ROMA TRE
UNIVERSITÀ DEGLI STUDI

PhD in "Biology applied to human health" (BASU)

Cycle XXVIII

THE ROLE OF NEUROGLOBIN IN THE HEMATOPOIETIC SYSTEM

PhD student: Dominga Cosentino

Supervisors: Prof. Francesco Grignani

Prof. Paolo Ascenzi

Coordinator: Prof. Paolo Visca

TABLE OF CONTENTS

RIASSUNTO

IL RUOLO DELLA NEUROGLOBINA NEL SISTEMA EMATOPOIETICO

Emoglobina e Mioglobina sono due proteine appartenenti alla famiglia delle globine in grado di trasportare e conservare l'ossigeno. Nel 2000 è stato identificato un terzo membro di questa famiglia: la Neuroglobina (Ngb), particolarmente espressa nel sistema nervoso, (da cui il nome) ma non solo, infatti la si ritrova anche nel tratto gastrointestinale, nel tessuto endocrino e nella retina. Si tratta di una proteina monometrica costituita da 151 amminoacidi (17 KDa) con un'alta affinità per l'ossigeno. Il gene della Ngb umana è situato sul cromosoma 14q24. Nel corso dell'evoluzione il gene della Ngb non ha subito molti cambiamenti e ciò ne sottolinea un ruolo rilevante. La Ngb, come tutti i membri della famiglia delle globine, è una emo proteina, con un gruppo prostetico eme, grazie al quale è in grado di legare reversibilmente l'ossigeno ed altri ligandi. L'atomo di ferro del gruppo prostetico può trovarsi nello stato ferroso (Fe^{2+}) o ferrico (Fe^{3+}). Sia nella forma ferrica che ferrosa la Ngb è esacoordinata con ligandi endogeni (istidina prossimale e distale) e O_2 o CO spiazzano l'istidina distale per legarsi essi stessi.

Nonostante i numerosi studi effettuati, non si conoscono ancora perfettamente quali siano tutte le funzioni della Ngb. Sicuramente è chiaro che la Ngb svolge una funzione protettiva nei neuroni in condizioni di ipossia ed ischemia. Inoltre la Ngb svolge un'azione protettiva in condizioni di stress ossidativo e perossidazione lipidica. Però il meccanismo completo con cui riesce a svolgere questa sua funzione neuroprotettiva non è stato ancora bene delucidato. Diversi studi hanno sottolineato come questa funzione possa essere collegata:

1-alla capacità della Ngb di eliminare tutte le specie reattive dell'ossigeno e dell'azoto;

2-alla sua elevata affnità per l'ossigeno che garantisce una riserva d'ossigeno utile in condizioni di ipossia;

3-alla sua capacità di intervenire nel meccanismo di trasduzione del segnale. Infatti la letteratura suggerisce che la Ngb nella forma ferrosa (legante l'O_2) viene subito convertita nella forma ferrica e solo quest'ultima è in grado di legare la subunità G_α della proteina G legata a sua volta al GDP e ne impedisce il rilascio di quest'ultimo, inibendone quindi l'attivazione. In questo modo la Ngb impedisce l'attivazione della trasduzione del segnale che porta poi alla morte cellulare, garantendo di conseguenza la sopravvivenza cellulare. E' noto che solo nella forma ferrica la Ngb può legare (oltre a G_α) la Flotillina-1, una proteina particolarmente espressa nei rafts, piccoli domini di membrana, ricchi in sfingolipidi e colesterolo che funzionano da piattaforme per concentrare e segregare

molecole segnale in modo da compartimentalizzare i processi cellulari. Nei rafts sono quindi presenti diverse proteine coinvolte nella trasduzione del segnale, tra cui le proteine G. La Flotillina è una proteina integrale di membrana che svolge un ruolo chiave nell'assemblaggio dei rafts e nella trasduzione del segnale. Se ne conoscono due isoforme: Flotillina-1 e -2. Il gene della Flotillina-1 è localizzato sul cromosoma 6 e codifica per una proteina di 47 KDa, particolarmente espressa nei neuroni, ma anche nella membrana dei fagosomi, degli endosomi e nel nucleo. La funzione della Flotillina è associata alla sua localizzazione nei rafts ed è coinvolta in molti processi cellulari: partecipa all'ingresso del glucosio nelle cellule stimolate con insulina, induce la rigenerazione dei neuroni, partecipa alla maturazione dei fagosomi, stimola la proliferazione di cellule cancerose di prostata e definisce un *pathway* endocitico Clatrina-indipendente.

In base a tutte queste informazioni lo scopo di questo lavoro è quello di andare a valutare innanzitutto se la Ngb sia espressa nelle cellule mieloidi e poi eventualmente capire quali siano le sue funzioni in questo sistema e se esista anche qui una relazione funzionale tra Ngb e Flotillina-1.

E' stata individuata un'ampia espressione della Ngb nelle cellule mieloidi utilizzate (THP1, U937 e HL60) ed anche in linee cellulari derivanti dal cancro ai polmoni (H1299, H1650 e H1975). Sulla base di questo rsultato siamo andati poi a valutare l'espressione della Ngb durante il differenziamento mieloide. Abbiamo riscontrato che l'espressione della Ngb, così come quella della Flotillina, aumenta durante questo processo ed in particolare la Ngb è espressa a più alti livelli in cellule macrofagiche. Visto che nei neuroni la Ngb ha una funzione protettiva nei confronti dello stress ossidativo, siamo andati a studiare l'espressione della Ngb durante una condizione di stress ossidativo che si verifica nelle cellule mieloidi, vale a dire durante la fagocitosi. Abbiamo osservato che l'espressione della Ngb aumenta nel corso di questo processo, così come la Flotillina-1, ed aumenta ancora di più durante la fagocitosi a carico di macrofagi maturi, che hanno un ruolo fondamentale nel processo stesso. Questi risultati suggeriscono un possibile coinvolgimento di queste due proteine in questo processo. Siamo andati poi a studiare l'espressione della Ngb in cellule mieloidi in condizioni che potrebbero indurre stress cellulare o apoptosi: durante la deprivazione di siero (testando diverse concentrazioni di siero al di sotto di quella utilizzata in condizioni standard) ed in seguito a trattamento con idrossiurea (un inibitore della sintesi di DNA). In entrambe le situazioni non abbiamo riscontrato nessuna varazione nei livelli di espressione della Ngb, suggerendo probabilmente un mancato coinvolgimento della Ngb stessa. Mentre abbiamo registrato un

