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REVIEW

# Protein conformational dynamics and phenotypic switching

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**Abstract**
Intrinsically disordered proteins (IDPs) are proteins that lack rigid 3D structure but exist as conformational ensembles. Because of their structural plasticity, they can interact with multiple partners. The protein interactions between IDPs and their partners form scale-free protein interaction networks (PINs) that facilitate information flow in the cell. Because of their plasticity, IDPs typically occupy hub positions in cellular PINs. Furthermore, their conformational dynamics and propensity for post-translational modifications contribute to "conformational" noise which is distinct from the well-recognized transcriptional noise. Therefore, upregulation of IDPs in response to a specific input, such as stress, contributes to increased noise and, hence, an increase in stochastic, "promiscuous" interactions. These interactions lead to activation of latent pathways or can induce "rewiring" of the PIN to yield an optimal output underscoring the critical role of IDPs in regulating information flow. We have used PAGE4, a highly intrinsically disordered stress-response protein as a paradigm. Employing a variety of experimental and computational techniques, we have elucidated the role of PAGE4 in phenotypic switching of prostate cancer cells at a systems level. These cumulative studies over the past decade provide a conceptual framework to better understand how IDP conformational dynamics and conformational noise might facilitate cellular decision-making.

**Keywords** Protein conformational dynamics · Intrinsically disordered proteins · Phenotypic switching · PAGE4 · Conformational noise · MRK hypothesis

## Introduction

Despite the initial skepticism regarding the existence of proteins that lacked structure, mainly because of the dominance of the structure/function paradigm that was predicated on the "lock-and-key" hypothesis formulated in the late nineteenth century by Emil Fischer (Uversky, 2021), it is generally held that intrinsically disordered proteins (IDPs) constitute a significant fraction of the proteomes of organisms across all three kingdoms of life (Ward et al, 2004; Xue et al, 2012a; Peng et al, 2015). Although IDPs, and intrinsically disordered regions (IDRs) within structured proteins, may lack rigid 3D structure, they can populate different conformations and, hence, exist as

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conformational ensembles (Tompa, 2011; Uversky, 2017; Wright and Dyson, 1999; Uversky & Dunker, 2010). Indeed, the unusually high degree of malleability facilitates their interactions with multiple partners (Uversky, 2015), and such interactions constitute a network referred to as protein interaction network (PIN). The PIN configuration defines a cell's phenotype and its ability to "make" decisions.

Although it was tacitly assumed that PINs are "wired" randomly, seminal work beginning in the late 1990s by Barabási and colleagues (Barabási and Albert, 1999; Barabási, 2009; Dehmamy et al, 2018) revealed that, indeed, cellular PINs are organized following a "scale-free" architecture. In such networks, the degree distribution *P(k)* exhibits a power-law behavior as a function of the degree *k*. A salient feature of scale-free networks is that they are robust to failure of random nodes. However, they are vulnerable to failure of hubs (Barabási and Albert, 1999). Furthermore, the fact that the organization and properties of the PINs are conserved during evolution (Rangarajan et al., 2015a) underscores their functional significance.

Consistent with their unique ability to interact with multiple partners by virtue of their plasticity, IDPs are typically found in hub positions defined as nodes with multiple interactions (defined as edges) in PINs (Dosztányi et al., 2006; Haynes et al., 2006; Gsponer & Babu, 2009**;** Patil et al, 2010; Hu et al., 2017) and play critical roles in many biological processes including transcription, splicing, translation, and signaling (Wright & Dyson, 2015; Bürgi et al., 2016; Shammas, 2017). Furthermore, they also regulate several key processes such as cell division (Galea et al, 2008; Yoon et al, 2012; Mitrea et al, 2012), circadian rhythmicity (Baggio et al., 2013; Hurley et al, 2013, 2016; Dong et al, 2016; Michael et al, 2017), and phenotypic plasticity (Xue et al, 2012b; Mooney et al, 2016). Therefore, it is not surprising that, under physiological conditions, the levels of IDPs are tightly regulated from transcript synthesis to protein degradation (Gsponer et al, 2008; Edwards et al, 2009; Babu et al, 2011; Uversky VN, 2014). Indeed, when dysregulated, IDPs have the potential to engage in multiple "promiscuous" interactions resulting in pathological states (Vavouri et al, 2009; Marcotte and Tsechansky, 2009). Remarkably, several proteins that are dysregulated in disease pathology such as the oncogenes MYC, c-Jun, c-Fos, and the cancer/testis antigens are IDPs (Iakoucheva et al, 2002; Uversky et al 2008; Rajagopalan et al, 2011; Babu MM, 2016). Yet, the molecular mechanisms by which IDPs accomplish their functions, and engage in promiscuous interactions, are not fully understood. When compared to number of IDPs, for example, in the human, where ~ 50% of the proteome is estimated to be comprised of IDPs (Dunker et al, 2001; Dyson and Wright, 2021), only a tiny fraction of IDPs have been characterized in significant detail.

Nonetheless, these studies have revealed that IDPs can transition from disorder to order upon binding to their cognate partners, a phenomenon referred to as, "coupled folding and binding" (Dyson & Wright, 2002; Sugase et al, 2007). However, while in some IDPs, such as the GTPase-binding domain (GBD) of the Wiskott–Aldrich syndrome protein (WASP) and the phosphorylated kinase-inducible domain (pKID) of the cAMP-response element binding (CREB) protein, which interacts with the KIX domain of the transcriptional coactivator CREB-binding protein (CBP), an ordered conformation is induced by the interacting partner – the "induced fit" hypothesis, the opposite may be true in other instances such as the α-MoRE located within the intrinsically disordered C-terminal domain of the measles virus (MeV) nucleoprotein called the $N_{TAIL}$, wherein the IDP ensembles populate multiple conformations a priori, and the ligand selects the most favored prefolded state from these conformations (Boehr et al, 2009). Nonetheless, it appears that, in many cases, a combination of the two extremes underlies the transition (Wang et al, 2013; Arai et al, 2015), suggesting that the intrinsic secondary structure propensities of the IDPs determine their binding mechanisms.

