**14. Scientific Abstract**

With the advent of next generation DNA sequencing technologies, the pace of discovery of cancer-associated sequence variants has greatly accelerated, leading to the realization that tumours (especially solid tumours) harbour hundreds of mutations. Ongoing statistical analysis across multiple laboratories worldwide is progressing rapidly, helping to identify which of these mutations are likely drivers of the cancer phenotype. However, in spite of rapid progress in mapping of cancer related signaling and interaction networks, there has been an increasing disconnect between the identification of a cancer variant, and the mechanistic understanding of transformation induced by the mutation. While the downstream effects of many of the mutations may be straightforward to explain (e.g., abrogation of protein expression or function for tumour suppressors), what happens at the molecular level as a consequence of a mutation is generally not well understood. Such molecular understanding is crucial for developing therapeutic interventions. Frequent consequences of cancer-related mutations are specific alterations of protein-protein interactions. As we have both appropriate structural data and technology to assess and modulate such interactions, an analysis of specific protein-protein interactions altered by cancer-associated mutations offers novel therapeutic avenues.

To ideally exploit this novel venue, we should be able to quantify of both loss and gain of interactions, be able to rapidly measure different sequence variants, and be have a robust, sensitive and accurate approach. In order to develop such a methodology, we have coupled affinity purification (AP) to a novel data-independent mass spectrometric acquisition (SWATH). We have then tested this strategy for the quantification of the changes imparted by melanoma-associated mutations in the kinase CDK4. In addition to confirming known interaction loss, we were able to also identify interaction gains in the same experiment, namely in the recruitment of HSP90 network components to the mutated proteins. We next used AP-SWATH to monitor the consequence of pharmacological inhibition of HSP90 on the recruitment of chaperones to the wild type and mutated CDK4 proteins and rapidly demonstrated that the mutant proteins are resistant to the inhibitor. This finding has obvious implications for patient care.

Based on these results, we are proposing to develop an experimental and computational pipeline to assess interactions changes that is scalable to optimally make use of ongoing genomic sequencing efforts. Our approach is flexible, and enables monitoring of interactome changes imparted by sequence variation and drug treatments, making it a potentially important systems biology tool.

In particular, we will first analyze a larger set of cancer associated variants for which the mechanism(s) of transformation is unknown, and will then perform in-depth studies of mutations in phosphatases. We will optimize the experimental and computational analysis platform to enable higher throughput analysis and provide cancer researchers with annotation of interactome changes imparted by mutations, and when possible, by the treatment with pharmaceuticals. This will greatly enhance our understanding of the effects of the mutations both on a cellular and molecular level, and provide the research community with leads for biological follow-up, thereby accelerating the development of therapeutic options.

**Public summary**

**10. Project summary**

Proteins inside each cell bind to each other to perform their activities. When mutations in a protein arise, many of these binding events are altered, and these can result in cancer. Recently, the scientific community has created excellent methods to identify the mutations, but this has thus far not been matched by analyses at the molecular level, including such binding events. Here, we are proposing to use innovative tools to systematically identify those binding events that are modulated after a mutation occurs. These tools should help bridge the gap between the descriptive information (what are the mutations?) provided by the sequencing of the genetic code, and functional information (what does this mutation do?).

**11. Previous research**

The Gingras lab has been developing new approaches to accurately quantify each of the proteins present in a biological sample using a machine called a mass spectrometer that can essentially identify proteins based on “fingerprints”. They are also recognized internationally for their studies of binding events between proteins. They have shown on a small test case that a new approach they developed can help in identifying how specific mutations can affect cancer development by changing how proteins bind to each other. Through collaborations, they now have access to large a collection of mutations identified in cancer cells. The Kim lab’s expertise is in defining how proteins interact with each other in a very precise manner. They develop computational tools which enable them to predict how a mutation could influence the binding of proteins to one another.

