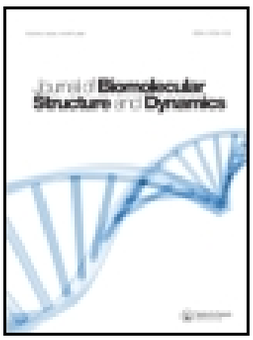

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Phenotypic suppression caused by resonance with light-dark cycles indicates the presence of a 24-hours oscillator in yeast and suggests a new role of intrinsically disordered protein regions as internal mediators

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ABSTRACT

The mutual interaction between environment and life is a main topic of biological sciences. An interesting aspect of this interaction is the existence of biological rhythms spanning all the levels of organisms from bacteria to humans. On the other hand, the existence of a coupling between external oscillatory stimuli and adaptation and evolution rate of biological systems is a still unexplored issue. Here we give the demonstration of a substantial increase of heritable phenotypic changes in yeast, an organism lacking a photoreception system, when growing at 12 h light/dark cycles, with respect to both stable dark (or light) or non-12 + 12 h cycling. The model system was a yeast strain lacking a gene whose product is at the crossroad of many different physiological regulations, so ruling out any simple explanation in terms of increase in reverse gene mutations. The abundance of intrinsically disordered protein regions (IDPRs) in both deleted gene product and in its vast ensemble of interactors supports the hypothesis that resonance with the environmental cycle might be mediated by intrinsic disorder-driven interactions of protein molecules. This result opens to the speculation of the effect of environment/biological resonance phenomena in evolution and of the role of protein intrinsically disordered regions as internal mediators.

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Oscillatory dynamics; entrainment; resonance; Kluyveromyces lactis; mutation

Introduction

Living organisms evolved on Earth in response to environmental stimuli of physical, chemical and biological origin. Changes of the biosphere were two-ways as environment can influence the evolution of organisms and organisms can in turn modify the environment. Adaptation to the changing environment is one of the leading processes in the development of life. With respect to the life cycles, environmental parameters can vary in a monotonic way (*i.e.* on time scales much longer than life cycles) or periodically, with frequencies comparable to those of internal biological oscillators (Winfree, 1967). Examples of such cycles are those associated to astronomic properties like day-night or season alternation. In particular, life evolved in parallel with light-darkness (LD) cycles of the 24 h period of Earth rotation generating living organisms with internal molecular clocks of circadian period. These cycles keep the physiology of the organism entrained to the day-night periodicity and are present in bacteria, fungi and higher eukaryotes, where metabolism and activities are dependent on LD cycles. Circadian clocks stem from transcription-translation feedback loops of clock genes and regulate many LD dependent cellular functions (Panda et al., 2002; Zhang et al., 2014).

Yeasts are unicellular fungi including *Saccharomyces cerevisiae*, an extensively studied model organism used in basic science and industrial applications. Some of *S. cerevisiae* internal cycles share common features with circadian clocks (see (Mellor, 2016) and (Causton, 2020) for recent reviewing). These cycles, called yeast respiratory oscillations (YRO) or yeast metabolic cycles (YMC), emerge when cells spontaneously synchronize in continuous cultivation and are coupled - *inter alia* - with the redox metabolism of the cell, with DNA synthesis and cell division. YROs are ultra-dian cycles with periods shorter than 24 h. It has been proposed that cell-produced sulphidric acid might be the molecular signal that synchronize cultured cells and allows temporal separation of metabolic activities (Tu et al., 2005). Interestingly, YRO is affected by light intensity, frequency and timing (Robertson et al., 2013). The presence of innate oscillators might be rather rooted in yeast cells but difficult to show in the ergodic system of standard batch cultivations (Bianchi, 2008). However, even in these cases of apparent unsynchronized

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growth, collective cyclic behaviors of the cell population can be detected, based on internal cellular dynamics and cell cross-talking (Tsuchyia et al., 2007; Romagnoli et al., 2011).

Differently, cyclic environmental parameters might be able to synchronize cells by an active entrainment process that keeps in phase the environmental parameter and the cellular output(s) controlled by the internal mechanism. This system has been used to show medium acidification as metabolic output of a hypothetical clock in *S. cerevisiae* (Eelderink-Chen et al., 2010). A different experiment based on entrainment of yeast (Edmunds et al., 1979) showed that, when yeast was grown at very low temperature, cyclic cell duplication and cyclic anabolic activities emerged. However, these results were never confirmed and other scientists (Robertson et al., 2013) excluded the presence in yeast of a circadian cycle regulated by light.

An interesting issue related to the presence of molecular clocks is the connection of organism fitness, reproduction, and population development with entrainment to environmental cycles. Mechanisms underlying this connection might be relevant for the selection of adapted genetic variants or species. For example, resonance with LD cycles has been shown to select *Synechococcus* species with endogenous clock of period closer to the environmental cycle (Ouyang et al., 1998). A different question is if the cellular mechanisms generating genetic and/or epigenetic variability might be under the control of endogenous oscillators, other than canonical clocks and, hence, increase the number of individual variants in the population under entrainment with environmental cycles.

In order to explore this issue, we used the genetic system of phenotype extragenic suppression in a haploid yeast strain with the deletion of a regulatory gene. To this aim, we selected the *KlMGA2* gene from the *Kluyveromyces lactis* yeast, because the KlMga2 protein participates in many different regulatory activities, possibly involving many other different regulatory factors. Due to the absence of the gene, the suppression of the phenotype associated with the deletion should be dependent on genetic or epigenetic variations occurring in the many other genes participating to the same pathways of the deleted gene. Our analysis on KlMga2 revealed that this is a multi-functional protein that contain high level of intrinsic disorder, with more than 23% of its residues being involved in disorder-based recognition, thereby serving as a foundation of its multi-functionality. The abundance of intrinsically disordered protein regions (IDPRs) in both the deleted gene product and in its vast ensemble of interactors supported the conjecture of both the spreading of oscillatory stimulus and the multiplicity of possible phenotypic reversion models.