In contrast to the above scenarios, some IDPs can stochastically switch among distinct conformational states suggesting that IDPs can alter the conformation of their ensembles while remaining disordered (Choi et al, 2011; Choi et al., 2019). Together, these observations suggest that despite being disordered, many IDPs are only marginally unstable and can easily transition to active conformations. On the other hand, it has also been observed that several IDPs (Chakrabortee et al, 2010; He et al, 2015; Kulkarni et al, 2017; Borgia et al, 2018) appear to remain largely disordered even while interacting with their biological targets to form what are known as "fuzzy" complexes (Sharma et al., 2015; Fuxreiter, 2018). Fuzzy binding is seen when the degree of disorder in the bound state of the IDP varies with the partner or cellular conditions such as polymorphic bound structures, conditional folding, and dynamic binding highlighting the structural continuum of complexes as well as their context-dependent interaction behaviors (Fuxreiter, 2020).

A recent report suggested that, in fact, frustration in such fuzzy complexes contributes to the versatility (one-to-many interactions), and high specificity but low affinity interactions, associated with IDPs (Freiberger et al., 2021). Complexes of IDP exhibit a high degree of local frustration, especially at the binding interface. However, the authors noted that the suboptimal interactions can potentially lead to multiple bound substates, each displaying distinct frustration patterns, which are differently populated in complexes with different partners. Therefore, IDPs appear to achieve specificity without a single common bound conformation, and the conflict between different

interactions is leveraged to control the binding to multiple partners. From the foregoing, it may be summarized that IDPs may explore myriad interaction mechanisms, ranging from induced folding to formation of fuzzy complexes where significant levels of disorder are preserved to polyvalent stochastic interactions (Uversky, 2018; Fuxreiter, 2020).

Prostate-associated gene 4 (PAGE4) is a remarkably prostate-specific protein in the normal human adult and is overexpressed in prostate cancer (PCa). It is also an IDP that appears to remain disordered when interacting with its partner (see below). Therefore, using PAGE4 as a paradigm, here, we discuss how its conformational dynamics, and consequently, conformational noise, can influence a PCa cell's decision to switch between an androgen-dependent and androgen-independent phenotype. These findings shed new light on how non-genetic mechanisms may contribute to phenotypic heterogeneity in the population and highlight important therapeutic implications.

## Conformational noise

The term noise in biology implies random variability in quantities arising in biological systems including isogenic systems. Therefore, cells in an isogenic population can display very different phenotypes in response to the same stimulus by switching their phenotypes (Huang S, 2009; Brock et al, 2009). In fact, phenotypic switching due to noise has been observed in development, stress response, pathological states such as cancer, and evolution (Mahmoudabadi et al, 2013; Jia et al., 2017).

Presently, noise in biology typically implies transcriptional noise mainly because gene expression is an intrinsically stochastic process which results in variability in protein levels between individual cells in a population (Raj and van Oudenaarden, 2009; Hansen et al, 2018). However, information transduced in cellular signaling networks also appears to be significantly affected by noise (Ladbury and Arold, 2012), particularly, noise contributed by the "non-functional" interactions of proteins (Kuwahara and Gao, 2013). This noise results from the intrinsic promiscuity of protein–protein interactions that modulate cellular signal transduction (Kontogeorgaki et al, 2017). Since a majority of the transcription factors and signaling molecules are IDPs that can engage in promiscuous interactions when dysregulated, they play a significant role in generating noise enhancing the potential to switch phenotypes. Furthermore, the overexpression of IDPs is observed to correlate with altered physiological (Vavouri et al, 2009) and pathological states (Iakoucheva et al, 2002; Uversky et al 2008; Rajagopalan et al, 2011; Babu, 2016).

## The MRK hypothesis

Almost a decade ago, we (Mahmoudabadi et al, 2013) proposed a model (the MRK model, Kulkarni and Kulkarni, 2019) to account for noise contributed by the conformational dynamics of IDPs. This noise referred to as "conformational noise" is defined as the random variability in the various confirmations sampled by the IDP ensemble which results in stochastic promiscuous interactions with other proteins. Although interconversions of conformations of the IDPs are in fast exchange, we postulated that the conformational preferences of the ensemble are impacted by post-translational modifications and, therefore, can have significant half-lives (in the order of several minutes to hours) contributing to conformational noise. Furthermore, the model showed that conformational noise can be an integral part of transcriptional noise, and therefore, IDPs could potentially amplify total noise in the system in response to intrinsic or extrinsic perturbations. Thus, conformational noise arising from the stochasticity of the promiscuous interactions initiated by the IDPs in response to a specific input allows the system to sample the network interaction space. This heuristic rewires the network and drives phenotypic switching to generate phenotypic heterogeneity (Fig. 1). Stated differently, the model suggests that IDPs uncover network configurations that are causal in phenotypic switching but are latent under normal conditions (Mahmoudabadi et al, 2013). Indeed, such stochasticity in phenotypic switching has been linked to cellular differentiation (Eldar & Elowitz, 2010; Nichol et al., 2016; Safdari et al, 2020), generation of induced pluripotent stem cells (iPS cells) via reprogramming (McArthur et al., 2008; Yamanaka, 2009; Wakao et al, 2013; Chung et al, 2014; Smith et al., 2015; Lin et al., 2018), emergence of cancer stem cells from non-stem cancer cells (Gupta et al, 2011; Sehl et al., 2015; Rambow et al., 2019), and emergence of chemoresistance (Kumar et al., 2019).

