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# Molecular Crowding Drives Active Pin1 into Nonspecific Complexes with Endogenous Proteins Prior to Substrate Recognition

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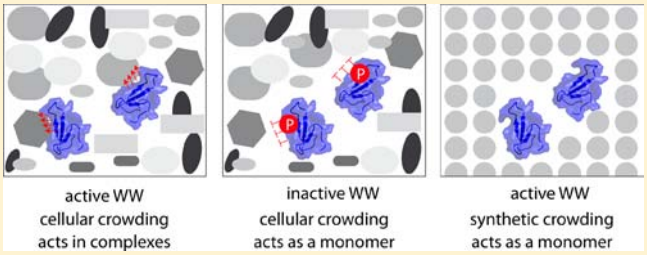
Supporting Information

**ABSTRACT:** Proteins and nucleic acids maintain the crowded interior of a living cell and can reach concentrations in the order of 200−400 g/L which affects the physicochemical parameters of the environment, such as viscosity and hydrodynamic as well as nonspecific strong repulsive and weak attractive interactions. Dynamics, structure, and activity of macromolecules were demonstrated to be affected by these parameters. However, it remains controversially debated, which of these factors are the dominant cause for the observed alterations *in vivo*. In this study we investigated the globular folded peptidyl-prolyl isomerase Pin1 in *Xenopus laevis* oocytes and in native-like crowded oocyte extract by in-cell NMR spectroscopy. We show that active Pin1 is driven into nonspecific weak attractive interactions with intracellular proteins prior to substrate recognition. The substrate recognition site of Pin1 performs specific and nonspecific attractive interactions. Phosphorylation of the WW domain at Ser16 by PKA abrogates both substrate recognition and the nonspecific interactions with the endogenous proteins. Our results validate the hypothesis formulated by McConkey that the majority of globular folded proteins with surface charge properties close to neutral under physiological conditions reside in macromolecular complexes with other sticky proteins due to molecular crowding. In addition, we demonstrate that commonly used synthetic crowding agents like Ficoll 70 are not suitable to mimic the intracellular environment due to their incapability to simulate biologically important weak attractive interactions.




## INTRODUCTION

The intracellular environment is heterogeneous and highly crowded with macromolecules, mostly proteins and nucleic acids, which reach concentrations on the order of 200−400 g/L.[1,2] The concept of intracellular molecular crowding suggests that macromolecules form, next to their functional interactions, nonspecific (attractive or repulsive) interactions with the crowded interior. Theoretical prediction of molecular crowding and its impact on protein behavior concluded that the increase in steric repulsion events with the crowders drives them into their most compact state.[3] A considerable amount of work has been performed both experimentally as well as by *in silico* simulations to characterize the effect of crowding on protein folding, aggregation, diffusion, and stability.[4−6] Furthermore macromolecular crowding affects viscosity, which was shown to tune protein activity and folding.[7,8] Fluorescence recovery after photobleaching (FRAP) measurements revealed that the diffusion coefficient of GFP in *E.coli* is 10 times less than in water.[9,10] While all these studies came to the conclusion that macromolecular crowding notably affects proteins, controversy exists whether hydrodynamic,[11] strong repulsive, or weak attractive interactions are the major determinant of protein property alteration.[12] In addition, the majority of investigations utilized artificial crowding mimetics, such as inert synthetic biopolymers, e.g., PEG, dextran, or Ficoll, which are commonly used to mimic cellular volume exclusion, viscosity, and hydrodynamic and repulsive interactions, however not weak attractive interactions.[13] Even the application of a single protein at very high concentration, e.g., 300−400 g/L bovine serum albumin (BSA) solution is still biased, neglecting the heterogeneous nature of the natural crowding environment in shape and chemical properties.

In-cell NMR spectroscopy is a unique technique to observe conformations, functions, and dynamics of macromolecules inside living prokaryotic or eukaryotic cells.[14−20] The method relies on the high sensitivity of the chemical shift to its surrounding, which is an ideal parameter to obtain structural information about the protein in its natural environment. It can be applied to prokaryotic cells like *E. coli*[21] as well as to eukaryotic cells by injection in *Xenopus laevis* oocytes[17] or in mammalian cells, insect cells, or in yeast by various techniques.[22−25] Interestingly, the backbone amide resonances of some proteins are not detectable by conventional in-cell NMR techniques, such as using the [$^{15}$N, $^{1}$H] SOFAST-HMQC experiment.[26,27] Broadening of NMR signals beyond the detection limit is often caused by slow rotational diffusion. However, the bacterial intracellular viscosity, e.g., was estimated

