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Proteomic Approaches to Unraveling the RB/E2F Regulatory Pathway

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1. Introduction

Correct entry into and progression through the cell cycle require an intact RB/E2F pathway, and its deregulation is now considered a general hallmark of cancer (Nevins 2001). Pioneer work in the early 90's showed that E2F activity is controlled through the temporal association of E2F factors with Retinoblastoma (RB) tumor suppressor proteins (pRB, p107 and p130), also called pocket proteins (Bandara & La Thangue 1991; Chellappan et al. 1991). The additional finding that RB activity is regulated through phosphorylations by cyclin dependent kinases (CDKs) provided the groundwork for the current model of cell cycle control (Weinberg 1995). According to this model, non-phosphorylated RB binds to E2F in G0/G1, leading to the repression of E2F target genes. Subsequent phosphorylation of RB by CDKs in mid-to late G1 disrupts its association with E2F. As a result, free E2F triggers the expression of target genes necessary for entry into and progression through the cell cycle (Burkhart & Sage 2008). This pathway is thought to be disrupted in most human cancers, either by activation of positively acting components such as G1 cyclins and CDKs, or the inactivation of negatively acting components such as RB and cyclin kinase inhibitors (Nevins 2001). The predicted consequence of deficient RB-mediated regulation is that E2F activity is constantly unleashed from the inhibitory effects of RB (DeGregori & Johnson 2006; Dimova & Dyson 2005; Iaquinta & Lees 2007).

Mammalian E2F is a family of related factors (E2F1-8), originally discovered for their pivotal role in transcriptional regulation of genes associated with DNA replication and G1/S progression (Attwooll et al. 2004; Trimarchi & Lees 2002). More recently, microarray expression profiling analyses and ChIP-chip analyses (chromatin immunoprecipitation coupled to microarray technology) in cells overexpressing individual E2Fs have revealed that the transactivation function of these factors exceeds beyond G1/S transition regulation. In fact, E2Fs regulate a wide spectrum of genes with diverse biological functions, including regulation of apoptosis, autophagy, mitosis, chromosome organization, macromolecule metabolism, or differentiation (Ma et al. 2002; Muller et al. 2001; Polager et al. 2008; Ren et al. 2002; Weinmann et al. 2002; Young et al. 2003). Thus, the role of E2F transcription factors in cellular physiology is probably more complex than it was originally thought to be.

Traditionally, the mammalian E2F family has been divided into “activators” (E2F1-3) and “repressors” (E2F4-8). However, recent *in vivo* data have questioned this oversimplified classification. Indeed, accumulating evidence suggests that most E2Fs can function both as activators as well as repressors, depending on the cellular context (Balciunaite et al. 2005; Iglesias et al. 2004; Infante et al. 2008; Lang et al. 2001; Lee et al. 2011; Ma et al. 2002; Morris et al. 2000; Muller et al. 2001; Polager et al. 2008; Young et al. 2003). Likewise, both oncogenic and tumor suppressor properties have been assigned to these factors (DeGregori & Johnson 2006; Johnson & DeGregori 2006). The mechanisms underlying this bimodal impact of individual E2Fs, and their implication in human cancer development remain to be elucidated. This is a particularly relevant point that needs to be addressed, since strategies based on E2F biology are being devised for the development of anticancer therapies (Kaelin, Jr. 2003). An additional level of complexity in the understanding of E2F function *in vivo* derives from the considerable functional overlap existing among several E2F members (Chen et al. 2009a; DeGregori & Johnson 2006). Nonetheless, the characterization of mouse models lacking individual E2Fs has revealed that these factors play unique roles in development, tissue homeostasis and tumor formation (Chen et al. 2009a; DeGregori & Johnson 2006; Trimarchi & Lees 2002).

Functional specificity of individual E2F factors is thought to be established through the regulation of distinct sets of target genes. In fact, there is growing evidence that this specificity is achieved by interaction of E2Fs with other proteins or by post-translational modifications (PTMs) on E2Fs or E2F-containing complexes. Much of this evidence has been gathered through proteomic approaches such as yeast two-hybrid screening, two-dimensional electrophoresis (2-DE) followed by mass spectrometry (MS) or shotgun proteomics (Figure 1). In this review, the application of proteomics in the study of RB/E2F regulatory pathway is summarized. Results derived from these experiments are expanding our current understanding of the RB/E2F biology in several important ways. They are piecing together the interactions within macromolecular complexes that regulate transcription of E2F target genes. Furthermore, they are helping define the mechanisms underlying RB/E2F-dependent control of cellular physiology and pathology.

2. Identification of proteins that interact with E2Fs

It has long been recognized that E2F activity is regulated through the association of E2F factors with specific protein partners. In fact, E2F1, the founder member of the family, was first identified as a sequence-specific DNA-binding activity that co-precipitated with RB (Chittenden et al. 1991). Recent development of non-hypothesis driven proteomic approaches has allowed a more extensive analysis of protein-protein interactions in the E2F field. Several methods have been successfully applied in the identification of RB/E2F interacting partners, in particular, yeast two-hybrid screenings and affinity purification coupled to MS.

2.1 Genome-wide yeast two-hybrid interaction screening

The yeast two-hybrid method is one of the most widely used methods for mapping protein-protein interactions. In this method, the “bait” protein is typically expressed in yeast as a chimeric protein fused to the DNA-binding domain of a known transcription factor (usually *Gal4*). All other “target” proteins that the bait protein is going to be screened against are expressed within the cell fused to the activation domain of this same transcription factor.

Fig. 1. A schematic diagram showing proteomic approaches to analyzing regulatory signaling pathways.

The interaction between the bait and target proteins brings into close proximity the DNA binding and activation domains of the transcription factor, resulting in the activation of a reporter gene (Fields & Song 1989). Given that the conditions applied in this methodology are not physiological, some of the detected interactions may not represent true interactions. Consequently, this experimental system is thought to yield a high false positive rate. Consequently, interactions detected by this system need to be further validated in an appropriate physiological system. Despite the mentioned drawbacks, it is also true that the yeast two-hybrid interaction screening provides a method to scrutinize protein-protein interactions within living cells, whereas other approaches measure protein interactions after the complexes have been removed from the cellular environment.