**12. Project description**

The Gingras and Kim labs will join efforts to provide a method to systematically identify changes in protein-protein interactions upon expression of proteins containing cancer associated mutations. They will first computationally predict which mutations are most likely to affect protein-protein interactions. Then, using new technology being developed in the Gingras laboratory, they will systematically test these; for this, they will use ~150 different mutant proteins. They will also predict which of the interactions can be disrupted by drugs, and test these predictions experimentally. What they are expecting to generate is both new biology on the mutations that they are studying that may explain how cancer arises, but also a methodology to systematically assess many more mutations in the future. They will distribute their results and methods to the scientific community, accelerating the discovery of the causes of cancer and of treatment options.

**13. Impact / relevance**

Understanding how a mutation causes cancer is critical to defining treatment options. For example, some drugs act by preventing the association of proteins with one another. This is the case of a drug that the Gingras lab used in a pilot study: in this case, they demonstrated that the mutant was resistant to the effects of this drug, which is important to know when treating patients that present with this particular mutation. The ultimate goal of this project is to accelerate the discovery of drug targets for cancers by providing more information about the mechanistic aspects of cancer development following protein mutation. While this is a long-term goal, immediate consequences on the life of Canadians living with cancers may result from one of our aims, which is to test known drugs for the changes they induce in protein binding events for non-mutated and mutated forms of proteins.

**16. Innovation statement**

The pace of discovery of cancer-associated mutations has been accelerating greatly in the past years, aided by major advances in DNA sequencing technologies, but the functional consequences of the great majority of these mutations are poorly understood. Several years ago, our collaborators (Vidal/Hill) began generating a collection of constructs encoding wild type and mutated alleles for proteins implicated in many diseases, including cancer, and shown using yeast-two hybrid technologies that several of these modulate specific protein-protein interactions.

The innovative aspects of our project rest on the development of new mass spectrometric approaches to accurately quantify altered protein-protein interactions in a scalable platform (Gingras), and the use of existing three-dimensional data and computational modeling to focus on mutations likely to affect interactions (Kim).

We have been working for many years on the development of affinity purification coupled with mass spectrometry to identify protein-protein interactions. While our previous approaches were appropriate for identifying static interactions, they largely failed at identifying differentially regulated interactions. A new concept in quantitative mass spectrometry, termed SWATH, enables the label-free quantification of all identifiable species in the mass spectrometer. Though it is very new (first publication in March 2012), the Lunenfeld has been an early adopter of the method, which we have optimized extensively. Because of its potential for monitoring interactome changes in a rapid, unbiased and accurate manner, we have coupled affinity purification (AP) to SWATH and validated the approach on cancer-associated mutations. Here, we will develop this approach into a scalable platform which will enable cancer biologists to rapidly identify interactions and pathways affected by cancer-associated mutations. This will be done in collaboration with AB-SCIEX and a number of computational experts, enabling us to create an innovative pipeline for functional analysis of cancer mutations.

19. **Table of contents**

**20. Proposal**

**Overview:**

Identification of changes in protein-protein interactions associated with sequence variants such as mutations identified as cancer drivers is critical to understanding the consequences of such variation. Technologically speaking, this requires a robust, sensitive and accurate quantification method: in our preliminary work, we have successfully coupled affinity purification (AP) to data-independent mass spectrometric acquisition (SWATH) to analyze the interactome changes imparted by mutations of the kinase CDK4. Here we propose to develop an integrated computational and experimental pipeline that will be scalable to make use of the flood of sequencing data. This project is possible and timely, based on our preliminary data, the availability of disease-associated mutant collections, a strong expertise in structural analysis of protein-protein interactions, and the collaborations we have established with computational biologists and a mass spectrometry vendor. We believe that the AP-SWATH pipeline we are proposing will greatly accelerate biological knowledge regarding the consequences of cancer-associated mutations.

