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Study of DNA replication in mammalian terminally differentiated cells upon cell cycle reactivation

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1. Glossary

ATM: Ataxia Telangiectasia Mutated
ATR: ATM and RAD3-related
BrdU: Bromodeoxyuridine
CDK: Cycline dependent kinase
Chk1: Checkpoint kinase 1
Chk2: Checkpoint kinase 2
Cip/Kip: CDK interacting protein/Kinase inhibitory protein
Cy3-dCTP: Deoxycytidine triphosphate coniugated with Cy3 fluorescent dye
dATP: Deoxyadenosine triphosphate
dCTP: Deoxycytidine triphosphate
dDK: Deoxyribonucleoside kinases
dCK: Deoxycytidine kinase
dGK: Deoxyguanosine kinase
dGTP: Deoxyguanosine triphopshate
dNTP: Deoxynucleotide triphophate
DSB: Double strand breaks
dTTP: deoxythymidine triphosphate
G_1: Gap1
G_2: Gap 2
GdR: Deoxyguanosine
GF: Grow factor
HSE: High speed extracts
INK4: Inhibitor of CDK4
LSE: Low speed extracts
M: Mitosis
MPF: M-phase promoting factor
MSC: Muscle satellite cells
Mt: Myotubes
NDPK: Nucleoside diphosphate kinase
NdR: Deoxyribonucleosides
NMPK: Nucleoside monophosphate kinase
P: Proliferating myoblasts

PI3K family: Phosphatidylinositol 3-kinase-related kinase protein family
pRb: Retinoblastoma protein
Q: Quiescent myoblasts
R point: Restriction point
rMt: Reactivated myotube by siRNA p21 and p27
RNR: Ribonucleotide reductase
S: Synthesys
SAMHD1: Sterile Alpha Motif and HD-domain containing protein
TD: Terminally differentiated
TdR: Deoxythymidine
TK1: Thymidine kinase 1
TK2: Thymidine kinase 2
XEE: *Xenopus* egg extracts

2. SUMMARY

Terminally differentiated (TD) cells are characterized by the permanent inability to proliferate. In culture, skeletal muscle myotubes (Mt), a model system of TD, can be forced to reenter the cell cycle by several means, including expression of adenovirus E1A, overexpression of cyclin D1 and CDK4/6, or depletion of CDK inhibitors. Nonetheless, cell cycle reactivation in Mt never results in a long-term survival and effective proliferation. Reactivated Mt suffer heavy DNA damage, and die by apoptosis or mitotic catastrophe. Critically, they are not able to fully duplicate their DNA.
The purpose of this thesis is to understand the molecular bases that prevent Mt to lead a regular and complete replication.
We investigated through functional (i.e., biochemical) problems and structural obstacles.
Functionally, we found that Mt attempt to duplicate their DNA with extremely low levels of deoxythimidine triphosphate (dTTP). This is explained by failure to upregulate thymidine kinase (TK) upon cell cycle reentry. Exogenous administration of deoxythymidine or expression of TK increased DNA synthesis, though it never attained completion.
We then used *Xenopus laevis* egg extracts (XEE) to study DNA replication in nuclei from Mt and proliferating (P) or quiescent (Q) myoblasts. XEE are able to complement any functional defect, highlighting structural obstacles. Maximal DNA replication in Mt nuclei was strikingly lower than in those from Q and P myoblasts, revealing the presence of a barrier that prevent Mt to replicate the whole genome. To investigate at which level of nuclear complexity this obstacle lies, we studied replication kinetics in naked and nucleosome-assembled DNA from Mt, in comparison with similar samples from P myoblasts. Together, all results showed that both functional and structural obstacles prevent full DNA duplication in myotubes. Furthermore, they suggest that hitherto unexplored peculiarities of their chromatin are ultimately responsible for the inability of these cells to proliferate.

3. INTRODUCTION

3.1. THE CELL CYCLE

The eukaryotic cell cycle is an ordered and regulated series of events that leads to the production of two daughter cells. It consists of two consecutive processes, mainly characterized by DNA replication and segregation of replicated chromosomes into two separate cells. Cell division can be subdivided into 4 traditional stages: Gap_1 (G_1), Synthesis (S), Gap_2 (G_2) and mitosis (M). The interlude between two M phases is defined interphase, period in which a cell spends most of its life cycle. In G_1 phase cells increase in size and prepare the next stage, synthesizing proteins necessary for DNA replication. During the S phase the amount of DNA doubles, whereas the transcription of RNA and protein synthesis decrease. The G_2 phase is characterized by rapid cell growth and strong protein synthesis. In mitosis the pairs of chromosomes condense and attach to fibers that pull the sister chromatids to opposite sides of the cell, in order to divide the nucleus. After this stage the cytokinesis occurs, leading to the division of the cytoplasm and the birth of two daughter cells with equal genome.
If a cell is subjected to adverse environmental conditions for replication, it may stop the cell cycle at the G_1 stage and enter a phase of relax, called G_0 (Baserga, R., and Rubin, R., 1993). This break may be reversible (typical of quiescence) or physiologically irreversible (typical of terminal differentiation). Even upon favorable conditions, however, the cell cycle cannot be repeated limitless. The number of duplications that a cell can perform is known as the Hayflick limit (Hayflick, L., 1965), from the name of the researcher who put in evidence this phenomenon, beyond which the cell becames senescent. Different cellular proteins, such as cyclins and cyclin-dependent kinases (CDK), are necessary to regulate the transistions above mentioned.

3.1.1. CYCLINS AND CYCLIN-DEPENDENT KINASES

The cyclin-dependent kinases (CDK), are a family of protein that can phosphorylate target substrates at specific serine or threonine sites. Until now, nine CDK have been identified and, of these, five are active during the cell cycle, i.e. during G1 (CDK4, CDK6 and CDK2), S (CDK2), G2 and M (CDK1). When activated, CDK induce downstream processes by phosphorylating selected proteins (Morgan 1995; Pines 1995). The expression of CDK is relatively constant during the cell cycle and their regulation depends mainly on the availability of cyclins, phosphorylation and dephosphorylation events and the presence of specific inhibitors (Ekholm, S.V., and Reed, S.I., 2000). Cyclins are proteins which levels rise and fall during the cell cycle and in this way they periodically activate CDK (Evans et al. 1983; Pines 1991).
During the transition from G_0 to G_1 phase, mitogenic signals converge primarily on the expression, assembly and activation of cyclin D/CDK complexes. Three cyclins D (D1, D2, D3) are known. They are expressed differently in different organs and tissues (Sherr, C.J., 1993). All the cyclins D can assemble with CDK4 or CDK6, causing a connection between the mitogenic signaling and the machinery of the cell cycle (Ekholm, S.V., and Reed, S.I., 2000). The progression of G_1 depends on the continous expression of these cyclins which, in turn, rely on mitogenic stimulation. There is a specific point of G_1 called "restriction point" (R point), beyond which the cell becomes independent of growth factors. The cyclin D/CDK complexes are no longer required until the conclusion of the cell division cycle and the following re-entry in the next G_1 phase (Matsushime, H. et al., 1994). Cyclin D/CDK phosphorylates and inactivates the retinoblastoma protein (pRb), p107 and p130. Once phosphorylated, these proteins release factors belonging to the E2F family, which activate the transcription of some genes necessary for the beginning of S phase, for the metabolism and the DNA replication (Trimarchi, J.M., and Lees, J.A., 2002). Cyclin D/CDK complexes have, also, a non-catalytic role in the progression of G_1 phase: they sequester specific cell

cycle inhibitors, releasing and activating the cyclin E/CDK2 complex, typical of the late G_1 phase (Sherr, C.J., and Roberts, J.M., 1995). The activity of cyclin E/CDK2 reaches its peak in the G_1/S transition of the cell cycle (Dulic, V. et al., 1992) decreasing rapidly in the early S phase, due to degradation by the proteasome (Welcker, M. et al., 2003). This complex can phosphorylate many proteins, among which pRb (in different sites than those recognized by cyclins D/CDK), and can regulate various substrates involved in the expression of histone genes, in the duplication of centrosomes and in the DNA replication (Yu, Q., and Sicinski, P., 2004). CDK2 can, also, forms complexes with cyclin A.

Cyclin A is involved in the progression from G_1 to S phase. The levels of this protein are high in S and G_2 phase and declines in early mitosis when it is degraded (Sherr, C.J., and Roberts, J.M., 1995). There are known two types of cyclin A (A1 and A2): cyclin A1 is expressed only in early embryonic stages of meiosis and in some types of cancer (Liu, D. et al. , 1998), while the cyclin A2 is present in all somatic proliferating cells (Murphy, M. et al., 1997).

The kinase activity of the cyclin A/CDK2 complex is exerted on substrates triggering the DNA replication (Coverely, D. et al., 2002) and on molecules which coordinate the end of S phase with the activation of the mitotic CDK (Mitra, J., and Enders, G.H., 2004).

Cyclin A can also regulate CDK1. The role of cyclin A-CDK1 in mitosis is poorly characterized and appears to be involved in the proper assembly of the complexes recognizing the origins of replication on chromatin (Li C.J. et al., 2004).

The cyclins B, called "mitotic cyclins" (Fung, T.K., and Poon, R.Y.J., 2005), bind CDK1 forming a complex called "M-phase promoting factor" (MPF). MPF phosphorylates and regulates fundamental substrates for the entry into mitosis (Fung, T.K., and Poon, R.Y.J., 2005).

Cyclins B begin to be expressed during S phase, reaching a peak in the G_2/M transition. Their degradation, just before the anaphase

(Fung, T.K. and Poon, R.Y.J., 2005), is required for the completion and exit from mitosis (Yam, C.H. et al., 2002).
There are known three types of cyclins B: B1, B2 and B3. Cyclins B1 and B2 are expressed in most proliferating cells and have a different cellular location: cyclin B1 colocalizes with microtubules, cyclin B2 is associated to the Golgi apparatus. The expression of cyclin B3, instead, is limited to the germline (Jackman, M. et al., 1995).

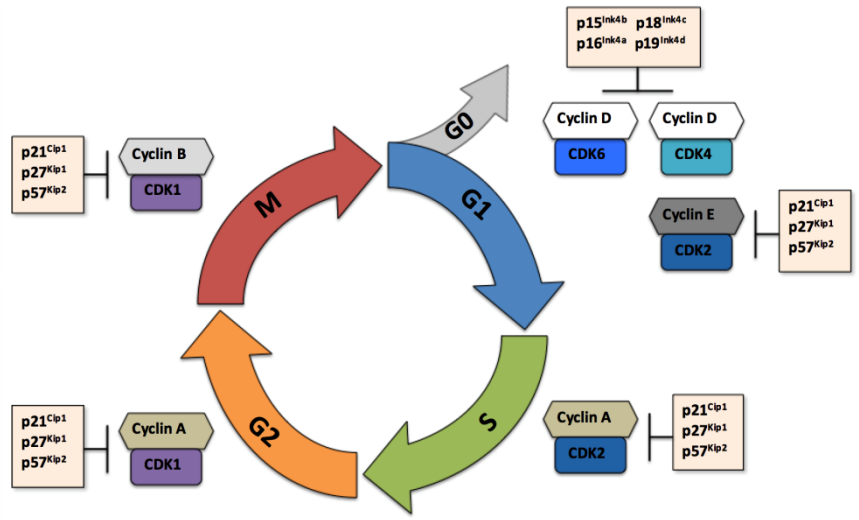

Figure 3.1 Exemplification of cell cycle and its regulators.

3.1.2. INHIBITORS OF CYCLIN-DEPENDENT KINASES

The kinase activity of the cyclin/CDK complex is regulated by inhibitory proteins called "Cyclin-dependent kinase inhibitors" (CKI). CKI inhibit CDK binding them directly or blocking the access of the substrate (or the ATP) to the kinase (Pavletich, N.P., 1999). In relation to their molecular structure and binding specificity, these inhibitors are divided into two families: the INK4 (from "Inhibitor of CDK4") and the Cip/Kip (from "CDK interacting protein/kinase inhibitory protein"), (Sherr, C.J. et al.,

1999). Proteins belonging to the family INK4 are: p15 (Hannon, G.J., and Beach, D., 1994), p16 (Serrano, M. et al., 1993), p18 (Guan, K.L. et al., 1994; Hirai, H. et al., 1995) and p19 (Cham, F.K.M. et al., 1995; Hirai, H. et al., 1995). Such inhibitors have multiple repetitions of ankyrin and bind only CDK4 and CDK6 preventing them to assembly with cyclins D (Sherr, C.J., and Roberts, J.M., 1999) and blocking the progression of the G_1 phase. The inhibitors family Cip/Kip include p21 (Gu, Y. et al., 1993; Harper, J.W. et al., 1993; El-Deiry, W.S. et al., 1993; Xiong, Y. et al., 1993; Dulic, V. et al., 1994; Noda, A. et al., 1994), p27 (Polyak, K. et al., 1994a, b; Toyoshima, H., and Hunter, T., 1994) and p57 (Lee, M.H. et al., 1995; Matsuoka, S. et al., 1995). These proteins can bind indifferently to CDK or cyclins, but with a greater affinity for the cyclin/CDK complex (Harper, J.W., 1997). The Cip/Kip inhibitors have no particular specificity for one cyclin/kinase complex and for this reason they play an important role in the regulation of all the phases of the cell cycle. It is described that these inhibitors are also able to facilitate the formation and the consequent activation of the same cyclin/CDK complex (Labear, J. et al., 1997); the balance between the activator and inhibitor activity is still a subject of debate. The most popular theory asserts that the different roles can depend on the concentration of these molecules: low levels seem to stimulate, while high levels appear to inhibit the activity of cyclin-CDK complexes. It, also, seems that different sites of binding (on cyclin or on kinase) may be involved in different functions (Nabel, E.G., 2002). In any case, INK4 and Cip/Kip implement a negative control of cell proliferation and deregulative events against these molecules play an important role in carcinogenesis (Harper, J.W., and Elledge, S.J., 1996).