Interaction proteomics has been helpful in exploring the intricate macromolecular interactions established by RB and E2F for the regulation of gene expression. Work from many laboratories has shown that RB mediates transcriptional repression through the recruitment of a large number of co-repressors, resulting in an alteration of chromatin conformation that hinders transcription. Most RB/E2F co-repressors, including histone deacetylases (HDAC1-3), nucleosome remodeling proteins (BRG1), DNA methyl transferases (DNMT1) or RBP1 have been identified through hypothesis-driven classical biochemical approaches (Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998). Interestingly, HBP1 and CtIP/CtBP co-repressors were discovered by yeast two-hybrid screening analyses using the pocket protein p130 as the bait (Meloni et al. 1999a; Tevosian et al. 1997). HBP1, a tumor suppressor member of the HMG family of transcription factors,

was found to function as a transcriptional repressor of N-MYC in association with RB in terminally differentiated muscle cells. This finding implies a role of this complex in the initiation and establishment of cell cycle arrest during differentiation (Tevosian et al. 1997). However, E2F proteins were not found in this repressor complex. By contrast, the complex formed by CtIP/CtBP and p130 also included E2F1, and provided an additional mechanism for RB/E2F-mediated repression (Meloni et al. 1999b). In agreement with the original findings, it has been recently shown that CtIP/CtBP plays a transcriptional co-repressor role in ZBRK1 expression. ZBRK1 is a zinc finger-containing transcriptional repressor that can modulate the expression of GADD45A to induce cell cycle arrest in response to DNA damage (Liao et al. 2010). It has been proposed that the contribution of RB to DNA damage-induced growth arrest may depend on the formation of this complex and loss of CtIP/CtBP-mediated repression could affect the cellular sensitivity to DNA damage. Conversely, CtIP/CtBP is able to activate the expression of a subset of E2F-target genes after its release from RB-imposed repression, implying that it can also function as an activator in other cellular contexts (Liu & Lee 2006).

Yeast two-hybrid screening has been particularly valuable to delve into the mechanistic basis for the functional specificity of E2F factors, particularly the so-called E2F “activators” (E2F1-3). This E2F subfamily exhibits a significant degree of functional redundancy among its members. However, E2F1 appears to be a stronger inducer of apoptosis than E2F2 and E2F3 (DeGregori et al. 1997; Hong et al. 2008; Kowalik et al. 1998; Lazzerini et al. 2005). The predominant role of E2F1 over the other E2F members in triggering apoptosis is thought to be conferred by unique protein partners that E2F1 associates with. The critical domain to specifically induce apoptosis has been shown to lie in the marked box of E2F1 (Hallstrom & Nevins 2003). Taking advantage of this knowledge, the E2F1 marked box has been used by Hallstrom and Nevins as the bait to screen for protein partners that could mediate E2F1-dependent apoptosis. JAB1 (c-JUN activating-binding protein) was identified as an E2F1-specific binding protein that functions synergistically with E2F1 to induce apoptosis coincident with an induction of p53 protein accumulation (Hallstrom & Nevins 2006). Interestingly, JAB1 association appears to regulate exclusively the apoptotic role of E2F1, as cell cycle entry is not affected by this E2F protein partner. In addition to JAB1, several more E2F1-interacting proteins were detected in this screen (Table 1), although their functional relevance in E2F1 function remains to be determined.

Remarkably, the E2F marked box has emerged as an important domain for mediating protein interactions that could dictate specificity of promoter recognition. For example, E2F2 and E2F3, but not E2F1 or E2F4, have been shown to interact specifically with RYBP (Ring-1 and YY1-binding protein) through their marked box. RYBP recruits these E2Fs to target promoters containing YY1 binding sites such as the CDC6 promoter. It has been proposed that the formation of an E2F2/3-RYBP-YY1 complex would facilitate the timely activation of CDC6 (Schlisio et al. 2002). An independent yeast two-hybrid screen with E2F3 as the bait discovered TFE3 (an E-box binding factor) as a protein that specifically interacts with E2F3. This association, which is dependent on the marked box of E2F3, facilitates transcriptional activation of the p68 subunit gene of DNA Pol α (Giangrande et al. 2003). Furthermore, this screen also yielded several more proteins that bound specifically the marked box of E2F3 (Table 1). Some of these proteins, such as CBP, RYBP or MGA had previously been shown to interact with E2Fs (Morris et al. 2000; Ogawa et al. 2002; Schlisio et al. 2002; Trouche et al. 1996), providing a strong validation of the screen. By contrast, E2F1, E2F2 and E2F4 are unable to bind TFE3 or to activate transcription of p68. Based on the characterization of all

E2F member	Interacting Protein	Function	Reference
E2F1	JAB1	Promotes apoptosis	Hallstron & Nevins 2006
	EAPP	Activates transcription of TK and represses transcription of p14 ^{ARF} . Promotes proliferation	Novy et al. 2005
	SKIP, CBP, TEF-5, B-MYB, MGA	n.d.	Giangrande et al. 2003
	MCRS1, E1F1, FHOD1, FLNA, SUI1, A-FABP, MX1, RNF2, PRMT3, RANBP9	n.d.	Hallstron & Nevins 2006
E2F2	RYBP	Activates transcription of CDC6	Schlisio et al. 2002
	B-MYB	n.d.	Giangrande et al. 2003
	ALIEN	n.d.	Escher et al. 2007
	EAPP	n.d.	Novy et al. 2005
E2F3	RYBP	Activates transcription of CDC6	Schlisio et al. 2002
	TFE3	Activates the p68 subunit gene of DNA Pol α	Giangrande et al. 2003
	RXR-BP, WNK1, PKI-B, SKIP, RYBP, CBP, TEF-5, B-MYB, MGA, SPIB	n.d.	Giangrande et al. 2003
	HSP86, A-FABP, MX1, RNF2, PRMT3, RANBP9	n.d.	Hallstron & Nevins 2006
	ALIEN	n.d.	Escher et al. 2007
	EAPP	n.d.	Novy et al. 2005
E2F4	ALIEN	n.d.	Escher et al. 2007
	MGA	n.d.	Giangrande et al. 2003
E2F5	ALIEN	n.d.	Escher et al. 2007
E2F6	ALIEN	n.d.	Escher et al. 2007

Table 1. E2F-specific binding partners identified through proteomic approaches. n.d.: not determined

these interactions it has been proposed that transcriptional regulation of specific E2F target genes can only be achieved when relevant interacting proteins act jointly forming a functional complex on the promoter (Freedman et al. 2009). Therefore, the unique marked box of each individual E2F factor appears to play a pivotal role in determining the specificity of interaction.