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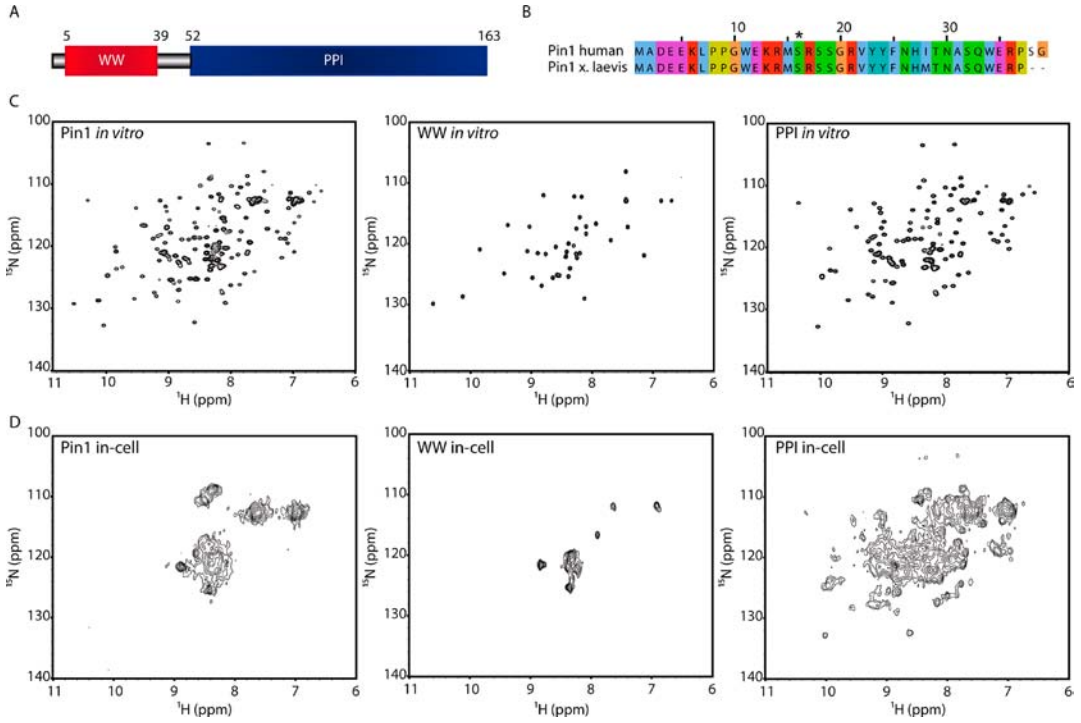


**Figure 1.** Pin1's WW domain forms nonspecific complexes with intracellular components of *X. laevis* oocytes. (A) Pin1 is a two domain protein consisting of a WW domain and a catalytically active PPI domain. (B) Alignment of the WW domain of human and *X. laevis* Pin1 demonstrates the high sequence identity. (C) Reference [$^{15}$N, $^{1}$H] SOFAST-HMQC spectra of purified Pin1, WW and PPI *in vitro*. (D) In-cell NMR [$^{15}$N, $^{1}$H] SOFAST-HMQC spectra of Pin1, WW, and PPI in intact living *X. laevis* oocytes.

to be in the range of 2−8 times that of water, suggesting that viscosity is not the reason why the backbone signals of some globular proteins remained invisible in these in-cell NMR studies.[4,26,28] Instead, various NMR spectroscopic investigations found that under intracellular and experimentally simulated crowding conditions, these proteins formed nonspecific weak attractive interactions with cellular components or protein crowders.[12,29] Nonspecific stickiness has been suggested to originate from the protein's surface property, exposing either charged[30] or hydrophobic patches[28] next to its functional site.

In this study the structure−function relationship of Pin1 (protein interacting with NIMA (never in mitosis A)-1), which is a unique peptidyl-prolyl cis/trans isomerase (PPI) and recognizes Pro-directed phosphorylated serine or threonine (pSer/Thr-Pro), is analyzed in the context of a native environment. Pin1 is a two domain protein consisting of an N-terminal Trp-Trp binding module (WW) domain responsible for protein−protein interaction and subcellular localization and a catalytically active C-terminal PPI domain. Protein phosphorylation is a key mechanism in cellular regulation and signaling.[31] Pin1 recognizes the phosphorylation signal and induces a conformational change in the substrate, which can alter its function and stability and presents a new mechanism to regulate protein activities in physiological processes as well as in disease.[32] Pin1 is up-regulated in many human cancers,[33] where its overexpression promotes tumor growth via cyclin D1 stabilization.[34] Pin1 is tightly regulated by phosphorylation in a cell cycle-dependent manner.[35] Polo-like kinase 1 (Plk-1) stabilizes Pin1 during mitosis by phosphorylation at Ser65[36] while death-associated protein kinase 1 (DAPK1), an inhibitory kinase, phosphorylates Pin1 at Ser71.[37] Another key regulation mechanism to control the functionality of Pin1 is the phosphorylation of Ser16 located in the WW domain by protein kinase A (PKA), which abolishes the capability of Pin1 to interact with its substrates, such as proteins involved in cell cycle progression and DNA transactivation, and disrupts the preferential subcellular localization to the nuclear speckle.[38] In addition, Pin1 was shown to be phosphorylated at Ser16 in Alzheimer's disease (AD).[39]

With a molecular weight of 19 kDa, Pin1 is highly suitable for NMR studies. Here we investigated the structure−function relationship of Pin1 in a native environment with in-cell NMR spectroscopy. We show that while intracellular macromolecular crowding drives functionally active Pin1 into nonspecific complexes with intracellular proteins prior to substrate recognition, phosphorylation of the Ser16 in the WW domain by PKA inactivates Pin1. At the same time, however this phosphorylation also abolishes Pin1's ability to reside in nonspecific complexes *in vivo*. Our data demonstrate the biological relevance and modulation of nonspecific weak attractive interactions as a consequence of macromolecular crowding under physiological conditions.