3.1.3. CHECKPOINTS

In order to ensure a faithful transmission of the genome to subsequent generations, preventing errors that can generate genomic abnormalities, the cell have developed several "checkpoints' (control points), which guarantee the successful completion of the current cell cycle phase before going through the next. These checkpoints can be activated as a result of DNA damage and are able to temporarily arrest the cell cycle allowing repair complexes to correct errors. If the cell fails removing the errors, a programmed cell death (apoptosis) occurs. The escape from apoptosis causes cell survival and, thus, the establishment of the damage and genomic instability, a key step for the neoplastic transformation (Murray, A.W., 1992). Four main control points are distinguished. The first checkpoint, which controls the passage through the G_1 phase, is called Restriction Point (R-point) and, as mentioned above, responds to growth factors and to the subsequent hyperphosphorylation of pRb by cyclin D/CDK4-6 complexes. R-point is the crucial control point which determines whether or not a cell can enter the cell cycle (Foster, D.A. et al., 2010). The checkpoint regulating the beginning of S phase (G_1/S transition) is important for the control of DNA integrity before its replication, while the checkpoint that controls the entry into M phase (G_2/M transition) bypasses the start of mitosis when a damage is detected or when the DNA replication is incorrect (Agarwal, M.L., et al., 1995). Finally, the checkpoint of the M phase (metaphase/cytokinesis) monitors the correct segregation of chromosomes, examining the right interaction between the mitotic spindle and chromosomes and their proper alignment along the metaphase plate (Nigg, E.A., 2001). The response to DNA damage is implemented by complex mechanisms that include sensors, transducers and effectors (Zhou, B.B., and Elledge, S.J., 2000). The sensors are proteins that perceive the damage and report it to the transducers, which amplify the signal and recruit effectors that block the cell cycle, and when it is possible, repair the damage. A class of sensors is composed by molecules belonging to

phosphatidylinositol 3-kinase-related kinase protein family (PI3K): Ataxia Telangiectasia Mutated (ATM) and ATM and RAD3-related (ATR) (Abraham, R.T., 2001). ATM responds mainly to double-strand breaks (DSB), often caused by ionizing radiation, while ATR responds to single-strand DNA caused by stalled replication forks (Shiloh, Y., 2003). Downstream of ATR and ATM, there are the Chk1 and Chk2 kinases ("Checkpoint kinase 1 and 2"), which modulate the activity of several cell cycle regulatory proteins, such as Cdc25 (phosphatase that activates cyclin-CDK complexes), p53 (a key protein which regulates apoptosis and repair), H2AX (whose phosphorylation is important for the correct removal of damages). The immediate consequence of checkpoint activation is therefore the cell cycle arrest in G_1, S or G_2/M, depending on when the damage occurred, allowing the cell to repair the error or to activate apoptotic mechanisms. (Lukas, J. et al., 2004).

3.2. NO PROLIFERATIVE STATES

Most of the cells that constitute an adult organism do not proliferate. They exit from the cell cycle entering in G_0 phase. Quiescent cells are able to revert this state, while terminally differentiated or senescent cells remain permanently in a no proliferative state.

3.2.1. QUIESCENCE

The quiescence is a temporary and reversible absence of proliferation. This cellular stage can occur in deprivation of growth factors, or when a cell looses the anchoring systems to the extracellular matrix or when it is inhibited by the contact with other cells. These situations arrest the proliferation in a different way, regulating a specific set of genes at trascriptional level. The modification of gene expression must constantly be maintained to ensure the permanent exit from the cell cycle (Coller, H.A. et al., 2006). For example, the absence of growth factors leads to a rapid

reduction of cyclins D expression (Guo, Y. et al., 2005). The arrest provoked by contact inhibition is determined by an increase in the levels of p27 without reduction of cyclin D (Polyak, K. et al., 1994; Hengst, L., and Reed, S.I., 1996). To restore proliferation it is sufficient removing the cause that determines the permanence in G_0 (Ekholm, S.V., and Reed, SI, 2000; Sherr, C.J., and Roberts, J.M., 2004). The quiescent fibroblasts, for example, have low levels of cyclins and CDK. After stimulation with growth factors, these levels return readily at the original concentration promoting fibroblasts to reenter the cell cycle (Kerkhoff, E., and Rapp, U.R., 1997; Ekholm, S.V., and Reed, S.I., 2000). It is known that the activity of cyclins and CKI in G_1 phase is to modulate pRb. Interestingly, pRb is not required for the induction of quiescence (Sage, J. et al., 2000), but its ablation reactivates quiescent and senescent cells (Sage, J. et al., 2003) demonstrating that the mechanism stimulating quiescence is partially different from that which maintains it.

3.2.2. REPLICATIVE SENESCENCE

In culture, fibroblasts can reach a maximum of 50 cell divisions before becoming senescent. This phenomenon is known as "replicative senescence", or the Hayflick limit (Hayflick, L., and Moorhead, PS, 1961). Replicative senescence is, thus, a state in which cellular proliferation is permanently arrested. It appears to be a fundamental feature of somatic cells, with the exception of most tumor cells and certain stem cells. Senescence (or cellular aging) occurs in response to different conditions, including the shortening of telomeres (terminal regions of chromosomes consisting of highly repeated DNA), DNA damage and oncogenes activation (Herbig, U. et al., 2004; Serrano, M. et al., 1997; Bartkova, J. et al., 2006). Unlike quiescence, in cellular aging the reduction of protein levels of cyclin D1 and cyclin E does not occur. These cyclins are able to bind their CDK, but the association generates an enzymatically inactive complex (Dulic, V. et al., 1993). It has been described that removing the tumor suppressor

p53 (Bischoff, F.Z. et al., 1990) or pocket proteins (pRb, p107, p130) (Shay, J.W. et al., 1991; Sage, J. et al., 2003) can prevent replicative senescence. p21 and p16 protein levels increase with the onset of senescence (Tahara, H. et al., 1995; Alcorta, D. et al., 1996). Furthermore, lowering the levels of these inhibitors can slow down the cellular aging before its onset or facilitate the process of immortalization (Brown, J.P. et al., 1997). In senescent cells, despite the presence of large amounts of cyclins, CDK are maintained in an inactive state by prevailing levels of CKI. The fundamental importance of CKI for maintaining replicative senescence has been confirmed by the discovery that the suppression of p21 or p16 induces proliferation in senescent cells (Pajalunga, D. et al., 2007a).

3.2.3. TERMINAL DIFFERENTIATION

The terminal differentiation is a post-mitotic state physiologically irreversible. This condition characterizes the cells that are no longer able to proliferate since they carry out highly specific functions, assuming particular morphological characteristics. In extreme cases, such as that of keratinocytes and erythrocytes, the terminally differentiated cells (TD) eliminate their nucleus, losing irreversibly the ability to divide. Other examples of TD cells are neurons, skeletal and heart muscle cells, adipocytes. The inability of TD cells to proliferate is an obstacle to the survival of tissues without a stem cell compartment. These tissues are particularly susceptible to cell loss caused by the onset of disease (Latella, L. et al., 2001). The molecular mechanisms that maintain the post-mitotic state are not fully elucidated. During terminally differentiation, G_1 phase cyclins decrease, while the levels of CKI increase in comparison to that found in proliferating cells (Kranenburg, O. et al., 1995). Thus, the balance is inclined towards the arrest of proliferation, allowing TD cells to withstand different mitogenic stimuli (Tiainen, M. et al., 1996).

3.2.3.1. MUSCLE DIFFERENTIATION

The skeletal muscle cells represent the classic example of terminal differentiation and an excellent experimental model due to their ease of growth in vitro. Different myogenic cell lines (myoblasts) are available and they can terminally differentiate when subjected to suitable conditions of growth. In the presence of a culture medium devoid of mitogenic signals or following contact inhibition, myoblasts exit irreversibly from the cell cycle and start to express a large number of muscle-specific genes becoming myocytes TD (Okazaki, K., and Holtzer, H., 1966). This ease of growth has allowed the performance of numerous studies to elucidate the mechanisms responsible for the induction and maintenance of muscle differentiation. The genes expressed during terminal differentiation include transcription factors of MyoD family (as Myf-5, myogenin, MRF-4) and numerous structural genes, as the myosin heavy chain and the muscle creatine kinase (Weintraub, H. et al., 1991).
MyoD is expressed in proliferating myoblasts, but it is inactivated by the presence of growth factors in the culture medium. Following deprivation of mitogenic factors, MyoD exerts its transcriptional activity on the promoters of specific muscle target, in particular p21 (Halevy, O. et al., 1995) and pRb (Martelli, F. et al., 1994). In skeletal muscle cells it has been shown that pRb plays a key role in the induction of the post-mitotic state, but not in its maintenance: the removal of the protein by terminal differentiated myotubes does not suppress the post mitotic status of these TD cells (Camarda, G. et al., 2004). This observation leads to distinguish permanently TD cells from those quiescent and senescent, where the maintenance of mitotic arrest depends on pRb. As opposed, the presence of p21 is not essential for the induction of muscle differentiation (Figliola, R., and Maione, R., 2004), but its activity is essential for myogenic maintenance (Pajalunga et al., 2007). The action of MyoD leads to the exit from the cell cycle. The cells become myocytes and fuse generating multinucleated cells called myotubes (Mt) (Okazaki, K., and Holtzer, H., 1966). During the

differentiation of skeletal muscle, the levels of cyclins D1, A, B are early lowered (Ohkubo, Y. et al., 1994; Tiainen, M. et al., 1996b)) while rapidly the levels of some CKI rise. It has been seen that there is a close correlation between the increase of many CKI and terminal differentiation of myotubes (Zabludoff, S.D. et al., 1998). Today it is clear that the cell cycle inhibitors have a role to maintain the proliferative block in skeletal muscle cells TD (Pajalunga, D. et al., 2007a). Several studies have found high levels of one or more CKI also in other terminally differentiated cell types, for example in granulocytes (Steinman, R.A. et al., 1994; Jiang, H. et al., 1994), in keratinocytes (Missero, C. et al., 1995), in oligodendrocytes (Durand, B. et al., 1997), in neurons and cardiomyocytes (Gill, R.M. et al., 1998; Zindy, F. et al., 1999), in adipocytes (Morrison, R.F., and Farmer, SR, 1999) and in plasma cells (Tourigny, M.R. et al., 2002). Despite the essential role of CKI in maintaining postmitotic state was assumed, the evidence in support of this thesis has been only correlative for a long time (Halevy, O. et al., 1995; Parker, S.B. et al., 1995).

3.2.4. CELL CYCLE REACTIVATION OF TERMINALLY DIFFERENTIATED CELLS

The terminally differentiated cells are totally unable to divide. However, this does not imply that they are refractory to proliferative stimuli. It was shown that myotubes stimulated with serum can exit from G_0 phase (Tiainen, M. et al., 1999), since they are able to increase early G_1 genes (c-myc, c-fos, c-jun, Id-1) expression, and then cyclin D1 levels. While quiescent myoblasts stimulated with serum proceed along the cell cycle expressing the late G_1 genes (cyclin E, PCNA, B-myb and cyclin A), myotubes are blocked in mid G_1 phase failing to raise protein levels of the late positive regulators (Tiainen, M. et al., 1996). Myotubes can reenter the cell cycle following the expression of retroviral oncogenes, which are not able to induce the S phase yet (Tiainen, M. et al., 1996). The re-entry into S phase does not take place even after the expression of high levels of E2F protein or cyclin

E/CDK2 complexes (key regulators for the G_1/S transition) (Pajalunga, D. et al., 1999).
It has been shown that TD cells, although physiologically refractory to proliferation, can be "forced" to synthesize DNA following the expression of DNA tumoral viruses oncogenes, such as SV40 Large T (Endo, T., and Nadal Ginard, B., 1989) or Adenovirus E1A (Crescenzi, M. et al., 1995). The oncogene E1A overcomes the block in G_1 phase, acting directly in the G_1/S transition. In myotubes infected by adenovirus, there is an increase in the levels of the late G_1 genes (cyclin E, PCNA, B-myb, cyclin A), but the early G_1 genes are not upregulated (c-fos, c-myc, Id-1 and cyclin D1). In several attempts to reactivate the cell cycle in TD cells, there was the important discovery that many terminally differentiated cell types (myotubes, neurons and adipocytes) can return to synthesize DNA by overexpressing cyclin D1/CDK4 complexes (Latella, L. et al., 2001). The activity of the cyclin D/CDK4-6 complex after the exogenous expression of both proteins was comparable to the levels found in proliferating myoblasts. This result suggests that the principal reason behind the inability of myotubes to proliferate is due to the tight negative control exercised by CKI over CDK4-6 kinases. Previous results strongly indicate that the high levels of cyclin D1 and CDK4-6 required for efficient induction of S phase are necessary to titrate the excess of CKI, in particular p21, p27 and p57 present in myotubes (Pajalunga et al., 2007). Moreover, the elevated expression of cyclin E/CDK2 complex, whose action follows the activation of cyclin D/ CDK4-6, does not produce the same result (Pajalunga, D. et al., 1999), suggesting that the cell cycle re-entry must involve mainly the activation of CDK4 and CDK6 (Latella, L. et al., 2001).
Recently it has been shown that it is possible to induce the reactivation of the cell cycle and DNA synthesis in postmitotic cells, quiescent, senescent or TD, by the removal of specific CKI, in the absence of growth factors. Consequent to this removal, the kinase activity associated with the cyclin D/CDK4-6 complex is restored. This result leads to reconsider the fundamental role of the

above mentioned kinases in cell differentiation: their activities must be down regulated to reach postmitotic state and, vice versa, they should be restored to overcome the proliferative block. These results indicate also that both the reversible and irreversible proliferative arrest are determined by the execution of an active common molecular program, which requires the constant expression of the cell cycle inhibitors (Pajalunga, D. et al., 2007). Despite the successes achieved in TD cells, their proliferation is currently impossible, regardless of the method used to obtain the reactivation. In particular, myotubes die following cell cycle reactivation by SV40 Large T (Endo, T., and Nadal-Ginard, B., 1989) or E1A (Latella, L. et al., 2001) expression or by CKI inhibition (Pajalunga, D. et al., 2007), while the inner ear cells are vulerable to cell death after the expression of E7 of HPV (Sulg, M. et al., 2009). It was found that muscle TD cells, unlike the quiescent cells, are intrinsically unable to complete chromatin replication (Fig. 3.2)

Reactivated myoutubes suffer DNA damage (Fig. 3.3) closely related to the beginning of S phase. They can die by apoptosis or be stopped in the G_2 phase (Pajalunga, D. et al., 2010).

Examples of proliferative arrest in G_2 include myotubes reactivated by cyclin D1/CDK4 expression and cardiomyocytes reactivated through E1A, E2F1 (Kirshenbaum, L.A. et al., 1996) or the intracellular domain of Notch2 expression (Campa, V.M. et al., 2008). Sometimes reactivated muscle TD cells (by CKI depletion o E1A expression) can enter in M phase, but unfortunately they have catastrophic mitosis (Fig.3.4).

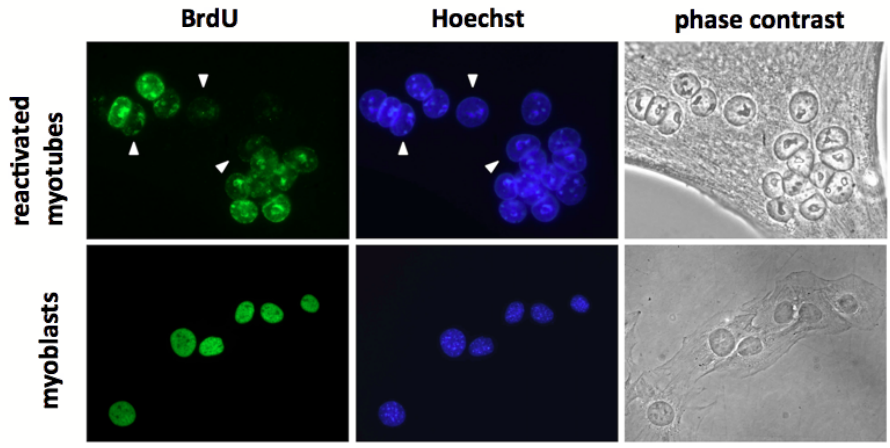

Figure 3.2 Immunofluorescence for BrdU. Reactivated myotubes by siRNA against p21 and p27 proteins, show an alterated pattern of BrdU incorporation (green) in comparison to the homogenous pattern of proliferating myoblasts (Pajalunga, D. et al., 2010).