Several important points characterize the MRK hypothesis. First, according to the model, the information that specifies the cell's phenotype resides in the configuration of its PIN. Second, cell fate is not determined a priori (is not deterministic), and hence, it is likely that each cell in the population has the potential to undergo specific phenotypic transition in response to the given input. Third, in response to a specific input, IDPs can rewire the network to uncover latent configurations and, thus, actuate a phenotypic switch. Fourth, the model proposes that, at least in some cases, upon withdrawal of the input, the PIN can rewire itself to the normal (default) network configuration, thereby reversing the phenotypic switch. Fifth, information can operate across spatiotemporal timescales. Thus, while information that operates over relatively short timescales

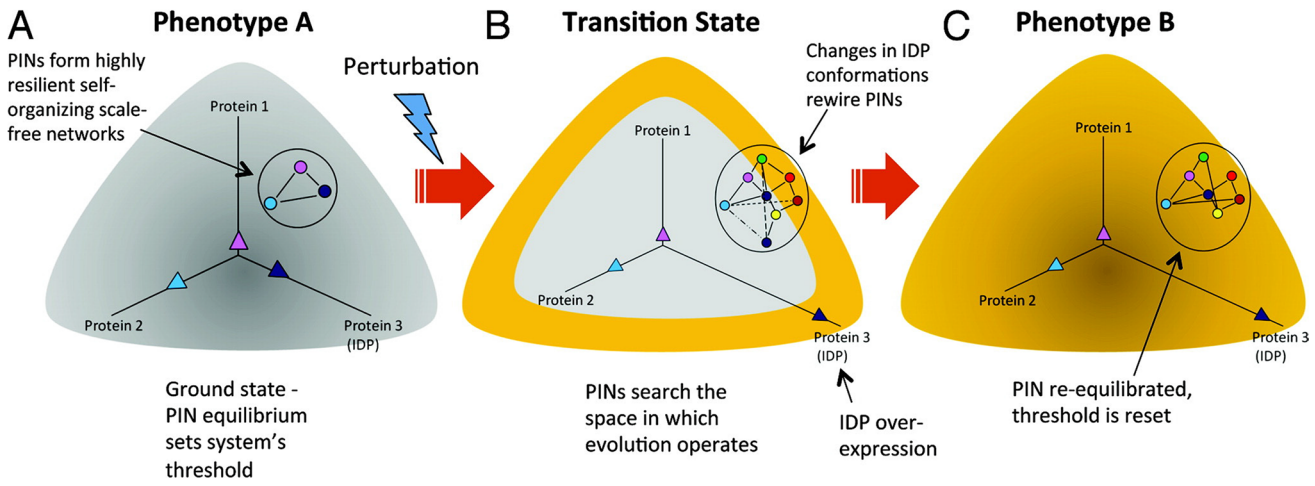


**Fig. 1** Rewiring of protein networks facilitates state-switching by activating latent pathways. (**A**) The state of a cell with phenotype A is depicted in grey and shows a simple protein network with three proteins (1−3), of which one is an IDP (indicated in dark blue), and expressed at different levels represented by the three vectors. This configuration represents the protein network's ground state threshold. (**B**) Depicts a transition state. A perturbation causes increased IDP expression (protein 3). Overexpression of the IDP results in promiscuity and the protein network explores the network search space shown by the various dashed lines. This transition state is depicted state in yellow around the grey area. (**C**) The state of the cell after it has transitioned to phenotype B from phenotype A represented in yellow. A particular configuration of the protein network that increased its fitness is "selected," which now represents the new ground state. Reproduced with permission from Mahmoudabadi et al. 2013

maybe retained within the PIN, information operating over longer periods such as cellular transformation, development, and evolution is transferred to the genome to ensure it is heritable (Sonnenschein et al., 2014),