**Introduction**

**Cancer-associated mutations: how interactomics can help reveal function**

In recent years, DNA sequencing of healthy and cancerous tissues has revealed a large number of genetic variations[[1](#_ENREF_1)]. With the current speed of next generation sequencing, new mutations are identified daily; yet, there is a dearth of data regarding the consequences of these mutations on cellular functions. While approaches such as RNA interference can help to identify the consequences of depletion of a complete protein, the consequences of sequence variations are more difficult to assess. At the molecular level, in addition to modifying the enzymatic activity of a protein or its expression level, there are many potential outcomes of sequence variation[[2-5](#_ENREF_2)]. One demonstrated consequence of sequence variation is a change in the interactions of the protein, which can contribute to the disease phenotype[[6](#_ENREF_6)] (Fig.1). Systematic assessment of protein-protein interaction disruption by yeast two hybrid (Y2H) technologies has revealed clear molecular signatures for certain mutants[[7](#_ENREF_7), [8](#_ENREF_8)]. However, there are limitations to Y2H, including the fact that it does not easily capture quantitative differences (they are scored as binding / no binding) and that the method in its current implementation is best at highlighting loss rather than gain of interactions.

**Affinity purification coupled to mass spectrometry (AP-MS)**

AP-MS has been used for many years for the identification of protein-protein interactions[[9](#_ENREF_9)] and is attractive since it can identify interactions in near-physiological conditions, providing a context in which a protein functions. While many groups (including ours[[10-29](#_ENREF_10)]) have employed AP-MS to identify static interactomes, limited publications have focused on the identification of differential interactions (reviewed in [[30](#_ENREF_30)]). In general, these studies have made use of quantitative proteomics approaches to discriminate between condition-specific interactions. Such quantitative studies have used various techniques, such as spectral counting (e.g.,[[31-34](#_ENREF_31)]), which is limited to monitoring relatively large changes in quantity for relatively abundant proteins, or the intensity of the ions in the precursor ion scans (MS1 or survey scan)[[35](#_ENREF_35)], whose measurements are often problematic. Alternatively, isotopic labeling has been employed for differential proteomics, and this has proven very useful, but fairly expensive and difficult to scale to large numbers of samples[[36](#_ENREF_36), [37](#_ENREF_37)]. Notably, all of these approaches require MS acquisition in a data-dependent manner (DDA), where the peptides to be sequenced are selected based on their relative abundance in the mass spectrometry MS1 survey scans. DDA introduces stochasticity in the process, which makes it difficult to make absolute conclusions in the absence of a peptide or protein across different samples, especially for lower abundance species[[38](#_ENREF_38)]. This is problematic for comparative quantification as is required for mapping sequence variant interactomes[[39](#_ENREF_39)].

**Towards better and faster MS measurements**

In recent years, a different paradigm for MS-based quantification of proteins has gained increased acceptance. Quantification at the MS/MS (MS2) level increases specificity and signal-to-noise ratios as compared to MS1[[40](#_ENREF_40)] and permits robust and sensitive quantification without requiring labeling[[41](#_ENREF_41), [42](#_ENREF_42)]. Standard MS2 quantification (known as selected reaction monitoring, SRM) however requires a fairly significant investment in assay development for each peptide and/or protein of interest, limiting its usefulness for systems biology experiments. Furthermore, the list of peptide species to be monitored must be predetermined, making it difficult to monitor the gain of interactions that would be generated by a mutation. Recently, a paradigm-changing MS acquisition method has been introduced that has the quantitative benefits of MS2 quantification without the limitations of the classical approaches. In this method, named data-independent acquisition (DIA)[[43](#_ENREF_43), [44](#_ENREF_44)], all precursor ions are fragmented independently of their signal in MS1 (Figs.2,3). A type of DIA that is particularly promising for the analysis of AP samples is termed SWATH (Sequential Window Acquisition of all THeoretical spectra)[[45](#_ENREF_45)]. In SWATH, the entire useful mass range is scanned over a ~ 3 second cycle with windows of incremental mass (here, 25 Da) and all precursors in each window are fragmented, resulting in a complete MS2 map of all compounds that are present in the sample. This map can then be used to detect and quantify proteins in the sample.