However, which new gene or which mechanism (genetic or epigenetic) was involved in the phenotypic suppression was beyond the scope of this work. In the same way we did not answer to the question if the observed entrainment could be defined as a proper 'clock' (and thus presenting features like temperature dependence or specific photoreceptors): we limited our investigation to the demonstration of a measurable population level response elicited by an external oscillation. As a matter of fact we observed that cells responded to the oscillatory character of the stimulus at a peculiar frequency and not to its energy content: this made mandatory to think of a resonance phenomenon and consequently demonstrated the existence of (still unknown) internal oscillators. Beside possible mechanistic hypotheses, we concentrated on the rate of generating cells with suppressed phenotype, hence on the increase in the rate of generation of new genetic/epigenetic variant clones as a function of the oscillating physical stimulus. One crucial point was the observed 'heritability' and thus the stability of phenotypic reversion. The heritable character of phenotypic reversion opens the way to the possible role of resonance phenomena in evolution.

Materials and methods

Selection of rag + reverted clones

The *K. lactis* GDK/*Klmga2*Δ strain (Micolonghi et al., 2012) was inoculated in liquid YPD medium (1% yeast extract, 1% peptone, 2% glucose) at a starting cell density of $4 \pm 1 \times 10^5$ cells/ml and grown for 48 h to $2 \pm 0.8 \times 10^8$ cells/ml (9 ± 1 duplications) under LD cycles. Cycles ranged from $2 + 2$ h to $24 + 24$ h; all light (LL) and all dark (DD) experiments were also performed; light intensity was 134 μM/s/m^2 (sunny outdoor ≈2000 μM/s/m^2, indoor ≈10 μM/s/m^2), and all cycle experiments started with a light phase. Two to eight independent experiments were performed for each cycle length. At the end of the experiment, cells were counted under microscope in a Burker chamber, cultures were opportunely diluted with sterile water and about $10^5 \div 10^6$ cells were plated onto 3 GAA plates (1% yeast extract, 1% peptone, 5% glucose, 5 μM antimycin A plus 2% agar for solid medium). Growth of the suppressed clones were scored after $4 \div 5$ days and recorded as number of Colony Forming Units (CFU) per plated cells (%).

Computational analysis of the KlMga2 protein

Intrinsic disorder predisposition of the KlMga2 protein (UniProt ID: Q6CMS9) was assessed by a set of commonly used per-residue disorder predictors from the PONDR family, such as PONDR® VLXT (Romero et al., 2001), PONDR® VSL2 (Obradovic et al., 2005; Peng, Radivojac, Vucetic, Dunker & Obradovic, 2006), and PONDR® VL3 (Peng et al., 2005) available at (http://www.pondr.com) and by the PONDR® FIT metapredictor (Xue et al., 2010) accessible at (http://original.disprot.org/metapredictor.php). The outputs of these tools are represented as real numbers between 1 (ideal prediction of disorder) and 0 (ideal prediction of order). A threshold of ≥0.5 was used to identify disordered residues and regions in a query protein. A protein region was considered flexible, if its disorder propensity was in a range from 0.2 to 0.5.

Complementary disorder analyses together with important disorder-related functional information were retrieved from the D^2P^2 database (http://d2p2.pro/) (Oates et al., 2013), which is a database of predicted disorder for a large library

of proteins from completely sequenced genomes (Oates et al., 2013). D^2P^2 database uses outputs of IUPred (Dosztanyi et al., 2005), PONDR® VLXT (Romero et al., 2001), PrDOS (Ishida & Kinoshita, 2007), PONDR® VSL2 (Obradovic et al., 2005; Peng et al., 2006), PV2 (Oates et al., 2013), and ESpritz (Walsh et al., 2012). The output of this platform is further supplemented by data concerning location of various curated posttranslational modifications and predicted disorder-based protein binding sites, known as molecular recognition features, MoRFs (Oates et al., 2013).

Information on the interactability of the subunits the KlMga2 protein was retrieved using Search Tool for the Retrieval of Interacting Genes; STRING, http://string-db.org/. STRING generates a protein-protein interaction (PPI) network based on predicted and experimentally-validated information on the interaction partners of a protein of interest (Szklarczyk et al., 2011). In the corresponding network, the nodes correspond to proteins, whereas the edges show predicted or known functional associations. Seven types of evidence are used to build the corresponding network, where they are indicated by the differently colored lines: a green line represents neighborhood evidence; a red line, the presence of fusion evidence; a purple line, experimental evidence; a blue line, co-occurrence evidence; a light blue line, database evidence; a yellow line, text mining evidence; and a black line, co-expression evidence (Szklarczyk et al., 2011). In this study, STRING was utilized in two different modes: to generate the PPI network of the KlMga2 protein and to produce the PPI network centered at the KlMga2 protein PPI network. Resulting PPI networks were further analyzed using STRING-embedded routine in order to retrieve the network-related statistics, such as number of nodes (proteins), number of edges (interactions), average node degree (average number of interactions per protein), average local clustering coefficient, which defines how close the neighbors are to being a complete clique. If a local clustering coefficient is equal to 1, then every neighbor connected to a given node N_i is also connected to every other node within the neighborhood, and if it is equal to 0, then no node that is connected to a given node N_i connects to any other node that is connected to N_i, expected number of edges (which is a number of interactions among the proteins in a random set of proteins of similar size), and a PPI enrichment p-value (which is a reflection of the fact that query proteins in the analyzed PPI network have more interactions among themselves than what would be expected for a random set of proteins of similar size, drawn from the genome. Such an enrichment indicates that the proteins are at least partially biologically connected, as a group).

Results

Deletion of KlMGA2 gene

The yeast *Kluyveromyces lactis* and the *KlMGA2* gene were chosen for our experiment. In yeasts, the Mga2 proteins regulate the expression of the fatty acid desaturase genes, necessary for the synthesis of the essential unsaturated fatty acids (Santomartino et al., 2017). Regulation occurs at the level of transcription. In *K. lactis*, the deletion of the *KlMGA2* gene (*Klmga2*Δ strain) causes many defects and phenotypes in addition to an altered fatty acid composition of membranes. These phenotypes involve: reduced expression of ergosterol biosynthetic genes and of glucose catabolic genes, altered mitochondrial morphology and respiration rate, extended longevity and increased resistance to oxidative stress, multi-budded cell morphology, reduced growth rate, sensitivity to the mitochondrial drug antimycin A on high (5%) glucose medium (rag^- phenotype) (Micolonghi et al., 2012; Ottaviano et al., 2015; Santomartino et al., 2019). This extended panel of defects deriving from the deletion of *KlMGA2* indicates that the KlMga2 protein participates in many different regulatory activities, possibly involving many other different regulatory factors. The suppression of a single phenotype in the deleted strain might therefore occur by a new mutation of a specific regulatory element. Eventually, two or more phenotypes might be suppressed simultaneously.

