Spatial Perspectives in The Redox Code – Mass Spectrometric Proteomics Studies of Moonlighting Proteins

Gabriella Pinto[1], Marko Radulovic[2] and

Jasminka Godovac-Zimmermann[1*]

[1]Division of Medicine, University College London, Center for Nephrology, Royal Free Campus, Rowland Hill Street, London, NW3 2PF, UK.
[2]Insitute of Oncology and Radiology, Pasterova 14, 11000 Belgrade, Serbia

* Corresponding author: J. Godovac Zimmermann, email:j.godovac-zimmermann@ucl.ac.uk

ABSTRACT:

The Redox Code involves specific, reversible oxidative changes in proteins that modulate protein tertiary structure, interactions, trafficking and activity, and hence couple the proteome to the metabolic/oxidative state of cells. It is currently a major focus of study in cell biology. Recent studies of dynamic cellular spatial reorganization with MS-based subcellular-spatial-razor proteomics reveal that protein constituents of many subcellular structures, including mitochondria, the endoplasmic reticulum, the plasma membrane, and the extracellular matrix, undergo changes in their subcellular abundance/distribution in response to oxidative stress. These proteins are components of a diverse variety of functional processes spatially distributed across cells. Many of the same proteins are involved in response to suppression of DNA replication indicate that oxidative stress is strongly intertwined with DNA replication/proliferation. Both are replete with networks of moonlighting proteins that show coordinated changes in subcellular location and that include primary protein actuators of the redox code involved in the processing of NAD^+/NADH, $NADP^+$/NADPH, Cys/CySS and GSH/GSSG redox couples. Small groups of key proteins such as {KPNA2, KPNB1, PCNA, PTMA, SET} constitute "spatial switches" that modulate many nuclear processes. Much of the functional response involves subcellular protein trafficking, including nuclear import/export processes, vesicle-mediated trafficking, the endoplasmic reticulum/Golgi pathway, chaperone-assisted processes and other transport systems. This is not visible to measurements of total protein abundance by transcriptomics or proteomics. Comprehensive pictures of cellular function will require collection of data on the subcellular transport and local functions of many moonlighting proteins, especially of those with critical roles in spatial coordination across cells. The proteome-wide analysis of coordinated changes in abundance and trafficking of proteins offered by MS-based proteomics has a unique, crucial role to play in deciphering the complex adaptive systems that underlie cellular function.

Keywords: Proteomics, Mass Spectrometry, Oxidative Stress, Moonlighting proteins, Redox Code

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I. INTRODUCTION

The accumulation of ever more data on the functional roles of individual proteins has led to increasing recognition of the importance of so called "moonlighting" or "multi-functional" proteins. Even proteins such as GAPDH that were once regarded as housekeeping proteins are now known to have multiple functional roles at very diverse subcellular locations depending on functional context (He et al., 2013,Sirover, 2012 ,Tristan et al., 2011). There are by now hundreds of similar examples. This has led to increasing recognition that new paradigms are necessary in cellular biology (Copley, 2012,Henderson& Martin, 2014,Jeffery, 2009,Shakib et al., 2005) and to the appearance of new databases that catalogue examples of moonlighting proteins and/or of protein-protein interactions at different subcellular spatial locations (Hernandez et al., 2014,Khan et al., 2014,Mani et al., 2015,Veres et al., 2015). Cells are not fixed aggregates of predefined molecular machines (e.g., protein-protein and other complexes) that are distributed to predefined locations/organelles (mitochondria, endoplasmic reticulum, etc.) and that are produced on demand by transcription. There are by now hundreds of examples of dynamic flux in the composition and in the subcellular location of the "machines" and the organelles. In hindsight, we might have recognized much earlier that the heterogeneous spatial partitioning of major cellular functions (energy, transcription, external signal sensing, etc.) to different subcellular locations requires such dynamic flux to enable cross-communication and ensure controlled cellular response to environment.

With the arrival of this change in paradigm, there is an increasingly obvious need for proteome-wide analysis of subcellular spatial fluxes of proteins (and other molecules) in response to cellular state. This is a serious technical challenge for which we presently do not have ideal tools. MS-based methods have the substantial advantage that thousands of proteins are measured concurrently in parallel in a single sample. Furthermore, quantitative changes of moderate magnitude for individual proteins can be determined reasonably accurately over extremely wide ranges of total abundances, e.g., for two proteins whose total abundance might vary by +1000-fold. These are important features because large numbers of gene expression studies, as well as theoretical considerations about the stability of complex adaptive systems (Levin,

2003,Whitacre& Bender, 2010), suggest that moderate, intertwined changes of many functionally related proteins are more the rule in cellular response than spectacular changes in a few proteins. Fluorescence-based methods are the main presently available alternative, using either antibodies (Brennan et al., 2010,Schwenk et al., 2007) or a variety of biological or chemical "tagging" methods (Cabantous et al., 2005,Dean& Palmer, 2014,Satori et al., 2013). The antibody methods have the potential advantage of using surface fixation to trap and analyze responses of "living" cells, but are fraught with potential difficulties with antibody availability, selectivity sufficient to deal with >1000-fold variation in abundance of different cellular proteins, the "accessibility" of target proteins in fixed cell samples, and sufficiently accurate quantitation of moderate changes for large numbers of proteins. Artefacts are known (Schnell et al., 2012). The "tagging" methods primarily analyze what might be termed "mutilated-but-surviving" cells and the need for independent confirmation of their results has been recognized, e.g., with (Green)-Fluorescent-Protein-tagged proteins (Costantini et al., 2015,Costantini& Snapp, 2013,Huh et al., 2003,Simpson et al., 2000,Starkuviene et al., 2004,Wurm& Jakobs, 2006). Both fluorescence approaches suffer from "selected-monitoring" limitations, that is, the monitored proteins are selected in advance and are usually only a few in number. As will be described in more detail below, this can be a crippling limitation for moonlighting proteins that can plug into various complex local interaction networks at different subcellular locations. Both fluorescence methods are also difficult to multiplex, and recent attempts at automated analysis of multiple, parallel preparations of presumably identical (but differently tagged) cell samples seem to be at the level of about 50 proteins (Wachsmuth et al., 2015).

Although all of these approaches have potentials for artefacts we contend that at present the MS-based methods seem to be our best bet for proteome-wide identification of network "hot-spots" that can provide appropriate target lists for further investigations. The major technical challenge for the MS-based methods is to show that reliable spatial distribution information can be ascertained following cell breakage and fractionation. The major initial scientific challenge is to clarify to what extent dynamic, correlated spatial redistribution of cellular proteins is a fundamental aspect of cellular function that requires targeted collection of data to complement other approaches such as correlations in gene expression. So far there are only a few

proteome-wide measurements of dynamic subcellular protein distribution. We therefore illustrate the challenges and some initial conclusions mainly with recent MS-based "subcellular spatial razor" studies of cell cycle arrest in response to either: (a) oxidative stress (OXS) engendered by exposure of cells to *tert*-butyl hydrogen peroxide (TBP); or, (b) repression of DNA replication at the origin activation checkpoint (OAC) by siRNA suppression of the CDC7 kinase that phosphorylates MCM replication licensing complexes (Baqader et al., 2014,Mulvey et al., 2013). The main focus here will be on the oxidative stress results and their relationship to the so-called Redox Code (Jones& Sies, 2015). The subcellular MS-based methods provide uniquely wide dynamic coverage of the proteome and provide evidence that response to oxidative stress is replete with moonlighting proteins that show coordinated changes in subcellular abundance/location in response to cellular oxidative state. Many of these same proteins also show changes for DNA replication/proliferation without direct inducement of oxidative stress. That is, moonlighting proteins appear to intertwine many aspects of DNA replication and oxidative stress.

II. A BRIEF REDOX CODE PRIMER

The earliest studies on cellular oxidative stress date from the 1940s when the term reactive oxygen species (ROS: superoxide radical, $^{\bullet}O_2^-$; hydrogen peroxide, H_2O_2; hydroxyl radical, $^{\bullet}OH$; and secondary organic species such as lipid peroxides) was first used. The basis for understanding of the etiologic importance of ROS in various diseases, including male infertility (MacLeod, 1943), retrolental fibroplasia in premature newborns (Campbell, 1951), in cumulative damage relative to aging (Harman, 1956), or in brain metabolism (Mann& Quastel, 1946), were predominantly set during those years. Since then, increasingly intense interest has been manifested by the very high number of publications on this topic. More than 130,000 English-language articles in PubMed deal with various aspects of oxidative stress, among which more than 50% are papers and 10% are reviews published during the last ten years. An ever increasing number of diseases have been linked to ROS, including diabetes (Kaneto et al., 2010), cardiovascular diseases (Sugamura& Keaney, 2011), and neurodegenerative syndromes such as Alzheimer's and Parkinson's diseases (Lin& Beal, 2006), as well as cancer (Finkel et al., 2007).

Today we know that cellular responses to "oxidative stress" (OXS hereafter) include a gradient of responses that depend on the degree and temporal persistence of the

perturbation (Fig.1). Such dependence is expected since the basic chemical and cellular processes themselves have a vast range of time scales. Species such as superoxide radicals with lifetimes of microseconds, or more stable derivatives such as hydrogen peroxide or lipid peroxides, presumably need to be present in high concentrations (acute stress) or have substantial abundance changes that persist over time (chronic stress) for systemic changes. Transcriptional/translational/degradational adaptation of protein or RNA abundances might require hours or even days to be fully realized. Adaptations that might have intermediate time scales include metabolic re-balancing, various kinds of protein post-translational modifications (PTMs), changes in translational control, and spatial translocation of proteins to different subcellular locations.

There are different types of cellular responses that represent gradients of complexity in the reorganization of cells (Fig. 1). Even under ambient, non-stressed, non-proliferative conditions, cellular molecular components experience oxidative damage that either needs to be repaired (e.g., DNA damage repair processes) or replaced (e.g., protein replacement by various types of selective autophagy processes). For extreme, acute oxidative stress the response might be cell death/elimination processes. Processes that probably fall in an intermediate regime might include morphological adjustments such as organelle replacement (e.g., mitophagy), nuclear reorganization, or exosomal secretion.

Although early work in the field tended to emphasize oxidative damage by highly reactive ROS, parallel work on what we will term “cellular oxidative status” (CEOX hereafter) has also made it clear that CEOX underlies important mechanisms in normal cellular physiology. The correlation of various aspects of the cell cycle with CEOX is an instructive example. A fundamental characteristic (Fig. 2) is that the cell cycle itself is a sort of redox couple with oxidative status for the G_0 and G_1 stages and reductive status for the S and G_2 stages (Burhans& Heintz, 2009,Chiu& Dawes, 2012,Menon& Goswami, 2007,Sarsour et al., 2009), (da Veiga Moreira et al., 2015,Tu et al., 2005,Yu et al., 2009). Present formulations of the redox code (Jones & Sies, 2015) emphasize use of the reversible electron accepting and donating properties of nicotinamide in NAD/NADP to provide organization of metabolism as well as to link metabolism to protein structure through kinetically controlled redox switches in the proteome. These switches include thiol/disulfide couples such as Cys/CySS and

GSH/GSSG that modulate protein tertiary structure, interactions, trafficking and activity. Much recent work has analyzed post-translational oxidation processes that involve Cys. A variety of methods provide chemical labeling of thiol groups at different stage of oxidation (Guo et al., 2014,Kim et al., 2015,Kramer et al., 2015,Tambor et al., 2012). In particular, "switch methods" for oxidized S-thiols and S-nitrosothiols to different thiol-reactive reagents have been combined with MS-based quantitative methods to monitor the higher levels of protein oxidation (Murray& Van Eyk, 2012). Although up to 4000 specific peptidyl-Cys residues (2% of the mammalian proteome) can be checked by a single experiment, there are two major limitations of this approach: (a) artifactual oxido-reduction events can occur during cell processing or organelle isolation, and the real oxidative state might not survive the purification step; and, (b) the dynamic nature of the modification often reflects only a relatively small portion of the proteins that are detected in a given cellular space/time. Similar considerations apply to reactive nitrogen species, but we limit examples to ROS in the following. Still other protein modifications include modification by lipid peroxidation products (Lin et al., 2015,Vasil'ev et al., 2014), carbonyl derivatization of proteins irreversibly modified by oxidative stress (Fedorova et al., 2014,Madian& Regnier, 2010) and methionine oxidation (Ghesquiere& Gevaert, 2014,Ghesquiere et al., 2011).