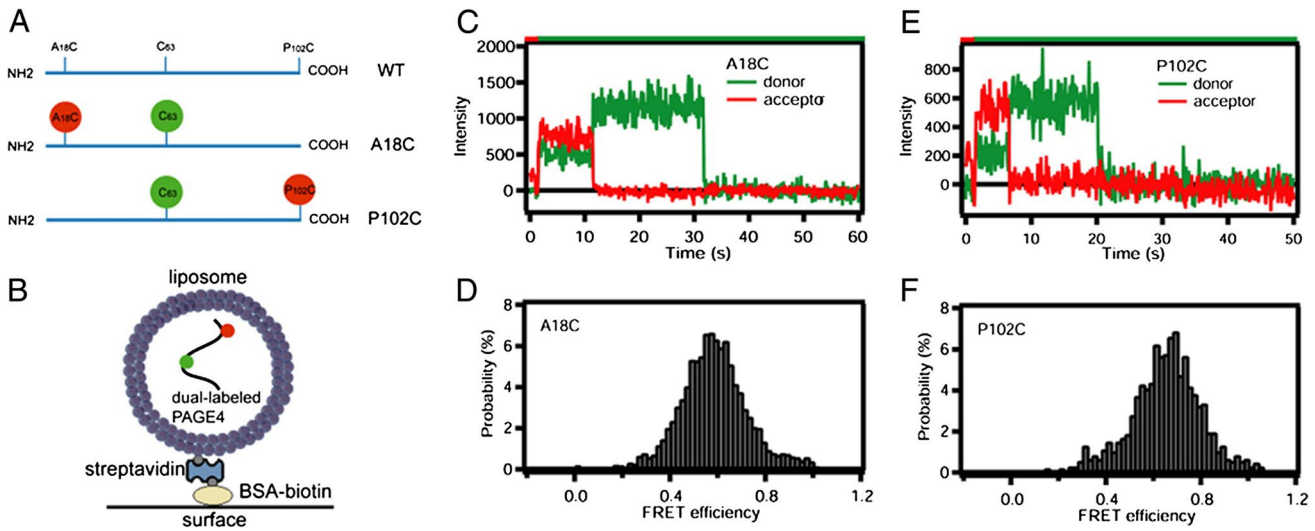


**Fig. 2** Single molecule FRET indicates that PAGE4 is an intrinsically disordered protein. (**A**) Schematic of the PAGE4 constructs with the native cysteine (green) and the introduced cysteine (red). Single PAGE4 protein molecules were encapsulated inside 100 nm diameter liposomes tethered to a quartz surface. (**B**) Shows a cartoon of this immobilization scheme (not to scale). Fluorescence emission time courses in the donor and acceptor spectral bands were collected and those indicating exactly 1 donor and 1 acceptor were further analyzed. Example intensity time courses showing anti-correlated donor/acceptor behavior upon photobleaching, which is characteristic of single molecules, are shown for the A18C/63C (**C**) and P102C/63C (**E**) FRET mutants. The color bar at the top indicates the illumination color. Red illumination at the start driving only acceptor fluorescence allows identification of molecules containing an active acceptor. The disappearance of red emission (with anticorrelated recovery of green) is photobleaching of the acceptor, and disappearance of green emission is photobleaching of the donor. Histograms assembled from all FRET active data points of over 300 molecules are shown for A18C/63C (**D**) and P102C/63C (**F**) PAGE4 mutants. These FRET signals agree with expectations based upon modeling PAGE4 as a highly flexible IDP. Reproduced with permission from Rajagopalan et al. 2014

Therefore, contrary to the prevailing wisdom that phenotype specification is deterministic, the MRK hypothesis advocates that stochasticity contributed by IDP conformational noise may be a confounding factor in specifying cell fate. Consistent with this line of thinking, several studies have shown that cells can reversibly switch phenotypes, such as, epithelial to mesenchymal transition (EMT), a drug-sensitive cell developing resistance and switching back to becoming drug sensitivity (Sharma et al, 2010; Al Emran et al, 2017; Su et al, 2017; Hammerlindl & Schaider, 2018; Sahoo et al, 2021) or a normal cell transforming into a malignant one and its reversal to normalcy (dormancy) (Shachaf et al, 2004; Shachaf & Felsher, 2005). A theoretical perspective (Rangarajan et al., 2015b) demonstrating how the oncogene c-Myc, an IDP, lends further credence to the MRK hypothesis.

## IDP conformational dynamics, noise, and cell decisions

PAGE4 is a small protein of 102 amino acids that is highly intrinsically disordered (Zeng et al., 2011; Rajagopalan et al, 2014; He et al, 2015) (Fig. 2). It primarily resides in the cytoplasm where it functions as a stress-response protein (Zeng et al, 2013). In response to stress, PAGE4 is upregulated and translocates to the mitochondrion and appears to suppress production of reactive oxygen species (ROS). Thus, overexpression of PAGE4 decreases the phosphorylation of MAP2K4, JNK, and c-JUN while increasing phosphorylation of ERK1/2. Taken together, these data indicate that under stress, PAGE4 appears to promote the survival of PCa cells by regulating MAPK pathway (Lv et al, 2019). In addition to serving as a stress response factor, PAGE4 is also a transcriptional regulator and potentiates transactivation by c-Jun (Rajagopalan et al, 2014).

The latter function of PAGE4 is modulated by the conformational dynamics of its differentially phosphorylated ensembles by kinases, namely HIPK1 and CLK2. HIPK1 is a stress-response kinase which phosphorylates PAGE4 at T51 and, to a minor extent, S9 (Mooney et al, 2014; He et al, 2015). Employing multidimensional NMR, small angle X-ray scattering, and single molecule Förster resonance energy transfer microscopy (Fig. 3), we determined that threonine phosphorylation, predominantly at T51, leads to compaction of the PAGE4 ensemble (radius of gyration, Rg, 34.7 ± 1.2 Å compared to non-phosphorylated where the Rg is 36 ± 1.1 Å) which is facilitated by the looping of the N-terminal region (He et al, 2015; Kulkarni et al, 2017; Lin et al., 2018; Lin et al., 2019). HIPK1-phosphorylated PAGE4 (HIPK1-PAGE4) acts as a strong potentiator of c-Jun which heterodimerizes with c-Fos to form the AP-1 transcription factor complex. AP-1 is a negative regulator

