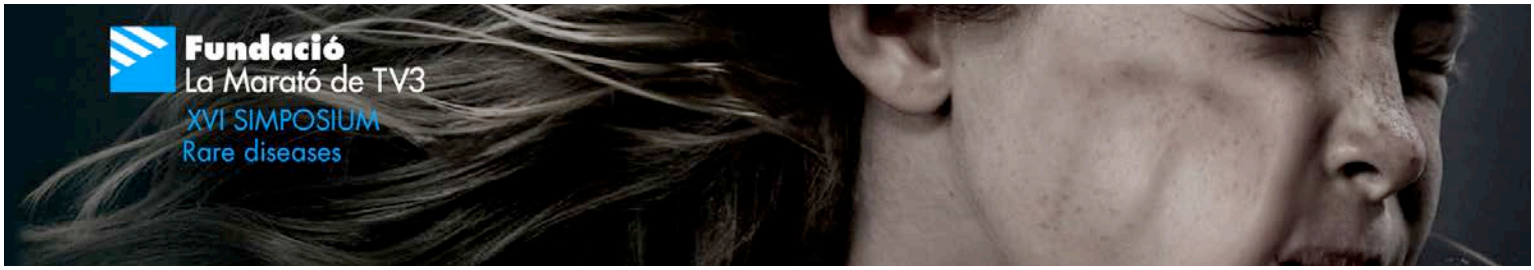

Role of the OR cyclin in ataxia telangiectasia

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1. Summary

Ataxia-Telangiectasia (AT) is an autosomal recessive disease characterized by neurological and immunological impairment, development of telangiectasias, tumour predisposition and hypersensitivity to gamma radiation. The disease is caused by mutations in the gene encoding ATM, a protein involved in the DNA damage response (DDR). Cyclin O is a protein that is also part of the DDR and it is necessary for apoptosis induced by DNA damaging agents. It forms kinase active complexes with Cdk1 and Cdk2. Cyclin O levels are regulated by the ATP-p53 axis and its downregulation leads to perturbations of the DDR and defective DNA damage-sensitive cell cycle checkpoints. Recently it has been described that cyclin O is necessary for centriole overduplication in multiciliated cells and its deficiency leads to diseases derived from the lack of functional cilia (ciliopathies). The project aimed to establish genetic and biochemical relationships between ATM, the related protein kinase DNA-PK and cyclin O to contribute to the understanding of AT pathology and to define putative therapeutic targets. It was also proposed to generate mouse models deficient in cyclin O and cyclin O/ATM to further investigate the genetic relationships between both proteins.

2. Results

Objective 1. Crosstalk between ATM and cyclin O in the DDR.
Objective 2. Biochemical mechanism of the crosstalk between ATM and cyclin O.

Previous results of the laboratory indicated that the gene encoding cyclin O is regulated by the ATM-p53 axis and that its downregulation leaded to perturbations of the DDR. The generation of cyclin O KO mice (objective 4) allowed us to establish cultures of cells deficient in cyclin O (such as embryonic fibroblasts, MEF, and activated T-cells) and study the DDR in these conditions.

The results obtained indicate that the loss of cyclin O leads to biochemical changes that impair the DDR affecting the DNA damage-dependent cell cycle checkpoints, in particular the G2/M. Thus, cyclin O KO MEFs show a quicker mitosis exit after the generation of double strand breaks (DSBs) in response to exposure to gamma radiation. On the other hand, they take longer to re-enter cell cycle when the DNA damage has been repaired, These findings indicate that cyclin O deficient cells have an altered G2/M checkpoint.

Biochemical evidence showed that both ATM and the related protein kinase DNA-PK show a deficient activation in cyclin O deficient MEFs in response to gamma radiation treatment. We also found that in these cells ATM substrate proteins involved in the DDR such as H2AX or Nbs1 showed an altered phosphorylation pattern compatible with a deficient activation of ATM and DNA-PK.

Unexpectedly Chk2, another kinase substrate of ATM, was phosphorylated normally while the ATR substrate kinase Chk1 was hyperphosphorylated in response to gamma radiation treatment in cyclin O deficient cells.