Over 300 different proteins (Go et al., 2011,Pan et al., 2014) are known to be subject to changes in functional activity as a consequence of Cys oxidation/reduction processes. This includes mitochondrial proteins (Mailloux et al., 2013), signalling systems (Forman et al., 2014,Ray et al., 2012) and nuclear hormone receptors involved in transcriptional activities (Carter& Ragsdale, 2014). Such Cys oxidation processes seem to constitute a PTM network that shows complexity and reversibility that might rival other PTM systems such as phosphorylation. Indeed, such PTM systems might be intimately intertwined (Fig. 2).

Cellular function is spatially heterogeneous with specialized functions distributed over different subcellular components, including mitochondria, the plasma membrane, the nucleus, the endoplasmic reticulum, etc. (Fig.2). Different subcellular compartments are known to be at different redox potential (Go& Jones, 2008,Hansen et al., 2006,Hansen et al., 2006,Jones, 2006) and communication between different subcellular organelles/locations is crucial to cellular response to environment.

Roughly 1-5% of the total cellular oxygen consumed during respiratory processes is converted in mitochondria to superoxide $^{\bullet}O_2^-$, detoxified to H_2O_2 by the mitochondrial manganese superoxide dismutase (MnSOD), and subsequently converted by glutathione peroxidase (GPX) into H_2O in normal conditions. Although ROS are localized in specific intracellular regions, they are recognized as potent messengers and signaling molecules (Ray, et al., 2012). The greater stability of hydrogen peroxide and its capacity to diffuse through mitochondrial membranes produces inhomogeneous fluxes also far from the original location. In its simplest form the redox code involves the generation of superoxide via the respiratory chain in mitochondria (Mailloux, et al., 2013), the subcellular diffusion of hydrogen peroxide as a spatial integrator (Antunes& Cadenas, 2000,Mishina et al., 2011) and discriminatory responses that are dependent on the distribution of different protein sensors/actuators to different subcellular locations (Go et al., 2015,Roos et al., 2013,Wani et al., 2014). Even in this simple form, the redox code requires exquisite subcellular spatial distribution of proteins. Reaction of Cys with hydrogen peroxide is an unfavourable reaction at physiological pH and the simple redox code proposes that protein 3D structure creates Cys residues with enhanced reactivity (Paulsen& Carroll, 2010). Specific proteins with appropriately sensitized Cys residues must then be distributed in a correlated manner to subcellular compartments with redox potentials appropriate for the proteins.

However, there are other cellular sources of superoxide, including the NOX family of proteins, xanthine oxidase, and various dehydrogenases (Bedard& Krause, 2007,Block& Gorin, 2012,Goncalves et al., 2015,Goncalves et al., 2014). These can be distributed over many subcellular locations. For example, NOX4 has been identified in the nucleus (Kuroda et al., 2005), plasma membrane (Xi et al., 2013), endoplasmic reticulum (Van Buul et al., 2005), specialized plasma membrane subcellular domains such as focal adhesions (Hilenski et al., 2004), and mitochondria (Ago et al., 2010,Block& Gorin, 2012). NOX4 is an integral membrane protein with six transmembrane helices and two heme groups (Bedard& Krause, 2007) and its distribution over such diverse subcellular locations suggests there is more than the simple redox code. There are an increasing number of examples of plasma membrane signalling processes in which NOX family proteins generate superoxide locally, i.e., in which at least the initial steps apparently do not require the participation of

hydrogen peroxide generated via mitochondria. Although these processes might be indirectly coupled to CEOX/OXS via mitochondrial influence on downstream signalling pathways (Fig. 2), the (dynamic) dispersion of the NOX proteins over many subcellular locations is a reminder that the high level functional organization of cells depends on exquisitely complex subcellular spatial distribution of proteins. The presence of NOX4 in mitochondria (Ago, et al., 2010,Bedard& Krause, 2007,Block& Gorin, 2012), already an ample source of superoxide, is another indication for further complexity.

Overall, the degree/temporal persistence of OXS/CEOX processes combine with diverse molecular mechanisms of very different time scales, with alterations at many subcellular locations and with gradients in the complexity of the cellular response. This gives rise to very complicated, highly intertwined transcriptomic, proteomic, and metabolic functional networks that remain challenging to decipher experimentally. In some ways, the aspects that are easiest to analyse are the opposite limiting situations of ambient "quality control" processes and of cell death processes.

A major motivation to study the intermediate/chronic regime of response to OXS/CEOX is the connections to various kinds of diseases. Disease implies chronic changes, and we suggest that this implies transition of cells to non-normal, quasi-stable states that avoid cell death processes but exhibit altered response to environmental cues from their tissue environments. If so, the involvement of cellular oxidative state in many types of diseases might depend on what kinds of non-normal, quasi-stable states can be reached through oxidative changes and how these states may interact with the cellular responses evoked by other types of environmental cues. In turn, these states and the processes by which cells transition to them represent potential therapeutic targets. Although we are gaining ever more information about the changes in individual proteins that are involved in response to oxidative changes, defining such states and transitions is still a formidable challenge. This is a main focus of this review, and we use the recent studies of cross-talk between oxidative stress and DNA replication/proliferation to illustrate the challenges. A major theme is that the dynamic distribution of crucial proteins to different subcellular locations/organelles is a key characteristic that is strongly intertwined for both processes.

III. COMPLEXITY AND SPATIAL PROTEOMICS OF CELLS

In the age of large-scale 'omics' that followed on the completion of human genome sequencing, the development of ever more powerful DNA sequencing methods, of DNA-microarray technology, and of proteomics led to two major large-scale methods to investigate cellular function and its relationships to diseases: (a) large-scale genome-wide association strategies (GWAS) that involve screening of large number of patients to identify genetic mutations correlated with disease; and (b) extensive investigation of differential gene expression via transcriptomics and proteomics**.** The impressive decrease of protein-coding gene number from up to 2 million (Pennisi, 2003) to 19,000 (Ezkurdia et al., 2014) estimated during the last ten years of human genome sequencing, has emphasized that much of cellular complexity is based on proteome-level mechanisms. These include different processing of primary RNA transcripts, translational control of expression, widespread post-translational modification of proteins and, increasingly, subcellular dynamics of "moonlighting" proteins. The concept that one protein $\Rightarrow$ one cellular location $\Rightarrow$ one function is definitively surpassed by the multiplicity of forms, functions, and locations for single proteins. Recently, GWAS approaches to complex diseases are less and less focused on individual associations, and more addressed at the biological pathways and networks suggested by genetic associations (Ramanan& Saykin, 2013). Thus, recent hypotheses that complex diseases might be influenced by a highly personalized combination of variants – some common and others rare, some protective and others deleterious – stimulate the integration of genetic associations with further investigations based on transcriptomic, metabolomics, and proteomic approaches in order to get pathway- and network-driven models that can explain the broad molecular underpinnings of disease (Perez de Diego et al., 2014,Scholz et al., 2012). Crucial, widespread processes such as translational control of expression, post-translational modifications including redox-related changes, and spatial switching of moonlighting proteins between different functions are essentially invisible to transcriptomics and must be addressed directly by proteomics.

The growing shift of interest from the simple identification of cellular proteins to the quantification of changes in subcellular protein abundance and localization has pushed the development of a new generation of proteomics strategies that combine organelle fractionation with quantitative MS-based methods. Organellar proteomics

aimed at establishing the resident proteins of organelles has been a popular application of proteomics and the basic methodology (Fig. 3) is well established (Domon& Aebersold, 2010).

Various protocols have been set up to isolate different organelles. Highly reproducible enrichment of a specific organelle by fractionation needs to be validated, with the aim to obtain subcellular proteins as representative as possible of the compartment under study. Hence, before proceeding with MS analysis, specific enzymatic assays, immunofluorescence microscopy, or Western blot analysis of proteins that are markers for a specific compartment are commonly applied (Baqader, et al., 2014). Shotgun proteomics is the most common approach to identify organellar proteins and typically follows strategies used in analysis of total protein content of cells (Fig. 3).

IV. THE BASIC SUBCELLULAR SPATIAL RAZOR

The by now large body of work on the proteomes of specific subcellular organelles has often been dominated by the concept that highly pure organelle preparations are required. While highly purified organelles allow determination of what might be termed "permanent resident" proteins, there is now substantial evidence that attempts to prepare "highly purified" subcellular organelles are a fatal mistake when studying dynamic cellular function. To illustrate this, consider the proteins nuclear respiratory factor 2 (NRF2 or NFE2L2) and hexokinase 1 (HK1). NRF2, which is crucial to transcriptional responses to oxidative stress (Baird& Dinkova-Kostova, 2011,Hayes& Dinkova-Kostova, 2014,Kaspar et al., 2009,Nguyen et al., 2009,Tebay et al., 2015), is normally tethered with its repressor KEAP1 to the outer mitochondrial membrane by binding interactions with PGAM5. This places NRF2 in proximity to mitochondrial sources of ROS. For appropriate functional contexts, it is released and transferred to the nucleus. In addition to its roles in glycolysis, HK1 has a known binding interaction with the mitochondrial membrane VDAC ion channel. This interaction is important in cellular energetics, anti-apoptotic activities and cancer metabolism (Robey& Hay, 2005,Robey& Hay, 2006,Robey et al., 2015) (Pastorino& Hoek, 2008,Pastorino et al., 2002,Pedersen, 2008,Shoshan-Barmatz et al., 2010). Both of these "peripheral" proteins are readily removed from mitochondria by stringent organelle purification methods, to thereby lose the ability to monitor crucial features of cellular function. Similar considerations apply to other organelles such as nuclei. We now know that the nucleus is not the shiny ball with a nuclear envelope

punctuated with a few nuclear pores as often seen in textbooks. Rather, it contains a highly complex "reticulum" (Malhas et al., 2011), the nuclear envelope can include complex vesicular structures that include endosomal proteins (Wu et al., 2014), large numbers of previously unsuspected proteins are associated with the nuclear envelope (Talamas& Capelson, 2015), specialized microdomains with crucial functional roles can be induced in the nuclear envelope (Al-Mehdi et al., 2012), and the involvement of ESCRTs (endosomal sorting complexes required for transport) in nuclear envelope functions has recently been established (Webster& Lusk, 2015). Much available evidence suggests that eventually we will establish that the nuclear envelope has many of the same kinds of complex, dynamic interactions that have been defined for the plasma membrane. In short, stringent organelle purification methods that strip peripheral proteins must be avoided for mitochondria, the nucleus, and similarly for other subcellular organelles.

