) or WT value (F); paired t test.
stretch-activated burst of Ca\(^{2+}\) sparks in both rat and mouse myocytes (fig. S7). If microtubules contribute to the activation of NOX, then disrupting microtubules should also block the stretch-dependent production of ROS. Treatment of cells with colchicine to depolymerize microtubules fully blocked the stretch-induced increase in DCF fluorescence, indicating that mechanotransduction through microtubules is required for stretch-dependent ROS production (Fig. 2, D and E). When stretch-dependent ROS production was inhibited, stretch often produced a slight decrease in the rate of ROS production, suggesting that in the absence of this pathway stretch may uncouple some baseline ROS production (Figs. 2D, 3B, and 4A).

NOX2 and NOX4 are the most abundantly expressed isoforms of NOX in adult cardiomyocytes (21). Membrane-bound NOX2 (also known as gp91\(^{phox}\)) requires association with cytosolic regulatory subunits, including p47\(^{phox}\), to produce O\(_2^-\), whereas NOX4 does not (22). Therefore, to examine the role of NOX2, we used a specific peptide inhibitor (gp91ds-tat) that prevents the interaction of gp91\(^{phox}\) with p47\(^{phox}\) (23). Inhibition of NOX2 by gp91ds-tat blocked stretch-dependent ROS production in a dose-dependent manner, and a control, scrambled peptide had no effect (Fig. 3A). To further confirm NOX2 as a molecular source of stretch-induced ROS production, we measured DCF signals in myocytes from NOX2\(^{-/-}\) mice. Myocytes lacking NOX2 expression did not show stretch-dependent production of ROS (Fig. 3, B and C). Recruitment of the small guanosine triphosphatases rac1 or rac2 to the NOX2 complex is also required for enzyme activation (16, 22). Specific inhibition of rac1, which binds microtubules (24), blocked stretch-induced ROS production (Fig. 3A).

For NOX2 to produce a ROS signal that rapidly influences Ca\(^{2+}\) release, we expected it to be located very close to the junctional SR (jSR) that contains the RyR2 clusters. To test this, we used the fluorescently labeled 6-carboxyfluorescein (FAM)-gp91ds-tat. FAM-gp91ds labeled the sarcolemma and intercalated disc structures at the periphery of myocytes and colocalized with t-tubule structures that penetrate the interior of the myocyte at the Z-line, which is in agreement with previous findings (Fig. 3D and fig. S8A) (25). In contrast, FAM-gp91ds showed no such Z-line localization in NOX2\(^{-/-}\) myocytes, confirming the specificity of this signal (Fig. 3E and fig. S8B). Co-localization analysis indicated that approximately 70% of the FAM-gp91ds signal from the interior of myocytes colocalized with the t-tubule marker Di-D (fig. S8D). NOX2 regulatory subunits also must be readily available to promote rapid signaling, which is supported by the preferential expression of heteromultimeric subunit complexes in t-tubule fractions (fig. S1) (19, 24).

If the ROS are produced locally at t-tubule structures and act rapidly, the measured increase in ROS should only occur in the stretched region of the cell because ROS are short-lived species typically confined to action within their immediate vicinity. ROS were measured with confocal line scans through the interior of myocytes (8 to 12 \(\mu\)m from the sarcolemma), in both the stretched (Fig. 3D, yellow dotted line) and nonstretched (within 15 \(\mu\)m of intercalated disc structures) (Fig. 3D, blue dotted line) regions of cells. No increase in ROS production was detected in the nonstretched region of the cell (Fig. 3A). Consistently, Ca\(^{2+}\) spark rate is unchanged in the nonstretched portion of a stretched cardiomyocyte (4). These findings suggest that the ROS responsible for oxidation of RyR2 are produced locally and probably do not arise from sarcolemma or intercalated disc sources. Taken together, the above implicates NOX2 as a source of rapid stretch-dependent ROS production at the jSR of cardiomyocytes (X-ROS signaling).

