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POSTER SESSIONS

CSI-01 – Cell Cycle & Checkpoints

SUN-001

Adaptations of the DNA replication licensing process in mouse embryonic stem cells

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Mouse Embryonic Stem Cells (mESCs) have the ability to proliferate and self-renew indefinitely in culture and, when stimulated, to differentiate towards all three germ layers. They exhibit an unusual cell cycle which consists mainly of S phase cells, a short G1 phase and a lack of major checkpoint responses. Maintaining genome stability is pivotal for embryonic stem cells, as they give rise to all mature cell types. In order for committed cells to maintain genome integrity, DNA replication is restricted to only once per cell cycle. This is accomplished through the assembly onto chromatin of multisubunit protein complexes which license DNA for a subsequent round of replication. Replication licensing takes part in G1 phase and consists of the loading of the hexameric MiniChromosome Maintenance 2-7 (MCM2-7) complex onto chromatin, a step which is dependent on the licensing factor Cdt1. We previously showed that the loading of MCM2-7 onto chromatin takes place in two waves in live mammalian cells: upon mitotic exit and at the G1/S phase transition.

We are interested to understand the molecular mechanisms which ensure genome stability in mESCs, and govern DNA replication licensing, and how these are compared to differentiated and cancer cells. We show that mESCs have a very short G1 phase and move to S-phase with high synchrony following mitotic arrest. During S-phase, replication factories are visualized by immunofluorescence against the replication protein PCNA. They show characteristic early, middle and late S-phase localization, reminiscent of replication factory dynamics in differentiated and cancer cells. Cdt1 is specifically destructed during the G1 phase of the mESC cell cycle and degraded following entry to S-phase. MCM2 and MCM7 can be immunodetected onto chromatin following mitotic exit and in S phase, and exhibit differential localization along the cell cycle. Following DNA damage by UV-irradiation in G1, Cdt1 is rapidly proteolysed, while changes in chromatin-loaded MCM proteins are evident. When mESCs are irradiated during mitosis, Cdt1 degradation is delayed until entry into G1, suggesting the Cdt1 is protected from proteolysis in mitosis. mESCs irradiated during mitosis or in G1 fail to progress to S-phase. We will be using functional live-cell imaging to assess licensing in mESCs by Fluorescence Recovery After Photobleaching (FRAP) of the GFP-tagged MCM protein subunits, through the cell cycle of uncommitted cells, or when cells are moving towards differentiation, with a concomitant lengthening of G1 phase.

Keywords: DNA replication licensing, mouse embryonic stem cells.

SUN-002

Analysis of the mechanism of DNA damage and replication arrest-induced histone mRNA decay

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Histone mRNA decay (HD) is the process which ensures that histone mRNA is rapidly degraded following completion of DNA replication at the end of S-phase. Strict coordination between histone protein production and DNA replication is essential for the correct packaging of newly replicated DNA, as imbalances can lead to deleterious effects such as genomic instability. Interestingly, histone mRNA stability is controlled by the presence of a stem-loop structure at the 3′ end of histone mRNA, and a protein, HBP/SLBP (hairpin/stem loop binding protein) which specifically binds to this structure, plays a major role in histone mRNA metabolism. Importantly, previous studies have shown HD is also one functional target of an intra S-phase checkpoint activated when DNA synthesis is inhibited, ensuring that histone mRNA is rapidly destroyed when global DNA replication is blocked. Consistent with this, replication stress-induced histone mRNA decay is blocked in the presence of inhibitors of the PIKK family of checkpoint protein kinases, implicating PIKK family members in the regulation of this process. Therefore, we aim to utilise a proteomic approach to to identify HBP/SLBP-associated proteins and post-translational status in order to elucidate the detailed mechanism of checkpoint activated HD. We have established isogenic cell lines stably expressing functional, tagged HBP/SLBP by using the Flp-In™ T-REx™ Expression system. Our results indicate that isogenic Flp-In HeLa cell lines inducibly expressing tagged forms of SLBP under the control of a doxycycline promoter are a useful model system for the molecular analysis of SLBP function during replication stress.

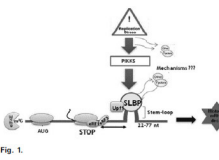

Fig. 1.

Keywords: hairpin/stem loop binding protein (HBP/SLBP), histone mRNA decay, Intra S-phase checkpoint.