Computational analysis of the intrinsic disorder predisposition, disorder-based functionality, and binding promiscuity of the KlMga2 protein

To gain better understanding of the reasons for these multifaceted consequences of the deletion of the *KlMGA2* gene, we conducted a computational analysis of the intrinsic disorder predisposition of the KlMga2 protein using several commonly used per-residue predictors of intrinsic disorder predisposition, such as PONDR-FIT, PONDR® VLXT, PONDR® VSL2, and PONDR® VL3 (Li et al., 1999; Romero et al., 2001; Obradovic et al., 2005; Peng et al., 2005; Peng et al., 2006; Xue et al., 2010) (Figure 1A).

We also used a D^2P^2 platform (http://d2p2.pro/) (Oates et al., 2013) that complement evaluations of the disorder predisposition of a query protein by IUPred (Dosztanyi et al., 2005), PONDR® VLXT(Romero et al., 2001), PrDOS (Ishida & Kinoshita, 2007), PONDR® VSL2B (Obradovic et al., 2005; Peng et al., 2006), PV2 (Oates et al., 2013), and ESpritz (Walsh et al., 2012) with some disorder-related functional information (Figure 1B). Results of these analyses are summarized in Figure 1, which clearly shows that the KlMga2 protein is expected to contain high levels of intrinsic disorder. In fact, almost 60% of the residues of this 1151 aminoacid-long protein are predicted as disordered, being assembled in four long disordered regions (residues 1-150, 377-583, 702-742, and 869-1055) and numerous short disordered regions. Figure 1B shows that these long, disordered regions include multiple disorder-based binding sites, known as molecular recognition features (MoRFs). MoRFs are specific protein regions that are disordered in their unbound state, but that can fold at interaction with specific partners (Romero et al., 2001; Dunker et al., 2002; Tompa, 2002; Daughdrill et al., 2005; Oldfield et al., 2005; Radivojac et al., 2007; Dunker et al., 2008; Dunker & Uversky, 2008; Uversky & Dunker, 2010; Uversky, 2011; Uversky, 2012; Uversky 2013), and which can be predicted by several computational means, including the ANCHOR algorithm (Dosztanyi et al., 2009; Meszaros et al., 2009) that utilizes the pair-wise energy estimation