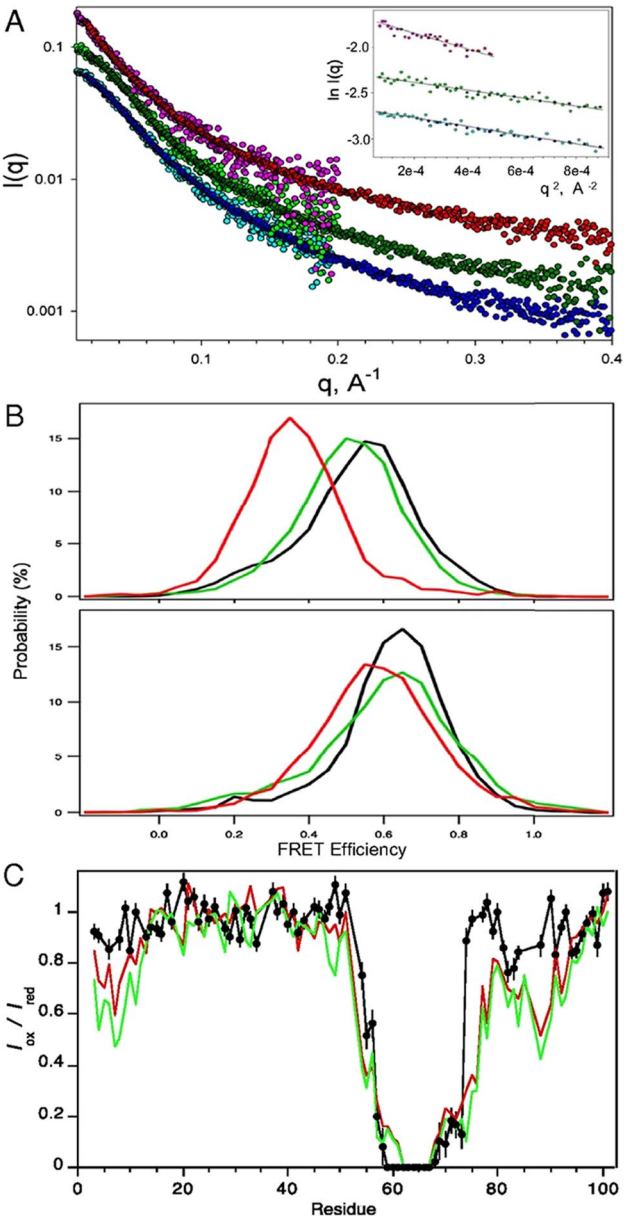


**Fig. 3** Conformational expansion of PAGE4 upon hyperphosphorylation by CLK2. (**A**) Experimental X-ray scattering data for the WT-PAGE4 (bottom curve, cyan/blue), HIPK1-PAGE4 (middle curve, light green/dark green), and CLK2-PAGE4 (top curve, pink/red). For each of the variants, the two colors denote independent data collections probing lower-q and medium-q ranges of the scattering data. The curves are offset for clarity. (*Inset*) Guinier fits of the lowest q data that yield model-free estimates of the ensemble-averaged radii of gyration for the three variants. (**B**) smFRET measurements. (*Upper*) Distributions of smFRET efficiency measurements for PAGE4 with donor and acceptor sites at positions 18 and 63 WT-PAGE4 (black), HIPK1-PAGE4 (green), and CLK2-PAGE4 (red). (*Lower*) Donor and acceptor sites are at positions 63 and 102 for WT-PAGE4 (black), HIPK1-PAGE4 (green), and CLK2-PAGE4 (red). (**C**) PRE data for CLK2-PAGE4 (black) with an MTSL spin label at C63. Results are compared with earlier observations for WT-PAGE4 (red) and HIPK1-PAGE4 (green). Reproduced with permission from Kulkarni et al. 2017

**Table 1** A summary of the size measurements of the PAGE4 phosphoforms from both the AAWSEM simulations and SAXA and smFRET experiments

| | SAXS ($R_g$) (Å) | | FRET efficiency (E) | | | | FRET RMS Dist (Å) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Res. 18–63 | | Res. 63–102 | | Res. 18–63 | | Res. 63–102 | |
| | EXP[a] | SIM[b] | EXP[a] | SIM[b] | EXP[a] | SIM[b] | EXP | SIM | EXP | SIM |
| Wild-type form | 36 ± 1.1 | 32.9 | 0.55 | 0.48 | 0.64 | 0.60 | 56 | 57.4 | 50 | 51.2 |
| HIPK1form | 34.7 ± 1.2 | 32.1 | 0.52 | 0.53 | 0.63 | 0.60 | 59 | 55.6 | 50 | 51.4 |
| CLK2 form | 39.8 ± 1.9 | 41.8 | 0.35 | 0.22 | 0.58 | 0.52 | 75 | 73.4 | 55 | 54.8 |

[a]*EXP*, experimental results [16]

[b]*SIM*, simulation results (this study)

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of the androgen receptor (AR) activity in PCa cells (Sato et al, 1997; Tilman et al., 1998), and AR, in turn, is a negative regulator of the CDC-like kinase 2 (CLK2) (Kulkarni et al, 2017). Therefore, inhibiting AR de-represses CLK2. CLK2 hyperphosphorylates PAGE4 (CLK2-PAGE4) at all S/T residues in the molecule leading to remodeling of the PAGE4 ensemble which now prefers to assume a more random coil-like confirmation (Rg, 49.8 ± 1.9 Å) (Table 1). Furthermore, in contrast to HIPK1-PAGE4, CLK2-PAGE4 attenuates c-Jun potentiation (Kulkarni et al, 2017).

Given the enormity of the conformational space of PAGE4, deriving an ensemble average picture representing its conformational plasticity represents a challenge. Therefore, to further understand the mechanisms driving the conformational diversity among different PAGE4 ensembles, we analyzed their simulated atomistic trajectories using the associative memory, water-mediated, structure and energy Model (AWSEM) forcefield, and the energy landscapes were elucidated using the energy landscape visualization (ELViM) method. This method identifies and compares the population distributions of different PAGE4 ensembles using the same effective phase space. The results showed a conformational ensemble with an extended C-terminal segment of non-phosphorylated "wild-type" (WT) PAGE4 to be predominant. Interestingly, this conformation exposes the T51 residue, underscoring its potential of undertaking a fly-casting mechanism while binding to its cognate partner (Fig. 4). In contrast, in the case of HIPK1-PAGE4, the compacted ensemble populates a conformation that sequesters the phosphorylated T51 which is consistent with the experimentally observed weaker affinity of HIPK1-PAGE4 for c-Jun (Mooney et al, 2014).

