Summary of RASSF symposium, "Clinical and Biological Implications for the role of the RASSF family of tumor suppressors", Banff, Alberta, Canada. February 4th to 8th , 2009.

Organizing committee: Dr. Shairaz Baksh, Dr. Eric O'Neill, and Dr. Gerd Pfeifer

The inaugural RASSF symposium was held on February 4 to 8th, 2009 at the Fairmont Banff Springs Hotel, Banff, AB, Canada. The aims of this symposium was to (1) bring together internationally recognized scientists and clinicians investigating the role played by a tumor suppressor gene family commonly silenced in numerous human cancers; (2) to discuss ideas and share scientific knowledge; (3) to provide a forum for young scientists in the field an opportunity to interact with experienced researchers; and (4) to advance our understanding of the field and stimulate collaborative ventures that will accelerate our ability to understand cancer detection and progression, development, and inflammation to mention a few. During the four days of the symposium, I think we achieved many of these goals during this inaugural meeting. We had 40 attendees that included 22 invited speakers, 11 trainees (graduate students or postdoctoral fellows), 2 technicians, 2 nurses and 3 principal investigators that were not speakers. Our attendees traveled from Japan, South Korea, England, Germany, Switzerland, Singapore, Canada and the United States. We had one poster session with 12 posters being presented that generated a lot of discussions during the poster session and during the breaks.

The symposium began with a brief welcome by the chair of the organizing committee, Dr. Shairaz Baksh, followed by the keynote address by Dr. Gerd P. Pfeifer. Dr. Gerd P. Pfeifer presented a historical perspective of the RASSF family of proteins. RASSF5 or Nore1 was the first RASSF family identified in 1998 followed by RASSF1 in 2000. Since then, 8 other RASSF related genes have been identified that share identify/similarity to the Ras binding domain (RBD) of RASSF1. Dr. Pfeifer outlined the complexities with the analysis of RASSF family members and described how epigenetic silencing by methylation was found for numerous RASSF family members including RASSF1, 2, 4, 5a and 5b depending on the cancer group. He went on to describe the varied functions of RASSF family members that included cell cycle control (RASSF1, 2, and 7), Ras signaling (RASSF1a, 1c, and 2 - 6), apoptosis (RASSF1a, 1c, 2 - 6), immune cell function (RASSF1a, RASSF5b and RASSF6) and microtubule stability (RASSF1, 5, and 7). This was a great introduction to the RASSF family of proteins that was followed by a reception that allowed our attendees to introduce themselves and make that first connection.

The symposium resumed the next day with another keynote address by Dr. Channing Der, a world leader in the Ras signaling. He delivered an introduction to the Ras family of GTPases and of the importance of these GTPases to the pathogenesis of numerous cancers. However, he pointed out that 13 signal transduction inhibitors approved for clinical application, none are against Ras, an oncogene mutated in >30 % of human cancers. One obvious approach for blocking Ras is to prevent GTP-binding, crucial for Ras oncogenicity. However, Dr. Der explained that the successful development of ATP-competitive inhibitors for protein kinases cannot be applied to develop similar inhibitors of the high picomolar affinity GTP binding to Ras. Thus, it might be better to target Ras by inhibiting its membrane localization or downstream effector signaling. He proceeded to describe several examples of how a Ras inhibitor may work to inhibit or reduce Ras activity. He ended his talk by describing how the Ral family of GTPases may be an important effector pathway that should be targeted for anti-Ras drug discovery. Dr. Der's seminar was a great introduction to the RASSF family of proteins as it gave us a perspective on GTPases and how we can utilize that information to better understand the function of some of the RASSF family of proteins.

The seminars continued with the presentation by Dr. Joseph Avruch describing his findings on how RASSF5/Nore1 can regulate MST1 and MST2 in response to Ras like GTPases, one of several seminars on the MST family of kinases. He described the phenotype of the *Mst1*$^{-/-}$ mice which exhibit low numbers of mature naïve T cells despite fairly normal thymic development. Two T cell autonomous defects appear to underlie this phenotype, i.e., 1) defective adhesion and migration due to impaired integrin clustering and 2) a markedly diminished threshold for the commitment to a proliferative response upon stimulation of the T cell antigen receptor, resulting in their spontaneous entry into an activated state *in vivo* that results in premature apoptosis. Mst1 null T cells exhibit radically reduced levels of Nore1B polypeptide despite unaltered Nore1B mRNA, and no phosphorylation of Mob1 in response to αCD3+αCD28. However, Lats1/2, FoxO1/3A and Yap1 phosphorylation were similar to wild type and Mst1 null T cells. Thus the Nore1B/Mst1 complex is a critical negative regulator of T cell activation. Dr. Geoffery Clark described his work looking at RASSF5A/Nore1A and its involvement in senescence. Overexpression of RASSF5/Nore1A resulted in