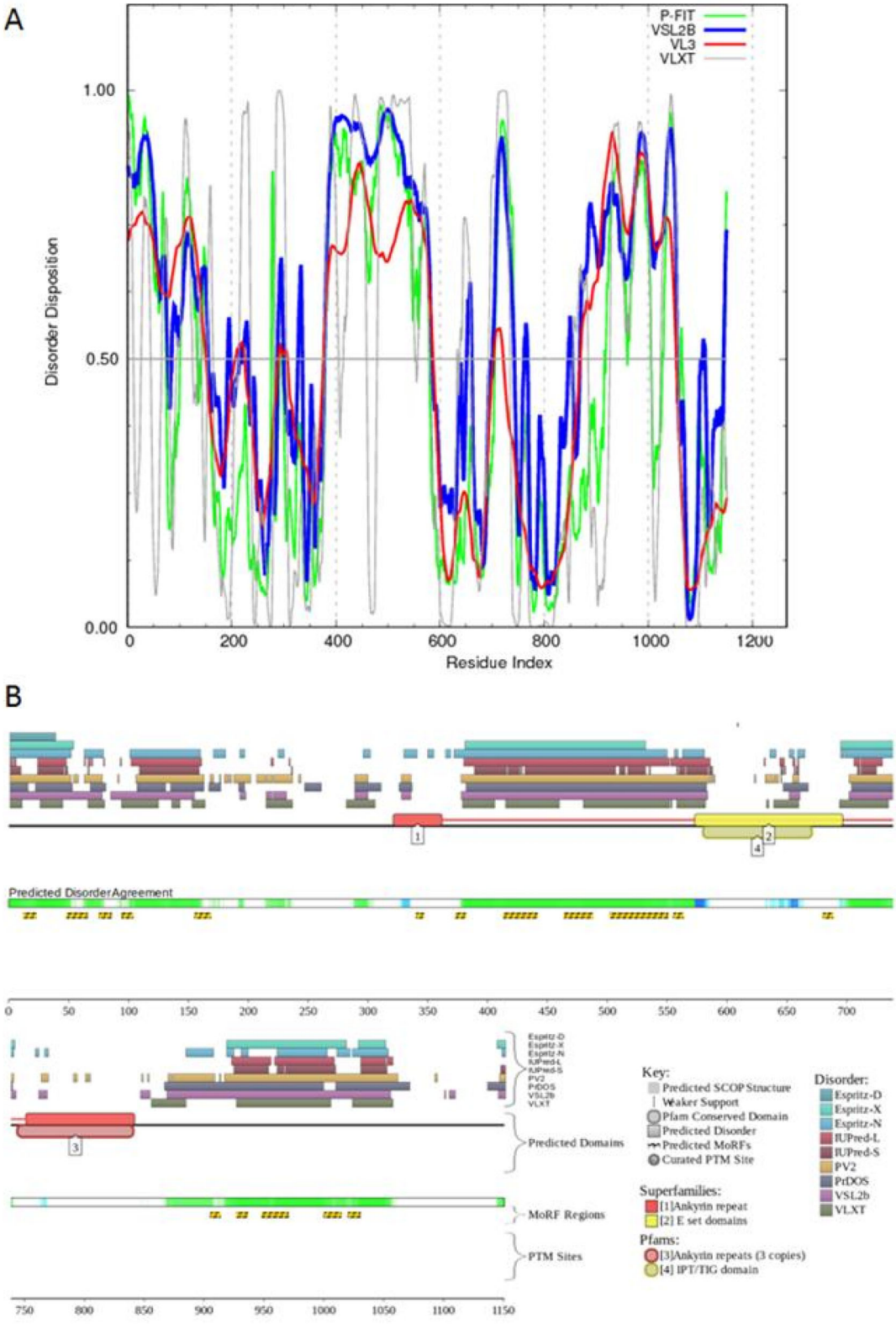

Figure 1. Multifactorial computational disorder analysis of the KIMga2 protein from *K. lactis* yeast. **1 A.** Intrinsic disorder profile of KIMga2 (UniProt ID: Q6CMS9) generated by the superposition of the outputs of PONDR® VLXT, PONDR® FIT, PONDR® VL3, and PONDR® VSL2. **1B.** Intrinsic disorder propensity and some important disorder-related functional information generated for the KIMga2 protein by the D^2P^2 database (http://d2p2.pro/)(Oates et al. 2013) Here, results of the evaluation of disorder predisposition by outputs of IUPred (Dosztanyi et al., 2005), PONDR® VLXT (Romero et al., 2001), PrDOS (Ishida & Kinoshita, 2007), PONDR® VSL2B (Obradovic et al., 2005; Peng, Radivojac, Vucetic, Dunker & Obradovic, 2006), PV2 (Oates et al., 2013), and ESpritz (Walsh et al., 2012) are enhanced by showing some disorder-related functional information, such as positions of the disorder-based interactions sites (MoRFs).

approach originally used by IUPred (Dosztanyi et al., 2005). Such disordered regions, which are able to undergo at least partial disorder-to-order transitions upon binding, are known to play crucial roles in recognition, regulation, and signalling (Wright & Dyson, 1999; Uversky et al., 2000; Romero et al., 2001; Dyson & Wright, 2002; Dyson & Wright, 2005; Oldfield et al., 2005; Mohan et al., 2006; Vacic et al., 2007; Uversky 2013; Uversky 2013). Figure 1B shows that the KlMga2 protein possesses18 MoRFs that include 268 of 1151 residues, indicating that at least 23.2% of the KlMga2 residues can be potentially involved in disorder-based recognition and thereby can serve as a foundation of the multi-functionality of this protein.

It is known that KlMga2 has several functional domains, such as ankyrin repeat regions (residues 322-362 and 752-842), E-set domain (residues 574-697) with embedded IPT/TIG domain (residues 581-666), and α-helical trans-membrane region (TM, residues 1074-1093). Ankyrin repeat is a 33-residue motif consisting of β-hairpin –α-helix– loop– α-helix fold (Zhang & Peng, 2000). Although proteins typically have four or more complete ankyrin repeats, which provide stabilizing interactions between adjacent modules, a minimal autonomous folding unit in such proteins was shown to contain two repeats (Zhang & Peng, 2000). Ankyrin repeat regions are found in many eukaryotic proteins, where they function as protein-protein interaction domains (Bork, 1993; Mosavi et al., 2004). In line with these observations, Figure 1B shows that the first ankyrin repeat (residues 322-362) of KlMga2 includes one of MoRFs (residues 340-346). IPT/TIG domains (Immunoglobulin-like fold, Plexins, Transcription factors, or Transcription factor Immunoglobin) have an immunoglobulin-like fold and are found in cell surface receptors, where they are used for protein-protein interactions (Chen et al., 2013), and in some transcription factors, where they serve as DNA binding domains (Liao, 2009). E-set domains also have immunoglobulin-like fold and often serve as carbohydrate-binding modules (Elleuche et al., 2017). Therefore, E-set domain might have multiple functions, ranging from protein-protein to protein-DNA, and to protein-carbohydrate interactions. Although E-set domain is mostly ordered in KlMga2, it contains a rather long IDPR (residues 639-659) and one of the MoRFs (residues 680-688).

The promiscuity of the KlMga2 protein is illustrated by Figure 2A representing STRING-generated protein-protein interaction network of this protein. This network contains 16 nodes connected by 39 edges. Proteins in this network, listed in Table 1, are associated with a wide spectrum of cellular processes, such as fatty acid biosynthesis and a number of concurrent activities.

Furthermore, when one adds a second shell of interactors (*i.e.* proteins interacting with proteins that directly bind to KlMga2), the resulting network increases to 499 nodes linked by 5531 edges (see Figure 2B). Proteins in this network are associated with 45 biological processes annotated by gene ontology (GO), including response to chemicals, response to stimuli, response to stress, proteolysis, protein catabolic process, protein metabolic process, cellular protein modification process, macromolecule modification, regulation of cellular component organization, reproduction of a single-celled organism, regulation of protein metabolic process, protein modification by small protein conjugation or removal, response to endoplasmic reticulum stress, ubiquitin-dependent ERAD pathway, meiotic cell cycle process, macromolecule localization, cell development, cytoskeleton organization, and cellular response to topologically incorrect protein.

These observations provide some logical support to the functional plasticity of the KlMga2 protein and explain why deletion of the *KlMGA2* gene has so many different phenotypical outputs. This large panel of interactors also provide an extended variety of potential extragenic suppressors involved in the phenotypic reversion of the deleted strain.

Restoring normal phenotype of Klmga2Δ strain via resonance with LD cycles

In our experiment, we took advantage of the reduced growth rate and of the rag$^-$ phenotype of the mutant strain (Figure 3A).

The reduced growth rate resulted in small sized colonies on standard YPD medium. The rag$^-$ phenotype corresponded to strongly impaired growth in selective GAA medium. The reduced growth rate could be ascribed to a general reduction of cellular fitness due to the various defects of the deleted strain. The rag$^-$ phenotype is usually due to defects in the glycolytic and/or fermentative pathways or in their regulation (Wésolowski-Louvel et al., 1992): the basis of this phenotype in *Klmga2*Δ strains has been analyzed in Ottaviano *et al*. (Ottaviano et al., 2015). We have noticed that the *Klmga2*Δ strain reverted spontaneously to the rag$^+$ phenotype with a reasonably high frequency (Figure 3B): rag$^+$ clones could be easily selected by plating cell cultures on GAA medium and were characterized by small or large colony size. Small colonies (more numerous) were suppressed only in the rag$^-$ phenotype, while large colonies (less numerous) were suppressed also in the slow growth phenotype. As stated above, we measured the rate of generation of suppressed clones when cells were cultivated under standard conditions (YPD medium and 29 ± 1 °C) but with light-dark cycles of various phase length.