Other proteins exhibit E2F-binding activity independently of the marked box, allowing the mapping of distinct functional domains within the E2Fs. A yeast two-hybrid interaction

screen with the amino-terminal region of E2F1 comprising the NLS but lacking the marked box led to the cloning of EAPP (E2F-associated phosphoprotein) (Novy et al. 2005). EAPP appears to potentiate the proliferative functions of E2F1 in several ways. It enhances the transcription of growth-correlated E2F-target genes like thymidine kinase whereas it represses the expression of the tumor suppressor p14^{ARF}, an E2F1-target gene that can mediate E2F-induced apoptosis. Of note, EAPP can also interact with E2F2 and E2F3a through their amino-terminal regions (Novy et al. 2005). Consequently, EAPP could mediate proliferation related redundant functions of the E2F “activators” through binding to their well-conserved N-terminal region.

E2F6 together with E2F7 and E2F8 have features that set them apart from other members of the E2F family. These factors lack RB-binding and transactivation domains, and pocket proteins are thought not to complex with E2F6-8 (Cartwright et al. 1998; Gaubatz et al. 1998; Trimarchi et al. 1998). Thus, there is a particular interest in identifying the proteins that interact with these non-classical E2F members and determining their mechanism for transcriptional regulation of target genes. Two independent yeast two-hybrid analyses have described distinct interactions between E2F6 and components of the Polycomb Group (PcG), known mammalian transcriptional repressors (Bunker & Kingston 1994). In the first study, E2F6 was shown to associate with members of the PRC1 complex, including some PcG proteins (RING1, MEL-18, MPH1, and BMI1) as well as RYBP (Trimarchi et al. 2001). The second screening identified the PcG protein EPC1 as a novel E2F6-binding protein, in complex with EZH2 and SIN3B (Attwooll et al. 2005). In both cases, the association of E2F6 with PcG proteins could account for an RB-independent mechanism for the recruitment of repressive complexes to E2F6 target promoters and the consequent transcriptional repression (Trimarchi et al. 2001). It should be mentioned that this work did not detect an interaction between RYBP and E2F2 or E2F3, as shown by Schlisio and co-workers (Schlisio et al. 2002). The basis for this discrepancy is not clear, but could reflect methodological differences. However, the common ability of E2F2, E2F3 and E2F6 to interact with RYBP, suggests that E2F2 and E2F3 may be able to repress a subset of target genes by RB-independent mechanisms.

2.2 Affinity purification coupled to mass spectrometry

Identification of protein-protein interactions is becoming increasingly easier since the extraordinary advances that have taken place in recent years in mass spectrometry (MS). Affinity purification coupled to mass spectrometry is currently the preferred method for screening protein-protein interactions, owing to the sensitivity, specificity and reliability of this approach (Gavin et al. 2002; Ho et al. 2002; Krogan et al. 2006; Sellers et al. 1998). In contrast to classical biochemical methods, MS-based proteomics is a discovery or explorative non-hypothesis driven science. However, it should be noted that the identification of protein complexes remains a significant challenge. This is because many interactions are transient and can be easily lost during sample preparation. Additionally, many proteins are expressed at low levels, and it remains difficult to purify them. In fact, isolating the protein complex is the most critical step in determining the success of a proteomic analysis. The most widely used method for protein complex isolation is antibody-based immunoprecipitation (IP), although MS is also compatible with other affinity purification methods used to map protein-protein interactions, such as oligoprecipitation and tagged protein precipitation by affinity (“protein pull-down”)(Gavin et al. 2002; Meng et al. 2006).

A wide range of RB/E2F partners have been identified to date by MS. Remarkably, the discovered proteins are mainly involved in transcriptional repression, which highlights the critical role of the RB/E2F network in the timely suppression of target gene expression. Combining immunological methods with an uncommon mass spectrometric technique named SELDI (surface-enhanced laser desorption/ionization) (Lehmann et al. 2005), co-repressor ALIEN was identified in E2F1 containing endogenous protein complexes (Escher et al. 2007). Additional co-immunoprecipitation experiments revealed specific interactions of ALIEN with E2F2 through E2F6. ALIEN is able to repress E2F1 transcriptional activity when tethered to target promoters containing E2F binding sites (Tenbaum et al. 2007). The mechanism for this repressive activity remains undetermined. An RB-dependent process has been proposed, based on the finding that ALIEN interacts with pRB/p107 and HDAC (Escher et al. 2007). However, ALIEN-mediated co-repression is also evident in cells lacking functional RB (Tenbaum et al. 2007).

Affinity purification coupled to MS analysis has been particularly useful in the identification of native RB/E2F transcriptional repressor complexes (Table 2). They were firstly characterized in *Drosophila melanogaster* (Korenjak et al. 2004). Subsequently, homologous complexes were identified in other animal species, including *Caenorhabditis elegans* and human (Harrison et al. 2006; Litovchick et al. 2007), emphasizing the importance of this pathway. Before the application of proteomic methods, a wide assortment of chromatin-modifying and binding complexes had been implicated in RB-mediated repression (Frolov & Dyson 2004). However, it is unclear which of the many reported interactions are biologically meaningful, due to the non-physiological methods employed for the screening. In an effort to characterize native RB/E2F repressor complexes in *Drosophila*, Brehm's group took advantage of the relative simplicity of the *Drosophila* dRB/E2F network, consisting of two pocket proteins, RBF1 and RBF2, and two E2F proteins, dE2F1 and dE2F2 that heterodimerize with a common partner, dDP. By classical chromatography followed by MS analysis of the resulting fractions, they isolated an RBF multisubunit complex, termed dREAM (Korenjak et al. 2004), which contains RBF1/2, dE2F2, dDP, dMyb and dMyb-interacting proteins (Mip/TWIT, CAF1p55, Mip40 and Mip120). Interestingly, an independent analysis of Myb-associated proteins, involving affinity chromatography and DALPC mass spectrometry analysis (direct analysis of large protein complexes) resulted in the identification of a similar complex in *Drosophila* that was called Myb-MuvB complex (Lewis et al. 2004).