***Here, we will systematically employ AP-SWATH to study cancer-modulated interactions. We will refine and disseminate an efficient pipeline that will accelerate the study of cancer mechanisms and partially bridge the gap between the genomics efforts and the biology of cancer.***

**Preliminary results: AP-SWATH analysis of CDK4 sequence variants**

In the past years, the Gingras lab has developed a robust experimental pipeline that exploits purification from a single epitope tag (often 3xFLAG), using proteins expressed at near endogenous levels to identify interaction partners[[9](#_ENREF_9), [11](#_ENREF_11), [13-18](#_ENREF_13), [20](#_ENREF_20), [22](#_ENREF_22), [23](#_ENREF_23), [27-29](#_ENREF_27), [46-50](#_ENREF_46)]. We have further created a database system called ProHits to manage all the MS interaction data[[51](#_ENREF_51), [52](#_ENREF_52)], and developed (with A Nesvizhskii) scoring tools to identify true interactors using semi-quantitative approaches[[24](#_ENREF_24), [53](#_ENREF_53), [54](#_ENREF_54)]. To develop the AP-SWATH quantitative proteomics approach, we used two melanoma-associated sequence variants for the cyclin-dependent kinase CDK4, R24C and R24H[[55](#_ENREF_55), [56](#_ENREF_56)] (Fig.4). These preclude association of CDK4 with a family of polypeptide inhibitors, the INK proteins[[57](#_ENREF_57)], resulting in unrepressed CDK4 activity and accelerated cellular proliferation[[7](#_ENREF_7), [58](#_ENREF_58)]. Consistent with the critical role of the CDK4-INK relationship in cancer, INK proteins are recurrently down-regulated in melanoma and other cancers[[59-61](#_ENREF_59)]. Quantification of SWATH data was accomplished by a targeted data extraction strategy using a spectral library[[45](#_ENREF_45)] which we built from the same samples by standard DDA. Spectra within this library were used to extract quantitative data from each SWATH run (Fig.3). The extracted data was subjected to statistical analysis[[41](#_ENREF_41)] using a strategy which evaluates the quality of each peak in MS2 and performs fold change calculation of pairwise samples at the level of MS2 peaks, peptides, and proteins. As expected, both R24C and R24H mutants largely lost interactions with members of the INK inhibitors (Fig.4). Interestingly, the CDK4 mutants also exhibited a markedly increased association with HSP90 proteins, the kinase co-chaperone CDC37, and several other components of the HSP90 network. We validated these regulated interactions by IP-western analysis, in all cases confirming the trends observed by AP-SWATH.

While an increased interaction between CDK4 mutants and HSP90 has never been reported, mutations in several other kinases lead to an increased interaction with CDC37 and HSP90[[62](#_ENREF_62), [63](#_ENREF_63)]. A model of addiction to HSP90 has been proposed for these mutant kinases that become dependent on CDC37-HSP90 folding for stability and activity[[64](#_ENREF_64)]. Consequently, disruption of the kinase-HSP90 interaction with HSP90 inhibitors has been proposed as a therapeutic avenue for cancers driven by oncogenic kinases (Fig.5). Using AP-SWATH, we next assessed the consequences on CDK4-HSP90 interactions of treatment with NVP-AUY922 (a potent HSP90 inhibitor currently undergoing clinical trial[[65](#_ENREF_65)]). As expected, treatment of the cells expressing wild type CDK4 with the HSP90 inhibitor resulted in marked dissociation of CDC37, HSP90 and several – but not all – other proteins by AP-SWATH and IP-western. Importantly, however, and as opposed to the report that other mutant kinases show increased sensitivity for HSP90 inhibitors, we found that the R24C and R24H mutants still significantly bound to CDC37 and HSP90 in the presence of the inhibitor, as compared to the wt; this was again confirmed by IP-Western (Fig.6), and by performing time course and dose-dependency studies (not shown). These results indicate that tumors driven by these CDK4 oncogenic kinases may not readily benefit from treatment with HSP90 inhibitors. Furthermore, our experiments set the stage for analyzing other drug-regulated interactions, including for other oncogenic kinase variants.