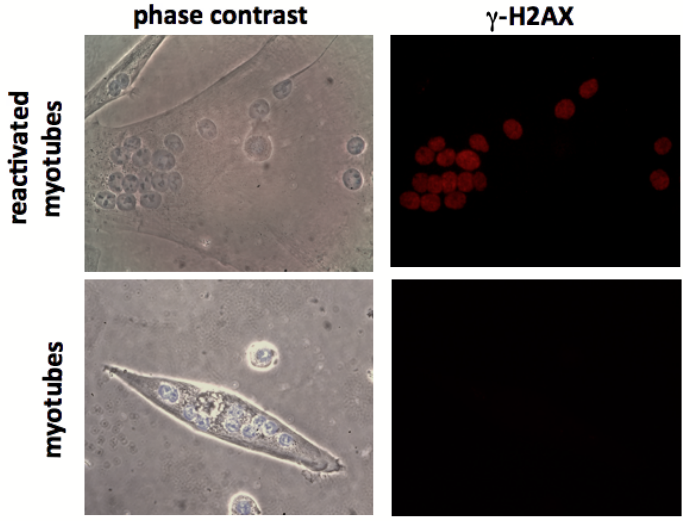

Figure 3.3 Immunofluorescence for γ-H2AX. Reactivated myotubes by siRNA against p21 and p27 proteins, are affected by DNA damage as reflected by the phosphorylation of histone H2AX (red) (S.Z. unpublished data).

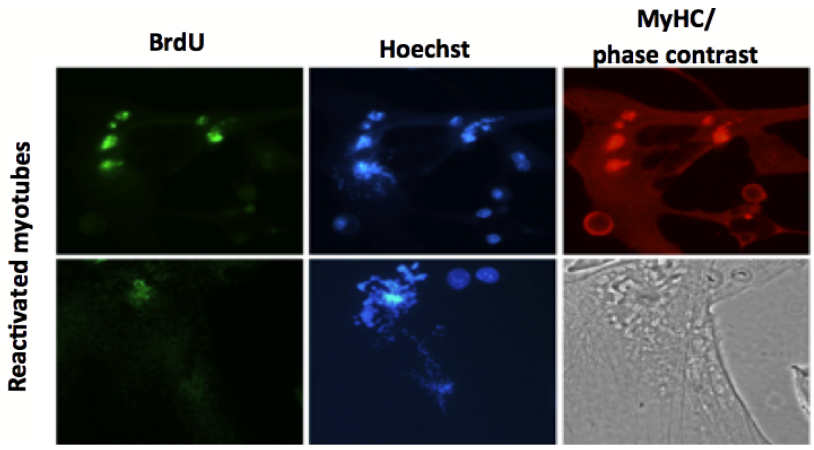

Figure 3.4 Immunofluorescence. Mitotic catastrophes of reactivated myotubes by siRNA against p21 and p27 proteins (Pajalunga, D. et al., 2010).

The reasons behind the persistent inability of TD cells to proliferate are object of study. DNA damage may be the cause or the effect of the partial genome replication. On one side it is possible that, following the replication attempt, the chromosomal damage may cause the activation of S-phase checkpoint and thus the arrest of DNA replication (Godfrey, V., and Prives, C. , 2005). Alternatively, the incomplete replication of DNA could be ascribed to the special characteristics of myotubes (as a particular organization of chromatin). Forcing DNA synthesis through structural obstacles could cause damage.

3.3. THE DEOXYRIBONUCLEOSIDE TRIPHOSPHATES (dNTPs)

To replicate their DNA and repair genomic lesions, mammalian cells require a balanced amount of the DNA precursors, the deoxyribonucleoside triphosphates (dNTPs); the two purine nucleotides: the deoxyadenosine triphosphate (dATP) and the deoxyguanosine triphosphate (dGTP); and the two pyrimidine nucleotides: the deoxycytidine triphosphate (dCTP) and the deoxythymidine triphosphate (dTTP). During S phase, dNTP pool increase from 20 to 30 folds and it is characterized by an elevated

turnover. In the other phases of the cell cycle the amount of dNTPs decrease, but is sufficient to support nuclear DNA repair and the mitochondrial DNA replication (Bogenhagen, D.F. and Clayton, D.A., 1976). The quantitative ratio between the individual nucleotides is fundamental to accurately replicate DNA. Thus, it was demonstrated that an imbalance of dNTP pool provokes a reduced fidelity in the chromatin replication leading to mutations, chromosomal aberrations and mutagenesis (Kunz, B.A. and Köhalmi, S.E., 1991; Kunz, B.A. et al., 1994).

3.3.1. METABOLIC PATHWAYS

dNTPs production occurs through two distinct via (Reichard, P., 1988): the *de novo* synthesis (neo-synthesis) and the salvage pathway (recovery). The *de novo* synthesis occurs cytoplasmically. From glucose and aminoacids dNTPs are generated for the duplication of nuclear and mitochondrial DNA. Many enzymes are involved in the production of the inosine monophosphate (IMP) and the uridine monophosphate (UMP), that are the precursors from which all the ribonucleoside 5'-diphosphates (NDP) derive. The ribonucleotide reductase (RNR) is a key enzyme of the *de novo* synthesis (Reichard, P., 1988; Nordlund, P., 2006). It catalyzes the reduction of NDP to 2'-deoxy-ribonucleosides 5'-diphosphate (dNDP) (Fairman, J.W. et al., 2011) and leads directly to the production of dADP, dGDP, dCDP, dUDP that are further phosphorylated by specific kinases.
RNR is finely regulated at trascriptional and post-trascriptional levels and by different subcellular localizations (Nordlund, P . and Reichard, P., 2006; Kolberg, M. et al., 2004). It exerts the maximum activity during S phase when the demand of dNTPs increases, supporting DNA replication. The active form of RNR is characterized by two copies of R1 subunit (88 kDa) and two copies of R2 (44 kDa) (Jordan, A. and Reichard, P., 1998). The catalytic site is assembled and activated when the two R2 subunits bind R1 molecules (Nordlund, P., and Reichard, P. 2006).

R1 contains two allosteric sites, mediating regulation of substrate specificity and activity. The specificity site regulates the correct balance of four ribonucleotides through their direct binding. The activity site regulates dNTP pool levels according to dATP and ATP concentrations. ATP activates RNR, while dATP inhibits the enzyme (Brown, C.N., and Reichard, P. 1969). R2 is expressed specifically in the S phase and has very short half-life (about 3 hours). In contrast, the levels of the subunit R1 are constant throughout the cell cycle, with a half-life of 20 hours (Eriksson, S. et al., 1984; Mann, G.J. et al., 1988). In cells that do not proliferate R1 can bind another subunit of RNR, the p53R2 (Tanaka, H. et al., 2000; Nakano, K. et al., 2000). This subunit has a high sequence homology with the R2 (80%), depends on p53 activity and its expression is found during all phases of cell cycle. The p53R2 is important for the repair of DNA damage in proliferating cells, and also for mitochondrial DNA replication and nuclear DNA repair in post-mitotic cells.

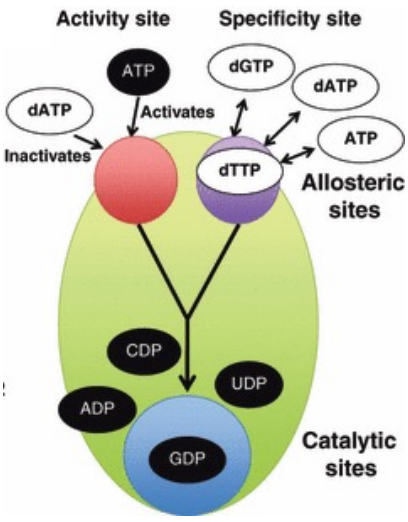

Figure 3.5 Allosteric regulation of RNR. RNR is activated by binding ATP or inactivated by binding dATP to the activity site located on the large subunit Rnr1 (R1). When the enzyme is activated, substrates are reduced if the corresponding effectors bind to the allosteric substrate specificity site as follows. When dATP or ATP is bound at the allosteric site, the enzyme accepts UDP and CDP into the

catalytic site. When dGTP is bound, ADP enters the catalytic site. When dTTP is bound, GDP enters the catalytic site. The substrates (UDP, CDP, ADP, and GDP) are converted to dNTPs. Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/46577223_fig1_Figure-1-Ribonucleotide-reductase-RNR-and-its-allosteric-regulation-A-The-enzyme

Unlike dATP, dGTP and dCTP, the dTTP synthesis follows a different and more complex pathway. It starts from the reduction of UDP and CDP, getting numerous other enzymes involved, including the deoxycytidine deaminase, thymidylate synthase and thymidylate kinase. The resulting dTDP is then further phosphorylated to dTTP from its specific nucleoside diphosphate kinase (Carreras, C.W. and Santi, D.V., 1995).
The salvage pathway is controlled by cytosolic and mitochondrial enzymes. dNTPs can be producted from DNA repair, mRNA degradation (Thelander L. and P. Reichard 1979). Otherwise, they can be derived from diet (Arner, E.S.J. and Eriksson, S. 1995) and hepatic metabolism (Fustin, J.M. et al., 2012). The cell can uptake deoxyribonucleosides (NdR) from the extracellular space through systems of cotransport with Na+ or by the membrane channels whose activity depends on the NdR concentration (Kong, W. et al., 2004). NdR inside the cell are mono-phosphorylated by deoxyribonucleoside kinases (dNK), losing the ability to cross the plasma membrane. In mammalian cells there are four dNK, each one with a specific distinct substrate which ensures the monophosphorylation of all the nucleotide precursors (Arner ESJ and S. Eriksson 1995). Thymidine kinase (TK1) phosphorylates deoxythymidine and deoxyuridine while deoxycytidine kinase (dCK) catalyzes the phosphorylation of deoxycytidine, deoxyadenosine and deoxyguanosine. The thymidine kinase 2 (TK2) differs from TK1 since it phosphorylates besides deoxyuridine and deoxythymidine also deoxycytidine. The deoxyguanosine kinase (dGK), instead, phosphorylates deoxyadenosine and deoxyguanosine (Eriksson, S. et al., 2002). TK1 and dCK have a cytosolic localization, while TK2 and dGK reside in the mitochondria. TK1 is active only during the S phase, while the other dNK are constitutively expressed and suffer slight

activity modulations during the cell cycle (Franzolin, E. et al., 2006; Leanza et al., 2008). The Timidine kinase 1 is regulated at transcriptional (Kim, Y.K. and Lee, A.S., 1991; Stuart, P. et al., 1985), post-trascriptional (Chang, Z. F., 1990; Lieberman H.B., 1988) and translational levels (Chou, W.L. and Chang, Z.F. 2001; Sherley, J.L. and Kelly, T. J., 1988). It is degradated in G2/M transition by APC/C-Cdh complex. It is important to know that in post-mitotic cells the TK1 trascription is inhibited, reducing the activity of the enzyme (Wintersberger, E. et al., 1992) The deoxyribonucleoside monophosphates undergo also into two reversible phosphorylations by nucleoside monophosphate kinase (NMPK) and nucleoside diphosphate kinase (NDPK), which transform deoxyribonucleoside monophosphates to diphosphates and then in triphosphates (Van Rompay, A. R. et al., 2000).
The levels of dNTPs are kept under tight control by an efficient pathway of degradation. Both the absence and the excess of the four different dNTPs can determine mutagenesis and genomic instability (Mathews, C.K., 2006). Therefore, catabolic enzymes must eliminate the excess of deoxyribonucleotides triphosphates. 5'deoxynucleotidases remove the phosphate in 5' position trasforming dNMP in NdR. 5'deoxynucleotidases carry out an important regolative function since they are able to increase cellular metabolic sensibility (Newsholme, E.A. et al., 1984: Gazziola, C. et al., 2001). Other enzymes like phosphorylases, deaminases and hydrolyses are involved in the dNTP metabolism. Recently it was described the role of sterile alpha motif and HD-domain containing protein (SAMHD1). SAMHD1 is a deoxyribonucleside triphospate hydrolyse, which activity is regulated by GTP binding. During G_1 phase, this protein can dephosphorylate dNTPs, removing their excess. SAMHD1 regulation is opposite respect to that of RNR. During S phase, in order to guaratee a right amount of nucleotides SAMHD1 is downregualted while RNR activity is very high. In G_1 phase the levels of SAMHD1 are elevated while that of RNR are very low (Franzolin, E. et al., 2013).

3.4. *Xenopus* Egg Extracts (XEE)

Xenopus is a genus of African frogs native of sub-saharan Africa. The two best-known species of this genus are *Xenopus laevis* and *Xenopus tropicalis*, which are commonly studied as model organisms for neuroscience, developmental biology, toxicology, and cell biology.
Unfertilized eggs from *Xenopus laevis* are used to product cell-free extracts, which are powerful tools to study, among other biological fields, cell cycle progression, chromatin structure and DNA replication. The first extracts were prepared by Lohka and Masui (Lohka, M., and Masui, Y. 1983) and were widely used after by Blow and Laskey, and Hutchison and colleagues, in the mid-80s.
Depending on the scientific question to be investigated, several types of extracts can be obtained. Xenopus unfertilized eggs arrested in metaphase of meiosis II are collected and crushed by sequential centrifugations. Low speed extracts (LSE) are prepared by a medium-speed centrifigation and contain the cytoplasm including light membranes, ribosomes and nuclear envelope. LSE extracts can be interphasic, if unfertilized eggs, before crushing, are activated by calcium ionophore mimicking the fertilization, or mitotic, if the eggs remain unactivated without calcium addition. LSE interphasic extracts are used to study chromatin assembly, pre-replication complex formation and loading on chromatin, origin firing and DNA replication, nuclear assemble. LSE mitotic extracts are employed to analyze chromosome condensation and mitotic spindle formation.
XEE can undergo an additional high speed centrifugation producing high speed extracts (HSE). This extra centrifugation separates the cytoplasm from membranes and ribosomes.
In our laboratory LSE interphasic extracts are used to study DNA replication in terminally differentiated cells. In the absence of an intact cell, these extracts recapitulate the key nuclear transitions of the eukaryotic cell cycle *in vitro* under apparently the same controls that exist *in vivo* (Blow J.J. et al, 1988).