SUN-420

Role of neutral sphingomyelinase in dopamine transporter trafficking

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Dopaminergic neurotransmission in the brain plays a central role in the control of movement, hormone release, and many complex behaviors. Dopamine (DA) uptake into presynaptic terminals is thought to be a key step in neurotransmission termination. Dopamine transporter (DAT) is Specific transport protein tightly control the availability of DA, in the synaptic cleft by mediating rapid transmitter reuptake at presynaptic terminals. DAT undergo highly regulated trafficking between the cell surface and endosomal compartments (Rao et al., 2011; Sager and Torres, 2011). Here, we suggest that neutral sphingomyelinase 2 (nSMase2) may regulate DA uptake and DAT trafficking. Previously we found that nSMase2 is a mediator of DA uptake (Kim et al., 2010). Interestingly, transfection of nSMase2 siRNA or pretreatment with the nSMase2-specific inhibitor GW4869 resulted in decreased DA uptake. Reciprocally, exposure of PC12 cells to cell-permeable C_6-ceramide induced a concentration-dependent increase in DA uptake. In mouse striatum and PC12 cells, we performed immunofluorescence staining to find out the localization of nSMase2 and DAT. In the dorsolateral mouse striatum, we observed colocalization of DAT and nSMase2. In PC12 cells, DAT are colocalized with nSMase2 in plasma membrane. We examined the physiological relevance of DAT–nSMase2 interaction by performing coimmunoprecipitation experiments in synaptosomal preparations from mouse striatum as described previously (Gorman et al., 2012). The enrichment of nSMase2 and DAT in striatum is more pronounced. In Immunoblotting with imunoprecipitation of DAT showed nSMase2, but imunoprecipitation of nSMase2 did not showed DAT. anti-nSMase2 antibody does not have good performance to pull down nSMase2 protein from supernatant. In nSMase activity assay with immunoprecipitated pellet, the IP pellet of anti-DAT antibody show nSMase activity to an extent equivalent to that achieved with the IP pellet of anti-nSMase2 antibody. These results indicate a physiological relevance of the DAT–nSMase2 interaction. Further analysis revealed that GW4869, nSMase2 inhibitor, decreased the surface level of DAT in PC12 cells stably expression DAT. In sum, our findings provide that physiological interaction between DAT and nSMase2 and, nSMase2 is suggested to facilitate relocalization of DAT on the presynaptic surface.

Keywords: Ceramide, Dopamine transporter, neutral sphingomyelinase 2.

SUN-422

Single particle tracking to study the binding of protein misfolded oligomers to membrane ganglioside GM1

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Recent results suggest an important role of ordered lipid domains of plasma membranes in amyloid aggregation process and its pathogenic effect. We investigated the contribution to toxicity of the membrane ganglioside GM1 in SH-SY5Y neuroblastoma cells exposed to oligomeric conformers of $A\beta_{42}$ and HypF-N endowed with different ultrastructural properties. By means of real-time single particle tracking, we show that misfolded oligomers bind GM1, decreasing its lateral diffusion on the plasma membrane of living cells. In turn, the biochemical response to the oligomeric species results from the membrane content of GM1 and its clustering. Overall, our results indicate an altered membrane raft mobility and clustering in neurons experiencing aberrant protein oligomers.

Keywords: Membrane GM1, Protein oligomers, Single particle tracking.

SUN-423

State-dependent H-bonds of exceptionally conserved asparagines in S6 helices of sodium and calcium channels: roles in channel gating and ligand action

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Background: Voltage-gated sodium and calcium channels play key roles in the physiology of excitable cells. The alpha-1 subunit of these channels folds from a polypeptide chain of four homologous repeats. In each repeat, the cytoplasmic halves of the pore-lining helices contain exceptionally conserved asparagines. Such conservation implies important roles, which are unknown. Inherited mutations of the asparagines are associated with some channelopathies. Engineered substitutions of the asparagines affect activation and inactivation gating and the action of pore-targeting drugs and toxins. In the absence of X-ray structures of eukaryotic sodium and calcium channels, underlying mechanisms are unclear.

Methods: X-ray structures of potassium and sodium channelds in the open and closed states were used as templates to build homology models of the open and closed Cav1.2 and Nav1.4 channels and some of their mutants. The models were optimized with the method of Monte Carlo-energy minimization and state-dependent contacts of the conserved asparagines were analyzed.

Results: In the homology models of the open and closed Cav1.2 and Nav1.4 channelds the asparagines do not face the pore. In the open, but not in the closed channels, the asparagine residue in a given repeat forms an inter-repeat H-bond with a polar residue, which is typically nine positions downstream from the conserved asparagine in the preceding repeat. The H-bonds, which are strengthened by surrounding hydrophobic residues, would stabilize the open channel and shape the open-pore geometry. According to calculations, the latter is much more sensitive to mutations of the asparagines, than the closed-pore geometry. Substitutions of the asparagines, their H-bonding partners, or surrounding residues change state-dependent inter-repeat contacts. The changes may affect channel activation, inactivation, and ligand action. Our models suggest the atomistic mechanisms behind a calcium channelopathy (the night blindness) and sodium channelopathies (Bragsada syndrome and infantile arrhythmias). We further propose that engineered substitutions of the conserved asparagines influence the state-dependent action of local anesthetics, antiarrhythmic agents, and steroidal agonists like batrachotoxin and veratridine by changing availability of the open channels and affacting the open pore geometry.

Conclusions: The exceptional conservation of asparagines in the inner helices of sodium and calcium channels is due to their involvement in inter-repeat H-bonds that stabilize the open state and shape the open pore geometry.

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Keywords: Channel gating, Molecular modeling, State-dependent drug action.