**Research objectives**

*Our preliminary data highlights a quantitative pipeline to study interactome changes in an unbiased manner. Here we propose to further extend this study and define what types of mutations are best analyzed with this approach.*

**Aim 1. Expand the pilot project**

Through a collaboration with the Vidal and Hill lab (see letter) we have access to a collection of ~4000 disease-related alleles, many of which are implicated in cancer. In a first step towards selecting additional baits for AP-SWATH analysis, we will refine the list of candidate allelic variants by a) restricting the list to mutants in which the link to cancer is clear (e.g., which are clearly cancer driver mutations); b) eliminating proteins that are localized in membranous components (these are problematic by AP-MS); c) selecting protein-protein interaction pairs for which a mutation is likely to disrupt an interaction (see details below); d) prioritizing proteins expressed endogenously in the model line used for AP-MS[[66](#_ENREF_66), [67](#_ENREF_67)]; e) from these, priority will be given to baits for which we already have reagents, including antibodies to the endogenous proteins.

The Vidal/Hill collection has already been enriched for their likelihood at altering interactions, but a systematic structural analysis of the possible impact of these mutants on altering interaction surfaces has not been performed so far. The Kim lab, which has ample expertise in this field and indeed pioneered some of these approaches[[68-70](#_ENREF_68)], will build a structure-based analysis pipeline to analyze these different mutants. We have already built a structure-based interaction network using available interactome data and high-quality structural data of known protein interactions (in databases such as the PDB[[71](#_ENREF_71)], 3DID[[72](#_ENREF_72)] and iPfam[[73](#_ENREF_73)]). Using this resource we will score all mutations by mapping them to the structure of the proteins and determining their interaction potential (based on position in the structure and disruption potential of the mutation[[68](#_ENREF_68), [69](#_ENREF_69)]). Many of the mutations that do not map onto our structure-based interaction network may lie in disordered regions and affect domain-peptide interactions. Such interactions tend to be of particular importance in cancer-related signaling pathways and are another focus of the Kim lab[[74](#_ENREF_74), [75](#_ENREF_75)]. We will hence also be able to evaluate their effect on the interactions mediated by them.

Using this prioritization and the criteria outlined above, we will define a set of 40 wt baits and a total of ~60 allelic variants (~100 independent cell lines). These will be processed by AP-SWATH; validation will be by AP-SRM[[41](#_ENREF_41)] and/or by IP-western (as in Fig.4) to yield quantitative information about changes in interactions between the variants.

**Aim 2. Study phosphatase mutant interactomes**

The Gingras lab has a long-standing interest in the analysis of PP2A-like serine/threonine phosphatases, and we have recently expanded our interaction studies to all 150 human phosphatases and ~25 regulatory subunits for which we have performed AP-MS on stable cell lines expressing all WT proteins (we are using the same cell lines for other functional genomics / proteomics studies). While the scaffolding subunit of PP2A (*PPP2R1A*/*PPP2R1B*) has long been known to be mutated in cancers[[76](#_ENREF_76), [77](#_ENREF_77)], recent reports have accumulated that implicate other phosphatases in cancer[[76](#_ENREF_76), [78-80](#_ENREF_78)]. Notably, PPP6C was identified as a driver in melanoma in two recent studies[[78](#_ENREF_78), [81](#_ENREF_81)]. Though many of these mutations are expected to abrogate phosphatase activity, some of the other mutations may regulate interactions. Since we have high density interaction maps for both PPP6C and the PP2A proteins (and antibodies to many components of the network), we will reconstitute each of the point mutations in the Flp-In T-REx system and perform a) phosphatase activity measurements (as in[[29](#_ENREF_29), [82](#_ENREF_82)]); b) interactome mapping by AP-SWATH; c) further study in relevant cancer cells (for example, in the case of PPP6C, we have raised efficient polyclonal antibodies which can be used for AP-SWATH from endogenous material, in this case, melanoma cells). This activity will be expanded during the timeframe of the grant to ~50 mutant phosphatase alleles.

