**SI Text**

**Expression and Purification of the Abp1p SH3 Domain and Its Ark1p peptide Binding Partner:** Two constructs based on the pET32b plasmid from Novagen (using NcoI and XhoI restriction sites) were used; each of the constructs contains a his-tagged thioredoxin gene followed by a TEV cleavage site and then either residues 535-592 of the yeast Abp1p protein (1-3) (corresponding to the Abp1p SH3 domain) or the residues KKTKPTPPPKPSHLKPK from the yeast protein Ark1p peptide (4). *E coli* BL21(DE3) cells transformed with the appropriate plasmid were grown in 1L (~95% $^{2}H_{2}O$) M9 minimal media with [U-$^{15}$N] $NH_4Cl$ and protonated glucose as the nitrogen and carbon sources, respectively. The constructs were purified under denaturing conditions using Ni-NTA resin and the protein was refolded by dialyzing into 50 mM sodium phosphate, 100 mM NaCl, pH 7.0, 4 mM DTT. Subsequently the His-tagged thioredoxin was cleaved using TEV protease.

To separate the His-tagged thioredoxin or any residual uncleaved fusion constructs from the Abp1p SH3 domain or the Ark1p peptide the cleavage mixture was passed through the Ni-NTA resin for a second time and the flow-through retained. The Abp1p SH3 domain was further purified on a 5 ml HiTrap Q column with the bound SH3 domain eluted using a 0 to 1M NaCl gradient. The pure protein was then dialyzed against NMR buffer, consisting of 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, and 1 mM $NaN_3$ (pH 7.0). The Ark1p peptide was further purified using reverse phase HPLC with a Phenomenex C18 preparatory column, using a 0 to 50% gradient of acetonitrile, to obtain a purity of > 95% as judged by mass spectrometry. The peptide was lyophilized and resuspended in a small volume of NMR buffer for further use.

**Expression and Purification of the G48M Fyn SH3 Domain:** The DNA sequence for the G48M Fyn SH3 domain (5) (residues 2-60, Thr 2 corresponds to Thr 84 of *G. gallus* Fyn SH3) was cloned into a pET-11M plasmid with a TEV protease cleavable N terminal hexa His-tag. This plasmid was used to transform BL21(DE3) cells for protein expression. The [U-$^{15}$N,$^{2}$H] G48M Fyn SH3 domain was prepared by growing cells in a (99.9%) $^{2}H_2O$ M9 minimal media with [U-$^{15}$N] $NH_4Cl$ and [U-$^{2}$H,$^{12}$C] glucose as the sole nitrogen and carbon sources, respectively. The protein was purified under denaturing conditions following the protocol for the Abp1p SH3 domain described above, followed by a further purification step on a Superdex 75 gel filtration column. The protein was then dialyzed into NMR buffer (50 mM sodium phosphate, 1 mM EDTA, 1 mM $NaN_3$, pH 7.0).

**Data Analysis:** Relaxation dispersion profiles, $R_{2,eff}(\nu_{CPMG})$, were generated from peak intensities, $I_1(\nu_{CPMG})$, in a series of 2D $^{1}$H-$^{15}$N correlation maps measured as a function of CPMG frequency, $\nu_{CPMG} = 1/(4\tau_{CP})$, where $2\tau_{CP}$ is the interval between consecutive refocusing pulses of the CPMG sequence. Peak intensities were converted into effective relaxation rates via $R_{2,eff}(\nu_{CPMG}) = -1/T_{relax} \ln(I_1(\nu_{CPMG})/I_0)$, where $I_0$ is the peak intensity in a reference spectrum recorded without the relaxation delay $T_{relax}$ (6). Dispersion profiles were recorded with $T_{relax}$ = 30 ms and 16-23 values of $\nu_{CPMG}$ ranging from 33.3 to 1000 Hz, along with 2-3 repeats for error analysis (Acquisition times of approximately 20 hours for each dispersion profile). Relaxation dispersion data was analyzed using a 2-site

exchange model, $A \underset{k_B}{\overset{k_A}{}} B$, with the best-fit model parameters extracted using in-house written software by minimization of the target function,

$$^2(\ ) = \frac{\left(R_{2,eff}^{clc}(\ ) \quad R_{2,eff}^{exp}\right)^2}{\left(\ R_{2,eff}^{exp}\right)^2}$$