In agreement with a role in transcriptional repression, the identified complexes localize to transcriptionally silent sites on polytene chromosomes and mediate stable repression of a specific set of E2F targets that have sex- and differentiation-specific expression patterns. The mechanism by which these repressive complexes mediate the silencing of their target genes has been controversial, and enzymatic and non-enzymatic modes of repression have been suggested (Korenjak et al. 2004; Lewis et al. 2004). The finding that dREAM only binds deacetylated histone H4, characteristic of repressed chromatin, suggests that dREAM complexes perform their repressive function through binding unmodified nucleosomes and therefore protecting them from activating modifications (Korenjak et al. 2004). This hypothesis was further supported by the identification of histone deacetylase Rpd3, the HDAC1 homolog in *Drosophila*, associated with the repressor complex (Lewis et al. 2004). Strikingly, at least seven, and possibly all dREAM subunits are related to *C. elegans* synMuv class B genes (Fay & Han 2000). These proteins encompass the DRM complex, genetically resolved by Harrison and collaborators, which controls vulval differentiation in the worm (Harrison et al. 2006). The similarity between the worm DRM and the fly dREAM and Myb-

E2F member	Complex	Function	Reference
Drosophila E2F2	dREAM: RBF1/2, dDP,dMyb, Mip/TWIT,CAF1p55, Mip40 , Mip120, Rpd3	Transcriptional repression of developmentally regulated genes	Korenjak et al. 2004
	Myb-MuvB: RBF1/2, dDP,dMyb, Mip40, Caf1p55, Mip130 , Mip120, dLin52, Rpd3, L(3)MBT	Transcriptional repression of developmentally regulated genes	Lewis et al. 2004
E2F1	CtIP/CtBP, p130	Repression of ZBRK1. RB-dependent growth arrest after DNA damage	Meloni et al. 1999 Liao Ching-Chun, 2010
	ALIEN, pRB/p107, CDK2	Repression of E2F1 expression and cellular proliferation	Escher et al. 2007 Tenbaum et al. 2007
E2F4/5	DREAM: p130, LIN9, LIN37, LIN52, LIN54 LIN53/RBBP4	Transcriptional repression of cell cycle genes in G0	Litovchick et al. 2007
E2F6	Complex: RYBP, RING1, MEL-18, MPH1, BMI1	not determined	Trimarchi et al. 2001
	Complex: EPC1, EZH2, SIN3B	Target gene repression in proliferating cells	Attwoll et al. 2005
	Complex: MGA, MAX RING1/2, MBLR, h-l(3)MBT-like, YAF2, HP1γ	Target gene repression in quiescent cells	Ogawa et al. 2002

Table 2. Multiprotein complexes encompassing E2F factors identified by proteomic approaches.

MuvB complexes indicates that the DRM complex likely acts in transcriptional repression of E2F targets, and implies a remarkable conservation in the mechanism of pocket protein function across species.

An integration of proteomic, genomic, and bioinformatic approaches has allowed DeCaprio’s group the identification and functional characterization of human DREAM, the homolog of the fly dREAM (Litovchick et al. 2007). To determine the composition of this complex the authors purified p130-associated proteins and applied a multidimensional protein identification method (MudPIT). This method couples biphasic and triphasic microcapillary columns to high performance liquid chromatography followed by tandem mass spectrometry analysis (Florens & Washburn 2006). The core components of the identified complex include p130, E2F4/5, DP1/2 and mammalian orthologs of synMuvB proteins LIN9, LIN37, LIN52, LIN54, and LIN53/RBBP4 (also known as LIN complex). Interestingly, the human DREAM repressor complex differs from the fly complex in that it

lacks MYB. In fact, MYB immunoprecipitates with LIN9, LIN37, and LIN54, but not with E2F or p130 in human cells, indicating the existence of two distinct transcriptional complexes incorporating either p130 or MYB. Indeed, several reports have demonstrated that the complex composition is dynamic and cell cycle phase-dependent. In quiescent cells LIN complex proteins associate with p130 and E2F4, and the complex binds to its target genes. In late G1/S phase, E2F4 and p130 dissociate and the LIN complex associates with MYB (Litovchick et al. 2007; Schmit et al. 2007), forming a new complex that is required for activation of G2/M genes. The dynamic interaction of the LIN complex with different DNA-binding proteins (E2F4 *vs.* MYB) supports a model in which DREAM is bound in G0 to E2F-regulated promoters via E2F4/p130 and in S-phase to G2/M promoters via MYB. Another finding that distinguishes the human complex from its fly and worm orthologs is that human DREAM does not appear to regulate target genes involved in development (Litovchick et al. 2007).

Mass spectrometry has also been applied to elucidate the mechanism by which E2F6 regulates transcriptional repression in quiescent cells. E2F6 was shown to interact directly with MGA and MAX (Ogawa et al. 2002). These proteins are known to bind as heterodimers to E boxes such as the MYC binding elements, and to antagonize MYC function (Hurlin et al. 1999). Given that MYC and E2F share common functions such as mitotic responses, cell cycle stimulation and induction of apoptosis, it has been proposed that E2F- and MYC-responsive genes could be co-regulated by E2F6 (Ogawa et al. 2002). In the complex containing E2F6-MGA-MAX, several Polycomb group proteins (RING1/2, MBLR, h-1(3)MBT-like protein, and YAF2) are also present, together with HP1 γ , a methyltransferase related to gene silencing in euchromatic loci (Horsley et al. 1996). The recruitment of E2F6 to its target genes could form a “platform” that is required for nucleating PcG proteins. Subsequent recruitment of HP1 γ to this platform would propagate chromatin inactivation, leading to entire repressed regions (Ogawa et al. 2002). It is remarkable that as many as three types of E2F6 repressor complexes containing different PcG proteins have been identified by proteomic methods (Table 2). The biological relevance for such diversity has not been clarified, although there is evidence that these complexes could be formed at different phases throughout the cell cycle. MAX and HP1 γ were found associated with E2F target promoters in G0, but not following re-entry into the cell cycle, suggesting a role for this complex in gene repression in quiescent cells (Ogawa et al. 2002). By contrast, the PcG protein EZH2 only forms complexes with E2F6-EPC1 in proliferating cells, suggesting that this complex regulates genes required for cell cycle progression (Attwooll et al. 2005).