increased percentage of senescence and shRNA to RASSF5/Nore1A resulted in decreased percentage of senescence. RASSF5/Nore1A may modulate senescence by associating with $p21^{CIP1}$. Endogenous RASSF5A/Nore1A appeared to be nuclear and colocalized to nuclear speckles and interacted with HIPK2 (homoeodomain interacting protein kinase 2) in order to modulate senescence. Dr. Donninger discussed his findings of RASSF2 association with K-Ras and he stated that RASSF2 levels were elevated in the brain, blood and lung. RASSF2 is frequently methylated in colon cancers and RASSF2 lacked the C1 domain of RASSF1A. RASSF2 was shown to associate with prostate apoptosis response gene 4 (PAR-4) and inhibited survival pathways including NFκB. PKA and PKB/Akt was shown to modulate the activity of PAR-4 and PAR-4 was found to induce cell death to a greater extent in cancer cells versus normal cells. In addition, RASSF2 was shown to promote the nuclear accumulation of PAR-4 and the loss of RASSF2 conferred partial resistance to PAR-4 mediated cell death. This session was informative and described a new molecular target for RASSF2 and further defined how RASSF5/Nore1A modulates MST1/2.

The next session described animal models of RASSF1A that included mice and *Drosphilia*. Dr. Stella Tommasi described the phenotype of the *Rassf1a*$^{-/-}$ mice. These mice are viable and fertile but were prone to spontaneous and induced tumourigenesis. In *Rassf1a*$^{-/-}$ mice, mitosis was delayed and frequently resulted in cytokinesis failure. She also described the generation of a *TP53*$^{-/-}$*/RASSF1*$^{-/-}$ mice. These double knockout animals showed increased tumour formation and reduced life span compared to single mutant mice, though loss of p53 appears to dictate the tumour spectrum. She also presented that the simultaneous deletion of p53 and RASSF1A resulted in increased levels of polyploidy. Dr. Louise van der Weyden showed that her *Rassf1a*-null mice displayed an increased incidence of tumours of the gastrointestinal tract (adenomas and adenocarcinomas) and aged *Rassf1a*-null mice showed an enhanced susceptibility to oesophageal achalasia compared with wild type littermates. The long latency to spontaneous tumour development in *Rassf1a*-null mice suggested additional somatic 'hits' are required before tumour development becomes overt. She showed that mutagens (such as irradiation) can reduce this latency, and that Rassf1a genetically interacts with Apc in tumourigenesis as *Rassf1a*-null mice on a *Min* (*Apc*$^{+/Min}$) background showed elevated levels of intestinal polyp formation and decreased survival compared with wild type littermates. To identify additional genes that co-operate with *Rassf1a* in tumourigenesis, she is now taking a forward genetics approach using somatic mutagens including retroviruses (*MuLV*) and transposons (*Sleeping Beauty*) in *Rassf1a* mice - these mutagens provide a molecular tag to allow easy identification of the disrupted gene(s) that promote tumour formation in co-operation with loss of *Rassf1a*.

Dr. Nicolas Tapon next described his results in dRASSF1$^{-/-}$ *Drosphilia* that demonstrated a smaller body size (15% smaller) with decreased cell numbers and enlarged rough eyes. dRASSF1 was shown to modulate the function of Hippo and an antagonistic relationship was found between Salvador and RASSF1. The loss of Hippo was found to result in increased tumour formation in *Drosphilia* and the loss of both RASSF1 and Hippo lead to a greater formation of tumours. Dr. Tapon also characterized a new member of the RASSF family, RASSF8 or Boa. Boa/dRASSF8 was shown to associate with ASPP (apoptosis stimulating protein of p53) and ASPP is required for the apical localization of Boa/dRASSF8. Dr. Tapon's data clearly demonstrated an important role for RASSF8 in *Drosphilia*.