To assay whether X-ROS signaling regulates intracellular Ca\(^{2+}\) release upon electrical stimulation, we modified a protocol used by Santana et al. (1996) to evaluate the activity of individual Ca\(^{2+}\) release units (CRUs) during depolarization of a myocyte (26). A CRU consists of a cluster of RyR2s in the jSR that produce a Ca\(^{2+}\) spark, which summate during electrical stimulation to produce the global increase in [Ca\(^{2+}\)] (5). A low concentration of nifedipine blocks a large portion of the Ca\(^{2+}\) influx that

![Fig. 2. ROS generation and microtubule integrity underlie stretch-activated Ca\(^{2+}\) sparks.](https://www.sciencemag.org/content/333/6037/9249/F1.large.jpg)
enters the cell through voltage-gated L-type Ca\(^{2+}\) channels during depolarization. This approach reduces the number of CRUs triggered by Ca\(^{2+}\)-induced Ca\(^{2+}\) release, thus enabling the visualization and quantification of fluorescence signals arising from individual CRUs in electrically stimulated myocytes (fig. S9, protocol details) (26). Using this protocol, field stimulation of a myocyte held at resting length triggered an immediate (<10 ms) spike in fluorescence in approximately 50% of the CRUs in a given line scan (Fig. 3F). Sites that demonstrated rapidly triggered Ca\(^{2+}\)-release were deemed active CRUs (Fig. 3, F and G, white bars). The remaining 50% of sites showed a delayed increase in fluorescence that probably

Fig. 3. NOX2 in the t-system produces stretch-dependent ROS that sensitizes nearby RyR2s. (A) Quantification of DCF slope before (black), during (red), and after (blue) stretch in rat myocytes treated with 1 μM gp91ds-tat–scramble peptide (n = 6 cells), 1 μM gp91ds-tat (n = 10 cells), 3 μM gp91ds-tat (n = 9 cells), 20 μM rac1 inhibitor (n = 7 cells), or in the nonstretched region of cells treated with scramble peptide (n = 6 cells). Data was normalized to prestretch slope. (B) DCF fluorescence time course with stretch in WT and NOX2\(^{-/-}\) myocytes (n = 6 cells). (C) Quantification of DCF slope before (black), during (red), and after (blue) stretch. (D) (Top) WT myocyte pre-incubated with 1 μM FAM-labeled gp91ds-tat shows sarcolemma, intercalated disc, and Z-line staining. (Bottom) High-magnification image of WT myocyte co-stained with Di-D (t-tubules) and FAM-labeled gp91ds-tat. Fluorescence plot-profile from region enclosed by dotted lines shows overlay of t-tubules and NOX2 inhibitory peptide. (E) (Top) NOX2\(^{-/-}\) myocyte pre-incubated with 1 μM FAM-labeled gp91ds-tat and imaged with identical settings as (D) shows only diffuse staining. (Bottom) High magnification image of NOX2\(^{-/-}\) myocyte co-stained with Di-D and FAM-labeled gp91ds-tat shows loss of t-tubule staining pattern for gp91ds. (F and G) Line-scan image of Fluo-4 signal from electrically stimulated rat ventricular myocyte in the presence of 2.5 μM nifedipine at resting length (F) and upon 8% diastolic stretch (G). Red mark denotes electrical stimulation (fig. S9, protocol details). White bars mark active calcium release units (CRUs) rapidly triggered by depolarization. (H) Quantification of the percentage of active CRUs triggered immediately (within 10 ms) upon depolarization in cells held at resting length (black) or upon stretch (red) in control myocytes (n = 11 cells) or those treated with 1 μM gp91ds-tat (n = 6 cells). *P < 0.05 compared with rest values; paired t test.
arose from the diffusion of Ca\textsuperscript{2+} from CRUs outside of the confocal image plane; these were deemed inactive CRUs (fig. S9, D and E). After physiologic stretch, the percentage of active CRUs rapidly triggered by field stimulation was significantly increased (Fig. 3, G and H). This is consistent with a stretch-dependent increase in RyR2 [Ca\textsuperscript{2+}] sensitivity enabling more reliable triggering of CRUs during electrical stimulation. Inhibition of NOX2 by gp91ds blocked the stretch-dependent increase in CRU triggering (Fig. 3H).