where $R_{2,eff}^{exp}$ and $R_{2,eff}^{exp}$ are experimental effective relaxation rates and their uncertainties, respectively, $R_{2,eff}^{clc}(\ )$ are calculated relaxation rates obtained by numerical integration of the Bloch-McConnell equations (7), $= \{x_1,\ldots,x_{npar}\}$ denotes the set of adjustable model parameters, and the summation is over the number of experimental data points. The global exchange parameters, $p_B$ (the population of the minor state) and $k_{ex} = k_A + k_B$ were extracted from simultaneous fits of TROSY, anti-TROSY and $^1$H CW decoupled $^{15}$N CPMG relaxation dispersion profiles (see text and below). Values of (the difference in $^{15}$N chemical shifts between states, ppm) and $D_{\mathrm{NH}}$ (the difference in dipolar couplings between states, Hz) were extracted on a per-residue basis (magnetic field independent) as were intrinsic relaxation rates, $R_{2,}^{Trosy}$, $R_{2,}^{anti\ \ Trosy}$ and $R_{2,}^{CW}$, for each static magnetic field (500 and 800 MHz). Extracted dipolar couplings are plotted in figures in the text so long as $> 0.2$ ppm (so that reasonably sized dispersions are quantified), if the reduced $^2 < 2$ for the residue, corresponding to a reasonable fit of the data, and in Fig. 4*c* if the error in $D_{\mathrm{NH}} < 5$Hz (see SI Fig. 10).

Data were analyzed using an in-house written program that is available upon request. In general, a complete description of the evolution of a 2-spin $^1$H-$^{15}$N spin-system that includes chemical exchange requires a 31 set basis. Here we use a reduced set

where the operators are spin-state selective $\{\hat{E},\ \hat{S}_i^A(\hat{E}\ \ 2\hat{I}_Z^A), \hat{S}_i^A(\hat{E}+2\hat{I}_Z^A), \hat{S}_i^B(\hat{E}\ \ 2\hat{I}_Z^B), \hat{S}_i^B(\hat{E}+2\hat{I}_Z^B)\}_{i=X,Y,Z}$, with $\hat{E}$ the identity operator and $\hat{S}_i(1\ \ 2\hat{I}_Z)$ and $\hat{S}_i(1+2\hat{I}_Z)$ corresponding to TROSY and anti-TROSY $^{15}$N magnetization components ($S$=$^{15}$N, $I$=$^{1}$H), with the superscripts A and B denoting whether the magnetization derives from the major or minor state, respectively. Conformational exchange is incorporated into this basis using the method of McConnell (7), with $R_{2,eff}^{calc}$ values calculated by numerically solving the resulting set of differential equations that describe the evolution of magnetization during the CPMG pulse train. Evolution during the P-element (see Fig. 2), including the effects of $^{1}$H spin-flips (due to external protons) that interconvert TROSY and anti-TROSY magnetization components, is explicitly taken into account. Evolution during the pulses is also accounted for. Phase cycling is included by repeating the simulations with the phases of the pulses varied as in the experiment. Magnetization at the start of the CPMG pulse train is partitioned between the two states (A and B) according to their populations. At the end of the simulated pulse train magnetization is returned to the Z-axis followed by a short delay (2-3/$k_{ex}$, set to what is used experimentally) during which magnetization equilibrates due to exchange. Linear combinations of Z-magnetization from states A and B are then selected for the calculation of $R_{2,eff}^{calc}$. The coefficients for the linear combination are those used to generate the eigenvector of the 2x2 exchange matrix whose eigenvalue (imaginary part) is closest to $_{A}$. So long as major and minor states are separated in chemical shift this is equivalent to selecting the major state. The effect of external protons during the CPMG element is taken into account using experimentally measured relaxation rates for $S_Z$ ($R_1$)

and $2S_ZI_Z$ ($R_{ZZ}$) (cross-relaxation rate between TROSY and anti-TROSY components is given by $1/2(R_{ZZ} - R_1)$). Errors in extracted parameters were estimated by performing Monte-Carlo simulations (8) using the experimental errors or by the covariance matrix method (8), with similar results obtained using both approaches.

***The importance of deuteration***: As described above, all samples used were prepared with high levels of deuteration. This increases sensitivity of spectra by reducing intrinsic relaxation rates of amide protons and eliminating potential $^1$H-$^1$H homonuclear scalar or dipolar couplings. The minimization of relaxation contributions from $^1$H spins external to the amide spin system ($^1$H spin flips) reduces the rate of interconversion between TROSY- and anti-TROSY magnetization components in the spin-state selective dispersion experiments. This has important implications for the extraction of reliable $D_{NH}$ values since it can be shown that, if not corrected for, spin flips will increase (decrease) measured values of $|D_{NH}|$ in cases where $D_{NH}$ and $_N$ are of the same (opposite) sign. Spin flips are explicitly included in fits of dispersion data, as described above, so that accurate values of $D_{NH}$ are extracted.