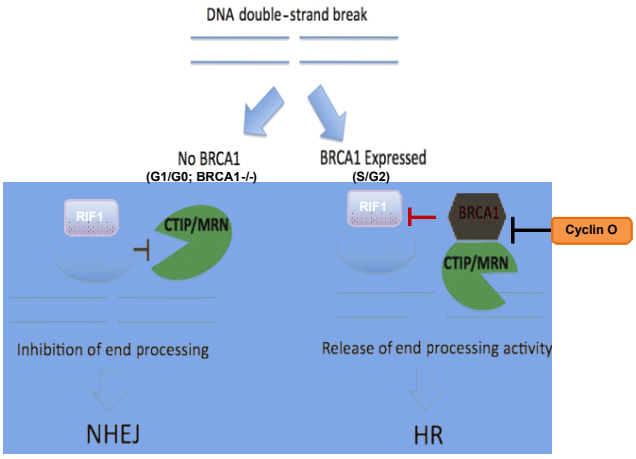
These observations, together with the fact that the phosphorylation of H2AX in response to gamma radiation comes back to normal levels in cyclin O KO cells where DNA-PK has been silenced and ATM has been inhibited using a small molecule specific inhibitor, suggest that ATM and DNA-PK have shared biochemical roles. These results indicate that in the absence of both kinases a third member of the family, ATR, would take over their functions in cyclin O deficient cells.

The regulation of ATR by cyclin O leads to the prediction that the resection rates of the DNA ends of the DSBs, the first step of the repair by homology-directed recombination (HDR), would be increased in cyclin O KO cells. This prediction was confirmed, indirectly at first by showing that these cells have a higher incorporation of phospho-RPA into the DNA and a higher rate of generation of single stranded DNA (ssDNA) after gamma radiation treatment. The direct proof

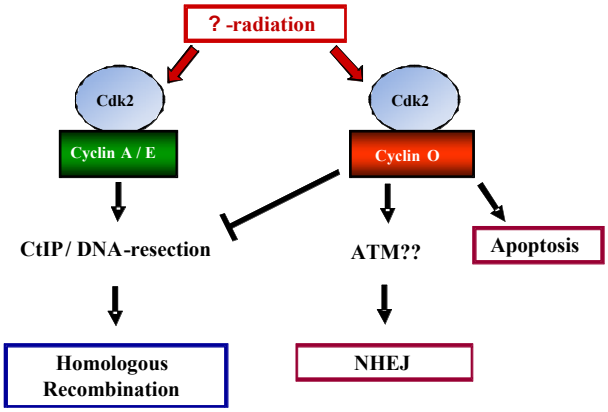
was to measure the length of the resected ends by using the SMART technique, which showed that the loss of cyclin O leads to the generation of longer ssDNA stretches in a gene dose dependent manner. By means of proteomic techniques we found that this effect is mediated by the cyclin O-dependent regulation of the phosphorylation levels of CtIP S326. This protein binds the DNA ends and should be phosphorylated in S326 in order to interact and recruit BRCA1 to the DSBs, where it is essential for HDR.

All these findings suggest that the loss of cyclin O would make the cells more prone to repair the DSBs by the high fidelity HDR mechanism instead of the faster but potentially mutagenic non-homologous end joining mechanism (NHEJ). We were able to prove that during the resolution of the G2/M checkpoint, cyclin O deficient cells enter M-phase with less DNA damage than WT cells, which is compatible with the longer recovery (=repair) time that it takes them and with the use of the HDR mechanism. This fact has been proved by using the rate of localization of HDR-related proteins to the DNA damage foci. This rate is increased in the case of cyclin O deficient MEFs. The direct measure of the DNA repair rate by HDR by using reporter constructs is also compatible with this hypothesis.

From this part of the project we can conclude that cyclin O would be regulating the cell's choice between the two main mechanisms of repair of the DSBs. Thus, through the preferential activation of the ATM/Chk2 pathway versus ATR/Chk1, cyclin O would inhibit the repair by HDR favouring the faster and mutation-prone NHEJ mechanism.