Results reported in Figure 4 show that average occurrence of suppressed clones ranged from 0.015% (16 + 16 h) to 0.049% (8 + 8 h) without significant difference to LL (0.031%) or DD (0.026%), except for the 12 + 12 h cycle that reached 0.2% occurrence.

This implies an approximately one order of magnitude increase in phenotype suppression corresponding to the 12 + 12 h cycle. This substantial increase scored a highly statistically significant difference with all other conditions ($p < 0.01$ with 6 + 6 and 8 + 8 h; $p < 0.001$ with LL, DD and the remaining LD cycles by Wilcoxon non-parametric test). This result suggests that yeast cells contain an endogenous 12 + 12 h oscillator that positively regulates mechanism(s) operating in phenotype suppression. These mechanism(s) could enhance in efficiency when the endogenous oscillator and the external entraining cycle resonate at the same frequency. Interestingly, LD cycles of 4 + 4 and 6 + 6 h, which

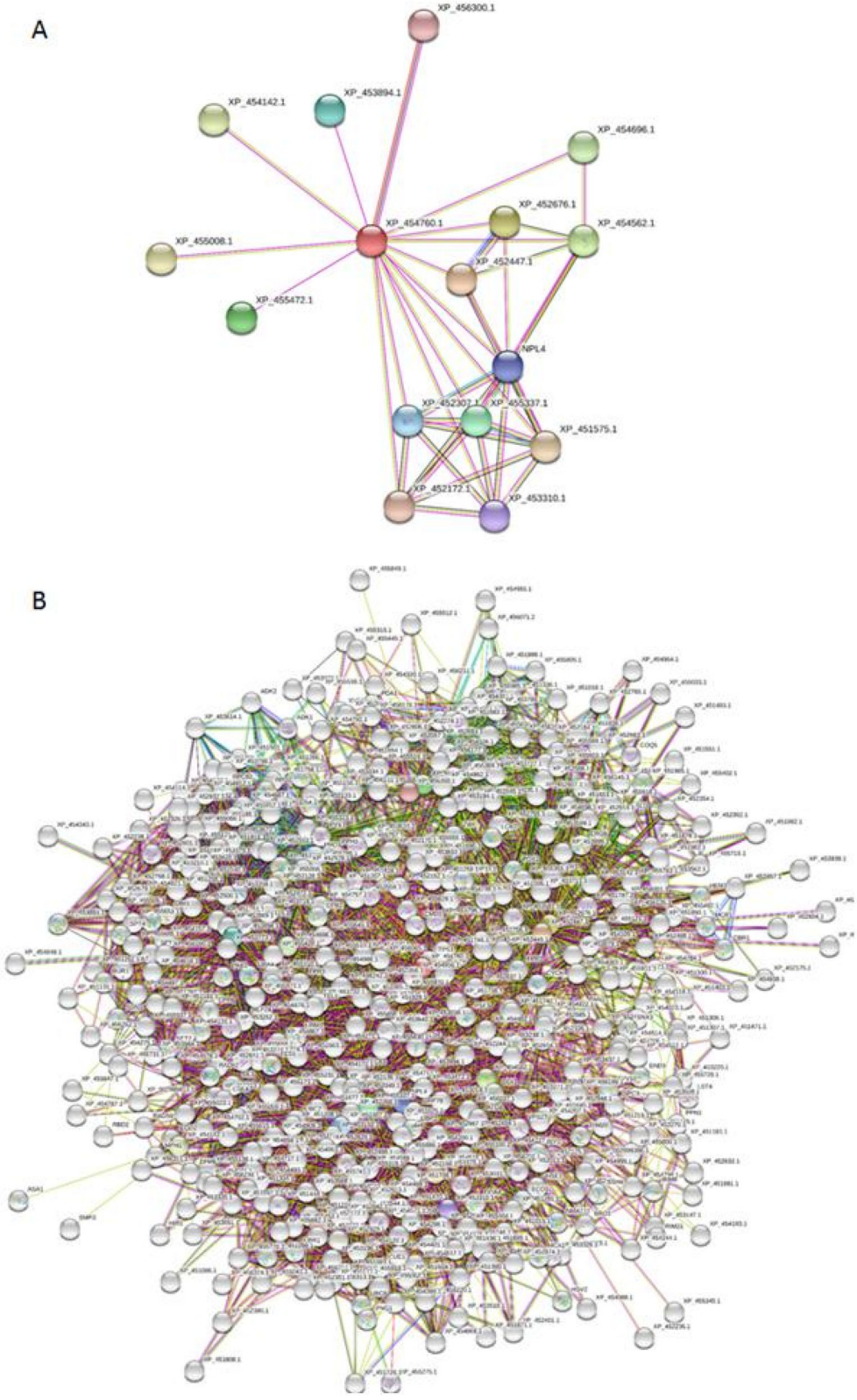

Figure 2. Analysis of the interactability of the KlMga2 protein from the yeast *K. lactis*. The analysis was performed by STRING (Search Tool for retrieval of interacting genes) resource, which is a web-based platform integrating publically available information on protein-protein interactions (PPIs) with computational predictions for a query protein (Szklarczyk et al., 2019). **1 A**. A network of 15 proteins from *K. lactis* interacting with the KlMga2 protein. A moderate confidence score of 0.4 was used to obtain this multi-protein interaction network. This network includes 16 nodes connected by 39 edges. Therefore, KlMga2-centered PPI network is characterized by an average node degree of 4.88 and has an averaged local clustering coefficient of 0.868. The average local clustering coefficient defines how close its neighbours are to being a complete clique; local clustering coefficient is equal to 1, if every neighbour connected to a given node N_i is also connected to every other node within the neighbourhood, and it is equal to 0 if no node that is connected to a given node N_i connects to any other node that is connected to N_i. Since the expected number of interactions among proteins in a similar size set of proteins randomly selected from a yeast proteome is equal to 17, this PPI network has significantly more interactions than expected, being characterized by a PPI enrichment p-value of $< 12.19 \times 10^{-6}$. **1B**. Extended PPI network of proteins involved in interaction with the *K. lactis* KlMga2. Here, proteins from the second shell (*i.e.* proteins interacting with the proteins that bind to KlMga2) are added. This network includes 499 proteins linked by 5,631 interactions, which is significantly more than the expected number of interactions among proteins in a similar size random set of proteins (3,091). It is characterized by an average node degree of 22.2, has an averaged local clustering coefficient of 0.482, and a PPI enrichment p-value of $< 10^{-16}$.