## RESULTS

**Severe NMR Signal Loss of Pin1 in Cells: Pin1's WW Domain Forms Nonspecific Complexes with Intracellular Components of *X. laevis* Oocytes.** To analyze the structural behavior of human Pin1 inside living cells, we injected Pin1 into *X. laevis* oocytes and performed in-cell NMR measurements. Human and *X. laevis* Pin1 share a high homology and a sequence identity of 87%, which is even higher in the WW domain (Figure 1A,B). Prior to injection, the quality of bacterially expressed human Pin1 was analyzed *in vitro* showing a well dispersed [$^{15}$N, $^{1}$H] SOFAST-HMQC spectrum (Figure 1C). Pin1 was manually injected into 200 *X.*


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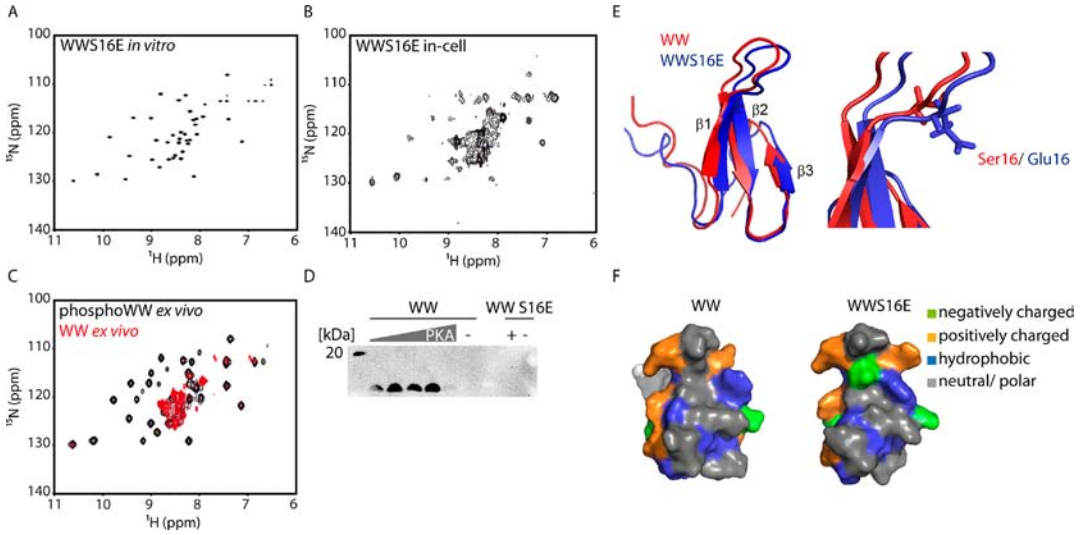


**Figure 2.** PKA phosphorylation of Ser16 inactivates Pin1's ability to form (non)specific interactions. (A) [$^{15}$N, $^{1}$H] SOFAST-HMQC spectra of the WW$^{S16E}$ obtained in dilute solution and (B) in living *X. laevis* oocytes. (C) NMR spectral overlay of the WW domain before (red) and after phosphorylation of Ser16 by the PKA. (D) The phosphorylation was verified by Western blot analysis with a pSer16 specific antibody. The WW domain carrying the S16E mutation was not phosphorylated. (E,F) The solution structures of WW in red and WW$^{S16E}$ in blue are virtually identical; surface charge representation indicated that the introduced negative charge of the WW$^{S16E}$ mutant blocks the binding pocket by the Glu residue at position 16.

*laevis* oocytes at a final concentration of 150 $\mu$M per oocyte, and [$^{15}$N, $^{1}$H] SOFAST-HMQC in-cell NMR measurements were recorded. At this relatively high Pin1 concentration all potential specific interactions with intracellular target proteins should be saturated, however, it was not possible to observe any backbone amide Pin1 signals in the corresponding 2D in-cell NMR spectrum, even with long recording times (159 min 128 scans/FID) (Figure 1D). The lack of signal in the spectrum suggested that the majority of Pin1 interacted with endogenous components of the oocyte resulting in long rotational correlation times and therefore broad or undetectable signals. The few peaks that were observed represent flexible unfolded regions of the injected protein as well as side chain amides (Figure 1D). Even oocyte lysis and the removal of insoluble parts failed to recover the backbone signals corresponding to Pin1 (Figure S1). Since Pin1 is a two domain protein consisting of a WW domain and a PPI domain (Figure 1A), we aimed to identify Pin1's nonspecific interaction site and thus analyzed the domains separately (Figure 1C,D). Interestingly, injecting the isolated PPI domain into oocytes resulted in a fairly complete [$^{15}$N, $^{1}$H] SOFAST-HMQC spectrum (Figure 1D), whereas the same experiment with the isolated WW domain (5 kDa) did not result in the detection of signals of the globular part of this domain. Consistent with the outcome received for the in cell measurements identical results were obtained in *X. laevis* oocyte extract (*ex vivo*) (Figure S1). Taken together these results suggest that the WW domain is mainly responsible for the observed nonspecific interactions that drive Pin1 into larger complexes *in vivo*, leading to a longer rotation correlation time and therefore to the severe signal loss.