considerevole aumento di espressione della Ngb in seguito a trattamento delle cellule mieloidi con H_2O_2 per indurre stress ossidativo. Questo risultato ci consente di ipotizzare che durante condizioni di stress ossidativo la Ngb, oltre ad una funzione neuroprotettiva, svolge la stessa funzione di protezione anche nelle cellule mieloidi. Diversi farmaci sono in grado di indurre stress ossidativo, uno di questi è l'isoniazide (Inh), farmaco anti tubercolare di prima linea. Inh inoltre è in grado di legare reversibilmente il Fe in forma ferrica e ferrosa del gruppo eme dell'emoglobina troncata del *Mycobacterium tuberculosis,* legame che perturba le proprietà del gruppo eme, renendo l'emoglobina incapace di svolgere le sue normali funzioni. Dato che anche la Ngb ha un gruppo eme, che viene convertito nella forma ferrica in condizioni di stress ossidativo per poter espletare la sua funzione neuroprotettiva, siamo andati a valutare l'espressione della Ngb in seguito a trattamento delle cellule mieloidi con varie concentrazioni di Inh. Abbiamo riscontrato un aumento di espressione della Ngb all'aumentare delle dosi di Inh somministrata. Avendo osservato che l'espressione della Ngb aumenta durante il differenziamento mieloide ed in condizioni di stress ossidativo, abbiamo pensato di combinare i due processi e vedere cosa accadesse alla Ngb. Abbiamo così osservato che l'espressione della Ngb aumenta ancora di più nel caso di stress ossidativo indotto in cellule mieloidi dfferenziate. In fine siamo andati a studiare la relazione tra Ngb e Flotillina-1 nel sistema ematopoietico. Prima di tutto siamo anadati a valutare se l'espressione della Flotillina-1 influenza quella della Ngb. Pertanto abbiamo prodotto cellule mieloidi sovra-esprimenti la Flotillina-1 e cellule in cui la Flotillina-1 è stata sileziata. Nel primo caso non abbiamo riscontrato nessuna variazione di espressione della Ngb, mentre abbiamo osservato una notevole diminuizione di espressione della Ngb nelle cellule in cui la Flotillina-1 era stata silenziata. L'assenza della Flotillina-1 impedisce l'aumento di espressione della Ngb anche nel caso di trattamento con H_2O_2 per indurre stress ossidativo. Questi risultati suggeriscono una possibile funzione protettiva e di stabilizzazione da parte della Flotillina-1 sulla Ngb. Per confermare questa ipotesi siamo andati a studiare se esiste un'interazione tra queste due proteine. Pertanto abbiamo messo a punto un esperimento di co-immunoprecipitazione utilizzando un anticorpo anti-Flotillina-1, seguito da Western Blotting con un anticorpo anti-Ngb. Questo è stato fatto in cellule mieloidi sia in condizioni basali che in seguito a trattamento con H_2O_2 per indurre stress ossidativo. Abbiamo ottenuto un risultato positivo, ossia un interazione, solo dopo trattamento con H_2O_2. Quindi in cellule mieloidi la Ngb interagisce fisicamente con la Flotillina-1 solo in condizioni di stress ossidativo.

In conclusione possiamo affermare che la Ngb è espressa in cellule mieloidi e di cancro al polmone; la sua espressione aumenta durante: il differenziamento mieloide, la fagocitosi e lo stress ossidativo; la sua espressione diminuisce in cellule in cui la Flotillina è stata silenziata; interagisce con la Flotillina-1 durante lo stress ossidativo.

In base ai risultati ottenuti sarebbe interessante andare a studiare il ruolo della Ngb nei progenitori ematopoietici sottoposti a fattori di crescita e di differenziamento verso la linea granulocitaria o monocitaria. Inoltre per rafforzare i dati che sostengono un potenziale ruolo protettivo della Ngb in condizioni di stress ossidativo in cellule mieloidi, andrebbe valutato l'effetto conseguente alla sua sovra-espressione e al suo silenziamento.

ABSTRACT

THE ROLE OF NEUROGLOBIN IN THE HEMATOPOIETIC SYSTEM

Hemoglobin and Myoglobin are proteins that belong to the globins family and are able to deliver and conserve oxygen. In the 2000 a new component of this family has been identified and named Neuroglobin (Ngb). Ngb is mainly expressed in the nervous system, from which it takes its name, but it is located also in the gastrointestinal tract, in the endocrine tissue and in the retina. Ngb is a monometric protein formed by 151 aminoacids (17 KDa) and has a high affinity for oxygen. The human Ngb gene is located in the chromosome 14q24. Over the evolution, the Ngb gene has been conserved and this suggests its relevant role in biological systems. Ngb, as all the globins family's members, is an heme protein that shows the heme prostetic group, by which can reversibly bind oxygen and other ligands. The iron atom in the prostetic group can have the oxidative state +2 (ferrous) or +3 (ferric). Both in the ferrous and in the ferric state the Ngb is hexacoordinated with other endogenous ligands (proximal and distal hystidine) and O_2 or CO crowd-out the distal hystidine to link themselves to the protein. Even though many studies have been conducted, it is not completely clear which functions the Ngb has. It is known that the Ngb plays a protective role in neurons under conditions of hypoxia and hyschemia. Moreover it plays, too, a protective function during the oxidative stress and lipidic peroxidation. However the mechanism by which Ngb can protect neurons has not been completely elucidated yet. Several studies have shown which mechanisms this function can be linked to: 1) the Ngb ability to scavenge reactive oxygen and nitrogen species; 2) its high affinity for oxygen that allows the organism to have a bulk of oxygen during hypoxia conditions; 3) its role in the signal transduction. In fact, the body of literature suggests that Ngb, in its ferrous state and linking oxygen, is quickly converted in the ferric state and only the latter is able to bind the G_α subunit of the G protein, that in turn is bound to the GDP. The link between the Ngb in the ferric state and the G protein does not allow the release of the GDP and consequently the activation of the G protein itself. This way Ngb halts the signal transduction that leads to cell death and guarantees the survival of the cells. Additionally, in the ferric state Ngb can bind Flotillin-1, a protein mainly express in rafts, small membrane domains, rich in sfingolipids e cholesterol, that function as platforms to concentrate and segregate signal molecules during cellular processes. Thus in rafts there are several proteins involved in the signal transduction, among which G proteins. Flotillin is an integral membrane protein that plays a key role in

assembling rafts and in the signal transduction. Two different isiform of Flotillin are known: Flotillin-1 and Flotillin-2. The Flotillin-1 gene is localized in the chromosome 6 and code for a 46 KDa protein, particularly expressed in neurons, but also present in the fagosomes membrane, in endosomes and in the nucleus. The function of Flotillin is associated with its localization in rafts and it is involved in many cellular processes: it participates in the glucose income in insulin-stimulated cells; it induces the neuronal regeneration; it participates in the fagosome maturation; it drives the proliferation of prostatic cancer cells and finally it defines an endocytic Clatrin-dependent pathway. According to this information, the aim of the project is to evaluate either the possible expression of Ngb in myeloid cells, and to analyze its function in this specific cell system, and to find a functional relationship between Ngb and Flotillin-1. In this project, a significant expression of Ngb has been individuated in myeloid cells (THP1, U937 and HL60) and in lung cancer cells (H1299, H1650 and H1975). According to these findings, the expression of Ngb during the myeloid differentiation has been analyzed. The expression of Ngb, as well as of Flotillin, increases during the differentiation. Additionally, Ngb is mainly expressed in macrophagic cells. Because of the neuroprotective role of Ngb during the oxidative stress, its expression has been evaluated during a specific condition of stress that occurs in myeloid cells, that is the fagocytosis. The Ngb level improves during the fagocytic process, as well as Flotillin-1, and increases even more during the fagocytosis realized by mature macrophages, which have a basic role in this process. These results suggest a possible involvement of these two proteins in this mechanism. Moreover, the Ngb expression has been analyzed in specific conditions that could have induced cellular stress and apoptosis, particularly during serum deprivation (by testing concentrations of serum lower than the concentration used in standard conditions) and after treatment with hydroxyurea (a DNA synthesis inhibitor). In both these situations there was not a modification in the Ngb expression, suggesting that probably the protein is not involved. Conversely, a remarkable increase of the Ngb expression in myeloid cells has been observed after H_2O_2 treatment, inducing oxidative stress. This result could suggest that, during oxidative stress conditions, Ngb may play a protective role in myeloid cells as well as shown in neurons. Different drugs can induce oxidative stress, such as Isoniazid (Inh), a first line anti-tubercolar drug. Inh is able to reversibly bind both the ferric and ferrous iron of the heme group in the truncated hemoglobin of the *Mycobacterium Tuberculosis.* This link modify the heme group properties, making the hemoglobin unable to perform its normal functions. The Ngb, too, has an heme group whose ferrous iron is converted in