Table 1. Interactors of *Kl*Mga2.

Pathway[a]	*Kl*protein[b]	*Kl*gene[c]	*Sc*gene[d]	Function[e]
Fatty acid biosynthesis	XP_452447.1	*KlOLE1*	*OLE1*	Acyl-CoA desaturase
	XP_452676.1	*KLLA0C10692g*	absent	Putative desaturase, unknown function (Santomartino et al., 2017)
Protein ubiquitination and degradation	XP_454562.1	*KLLA0E13575g*	*RSP5*	E3 ubiquitin-protein ligase
	XP_452307.1	*KLLA0C02475g*	*UFD1*	Proteasomal protein degradation
	XP_453310.1	*KLLA0D05599g*	*YOD1*	Protein deubiquitination
	XP_452172.1	*KLLA0B14410g*	*DOA1*	Ubiquitin-mediated protein degradation
	XP_455337.1	*KLLA0F05676g*	*CDC48*	ATP-dependent translocation of ubiquitinated proteins
	XP_455008.1	*KLLA0E23409g*	*YTA6*	ATPase, Cdc48 family
	XP_455058.1	*KLLA0E24575g*	*NPL4*	Cdc48-dependent protein degradation, transcription regulation
Phosphorylation	XP_455472.1	*KLLA0F08635g*	*HSD1*	CTP-dependent ER diacylglycerol kinase, lipid metabolism
Vacuolar protein targeting and cell cycle regulation	XP_453894.1	*KLLA0D18799g*	*VPS64*	ER protein, unknown function
Secretion	XP_451575.1	*KlSEL1*	*UBX2*	Secretion lowering protein, ubiquitinated protein metabolism, lipid homeostasis
Transcription regulation	XP_454142.1	*KLLA0E04379g*	*SAP1*	ATPase of the AAA family SIN1-associated protein SAP1
	XP_454696.1	*KLLA0E16567g*	*ESS1*	Peptidyl-prolyl *cis-trans* isomerase, RNA PolII complex

[a]General cellular pathway or function.
[b]Protein id.
[c]*K. lactis* gene name or systematic ORF name (http://gryc.inra.fr).
[d]*S. cerevisiae* gene name.
[e]Relevant specific protein function.

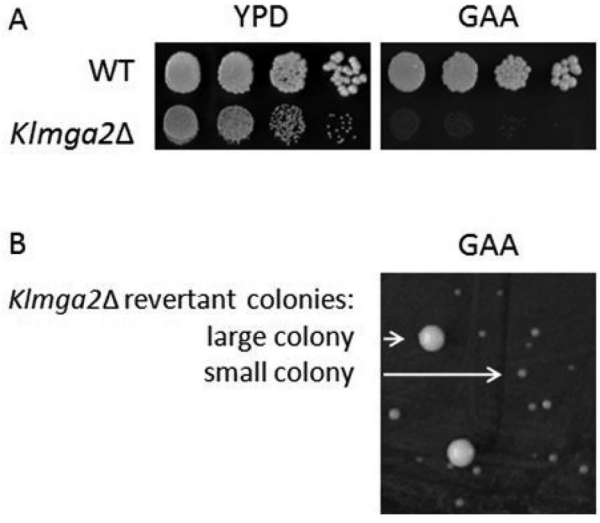

Figure 3. Phenotypes and reversion of the *Klmga2*Δ strain. **1 A**. Figure reports serial dilutions of the wild type (WT) and of the *Klmga2*Δ mutant strains plated on complete medium (YPD) and antimycin A-containing selective plates (GAA), to obtain single colonies. The figure highlights the slow growth phenotype of the mutant strain (small colony on YPD) in comparison with the wild type (large colony) strain, and the rag$^-$ phenotype (strongly reduced growth on GAA; see also supplementary material Figure SM1) of the mutant strain. **1B**. Appearance of extragenic-suppressed reverted clones in the mutant cell population on GAA medium. Arrows point to typical large and small colony clones.

were the closest to the *Klmga2*Δ strain duplication time (5.5 h), did not produce suppression increase, suggesting that the cell resonating oscillator was not related to the general cell metabolism or physiology but rather to an environmental adaptation to the Earth rotational frequency.

Statistical analysis also showed that the number of suppressed clones (CFU) did not correlate neither with the number of cycles (Pearson $r = 0.183$) nor with the length of cycles (Pearson $r = 0.152$). We can explain these two observations assuming that the decisive feature of the stimulus is only its cyclic nature with a specific frequency; *i.e.* we have a resonance phenomenon. Furthermore, Figure 4 shows the variability bars corresponding to the square root of the variance of the CFU. The variance (at odds with standard error that is related to the uncertainty of the mean) follows the natural fluctuations of the system and in the 12 h case we had the largest bar. The large variance at the peak frequency is typical to that of a system approaching a phase transition. In phase transitions, every system (gas, metal and so on) has a physical quantity that quantifies the response to an external stimulus, the so-called susceptibility. It can be showed (Stanley, 1971) that susceptibility is proportional to the system correlation (to the variance, as in our case) and to the reciprocal of the difference between a critical parameter (in our case, the light-dark period) and a critical value (12 h). In summary, as the system tends toward the frequency peak, the variance (the fluctuation around the mean value) diverges, so the response diverges. In our particular yeast case, the response variable was the amount of phenotypic suppression that, as expected, diverged at the characteristic frequency inducing transition.

Both small and large colony rag$^+$ suppressed clones were produced when the small colony-rag$^-$ *Klmga2*Δ strain was cultivated under LD cycles. Small-rag$^+$ colonies were the by far majority of suppressed clones and consequently their number strongly correlated (Pearson $r = 0.998$, $R^2 = 0.995$) with the total number of colonies (Figure 5A).