Deciphering the protein interactome of upstream components of the RB/E2F pathway has also led to the characterization of new functions of the pathway. This is the case of a recently work published by Sicinski’s group in which cyclin D1 protein partners were characterized by immunoaffinity purification coupled to MS analysis (Jirawatnotai et al. 2011). Identification of cyclin D1 interactors revealed a network of DNA repair proteins, including RAD51, a key DNA recombinase that drives the homologous recombination process in response to DNA damage (Baumann & West 1998). Remarkably, the finding that Cyclin D1’s function in DNA repair appears to be independent of its kinase activity could have clinical applications. For instance, a large pool of RB-negative cancers, which do not require D-cyclins for proliferation, may still be benefited by therapeutic interventions targeting cyclin D1 in combination with radiation treatment. This work clearly shows how interacting-proteomics can fundamentally change our understanding of signaling networks

and how combining multiple approaches is possible to unveil novel therapeutic strategies to different disorders such as oncogenic malignancies.

3. Post-translational modifications on E2F transcription factors

Post-translational modifications (PTMs) are gene non-template chemical modifications occurring at distinct amino acid side chains of proteins. PTMs can change the size, charge-state, structure or conformation of proteins. As a result, PTMs influence several aspects that directly or indirectly affect protein function. Sub-cellular localization (Mueller et al. 2009), protein half-life (Min et al. 2010) or binding affinity to other molecules such as nucleic acids, lipids or other proteins (Takasaki et al. 1999) are usually conditioned by these modifications. Consequently, PTMs play a key role in functional proteomics and are involved in the modulation of almost every single cellular process. Not surprisingly, malfunctions on these critical cellular processes have usually been related to several diseases (Ko et al. 2010; Song et al. 2010). More than 300 types of PTMs have been described to date and the number and variety of identified modifications is continuously increasing (Zhao & Jensen 2009). Some modifications such as phosphorylation, acetylation, glycosylation and methylation are very common and are found in almost every protein while others, for example biotinylation, are very rare. Given the high abundance, dynamism and diversity of PTMs, they likely constitute one of the most complex regulatory mechanisms in eukaryotic cells.

The E2F transcription factors are not an exception to such modifications, as they are known to be regulated by a variety of PTMs. In the mid 90's *in vitro* assays showed that ubiquitination occurring at the C-terminal end of E2F1 is determinant in mediating cell cycle-dependent degradation of this transcription factor by the ubiquitin/proteasome pathway (Hofmann et al. 1996). Additionally, two independent studies concluded that E2F1-3 but not E2F4-6 can be acetylated by p300/CBP acetyltransferases. Further *in vitro* acetylation assays using deletion mutants of E2F1, restricted the site of acetylation to three conserved lysine residues located at the N-terminal region of its DNA binding domain. This modification could be reversed by HDAC1, and was found to influence the DNA-binding ability of E2F1 and consequently its transcriptional activity (Martinez-Balbas et al. 2000; Marzio et al. 2000). More recently, Set9 methyltransferase and LSD1 demethylase have been shown to regulate E2F1-mediated p53-independent cell death (Kontaki & Talianidis 2010; Xie et al. 2011). Interestingly, a complex cross-talk between different PTMs was found on E2F1. Precisely, Set9-catalyzed methylation at Lys185 inhibits acetylation and phosphorylation events involved in the stabilization of E2F1, while ubiquitination, known to favor protein degradation, is stimulated (Kontaki & Talianidis 2010). Although these diverse PTMs have been described to affect the biological functions of E2Fs, phosphorylation has been the most studied modification.

3.1 Biochemical analysis of phosphorylations

Phosphorylation of proteins is a highly dynamic process involved in the regulation of several essential cellular functions such as cell cycle regulation, differentiation and signal transduction. Given its biological relevance, the identification and kinetic studies of protein phosphorylation have become a major challenge in molecular biology. Pioneer works describing phosphorylation events on E2F were mainly based on results obtained from electrophoretic mobility shift assays (Peeper et al. 1995) and *in vitro* kinase assays (Lin et al. 2001; Vandel & Kouzarides 1999). A major common feature of these studies is that they were

hypothesis-driven, that is, the identification of the PTMs was, to an extent, a targeted approach. Moreover, each work was focused on individual proteins where a single or a few modifications were described at a time. Consequently, the information about PTMs affecting the biological function of E2Fs has been considerably limited. Indeed, these classical approaches have led to the identification and characterization of merely 7 phosphorylations on E2F1 over a period of 10 years, all of which are registered in the Phosphosite database (Hornbeck et al. 2004). Phosphorylation of E2F1 at Ser332 and Ser337 disrupts its interaction with RB to become transcriptionally active (Fagan et al. 1994). By contrast, phosphorylation at Ser375 increases its ability to interact with RB and, consequently, decreases its DNA binding capacity (Peeper et al. 1995). Phosphorylation of E2F1 residues Ser403 and Thr433 by TFIIF kinase in S-phase triggers rapid degradation of the transcription factor (Vandel & Kouzarides 1999). Finally, two residues on E2F1, Ser31 and Ser364, can be phosphorylated in response to DNA damage by ATM/ATR and Chk1 kinases, respectively. Both phosphorylations are involved in E2F1-mediated apoptosis (Lin et al. 2001; Stevens et al. 2003). Phosphorylation of E2F3a at Ser124 and its role in DNA-damage induced apoptosis has also been described by classical biochemical approaches (Martinez et al. 2010).

3.2 MS-based identification of phosphorylations

Recent advances in mass spectrometry have largely overcome the disadvantages faced by classical biochemical approaches allowing the large-scale identification of phosphorylated peptides and the more challenging localization of them within the peptide sequence. In order to decipher the biological meaning of site-specific phosphorylation events in the regulation of cellular processes, quantitative phosphoproteomics studies have produced a considerable body of work during the last 5 years. As a result, thousands of new phosphorylations have been identified (Huttlin et al. 2010; Monetti et al. 2011), some of them corresponding to E2F family members.

Due to the low stoichiometry of site-specific phosphorylation, refinement of phosphopeptide enrichment methodologies has been crucial for the fruitful characterization of this type of PTM by MS. Currently, there are several phosphopeptide enrichment methods available, such as strong cation exchange chromatography (SCX) (Beausoleil et al. 2004), hydrophilic interaction chromatography (HILIC) (Boersema et al. 2007), immobilized metal affinity chromatography (IMAC) (Li & Dass 1999) and titanium dioxide enrichment method (TiO₂) (Larsen et al. 2005; Pinkse et al. 2004). They all manage to separate phosphorylated peptides from non-phosphorylated ones taking advantage of the properties that the negative charge of the phosphate groups confer to phosphopeptides. Antibody-based enrichment approaches have also been demonstrated to be very efficient and useful for phosphoprotein/phosphopeptide enrichment (Choudhary et al. 2009). For optimal analysis of the phosphoproteome these methods are usually combined in multi-stage enrichment strategies (Thingholm et al. 2008). In addition, implementation of quantitation methods has opened new perspectives in studies of complex and dynamic biological signaling networks. In the past decade, highly accurate techniques based on stable isotope labeling for protein quantitation by MS have been developed. Stable isotope labeling of amino acids in cell culture (SILAC) (Ong et al. 2002) and iTRAQ (Ross et al. 2004) are currently the most frequently used techniques in quantitative MS-based phosphoproteomics.