They are able to duplicate exogenous DNA molecules (Tutter A., 2006) since they contain all the replication factors necessary to DNA synthesis. DNA added to the extracts is first assembled into chromatin and then into structures corresponding to interphase nuclei. Once nuclear assembly is complete, the DNA is efficiently replicated in a semi conservative way (fig 3.6)

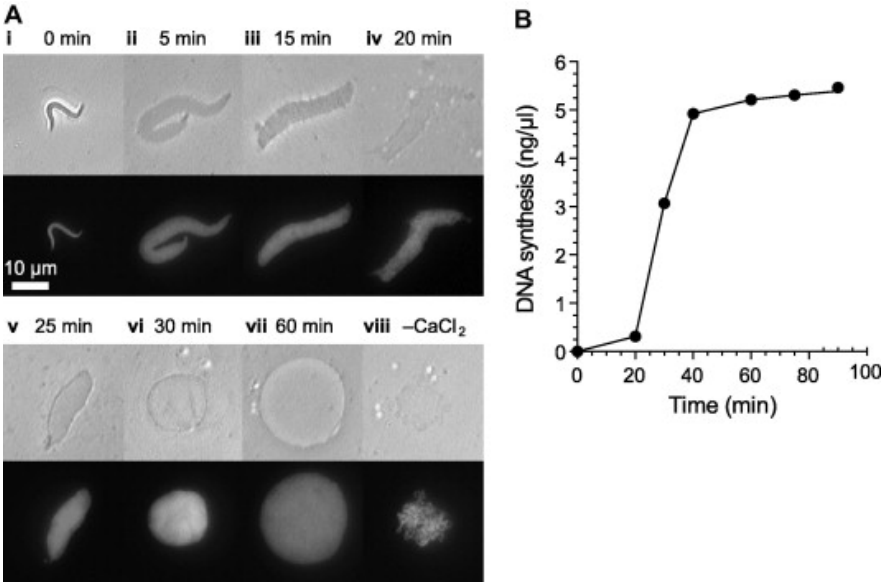

Figure 3.6 Nuclear assembly and DNA replication in *Xenopus* egg extracts. (A) Timecourse of nuclear formation in *Xenopus* egg extracts. Sperm nuclei incubated in metaphase arrested *Xenopus* egg extracts released into interphase by addition of $CaCl_2$. (B) The replication kinetics of sperm nuclei added to interphase egg extracts. Figure from Gillespie, P.J. et al. 2012.

2. AIMS

Today, reactivating the cell cycle of TD cells is achievable. Although the reactivated cells can carry out mitosis and even cytokinesis, S phase represents a major hurdle, since DNA duplication is not completed, resulting in cell death. The ability to induce proliferation into terminally differentiated cells such as neurons, cardiomyocytes, or endocrine cells would have a major impact on the therapy of inherited and acquired diseases and damaged tissues.
Skeletal muscle cells are a prototypic example of terminal differentiation, being extremely resistant to a wide variety of mitogenic stimuli. It is easy to culture them in large numbers. Their differentiation program, extensively studied, can be readily triggered in culture and normally requires cell cycle exit to be executed. For these reasons, they constitute an excellent model system to study terminal differentiation. Indeed, at least in some cases, it has been shown that methods to reactivate the cell cycle in myotubes attain the same result in other TD cells.
Understanding the reasons behind the inability of myotubes to fully replicate their genomes is the objective of this thesis. Theoretically, two classes of problems might impair DNA replication: functional and structural obstacles. With “functional problems”, we indicate biochemical defects in the replication machinery; the expression “structural obstacles” refers to any unusual conformation of the genetic material that might physically prevents DNA replication.
Since both a deficiency and a surplus of dNTPs can cause genomic instability, DNA damage, and mitotic catastrophe (Kunz, B. 1994), we explored the nucleotide pool in reactivated myotubes and its potential impact on DNA replication.
To determine whether a structural obstacle to DNA replication does exist in myotubes, we chose to use cell-free extracts from *Xenopus laevis* unfertilized eggs. These extracts autonomously duplicate any type of genetic material, including purified DNA (Blow J.J et al 1988). We wished to study DNA replication in myotube nuclei incubated in XEE, in comparison with nuclei from

myoblasts. We reasoned that, since XEE provide the entire DNA replication machinery, incomplete DNA synthesis in Mt nuclei would imply the presence of a structural impediment. To begin to dissect the nature of such hypothetical obstacle, we subdivided the nucleus into different levels of complexity: naked DNA, DNA arranged into nucleosomes, and intact chromatin. Once purified, these distinct samples will be incubated in XEE to evaluate their replicability. The level at which the obstacle lays should then become clear.

4. RESULTS

To study, at the functional level, the inability of myotubes to complete replication, we quantified dNTP pool in proliferating murine myoblasts and in myotubes, either resting or reactivated by RNA interference against p21 and p27 CKIs (Pajalunga et al., 2007). We evaluated the percentage of cells double-positive for 5-bromo-2'-deoxyuridine (BrdU) and myosin heavy chain (MHC) in the culture to measure reactivation efficiency (30/40%). rMt were analyzed at 28, 30 and 36 hours after cell cycle reactivation.
Proliferating myoblasts exhibited a typical balance of the four dNTPs, while resting myotubes showed the expected low levels (Frangini et al., 2013) (Fig. 5.1).
In rMt the amounts of deoxytimidine and deoxyguanosine triphosphate (dTTP and dGTP) remained very low, while, in contrast, the levels of deoxycytidine and deoxyadenosine triphosphate (dCTP and dATP) were markedly higher than the levels found in proliferating myoblasts (Fig. 5.1 and Table 1).

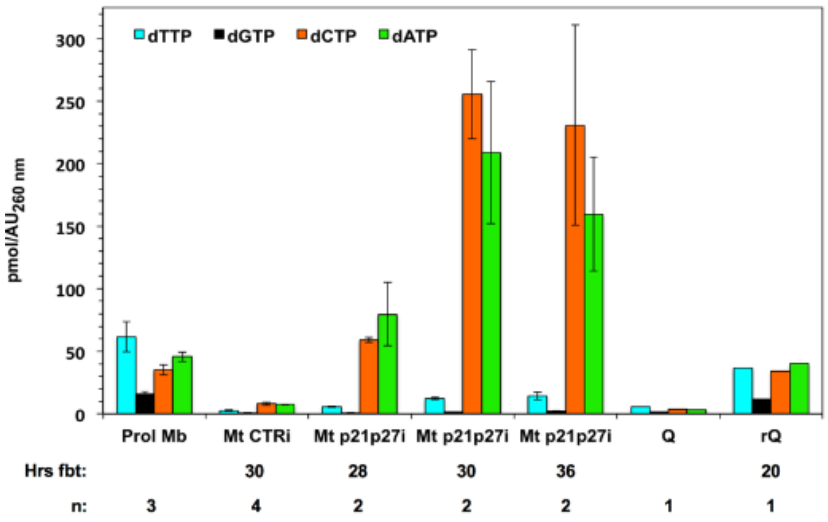

Figure 5.1 Reactivated myotubes display low levels of dTTP and dGTP. Myotubes were transfected with siRNAs to p21 and p27 (Mt p21p27i) or to GFP as a control (Mt CTRi) and harvested at the indicated times from the beginning of treatment

(Hrs fbt). Reference samples include proliferating myoblasts (Prol Mb), myoblasts cultured in suspension to attain quiescence (Q), and formerly quiescent myoblasts reactivated by replating (rQ). The dNTP pool was extracted with 60% methanol and individual dNTPs quantitated by a DNA polymerase-based assay. Data are presented as averages ± SEM of the indicated numbers (n) of independent experiments

Table 1. Statistical evaluation of dNTP measurements

		p (Student's t test)			
Samples	**dTTP**	**dGTP**	**dCTP**	**dATP**	**Comparison**
Mt p21p27i	< 0.01	< 0.01	< 0.01	< 0.05	Prol Mb
Mt p21p27i 28h	<0,05	<0,05	<0,05	N.S.	Prol Mb
Mt p21p27i 30h	<0,05	<0,05	<0,05	<0,05	Prol Mb
Mt p21p27i 36h	<0,05	<0,05	<0,05	<0,05	Prol Mb
Mt p21p27i GdR+TdR	< 0.01	< 0.01	N.S.	N.S.	Mt p21p27i
Mt p21p27i GdR	< 0.01	< 0.05	N.S.	N.S.	Mt p21p27i
Mt p21p27i TdR	< 0.05	< 0.05	N.S.	N.S.	Mt p21p27i
Ad-TK 20h	< 0.05	N.S	< 0.05	< 0.05	Prol Mb
Mt p21p27i Ad-Ctr 20h	< 0.05	< 0.05	< 0.05	< 0.05	Prol Mb
Mt p21p27i Ad-TK 20h	< 0.05	< 0.05	N.S.	< 0.05	Prol Mb
Ad-TK 30h	< 0.05	-	< 0.05	< 0.05	Prol Mb
Mt p21p27i Ad-Ctr 30h	< 0.05	< 0.05	< 0.05	< 0.05	Prol Mb
Mt p21p27i Ad-TK 30h	< 0.05	< 0.05	< 0.05	< 0.05	Prol Mb

Prol Mb: proliferating myoblasts; Mt p21p27i: myotubes reactivated by p21 and p27 RNAi; Mt p21p27i GdR, TdR, or GdR+TdR: myotubes reactivated in the

presence of 500 μM deoxyguanosine, 100 μM thymidine, or both; Ad-TK: resting myotubes infected by adenovirus carrying TK of herpes simplex virus; Mt p21p27i Ad-Ctr: myotubes reactivated by p21 and p27 RNAi and infected by control adenovirus; Mt p21p27i Ad-TK 20h: myotubes reactivated by p21 and p27 RNAi and infected by adenovirus carrying TK of herpes simplex virus N.S.: not significant.

To ascertain that quiescent, proliferation-competent cells promptly expand their nucleotide pool upon reentry into S phase, we performed the same analyses in myoblasts rendered quiescent in suspension culture (Milasincic et al., 1996). As expected, in a single experiment, the low amounts of the four dNTPs found in quiescence promptly rose to proliferation levels upon cell cycle reactivation by reestablishment of substrate adhesion (Fig 5.1).
Since rMt cannot completely duplicate DNA, these data prompted us to ask whether restoring proliferation-like levels of dTTP and dGTP would extend DNA replication.
Two general, opposite, and complementary ways to increase dNTP levels are blocking their catabolism or enhancing their biosynthesis.
Initially we took the first approach by knocking down, with a short interfering RNA (siRNA), SAM domain and HD domain-containing protein 1 (SAMHD1), a key nucleotide catabolic enzyme that coordinates the dNTP pool with the cell cycle (Franzolin et al., 2013). Western blotting indicated an efficient knockdown (Fig. 5.2).
A preliminary experiment was performed to analyze dNTP pool in rMt interfered for SAMHD1. We observed an increase in the levels of dCTP at 30 and 36 hours after cell cycle reactivation. However, the dTTP and dGTP pools were not affected by this manipulation, suggesting that their shortage may be determined by deficient synthesis, rather than high catabolism (Fig. 5.3). Hence, this approach was not further pursued.

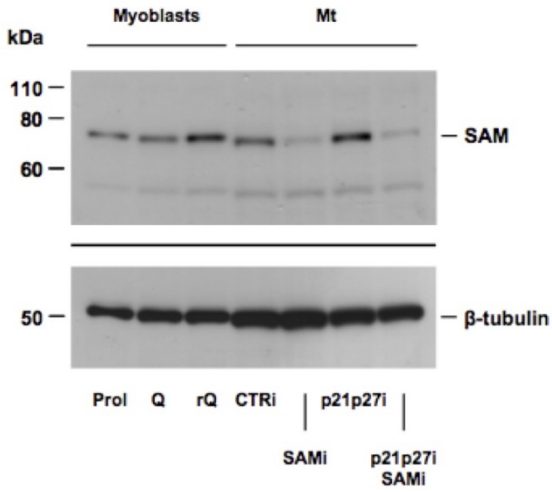

Figure 5.2 Down regulation of SAMHD1. Western blot showing SAMHD1 (SAM) in myoblasts or myotubes under the indicated conditions. Myoblasts have been included as a reference for SAMHD1 levels in proliferating, quiescent, or reactivated conditions.

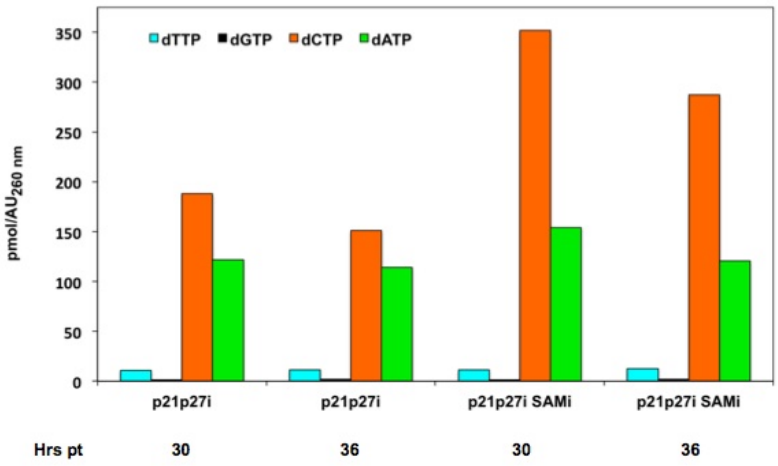

Figure 5.3 Suppression of SAMHD1 does not increase dGTP or dTTP levels. dNTP levels in p21p27i: myotubes reactivated by p21 and p27 RNAi analyzed at 30 and 36 hrs post transfection; p21p27 SAMi: myotubes reactivated by p21 and p27 RNAi and treated with RNAi against SAMHD1, analyzed at 30 and 36 hrs post transfection.

Thus, we adopted an alternative approach to remodel the dNTP pool. We supplemented the medium of rMt with 500 μM deoxyguanosine (GdR) and/or 100 μM deoxythymidine (TdR). The concentrations were chosen after pilot experiments to obtain dTTP and dGTP levels roughly similar to those found in proliferation. The administration time was critical. We observed that early treatment with nucleosides inhibited S phase entry in rMt and found that the optimal time for addition was 17 hours after CKI siRNA transfection, closer to the beginning of S phase (ranging from 20 to 28 hrs post-transfection). Figure 5.4 shows that exogenous addition of GdR+TdR increased both dGTP and dTTP. Moreover, we obtained that TdR alone was able to increase both deficient nucleotides. In contrast, the sole addition of GdR exclusively raised dGTP levels (Table 1).