The large-rag$^+$ colonies were fewer and not correlated to the total of number of colonies (Pearson $r = 0.478$, $R^2 = 0.228$). This result points to the fact that large colonies are an independent population with respect to the 'bulk' of colonies and their number is not affected by plating efficiency. Of course, the occurrence of large-rag$^-$ clones could not be determined because of the experimental design intended to select only rag$^+$ colonies. Occurrence of large and small rag$^+$ colonies was not correlated each other (Pearson $r = 0.416$, $R^2 = 0.173$; Figure 5B), indicating that the events generating suppression of the rag$^-$ phenotype and of the slow growth phenotype were independent. Interference of plating efficiency with number of colonies (Figure 5C) was assayed by calculating the correlation coefficient between large, small

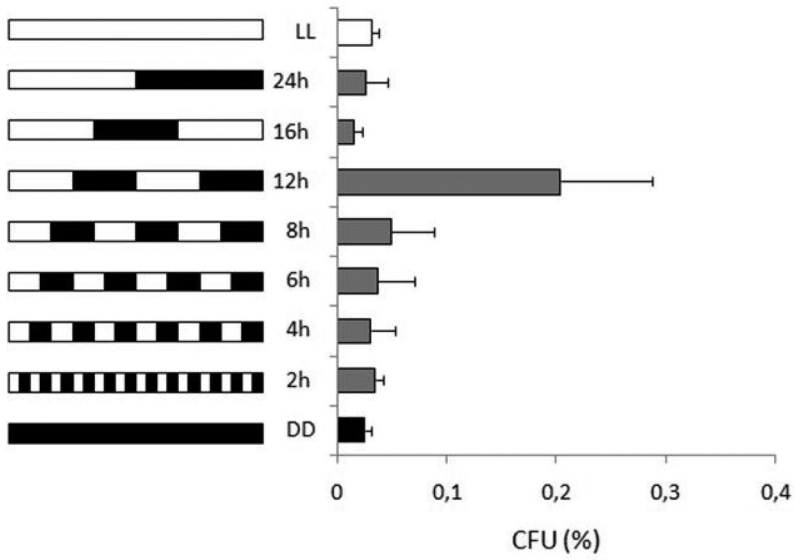

Figure 4. Rate of phenotypic suppression in *Klmga2*Δ strain. The average percentages (CFU) of generation of suppressed cell colonies are reported in the histograms together with relative among experiments standard deviations. Length of light (dark) periods are indicated in hours (h). All light and all dark incubation experiments are indicated as LL and DD, respectively. The left part of the figure reports the scheme of light-dark alternation. Percentage of CFU at $t = 0$ ranged from 0.0005 to 0.001 (supplementary material Figure SM2).

and total rag$^+$ colonies per plate and the total number of plated cells. No relevant correlation could be found with the number of plated cells with the three classes of large-rag$^+$ (Pearson $r = 0.324$), small-rag$^+$ (Pearson $r = 0.408$) and total colonies (Pearson $r = 0.419$) so ruling out any relevant confounding effect due to plating variability.

The suppression of phenotypes was stable: cells picked from small or large rag$^+$ colonies were grown without selective pressure (YPD medium) for 24 h, opportunely diluted and plated onto YPD plates to allow growth of hundreds single colonies. YPD plates were then replicated onto GAA plates and scored for rag$^+$ phenotype or rag$^-$ back reversion: all colonies were rag$^+$ indicating the stability, and thus the heritability, of the genetic/epigenetic suppression. Furthermore, large-rag$^+$ colonies yielded only large-rag$^+$ colonies suggesting that also the reversion of the *Klmga2*Δ strain to large colony phenotype, which was the wild type rate of growth, was stable.

Finally, in order to establish the long-time period of cell composition of the culture in terms of the various phenotypes (original mutant strain, small-colony rag$^+$ suppressed, large-colony rag$^+$ suppressed), we set up a prolonged cultivation in non-selective YPD medium at constant light (10 μM/s/m^2, as for routine yeast cultivation in the thermostatic room) by several serial passages of dilution (1:1000) of 3-4 days grown cultures into fresh medium. At each step, the percentages of large- and small-colony rag$^+$ cells were determined by plating opportune dilutions onto GAA plates. Results (supplementary material Figure SM3**)** showed that the reverted rag$^+$ cell fraction reached the 63% of the total cell population, with the majority (53%) of large-colony rag$^+$ cells, after 10 passages in fresh medium. Our experiment was set to select only Rag + reverted clones. Hence, other best fitted reversions, like as large-colony rag$^-$ cells, could not be evidenced but could be a relevant part of the final culture composition. Notably, the predominance of the reverted cells in the population could be eventually reached with a minor number of passages in 24 h 12 + 12 LD cycles incubation, being the frequency of reversion ten-fold higher in that condition. We also noticed the accumulation of a relevant number of unviable cells, unable to generate colonies, along the experiment (not shown). This finding could be correlated with the fact that the deletion of *KlMGA2* causes alteration of the chronological life span (Santomartino et al., 2019) and hence in the accumulation of unviable cells in long time culturing.

Discussion

Yeast does not seem to be a light-dependent organism: no recognized light-responsive protein is present in the *K. lactis* or *S. cerevisiae* proteome, yeasts do not contain photo-receptive molecules and their metabolism and physiology have no evident relation with light-dark alternation. As far as the typical fungal mechanism of light sensing is concerned, both yeasts lack the essential light responding proteins (WC1, WC2, and Frq) of the circadian cycle (Salichos & Rokas, 2010). However, yeast cells are transparent to light, and light can influence cellular mechanisms in many ways. Light alters the redox equilibrium of the cell by inducing the synthesis of hydrogen peroxide. As a consequence, the stress-sensing transcription factors Crz1 and Msn2 move to the nucleus (Bodvard et al., 2011; Bodvard et al., 2013; Bodvard et al., 2017). Light effects on the redox equilibrium also affect respiration and the YRO (Robertson et al., 2013) suggesting that cellular metabolism and physiology might have evolved in yeast following the adaptation to the cyclic harmful effects of photons during daytime.