***NMR methodology*:** $^{15}$N TROSY- and anti-TROSY-based CPMG relaxation dispersion pulse schemes for the quantification of $^1$H-$^{15}$N dipolar couplings in invisible states of proteins are illustrated in Fig. 2. We also include the $^1$H-decoupled $^{15}$N CPMG-based relaxation dispersion pulse scheme (denoted CW CPMG), that has been described previously (9), for completeness (SI Fig. 5). In the discussion that follows we focus on the TROSY/anti-TROSY experiments. Both of these sequences build on a pulse scheme

developed by Loria *et al.* (10) that makes use of the TROSY effect for recording $^{15}$N dispersion profiles of high molecular weight proteins dissolved in isotropic solution and below we describe the unique features that enable the extension of the methodology to studies of proteins that are partially aligned.

In both experiments of Fig. 2 magnetization is initially transferred from $^{1}$H to $^{15}$N and during the subsequent delay, $_{eq}$, the magnetization of each exchanging state is restored to its equilibrium level, prior to the start of the CPMG pulse train. This can be accomplished in the case of a two-site exchanging system $A \underset{k_B}{\overset{k_A}{}} B$, for example, by choosing $_{eq} \sim 2\text{-}3/k_{ex}$, where $k_{ex} = k_A + k_B$, as is described more fully elsewhere (9). Exchange of magnetization occurs during the subsequent constant-time CPMG pulse train (11) between points *a* and *b*. As described in the text the flow of magnetization in the experiments is such that in the TROSY scheme only the TROSY components that are present during the CPMG element are selected and recorded during ($t_1$,$t_2$). In contrast, insertion of a $^{1}$H 180$^{o}$ pulse at point *c* in the anti-TROSY scheme (Fig. 2) interconverts TROSY and anti-TROSY magnetization components, with the TROSY component subsequently selected. In this manner magnetization that is anti-TROSY during the CPMG element is subsequently converted to the TROSY variety prior to ($t_1$,$t_2$) evolution. This leads to substantial benefits in resolution and sensitivity over data sets recorded where the anti-TROSY components have evolved throughout.

Central to the performance of both TROSY and anti-TROSY experiments is the P-element in the center of the CPMG pulse train (10) that has been modified to account for the fact that the effective coupling during this duration, $J_{eff} = J_{NH} + D_{NH}$ varies in a site-specific manner (since the $^{1}$H-$^{15}$N dipolar coupling, $D_{NH}$, but not the scalar coupling, $J_{NH}$, is

variable). As described by Loria *et al.* (10) the P element minimizes cross-relaxation between TROSY- and anti-TROSY components by selectively inverting one of them so that the effective intrinsic relaxation during the CPMG interval approximates single exponential. There is a second benefit; we show below that the P-element leads to a 'self compensation' of radio frequency (rf) pulse imperfections during the CPMG scheme so that *N* (Fig. 2) can be either odd or even. Consider the evolution of magnetization during the CPMG pulse train with explicit neglect of relaxation and chemical exchange contributions that do not impact on the results below. We can write the evolution of the magnetization operator $\hat{O}_p$ as

$$\hat{R}\hat{O}_p\hat{R}^{\ 1} \qquad [1]$$

where

$$\hat{R} = \hat{K}'\hat{P}\hat{K} \qquad [2]$$

and

$$\hat{K} = \{\exp(\ i(2\ J_{NH}\hat{I}_Z\hat{S}_Z + \ \hat{S}_Z)\ _{CP})\exp(\ i(\ _1\hat{S}_Y + \ \hat{S}_Z)\ _p)\exp(\ i(2\ J_{NH}\hat{I}_Z\hat{S}_Z + \ \hat{S}_Z)\ _{CP})\}_N \qquad [3]$$

$$\hat{K}' = \{\exp(\ i(2\ J_{NH}\hat{I}_Z\hat{S}_Z + \ \hat{S}_Z)\ _{CP})\exp(\ i(\ _1\hat{S}_X + \ \hat{S}_Z)\ _p)\exp(\ i(2\ J_{NH}\hat{I}_Z\hat{S}_Z + \ \hat{S}_Z)\ _{CP})\}_N \qquad [4]$$