Cyclin O regulates the cell's choice of the DNA repair mechanism mediated by BRCA1-CTIP. Adapted from Aly and Ganesan, 2011.



The composition of the Cdk2 complexes determines the cellular response to DNA damage.

Objective 3. The role of cyclin O in the endoplasmic reticulum (ER) stress pathway.

Stimuli such as oxidative and reductive stress or the release of Ca^{2+} from the ER stores lead to the activation of the ER stress pathway. The work done during all these past years has shown that cyclin O is induced as part of the response to stimuli that cause ER stress due to the accumulation of misfolded proteins. In order to investigate its biochemical role in this pathway we have used the abovementioned tools derived from cyclin O KO mice (objective 4).

Our results show that cyclin O would be involved the activation of the stress kinase (JNK and p38) pathway as a consequence of the instauration of the ER stress. In the ER stress pathway it has been well established that JNK and p38 are activated via activation of the ER transmembrane protein IRE1. This protein interacts with TRAF2, which activates the MAP3K Ask1, which will activate JNK and p38 through activation of MEK4/7 and MEK3/6, respectively. However our results show that this is not the only activation pathway of JNK/p38 in response to ER stress. We have identified a cyclin O-dependent, Ask1-independent alternative activation pathway of the stress kinases in response to ER stress.

The fact that ATM loss leads to the instauration of oxidative stress in neurons leads us to think that the inhibition of the cyclin O complexes would avoid the activation of the ER stress pathway that leads to cell death. This oxidative stress has been proposed as the main cause of the neuronal death that causes cerebellar degeneration in AT patients. Taking this into account, cyclin O could be a suitable therapeutic target to design inhibitors able to block cell death induced by oxidative/reticular stress.

Objective 4. In vivo study role of cyclin O in AT tumorogenesis and neurodegeneration.

The fourth objective of the project aimed to generate cyclin O knockout mice to study its role *in vivo*. It was also proposed to generate ATM/cyclin O double KO mice by intercrossing the mouse strains. ATM KO mice recapitulate some of the clinical features of the human patients such as the cancer predisposition or the sterility, but they are not suitable models to study neurodegeneration.

Cyclin O KO mice are viable and are born with a near-Mendelian frequency, ruling out embryonic lethality. The most prominent feature of these mice is that 80% of the homozygotes and about 10% of the heterozygotes develop severe hydrocephalus within the first five weeks of postnatal life. If they survive this

period, the skull starts to be calcified and the brain can adapt to the increasing intracranial pressure and the KO mice can live without apparent pathologies for as long as WT mice.

By using MRI we found that cyclin O KO mice develop non-obstructive hydrocephalus that affects only the lateral (first and second) ventricles. The phenotype is 100% penetrant, all the homozygous mice even the long time survivors are severely hydrocephalic. Surprisingly the mice are clearly haploinsufficient since all heterozygous mice develop hydrocephalus, although more slowly than the homozygous mice.

In addition to the hydrocephalus, cyclin O KO mice show signs of neuronal distress and damage. They also show a thin brain cortex with disorganized structure, losing the typical 6-layer configuration. Both homo- and heterozygous mice show neuronal loss in the hippocampus and severe defects in its folding.

Concerning the crosses to the ATM KO mice, the MRI study could not be completed because of the difficulty in getting enough double KO mice, although we found that they are viable. The analysis of some gene combinations showed that ATM KO mice have smaller lateral (first and second) and third ventricles. However, the fourth ventricle is enlarged, perhaps reflecting neuronal loss at the level of the cerebellum, a hallmark of the human disease. The loss of a single allele of cyclin O (ATM hom/cyclin O het) is enough to completely rescue the phenotype, having all the ventricle volumes indistinguishable from the WT/WT brains. These results prove that ATM and cyclin O interact genetically.

Regarding the tissues outside the CNS, in agreement with previous results cyclin O KO activated T-cells and fibroblasts (all proliferating cells) are highly radioresistant. On the contrary, quiescent cells such as thymocytes are as radiosensitive as in the case of the WT.