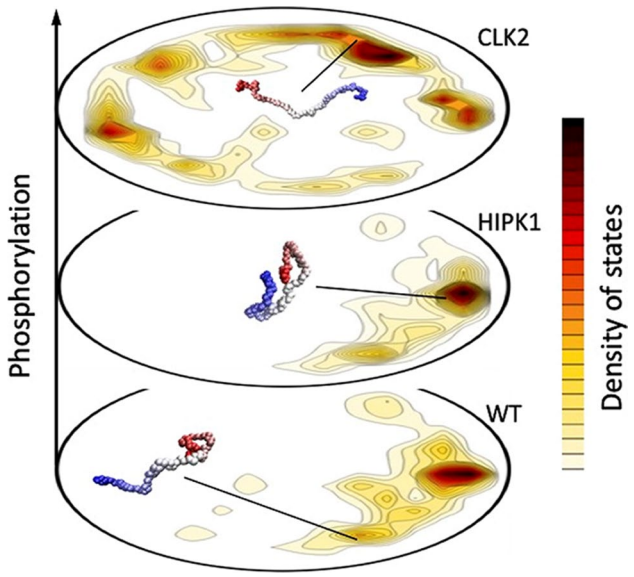


**Fig. 4** Employing the energy landscape visualization method (ELViM), different PAGE4 ensembles are represented in one single conformational phase space. The density of states, shown in the contour plots, varies according to the physical–chemical conditions, which in this case is the PAGE4 phosphorylation state. Each free energy valley can be characterized by specific conformations that entail particular binding affinities, typical of the promiscuous behavior of IDPs. For WT-PAGE4, through a fly-casting mechanism, the C-terminal region is extended, allowing the binding to its cognate partner. For the HIPK1-PAGE4, the lower free energy of the compact state decreases the affinity for c-Jun. Finally, the dominant extended conformations of CLK2-PAGE4 inhibit any binding affinity

## Oscillatory dynamics of the HIPK1-PAGE4-AP1-AR-CLK2 circuit guides cellular decisions

Mathematical modeling of the HIPK1-PAGE4-AP1-AR-CLK2 circuit in PCa cells (Kulkarni et al, 2017; Lin et al, 2018; Salgia et al., 2018; Lin et al., 2019) suggested that these interactions between HIPK1, PAGE4, the AP-1 transcription factor complex, androgen receptor (AR), and CLK2 form a negative feedback loop which may give rise to oscillations in intracellular levels of the different conformational ensembles of PAGE4 as well as AR activity (Kulkarni et al, 2017) (Fig. 5). Therefore, in cells that express both HIPK1 and CLK2, the feedback loop can lead to "dynamic" regulatory circuits due to changes in PINs. Thus, conformational noise that is contributed by differential phosphorylation of PAGE4 can result in cell-to-cell variability due to rewiring of the network circuit and promote phenotypic heterogeneity in a population of androgen-dependent PCa cells. Thus, it follows that due to the oscillatory dynamics, a cell can exhibit a varying degree of androgen dependence at different time points. Thus, even non-synchronous oscillations