**Aim 3. Expand to drug-regulated interactions**

As interactors (especially regulated binders) are identified in Aims 1 and 2, they will be screened against pharmacological compound databases to see whether they are the targets of any drugs. If this is the case, the drugs will be tested for their effect(s) on the interactions, as we have done in Fig.6 for the HSP90 drugs. This will be done by first screening for possible drug hits (the Kim lab has related projects to do just this, using a number of accessible web tools, including DrugBank[[83](#_ENREF_83)] and Therapeutic Target Database, TTD[[84](#_ENREF_84)]). For initial test screening, compounds can be cherry picked from the large collections available at the Lunenfeld SMART facility (see letter from J Dennis), and larger amounts will be purchased as needed. This last aim should help us accelerate the process of finding drugs that modulate interactions, enabling us to propose steps towards therapeutic modulation of protein-protein interactions, thereby expanding the arsenal of anti-cancer agents.

**Perspectives**

As we are working towards the completion of Aims 1 and 2, we will be continuously revisiting our experimental and computational pipeline. We are working with mass spectrometry vendor AB Sciex, SLRI colleague T Pawson and computational proteomics expert A Nesvizhskii to refine the SWATH approach and improve the MS data handling. Our proposal benefits from the complementarity in expertise of the lead PIs, our collaborators and our lab members (PDF Couzens and student Veri will perform the experimental objectives assisted by several other Gingras lab members while PDF Teyra will be tasked with the computational predictions of regulated interactions).

We (Kim) will also reanalyze the data generated in Aims 1 and 2 to improve our ability to predict interaction modulation by mutation. Since there is currently a dearth of high quality quantitative interaction proteomics data to analyze the consequences of a mutation on interactome changes, our dataset will enable us to revise the scoring approach and provide an improved stratification strategy for future studies, both within the context of model systems, and with cell lines, xenografts and tissues directly derived from the patient samples.

Taken together, our project will develop an efficient platform to enable higher throughput analysis and provide cancer researchers with annotation of interactome changes imparted by mutations, and when possible, by treatment with pharmaceuticals. This should greatly accelerate the knowledge of the biology of the mutations, and provide the research community with leads for biological follow-up, thereby accelerating the development of therapeutic options. For example, the data generated here will provide a number of highly valuable targets that will be further studied in a related project from the Kim lab that aims at generating peptide-based inhibitors to cancer-related protein-protein interactions using a combination of computational and combinatorial chemistry approaches. By focusing on the interactions that are specifically mutated (e.g., strengthened) in cancer cells and whose inhibition would be of particular value therapeutically, target prioritization for inhibitor development would be improved.

**21. Vision Statement**

What we envision as main outcomes of the project are a) the generation of valuable data on the interactions modulated across ~150 protein forms; b) the creation of a robust pipeline which enables us to combine structural information and experimental data to systematically map the changes in interactomes imparted by cancer associated mutations. As there is an increasing push towards DNA sequencing to identify new mutations, it will be essential to develop methodologies that enable to zoom in on mutations that can affect interactions and to define which of these are regulated interactions. While some of the interactions may be themselves currently “druggable”, as in the case of the HSP90 inhibitors used in our preliminary study, others may become attractive targets for future compound development. We therefore see our studies as helping to bridge the gap between the genomics field and the bedside.

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