ferric iron under oxidative stress conditions to protect neurons. Thus, the Ngb expression has been evaluated after treatment with different concentrations of Inh in this cellular system and consequently a dose-dependent increase of Ngb expression has been observed. Afterwards, combining the myeloid differentiation with oxidative stress conditions (H_2O_2-induced), a higher Ngb level has been measured in mature myeloid cells compared to the Ngb expression in undifferentiated cells. Finally the functional relationship between Ngb and Flotillin-1 in the hematopoietic system has been studied. First of all the possible influence of Flotilin-1 on Ngb has been evaluated. According to this purpose, over-expressing Flotillin-1 cells and Flotillin-1 knockdown cells have been generated. In the first condition no variations in the expression of Ngb have been observed, in the latter a remarkable decrease in the protein have been noticed. The lack of Flotillin-1 prevent Ngb from increasing also in H_2O_2-induced oxidative stress conditions. This data suggests a possible protective and stabilizing function of Flotillin-1 on Ngb. To confirm this hypothesis, the physical interaction between these two proteins has been investigated by setting up a co-immunoprecipitation assay with an antibody anti-Flotillin-1, and then a Western Blotting with an antibody anti-Ngb, in myeloid cells both in basal conditions and after an H_2O_2-induced oxidative stress treatment. The results obtained showed that, in myeloid cells, the physical interaction occurs only after H_2O_2 treatment, thus in conditions of oxidative stress. In conclusions, to sum the data collected during this project: Ngb is expressed in myeloid cells and in lung cancer cells, too; its expression increases during the myeloid differentiation, the fagocytosis and the oxidative stress; additionally, its level tends to diminish in Flotillin-1 knockdown cells; and finally, Ngb interacts with Flotillin-1 during the oxidative stress. According to these results, further studies could be conducted to elucidate and better understand the functions of Ngb in different cellular systems. As examples, the role of Ngb could be analyzed in myeloid progenitors treated with growth factors and induced to differentiate in granulocytes or monocytes. Moreover, as a support to the hypothesis that Ngb has a potential protective effect in myeloid cells under oxidative stress conditions, consequences of the silencing or over-expression of Ngb could be evaluated.

INTRODUCTION

1.NEUROGLOBIN

Hemoglobins and Myoglobins are related protein families that function in oxygen transport and storage. In 2000 a third globin type has been identificated, predominantly expressed in the brain and consequently named Neuroglobin (Ngb). Mouse Ngb is a monomer with a high oxygen affinity [half saturation pressure P_{50}=0,9 to 2.2 Torr (0,12-0,29 kPa)]. The human Ngb gene (NGB) is located on the chromosome 14q24 and it is constituted by a unique exon-intron. The promoter region contains several putative Sp1-binding sites and at least three transcription starting points but there is not a TATA domain. NGB gene has three introns at positions B12-2, E11-0 and G7-0, of which B12-2 and G7-0 are conserved in Hemoglobins and Myoglobins.

Both mouse and human genes code for proteins of 151 amino acids (17 KDa) that are 94% identical, that is a higher conservation than that between the orthologous Hemoglobins or Myoglobins of these species (77-85 % identity). Even if this proteins belong to the globin superfamily, they have a low amino acid sequence similarity with vertebrate Myoglobins (<21% identity) and Hemoglobins (<25% identity) insinuating a distinct evolution and function (Burmester T. et al; 2000).

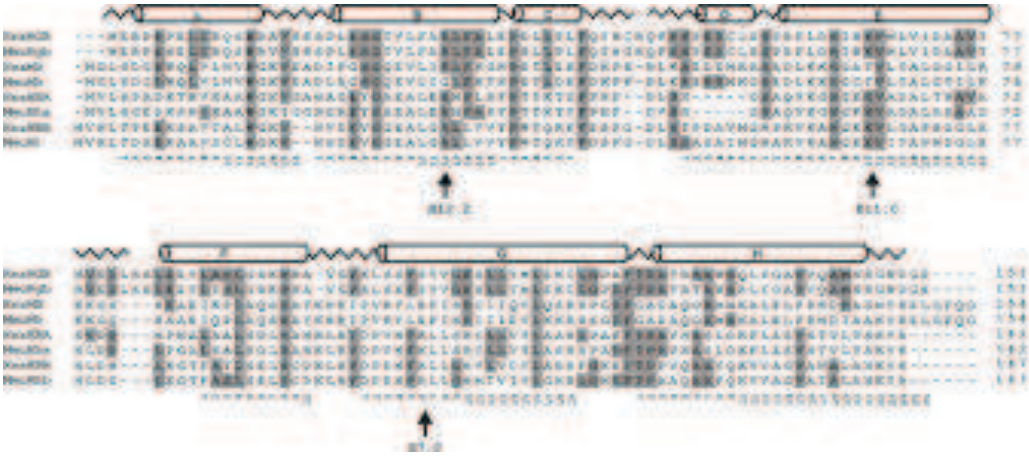


Figure 1: *Comparison between Human Ngb, mouse Ngb, Myoglobins and Hemoglobins α and β. (Burmester T. et al. Nature, 2000)*

Ngb as well as all members of globins superfamily is a heme protein, with a heme prosthetic group (Fe^{2+}-protoporphyrin IX), by which reversibly binds oxygen or other ligands. The iron atom in the heme prosthetic group exists in either the ferrous (Fe^{2+}) or ferric (Fe^{3+}) redox state. Both the ferrous and ferric Ngbs forms are hexacoordinated to

endogenous ligands (namely proximal and distal histidine residues), and O_2 or CO displace the distal His residue to produce ferrous O_2- or CO-bound Ngb (Dewilde et al., 2001).