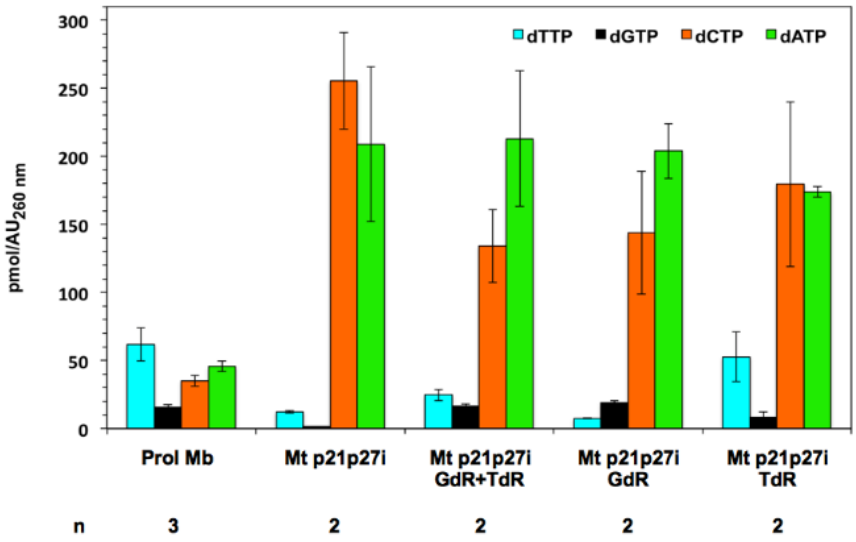

Figure 5.4 Supplementation of GdR or TdR. Myotubes were transfected with p21 and p27 siRNAs as in Figure 5.1. GdR (500 μM) and/or TdR (100 μM) were added to the culture medium 17 hrs later. The dNTP pool was quantitated as in Fig. 5.1 30 hrs fbt. Values for proliferating myoblasts and myotubes reactivated by p21 and p27 RNAi are the same shown in Fig. 5.1 and are reproduced here for ease of reference.

To explore the connection between DNA replication and dNTP pool in rMt, we performed *in situ* microfluorimetric measurements of DNA content in individual nuclei, as reflected by 4',6-diamidino-2-phenylindole dilactate (DAPI) fluorescence intensity. This analysis was made to quantify the extent of DNA replication in all of the above conditions.
rMt with GdR+TdR increased significantly DNA synthesis (DNA content 52% above controls) (Fig. 5.5 and Table 2)

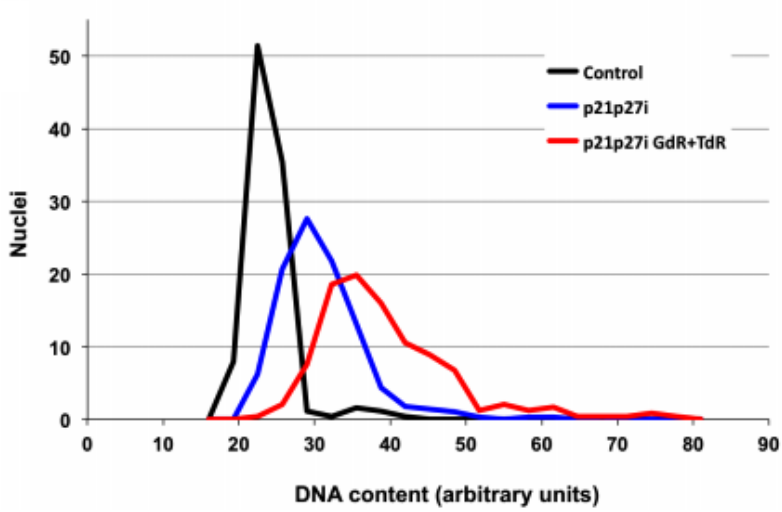

Figure 5.5 Higher dTTP and dGTP levels allow extended DNA replication in reactivated myotubes. Myotubes were transfected as in Figure 5.4; at the end of S phase, 30 hrs later, the cells were immunostained for MCM7, a marker of cell cycle reactivation, and counterstained with DAPI. Nuclear DNA content was evaluated by microfluorimetric measurement of DAPI fluorescence in individual, MCM7$^+$ myotube nuclei. On average, 209 nuclei per sample were evaluated (range 112-285) and their DNA-content distribution is shown by the corresponding curve. Control TD myotube nuclei were uniformly MCM7$^-$ and their DNA content was determined without selection.

To investigate the distinct contribution of each of the two deoxynucleosides, we treated rMt with either GdR or TdR. Administration of GdR, in agreement with its incapacity to increase dTTP levels, was not able to increase rMt DNA synthesis

in a significant way. In contrast, treatment with TdR alone, which raised both dTTP and dGTP levels, increased rMt DNA content to 56% above control myotubes, mimicking the combined action of GdR+TdR (Fig. 5.6 and Table 2). These data show how the DNA replication in rMt is influenced by the intracellular levels of TdR. Indeed, administration of deoxythymidine alone is sufficient to double the amount of the duplicated DNA.

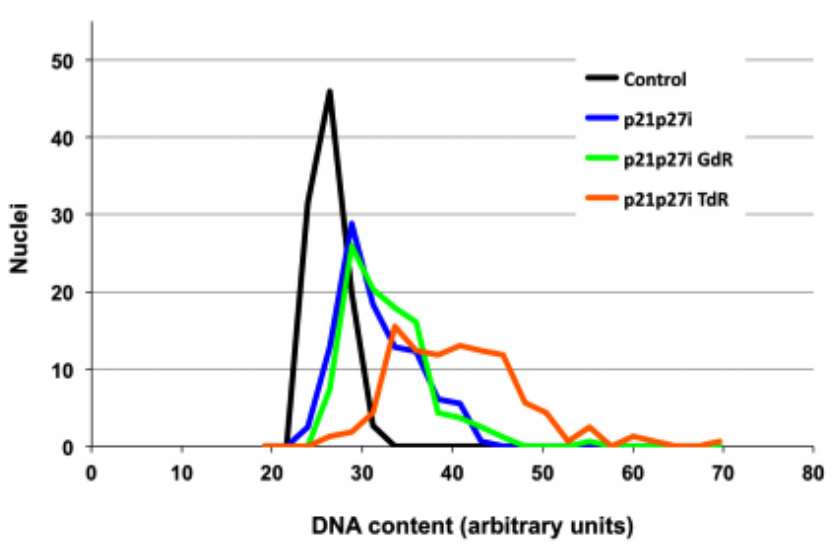

Figure 5.6 TdR alone is sufficient to extend DNA replication. Myotubes were transfected as in Figure 5.4; at the end of S phase, 30 hrs later, the cells were immunostained for MCM7, a marker of cell cycle reactivation, and counterstained with DAPI. Nuclear DNA content was evaluated by microfluorimetric measurement of DAPI fluorescence in individual, $MCM7^+$ myotube nuclei. On average, 209 nuclei per sample were evaluated (range 112-285) and their DNA-content distribution is shown by the corresponding curve. Control TD myotube nuclei were uniformly $MCM7^-$ and their DNA content was determined without selection.

Table 2. DNA content in myotube nuclei and statistical analyses

Samples	DNA content (% increase above 2n)	S.D.	n	p (Student's t test)	Comparison
CTRi	0		4		
CTRi GdR	3	0.7	2	N.S.	CTRi
CTRi TdR	1	2.1	2	N.S.	CTRi
p21p27i	27	2.5	4	<0.05	CTRi
p21p27i GdR+TdR	52	11.0	20	<0.05	CTRi
p21p27i GdR	29	3.4	2	<0.05	CTRi
p21p27i TdR	56	6.5	2	<0.05	CTRi

CTRi, CTRi GdR, CTRi TdR Myotubes transfected with siRNAs to GFP as a control and where mentioned, with 500 μM deoxyguanosine or 100 μM thymidine; Mt p21p27i: myotubes reactivated by p21 and p27 RNAi; Mt p21p27i GdR, TdR, or GdR+TdR: myotubes reactivated in the presence of 500 μM deoxyguanosine, 100 μM thymidine, or both; Data are presented as averages ± SEM of 2-4 experiments. N.S. non significant, S.D. standard deviation.

Since reactivation of cell cycle in terminally differentiated cells induce also DNA damage (Pajalunga et al., 2010), we investigated whether administration of exogenous deoxynucleosides could able to reduce genomic lesions. To this aim, we performed microfluorimetry for γ-H2AX, a marker of double-strand DNA breaks, on rMt with or without addition of GdR+TdR. Deoxynucleoside supplementation caused a significant reduction in γ-H2AX staining (Fig. 5.7 and Fig. 5.8). This result indicates that at least part of the damage incurred by myotubes in the course of cell cycle reactivation is due to insufficient availability of deoxynucleotides.

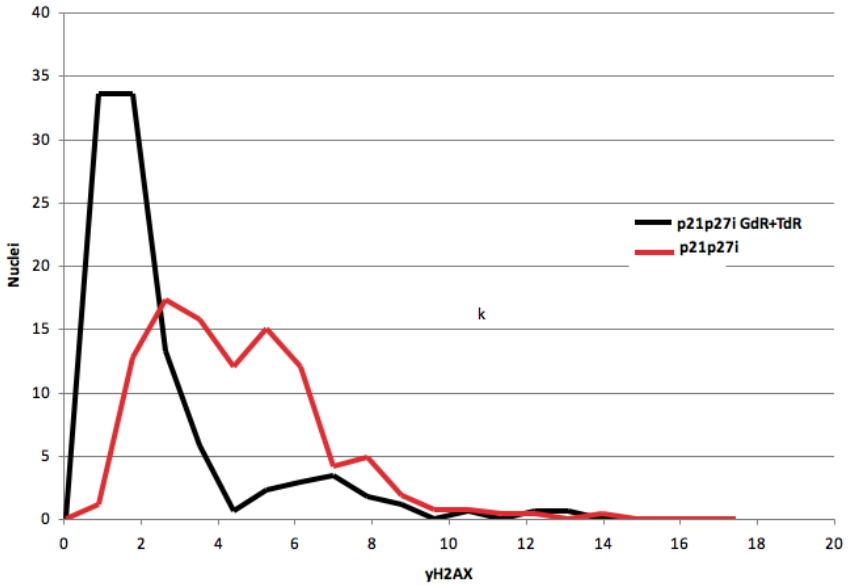

Figura 5.7 Myotubes, treated as indicated, were subjected 30 hrs fbt to immunofluorescence for Ki67, a marker of cell cycle reactivation, and γ-H2AX staining. Intensities of individual Ki67$^+$ nuclei were measured by microfluorimetry. Control myotubes displayed no detectable γ-H2AX fluorescence and are not reported in the graph. It is shown an average of 2 independent experiments S.D. 4,70. Myotubes p21p27i GdR+TdR have the 40% of DNA damage reduction.

To understand the reason of the relative lack of some nucleotides in rMt, we analyzed by quantitative, real-time PCR (qPCR) the expression pattern of a number of enzymes involved in their biosynthesis. Figure 5.9 shows that, as expected of non-proliferating cells, resting myotubes replace the ribonucleotide reductase R2 subunit with p53R2 (Hakansson et al., 2006). At least three enzymes involved in deoxythimidine synthesis (dCMP deaminase, DCTD; thymidilate synthase, TS; thymidine kinase 1, TK1) were strongly dowregulated in these myotubes (Table 3). Their expression barely rose upon cell cycle reentry, suggesting an explanation for the very low levels of dTTP found in rMt.

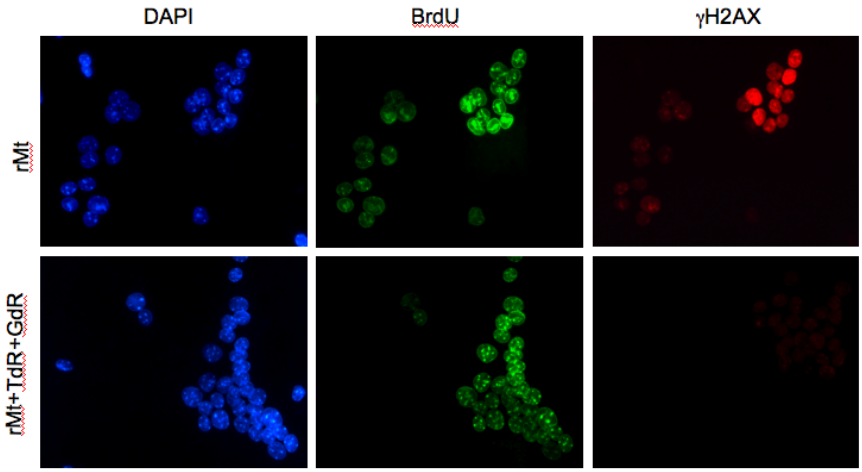

Figure 5.8 Immunofluorescence for γ-H2AX. p21p27i reactivated myotubes were subjected 30 hrs fbt to immunofluorescence for γ-H2AX and BrdU staining with or without TdR+GdR.

As a control, we performed the same analyses in quiescent myoblasts. They displayed a slight downregulation of the thymidine synthesis enzymes and, upon cell cycle reactivation, promptly returned to an expression pattern similar to that of proliferating cells (Fig. 5.10 and Table 3).

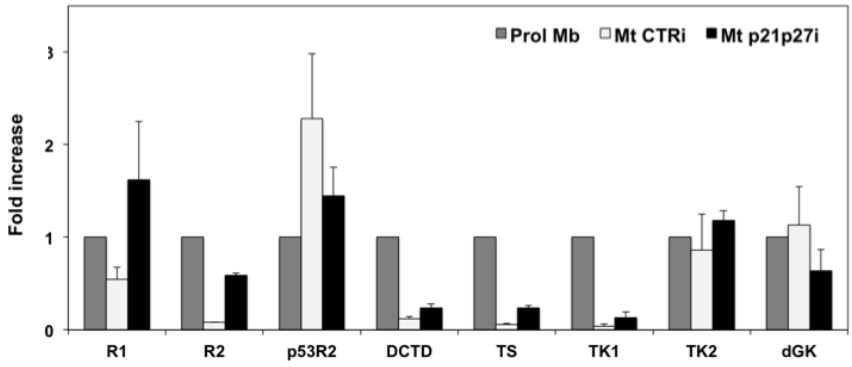

Figura 5.9 Thymidine synthetic enzymes are not upregulated in reactivated myotubes. Myotubes were reactivated by CKI depletion. The mRNA levels of several dNTP synthetic enzymes were quantitated by qPCR in myotubes treated as indicated, proliferating myoblasts, and quiescent myoblasts or myoblasts

reactivated after quiescence. In all cases, reactivated cells were harvested in the mid of S phase. Histograms show fold increases in comparison with proliferating myoblasts, made equal to 1 (averages ± SEM of two independent experiments). R1, R2, and p53R2 indicate the corresponding ribonucleotide reductase subunits; DCTD, deoxycytidine monophosphate deaminase; TS, thymidilate synthase; TK1 and TK2, thymidine kinases 1 and 2; dGK, deoxyguanosine kinase.