The next sessions focused on the involvement of RASSF family members in cell death. Dr. Yutaka Hata described the important role for RASSF6 in TNFα and okadaic acid-induced cell death. RASSF6 was demonstrated to may have a role in endonuclease G release. MST2 can associate with RASSF6 and block RASSF6-mediated cell death. Dr. Hata commented on the fact that RASSF2, 3, 4, and 6 can inhibit MST2 activity but RASSF1A cannot. Dr. Victor Yu continued to describe the role of MOAP-1 in cell death and how MOAP-1 and RASSF1A were pulled into microtubules. He described the association of MOAP-1 with Baxβ, a 218 amino acid primate specific isoform of Bax that was highly regulated by the proteosome machinery. MOAP-1 is regulated by the ubiqutin-proteosome machinery and had a high turnover in the cell (half life of 25 minutes) and, specifically, apoptotic signals suppressed the polyubiqutination of MOAP-1. They have identified the ring finger containing protein TRIM39 as a MOAP-1 binding protein. Lastly, they found that the expression of RASSF1A or RASSFC was found to stabilize the expression of MOAP-1.

Dr. Andrew Chalmers described the role of a relatively new RASSF family member, RASSF7, and how it is involved in mitotic regulation in *Xenopus*. The expression of RASSF7 was found to be increased in some cancer types with localization to the centromeres and is a non-typical RASSF family member with an N-terminal Ras binding domain. The loss of RASSF7 in *Xenopus* resulted in a stunted growth, cell death in the embryos and lack of mitotic spindle formation affecting cell growth. This session was completed by Dr. Brian Hemmings describing his work with NDR and RASSF1A. MOB1 is important to the activity of NDRs and RASSF1A was found to function upstream of the MOB1/NDR1 complex. NDR1 was found to become activated up apoptotic stimuli and the presence of overexpressed RASSF1A was found to inhibit the activity of NDR1. NDR1 deficiency in *C. elegans* and *Drosphilia* resulted in embryonic lethality, but not in mice (mainly because of NDR2 in mice). However, *Ndr1*$^{-/-}$ mice develop high grade lympohmas as they age that is more prevalent in females versus the males. In mice, NDR2 is abundantly expressed in the colon and brain and the loss of NDR2 in mice results in the increase in expression of NDR1 in the colon and lung. He

further showed that the *Ndr1*$^{-/-}$/*Ndr2*$^{-/-}$ mice is embryonic lethal around E12. Both of these kinases have important roles in development and RASSF1A is an upstream modulator of the NDR pathway.

RASSF1 has been demonstrated to be important in modulating the MST1/MST2 family of sterile 20 kinases (part of the MAPKKKK family of proteins). The next few seminars highlighted how important RASSF1 is to the Hippo signaling pathway. Drs Nishina and O'Neill investigated RASSF1A/MST biology by utilizing knockout mice and mammalian cells to carry out their research, respectively. Dr. Hiroshi Nishina demonstrated that MST1 can induce chromosomal condensation and that RASSF1C can associate with DAXX (a DNA damage modulator) and couple nuclear DNA damage to JNK activity changes in the cytosol. DAXX can also promote the movement of RASSF1C from the nucleus to the cytosol. They characterized the MKK4/MKK7 double knockout mice that failed to activate JNK. RASSF1C and DAXX may play important roles during DNA damage and nuclear condensation. Dr. Eric O'Neill characterized RASSF1A/MST1 associations and found that RASSF1A and Raf-1 have overlapping binding sites on MST2. He also highlighted the importance of the polymorphism to RASSF1A, S131A, the ATM phosphorylation site containing residue. He demonstrated that S131 of RASSF1A was required to stabilize p73 and that RASSF1A/MST was upstream of LATS and YAP control of p73 transcriptional activity. This pathway may have a potential role in DNA damage control. Dr. Luis Miguel Martins continued on the theme of MAP kinases by presenting data characterizing MAP4K3, a target for the ubiqutin-proteosome system and a suppressor of DNA damage induced cell death. MAP4K3 is downregulated in human pancreatic cancers and may function to stabilize PUMA mRNA, a BH3-domain protein. Overexpression of MAP43K results in increased caspase activity and Bax activation suggesting a role in cell death.