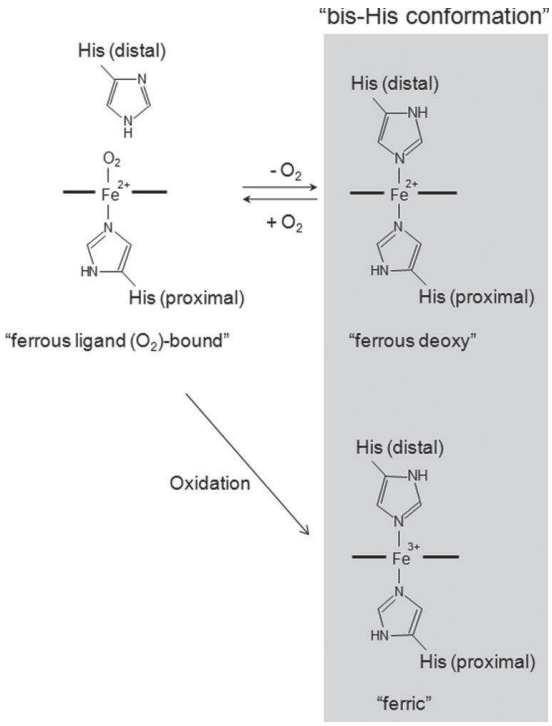


Figure 2: *Illustration of the ferrous and ferric form of Ngb (Watanabe et al.,Journal of Biological Chemistry, 2012).*

It is highly expressed in brain neurons but also in the peripheral nervous system, endocrine tissues and retina (Reuss et al., 2002). Ngb has a neuroprotective role against hypoxic/ischemic insults that occur in neurons. In fact antisense-mediated Ngb knockdown rendered cortical neurons more vulnerable to hypoxia, whereas Ngb over-expression gives protection of cultured neurons against hypoxia (Sun et al., 2001).

In animal stroke models, Ngb over-expression reduced infarct size (30% compared to wild type) in rats after a middle cerebral artery occlusion (MCAO), and the outcome was reversed by Ngb knockdown thanks to anti-sense oligonucleotide (Sun et al., 2003). The reduction of brain infarction in Ngb-over-expressing transgenic mice can be sustained up to 14 days after ischemia (respect to wild type), underlined that Ngb over-expression offers neuroprotection against transient focal cerebral ischemia (Zhanyang et al., 2012), but the involved mechanisms it is not yet completely clear.

Ngb has also a protective role against other neurological disorders, like β-amyloid and NMDA toxicity (Khan et al., 2007; Li et al., 2008) and then protects retinal ganglion cells (RGC) against ocular Hypertension and glaucomatous damages (Wei et al., 2011).

Therefore it has been widely accepted that Ngb has a protective role, but the underlying mechanism remains poorly defined. An initial evidence suggests that the neuroprotective role may be largely linked to its ability to bind O_2 and NO. In addition, putative signal transductions and a mitochondrial function preservation may also be involved.

Several studies have indicated that Ngb neuroprotective function is related to its ability in scavenging reactive species, in fact Ngb can bind nitric oxide (NO) with a high intrinsic affinity and a low dissociation rate (Van Doorslaer et al., 2003). In addition, Ngb-O_2 reacts with NO quickly to produce NO_3^- and ferric Ngb (HNgb). This pathway would dispose of NO and may protect cellular respiration by the inhibitory effect of NO on cytochrome c oxidase activity (Moncada et al., 2002; Brunori et al., 2008).

Furthermore, Ngb over-expression rendered neuroblastoma cells more resistant to NO-induced cell death, underlined Ngb ability in neutralizing neurotoxic effects of reactive nitrogen species (Jin et al., 2008).

Ngb also has a protective function against other oxidative challenges in neurons, such as H_2O_2-induced oxidative stress or lipid peroxidation (Fordel et al., 2006; Sun et al., 2005).

HNgb binds the GDP-bound form of the α subunit of G protein (G_α), competing with $G_{\beta\gamma}$ subunit (to binds G_α) (Kitatsuji et al., 2007; Wakasugi et al., 2003).

G proteins are heterotrimeric proteins constituted by a Gα subunit with GTPase activity and a $G\beta_\gamma$ dimer, and belongs to a protein family whose signal transduction functions depend on the binding of guanine nucleotides (Gilman et al., 1992; Simon et al., 1991). Ligands- or signal-activated G protein-coupled receptors (GPCRs) cause GDP release from Gα to bind GTP. The GTP bind to Gα causes conformational changes that comport dissociation of GTP-Gα from $G\beta_\gamma$, that can separately regulate the activity of different effector molecules (adenylyl cyclase, ion channels,..). Thanks to the intrinsic GTPase activity of Gα (which hydrolyzes bound GTP to GDP), the signal transduction is turned off and GDP-Gα reassociated with $G\beta_\gamma$. Gα proteins are divided in four families: $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$(Hepler et al., 1992; Simon et al., 1991).

Ferric HNgb binds exclusively the GDP-bound form of $G\alpha_{i/o}$ and functions as a guanine nucleotide dissociation inhibitor (GDI) for $G\alpha_{i/o}$ (Wakasugi et al., 2003). On the contrary ferrous-ligand-bound HNgb under normoxia does not interact with $G\alpha_{i/o}$ nor has GDI activity (Kitatsuji et al., 2007; Wakasugi et al., 2003). So HNgb may be an oxidative

stress-responsive sensor for signal transduction in the brain (Wakasugi et al., 2003 and 2005).

Moreover, Ngb exists in lipid rafts only during oxidative stress. In fact, the addition of a lipid rafts distruptor (MβCD) reduces neuroprotective activity of HNgb, that is restored by reconstruction of lipid rafts. This suggests that lipid rafts are fundamental for Ngbs neuroprotection (Watanabe et al., 2012).

Wakasugi et al in 2004 have demonstrated that Ngb interacts with Flotillin-1 by using a yeast two-hybrid screening, evidence that was confirmed by GST pull-down assays. Flotillin-1 is a lipid raft microdomain-associated protein, and also $G\alpha_{i/o}$ exists in lipid rafts (Moffet et al., 2000; Yuyama et al., 2007). Therefore, Flotillin-1 recruits HNgb to lipid rafts, where HNgb binds $G\alpha_{i/o}$ and acts as a GDI for $G\alpha_{i/o}$ preventing neuronal death (Watanabe et al., 2012).

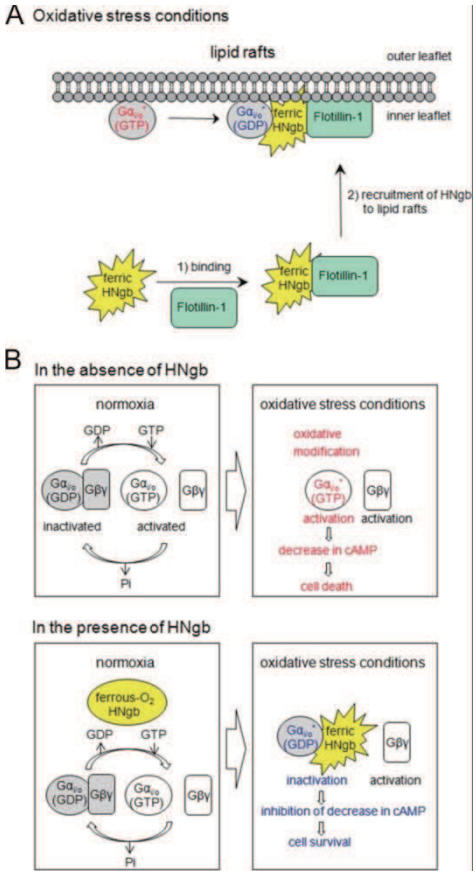


Figure 3: *Neuroprotective mechanism of HNgb (Watanabe et al., Journal of Biological chemistry, 2012).*