**PKA Phosphorylation of Ser16, Located In the WW Domain's Substrate Recognition Pocket, Inactivates Both, Unique Substrate Recognition and Nonspecific Stickiness of Pin1.** PKA is known to phosphorylate Pin1 at Ser16 in the WW domain, which results in the abrogation of substrate recognition and the disruption of subcellular localization to the nuclear speckle.[38] To test if the substrate recognition site located in the WW domain is also responsible for the observed nonspecific interactions, we mutated Ser16 to Glu16 in the WW domain (WW$^{S16E}$) to mimic the phosphorylated and inactivated Pin1 state. Figure 2A,B shows [$^{15}$N, $^{1}$H] SOFAST-HMQC spectra of WW$^{S16E}$ that were obtained in dilute solution and under in-cell conditions, respectively. In contrast to the wildtype WW domain, the spectrum of the mutant showed all expected backbone amide resonances *in vivo*, suggesting that WW$^{S16E}$ lost its property to stick to endogenous components of the oocyte (Figure 2A,B). To rule out the occurrence of signals as a result of protein leakage during the in-cell NMR measurement, the buffer surrounding the oocytes was analyzed under the same NMR condition. The measured spectrum was devoid of any WW$^{S16E}$ signals observed under the *in vivo* condition, suggesting that the protein had not leaked out during the in-cell NMR experiment (Figure S2A). To validate the biological relevance of the phospho-mimic mutant and to test whether Pin1's nonspecific stickiness can also be switched off in the context of a physiological interaction, we phosphorylated the WW domain at Ser16 by PKA (WW$^{S16p}$) and measured spectra of the phosphorylated domain in *X. laevis* oocyte extract. Analysis of the 2D SOFAST-HMQC NMR spectra revealed that in contrast to the nonphosphorylated WW domain, WW$^{S16p}$ exhibited a well dispersed and resolved [$^{15}$N, $^{1}$H] SOFAST-HMQC spectrum (Figure 2C). The phosphorylation state of the WW domain was verified by Western blot analysis with a pSer16-Pin1 specific antibody (Figure 2D). Furthermore we analyzed the behavior of the WW$^{W34A}$ mutant, which is another active site mutant that abolishes substrate recognition,[40] however without the introduction of a negative charge. In agreement with the results measured for the WW$^{S16E}$ mutant and WW$^{S16p}$, both WW$^{W34A}$ as well as Pin1$^{S16E}$ exhibited a well resolved [$^{15}$N, $^{1}$H] SOFAST-HMQC spectrum in *X. laevis* oocytes extract (Figure S2B–E). Furthermore WW$^{W34A}$ injected in *X. laevis* oocytes resulted in a well resolved complete [$^{15}$N, $^{1}$H] SOFAST-HMQC spectrum (Figure S2F). Taken together, these results suggest that while the substrate recognition site of Pin1, in its nonphosphorylated active state,


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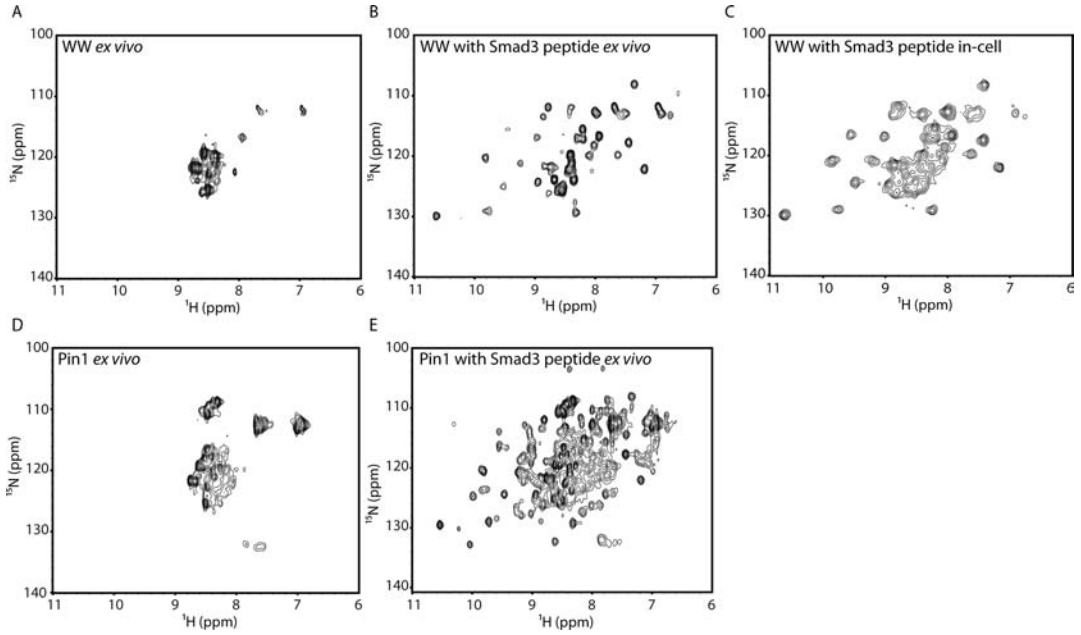


**Figure 3.** Nonspecific stickiness is lost upon substrate recognition. (A) NMR spectra of the WW domain in *X. laevis* oocyte extract (*ex vivo*) before and (B) after addition of the phosphorylated Smad3 peptide. Virtually identical results are obtained intact living cells (C). Similar results have been acquired for the full length protein in *X. laevis* oocyte extract (D,E).

complexes via nonspecific and specific interactions with components of the cellular interior, phosphorylated $WW^{S16p}$ and therefore inactivated Pin1 exist within a monomeric state, incapable of forming both nonspecific and specific interactions via its substrate binding pocket.