These cellular features dictate that, for MS studies of dynamic protein changes, the fractionation methods should be as simple and mild as possible, but that compensatory procedures are necessary to minimize possible artefacts. We introduced the two-compartment nucleus-cytoplasm "subcellular spatial razor" (Fig. 4A) to deal with this situation (Baqader, et al., 2014,Mulvey, et al., 2013,Pinto et al., 2014). A crucial feature is that all experiments are performed as differential analyses that use isotope labelling, typically SILAC (Ong et al., 2002), to differentiate between perturbed/unperturbed cells. These are mixed and co-fractionated, and only proteins that show large differential changes are accepted as significant changes in response to perturbation. This reduces artefacts caused by the cell breakage/fractionation protocol, but the data should of course still be subjected to screening for reproducibility over replicates and for adequate quantitation. A second crucial feature is that the MS data that are collected (SILAC ratios S_n, S_c, S_t) are robustly over-determined and this redundancy allows checks for consistency in the data. In particular, conservation of mass dictates that for the 3D orthogonal basis set $\{S_n/S_t, S_c/S_t, S_t\}$, changes in total abundance $\{S_t\}$ are expressed on an orthogonal axis from a distribution plane $\{S_n/S_t, S_c/S_t,\}$ that is independent of changes in total abundance. All data points should lie within two quadrants of the space (Fig. 4B,C).

The spatial razor also provides a natural formulation to look at several crucial characteristics of the data: (a) are the data consistent with selective trafficking of

specific proteins in response to perturbation (plots of S_n/S_t or S_c/S_t vs. number of SILAC ratio counts, Fig 4D); and, (b) do changes in its compartmental abundance simply mirror the changes in total abundance of a protein (plots of S_t vs. S_n/S_c) (Baqader, et al., 2014,Mulvey, et al., 2013,Pinto, et al., 2014,Qattan, et al., 2012,Tudzarova, et al., 2010)? For example, for TBP-induced oxidative stress the nucleus contains small, but roughly equal numbers of proteins from mitochondria or the endoplasmic reticulum with substantially increased/decreased nuclear fraction (Fig 4D). However, large numbers of abundant proteins from mitochondria/endoplasmic reticulum show no change in nuclear fraction. When coupled with high reproducibility, this is very strong evidence that the changed proteins represent selective trafficking. Further confidence is engendered by results that show that the identities of the changed proteins vary with the kind of perturbation applied (Baqader, et al., 2014,Mulvey, et al., 2013). In short, the subcellular spatial razor strategy provides an appropriate framework to look at trafficking of proteins to/from a target organelle. We note that, for all cell types/perturbations to which the subcellular spatial razor strategy has so far been applied (Mulvey, et al., 2013,Pinto, et al., 2014) there is little correlation between changes in total abundance (S_t) and in redistribution between compartments (S_n/S_c). That is, cells combine transcriptional/translational/degradational changes in the total abundance of a protein with changes in its nuclear/cytoplasmic distribution to achieve the local compartmental changes that underlie cellular response to perturbation. This is in concert with the new "moonlighting" paradigm of cellular function, and has profound consequences for the ways in which we should analyze and think about cellular function.

V. DIFFERENTIAL ANALYSIS WITH A JOINT SPATIAL RAZOR FORMULATION

Current wisdom often implies that the most desirable way to examine dynamic cellular properties are experimental time series of the responses to a single type of perturbation. In fact, the limitations of this approach have already been discussed in papers that justify the need for theoretical prediction of fluxes and protein "mislocation" in disease models (Lee et al., 2013) – the approach leads to an almost infinite set of possible experiments. We suggest that a more-productive strategy is to drive cells into different (semi)-stable regions of a response landscape (phase

changes) and identify the major differences between two such states. This provides unbiased “hot spot” characterizations of an exceedingly complex overall network that provide focus for further investigations (see below). In this framework, the checkpoints of the cell cycle become a very valuable experimental tool. We were able to induce cell cycle arrest with two completely different cellular perturbations, neither of which involves direct manipulation of cyclins. This provides a means to compare multiple (semi)-stable states relative to the basal state of cells with parallel applications of the subcellular spatial razor (Fig. 5A,B).

A first application of this strategy was an overall assessment of the importance in cellular function of the dynamic redistribution of proteins between different subcellular locations. By comparing the total and compartmental changes in protein abundance for 4048 proteins (Fig. 5C) between TBP-induced oxidative stress (OXS hereafter) and CDC7-kinase-induced repression of DNA replication at the origin activation checkpoint (OAC hereafter), a 401-set of proteins (Fig. 5D) that show significant changes in response {S_n, S_c, S_t or S_n/S_c} to one or both of the perturbations was defined (Radulovic et al., 2016). The strongest response for OXS was a very large increase in total and nuclear abundance of HMOX1 (Fig 5C). The increased total abundance is a well-known response to OXS that is thought to involve NRF2 transcriptional activities (Hayes& Dinkova-Kostova, 2014) and the increase in nuclear abundance of HMOX1 has recently been observed in other cell types (Biswas et al., 2014). Surprisingly, similar strong increases for HMOX1 are observed for OAC, i.e. by repressing DNA replication. This is a first indication for strong intertwining of the OXS/OAC responses. For other proteins, the characterization of responses that are the SAME, OPPOSITE, MIXED, or UNIQUE to one of the two perturbations (Fig. 5) provides a profound view into the complex, spatially distributed interrelationships between cellular oxidative status and DNA replication/proliferation. For example, changes in SHMT2 (one-carbon metabolism) and XRCC5 (DNA repair) are predominantly for OAC and for both proteins the major mechanism is N $\rightarrow$ C redistribution that results in strong increases in cytoplasmic abundance (Fig. 5E). Conversely, changes for ICT1 (mitochondrial translation) and PSMD5 (proteasome) are dominantly for OXS. Proteins such as FTL (iron storage), MARCKS (cytoskeletal modulation), and PCNA (multiple activities including DNA replication) show similar behavior for OXS/OAC, whereas PMPCA (mitochondrial import, proteolysis) and

HSPH1 (immune system regulation) show opposite behavior for OXS/OAC (Fig. 5F). The combination of two compartments, two mechanisms (total abundance, redistribution) and differing basal distribution results in complex, mixed behavior for other proteins such as CANX (multiple activities, including calcium binding, immune system modulation, protein folding processes) and HSPA9 (multiple activities including mitochondrial import and binding of p53). For both of these proteins (Fig. 5E), the combination of changes in total abundance and redistribution results in dominant compartmental changes in the nucleus for OXS, but in the cytoplasmic compartment for OAC. Overall, the proteins in the significant 401-set revealed several crucial characteristics (Radulovic et al, 2016). (a) Cells combine changes in total abundance and in trafficking to achieve the local, compartmental changes in protein abundance that are the real basis of cellular response. (b) OXS and OAC are strongly intertwined - within the 401-set, 245 proteins showed appreciable changes of varying magnitude for both (semi)-stable states, with only 67/89 proteins more specific to OAC/OXS respectively. (c) Proteins that are components of many subcellular structures, including mitochondria, the plasma membrane, the endoplasmic reticulum, the extracellular matrix, etc., are involved in the responses. (d) The changed proteins correspond to a wide diversity of functional processes. (e) Cells make use of diverse mechanisms including nuclear import/export systems, vesicle-mediated trafficking and chaperone-assisted processes to redistribute proteins. (f) There may be relatively small numbers of crucial proteins that dominate cellular spatial coordination – a 49-set of proteins that show very strong changes for both OXS and OAC was identified. (g) Correlated changes in small numbers of proteins can lead to complex, dynamic control axes in specific locations such as the nucleus.

The networks involved in the cellular responses are very complex. Use of only curated, well-documented binary interactions from STRING (Szklarczyk et al., 2015), REACTOME (Croft et al., 2014), and CORUM (Ruepp et al., 2010), the overall interaction network contains 134,850 binary interaction pairs between the 4048 proteins (Radulovic et al., 2016). However, because a large majority of the proteins show no or very modest changes for either OXS or OAC, the network “hot-spots” between the two (semi)-stable states can be analyzed in terms of local control axes with a “joint spatial razor” approach. We illustrate this here with the nuclear {KPNA2, KPNB1, PCNA, PTMA, SET} spatial switch that was identified.

VI. THE NUCLEAR {KPNA2, KPNB1, PCNA, PTMA, SET} SPATIAL SWITCH

A striking result of the joint spatial razor analysis was that proliferating cell nuclear antigen (PCNA) shows little change in total or cytoplasmic abundance, but a strong decrease in nuclear abundance for both OXS and OAC (Fig. 5F). Correlations to other proteins were identified in two ways. First, the functionally well-defined human protein complexes contained in the CORUM database were scanned for complexes that contain PCNA. This identified eight protein constituents of three key PCNA complexes: {PCNA, RFC2-5}, {PCNA, XRCC5,6} and {PCNA, DNMT1, EHMT2}, all of which except EHMT2 were included in the 401-set (Fig. 6). The {PCNA, RFC2-5} and {PCNA, DNMT1, EHMT2} complexes are both intimately involved in DNA replication. A scan of the CORUM complexes with these proteins identified a further 77 nuclear complexes that contained 160 proteins related to PCNA, but only two further proteins that were included in the 401-set: DNA-dependent protein kinase catalytic subunit (PRKDC) and Vigilin (HDLBP). The {PRKDC, XRCC5, XRCC6} complex is best known for DNA damage repair processes, but PRKDC has also been shown to be involved in numerous other activities including innate immune response, response to hypoxia, metabolic control, and transcriptional control (Goodwin& Knudsen, 2014). The vigilin {HDLBP, PRKDC, XRCC5, XRCC6} complex is thought to participate in chromatin silencing (Wang et al., 2005), but may also have other activities (Radulovic et al., 2016). Notable is that PCNA, DNMT1 (DNA/histone methylation, transcription), and to a lesser extent, the RFC2-5 complex (DNA replication) all show SAME reduced nuclear abundance for both OXS and OAC (Fig 6). Changes in XRCC5,6 and PRKDC are primarily for OAC and HDLBP shows OPPOSITE behavior that is most strongly reflected in its cytoplasmic abundance.

An overlap matrix for the 77 nuclear complexes that contain 160 proteins related to PCNA reveals that proteins in the 401-set of significant changes are exclusively in the three core complexes or the associated vigilin complex (Fig. 7). A striking feature is that the three core PCNA complexes are *not* subsumed into larger complexes. Instead, subsets of the three core complexes participate in many other "peripheral" complexes. That is, the dominant feature of the response to OXS and OAC may be dynamic redistribution of the amounts of the different "peripheral" nuclear complexes as a

consequence of changes in total abundance and compartmental distribution of a few constituents of the three core complexes. Many other crucial transcriptional and chromatin remodelling complexes do not show significant changes in total or compartmental abundance for the individual proteins (Radulovic et al, 2016), even though OXS and OAC are both strong perturbations that lead to cell cycle arrest. A salient feature is that significant changes in abundance/location are seen only for the core protein complexes and these proteins appear to define four mostly independent functional branches (Fig. 7). Another salient feature is that changes in total abundance and in compartmental redistribution are intimately intertwined in the control axis.