Ngb expression is confined to metabolically active and oxygen-consuming cell types (Burmester et al., 2004). At the subcellular level, Ngb is associated with mitochondria, linked it to the oxidative metabolism (Burmester et al., 2007). Mitochondria have a key roles in energy production, ROS homeostasis and cell death signaling. They respond to various insults to cells and its dysfunction is related with a large variety of clinical phenotypes. Mitochondria comprise a central locus for energetic perturbations and oxidative stress in hypoxia/ischemia (Nicholls et al., 2000; Sims et al., 2002). Ngb over-expression improves mitochondrial function and reduces oxidative stress after hypoxic insults that occur in neurons (Liu et al., 2009). Ngb might affect both mitochondrial functions and free radical generations as its potential neuroprotective mechanisms. Hypoxia and OGD (Oxygen-Glucose Deprivation) develop mitochondrial depolarization (Larsen et al., 2006). The mitochondrial permeability transition pore (mPTP) is a protein pore created across the inner and outer membrane of the mitochondria in pathological conditions (such as stroke). During hypoxia/ischemia, mPTP opening determined release of Cyt c from mitochondria to cytosol (Zhang et al., 2008) and next activation of caspase-dependent or -independent apoptosis pathways (Petit et al., 1998; Zhu et al., 2002). Ngb over-expression is correlated with reduced mPTP opening, and decreased cytochrome c release, highlighting an inhibitory role of Ngb in OGD-induced mPTP opening, that is one of the major causes of cell death in a variety of tissue ischemic damages, like heart attack and stroke. Therefore, Ngb inhibitory effect in mPTP opening may be an important mechanism of Ngb neuroprotection. Several Ngb-binding proteins have been identified by yeast two-hybrid assay: Na/K ATPase β1, cytochrome c1, ubiquitin C, voltage-dependant anion channel (VDAC) and a few more (Yu et al., 2012), that some are biologically relevant for neuronal function and survival (Yu et al., 2012).

Ngb gene expression is tissue-specific and Ngb mRNA is widely distributed throughout the adult rat brain, including cerebral cortex, hippocampus, and subcortical structures (thalamus, hypothalamus, olfactory bulb, and cerebellum) (Wystub et al., 2003; Zhang et al., 2002; Geuens et al., 2003). Ngb protein distribution is compatible with its mRNA localization and the subcellular immunoreactivity is restricted to the cytoplasm. The highest Ngb expression is in the retina with a concentration 100-fold higher than in the brain (Schmidt et al., 2003). Ngb mRNA was observed in the perikarya of the nuclear and ganglion layers of the neuronal retina, whereas the protein was present mainly in the plexiform layers and in the ellipsoid region of the photoreceptor inner segment (Hundahl et al., 2005). The distribution of Ngb correlates with the subcellular localization of

mitochondria and with the relative oxygen requests. So Ngb supplies oxygen to the retina, as well as Myoglobin in the myocardium and skeletal muscle.

The expression of Ngb gene is up-regulated in:

1) hypoxic/ischemic conditions in cultured cells (Schmidt-Kastner et al., 2006; Shao et al., 2009) and stroke animals brain (Sun et al., 2001 and 2003, Shang et al., 2006; Fordel et al., 2007)
2) in the cerebellum of mouse pups in response to hypoxic-ischemic insults caused by maternal seizures during intrauterine life (Lima et al., 2011)
3) in the cortical peri-infarct region in stroke patients (suggesting its clinical relevance for endogenous neuroprotection) (Jin et al., 2010)

While expression level decreased to about a half in aged rats (24 months) compared to young ones (3, 12 months) in various regions of brain, implying the pathophysiological importance of Ngb in age-related neurodegenerative diseases (Sun et al., 2005).

Transcription factors Sp1 and Sp3 can bind to the human Ngb promoter region causing transactivation of Ngb promoter (Zhang et al., 2011). Transcription factors, members of the NFkB family (p65, p50, cRel), Egr1, and Sp1, bind the Ngb promoter, causing basal Ngb expression. Moreover, NFkB (p65) and Sp1 as well as Hif1, were also responsible for hypoxia-induced up-regulation of Ngb expression (Yu et al., 2012).

Globins family

The term globin regroups a variety of proteins, structurally and phylogenetically related but present in organisms of developmental levels also very different. All the members of this family share the typical globin fold and the presence of the prosthetic group heme, that allows them to bind oxygen and other small diatomic molecules. The most famous representatives of this family are undoubtedly Hemoglobin (Hb), the more studied protein in the world, present in red blood cells and responsible for transporting oxygen (necessary for the oxidative respiration of the cells) through the blood in vertebrates, and Myoglobin (Mb), the first protein whose structure has been solved by X-ray (by John Kendrew and Max Perutz in 1958) and whose function is to store oxygen in the muscles tissue. But recent studies have revealed the existence of a high number of members of this family, classified on the basis of structural, functional and evolutionary parameters and present not only in animals but also in bacteria, protozoa, plants and mushroom.

Globins are small metalloproteins that generally comprise around 150 amino acids, but may also have N- and/or C-terminal extensions. Most globins cover eight α-helical segments (named A through H) with a characteristic 3-over-3 α-helical sandwich structure

(referred to the globin fold) and with a heme prosthetic group (Fe^{2+}-protoporphyrin IX), by which they reversibly bind oxygen and other ligands (Perutz 1979; Bolognesi et al., 1997).

Figure 4: *Example of the globin fold in tertiary structure. (Pesce et al.,* ***Biochemistry and Molecular Biology Education,*** *2001).*

While their overall structures are conserved, the primary sequences of globins often change. In fact, only the proximal histidine adjacent to the Fe^{2+}, is present in all globins, and most of them also show a phenylalanine in the inter-helical region CD1, which stabilizes the heme.

Although the globin polypeptide chain consists of about 150 residues, lately it was discovered a "truncated" hemoglobin, shorter than about 20-40 amino acid residues and having a three-dimensional structure partially different. These so-called truncated hemoglobins are expressed in eubacteria, cyanobacteria, protozoa and plants, and were divided into three phylogenetic groups (I, II, III) according to an amino acid sequence analysis. Their structure is different compared to the classic folding globin, although it retains some features. The pairs of antiparallel α-helical B-E and G-H form a sandwich "two-over-two", which can be considered as a reduction to the minimum of the "three-over-three" globin fold (Pesce et al., 2001). The helix A and the region CD-D are almost completely absent and the helix F is partly replaced by a long pre-helix loop. A characteristic common to all "truncated" hemoglobins is the presence of an apolar tunnel that connects the distal heme pocket with the solvent; this channel might have a role in facilitating the binding of gaseous ligands.

A further subgroup is called "mini-hemoglobins" and is characterized by a sequence even shorter. One of which is the hemoglobin of marine worm Cerebratulus lacteus, the shortest hemoglobin known so far, that consists of only 109 amino acids. Deletions respect to the

classic folding globin affecting different areas. Even the mini-hemoglobin have a hydrophobic channel within the protein matrix (Pesce et al., 2002).