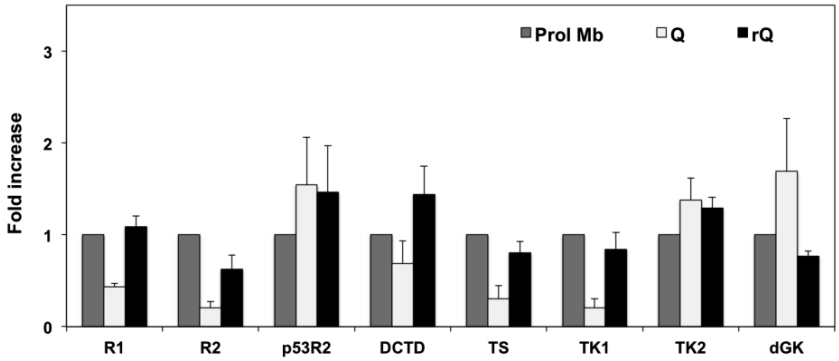

Figura 5.10 Biosynthetic enzymes are upregulated in reactivated quiescent myoblasts. The mRNA levels of several dNTP synthetic enzymes were quantitated by qPCR in proliferating myoblasts, and quiescent myoblasts or myoblasts reactivated after quiescence. Reactivated quiescent cells were harvested in the mid of S phase. Histograms show fold increases in comparison with proliferating myoblasts, made equal to 1 (averages ± SEM of two independent experiments).

Table 3. Statistical evaluation of gene expression in reactivated myotubes or myoblasts, compared to proliferating myoblasts.

p (Student's t test)								
Samples	**R1**	**R2**	**P53R2**	**dCTD**	**TS**	**TK**	**TK2**	**DGK**
Mt p21p27i	N.S.	<0,05	N.S.	<0,05	<0,05	<0,05	N.S.	N.S.
Qr	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	<0.05

Mt p21p27i: myotubes reactivated by p21 and p27 RNAi; N.S.: not significant.

Altogether, these results indicate that low expression of enzymes converging on dTTP biosynthesis are responsible for the limiting amount of this nucleotide in rMt.

We focused our attention on TK1. Indeed this enzyme is encoded by a highly cell cycle-dependent gene, whose transcription is strongly downregulated in non-proliferating cells and sharply increases at the G_1/S boundary, upon cell cycle reentry. We asked if TK1, upon cell cycle reactivation, could execute its function in rMt. We used a kinase assay to measure TK1 activity. We found that in rMt it only moderately increased, compared with resting myotubes, and never reached the TK1 levels found in proliferating myoblasts (Fig. 5.11).

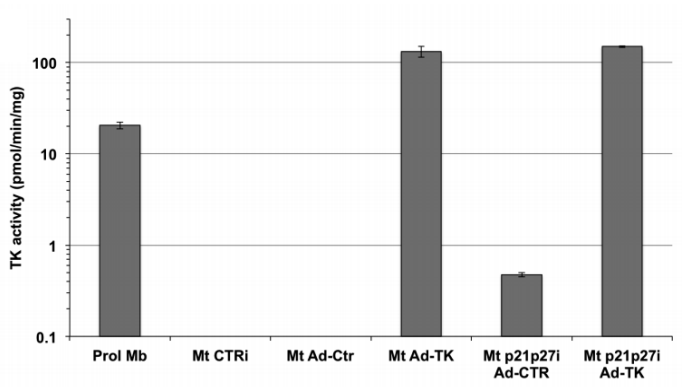

Figure 5.11 TK activity is low in reactivated myotubes. Total TK activity in myoblast or myotubes, treated as indicated and collected 30 hrs fbt. *In vitro* kinase assays were performed on total proteins extracted from the different samples, using 3^{H}TdR as substrate. The reactions were incubated at 37°C and a specific inhibitor of TK2 activity (KIN109) was used, in order to quantify only the amount of 3^{H}TMP produced by TK1. Prol Mb: proliferating myoblasts; Mt p21p27i: myotubes reactivated by p21 and p27 RNAi; Mt p21p27i GdR, TdR, or GdR+TdR: myotubes reactivated in the presence of 500 μM deoxyguanosine, 100 μM thymidine, or both; Ad-TK: resting myotubes infected by adenovirus carrying TK of herpes simplex virus; Mt p21p27i Ad-Ctr: myotubes reactivated by p21 and p27 RNAi ad infected by control Adenovirus; Mt p21p27i Ad-TK: myotubes reactivated by p21 and p27 RNAi and infected by adenovirus carrying TK of herpes simplex virus. The scale is logarithmic to accommodate widely different values. TK activities measured in myotubes are all statistically significant, in comparison with myoblasts ($p < 0.05$ by Student's t test).

Thus, we attempted to overexpress thymidine kinase 1. To this aim, rMt were infected with a recombinant adenovirus carrying herpes simplex thymidine kinase (Ad-TK) or a control virus. Ad-TK muscle cells exhibited extremely high levels of dTTP,

irrespective of cell cycle reentry. Interestingly, they also showed a marked increase in dGTP (Fig 5.12, Table 1). As expected, control-infected rMt displayed limited DNA replication. In contrast, in rMt forcibly expressing TK, DNA synthesis was dramatically extended, though not reaching completion (Fig. 5.13 and Table 4).

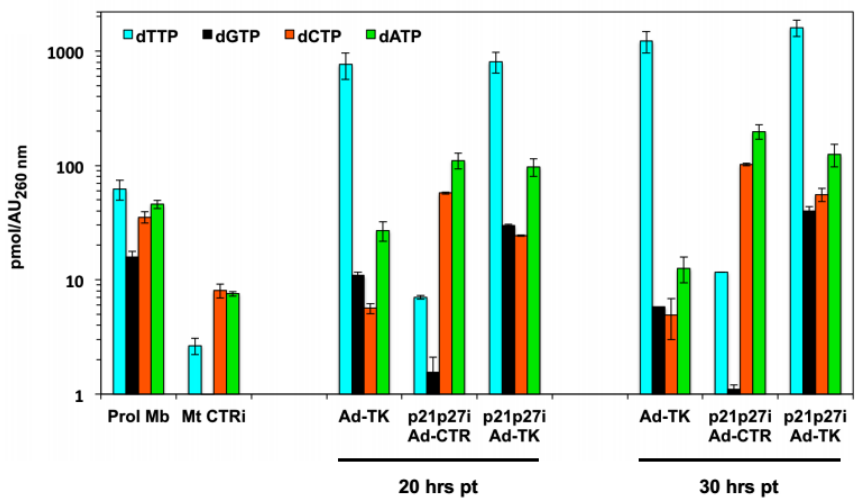

Figure 5.12 TK1 overexpression increase both dTTP and dGTP. dNTP pools measured at the indicated times in myoblasts or myotubes treated as shown.

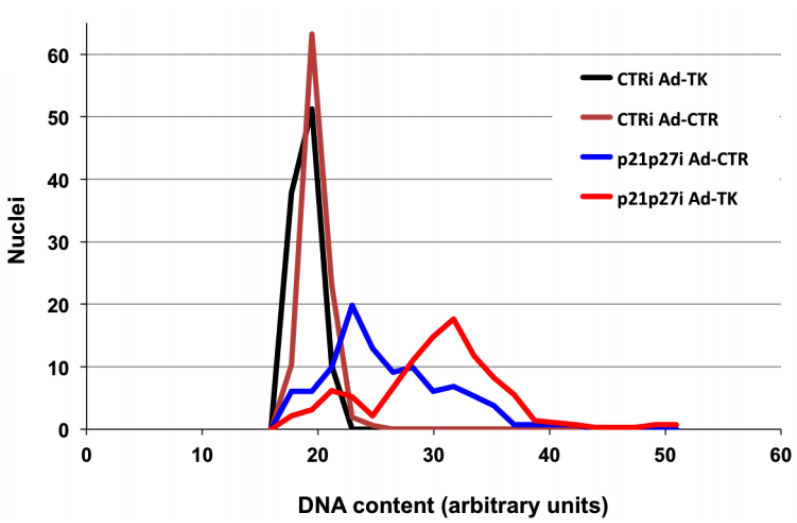

Figure 5.13 Exogenous thymidine kinase 1 expression extends DNA synthesis in reactivated myotubes. Microfluorimetric measurements of DNA contents in the indicated cell samples, harvested 30 hrs fbt. CTRi, interference with control siRNA; Ad-CTR, infection with control adenovirus; Ad-TK, infection with the adenovirus expressing herpes simplex virus TK.

Table 4. DNA content in reactivated myotubes, as pecentage increase above controls and statistical measurements

Samples	DNA content (% increase above 2n)	S.D.	n	p (Kolmogorov-Smirnov Test)	Comparison
CTRi	0	0	2		
CTRi Ad-CTR	2	2.8	2	N.S.	CTRi
CTRi Ad-TK	0	0	2	N.S.	CTRi
p21p27i Ad-CTR	24	9.9	2	< 0.000001	CTRi
p21p27i Ad-TK	56	4.9	2	< 0.000001	CTRi

N.S. not significant

We sought to confirm the role of TK1 in dNTP pool synthesis and DNA replication in rMt. For this purpose, rMt underwent TK1 RNAi. To demonstrate the efficacy of the knockdown, in the absence of suitable antibodies against the murine enzyme, we resorted to a functional assay. Figure 5.14 shows that EdU incorporation into rMt largely depends on TK1 expression, as low as it might be in these cells. Myotubes were similarly reactivated by CKI suppression, independently of TK1 interference, as shown by the expression of the cell cycle-dependent protein MCM7. However, in TK1-knockdown cells, EdU was nearly undetectable. TK1 knockdown markedly reduced DNA replication in myotubes reactivated by CKI RNAi (Fig. 5.15), confirming the crucial role of this enzyme in limiting DNA synthesis. Altogether, these results show that inadequate expression of enzymes involved in dTTP anabolism is responsible for low dNTP levels and confirms that expanding the dNTP pool results in enhanced DNA synthesis.

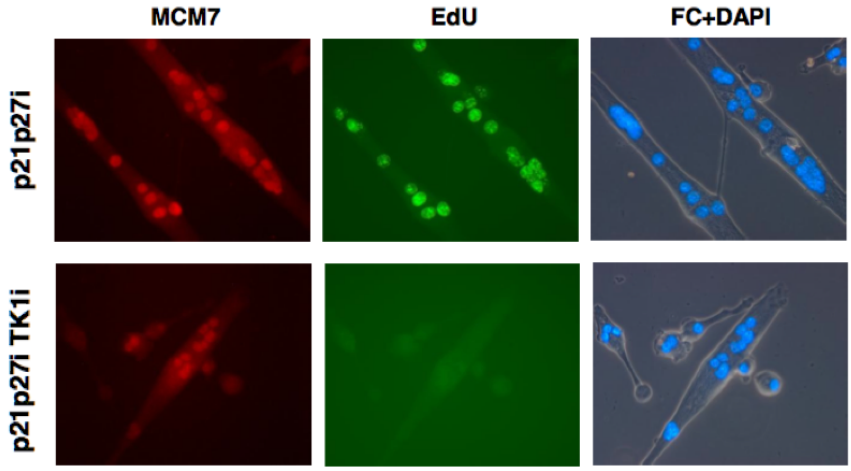

Figure 5.14 TK1 suppression impairs EdU incorporation. Double immunofluorescence for MCM7 and EdU in p21p27-depleted myotubes, additionally subjected to interference with TK1 or control siRNA. Cultures were fixed during S phase and stained as indicated. FC, phase contrast.

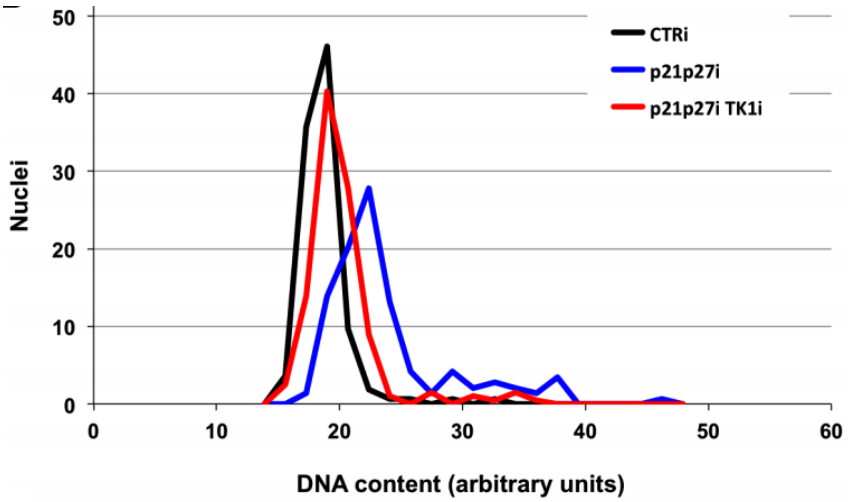

Figure 5.15 Suppression of TK1 impairs DNA replication in reactivated myotubes. Microfluorimetric measurements of DNA contents in the indicated cell samples, assayed 31 hrs fbt.

To investigate whether structural obstacles prevent myotubes from completing DNA duplication, we used *Xenopus* egg extracts (XEE) to drive *in vitro* replication assays. These extracts contain all the factors necessary to replicate DNA in any form (Tutter AV, et al 2006). We reasoned that any biochemical defects underlying incomplete DNA replication in myotubes would be ignored by the self-sufficient XEE replication machinery. In contrast, incomplete DNA replication in these conditions would constitute a strong indication of the presence of structural impediment(s).
Thus, we compared the replication kinetics of myotube and myoblast nuclei in XEE. Nuclei, isolated from proliferating (P) and quiescent (Q) myoblasts or myotubes, were incubated in XEE to quantify the amount of DNA synthesized during a period of 5 hours. Q nuclei represent the most appropriate control, since they come from a synchronous population lying in G_0 phase, like skeletal muscle TD cells. P nuclei were also included as a reference. However, a fraction of them have already gone through S phase *in vivo* and cannot re-replicate their DNA; thus, P nuclei,

on average, cannot attain 100% replication. Myotube nuclei started DNA replication more than 60 minutes later than Q ones. Furthermore, while Q nuclei essentially completed DNA replication within five hours, myotube DNA synthesis remained conspicuously incomplete (Fig. 5.16).

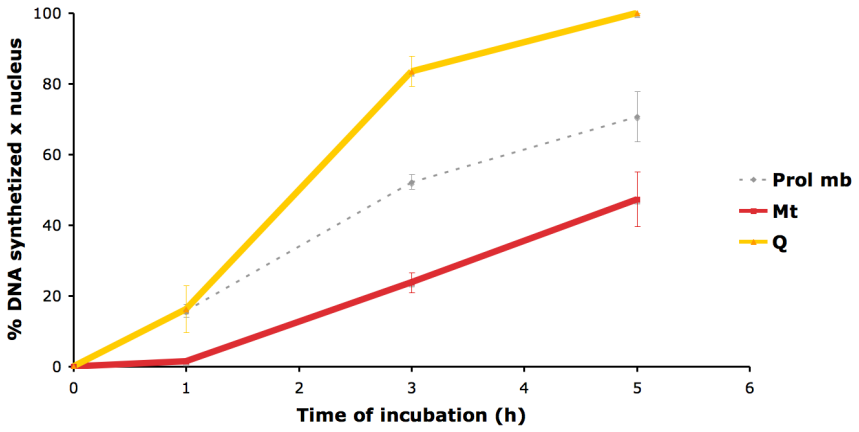

Figura 5.16 Myotube nuclei do not completely replicate their DNA in XEE. Quantification of replicated DNA (per nucleus) in nuclei isolated from myotubes and quiescent or proliferating myoblasts. Nuclei were incubated in XEE in the presence of dTTP^{3H} to follow replication kinetics. Aliquots were collected at the indicated time points. The amount of radioactivity per sample is a measure of the replicated DNA. In parallel, the same analyses were performed with a fluorescent tracer (Cy3-dCTP), to visualize the percentage of nuclei replicating their DNA (typical range, 70-90%). Radioactive measurements were normalized to the number of Cy3-dCTP^{+} nuclei and were plotted as percent of maximal DNA synthesis. Values are shown as means of three independent experiments with SE (error bars). At all time points, the differences between myotube and quiescent cell nuclei were statistically significant ($p < 0.05$ by Student's t test).