To clarify whether the negative charge introduced by the presence of the phosphorylated Ser16 leads to a conformational rearrangement in Pin1, we determined the structures of the WW- and the phospho-mimic $WW^{S16E}$ domain by NMR spectroscopy (Table S1). Figure 2E shows the superimposed structures of $WW^{S16E}$ and WW. Structural analysis revealed that the presence of the glutamate introduced a repulsive steric hindrance in direct proximity to the substrate binding pocket in the WW.[41] Overall the structures of WW and $WW^{S16E}$ are very similar and show no major structural differences. However in contrast to the wildtype, the flexibility of the first loop, comprising the amino acids 16−21, seems to be notably different in the mutant. This is substantiated by the fact, that in the wildtype protein peaks for Arg17, Ser18 and Ser19 in [$^{15}$N, $^{1}$H] TROSY-HSQC spectra were not observed at 18 °C and at 4 °C. In contrast to the wildtype, Ser19 is visible at 18 °C and the whole loop is detectable at 4 °C in the $WW^{S16E}$ mutant (Figure S3). Recently, it has been shown that the phosphorylation of Ser16 induces a loss in flexibility of the phosphor binding region and that the phosphorylated WW domain contains a new hydrogen-bond network which stabilizes the binding loop.[42] The surface charge analysis revealed that the stickiness of the binding pocket of Pin1 is not solely due to a hydrophobic patch (Figure 2F). Instead the active site consists of a mix of positively charged arginines stabilizing the phosphate group of the pSer/pThr-Pro recognition site and the hydrophobic Trp34 stabilizing the proline. This is massively disturbed by the introduction of a negative charge at Ser16 in the phospho-mimic mutant.

**Nonspecific Stickiness Is Lost upon Substrate Recognition.** To obtain more insight into the strength of the observed nonspecific interactions of Pin1 with intracellular components, a substrate competition study was performed in *X. laevis* oocyte extract. First the backbone amide region of the WW domain was measured in oocyte extract (Figure 3A). After the measurement, the phosphorylated Smad 3 peptide IPEpTPPG was added in a 2:1 ratio to the WW domain present in the oocyte extract, and [$^{15}$N, $^{1}$H] SOFAST-HMQC spectral acquisition was repeated (Figure 3B). While the WW domain showed the anticipated backbone amide resonance-loss in oocyte extract, due to its stickiness, addition of the peptide resulted in visible backbone amides (Figure 3A,B). Importantly, the detected peaks revealed the peptide bound form of the WW domain (Figure S4), in which the binding pocket is occupied by the peptide, inhibiting interactions with cytosolic components of *X. laevis* oocytes and enabling free rotation. Injection of the preformed Smad3-peptide-WW complex in *X. laevis* oocytes and subsequent in-cell NMR analysis validated the results obtained in extract (Figure 3C). Finally, virtually identical results were obtained for the full length protein in *X. laevis* oocyte extract (Figure 3D,E). The outcome of these experiments demonstrates that the substrate recognition site of Pin1, which is located in the WW domain, performs both specific (strong attractive) and nonspecific (weak attractive) interactions with components of the cellular interior. These data demonstrate that due to its stickiness, active Pin1 resides in macromolecular complexes prior to substrate recognition.

**Protein Crowders Promote Pin1's Nonspecific Stickiness.** Despite many investigations there is disagreement which factor(s), e.g., viscosity, hydrodynamic interactions, strong repulsive or weak attractive interactions, have the highest influence on proteins under crowded conditions.[12,43] To evaluate which of these nonspecific physiochemical factors promote Pin1's stickiness, we studied the structural behavior of WW and $WW^{S16E}$ in the presence of high concentrations of glycerol, Ficoll 70, BSA, or ovalbumin, imitating the effects of (i) viscosity; (ii) viscosity, hydrodynamic and strong repulsive interactions; and (iii) viscosity, hydrodynamic, strong repulsive and weak attractive interactions, respectively.