Figure 5: *Two -over -two fold globin in hemoglobin truncated Paramecium caudatum. (Pesce et al., Biochemistry and Molecular Biology Education, 2001).*

The quaternary structures of globins is very various, from simple to complex monomers consisting of hundreds of subunits. The most famous example of monomeric globin is Myoglobin. Hemoglobin is instead a tetramer, more precisely a "dimer of dimers", composed by two α subunit and 2 β subunits. It is interesting to note that this structural difference is reflected on the function. Hemoglobin binds oxygen in the lungs, transports it in the organism, transfers it to tissues, and binds CO_2 thanks to amino-terminal groups of the four globin chains. Myoglobin, present in muscle tissue, stores oxygen and releases it during intense aerobics activity. At the oxygen partial pressures of the pulmonary alveolus (PpO_2~100 mmHg), the Hemoglobin in the blood is almost completely saturated with oxygen. While in tissues (PpO_2 ~25-30 mmHg) Hemoglobin largely releases oxygen that binds Myoglobin for which has a great affinity at those partial pressures of oxygen. The binding process and the release of O_2 by Hemoglobin is closely related and controlled by its quaternary structure. Hemoglobin indeed can exist in two quaternary forms, a high affinity for oxygen (R form) and a low affinity (T form). The deoxy protein is in a low affinity state, the binding of the first oxygen induces a conformational change (to the R form) which increases the affinity for the binding sites. This conformational change involves the heme group and, by the binding to the proximal histidine of the F helix, the change involves the tertiary and quaternary structure of the hemoglobin, and facilitates the binding of other molecules of oxygen in the free binding sites of the protein (cooperative binding).

Myoglobin, in addition to the main function of supplying oxygen, has a role as scavenger of intracellular NO (strong inhibitor of cytochrome c oxidase, terminal enzyme of the respiratory chain) in cardiac muscles and skeleton (Brunori et al., 2001).

Even dimeric globin are quite widespread in nature. Some examples are: Cytoglobin, rice hemoglobin HB1, hemoglobins of Scapharca and Vitreoscilla.

While the majority level of complexity in vertebrates is constituted by tetrameric structures, in invertebrates there is a wider variety of aggregation states. They were in fact identified both multisubunit hemoglobins, in which the number of monomers can be up to 144 units, and multidomain hemoglobins. These are proteins originated by gene duplication and containing more domains having globin folding. The multidomain proteins can in turn aggregate to form multidomain multisubunit hemoglobin. So globin fold, in these cases, represents a sort of "brick" for the construction of complex aggregates, which show interesting symmetries in the structure. There are also the so-called "globin fusion" in which the globin domain is combined with a non-globin domain. From a phylogenetic analysis, it seems likely that these proteins have evolved following the merger of a primitive globin gene with the gene coding for another protein having transduction or enzymatic function. A subset of these are Flavohemoglobins, expressed in bacteria and pathogenic mushrooms, and consist of a "classic" globin domain fused to a domain containing a group FAD and NADH with oxydoreductase activities. In Escherichia coli, the flavohemoglobin Hmp is expressed following an increase in the concentration of O_2 and NO and catalyzes the oxidation of oxygen-dependent NO. The nitro oxidation reaction occurs at the level of heme globin, thanks to electrons provided by one molecule of NADH. The intramolecular electron transfer occurs through the group FAD.

The large diffusion of Hemoglobin in the living realm allows to assume that the origin of these proteins is very old. The study of functions carried out in various organisms also shows that hemoglobin may have other functions different from the transportation of oxygen, such as catalytic functions, detoxification or sensors gaseous ligands. The role of iron-porphyrin group in the electron transfer has established soon at the evolution, as shown by the ubiquity of cytochromes, involved in many oxide-reductive reactions. The atom of porphyrin iron, during these reactions, cycles from 2+ oxidation state to 3+. At some point in the evolution, estimated around about 1800 million years ago, oxygen began to accumulate in the atmosphere and probably hemo proteins developed some of the protective capabilities against this potential toxic agent (Kiger et al., 2002). Some Hemoglobins have assumed the role of scavenger also of other substances, such as NO and

CO, through redox catalytic activity. Flavohemoglobins, truncated hemoglobin of Mycobacterium tuberculosis, hemoglobin of Ascaris suum and even Myoglobin of vertebrates, are examples of hemoglobins still existent that retain this catalytic role. Available oxygen, as a final electron acceptor, has probably boosted the evolution of heme proteins that can bind reversibly. This type of function, unlike the cytochromes, requires the iron atom in oxidation state 2+. These primitive carrying oxygen hemoglobins were supposedly expressed in small amounts within cells and facilitated the supply of oxygen for the oxidative respiration. With the evolution of multicellular organisms, we have developed specialized structures, like erythrocytes, in which they expressed high levels of hemoglobin responsible for the transport of oxygen (Hardison et al., 1996). In today's organisms, the transport of oxygen is probably the main role of globin, but not the only one: some globins act as oxygen sensors, other as final acceptors of electrons in redox cycles. In invertebrates, in particular, there are hemoglobins involved in the acquisition of sulfur, which is used as electron acceptor in the respiratory chain, or have a role in phototropism (Kiger et al., 2002). The hypothesis of the existence of an ancestral hemoglobin, from which all hemoglobin present in prokaryotes, mushrooms, plants and animals derived is evaluated. The analysis of genes coding for different hemoglobins widespread in nature seems to reinforce this hypothesis. From these studies the existence of a gene coding for an ancestral hemoglobin dating back to more than 1500 million years ago has been hypothesized, before the divergence between plants and animals, as shown by the figure below.