These data indicate that the inability of myotubes to complete DNA replication is due, in part, to the presence of structural obstacles.

To begin to identify the molecular nature of such obstacle, we conceptually subdivided the nucleus into different levels of complexity: naked DNA, DNA arranged into nucleosomes, and intact chromatin. To determine at which of these levels of complexity the obstacle lies, we thought to study the replication

kinetics in XEE of different preparations of genetic material from myotubes and myoblasts (Fig 5.17).

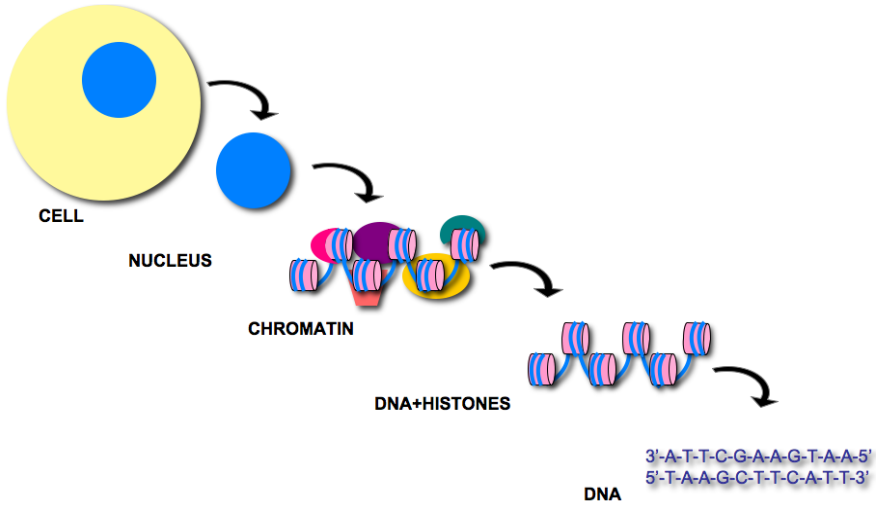

Figure 5.17 Schematic illustration of different levels of complexity taken into account to study the structural obstacle.

By incubating DNA from myoblasts and myotubes in XEE, we observed that the amount of DNA synthesized by the two samples was similar (Fig. 5.18 and Table 6). This means that, at the level of purified DNA, there is no barrier prohibiting full DNA replication. At the next complexity level, nuclei were purified and treated with 1M NaCl. High salt concentrations detach proteins not tightly bound to DNA.

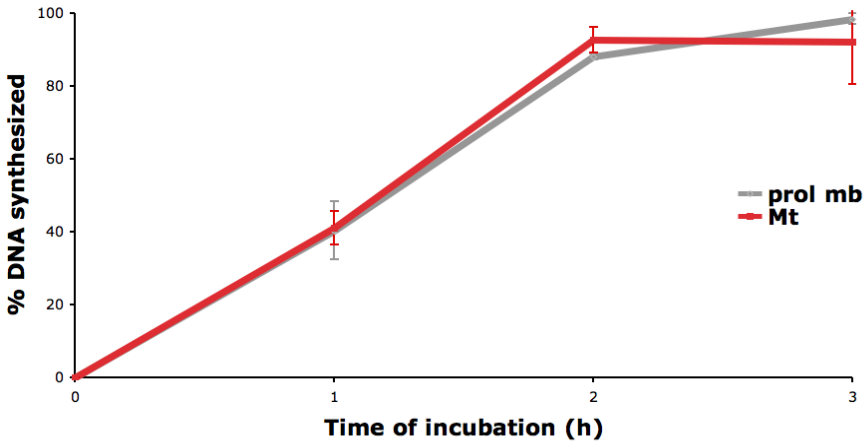

Figure 5.18 Myotube nacked DNA replicate as much as proliferating samples in XEE. Densitometry of replicated DNA from myotubes and proliferating myoblasts. Nacked DNA was incubated in XEE in the presence of biotinylated-dUTP to follow replication kinetics. Aliquots were collected at the indicated time points. Dot blot, incubation with streptavidin-peroxidase and chemiluminescent analyses were performed. Mt: myotubes DNA; Prol mb: myoblasts DNA. Values are shown as means of three independent experiments with ESs (error bars). At all time points, the differences between myotube and proliferating DNA were not statistically significant (p >0.05 by Student's t test).

To probe the results of such selective detachment, we performed an immunofluorescence against H1, the histone most weakly bound to DNA, and against pRB, one of the non-histone proteins most strongly attached to DNA (Fig 5.19). The presence of H1 and the absence of pRb confirmed a successful preparation. This conclusion has anyway to be proved by Western blot analyses.

Preliminary results were obtained by studying the replication kinetics in XEE of salt-treated nuclei, using a fluorescent nucleotide (Cy3-dCTP) as a tracer. The percentages of dye-positive nuclei was calculated.

NaCl-treated nuclei from proliferating cells and myotubes displayed similar percentages of labeled-dCTP incorporation (Fig 5.20).

These results are going to be complemented by quantitative assessments of DNA replication.

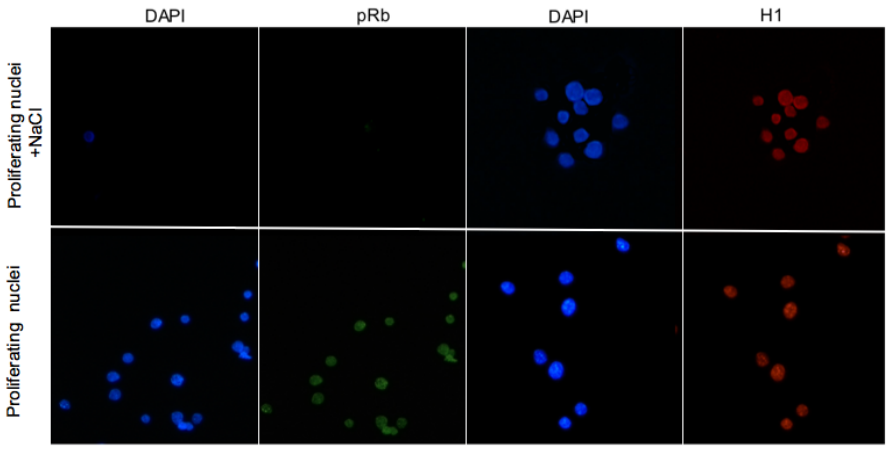

Figure 5.19 High salts treatment detaches pRB protein from DNA maintaining H1 bound to chromatin. Immunofluorescence for pRb (green) and H1 (red) proteins.

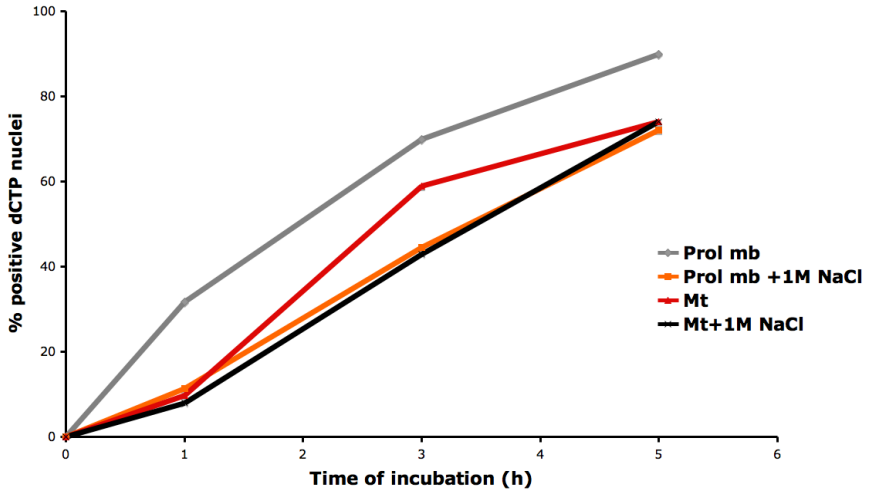

Figure 5.20 Salt treated nuclei from myotubes and proliferating cells show the same replication kinetic in XEE. Nuclei were extracted from the above mentioned cells.

They were treated with 1M NaCl and then incubated in XEE with Cy3-dCTP to follow the reaction. Aliquots were collected at indicated time points. Cy3-dCTP$^+$ nuclei were counted and plotted. Untreated nuclei from myoblasts and myotubes were used as controls. Prol mb: myoblast nuclei; Mt: myotube nuclei; Prol mb+1M NaCl: myoblast nuclei treated with 1M NaCl; Mt+1M Nacl: myotube nuclei treated with 1M NaCl.

5. DISCUSSION

TD cells can be forced to re-enter the cell cycle by several means, but they never attain proliferation, due to incomplete DNA replication associated with DNA damage. We regard the incompetence to fully replicate DNA as the last hurdle to overcome to finally achieve the goal of TD cell proliferation.
The aim of this thesis was to understand the reason behind defective DNA duplication, distinguishing between functional and structural problems. I presented evidences that both classes of causes affect cell cycle reactivation in myotubes.
We found that reactivated myotubes attempt to replicate their DNA with an aberrant dNTP pool (Fig 5.1). dTTP and dGTP amounts turn out to be very low, while dATP and dCTP reach very high levels, in comparison to those found in proliferating myoblasts. In reactivated myotubes, exogenous administration of GdR and TdR determines an increase in the levels of the corresponding dNTPs, resulting in the extension of the DNA replication (Fig. 5.4 and Fig 5.6). Improving DNA synthesis lowers DNA damage (Fig. 5.7), as indicated by a decrease in H2AX phosphorylation.
The contributions of TdR and GdR are not equal, since when cells are incubated with TdR, the concentrations of dGTP and dTTP rise in concert, enhancing DNA replication. In contrast, increasing dGTP alone is ineffective. These results can be explained by the role of dTTP which, through RNR, induces GDP reduction.
The structure of the enzyme network regulating dNTP concentrations is altered. Insufficient expression of genes encoding key dTMP synthetic enzymes, prominent among which TK1, drastically hampers the production of dTTP (Fig. 5.9). Thus, the pathway converting dCMP to dTMP is virtually inactive in myotubes. Such inactivity causes accumulation of dCTP and lack of dTTP, which is not available for its twofold function as DNA precursor and allosteric stimulator of GDP reduction. Even though we did not measure RNR activity, we can suppose that such enzyme is active in reactivated myotubes. Indeed, cell-cycle reactivation restores both the transcription (Fig. 5.9) and the

translation (data not shown) of RNR subunits in myotubes. Furthermore, p21p27i cells treated with HU, a specific inhibitor of RNR, show a great reduction in BrdU-positive myotubes (35% reduction) (data not shown).
The role of TK1, whose activity is not restored upon cell cycle reactivation (Fig 5.11), is confirmed by its dowregulation, which further reduces the extent of DNA replication (Fig. 5.15). Conversely, reactivated myotubes expressing exogenous TK1 display significantly extended DNA synthesis (Fig. 5.13).
The relevance of dTTP in TD cells is validated by E1A-mediated cell cycle reactivation in myotubes. In fact, E1A expressing myotubes show increased levels of dTTP biosynthetic enzymes, enlarged dTTP pool, and accordingly, they extend DNA replication to 25% more than p21p27i myotubes (data not shown). Such results confirm the fundamental role of dTTP in the extension of DNA replication in reactivated TD cells. It is worth noting that herpes simplex virus type I, whose natural host is the neuron, carries its own ribonucleotide reductase and TK-encoding genes (Roizman 2001). This suggests that persistent TK silencing is a feature that characterizes other TD cell types, besides myotubes.
To generalize the data presented in this thesis, we propose to study dTTP significance in other TD cells, such as keratinocytes.
Despite the restoration of the dNTP pool to levels quite similar to those of proliferating cells, myotubes remain unable to fully duplicate their DNA. The XEE experiments provide a conceptual explanation. Myotube nuclei replicate only half of their genetic material, despite being incubated in the presence of all components necessary for DNA synthesis (Fig. 5.16). This indicates that a structural obstacle hinders DNA replication in myotubes. We presume that such obstacle does not take place in specific genomic loci, but it occurs randomly. Indeed, preliminary genomic hybridization studies suggest that reactivated myotube nuclei fail to duplicate their own DNA with a stochastic pattern, namely, no preferential hyporeplicated loci are found (data not shown).

Further XEE experiments reveal that the barrier is not present in naked DNA. Indeed, DNA purified from myotubes is replicated to the same extent as that from proliferating cells (Fig. 5.18).
Once defined the nuclear complexity level on which the replication block lays, we will analyze the corresponding material by mass spectrometry. This approach will help us highlight the differences in chromatin protein constituents between proliferating cells and myotubes. These differences can be acted upon in order to achieve completion of DNA replication in TD cells.

7. MATERIALS AND METHODS

7.1. CELL CULTURE

Muscle satellite cells (MSC) were isolated as described by Rando T.A. in 1994. The MSC were grown on gelatin-coated dishes in growth medium (GM): Ham's F-10 medium (Life Technologies) supplemented with 20% fetal bovine serum (FBS), 3% chicken embryo extract, and 2.5 ng/ml basic-FGF, antibiotics (10,000 units/ml of Penicillin and 10,000 g/ml of streptomycin, GIBCO). Differentiation was induced by plating cells on gelatin-coated dishes in differentiation medium (DM): Dulbecco's Modified Eagle Medium (Life Technologies) supplemented with 10% FBS and antibiotics. After 48 hours, the vast majority of the cells was differentiated (differentiation index higher than 95%).
MSC were made quiescent by culturing them in suspension on semisolid medium (GM with 0.7% Noble Agar (Life Technologies) for 22 hrs. Cell cycle reentry was triggered by replating them on gelatin-coated dishes in liquid GM.
The plates were kept at room temperature (38°C) and at 6.7% of CO_2. The differentiated cells were detached from the culture plate by treatment with 1X trypsin (Gibco) for 2 minutes at 37°C. To mark the newly synthesized DNA, proliferating myoblasts and reactivated myotubes were cultured in the presence of 20 nM 5-bromo-2'-deoxyuridine (BrdU).