The next few seminars focused on the diverse roles for RASSF1A and how these roles might be modulated by upstream factors. Dr. Leyuan Liu presented his work investigating the role of C19ORF5 and RASSF1A in autophagy, and, specifically, mitophagy. They demonstrated that C19ORF5 can associate with LC3, a mammalian homolog of an autophagic protein, and may form a complex with RASSF1A on microtubules. Taxol can increase the amount of C19ORF5 in mitotic cells. These proteins together might be important for mitotic cell death. Dr. Dae-Sik Lim's presentation continued to focus of the role of RASSF1A in mitosis and described the important of S131A of RASSF1A and how it is important in being recognized by ATM. RASSF1A can associate with MDM2 and interfere with G0/G1 phase transition and p53 stability. It is targeted for degradation by Skp2 but resynthesized during mitosis. He also showed that S203 is a kinase phosphorylation site modulated by Aurora kinase A. Dr. Lim further demonstrated that WW45 can associate with MST1/2 in order to activate LATS to inhibit YAP activity and *WW45*$^{-/-}$ mice die at E17.5 from perinatal lethality and intestinal hyperplasia. The generation of the WW45$^{-/-}$ mice will greatly aid in understanding the RASSF1A/MST signaling pathway. Dr. Huang continued to explore phospho-regulation of RASSF1A by Aurora kinase A on T202 and S203. The phosphorylation of RASSF1A on T202/S203 may modulate microtubule binding and stability, and Aurora kinase A overlaps with RASS1A to centromeres and mitotic spindles. There is a growing body of literature to suggest phospho-regulation of RASSF1A and how they may modulate the function of RASSF1A.

The last sessions of the RASSF symposium highlight the clinical implications for RASSF family members and how we can utilize what we know about the molecular mechanisms and interacting partners to RASSF family members to better understand the *in vivo* function for RASSF1A. Both Dr. Delvac Oceanby and Dr. Junichi Sadoshima presented studies to demonstrate the importance of RASSF1A and MST1 in the function of the heart and how the heart responds to insults, such as hypertrophy and ischemia. Hearts from *Rassf1a*$^{-/-}$ mice appear normal, but when given a hypertrophic insult *Rassf1a*$^{-/-}$ mice did not respond well and higher levels of hypertrophic markers such as ANP and BNP. MST1 transgenic overexpression mice have enlarged hearts and respond very well to hypertrophic insult. It was suggested that RASSF1A may modulate hypertrophy by utilizing MST1. These findings demonstrated the importance of the RASSF1A/MST pathway and the need to understand upstream regulators and downstream effectors for this pathway. Dr. Tatsuo Kinashi presented his work in trying to understand how RASSF5B/Nore1B/RAPL-Rap1 signaling pathway was involved in immune cell trafficking and proliferation. Rap1 activation occurred in a PLCγ-dependent manner and there was extensive colocalization of RAPL with LFA-1 at the immunological synapse. *Rapl*$^{-/-}$mice had hypoplastic lymphoid organs and impaired migration to lymph nodes. In addition, dendritic cells obtained from RAPL$^{-/-}$mice also demonstrated impaired migration. As observed for RASSF5A/Nore1A, RAPL was demonstrated to associate with MST1. Furthermore, *Mst1*$^{-/-}$ mice also had hypoplastic lymphoid organs and reduced numbers of T and B cells in the lymph nodes and spleen. The last talk of the symposium was delivered by Dr. Shairaz Baksh. He described recent work characterizing innate immunity in the *Rassf1a*$^{-/-}$ mice following pathogenic insult. He suggested that RASSF1A may be an interesting modulator of NFκB activity and inflammation. *Rassf1a*$^{-/-}$ mice die rapidly following intra-peritoneal injections of pathogenic components. *Rassf1a*$^{-/-}$ mice have elevated production of pro-inflammatory cytokines following intra-peritoneal injections of pathogenic components resulting in the colon architecture destruction and severe symptoms of human inflammatory bowel disease. RASSF1A joins RASSF2 and RASSF6 in a role to possibly modulate NFκB activity and NFκB-directed inflammation.

Dr. Baksh also delivered the closing remarks to the meeting. The conference attendees commented that the meeting was well organized, scientifically very successful, and gave attendees many opportunities for discussions and generated new ideas and collaborations. In addition, the venue and hotel was outstanding. A follow up meeting would be needed and it was tentatively scheduled for the summer 2011 in Oxford, England.