The first global *in vivo* SILAC-based quantitative phosphoproteomic study to be reported was performed by Mann's group. It combined SCX and TiO₂ enrichment of phosphopeptides followed by LC-MS/MS analysis to quantify changes in phosphopeptide

levels in response to EGF treatment (Olsen et al. 2006). From the 6,600 phosphosites corresponding to 2,244 proteins that were detected in this study, the previously described E2F1 phosphoserine-375 (pSer375) (Peeper et al. 1995) was identified. This large-scale analysis showed that the phosphorylation state of E2F1 Ser375 is independent of EGF treatment, since the amount of phosphopeptide remained constant over time after the addition of the stimulus (Olsen et al. 2006).

Similar studies carried out by Dephoure and colleagues combined SILAC-based quantitation with SCX followed by TiO₂ and IMAC to assess the quantitative atlas of mitotic phosphorylation on HeLa cells. MS analysis of enriched phosphopeptides revealed the presence of E2F1 pSer375 in G1 phase cell lysates. In addition, two new phosphorylations were identified on E2F2 and a single one on E2F7. E2F2 residues Ser133 and Tyr130 were shown to be phosphorylated during the M phase of the cell cycle while E2F7 Ser410 was found to be phosphorylated in G1 phase (Dephoure et al. 2008).

Additional quantitative phosphoproteomic studies have reported the identification of a large number of phosphoresidues on various members of the E2F family, some of which had not been reported before (Figure 2). To decipher the phosphorylation dynamics occurring during early differentiation of human embryonic stem cells, a SILAC-based quantitation combined with SCX and TiO₂ followed by MS analysis was performed (Van et al. 2009). Of the 5,222 identified proteins, 1,399 were phosphorylated in 3,067 residues. Two phosphorylated residues, pSer16 and pThr14 precisely, corresponded to E2F4 and were first described in this work. Quantitative phosphoproteomic analysis of T cell receptor signaling revealed the existence of 2 new phosphorylations on E2F family members. Apart from the phosphorylations at Ser375 and Ser332, previously described to affect E2F1 binding affinity for RB (Fagan et al., 1994; Peeper et al., 1995), a new phosphorylated serine at position 340 was detected on E2F1. Moreover, phosphorylation on E2F2 Ser117 was also observed in this analysis (Mayya et al. 2009).

A quantitative phosphoproteomic analysis focused on dissecting the rapamycin-dependent activation of oncogenic cascades also identified several new phosphorylation sites on E2Fs (Chen et al. 2009b). Differentially SILAC labeled cells were cultured in the presence or absence of rapamycin, and stimulated with EGF. Both protein lysates were combined, digested and phosphopeptides were subjected to double IMAC purification followed by MudPIT LC-MS/MS analysis. Of the 6,175 phosphosites identified in this screening, 3 corresponded to E2F8. For the first time, E2F8 Ser355, Ser357 and Ser358 were found phosphorylated. Quantitative phosphoproteomics was also applied in mouse cells to unravel the signaling cascade initiated in response to the oncogenic mutant receptor tyrosine kinase Flt3. This kinase has been involved in the development of several hematopoietic malignancies (Stirewalt & Radich 2003). A total of 14,700 phosphosites were identified, of which 1 corresponded to E2F7 (pTyr416) and 5 to E2F8 (pSer20, pSer71, pSer102, pSer358 and pSer429) (Choudhary et al. 2009). The Tyr416 residue of mouse E2F7 is not conserved in humans. By contrast, 4 of the 5 novel murine E2F8 phosphosites identified in this screening are conserved in humans, suggesting that they may be biologically relevant (Choudhary et al. 2009). In fact, pSer71 of E2F8 has recently been detected in the phosphoproteome of human embryonic stem cells undergoing differentiation (Rigbolt et al. 2011). In this report, an additional phosphosite on E2F8, pSer316, was described. Recently, in an attempt to elucidate the mechanism that regulates the switch of the MuvB core from B-MYB to DREAM, phosphorylation of E2F4 at Ser384 was detected. This phosphorylation could contribute to entry of cells into quiescence (Litovchick et al. 2007).

Fig. 2. Phosphorylations detected in human E2F family members. In black phosphorylations detected by classical biochemical methods; in red, phosphorylations detected by MS; in bold red, phosphorylations detected by both methods. S: Serine; T: Threonine; Y: Tyrosine.

Novel methodological approaches in SILAC-based quantitative phosphoproteomics include a double phosphopeptide enrichment using IMAC followed by HILIC. This kind of strategy has allowed the identification of pSer7 on E2F8 (Yao et al. 2011). Furthermore, quantitative data indicated that protein SUMOylation influences phosphorylation detected at residue Ser7, since inhibition of SUMOylation was found to increase the amount of pSer7. This new evidence of cross-talk between different PTMs underscores once more the complexity of PTMs and the relevance of the combinatorial PTM patterns.

4. Protein profiling to elucidate E2F function

A large number of potential E2F targets have been discovered recently through the application of high throughput transcriptomic analyses. However, mRNA abundance poorly correlates with protein levels. This discrepancy can in part be explained by several post-transcriptional mechanisms such as alternative splicing of pre-mRNAs, microRNA mediated regulation, or selective degradation and post-translational modifications of proteins. Therefore, protein expression analyses should be carried out in addition to mRNA expression analyses to achieve a full picture of E2F regulated pathways.