Recently we have been able to understand the rational behind the development of hydrocephalus by cyclin O KO mice. cyclin O is an essential player in the process of formation of cilia in multiciliated cells, present in airway epithelia, the oviduct and in the ependyma, the epithelium lining the brain's ventricles. The cilia are essential in the ependyma for the recircularization of the cerebrospinal fluid (CSF). If they are absent or do not work properly, the CSF accumulates and originates the hydrocephalus. We have been able to demonstrate that cyclin O KO mice have a severe defect in the generation of multiciliated cells. Only very sparse, morphologically aberrant non-functional cilia are present in these epithelia. These facts confirm the essential role of cyclin O in ciliogenesis and suggest that it is one of the genes whose mutation can generate two other rare diseases: Primary Ciliary Diskynesia ORPHA244 and Normal Pressure Hydrocephalus ORPHA314928.

In addition to this and in agreement with the molecular data described in objectives 1 and 2, cyclin O KO mice show a lower incidence of spontaneous tumours compared to WT mice and the tumour spectrum is, most likely, also different.

3. Relevance and possible implications

This research project has been crucial to determine the relationships between a new member of the cyclin family (cyclin O) and the DNA damage response. We have proved that cyclin O is a regulator of ATM and of the DDR.

Our biochemical and genetic evidence suggests that inhibition of the cyclin O complexes would result in an impairment in the function of ATM. However, if they are inhibited together with DNA-PK, the cell is forced to use ATR to take over their functions.

The experiments carried out with cyclin O deficient cells show that this protein is crucial for the cell's decision to choose between the two main mechanisms of repair of the DSBs: the non-homologous end joining (NHEJ) and the homology-directed recombination (HDR). NHEJ is a fast and efficient process to repair the DSBs that are the most deleterious DNA lesions for the cell, but it is a mechanism that can cause mutations and translocations. HDR is an error-free mechanism, but it can only work in those phases of the cell cycle where a non-damaged sister chromatid is available to recover the lost information (end of S, G2 and M).

The inhibition of cyclin O complexes favours the resection of the broken ends of the DNA at the DSBs and, as a consequence, their repair by HDR. The lower incidence of spontaneous tumours in cyclin O KO mice is in agreement with the molecular findings indicating that the cells from these mice are skewed to use the error free HDR mechanism to repair the DSBs.

We can anticipate that a selective inhibitor of the cyclin O complexes with Cdk1 and Cdk2 would have antimutagenic and antitumoral properties. In the context of AT patients, the lack of a functional ATM protein leads to a collection of pathologies derived from the impossibility to repair the DSBs using HDR (lymphomas, sterility, etc.) The pharmacological inhibition of the cyclin O complexes in combination with DNA-PK inhibitors would force the cell to use ATR to fulfil the function of both kinases allowing to the use of HDR to repair the DNA.

Preliminary MRI studies show that the loss of a single cyclin O allele in ATM KO mice leads to the reversion of the abnormalities in the sizes of the ventricles of ATM homozygous mice. If we were able to demonstrate that this observation is due to the preservation of the neurons in the cerebellum, then inhibitors of the cyclin O complexes could be useful to treat the neurodegeneration of the AT patients that results in ataxia.

Our previous results show that cyclin O can form active complexes with Cdk1 and Cdk2. Cdk1 and Cdk2 inhibitors are available and are being used in clinical trials as antitumour agents. The problem of these drugs is that they do not discriminate between the Cdk2-cyclin A and Cdk2-cyclin E complexes needed for the normal cell cycle from the Cdk2-cyclin O complexes. However, in recent years progress has been made in the identification of molecules able to selectively inhibit Cdk1 or Cdk2 in spite of their high degree of homology. Perhaps in the near future new molecules will be developed that are able to selectively inhibit the different Cdk-cyclin complexes. This would open the door to check if our predictions hold true and we can bring a little hope to the people affected by this devastating and so far incurable disease.

4. Literature produced

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