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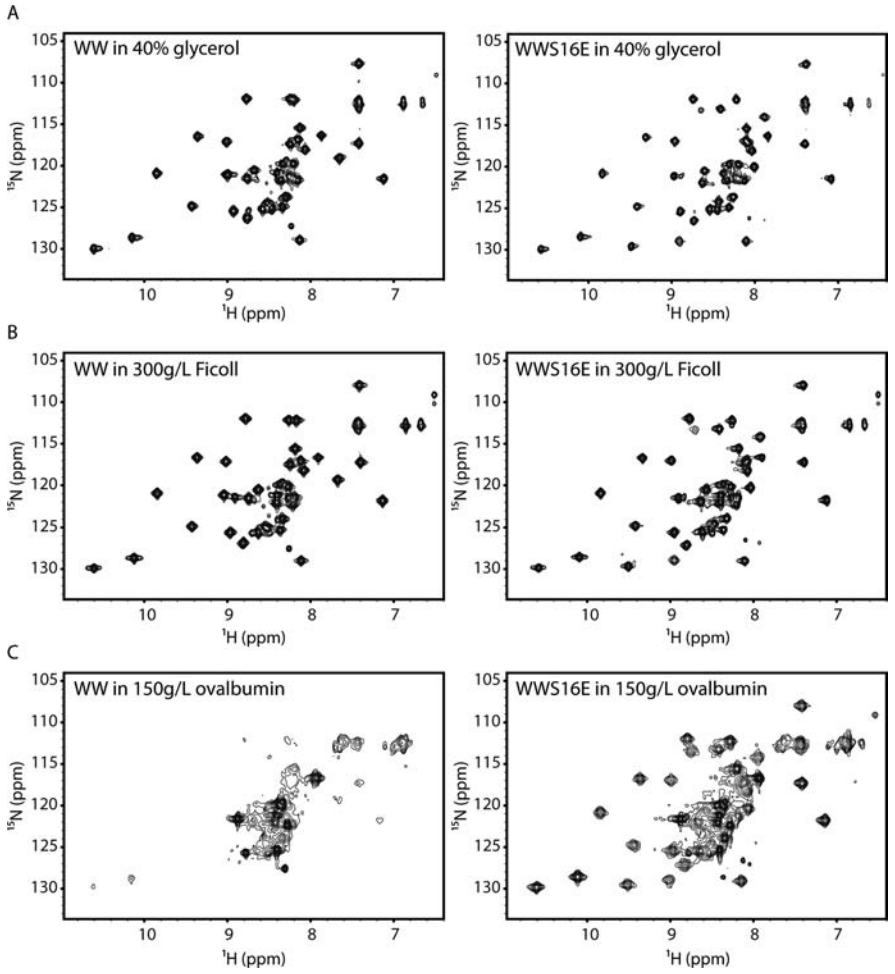


**Figure 4.** Protein crowders promote Pin1's nonspecific stickiness. WW and $WW^{S16E}$ were measured in (A) 40% glycerol, (B) 300 g/L Ficoll 70, and (C) 150 g/L ovalbumin. While 40% glycerol and 300 g/L Ficoll 70 had no influence on WW and $WW^{S16E}$, the presence of 150 g/L of ovalbumin impaired the detection of the backbone signals of the WW domain.

Figure 4A,B shows NMR spectra obtained in 40% glycerol and 300 g/L Ficoll 70, respectively, demonstrating that neither of these solutes can correctly simulate the crowding conditions as observed in the oocyte and extract. Since the WW domain is detectable in Ficoll 70 and glycerol, enhanced viscosity and hydrodynamic and strong repulsive interactions, although present *in vivo* as a consequence of macromolecular crowding, are not the factors that drive active Pin1 into transient complex formation. In contrast, employing ovalbumin or BSA as macromolecular crowding environment resulted in the disappearance of the wildtype WW domain backbone amide resonances, whereas the $WW^{S16E}$ resonances remained unaffected (Figures 4C and S5). In summary, these results suggest that the observed stickiness of active Pin1 is related to the WW domain and its property to form nonspecific weak attractive interactions with endogenous proteins as a direct effect of macromolecular crowding.

## ■ DISCUSSION

In contrast to the dilute solution in which a protein's structural and functional behavior is commonly characterized, protein interactions under physiological conditions take place within a heterogeneously crowded environment. The state of the crowded interior, is dominantly maintained by macromolecules, e.g., proteins, RNA and DNA, however not small molecules.[44] Macromolecular crowding leads to volume exclusion, with fundamental consequences for factors, such as viscosity, water activity, hydrodynamic interactions as well as strong repulsive and weak attractive interactions between the crowders. Molecular crowding effects on proteins and nucleic acids were extensively studied under simplified *in vitro* conditions, e.g., in the presence of a physiological salt solution supplemented with a particular synthetic or natural crowder.[3,45] While the majority of these studies concluded that molecular crowding significantly affected proteins or nucleic acids, it is not entirely clear yet to what extent and which of these factors were responsible for the observed property alterations *in vivo*, e.g., macromolecular compaction, impaired translational and rotational diffusion, and positive as well as negative impact on protein stability.[46,47] Importantly, recent evidence points to the experimental conditions of *in vitro* molecular crowding investigations as a source for the inconsistent experimental outcomes, in particular the choice of the crowder such as synthetic molecules (Ficoll or PEG) vs more natural crowders (BSA or ovalbumin) or measurements in cellular extracts or in living cells.[4,48,49]

In the study presented here in-cell NMR spectroscopy was used to characterize the structural behavior of Pin1 under native macromolecular crowding conditions inside living *X. laevis* oocytes and in native-like crowded oocyte extract. In