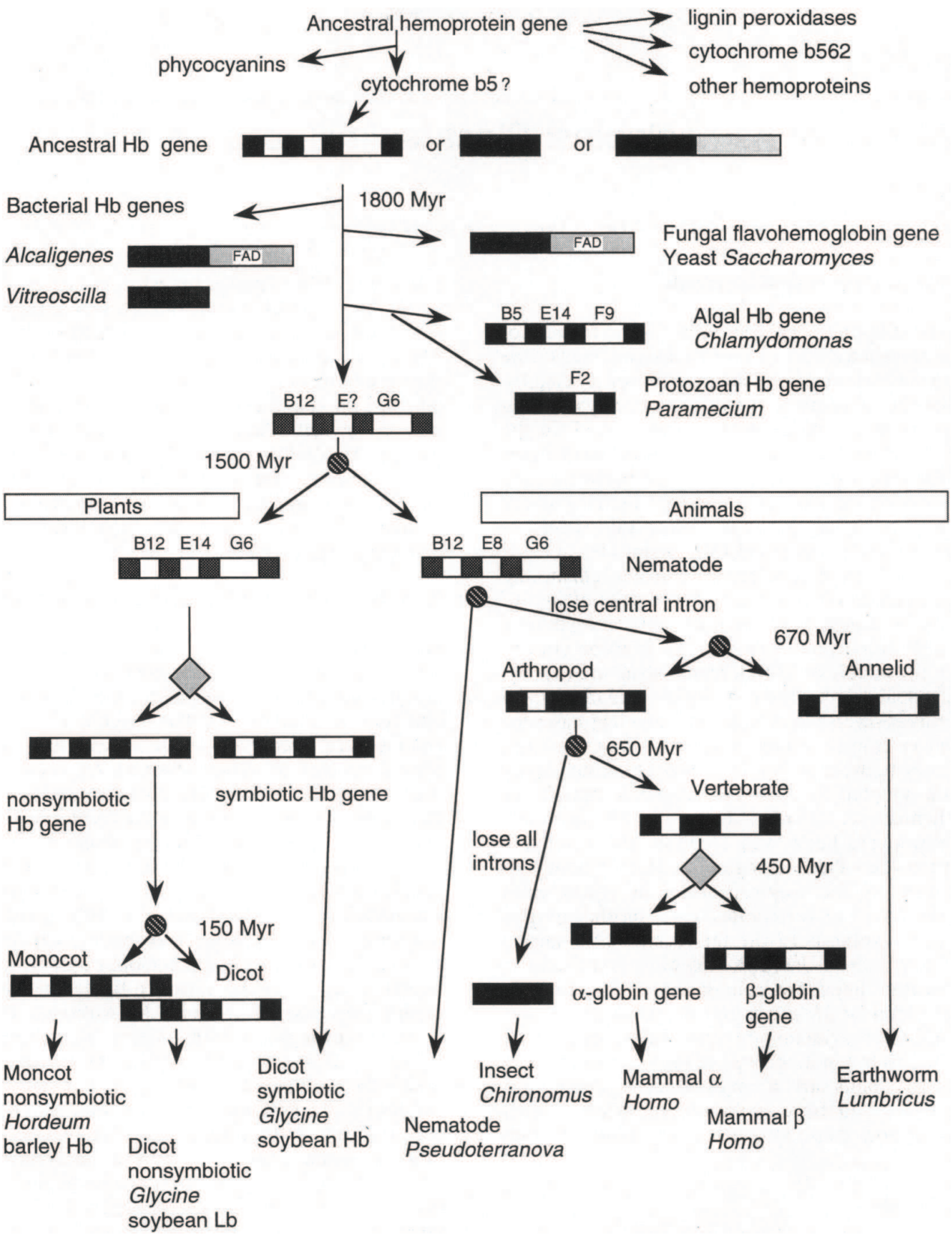


Figure 6: *Scheme for the origin of the family of hemoglobin from a common ancestor (Hardison et al., PNAS, 1996).*

The function of many hemoglobins is unknown. Studies on the spectroscopic property, investigations to measure the affinity for gaseous ligands, determination of three-dimensional structures through X-ray analysis are some of the methods used to derive structural and functional informations of these proteins.

Heme group

A common feature of the super family of globins is the presence of heme, a prosthetic group (i.e. non-protein) that can be bound to the protein both with a covalent and with a non-covalent bound, although in most cases this bond is rather weak. The Fe-protoporphyrin IX is the prosthetic group of globin; it is derived from an organic macromolecule, the porphyrin (a macromolecule heterocyclic, highly conjugated, it's composed of four pyrrole subunits interconnected through their coals at by means of methine bridges=CH), which coordinates an iron atom. In the Fe-protoporphyrin IX tetrapirrolic ring is replaced with two vinyl groups in position 2 and 4, two propionyl groups in position 6 and 7 and four methyl groups in the remaining positions.

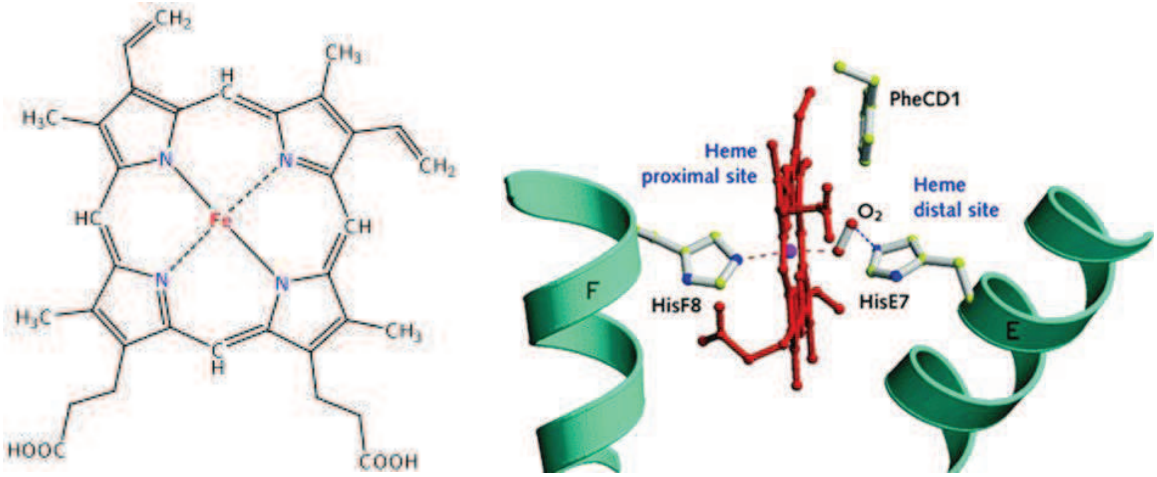


Figure 7: *A diagram of the heme group B and the direct link between the heme group and the side chain of proximal histidine.*

The iron atom is coordinated by four nitrogen atoms of the pyrrole rings, all of which are planar; in all globins, too, Fe atom of the tetrapirrolic ring ties a histidine residue, named proximal (HisF8, using the numbering used for the classical HbA), which instead acts as a Lewis base donating an electron pair to "coordinate" the iron, which acts as a Lewis acid. The proximal histidine is the only residue conserved in all known globins, along with a phenylalanine (CD1) implicated in the stabilization process of heme within the protein

matrix. For this stabilization process hydrophobic interactions of the prosthetic group shape with the side chains of leucine, isoleucine, valine and fenilalanine residues are also important. The iron atom is directly bound to the pyrrole nitrogen in a coordination compound (or complex); in fact all the cations of any metal in the periodic table are capable of accept electronic density and can therefore coordinate around itself electron donor groups, exceeding in number the own number of oxidation (charge electricity).

The ability to form directed and enough strong bonds, by accepting electronic couples from surrounding molecules or ions, it is a characteristic of transition metals. The coordinative bond is usually of medium strength; it ranks among intermolecular interactions energetically weak present in solids, ionic links and covalent bonds, the strongest type of chemical known bond. The geometry of the compound is the simplest and as more symmetric as possible: for example, if the metal has six identical coordinates molecules, it will form a regular octahedron.