Two-dimensional gel electrophoresis (2-DE) combined with protein identification by MS has been the most popular method in expression proteomics. In this global protein profiling approach, 2-DE is used to separate proteins within a complex mixture based on their pI and molecular weight. Subsequent staining and image analysis can detect differences in spot intensities, rendering a list of proteins that are differentially expressed between the conditions under comparison. The resulting spots are then excised and analyzed by MS (Görg et al. 2004). Global protein expression analyses of cells that either overexpress or are deficient for individual E2Fs have emerged as a useful methodology to elucidate the spectrum of E2F activity. Pützer's group analyzed protein expression in p53-deficient osteosarcoma cells (Saos-2) expressing E2F1 fused to the murine estrogen receptor ligand-binding domain (ER). This construction permitted conditional activation of E2F1 after addition of 4-hydroxytamoxifen (4-OHT) to the cultures (Stanelle et al. 2002). 2-DE analysis of E2F1 induced and non-induced Saos-2 cells' proteome led to the identification of 33 novel differentially regulated E2F1 target proteins by MALDI-MS (Li et al. 2006b), 15 upregulated and 18 downregulated. Only eight of these differentially regulated proteins harbor E2F-consensus sites in their promoter (Rabinovich et al. 2008): SRSF1, TUBB, GDI2, H2B.1, TCP1 γ , HNRNPA2/B1, HNRNPK and MATR3. Nevertheless, since E2F1 overexpression was the only stimulus applied to the culture, most of the identified target genes are probably E2F1 targets. Functional analysis of E2F1-regulated target genes suggests that E2F1 plays predominantly a negative role in Saos-2 cellular proliferation. By altering the balance between anti- and pro-apoptotic Bcl-2 family members, E2F1 would render cells susceptible to both mitochondrial apoptosis and ER-stress related death signals (Li et al. 2006b). These results are consistent with the findings described by several groups, including ours, in mouse models lacking E2F1, and favor the hypothesis that E2F1 plays anti-proliferative and pro-apoptotic roles *in vivo* (Field et al. 1996; Garcia et al. 2000; Yamasaki et al. 1996).

A similar pro-apoptotic role has been attributed to E2F1 in proteomic analyses involving p53-wild-type osteosarcoma cells (U2OS) carrying an inducible E2F1 transgene. 2-DE followed by MS led to the identification of 76 proteins, 53 overexpressed and 23 suppressed after E2F1 induction (Liontos et al. 2009). Many of them appear to be potentially novel E2F1-regulated targets. As many as 63% and 77% of the overexpressed and suppressed proteins, respectively, harbor E2F1 responsive elements in their promoters. The spectrum of identified E2F1-regulated proteins included chaperones, metabolic enzymes, proteins associated with RNA processing, components of the protein degradation/turnover machinery, cytoskeletal and motor/contractile related proteins, regulatory and cell signaling molecules, transport carriers and channels, as well as putative oncogenes. A significant number of the identified E2F1 downstream targets are part of pro-apoptotic signaling cascades regulating ATM and/or p53. In agreement with an oncosuppressor function for E2F1 *in vivo*, the authors reported a positive correlation between E2F1 expression and DNA damage response and apoptosis in primary osteosarcoma tumors with wild-type p53. This picture is contrary to the general view that the increased levels of E2F activators, such as those observed in many cancer types, are mediating uncontrolled proliferation (Chen et al. 2009a). Whether deregulated expression of E2F proteins promotes or limits cancer progression has not been unequivocally established. Other factors, such as RB or p53 status may determine the final outcome of E2F-dependent activity in each particular context.

Protein profiling analyses performed by our group are also helping to unveil the physiological role of E2F2. A repressor function for E2F2 is emerging from these studies, in agreement with DNA microarray and functional data. When quiescent E2F2^{-/-} and wild-

type T lymphocyte expression profiles were gathered by 2-DE followed by MS, we identified a set of deregulated proteins involved in TCR-mediated signaling, cell survival and stress responses (Azkargorta et al. 2006). The aberrant expression of these proteins was linked to the hyperproliferative phenotype that characterizes E2F2-deficient cells (Iglesias et al. 2004; Infante et al. 2008; Murga et al. 2001; Zhu et al. 2001). Interestingly, comparative proteomics has also revealed novel pathways regulated by E2F2. We have recently found that mediators of the Aryl-hydrocarbon receptor (AHR) are aberrantly expressed in the proteome of proliferating E2F2^{-/-} lymphocytes relative to wild-type counterparts (Azkargorta et al. 2010). Consequently, E2F2^{-/-} cells exhibit an increase in their sensitivity to dioxin-triggered apoptosis. These results suggest that E2F2 modulates cellular sensitivity to xenobiotic signals through the negative regulation of the AHR pathway.

Remarkably, a comparison of proteome and transcriptome profiling results derived from the same cellular systems has shown a clear discrepancy in individual targets regulated by E2Fs (Azkargorta et al. 2006; Azkargorta et al. 2010; Infante et al. 2008; Li et al. 2006a; Lontos et al. 2009; Muller et al. 2001). These discrepancies may be explained by the different methods of assay/sample preparation, different detection sensitivity, alternative splicing, post-transcriptional regulation, PTMs, selective degradation of proteins, and the time discrepancy between mRNA and protein expression. Thus, proteomics and DNA-based technologies should be considered as complementary approaches.

4.1 The RB/E2F pathway in cancer protein profiling

Transcriptomic analyses have demonstrated a commonly conserved RB/E2F-dependent “proliferation signature” in cancer cells, supporting a role for this pathway in regulation of cellular proliferation in normal as well as tumor cells (Whitfield et al. 2006). Given the prevalence of this signature in cancer, a similar finding would be expected at the protein level. However, most 2-DE/MS-based proteomic analyses of tumor cells performed to date have not displayed this pathway unequivocally, probably due to the limitations of the technology. An important drawback of traditional 2-DE-based proteomic methods is that they can only reveal the presence of highly abundant proteins within the cells, whereas proteins that function at low levels, such as certain transcription factors and checkpoint/regulatory proteins, are not easily identified if no pre-enrichment and/or pre-fractionation steps are applied (Gygi et al. 2000). In general, the number of proteins identified in these experiments is quite low, in the range of 100-300 proteins per experiment. This number is several orders of magnitude lower than the thousands of genes that can be interrogated by DNA microarray analyses. Consequently, cancer proteome profiles gathered to date with this technology should, in general, be considered as preliminary.