Further analysis was based on additional interactions contained in the STRING and REACTOME databases. The binary interaction network for the 49-set of proteins with strong changes for OXS and OAC included two further proteins with direct interactions with PCNA. SET and PTMA show strong MIXED/OPPOSITE behavior for OXS/OAC, notably with substantial increases in total/cytoplasmic abundance for OXS (Fig. 8A). The local interaction network for these three proteins included 54 other proteins and 306 edges that represent binary interactions (Fig. 8B). The interactions deduced from the CORUM complexes involving PCNA (Fig. 7) correspond to strong changes in the interaction potentials. However, the four branches (Fig. 7) are only modestly connected to other systems, i.e., the interaction network is consistent with transient distribution of PCNA over these branches. PCNA has numerous additional interactions that seem to be intertwined with a nuclear import/export system that involved KPNA2, KPNB1, and XPO1. This system also involves SET and PTMA, each of which is connected to other parts of the interaction network. These interactions have been broken down into subtypes such as co-expression, binding, etc. and further analyzed for OXS and OAC (Radulovic et al., 2016). Here, we note that the total changes in interaction potentials are a complex, intertwined network of a type that might be expected for a stable complex adaptive system. Importantly, many of the interaction potentials are strongly different for the nuclear compartment (Fig. 8B). Nuclear import/export of PCNA, SET, PTMA, and other proteins produces a quantitative nuclear interaction set that is not equivalent to changes in the total abundance of the proteins. In short, compartmental redistribution alters many nuclear interactions in ways that are invisible to measurements of gene expression. Proteins such as PCNA, SET and PTMA are also known to be

moonlighters with defined functions at many other cellular locations (Fig. 8C). Their compartmental redistribution is expected to result in changes across the cell, and these proteins might be critical in higher order spatial organization of cellular response. Also noteworthy is the intimate involvement for OXS/OAC of the CCT protein folding/membrane fusion complex and a series of specific heat shock protein chaperones (Fig. 8B).

Similar joint spatial razor analysis has been carried out for proteins involved in caveolae, extracellular matrix remodelling, TGFβ signalling, IGF pathways, emerin complexes, mitochondrial protein import complexes, protein transport systems, spliceosomes, proteasomes, ribosomes, etc. We refer readers to those analyses (Radulovic et al., 2016). Here, we show selected sets of proteins contained in the previously reported Supplementary Tables (Radulovic et al., 2016) to illustrate some general features related to present formulations of the redox code.

VII. THE PRIMARY ACTUATORS OF THE REDOX CODE

The redox code emphasizes four redox couples: NAD^+/NADH, $NADP^+$/NADPH, Cys/CySS, and GSH/GSSG (Jones& Sies, 2015). A variety of proteins related to processing of these couples were monitored (Fig. 9) and show interesting features.

Of 31 monitored proteins that are related to the Cys/CySS and GSH/GSSG couples or to peroxidases (Fig. 9A), only three were included in the most-significant changes detected for OXS: Prostaglandin G/H synthase 1 (PTGS1, OXS), Microsomal glutathione S-transferase 3 (MGST3, SAME), and Thioredoxin-dependent peroxide reductase (PRDX3, MIXED). Thioredoxin (TXN) and a variety of other proteins (GSTK1, GSTM3, GSTO1, GSTP1, PRDX1, PRDX2, PRDX4, PRDX5, PRDX6, SOD1, SOD2, etc.) showed at most smaller changes (Fig 9A). For OAC, the strongest changes were for MGST3, PRDX3, and Isoform 3 of Thioredoxin reductase 1 (TXNRD1, OAC). The behavior of the two common proteins MGST3 (SAME, dependent on total abundance) and PRDX3 (MIXED, dependent on N $\rightarrow$ C transfer for OXS and on N $\rightarrow$ C transfer plus increased total abundance for OAC) is suggestive of another spatial switch in the nucleus. Interestingly, PTGS1 and MGST3 are both involved in lipid metabolism.

Further indications for a spatial switch are provided by the 155 proteins related to the NAD^+/NADH and $NADP^+$/NADPH couples, with 17 showing strong changes (Fig.

9B). A general trend for OXS is decreased nuclear abundance dominated by N → C trafficking with few significant changes in the cytoplasmic compartment (Fig. 9C). Conversely, for OAC there is a general trend of increased cytoplasmic abundance with limited changes in nuclear abundance. Several proteins (CYB5R3, HADHA, PRDX3, PYCR1) show this pattern for both OXS and OAC, while others primarily show an increase in cytoplasmic abundance for OAC (ALDH2, DLD, GLUD1, HIBADH, HSD17B10, IDH3A, TXNRD1). Overall, of the strongest changes, 13 of 17 are dominantly associated with the NAD^+/NADH and $NADP^+$/NADPH couples, two with the Cys/CySS and GSH/GSSG couples and two proteins (PRDX3, TXNRD1) are associated with both. Spatial switching is clearly important for OXS and OAC. For the direct actuators and the nucleus, processes that redistribute proteins involved in the NAD^+/NADH and $NADP^+$/NADPH couples appear to predominate over proteins involved in Cys modifications. The latter might, however, be crucial to nuclear import/export of many other proteins. How the redistribution of proteins potentially can produce local changes in superoxide and NADH/NADPH might be functionally important is a topic that should be further investigated.

VIII. PROTEIN TRANSPORT MECHANISMS

There are strong indications that protein transport mediated via the endoplasmic reticulum (ER)/Golgi pathway and via vesicle-mediated transport is also important to the OXS response. Compared to the cytoplasm, the redox potentials of mitochondria/ER are more reducing/oxidizing respectively (Jones& Sies, 2015). However, mitochondria and the ER are in direct contact via the MAM (mitochondrial associated membrane) region of the ER that is important for both Ca^{2+} homeostasis and lipid metabolism (de Brito& Scorrano, 2010,Fujimoto& Hayashi, 2011,Lynes et al., 2012). The ryanodine/Ca^{2+} release channel is known to be subject to redox control (Sun et al., 2013). Substantial numbers of proteins involved with mitochondria and the ER were observed to show changes in total and compartmental abundance in connection with OXS (Fig. 4D). Of the 195 ER proteins quantified for both OXS/OAC, 31 showed significant changes for one or both perturbations and revealed considerable potential for cross-talk between OXS and OAC (Fig 10A). Some ER proteins with strong changes have previously been shown, e.g., HMOX1 (Fig. 5B), SET (Fig. 6), MGST3 (Fig.8A). Others will be shown below in connection with vesicle-mediated trafficking, e.g., BCAP31, LMAN1, and various RAB proteins

(RAB1A, 2A, 14). Because these first joint spatial razor analyses focused on the nucleus and do not directly measure mitochondrial/ER interactions, we comment here only on a few additional proteins that showed interesting changes for OXS (Fig. 10B).

ERO1 is a key contributor to *de novo* protein folding in the ER (Sevier& Kaiser, 2008). ERO1L was the dominant species in IMR90 cells, and substantial SAME increases in total/cytoplasmic abundance were observed for OXS and OAC, i.e.,protein folding capacity appears to augment for both perturbations. Very strong SAME changes in total and compartmental abundance were observed for syntenin 1 (SDCBP), which is a well-known moonlighting protein with wide-ranging activities in the ER, nucleus, cytosol, and plasma membrane. POR (NADPH-cytochrome P450 reductase/NADPH-hemoprotein reductase) showed strong SAME increase in total abundance, but strong decrease in nuclear abundance for OXS. These strong SAME changes are further evidence of strong intertwining of OXS and OAC. Some proteins were more specific for OXS, e.g., SQSMT1 and PTGS1. Caveolin (CAV1) and PTRF (cavin 1) both showed reduced cytoplasmic abundance for OXS, as described previously in the context of other caveolar proteins (Radulovic et al., 2016). Several PTM systems involved in the very elaborate processing, stabilizing and targeting of the collagen triple helix to the extracellular matrix were monitored. An unexpected, if moderate, change was the OPPOSITE changes in nuclear abundance for prolyl-4-hydroxylase (P4HA1) and prolyl-3-hydroxylases 1,3 (LEPRE1, LEPREL2). No significant changes were detected for procollagen-lysine 5-dioxygenases (PLOD1,2,3), but changes in compartmental abundance for OAC only were detected for the glycosyl transferases Procollagen galactosyltransferase 1 (GLT25D1), GDP-fucose protein O-fucosyltransferase 2 (POFUT2), and Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 (RPN1). These might be related to the distinctive differences between OXS/OAC for various types of collagens that we have described previously (Radulovic et al., 2016). The strong OPPOSITE behavior of RRAS2 is also noteworthy.

Overall, these results suggest that the intimate involvement of the ER in protein trafficking can be strongly influenced by redox status and, somewhat unexpectedly, repressing DNA replication can influence some of the same proteins. This might be an important axis for the widespread redistribution of proteins involved in many subcellular locations, including the plasma membrane and the extracellular matrix,

that we have described in more detail (Radulovic et al., 2016). A number of these proteins involve oxido-reductase activity and/or iron homeostasis. Interestingly, there are indications for at least some systems that local H_2O_2 generated directly in the ER is more important than diffusion of H_2O_2 generated in mitochondria (Kakihana et al., 2012).

The data also contain also substantial evidence for important roles of vesicle-mediated trafficking. Of 157 proteins annotated by GO to vesicle-mediated transport (GO:0016192) or to post-Golgi vesicle-mediated transport (GO:0006892), only a minority show appreciable changes (Fig.11A). Changes for constituents of the AP2 complex (clathrin-mediated endocytosis) and the AP3 complex (endosomal/lysosomal transport) were observed only for OAC. In contrast, components of the coatomer complex (COPA, COPB1, COPG1; Golgi vesicle transport) show SAME increased nuclear abundance as a result of C $\rightarrow$ N redistribution for both OXS and OAC (Fig. 11B). The strongest changes were for the ferritin complex (FTL and FTH1) as a result of strong SAME increase in total abundance. These proteins are crucial to iron homeostasis and, interestingly, FTL and FTH1 both seemed to equilibrate readily between the nuclear and cytoplasmic compartments for both OXS and OAC despite the strong changes in total abundance. Decreased nuclear abundance only for OXS was observed for a series of RAB proteins (RAB1A, RAB2A, RAB6A, RAB14, RAB34) and for proteins involved in SNARE-related vesicle trafficking (SNAP23, VAMP3, STX7, STX12). Other RAB proteins that are not currently annotated to vesicle-mediated trafficking showed similar behavior (Radulovic et al., 2016). Overall, for OXS distinctive increases and decreases in nuclear abundance were observed for specific, different groups of proteins associated with different types of vesicle-mediated transport processes (Fig. 11B). With the exception of the ferritin subunits, all of these changes arise from changes in subcellular distribution rather than in total protein abundance. They seem to represent specific, directed nuclear transfer processes since the different proteins do not show significant changes in cytoplasmic abundance.

IX. ADVANTAGES AND PERSPECTIVES OF THE MS-BASED JOINT SPATIAL RAZOR APPROACH

Comprehensive pictures of cellular function will require collection of data on the subcellular transport and local functions of many moonlighting proteins, especially of

those with critical roles in spatial coordination across cells. Analyzing this is a serious technical challenge. “Selected monitoring” of a few proteins as is common in fluorescence experiments with tagged proteins can only provide incomplete and necessarily ambiguous “local explanations”. These ignore the encapsulation of a local fragment network in a much more complex network involving many more proteins. For example, since conservation of mass applies to cell biology, activation of the SET-RAC1-PP2A fragment network that influences cell migration (Fig. 8C) implies correlated changes for many other activities at many different subcellular locations. The very substantial intertwining of different, complex functional networks and the transfer of proteins between different local spatial/functional networks requires quantitative monitoring of whole networks rather than of a few individual proteins to distinguish direct, dominant mechanisms from indirect, propagated effects. The magnitudes of the responses in highly intertwined networks that cover diverse spatial locations are crucial. Assembly of hundreds or even thousands of parallel “local” experiments on presumably identical (but differently tagged) cells seems unlikely to provide quantitative interpretations of functional networks that involve moderate, but coordinated changes in hundreds of proteins. The quantitative MS-based joint spatial razor approach applied to analysis of multiple semi-stable states provides an effective means to prioritize nuclear features and proteins that should be further investigated. Such prioritization of the key proteins and networks that show the strongest responses is crucial. For example, the described experiments identified >100 proteins that have known functions in mitochondria, the plasma membrane, the ER, etc. and that show appreciable nuclear changes for OXS/OAC, but whose nuclear functions are ambiguous or unknown. This is suggestive of complex, inter-compartmental feedback loops (Radulovic et al., 2016). We anticipate that focussed, in-depth investigation of these proteins using confirmatory methods such as fluorescence tagging or protein-protein binding interactions will identify new nuclear functional networks. That is, the unbiased surveys provided by MS-based subcellular spatial razor methods can provide strong, new focussing for efforts by the many researchers with interests in specific subnetworks.