$$\hat{P}_{TR} = \exp(\ i\ \hat{I}_X)\exp(\ i\frac{}{2}\hat{S}_X)(\exp(\ i(2\ J_{NH}\hat{I}_Z\hat{S}_Z + \ \hat{S}_Z)\ _b)\exp(\ i\ \hat{S}_X)\exp(\ i\ \hat{I}_X)$$
$$\exp(\ i(2\ J_{NH}\hat{I}_Z\hat{S}_Z + \ \hat{S}_Z)\ _b)\exp(\ i\frac{}{2}\hat{S}_Y) \qquad [5]$$

$$\hat{P}_{A\ TR} = \exp(\ i\ \hat{I}_X)\exp(\ i\frac{}{2}\hat{S}_X)(\exp(\ i(2\ J_{NH}\hat{I}_Z\hat{S}_Z + \ \hat{S}_Z)\ _b)\exp(\ i\ \hat{S}_Y)\exp(\ i\ \hat{I}_X)$$

$$\exp(\ i(2\ J_{NH}\hat{I}_Z\hat{S}_Z + \ \hat{S}_Z)\ _b)\exp(\ i\frac{}{2}\hat{S}_Y)$$

[6]

In Eqs. [3-6] $\hat{S}_Z$ is the $^{15}$N Zeeman Hamiltonian where (rad/sec) is the offset of spin S ($^{15}$N) from the rf carrier in the rotating frame, $2\ J_{NH}\hat{I}_Z\hat{S}_Z$ is the scalar coupling Hamiltonian connecting spins *I* and *S*, $_1$ is the $^{15}$N rf field strength (rad/sec), $_p$ is the duration of each refocusing pulse of the CPMG train and $\hat{P}_{TR}$, $\hat{P}_{A\ TR}$ are the operators for P-elements for the TROSY and anti-TROSY experiments, respectively. We have neglected pulse imperfections in the $^{15}$N and $^1$H 180° pulses in the P-element in the analysis below, but not for the pulses that make up the individual CPMG elements. Experimentally effects of imperfections in the central pulses of the P-element can be minimized by including a pair of surrounding gradients. It is worth noting that the 90° $^{15}$N pulses at each end of the P-element are phase cycled and the effects of the phase cycle are taken into account in what follows (see legend to Fig. 2). It follows that

$$\hat{R} = \hat{K}'\hat{P}\exp(\ i\ /2\hat{S}_Z)\hat{K}'\exp(i\ /2\hat{S}_Z)\,. \qquad [7]$$

where $\hat{P}$ $\{\hat{P}_{TR},\hat{P}_{A\ TR}\}$ depending on whether the TROSY or anti-TROSY experiment is selected. Consider first the TROSY experiment. Then $\hat{O}_P$ in Eq. [1] is $\hat{S}_Y(\hat{1}\ \ 2\hat{I}_Z)$ and we note that

$$\hat{K}'\exp(i\ /2\hat{S}_Z)\hat{O}_p\exp(\ i\ /2\hat{S}_Z)(\hat{K}')^{\ 1} \qquad \begin{matrix}\hat{S}_X(\hat{1}\ \ 2\hat{I}_Z)\\ \hat{S}_Y(\hat{1}\ \ 2\hat{I}_Z)\\ \hat{S}_Z(\hat{1}\ \ 2\hat{I}_Z)\end{matrix}\ , \qquad [8]$$

where the appropriate multiplicative factors associated with each of the three terms that account for pulse imperfections have been neglected. Eq. [8] simply reflects the fact that S pulse imperfections and evolution during the CPMG train will lead to the interconversion of $\{\hat{S}_X(1 \quad 2\hat{I}_Z), \hat{S}_Y(1 \quad 2\hat{I}_Z), \hat{S}_Z(1 \quad 2\hat{I}_Z)\}$. A similar equation holds for $\hat{O}_p = \hat{S}_Y(\hat{1} + 2\hat{I}_Z)$ that is of relevance to the anti-TROSY scheme. Eq. [8] can be simplified by considering the following relations

$$\hat{P}_{TR} \exp(\ i \diagup_2 \hat{S}_Z) \begin{matrix} \hat{S}_X(\hat{1} \quad 2\hat{I}_Z) \\ \hat{S}_Y(\hat{1} \quad 2\hat{I}_Z) \\ \hat{S}_Z(\hat{1} \quad 2\hat{I}_Z) \end{matrix} \exp(i \diagup_2 \hat{S}_Z)\hat{P}_{TR}^{\ 1} = \begin{matrix} \hat{S}_X(\hat{1} \quad 2\hat{I}_Z)\sin \\ \hat{S}_Y(\hat{1} \quad 2\hat{I}_Z) \\ \hat{S}_Z(\hat{1} \quad 2\hat{I}_Z)\sin \end{matrix} \qquad [9.1]$$