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agreement with the majority of earlier in-cell NMR studies,[27,29,50] investigating globular folded proteins, we also failed to observe Pin1's backbone amide resonances under in-cell and *ex vivo* conditions. Employing NMR spectroscopy, the Pielak and the Gierasch lab independently demonstrated on various globular folded proteins with a low net charge under physiological conditions that signal loss is caused by the protein's slowed rotational tumbling.[4,28] They proposed that exclusively weak attractive interactions between the crowders and the protein under investigation lead to the slowed rotational movement.[4]

Our systematic in-cell NMR analysis of Pin1 and its PPI and WW domain in-cell revealed that the substrate recognition site, which is located in the WW domain, constantly forms weak attractive nonspecific interactions with endogenous components of *X. laevis* oocytes. In principle signal loss could also be due to the formation of specific complexes if they are large enough to significantly increase the rotational correlation time. However, at an intracellular concentration of 150 $\mu$M of Pin1 as used in these investigations it is far more likely that the concentration of specific substrates is far below the concentration of Pin1 and that the observed line broadening is due to nonspecific interactions. This interpretation is supported by the experiments with BSA and ovalbumin which are not substrates of Pin1. Interestingly, inactivation of Pin1 via PKA phosphorylation at Ser16 abrogated both substrate recognition and the nonspecific stickiness, suggesting that the nonspecific stickiness forced active Pin1 to permanently reside in macromolecular complexes, whereas the phosphorylated inactivated form diffused as a monomer under cellular conditions. Phosphorylation of Ser16 located in the loop between $\beta$1 and $\beta$2 of the WW-domain is physiologically highly relevant, since it abolishes substrate recognition and changes subcellular localization.[38] In Alzheimer's disease (AD) high levels of Ser16 phosphorylated inactivated Pin1 are found in brain tissues of Alzheimer patients, especially in the most insoluble hyperphosphorylated tau fraction of AD brain tissue.[39]

Our NMR study of differently inactivated substrate recognition, namely the phospho-mimic $WW^{S16E}$ and the $WW^{W34A}$ domains further confirmed Pin1's dual interaction preferences in substrate recognition. Structure determination of the $WW^{S16E}$ domain and analysis with respect to the wildtype revealed that the $WW^{S16E}$ domain, maintained its globular three $\beta$-strand fold. Of note, our *in vitro* NMR measurements of the $WW^{S16E}$ domain recorded at different temperatures showed that the dynamics of the loop between $\beta$1 and $\beta$2 is different from the wildtype, which is in agreement with the recently published model of the $WW^{S16p}$ domain by Smet-Nocca and co-workers.[42]

A small molecule, a synthetic crowder, and protein crowders were employed to evaluate which factor of cellular molecular crowding, viscosity, hydrodynamic interactions, strong repulsive or weak attractive interactions, respectively, forced active Pin1 to be constantly engaged in transient macromolecular complexes. These investigations clearly showed that Pin1 exclusively formed nonspecific weak attractive interactions with the protein crowders, suggesting that active Pin1 permanently interacted with endogenous proteins of the *X. laevis* oocyte. In contrast, the synthetic inert crowder employed here, Ficoll 70, could not mimic this nonspecific interaction phenotype of Pin1, which is in agreement with the recent observations by the Pielak and the Elcock groups.[4,44,51] A similar behavior was observed for the transcriptional repressor MetJ, which was shown to nonspecifically associate with genomic DNA pointing to a search mechanism for identifying and binding target sequences.[52] In accordance with the accumulated examples of proteins with undetectable amide backbone signals under liquid in-cell NMR conditions, we believe that most of the mildly charged cellular proteins, exposing a certain degree of hydrophobicity on their surfaces, adopt nonspecific attractive interactions under cellular conditions, suggesting that they permanently reside in macromolecular complexes with endogenous proteins prior to substrate recognition as originally postulated by McConkey.[53] Future studies have to analyze the effects of these weak transient nonspecific interactions and their impact on protein functionality.

## ■ CONCLUSION

The investigation presented here reveals that Pin1 uses its WW domain to constantly interact with other cellular components in *X. laevis* oocytes, while the catalytic domain shows a significantly lower level of nonspecific stickiness. These nonspecific weak attractive interactions as well as specific substrate recognition can be abrogated by phosphorylation of Ser16 in the WW domain by PKA. The observed nonspecific interactions can also be suppressed by binding of a phosphorylated peptide derived from a known specific substrate of Pin1. In addition, we demonstrate that synthetic crowding agents like Ficoll 70 fail to imitate the intracellular environment correctly due to their incapability to perform physiologically relevant weak attractive interactions with the protein under study.