We next measured stretch-dependent ROS production and [Ca\textsuperscript{2+}]\textsuperscript{i} regulation in the mdx mouse. mdx myocytes demonstrated significantly increased ROS production with stretch when compared with WT controls (Fig. 4, A and B). This effect was blocked by the NOX2 peptid inhibitor. Consistent with the large response in mdx myocytes, X-ROS signaling components were up-regulated in the dystrophic heart. Specifically, NOX2 expression and activity are increased (11), and we observed increased expression of all tubulin subunits, with a concomitant increase in microtubule network density (fig. S10).

In mdx heart cells, the occurrence of Ca\textsuperscript{2+} waves was increased at rest and further increased after stretch (Fig. 4C). Acute inhibition of NOX2 with gp91ds-tat blocked the stretch-induced waves (Fig. 4C). NOX2 inhibition did not affect the increased Ca\textsuperscript{2+} wave activity at rest (Fig. 4C), which is consistent with other chronic pathways stably promoting wave generation in the basal state (10, 27). A more dramatic form of aberrant Ca\textsuperscript{2+} regulation was also observed in mdx myocytes, in which stretch triggered Ca\textsuperscript{2+} oscillations. This behavior ceased soon after the stretch was released, which is consistent with aberrant reversible signaling and not mechanical damage (Fig. 4E). Inhibition of NOX2 reduced this behavior (Fig. 4D). Very large mechanical stresses of mdx myocytes, whether applied as osmotic shock (17) or large strain stretch (20%) (7), can lead to massive Ca\textsuperscript{2+} influx through membrane micro-tears, triggering rapid cell death. We used physiologic 8% stretch (3) and never observed myocyte death. Stretch that is physiologic in WT myocytes thus appears to be a pathological trigger in mdx heart cells through NOX2 ROS production.

ROS can drive the downstream production of other reactive oxygen or nitrogen species that also contribute to cardiac Ca\textsuperscript{2+} signaling and pathology. In DMD, chronic RyR2 S-nitrosylation is linked to “leaky” RyR2s and Ca\textsuperscript{2+}-dependent arrhythmia (10). O\textsubscript{2}\textsuperscript{-} (the ROS produced by NOX) is an upstream prerequisite for the S-nitrosylation of RyR2. ROS also function as an upstream regulator of various transcriptional and signaling defects underlying DMD cardiomyopathy (11, 12, 17). Enhanced X-ROS signaling with each diastolic stretch in mdx myocytes (Fig. 4A) would promote a chronic oxidative environment that likely contributes to disease progression.

The mechanical distortion produced by physiologic stretch regulates NOX2-dependent ROS production through an intact microtubule network (X-ROS signaling). ROS produced by stretch is strategically localized to the SR to permit rapid redox modification of RyR2 and regulation of cardiac Ca\textsuperscript{2+} signaling. That ROS can dynamically tune RyR2 sensitivity in a physiological context reveals a signaling pathway that when dysregulated contributes to the defective [Ca\textsuperscript{2+}]\textsuperscript{i} handling inherent to cardiomyopathy in DMD. Given the predominant role of oxidative stress (14, 18) and cytoskeletal dysfunction (28) in the progression of cardiomyopathy, X-ROS signaling may have important pathophysiological effects in the context of heart disease.

### References and Notes

8. Materials and methods are available as supporting material on Science Online.


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Supporting Online Material
www.sciencemag.org/cgi/content/full/333/6048/1440/DC1
Materials and Methods
Figs. S1 to S11
References (29–31)
Movies S1 and S2
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**Chromosome Organization by a Nucleoid-Associated Protein in Live Bacteria**

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Bacterial chromosomes are confined in submicrometer-sized nucleoids. Chromosome organization is facilitated by nucleoid-associated proteins (NAPs), but the mechanisms of action remain elusive. In this work, we used super-resolution fluorescence microscopy, in combination with a chromosome-conformation capture assay, to study the distributions of major NAPs in live *Escherichia coli* cells. Four NAPs—HU, Fis, IHF, and StpA—were largely scattered throughout the nucleoid. In contrast, H-N5, a global transcriptional silencer, formed two compact clusters per chromosome, driven by oligomerization of DNA-bound H-N5 through interactions mediated by the amino-terminal domain of the protein. H-N5 sequestered the regulated operons into these clusters and juxtaposed numerous DNA segments broadly distributed throughout the chromosome. Deleting H-N5 led to substantial chromosome reorganization. These observations demonstrate that H-N5 plays a key role in global chromosome organization in bacteria.