An RB/E2F dependent signature was appreciable in the proteome of cervical cancer cells with high-risk HPV infection analyzed by 2-DE and MS. Differentially expressed proteins in cervical cancer cells were identified and functionally classified as proteins involved in the regulation of the cell cycle, general genomic stability, telomerase activation and cell immortalization (Choi et al. 2005). A significant proportion of these genes (30%), particularly those present in the nuclear fraction, are well-known E2F transcriptional targets (PCNA, CDC25A, MCM3,4,8, CHRC-1), most of which were upregulated. As exceptions, XRCC2 involved in DNA repair and CASP-2 involved in apoptosis induction, which were downregulated. These results are consistent with a model in which functional inactivation of RB by HPV oncoprotein E7 unleashes E2F and triggers aberrant E2F-dependent gene expression (McLaughlin-Drubin & Munger 2009). By contrast, a similar type of analysis

performed with retinoblastoma tumors, whereby both copies of the RB1 gene are inactivated, did not identify any E2F targets among the 27 differentially expressed proteins (Mallikarjuna et al. 2010). In this work, more aggressive tumors showed significantly higher expression of CRABP2, APOA1, PRDX6 and RCVRA, and lower expression of CRABP1. Differentially expressed proteins were shown to be involved in metabolic process, transport activity, response to oxidative stress, development, and cell signaling and transduction, reflecting the important role of these processes during retinoblastoma progression.

In general, 2-DE/MS-based proteomic analyses of cancer cells have barely yielded a handful of E2F-target genes among the differentially expressed proteins. For example, the comprehensive proteome profiles of mouse lung adenocarcinoma cell lines, have revealed 82 and 40 unique proteins significantly up- or down-regulated respectively in highly metastatic cells compared to low metastatic controls (Zhang et al. 2008). Several of the proteins are involved in proteasome, cell-cycle and cell-cell communication pathways. Among them, several E2F targets have previously been associated with cancer development and metastasis: KRT8, the main cytokeratin in lung cancer, MCM7, a highly informative biomarker for cervical cancer, or ANX4, overexpressed in pancreatic adenocarcinoma. Similarly, a comparative proteome analysis of human lung squamous carcinoma and paired normal bronchial epithelial tissues revealed some deregulated E2F target genes in a list of 68 proteins that were identified by MS (LTBP4, GNB1L, MDM2, IRS1)(Li et al. 2006a).

As 2-DE/MS methodology is being replaced by more powerful techniques, the number of proteins that can be identified in a given proteome is increasing rapidly, allowing for a more accurate definition of the pathways involved in cancer. A recent report has described the use of 1-D gel electrophoresis followed by tryptic in-gel digestion and chromatography coupled to MS. This shotgun proteomic approach revealed changes in the expression levels of 281 proteins in meningiomas compared to normal human arachnoidal cells (Saydam et al. 2010). Interestingly, a highly significant functional network involved in DNA replication, recombination and repair, and in cell cycle was exclusively expressed in cancer cells, arguing for a prevalent RB/E2F signature in the meningioma proteome. Similar results have been gathered recently with high-risk neuroblastoma proteomes analyzed by another type of shotgun proteomics approach. Quantitative global protein expression profiling performed using isotope-coded affinity tags (ICAT) followed by LC-MS/MS analysis identified a total of 1,461 proteins that were differentially expressed in neuroblastoma. Again, pathway analysis of these proteins showed enrichment in the RB/E2F regulated network (Chen et al. 2010). These results underscore the power of shotgun proteomics, and suggest that many novel insights regarding RB/E2F function will be unraveled by this approach.

5. Future perspectives

Much has been learned on RB/E2F-mediated regulation of gene transcription by proteomic approaches to date. The identification of E2F interacting proteins as well as transcriptional complexes encompassing RB and E2F has helped elucidating the regulation of this pathway. However, the current knowledge is still too limited to fully understand the complex RB/E2F-regulated network. Further work involving high throughput proteomic approaches should help elucidate the nature of the diverse macromolecular complexes that are thought to harbor RB/E2F, and the role of these complexes in different biological settings.

Development of modified peptide enrichment methods together with recent advances in MS has allowed large-scale analyses of PTMs, providing a large pool of new PTM

identifications. With regard to E2Fs, MS-based phosphorylation analyses have greatly increased the number of phosphorylated residues identified within this transcription factor family, and many more will probably be identified in the future. However, they will need to be validated and functionally characterized *in vivo* in order to decipher the influence of each individual PTM on E2F activity. As works focused on MS-based identification are gaining relevance, other types of PTMs such as acetylations and ubiquitinations, will probably be identified on E2Fs in a short period of time. Furthermore, multiple PTMs should be possible to analyze in the same system, producing direct data on their crosstalk at the global level in regulating E2F function. In addition, implementation of quantitative methods will allow studying the dynamics of site-specific PTMs on E2Fs, which was almost unachievable a decade ago. Defining their kinetics will be crucial to understand how PTMs influence on protein regulation and function. This explosion of information, still very descriptive, opens a huge range of possible new studies in the field of RB/E2F network.

Several differential proteomic analyses have been performed to examine how the presence/absence of each individual E2F is reflected in the proteome. The strategy to accomplish this objective will surely move from the low scale approach that represents 2-DE/MS used so far, to gel-free high throughput strategies. This approach has been widely adopted in proteomics lately, owing to its superior performance compared to 2-DE technology. Advances in sample preparation, including novel fractionation methods, together with more powerful mass spectrometers have been proved to qualitatively and quantitatively assess changes in large scale protein analyses. Indeed, large-scale protein expression analysis of cancer cell proteomes by shotgun proteomics promises to be valuable for investigating mechanisms of cancer transformation (Chen & Yates, III 2007). Nonetheless, the limitations of global proteomic analyses in identifying and quantifying low abundant proteins such as transcription factors will push the emergence of hypothesis-driven strategies based on targeted proteomic approaches.

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Biomedical research has entered a new era of characterizing a disease or a protein on a global scale. In the post-genomic era, Proteomics now plays an increasingly important role in dissecting molecular functions of proteins and discovering biomarkers in human diseases. Mass spectrometry, two-dimensional gel electrophoresis, and high-density antibody and protein arrays are some of the most commonly used methods in the Proteomics field. This book covers four important and diverse areas of current proteomic research: Proteomic Discovery of Disease Biomarkers, Proteomic Analysis of Protein Functions, Proteomic Approaches to Dissecting Disease Processes, and Organelles and Secretome Proteomics. We believe that clinicians, students and laboratory researchers who are interested in Proteomics and its applications in the biomedical field will find this book useful and enlightening. The use of proteomic methods in studying proteins in various human diseases has become an essential part of biomedical research.

How to reference

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