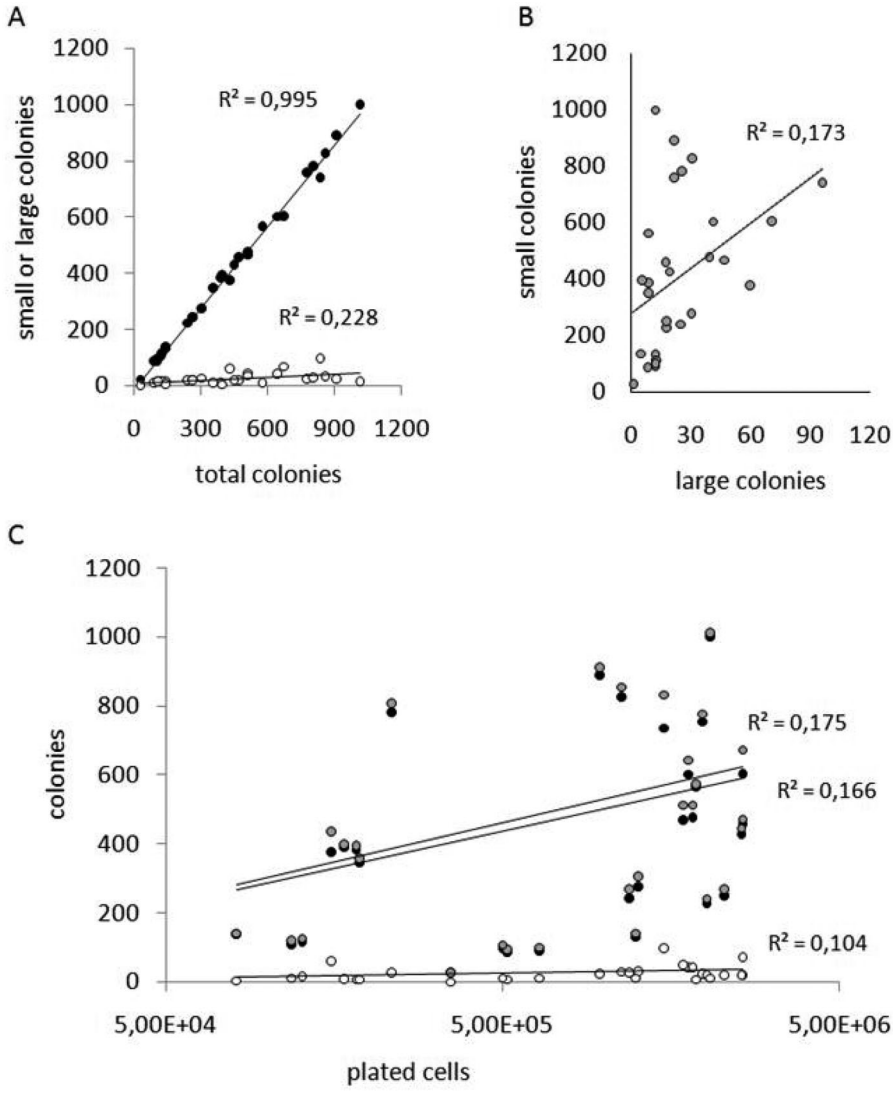

Figure 5. Correlation analysis. **5 A**. Correlation between the total number of colonies in each experiment and the number of small (black dots) or large (white dots) colonies, respectively. **5B**. Correlation between the number of small and large colonies (gray dots). **5 C**. Correlation between the total number of plated cells and the number of small (black dots), large (white dots) and total (gray dots) colonies, respectively. Trend lines with R^2 values are shown for each graph.

One open question is if yeast possess or not a canonical molecular clock of circadian frequency governing cellular functions. Our results indicate that, whatever the underlying mechanism of light reception and signal transduction, a 12 + 12 h environmental LD cycle resonates with an endogenous light-sensitive yeast oscillator that generates a clear biological response. This resonance phenomenon affects the efficiency of cell activities involved in the genome stability and/or the gene expression pathways involved in phenotypic suppression thus introducing an increased variability in the growing cell population. Conversely, the increased genetic suppression at 24 h environmental LD cycling indirectly demonstrates the existence in yeast of an endogenous light-dependent oscillator, that governs basic cellular processes like generation or stabilization of mutant cells or activation/inactivation of gene expression.

On a more general ground, we gave a proof-of-concept of an external environmental cyclic factor that, resonating with an internal biological oscillator, drastically alters the adaptation and evolutionary potential of a biological system, with the trend of a phase transition, consistently with the conjecture reported in Damasco and Giuliani (Damasco & Giuliani, 2017), thus adding a new dimension in evolutionary studies.

Intrinsically disordered regions (IDPRs) exist as highly dynamic conformational ensembles consisting of multiple rapidly interchangeable structures (Dunker et al., 1998; Wright & Dyson, 1999; Uversky et al., 2000; Dunker et al., 2001; Tompa, 2002; Daughdrill et al., 2005; Uversky; Uversky 2013). It is tempting to hypothesize that some of the intrinsically disordered proteins or hybrid proteins, which, similarly to the *K. lactis* protein KlMga2, contain both ordered domains and IDPRs could each other interact to promote phenotypic suppression. In fact, disordered proteins are characterized by highly heterogeneous spatiotemporal organization. Such molecules simultaneously contain independently foldable units (foldons), disordered regions that can fold upon the interaction with binding partners (inducible foldons), non-foldable protein regions (non-foldons), regions that are always in a semi-folded form (semi-foldons), disordered regions that can differently fold at interaction with different binding partners (morphing inducible foldons), and

ordered regions that have to undergo order-to-disorder transitions to become functional (unfoldons) (Uversky 2013; Uversky 2013; Uversky 2013; Jakob et al., 2014).They also show unique ability to be engaged in numerous interactions with various partners and thereby play a number of crucial roles in regulation of signalling pathways and in the control and coordination of transcription, translation, and the cell cycle (Wright & Dyson, 1999; Dunker et al., 2002; Dunker et al., 2002; Uversky 2002; Uversky 2002; Uversky, 2003; Uversky & Dunker, 2013; Uversky, 2015; DeForte & Uversky, 2016). They are capable to sense and react to subtle changes in their environment (Uversky 2013). Combination of all these features makes disordered proteins to be perfectly suited for by-passing the lack of Mga2 allowing for alternative modes ending up in a physiological regulation of global metabolism.

The recognition of the ability of external oscillatory stimuli to drastically increase the organisms' heritable phenotypic plasticity could have both theoretical (evolution mechanisms) and biotechnological (generation of wide phenotypic space of microbial strains) consequences.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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