## ■ MATERIAL AND METHODS

**Plasmids, Protein Expression and Purification, and Peptide Synthesis.** DNAs comprising the genes of Pin1 and PPI and WW domains were cloned into a modified pET29b-vector (Novagen) containing a N-terminal $His_6$-tag followed by a Tobacco etch virus (TEV) protease cleavage site. Site-directed mutagenesis was performed by quick change PCR to introduce the desired mutations. Uniformly $^{15}N$- and $^{15}N$, $^{13}C$-labeled samples were expressed in *E.coli* strain BL21 T7 express (NEB) in M9 minimal medium containing 1g/L $^{15}NH_4Cl$ and 2g/L $^{13}C_6$-Glucose. Cultures were grown to $OD_{600}$ of 0.6 at 37 °C and induced with 1 mM IPTG followed by a subsequent growth period of 3h at 37 °C. Harvested cells were lysed via sonication in 25 mM Tris pH 7.8 and 20 mM NaCl and supplemented with protease inhibitors. Proteins were purified by Ni-affinity chromatography with Ni-sepahrose fast flow (GE-Healthcare). The $His_6$-tag was cleaved off using TEV protease, followed by a reverse Ni-affinity chromatography step. Afterward samples were dialyzed against 25 mM Hepes pH 7.6, 50 mM NaCl, and 1 mM DTT and concentrated to 1−3 mM stock solutions. The Smad 3 peptide[41] IPEpTPPG was synthetized in house.

**In-Cell NMR Spectroscopy and Oocyte Extract Preparation.** The ovary from female *X. laevis* frogs was dissected and follicular cells as well as the connective tissue were removed by collagenase treatment. In-cell NMR samples were prepared by injecting 50 nL of the 3 mM [*U*-$^{15}N$] protein stock solution (150 $\mu$M intracellular concentration) into each oocyte. After injection oocytes were washed thoroughly with Ori-$Ca^{2+}$ buffer (5 mM Hepes pH 7.6, 110 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$) and allowed to recover for 1 h at 18 °C. For one NMR sample 200−250 oocytes were injected. The oocytes were collected and transferred into a Shigemi tube without the plunger and kept in a total volume of 1 mL of Ori buffer containing 10% of $D_2O$. For the confirmation of cell integrity oocytes were separated from the surrounding buffer after the in-cell NMR measurement and checked for protein leakage into the supernatant. Lysate and cleared lysate were prepared as follows: The oocytes were transferred into a 1.5 mL tube and mechanically crushed

on ice with a plunger. Insoluble fractions were removed by centrifugation at 20 000 *g* at 4 °C for 30 min and the supernatant was used for NMR measurements. The lysate was heated to 95 °C for 10 min for cleared lysate preparation. Precipitated proteins were removed by centrifugation. For larger amounts of extract the oocytes of one whole ovary were sorted and transferred into several 2 mL tubes. As described before they were mechanically crushed and insoluble fractions were removed by centrifugation. The extract was then collected, aliquoted, and stored until needed at −80 °C.

**NMR Measurements and Analysis.** All NMR experiments were conducted at a sample temperature of 18 °C if not indicated otherwise on Bruker Avance 800, 700, and 600 MHz spectrometers equipped with cryogenic triple resonance probes. Two-dimensional ($^{15}$N−$^{1}$H) correlations for in-cell NMR resulted from SOFAST-HMQC experiments.[54] Backbone and side chain resonances were assigned using TROSY-type[55,56] HNCA, HNCACB, HNCO, HN(CA)CO, (H)CC(CO)NH-TOCSY, H(CCCO)NH-TOCSY experiments of [*U*-$^{15}$N,*U*-$^{13}$C]-WW and [*U*-$^{15}$N,*U*-$^{13}$C]-WW$^{S16E}$. Titration experiments were performed with unlabeled Smad3 peptide IPEpTPPG added to the $^{15}$N labeled WW-domain in 10 steps with protein/peptide ratios between 1:0.1 and 1:6. For measurements in *X. laevis* extract, 120 μL extract aliquots were thawed, and protein of a 1.5 mM stock solution was added to a final concentration of 145 μM. For peptide competition experiments the peptide was added in a molar ratio of 1:2 of protein to peptide. Spectra processing was performed with TopSpin 3.0 software (Bruker) and for analysis UCSF SPARKY 3.114 (San Francisco, CA) was used. The chemical shifts of WW and WW$^{S16E}$ were deposited in the Biological Magnetic Resonance Data Bank (BMRB) with accession numbers 19258 (wildtype WW domain) and 19259 (WW$^{S16E}$ domain). The final structures were deposited in the Protein Data Bank (PDB) with accession codes 2m8i (wildtype WW domain) and 2m8j (WW$^{S16E}$ domain).

***In vitro* Phosphorylation.** 50 μM of the WW domain were *in vitro* phosphorylated by 250 kunits of cAMP-dependent PKA, catalytic subunit (NEB) in a reaction volume of 500 μL in the presence of 50 mM Tris pH 7.5, 500 μM ATP, and 10 mM $MgCl_2$. The reaction was incubated overnight at 30 °C in a slide-A-lyzer and dialyzed against 50 mM Tris pH 7.5, 500 μM ATP, and 10 mM $MgCl_2$ to remove the glycerol from the reaction. Phosphorylated WW domain was concentrated to 25 μL and added to *X. laevis* oocyte extract containing phosStop (Roche) and 10% $D_20$. Phosphorylation was validated by Western blotting with a pSer16-WW specific antibody.

**Western Blotting.** Western blotting was performed as previously described.[57] The following antibody was used: anti-pSer16WW (Epitomics).

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Further experimental data as well as detailed description of the structure calculation and determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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