Overall, for global identification of “hot-spots” in enormously complex functional networks, the MS-based proteomics methods provide unique quantitative capabilities that cannot be even approached by any other currently available technology. This type

of approach needs to be further developed to include other subcellular locations, to include the plethora of post-translational modifications that are only visible to proteomics methods, and to be applied to a wide variety of cell types and functional contexts.

X. CONCLUSIONS

Although much progress has been made, for eukaryotic cells we are still far from having good quantitative models of their function. The arrival of the "moonlighting" paradigm represents a recognition that much of cellular function is at the proteome level and includes crucial mechanisms that are visible only very indirectly if at all with genomic approaches such as GWAS or measurements of global differential expression by transcriptomics or proteomics. The moonlighting paradigm also represents an indirect recognition that the spatial distribution of different cellular functions to different subcellular locations requires exquisitely controlled, fluid, spatial redistribution of cellular components to achieve regulation of function across cells. The cell biology revealed by spatial cross-talk between oxidative stress and DNA replication has been considered in more detail elsewhere (Radulovic et al., 2016). In the following, we outline why subcellular MS-based proteomics has a unique role to play in furthering understanding of cell biology.

Despite the accumulation of vast amounts of genomics data or of differential expression data, computational attempts to predict gene function so far have a rather limited degree of success (Lehtinen et al., 2015,Pavlidis& Gillis, 2012,Pavlidis& Gillis, 2013,Piro& Di Cunto, 2012). Conventional cell biology approaches regularly identify new functions for proteins and, indeed, the predictive computational methods are largely anchored by conventional cell biology results. Furthermore, large-scale gene expression data or large-scale protein-protein binding data typically only "explain" a minority of experimental data. For example, a more complete, quantitative, binary interaction analysis of the 401-set has been presented (Radulovic et al., 2016). As shown in Fig. 12, the combination of changes in total abundance and in compartmental redistribution leads to very complex changes in binary interaction potentials between different proteins in both the nuclear and cytoplasmic compartments. This emphasizes that protein trafficking strongly modulates functional interactions in specific compartments. Such changes cannot be detected by measurements of total cellular abundance of proteins or by transcriptomics. STRING,

REACTOME and CORUM contain a total of 134,850 curated binary interaction pairs between the 4048 proteins monitored in the OXS/OAC joint spatial razor experiments. There are 3472 binary interactions for the 401-set proteins. Of these latter interactions, 26% correspond to correlations in gene expression, 47% to binding interactions and the union of expression and binding corresponds to 56% (Radulovic et al., 2016). That is, despite collection of very large amounts of high throughput data of these types, a major fraction of current knowledge is based on aggregation of large numbers of conventional cell biology experiments. Conversely, conventional experiments are very incomplete and the OXS experiments identify substantial numbers of proteins whose involvement in oxidative stress was previously unknown and/or difficult to predict from prior knowledge. This includes spatial changes for proteins involved in crucial redox couples as well as systematic changes in transport pathways.

We suggest that spatial dynamics of proteins is a critical missing link in systems biology analysis of cellular function. The OXS results presented here indicate that the redox code goes well beyond the level of post-translational modifications of sensitive proteins and is replete with moonlighting proteins. This should not be a surprise. Dynamic changes in the abundance, form and distribution of proteins are essential for cellular function. For example, large proportions of the constituent proteins of mitochondria or the plasma membrane are known to be directed to the location by protein transport/import systems and to be recycled by processes such as autophagy. Recent evidence suggests that even organelles such as mitochondria may have constitutive export of proteins (Mohanty and McBride, 2013, Soubannier et al., 2012). Oscillatory circadian rhythms (Aguilar-Arnal and Sassone-Corsi, 2015), oscillations in response to oxidative state (Causton et al., 2015, Hoyle and O'Neill, 2015, Pekovic-Vaughan et al., 2014), superoxide bursts in mitochondria, (Wang et al., 2008, Xu et al., 2013) and pH bursts in endosomes (Lamb et al., 2009) are among the many other instances of dynamic behavior. Treadmilling of cadherins in the maintenance of cellular polarity and the possible involvement of this and other endocytotic processes in epithelial-to-mesenchymal transitions is another example (Corallino et al., 2015). The present data suggests that many such dynamic processes will be reciprocally coupled to processes such as DNA replication, oxidative state and the cell cycle. By focussing on the need for efficient inter-compartmental communication in a spatially

heterogeneous system, MS-based approaches such as the subcellular spatial razor methods provide powerful tools for identifying the dominant proteins and processes involved in high order spatio-functional coupling across cells.

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Figure Captions.

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Figure 7. Mapping of 401-set proteins to an r = 2 overlap matrix for 77 complexes found with the indicated search set. The assignment of the proteins to different experimental sets is indicated by the legend at lower right. The numbers enclosed in diamonds indicate the number of highly overlapped CORUM complexes that were combined to simplify visualization. For the four indicated branches the table inset shows the number of: unique proteins, total proteins (diagonal), and shared proteins (off-diagonal) as well as the total number of proteins that are contained in one to four branches.

Figure 8. The {KPNA2, KPNB1, PCNA, PTMA, SET} switch. (A) Joint spatial razor plot for the PCNA nuclear branch points (Fig. 7) and for proteins of the switch. (B) Binary interaction potentials for proteins A,B for total abundance changes ($S_t^A * S_t^B$) and nuclear abundance changes ($S_n^A * S_n^B$) for OXS for 54 proteins with binary interactions with one or more of PCNA, PTMA and SET. Green nodes are proteins in panel A. Edges are colored according to the scale. (C) Moonlighting activities ascribed to PTMA, SET and PCNA in the nuclear and cytoplasmic compartments. For SET, the red dashed box shows a local network fragment involved in cellular migration (Ten Klooster et al, 2007). The fragment network includes transport between the nucleus, cytosol and plasma membrane, nuclear phosphorylation of SET, cytoplasmic interaction of SET-RAC1 and plasma membrane interaction of SET-RAC1-PP2A. It may include redox modification of RAC1 with prostaglandin at the plasma membrane (Wall et al, 2015). Since SET, RAC1 and PP2A all have other activities, the evaluation of the functional importance of this fragment requires its quantitative encapsulation in a more extensive network. Inhibition of cytoplasmic PP2A phosphatases by SET is also important for hematologic malignancies (Haesen et al., 2014) and for hyperphosphorylation of tau in neuronal cells and Alzheimer's patients (Chasseigneaux et al., 2014). Distribution between the nucleus, cytoplasm and plasma membrane also toggles inhibition or promotion of axon growth and regeneration (Trakhtenberg et al., 2014).

Figure 9. Proteins involved in redox couples. (A) 31 proteins involved in the Cys/CySS and GSH/GSSG couples or peroxidases. (B) 155 proteins involved in the

NAD^+/ NADH or $NADP^+$/NADPH couples. (C) Proteins in panel B with significant changes for OXS.

Figure 10. Response to OXS/OAC of endoplasmic reticulum proteins. (A) 195 proteins annotated by GO to ER. (B) 15 ER proteins with significant changes for OXS. Additional ER proteins (HMOX1, SET, MGST3, BCAP31, LMAN1, RAB proteins) are shown in other figures (see text).

Figure 11. Proteins involved in vesicle-mediated transport. (A) 157 proteins annotated by GO to vesicle-mediated transport. (B) 16 proteins from panel A with significant changes for OXS.

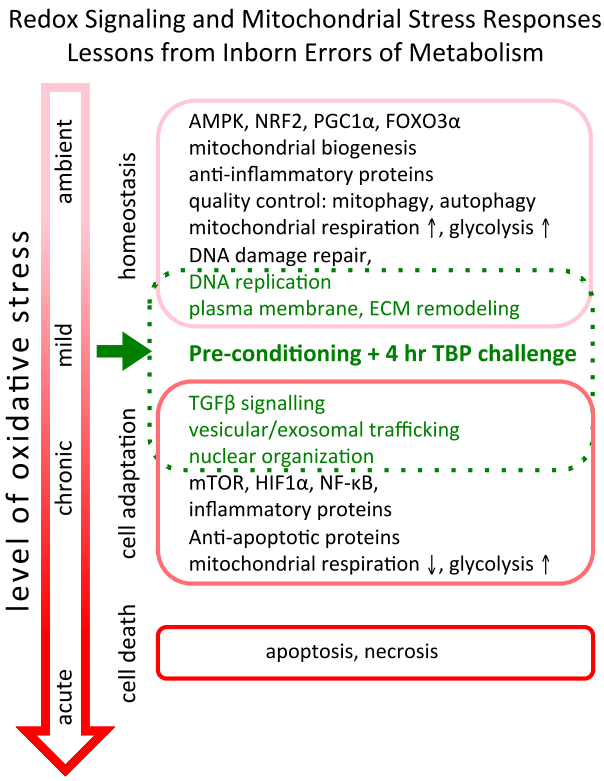
Figure 12. Nuclear and cytoplasmic interaction networks for the 401-set for OXS. The nodes correspond to 401 proteins with significant changes for OXS/OAC. The edges correspond to 3472 binary interactions from STRING, REACTOME and CORUM. For two proteins A,B the edges are colored according to the scale for the interaction potentials $(S_n^A \cdot S_n^B)$ and $(S_c^A \cdot S_c^B)$. The proteins were clustered to seven functional groups (table) with the T-fit algorithm of the Cytoscape plug-in Clust&See. The 401-set proteins have many interactions within their root cluster, but also many cross-cluster interactions.

Author's CVs and photos

Gabriella Pinto holds a first PhD in Biotechnology Sciences at Department of Organic Chemistry and Biochemistry and a second PhD at Department of Agriculture. She worked as a post-doctoral scientist at University College London, UK, within the "Proteomics & Molecular Cell Dynamics" research group led by Prof. Jasminka Godovac-Zimmermann. She worked at University of Naples "Federico II", National Research Council (CNR) of Italy and University College London, UK. Over the years, she worked in food proteomics, phosphoproteomics and large scale quantitative sub-cellular proteomics in breast cancer.

Marko Radulovic has a PhD in Immunology and currently holds a Research Professor post at the Institute for Oncology and Radiology in Belgrade, Serbia. He also worked at Max Planck Institute for Experimental Medicine in Germany, University College London and Imperial College in United Kingdom. His research interests are mainly in the computational analysis of tumour histomorphology and of cancer-related high-throughput proteomics data.

Jasminka Godovac Zimmermann is Professor at University College London, UK and Head of the UCL Proteomics&Molecular Dynamics Group. She is protein chemist trained for PhD at Max-Planck Institute for Biochemistry in Martinsried, Germany. Subsequently, she worked in Australia, Germany and UK where she established and directed centers for protein and proteomics research. Currently she works on breast cancer proteomics and the complexity of eukaryotic proteomics.