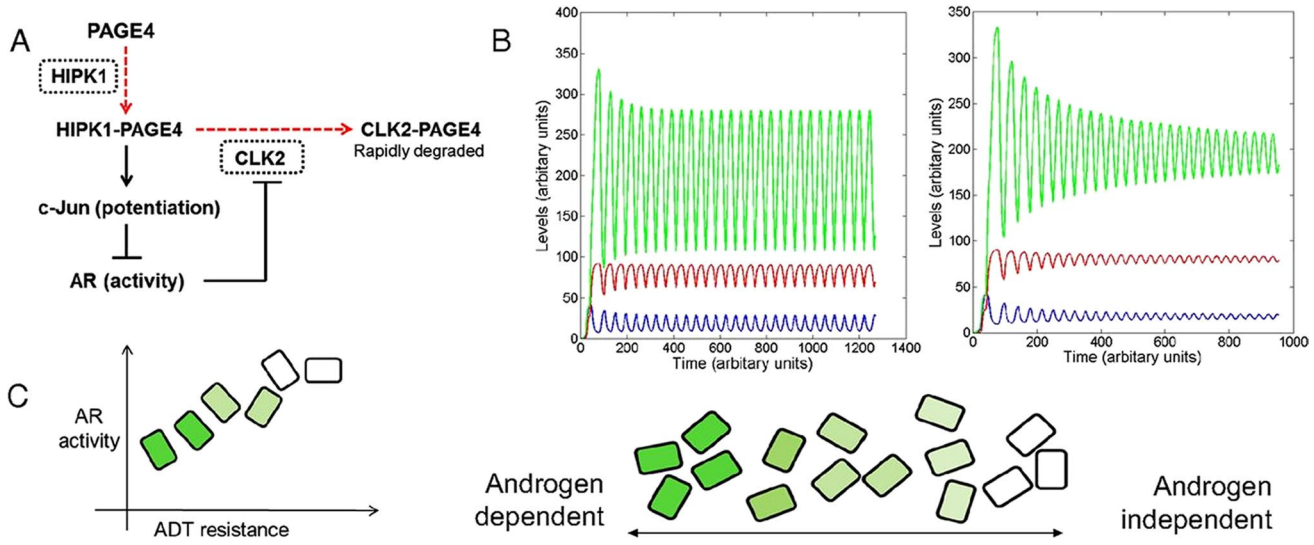


**Fig. 5** Modeling the PAGE4/AP-1/AR/CLK2 regulatory circuit. (**A**) Regulatory circuit for PAGE4/AP-1/AR/CLK2 interactions. Dashed red lines denote enzymatic reactions, and solid black lines denote non-enzymatic reactions. CLK2 and HIPK1, the two enzymes involved, are shown in dotted rectangles. (**B**) Dynamics of the circuit showing sustained and damped oscillations for HIPK1-PAGE4 ($PAGE4_M$, shown in blue), CLK2-PAGE4 ($PAGE4_H$, shown in red), and CLK2 (shown in green). (**C**) Distribution of androgen dependence for an isogenic population over a spectrum, as indicated by the shade of green. Dark green boxes denote highly androgen-dependent (i.e., ADT-sensitive) cells, and white boxes denote androgen-independent cells. Reproduced with permission from Kulkarni et al. 2017

can generate heterogeneity in an isogenic population by a non-genetic, IDP conformation-based mechanism. These oscillations can be dampened by depriving the system of androgen but may be reinstituted if deprivation is withheld or administered intermittently, suggesting that PCa cells can potentially transition from an androgen-independent to an androgen-dependent phenotype reversibly (Lin et al., 2018).

Corroborating these observations, a recent study (Lv et al, 2019) reported that PAGE4 overexpression in androgen-dependent (LNCaP) and independent (DU145) cells treated with hydrogen peroxide (H2O2) suppressed production of reactive oxygen species (ROS) in response to stress. However, co-expressing PAGE4 and CLK2 in these cells attenuated the ability of PAGE4 to suppress ROS suggesting that hyperphosphorylation inactivates PAGE4. On the other hand, co-expression of HIPK1 with PAGE4 reduced ROS production after H2O2 treatment in LNCaP. But in DU145 cells, co-transfection of HIPK1 and PAGE4 increased ROS suggesting that HIPK1 may impact PAGE4 function in a cell type-dependent manner.

## Coupled feedback loops involving PAGE4, EMT, and Notch signaling, and non-genetic heterogeneity in PCa cells

It is now well-recognized that non-genetic mechanisms can give rise to functional heterogeneity. However, the design principles of the regulatory networks are not fully understood. Therefore, we (Singh et al., 2021) examined coupled dynamics of feedback loops involving oscillations in and AR signaling mediated through PAGE4, multistability in EMT, and Notch-Delta-Jagged signaling mediated cell–cell communication, each of which can generate non-genetic heterogeneity through multistability and/or oscillations. Interestingly, we found that different coupling strengths between AR and EMT signaling can lead to monostability, bistability, or oscillations in the levels of AR, as well as propagation of oscillations to EMT dynamics (Fig. 6). More specifically, we observed that depending on the relative strengths of the effect of ZEB1, an EMT inducer, on AR and *vice-versa*, the stand-alone dynamical features of EMT and AR circuits (multistability and oscillations) could percolate to the other circuit. In other words, the EMT circuit exhibits oscillations, and/or the AR circuit exhibits multistability. While multistability in EMT has been reported previously (George et al., 2017; Karacosta et al., 2019; Ruscetti et al., 2016), this is the first report to suggest oscillations in EMT.

The bidirectional coupling between AR signaling and EMT suggests a potential link between progression towards a partial or full EMT with significant therapeutic implications. Thus, while the epithelial phenotype usually co-occurs with PAGE4 oscillations, transitions to hybrid E/M or mesenchymal phenotypes quench these oscillations and promote low AR levels. Therefore, EMT induction can potentially promote therapy resistance by stabilizing an androgen-independent PCa phenotype through the ZEB1-AR signaling