The structure of the bacterial chromosome and the molecular mechanisms underlying its organization are poorly understood, in part due to the lack of appropriate tools for visualizing the chromosome in vivo. Fluorescence microscopy experiments have shown that DNA occupies only the central part of the bacterial cell, referred to as the nucleoid (1), but the diffraction-limited optical resolution prevents a detailed characterization. Ultrastructural characterization of the nucleoid by electron microscopy has provided varying results depending on the procedures used to fix, dehydrate, and embed the cells (1, 2). Recently, labeling of specific gene loci with the use of fluorescence in situ hybridization and fluorescent repressor-operator systems has allowed imaging of individual gene positions, and their relationship to DNA replication and segregation, in fixed and live bacterial cells (3–5). However, these studies probe only one set of specific loci at a time, and the global chromosome organization remains unclear.

In bacteria, major nucleoid-associated proteins (NAPs) are the most abundant factors that associate with the chromosome (6, 7). In *Escherichia coli*, major NAPs include H-N5, HU, Fis, IHF, and StpA (6). Each of these NAPs binds up to hundreds of specific sites per chromosome (6, 8, 9). Moreover, because of their substantial nonspecific DNA-binding affinity, the majority of cellular NAPs are bound to the chromosomal DNA with a coverage of roughly one NAP per 100 base pairs of DNA (10). NAPs have two major functions: gene regulation and chromosome organization (7). In particular, H-N5 preferentially binds to AT-rich sequences (8, 9, 11–13), functions as a global transcriptional silencer of genes with high AT content (14, 15), and is thought to reside at the center of the nucleoid (16). The oligomerization of H-N5 can promote higher-order DNA structures in vitro (17), potentially through DNA looping, bridging, and/or stiffening (18–20). These biophysical properties of NAPs and their numerous binding sites on DNA have led to the hypothesis that NAPs potentially act as chromosome organizing centers (21). However, whether the implicated higher-order DNA structures induced by NAPs exist in vivo and how the chromosome is globally organized by the NAPs remain unknown.

In a live bacterial cell, a single protein, upon binding to the less mobile structures such as the cell membrane or chromosome, can be detected and localized against a strong cellular autofluorescence background (22–24). However, the diffraction-limited optical resolution limits this imaging approach to proteins with low copy numbers in the cell (24). To obtain the subcellular distribution and organization of the abundant bacterial NAPs, sub-diffraction-limit image resolution is required.

Here, we used localization-based super-resolution imaging [stochastic optical reconstruction microscopy (STORM) or photoactivated localization microscopy] (25–29) to survey the subcellular distributions of major NAPs: H-N5, HU, Fis, IHF, and StpA. Unless otherwise specified, we tagged the target of interest with a monomeric photoactivatable fluorescent protein, mEos2 (30). We then created *E. coli* strains in which the fusion proteins were expressed from their native promoters at the endogenous loci, allowing the targets to be fully labeled and expressed approximately at the wild-type (WT) level (table S1) (31). All of these mEos2 fusion strains exhibited the same growth rates (cell-doubling times) as the wild type (31). Cells were imaged in a M9 minimal medium supplemented with glucose at room temperature shortly after they were removed from the 37°C culture at the early log phase (31).

To acquire a super-resolution image, we used a weak 405-nm light to activate the mEos2 molecules such that only an optically resolvable subset of molecules were activated at any given instant. We then illuminated the activated molecules with a 561-nm light and used astigmatism imaging to determine their centroid positions in all three dimensions, as previously shown in three-dimensional (3D) STORM (32). The molecular localizations accumulated over time allowed a