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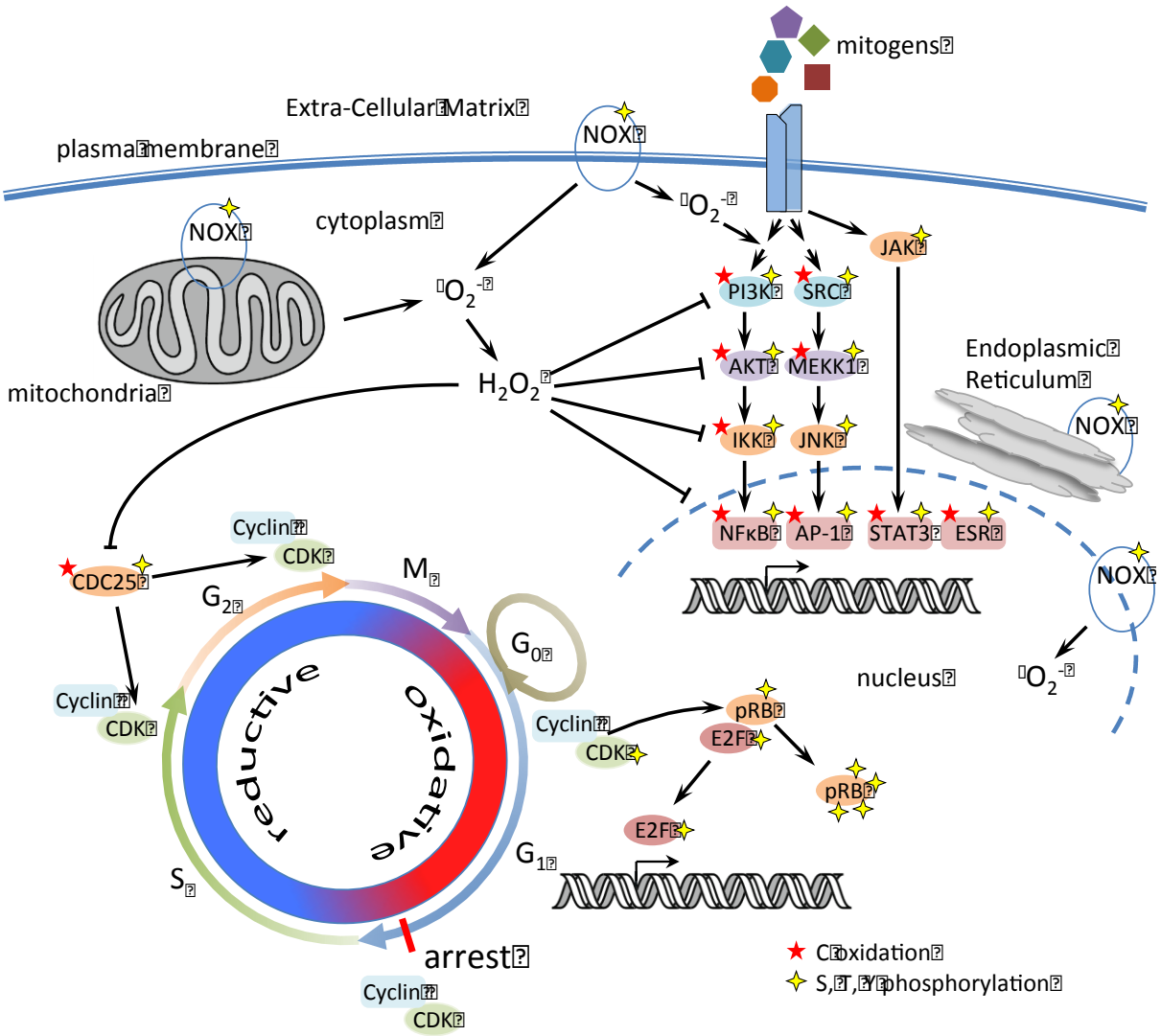

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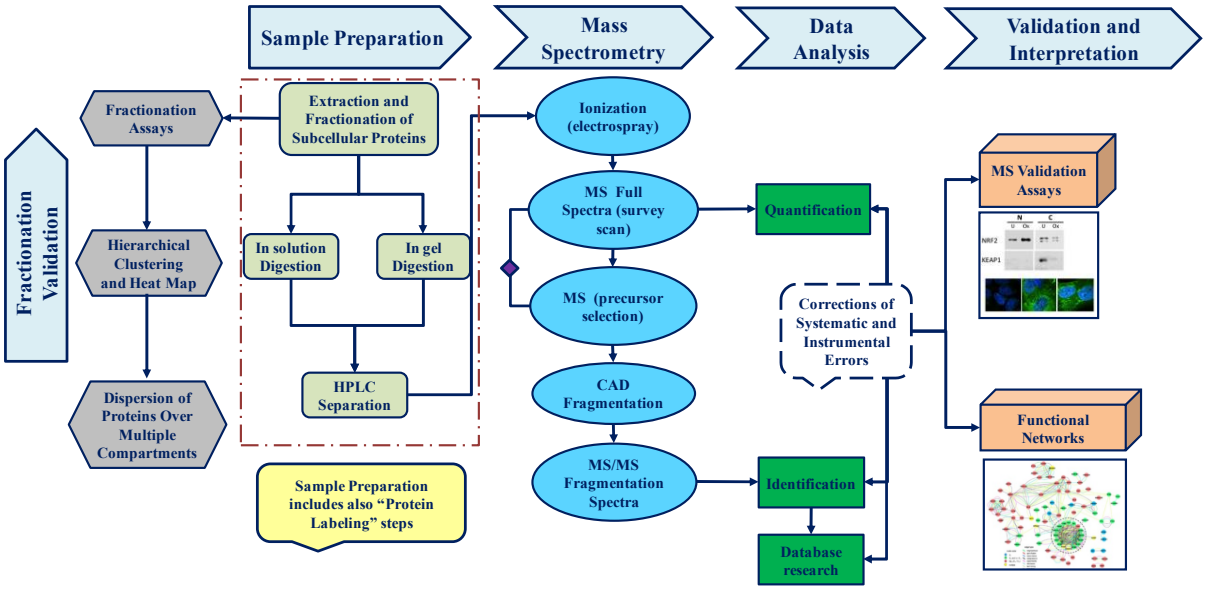

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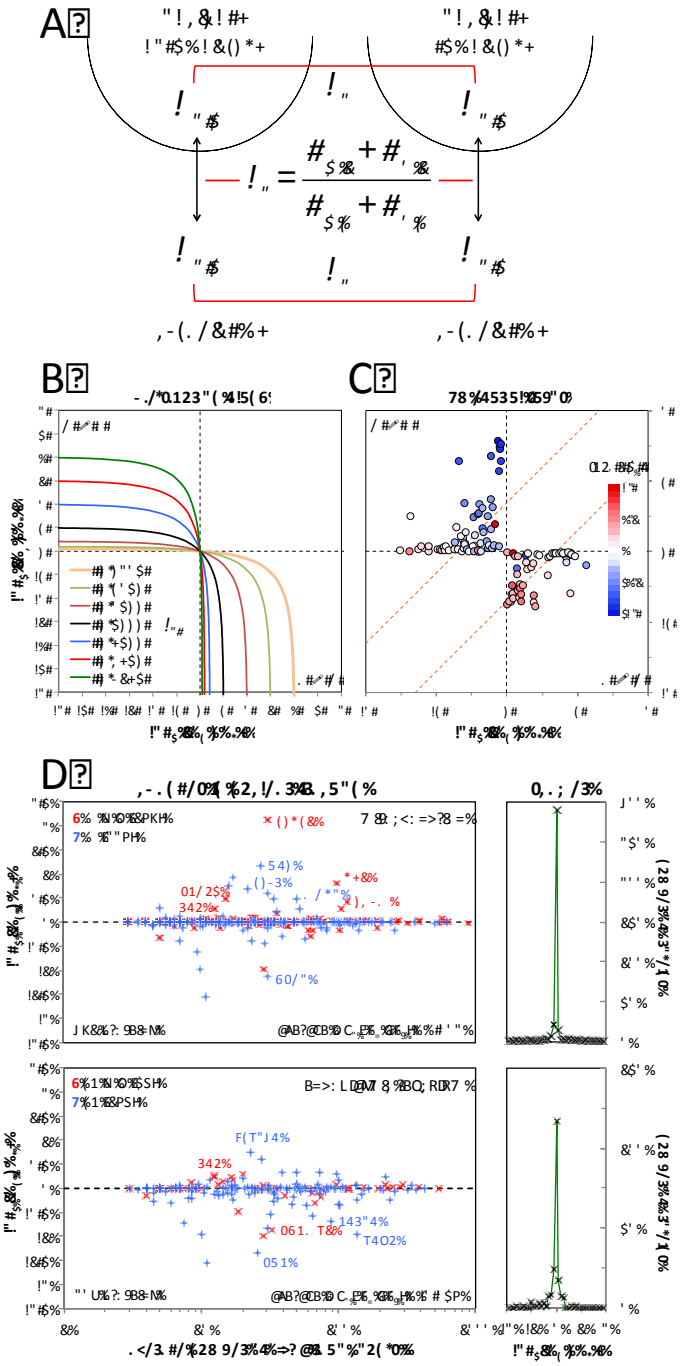

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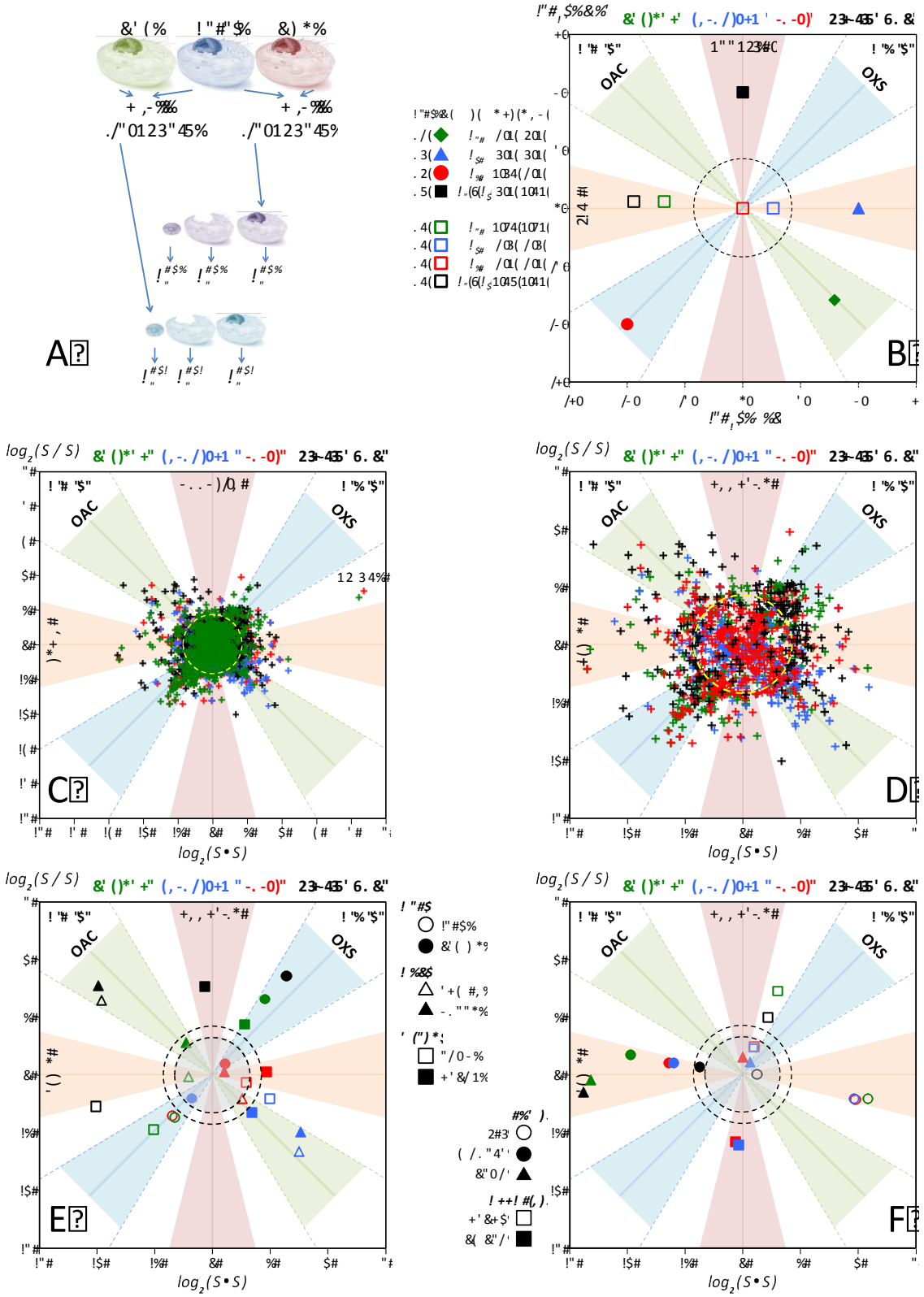