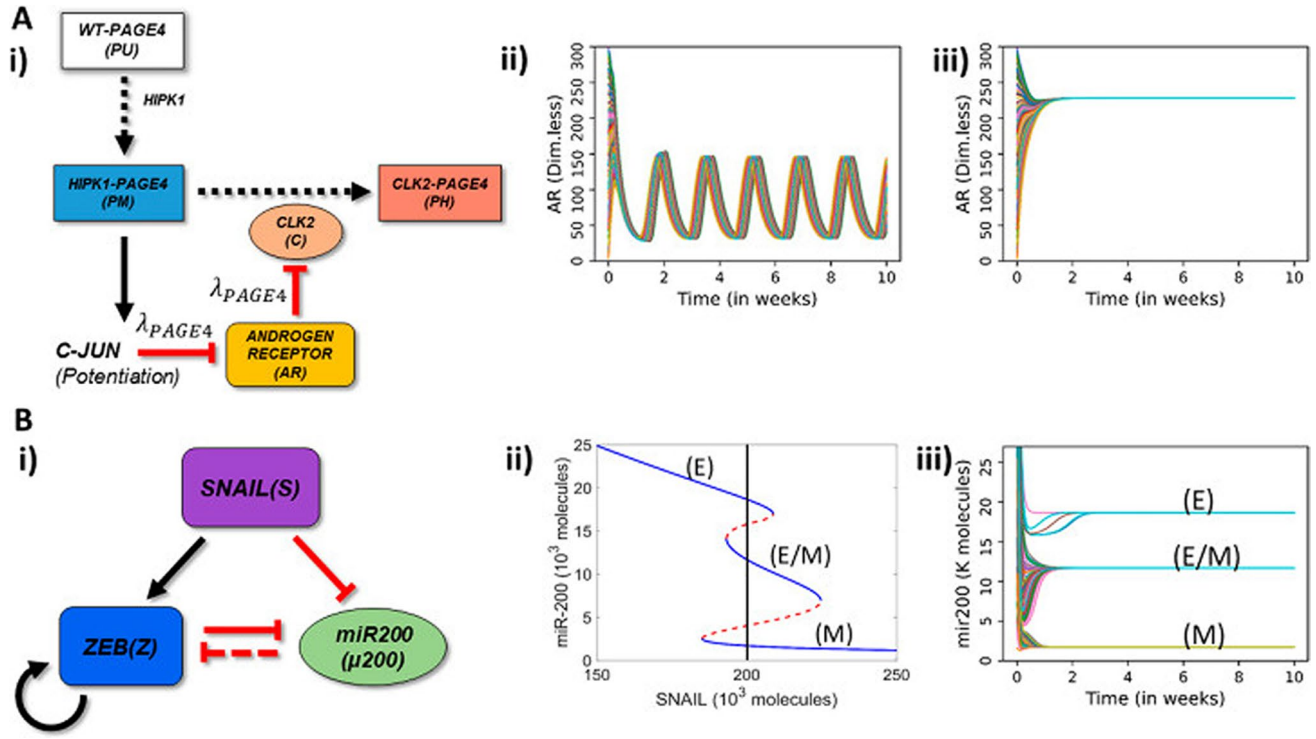


**Fig. 6** Schematic representation of PAGE4-AR and EMT circuits and their stand-alone dynamics. (A) (i) Schematic representation of PAGE4-Androgen Receptor (AR) circuit: The enzyme HIPK1 double phosphorylates WT-PAGE4 and forms the HIPK1-PAGE4 complex which can be further hyperphosphorylated by CLK2 enzyme. Solid arrows show activation, dotted arrows show phosphorylation and red hammer heads show inhibition. In turn, the HIPK1-PAGE4 complex regulates CLK2 levels via the intermediates c-JUN and AR. A strong inhibition of AR by c-JUN and that of CLK2 by AR leads to oscillations (λPAGE4 = 0.1) (ii) or a single steady state (mono-stability) (λPAGE4 = 0.9) (iii). (B) (i) EMT circuit: ZEB and microRNA-200 form a mutually inhibiting loop while SNAIL acts as an external EMT inducer. Solid arrows show transcriptional activation, dashed line show microRNA-mediated inhibition, and solid hammerheads show transcriptional inhibition. (ii) Bifurcation diagram of microRNA (miR)-200 as a function of SNAIL shows tristability, bistability or mono-stability depending on SNAIL levels. Blue and red curves show stable and unstable states respectively. The vertical black line depicts the SNAIL level (=200,000 molecules) used in panel (iii). (iii) Dynamics of miR-200 for SNAIL = 200 K showing the existence of three states-epithelial (high miR-200; 20 K molecules), mesenchymal (low miR-200; ~ 12 K molecules). In panels A—ii, A—iii, B—iii, different curves depict AR and miR-200 dynamics starting from multiple randomized initial conditions. Reproduced with permission from Singh et al. Entropy (Basel). 2021 Feb 26;23(3):288

axis. Similarly, coupling between AR and ZEB1 implies that EMT can promote a drug-resistant state (Zheng et al., 2015; Fischer et al., 2015). Conversely, a switch from drug-sensitive to drug-resistant state can also trigger EMT. Taken together, these results reveal the emergent dynamics of coupled oscillatory and multistable systems and shed new light on potential mechanisms of non-genetic heterogeneity and cellular decision-making that are actuated by IDP conformational dynamics.

## Conclusions and future directions

From the foregoing, it appears plausible that conformational noise contributed by IDP conformational dynamics is an additional source of noise that has hitherto remained unappreciated. As propagators of conformational and transcriptional noise, IDPs rewire PINs and unmask latent interactions in response to perturbations. Further, it may also be noted that IDPs could likely relay, and even amplify, other types of intrinsic and extrinsic noise and perturbations in the system. Therefore, noise-driven activation of latent pathways actuated by IDPs drives phenotypic switching and, thus, generates heterogeneity via non-genetic mechanisms as postulated by the MRK hypothesis.

Our cumulative efforts have provided empirical evidence for many aspects of the MRK hypothesis. However, a quantitative measure of conformational noise that is implied to originate from conformational dynamics of an IDP ensemble is still lacking. Nonetheless, the identification of the conformational preferences of the various phosphorylated forms of PAGE4 could be leveraged to authenticate conformational noise. For example, hyperphosphorylation of all 8 S/T residues in PAGE4 by CLK2

results in an almost random coil-like conformation that is non-functional. However, it is conceivable, perhaps highly plausible, that some S/T residues are more critical in unfolding the polypeptide than others. Hence, the relative abundance of these phosphoforms, the dynamics of CLK2, and the as yet unidentified phosphatase that dephosphorylates these S/T residues can impinge on the half-lives and the activity of the differentially phosphorylated ensembles of PAGE4 and, therefore, contribute to conformational noise. Interestingly, in the case of the Elk-1 transcription factor, multisite phosphorylation of 8 S/T residues also leads to opposing effects on its transcriptional activation potential. However, time-resolved NMR spectroscopy revealed that phosphorylation proceeds at significantly different rates (with differences ranging from > 30 min to 3 h), and while phosphorylation at the fast and intermediate sites promoted transactivation by Elk-1, phosphorylation at the slow site opposed it (Mylona et al., 2016), lending further support to the concept of conformational noise. Further research on PAGE4 and phenotypic switching in PCa that is currently under way in our respective laboratories should help gain deeper insight into IDP conformational noise and cellular decision-making.

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## Declarations

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