7.2. RNA INTERFERENCE.

Myotubes were transfected with the HiPerFect transfection reagent (QIAGEN) combined with siRNA. siGenome duplex (Dharmacon) siRNAs were used to interfere with murine CDKN1A (p21) and CDKN1B (p27) transcripts. The antisense sequences for p21 and p27 were 5'-P.GAACAGGUCGGACAUCACCUU-3' and 5'-P.UAUCCCGGCAGUGCUUCUCUU-3', respectively. The antisense sequence to mouse SAMHD1 was 5'-AUAGAAGUGAACACGAUCGAT-3' (Qiagen). Interference

with murine TK1 and DCTD was carried out using Dharmacon siGenome SMART pools M-042809-00 and M-058063-02, respectively. The siRNA to GFP (antisense sequence: 5'-GGUGCGCUCCUGGACGUAGCCUU-3', Sigma Aldrich) was transfected as a control. Transfection complexes were kept in the culture medium throughout the experiment.

7.3. DEOXYNUCLEOSIDE COMPLEMENTATION

GdR and TdR (Sigma Aldrich) were added, singularly or in combination, to rMt culture medium at final concentrations of 500 and 100 μM, respectively, 17 hr after siRNA transfection. GdR and TdR concentrations were chosen on empirical bases, taking into account the instability of GdR and the potential cell cycle-blocking effects of high levels of TdR.

7.4. dNTP POOL ANALYSIS

This procedure is carried out in collaboration with Dr. Chiara Rampazzo, from the University of Padua. dNTP pools were extracted from cell cultures with 60% ice-cold methanol for 1 hr. Cells left on the plate were dissolved in 0.3M NaOH and the A260nm of the lysates was used as an index of cell mass (Rando TA, et al., 1994). dNTP pool quantitations were performed by an in vitro modified DNA polymerase-based assay, as described (Ferraro P, et al., 2005). Two different aliquots of each pool extract were analyzed and pool sizes were normalized by the A260nm of the NaOH lysates (Frangini M, at al., 2013).

7.5. IMMUNOFLUORESCENCE AND WESTERN BLOTTING

Immunofluorescence was performed on cells (or on nuclei) fixed with 4% formaldehyde and permeabilized with 0.25% Triton X-100 (Sigma Aldrich). For BrdU staining, DNA was denatured with 2M hydrochloric acid. Primary mouse monoclonal antibodies were

used to detect: BrdU (Bu20a clone, DAKO); MCM7 (DCS-141 clone, Santa Cruz); myosin heavy chain [MF20, (Bader et al., 1982)]; γ-H2AX (JBW301 clone, Millipore); pRB (Pharmigen); H1 (S.Cruz) . Ki67 was detected with a rabbit policlonal antibody (Abcam). The modified thymidine analogue EdU, incorporated into newly synthesized DNA, was revealed by Click-iT Imaging kit (ThermoFisher Scientific). AlexaFluor 488- or 594-conjugated secondary antisera to mouse or rabbit IgG (Life Technologies) were used. Nuclei were counterstained with 0.2 μg/ml DAPI for 20 min.
To prepare total cell extracts, cells were harvested, washed in PBS, and lysed with RIPA buffer, containing 500mM NaCl. Proteins were separated on gradient, 4–12% polyacrylamide gels and analyzed by western blotting with the following antibodies: anti-SAMHD1, anti-Cyclin A2 (C-19, Santa Cruz), anti-Pax7 (Developmental Studies Hybridoma Bank cat. no. pax7, RRID:AB_528428), anti-Myogenin (FD5 hybrodoma), anti-β-tubulin (2-28-33 clone, Sigma Aldrich).

7.6. DNA QUANTITATION AND γ-H2AX EXPRESSION

Microfluorimetry was used to quantitate DNA in myotube nuclei stained with DAPI, as multinucleated myotubes are not readily amenable to cytofluorimetry. rMt were allowed to synthesize DNA until shortly before M phase or cell death. The cells were then fixed, immunostained for MCM7 (a cell cycle-dipendent marker of reactivation), and counterstained with DAPI (Diffley, 2011). To assess the extent of DNA replication in rMt and exclude non-reactivated ones, DAPI fluorescence intensity was measured solely in MCM7-positive nuclei. Control myotubes were subjected to the same treatments, but DAPI microfluorimetry was performed on all nuclei, which were uniformly MCM7-negative. Random myotube images were captured at room temperature, using an Axioskop 2 Plus fluorescence microscope (Carl Zeiss) equipped with a Neofluar 40X objective (NA 0.75), and an AxioCam camera (Carl Zeiss). Images were capured with the ZEN 2012 software (Carl

Zeiss). The images were analyzed with the public domain software ImageJ (v. 1.43u). DAPI fluorescence intensity (proportional to DNA content) was measured in all well-defined individual nuclei in the captured images, with local background subtraction. Fluorescence values, in arbitrary units, were distributed into 20, equally-wide intensity channels and the percentages of total nuclei falling into each channel were plotted. The curves thus obtained were integrated to calculate the average fluorescence intensity (AFI) of the sample. In each experiment, the AFI of control myotube nuclei was made equal to 100% and the percent increase in DNA content in rMt nuclei was calculated. γ-H2AX levels were similarly measured, except that reactivated nuclei were detected by Ki67 immunofluorescence.

7.7. GENE EXPRESSION ANALYSIS BY QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from cell cultures with TRIzol reagent (Thermo Fisher Scientific) and cDNA was synthesized from 2 μg of RNA using the High Capaciy cDNA Reverse Transcriptiion Kit (Applied Biosystem), according to the manufacturer's instructions. qPCR was performed with 0.3μl of cDNA (from a 20-μl total reaction volume), using SYBR Green cyanine dye (SensiMix, Bioline), on a 7500 Fast Real-Time PCR System (Applied Biosystems). The specificity of qPCR reactions was verified by the melting-temperature method. Each gene was assayed in triplicate and the mean threshold cycle (Ct) value was calculated. The Pctk1 housekeeping gene (hg), found to be homogeneusly expressed in all samples, was used to normalize target gene (tg) results. Proliferating myoblasts were used as reference (Ctref). Gene expression fold-change was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Cttg - Cthg) - (Ctref - Cthg)$, setting to 1 the expression of tg in proliferating myoblasts.
The following primers were used:
RNR-R1 (5’-CCCAATGAGTGTCCTGGTCT-3’, 5’-GTTCTGCTGGTTGCTCTTCC-3’);

RNR-R2 (5'-CCTACTAACCCCAGCGTTGA-3', 5'-GTTTCAGAGCTTCCCAGTGC-3');
RNR-p53R2 (5'-ACACACGCACACACCACCTGTA-3', 5'-TGACAAATGGGAAGCACAGAGC-3');
DCTD (5'-GAAGCAAGGACCCAAGTTTCC-3', 5'-CCTTCACATCAGCCGAGTTCT-3');
TS (5'-ATGTGGTGAATGGGGAACTGT-3', 5'-GAGCTTTGGGAAAGGTCTTGG-3');
TK1 (5'-CGATGTGACCCAGGAGTCC-3', 5'-GGAGAGTGTGGTGAAGCTCA-3');
TK2 (5'-TGTCCTTGTGTGAGAGAGCAGT-3', 5'-CTCCAGGGTATACGGTCATCAT-3');
dGK (QuantiTect primer assay, Qiagen).

7.8. THYMIDINE KINASE ENZYMATIC ASSAY

Cultures of exponentially growing myoblasts and myotubes were rinsed three times with ice-cold 0.9% NaCl, drained, and collected by scraping in ice-cold lysis buffer (10 mM Tri-HCl, pH 7.5, 0.5% Triton X-100, 2 mM EDTA, 1 mM DTT), supplemented with 0.2 M NaCl and protease inhibitors. Lysates were then centrifuged (16,000 g at 4°C for 30 min) and the supernatant was aliquoted and stored at -80°C. Protein concentration was determined by the Bradford colorimetric assay. TK activity was assayed as described by Franzolin E, et al., 2006. The substrate was 1 μM [3H]TdR (Perkin-Elmer Life Sciences) (800 cpm/ pmol) and two different aliquots of extracts were used to check for proportionality of the assay. We expressed TK activity as pmol product min^{-1} mg^{-1} protein.

7.9. PREPARATION OF *XENOPUS LAEVIS* INTERPHASE EGG EXTRACTS (XEE)

XEE were provided in the context of a collaboration with Dr.Vincenzo Costanzo.

Female frogs were injected with 300U and 600U of Human Chorionic Gonadotropin respectively 24h and 16h before eggs collection and let in a 0.1M NaCl bath. After collection eggs were dejellied by several washes with the Dejellying Buffer (20mM Tris-HCl pH 8.5, 110 mM NaCl, 5 mM DTT) and then rinsed 3 times with MMR buffer (5 mM Hepes-NaOH pH7.5, 100 mM NaCl, 0.5 mM KCl, 0.25 mM MgSO4, 0.5 mM CaCl2, 25 µM EDTA). Dejellied eggs were then incubated with 2 μl of 10 mM Calcium ionophore A23187 (Sigma). After 5-10 minutes eggs were washed three times with MMR buffer and then rinsed twice with ice-cold S buffer (50 mM Hepes-NaOH pH 7.5, 50 mM KCl, 2.5 mM MgCl2, 250 mM sucrose) freshly supplemented with 2 mM β-mercaptoethanol and 15 µg/ml Leupeptin. Activated eggs were packed by centrifugation (few seconds at 3300 x g in a microcentrifuge) to get rid of excess buffer and then crushed by centrifugation for 10 minutes at 16100 g. Crude extract was separated from yolk and insoluble material, supplemented with 40μg/ml of Cytochalasin B and then ultracentrifuged for 18 min at 70000 rpm at 4°C with a TLA100 rotor (Beckman). The final extract, obtained by mixing the clarified protein extract and the membranes fraction was supplemented with 200 µg/ml Cycloheximide (Calbiochem).

7.10. NUCLEI PREPARATION

Cells were collected and centrifugated to obtain a pellet. The pellet was resuspended with IPO Buffer (10 mM K-Hepes (pH 7.5), 2 mM KCl, 2mM MgCl2,1 mM DDT, 1 mM PMSF, 1 mM protease inhibitors)). Samples were incubated for 60 min on ice and then centrifuged at 250 g for 4 min at + 4°C. To eliminate cellular debris, cells were incubated at room temperature (2 min for myoblasts and 4 min for myotubes) with trypsin (Gibco, 0.0025% and 0.00375% respectively for the myoblasts and myotubes). At the end of the process, nuclei were permeabilized with 0.2% Triton X-100 for 5 min. After centrifugation at 250 g for 3 min at + 4°C, nuclei were washed with ISO Buffer (10 mM K-Hepes (pH 7,5),

25 mM KCl, 2 mM MgCl2, 75 mM sucrose, 0,01% of NP-40 (nonyl phenoxypolyethoxylethanol)) and centrifuged at 900g at + 4°C for 3 min. Nuclei were counted (throught Burker chambers) and supplemented with 10% DMSO (dimethylsulfoxide). They were, then, stored at -80°C.

7.11. DNA EXTRACTION

DNA was purified using the QIAamp genomic DNA Kit (Quiagen) according to the manufacturer's instructions. The protocol was modified adding a step of RNA digestion with Rnasi 1 μg/ml.
DNA was quantified with the spectrophotometer Nanodrop (Termo Scientific).

7.12. REPLICATION ASSAY IN XEE

The genomic material (nuclei or DNA) was added to a mix (replication mix) consisting of:

30 µl XEE
2 µl energy mix (20x; 0,2 mg/ml creatine phosphokinase (Sigma), 2 mg/ml phosphocreatine (Sigma), 20 mM MgCl 2, 2 mM EGTA)
2 µl dTTP-3H(Perkin Elmer, 1 mCi) or 2 µl Biotin-16-dUTP (Roche, 1 mM)

The mix was incubated at 23°C for the time of interest.

7.13. DNA REPLICATION QUANTIFICATION IN XEE

16 µl of Stop Buffer (80 mM di Tris-HCl (pH 8), 8 mM di EDTA (pH 8), 0,1% H3PO4, 5% SDS, 50% Glycerol, 0.025% Blue-Bromofenolo, 1 mg/ml Proteinasi K, 200 µg/ml Rnasi) were added to 4µl from the replication mix (see 'replication assay in XEE').
The samples were incubated overnight at 37°C.

For radioactive material: the samples were transfered on glass microfilters (Whataman GF/C 2.5). The filters were incubated with 5% cold trichloroacetic acid (TCA) and 2% cold inorganic pyrophosphate (PPi) for 20 min at +4°C. After 4 washings in 5% cold TCA, the samples were treated with 100% ethanol and transfered to vials with liquid scintillation (Optifluor, Perkinelmer). Radioactivity (index of the quantitiy of DNA replicated) was evaluated with Betacounter (Beckman). The protocol of Menut et al., 1999, was used to quantity the DNA replicated.
For biotinylated material: the samples were transfered on a membrane (Optitrain BA-S 85", 0,45 μm) throught a dot blot microfiltration apparatus (BIO-RAD). The membrane was washed with 0,16M HCl for 3 times. The membrane was crosslinked (+80°C for 2 hours) and incubated with 1% Bovine Serum Albumin (BSA, Sigma) for 1hour. Streptavidin-Peroxidase from Stepromyces avicinii (Sigma, 1:10.000) coupled to horseradish peroxidase (HRP) was used to detect biotinylated material. The membrane was later treated with WesternBright ECL and visualized throught the ImageQuant Las 4000 (EG Healthcare). The densitometric analyses were made with ImageJ (NIH software).

7.14. VISUALIZATION OF REPLICATING NUCLEI IN FLUORESCENCE

1μl of Cy3-dCTP (GE Healthcare, 25nmol) (mixed to the replication mix) was used to follow the replication kinetics. At different time points 5μl from the samples were taken and mixed with 5μl of XEE fixative (3x Buffer A (45 mM Pipes pH 7,2 + 45 mM NaCl + 240 mM KCl), 4% formaldehyde, 0,02 mg/ml DAPI, 50% glycerol). Samples were transfered on cover slips. Nuclei were visualized using an Axioskop 2 Plus fluorescence microscope (see 'DNA quantitation and γ-H2AX expression'). The percentage of nuclei incorporating Cy3-dCTP was counted.

7.15. SALTING OUT WITH NaCl

Nuclei were treated with 1M NaCl for 10 min. To remove NaCl, samples were washed with ISO Buffer (see 'Nuclei Preparation') and centrifugated at 250 g for 3 min.

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9. List of pubblications

Deborah Pajalunga, Elisa Franzolin, Martina Stevanoni, Sara Zribi, Nunzia Passaro, Aymone Gurtner, Samantha Donsante, Daniela Loffredo, Lidia Losanno, Vera Bianchi, Antonella Russo, Chiara Rampazzo, and Marco Crescenzi '**A defective dNTP pool hinders DNA replication in cell cycle-reactivated terminally differentiated muscle cells'** accepted in cell death and differetiation.