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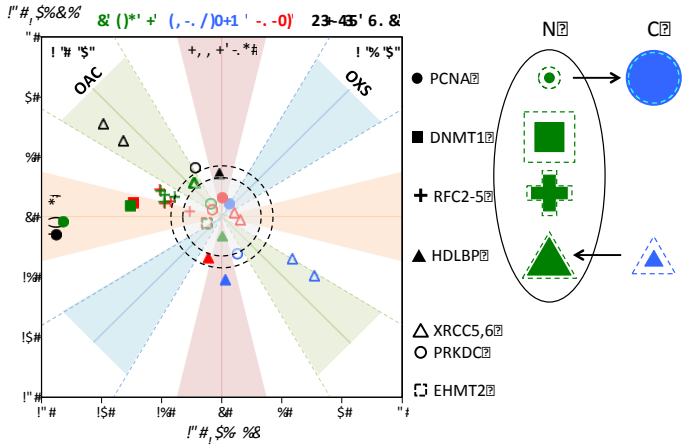

Fig. 6. Changes in total and compartmental abundance for core protein complexes containing PCNA. *Left*. Joint spatial razor plot for the constituent proteins of three complexes: (PCNA, RFC2-5), (PCNA, DNMT1, EHMT2) and (PCNA, XRCC5,6). *Right*. Cartoon of the changes in abundance relative to basal abundance for the nuclear and cytoplasmic compartments for OXS. The relative size of the dotted (basal) and solid (perturbed) symbols denotes the direction of the change in abundance in each compartment for the individual proteins. The arrows indicate the direction of compartmental redistribution. The relative changes are sensitive to the basal distribution between compartments, e.g. basal PCNA is predominantly in the cytoplasmic compartment. RFC2-5, DNMT1 and EHMT2 were detected only in the nuclear compartment.

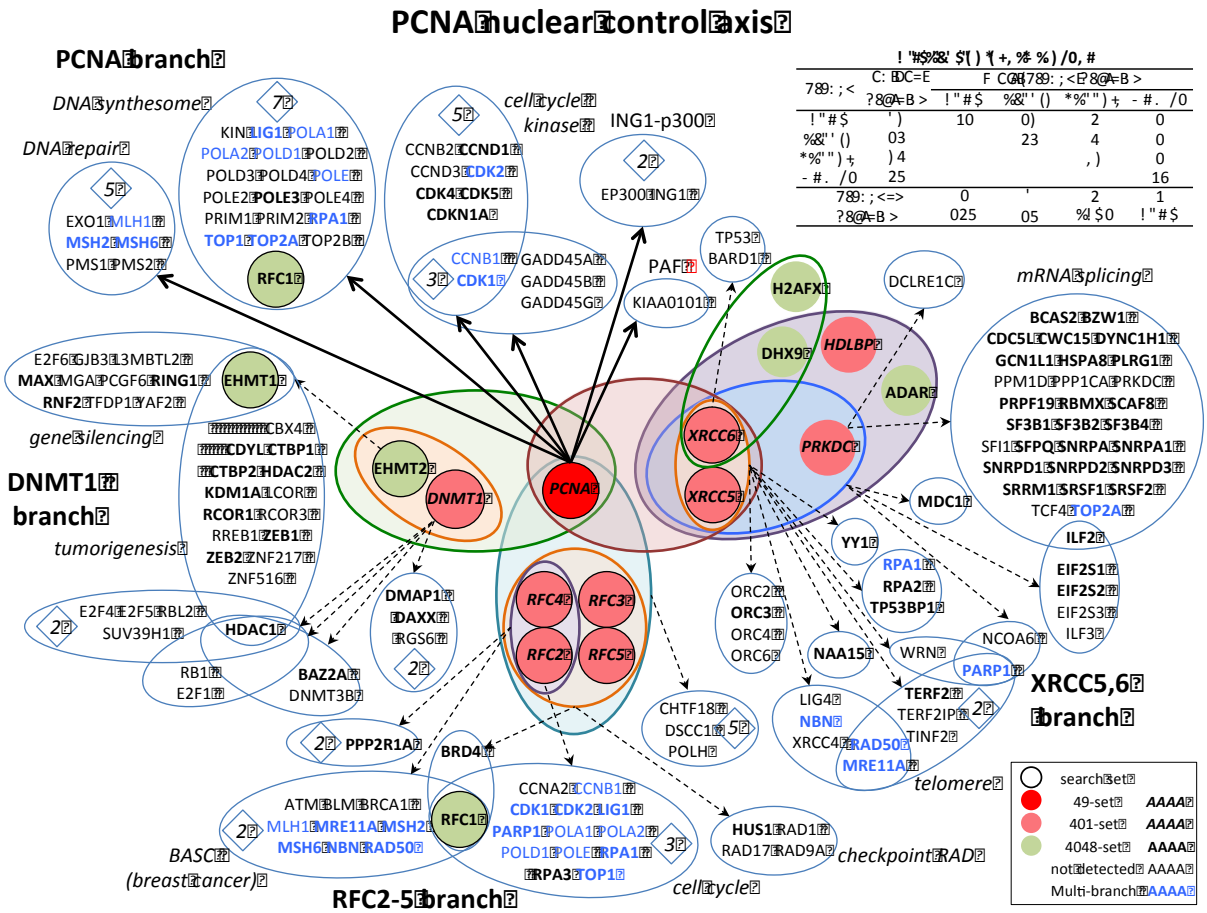

Fig. 7. Mapping of 401-set proteins to an r = 2 overlap matrix for 77 complexes found with the indicated search set. The assignment of the proteins to different experimental sets is indicated by the legend at lower right. The numbers enclosed in diamonds indicate the number of highly overlapped CORUM complexes that were combined to simplify visualization. For the four indicated branches the table inset shows the number of: unique proteins, total proteins (diagonal), and shared proteins (off-diagonal) as well as the total number of proteins that are contained in one to four branches.

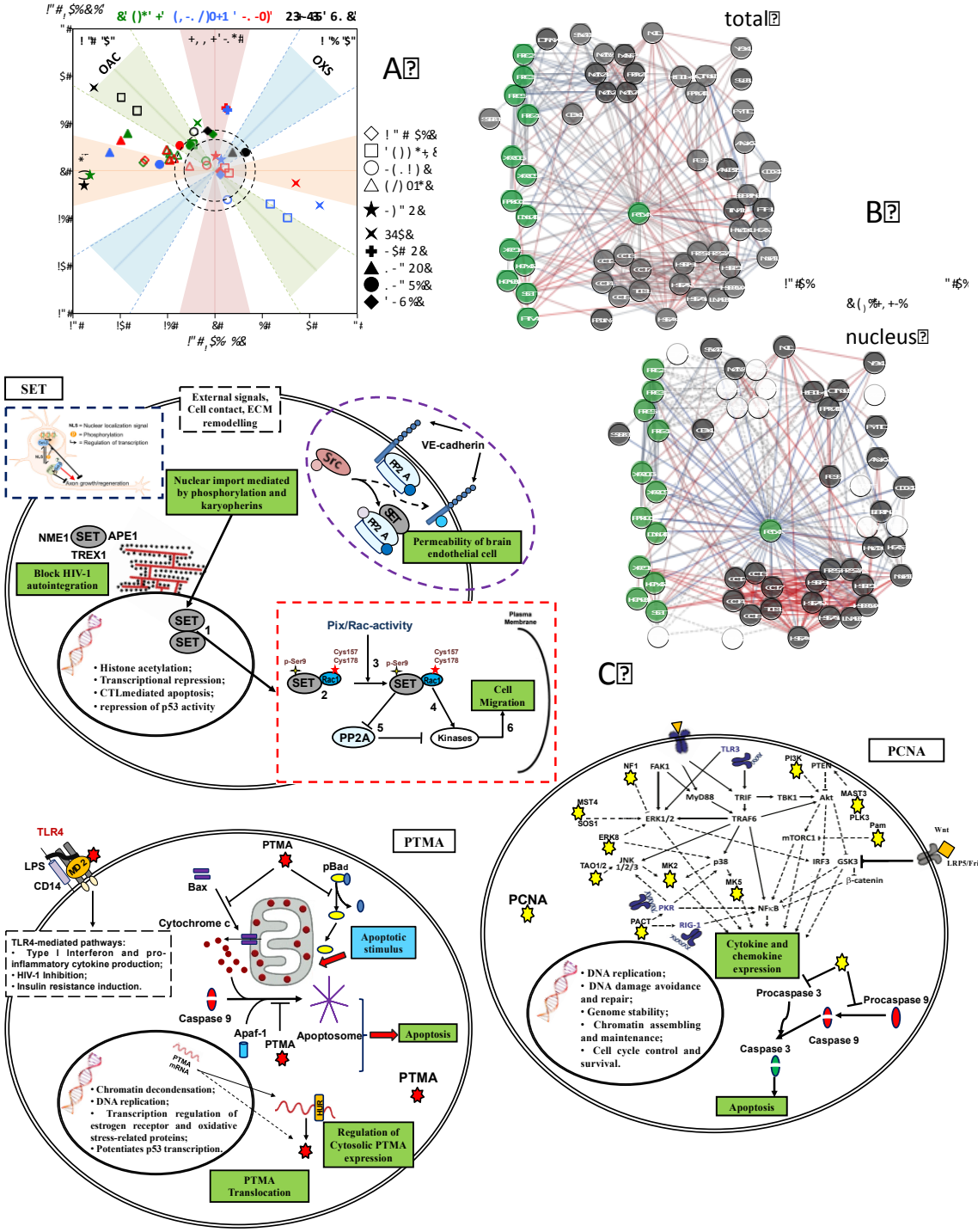

Figure 8. The {KPNA2, KPNB1, PCNA, PTMA, SET} switch. (A) Joint spatial razor plot for the PCNA nuclear branch points (Fig. 7) and for proteins of the switch. (B) Binary interaction potentials for proteins A,B for total abundance changes ($S_t^A * S_t^B$) and nuclear abundance changes ($S_n^A * S_n^B$) for OXS for 54 proteins with binary interactions with one or more of PCNA, PTMA and SET. Green nodes are proteins in panel A. Edges are colored according to the scale. (C) Moonlighting activities ascribed to PTMA, SET and PCNA in the nuclear and cytoplasmic compartments. For SET, the red dashed box shows a local network fragment involved in cellular migration (Ten Klooster et al, 2007). The fragment network includes transport between the nucleus, cytosol and plasma membrane, nuclear phosphorylation of SET, cytoplasmic interaction of SET-RAC1 and plasma membrane interaction of SET-RAC1-PP2A. It may include redox modification of RAC1 with prostaglandin at the plasma membrane (Wall et al, 2015). Since SET, RAC1 and PP2A all have other activities, the evaluation of the functional importance of this fragment requires its quantitative encapsulation in a more extensive network. Inhibition of cytoplasmic PP2A phosphatases by SET is also important for hematologic malignancies (Haesen et al., 2014) and for hyperphosphorylation of tau in neuronal cells and Alzheimer's patients (Chasseigneaux et al., 2014). Distribution between the nucleus, cytoplasm and plasma membrane also toggles inhibition or promotion of axon growth and regeneration (Trakhtenberg et al., 2014).