$$\hat{P}_{A\ TR} \exp(\ i \diagup_2 \hat{S}_Z) \begin{matrix} \hat{S}_X(\hat{1} + 2\hat{I}_Z) \\ \hat{S}_Y(\hat{1} + 2\hat{I}_Z) \\ \hat{S}_Z(\hat{1} + 2\hat{I}_Z) \end{matrix} \exp(i \diagup_2 \hat{S}_Z)\hat{P}_{A\ TR}^{\ 1} = \begin{matrix} \hat{S}_X(\hat{1} + 2\hat{I}_Z)\sin \\ \hat{S}_Y(\hat{1} + 2\hat{I}_Z) \\ \hat{S}_Z(\hat{1} + 2\hat{I}_Z)\sin \end{matrix} \qquad [9.2]$$

where $=2\ J_{\text{eff}\ b}$, $_b = 1/(4|J_{NH}|)$, $J_{NH} < 0$ in $\hat{P}$ and the phase cycling of the 90 $^{o\ 15}$N flanking pulses has been taken into account. These pulses eliminate magnetization components that result from the fact that $J_{\text{eff}} \neq J_{NH}$ and that are proportional to cos . Since cos $\sim (\ + \ /2)$ for $\sim - \ /2$ such unwanted terms can be considerable (cos $\sim 0.3$ for values of $D_{NH}$ on the order of 15 Hz and $J_{NH}$ = -93 Hz). By contrast, because $J_{\text{eff}} \neq J_{NH}$ the value of sin deviates from –1 but only to second order, sin $\sim -1 + (\ + \ /2)^2/2$; thus sin is much more tolerant of the inevitable missetting of the $_b$ delay (sin =-0.97,-0.91 for $D_{NH}$ = 15 and 25 Hz, respectively). Replacing sin by –1 in Eq. [9] it follows that

$$\hat{P}\exp(\ i\ \diagup_2 \hat{S}_Z)\ \begin{matrix}\hat{S}_X(\hat{1}\mp 2\hat{I}_Z)\\ \hat{S}_Y(\hat{1}\mp 2\hat{I}_Z)\\ \hat{S}_Z(\hat{1}\mp 2\hat{I}_Z)\end{matrix}\ \exp(i\ \diagup_2 \hat{S}_Z)\hat{P}^{\ 1}\quad \exp(\ i\ \hat{S}_Y)\ \begin{matrix}\hat{S}_X(\hat{1}\mp 2\hat{I}_Z)\\ \hat{S}_Y(\hat{1}\mp 2\hat{I}_Z)\\ \hat{S}_Z(\hat{1}\mp 2\hat{I}_Z)\end{matrix}\ \exp(i\ \hat{S}_Y) \quad [10]$$

where $\hat{P}=\hat{P}_{TR}$ if $\{\quad \hat{S}_i\ (\hat{E}\quad 2\hat{I}_Z)_{i=X,Y,Z}\}$ are considered and $\hat{P}=\hat{P}_{A\ TR}$ for $\{\hat{S}_i\ (\hat{E}+2\hat{I}_Z)_{i=X,Y,Z}\}$. Substituting Eq. [7] into Eq. [1] and using Eq. [10] it can be shown that

$$\begin{aligned}\hat{R} &= \hat{K}'\exp(\ i\ \hat{S}_Y)\hat{K}'\exp(i\ \diagup_2 \hat{S}_Z)\\ &= \hat{K}'\exp(\ i\ \hat{S}_Y)\hat{K}'\exp(i\ \hat{S}_Y)\exp(\ i\ \hat{S}_Y)\exp(i\ \diagup_2 \hat{S}_Z).\\ &= \exp(\ i\ \hat{S}_Y)\exp(i\ \diagup_2 \hat{S}_Z).\end{aligned} \quad [11]$$

Eq. [11] establishes that, so long as the appropriate P-element is chosen, evolution due to chemical shift and/or scalar couplings is refocused in the general case of pulse imperfections during the CPMG train (providing sin ~-1), independent of whether *N* is even or odd. Thus, the minimum $_{\text{CPMG}}$ value is 1/ $T_{relax}$, where $T_{relax}$ is the duration of the CPMG element.

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