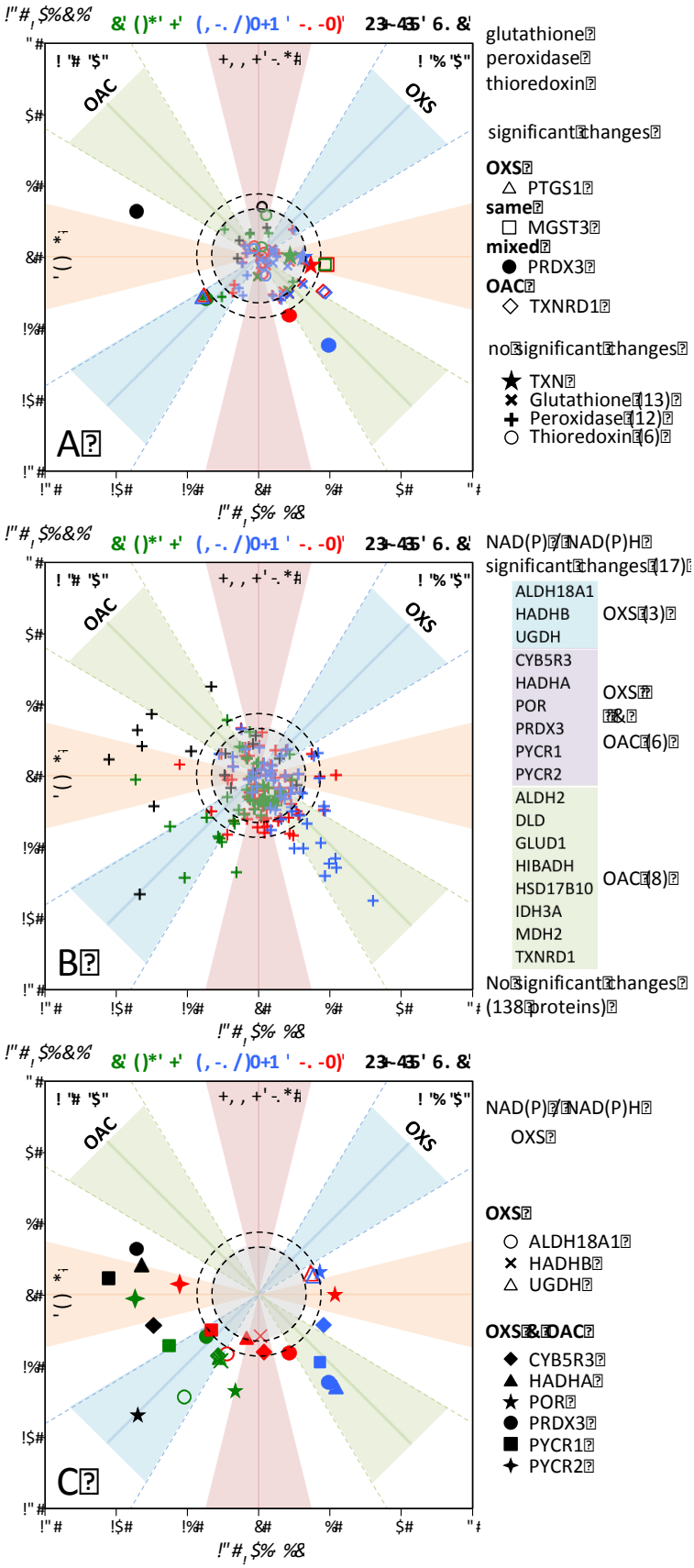

Figure 9. Proteins involved in redox couples. (A) 31 proteins involved in the Cys/CySS and GSH/GSSG couples or peroxidases. (B) 155 proteins involved in the NAD^+/ NADH or $NADP^+$/NADPH couples. (C) Proteins in panel B with significant changes for OXS.

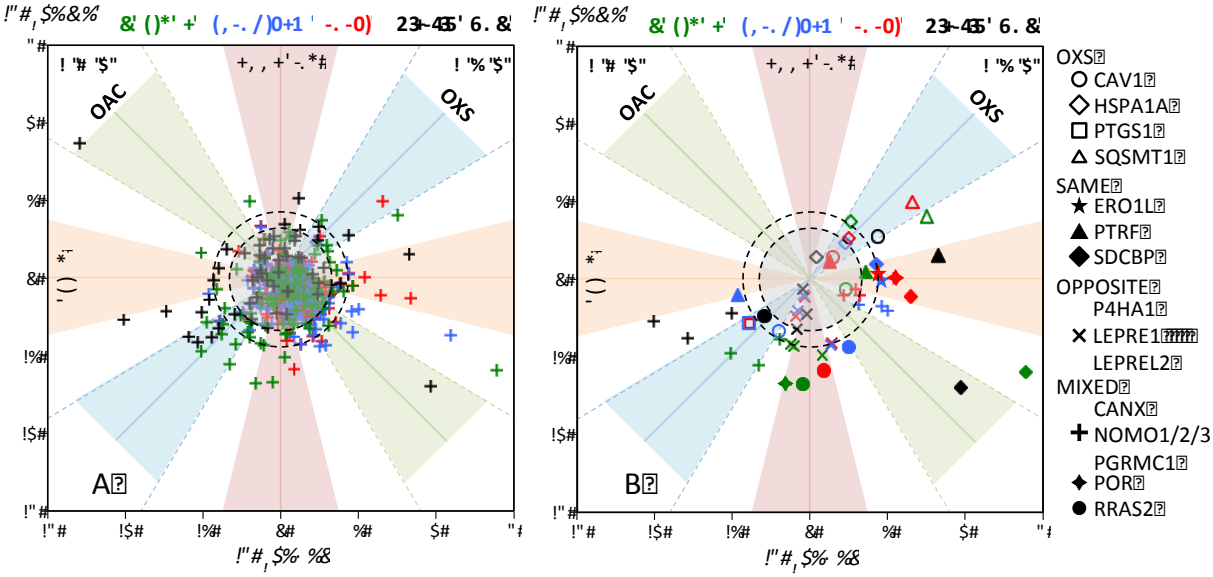

Figure 10. Response to OXS/OAC of endoplasmic reticulum proteins. (A) 195 proteins annotated by GO to ER. (B) 15 ER proteins with significant changes for OXS. Additional ER proteins (HMOX1, SET, MGST3, BCAP31, LMAN1, RAB proteins) are shown in other figures (see text).

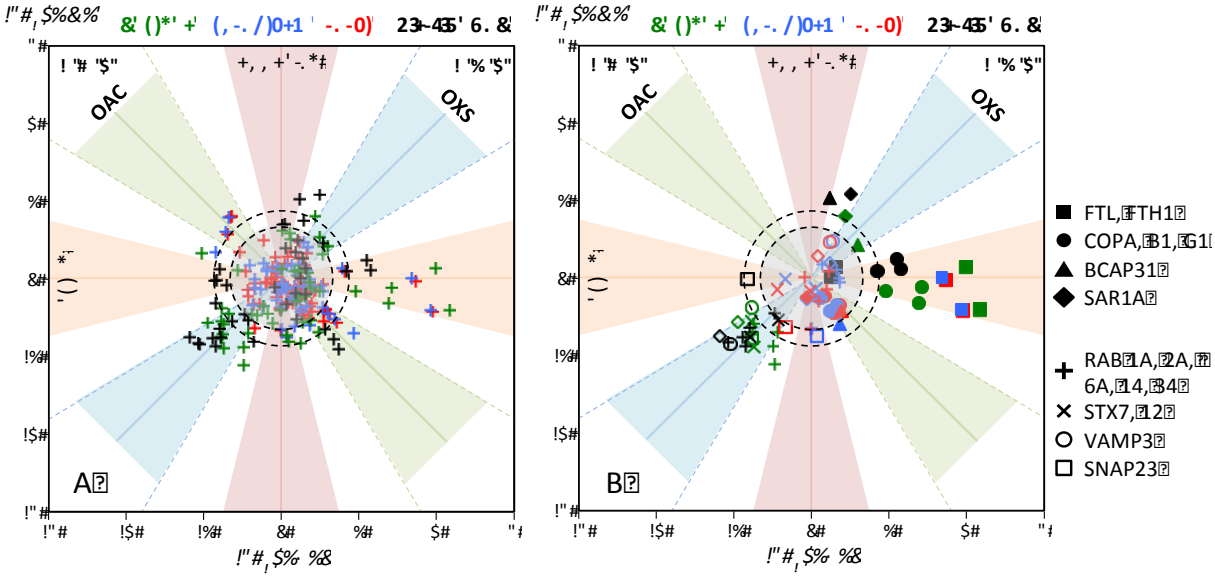

Figure 11. Proteins involved in vesicle-mediated transport. (A) 157 proteins annotated by GO to vesicle-mediated transport. (B) 16 proteins from panel A with significant changes for OXS.

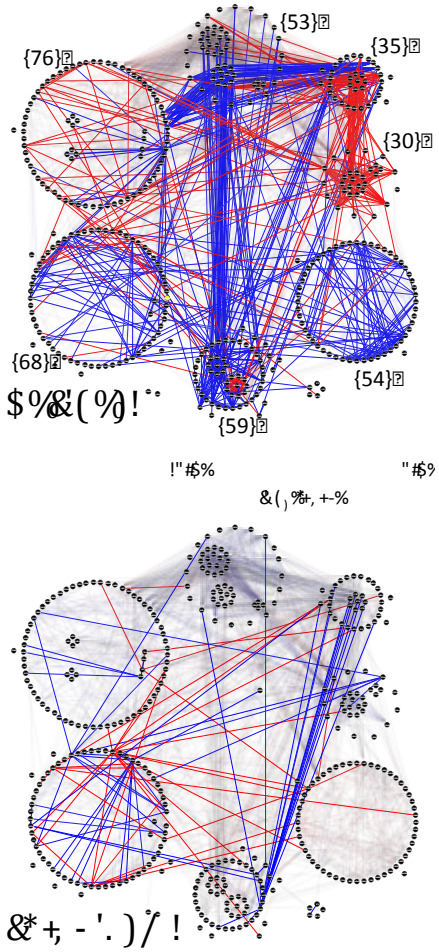

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Figure 12. Nuclear and cytoplasmic interaction networks for the 401-set for OXS. The nodes correspond to 401 proteins with significant changes for OXS/OAC. The edges correspond to 3472 binary interactions from STRING, REACTOME and CORUM. For two proteins A,B the edges are colored according to the scale for the interaction potentials $(S_n^A \cdot S_n^B)$ and $(S_c^A \cdot S_c^B)$. The proteins were clustered to seven functional groups (table) with the T-fit algorithm of the Cytoscape plug-in Clust&See. The 401-set proteins have many interactions within their root cluster, but also many cross-cluster interactions.