The theory on the energetic structure of coordination compounds is called theory of crystal field and is based on the description of the ionic metal-ligand bond. It describes the complex as a central metal positively charged and negatively charged ligands, which approach the central metal disrupting it from the energy point of view. The entity of perturbation will be different from the various d orbital, depending on their spatial orientation compared to that of perturbing species. This breaks the symmetry energy of electrons in the d orbitals: in the octahedral field two groups of orbitals, whose energetic difference is called "separation energy" of the crystal field and is denoted by Δ_0, are formed. Depending on the extent of this magnitude, that is compared with the electron-electron repulsion that takes place when two electrons are in the same orbit, configurations for different electronic ground state are possible. If the separation, that occurs between two orbitals, is not very high, a high spin elettronic disposition is favourite. But, if the disturbance is very strong and there is therefore a great separation between orbitals, it can be energetically cheaper to pair the binding electrons in a lower energetic orbital (low spin). The iron coordination number, that is the maximum number of coordination bonds, is six: there may be six atoms (called ligands) around the iron that bring into sharing the binding electrons. In fact iron has six electrons of the state 3d in 5 orbitals and four out of five electrons are unpaired. When iron (ferrous state) binds with the four pyrrole nitrogen atoms has no more unpaired electrons and the bonds that it forms become covalent links.

The iron atom within the heme group can exist in oxidation state Fe^{2+} (also called Fe II, reduced or ferrous state), which has six valence electrons in the d orbitals, or Fe^{3+}, (Fe III,

oxidized or ferric state), with 5 valence electrons. In both oxidation states the coordination number of the iron is six. As mentioned before, iron ligands must have an octagonal coordination geometry; porphyrin provides four coordination bonds and another is created with the proximal histidine: so remains a free coordination bond. In most of the heme proteins, in the absence of exogenous ligands this coordination site is empty (or occupied by a water molecule bound very weakly) and can be then used to reversibly bind O_2, but also other molecules such as CO and NO, or ions such as OH^-, CN^- or N_3. When one of these molecules binds it, they complete the octagonal coordination of the iron atom. There is a close connection among the coordination number, molecular structures and magnetic properties of heme. When the iron is in the form of free ion, all its d orbitals have the same energy; within the heme group, the ion iron is bound to protoporphyrin and histidine: these species magnetically disturb d orbitals, by creating the separation of levels as discussed above. There are five types of d orbitals, called d_{xy}, d_{xz}, d_{yz}, d_{x2-y2}, d_{z2}. Under the influence of the "crystal field", the energetic level of these orbitals tend to split; about orbitals d_{x2-y2} and d_{z2}, that point in the direction of ligands, there is an increase in the energy due to the repulsion between the electrons of ligands and those present in these orbitals. The other three orbitals d_{xy}, d_{xz}, d_{yz} not point directly towards ligands therefore repulsion and its variation is less energetic.

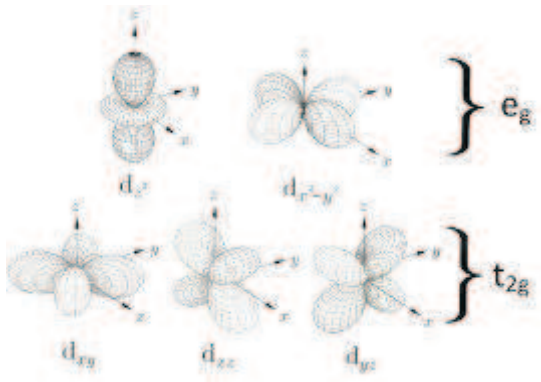


Figure 8: *Spatial orientation of d orbitals.*

In configurations with low spin, 6 (Fe^{2+}) or 5 (Fe^{3+}) valence electrons are all in orbital d_{xy}, d_{xz}, d_{yz}, and antibonding orbitals d_{x2-y2}, d_{z2} are empty. When the complexes are in high-spin orbitals d_{x2-y2}, d_{z2}, each orbital contains an electron. Between high and low spin there is also a difference in the length of iron-ligand bonds, which in the first case are longer; this applies both to axial bonds and to the porphyrin macrocycle, which then widens.

Furthermore in the forms with 6c the iron atom is nearly located in the heme's plane, instead in those with 5c it is out of the heme's plane but in the fifth ligand, the distal histidine, causing a contraction of the cavity of the porphyrin. This happens because in the complex with 5c the perturbation of the field is so high that the state with minimum energy is at high-spin and the emic iron is too large (its radius is 92 pm in human hemoglobin A) to insert the porphyrin in the ring. When the O_2 binds, the field perturbation grows and increases the separation of levels enough to make the shape more prone to a low spin: the radius of the iron is contracted to 75 pm, and the iron is in the tetrapirrolic ring. Thus the distal histidine approaches to the heme and in the human HbA always generates the sequence of structural changes that favor the following links with O_2. For other axial ligands, the field strength can be minor and high-spin forms can be observed even in a hexacoordinated state.

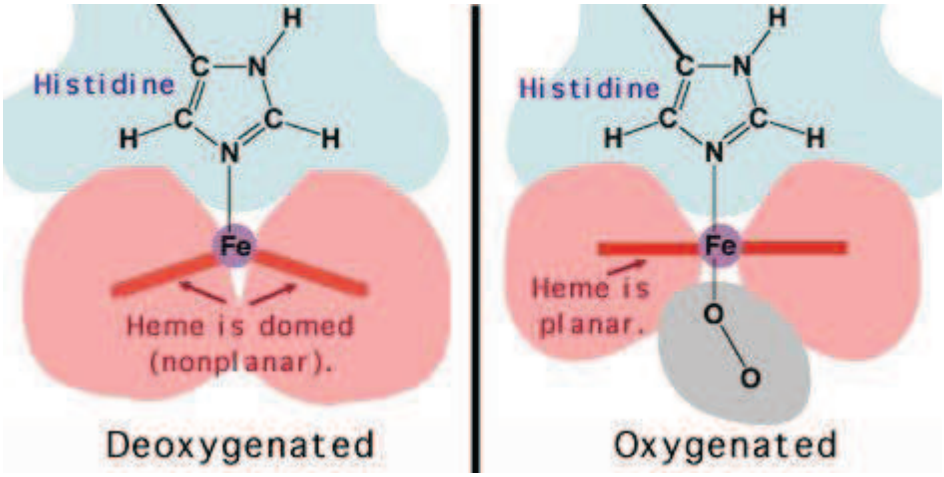


Figure 9: *Heme in deoxygenated and oxygenated state.*

Hemoglobin and Myoglobin are the best characterized examples of pentacoordinated globins, as discussed before. By forming five coordination bonds, Fe^{2+} is in a high-spin state and is able to reversibly bind one molecule of O_2 through the free valence of coordination.

Up until recently, the unbound form of the heme group was thought to be pentacoordinated in all globins.

Therefore, a large curiosity arose about the discovery of so-called "hexacoordinated hemoglobin" in which, even in the absence of ligands, the atom of iron porphyrin, both ferrous and ferric, is involved in six bounds with four pyrrole rings: one with the proximal

histidine and one with another compound (generally another histidine) on the distal surface in the traditional site of oxygen or other exogenous ligands link. This characteristic ensures that the iron is in a state of low spin even when the heme is not bound to exogenous ligands. Hemoglobins, functionally active in pentacoordinate form, may undergo irreversible. Changes that lead to the formation of derivatives with intramolecular hexacoordination but inactive against their physiological ligands. Also in different hexacoordinated hemoproteins not belonging to the globin family, but having often a redox or catalytic function, the endogenous ligand generally can not be displaced by exogenous ligands. On the contrary, hexacoordinated hemoglobins show their biological functions in this state of coordination, by reversibly binding small diatomic ligands, such as O_2, CO and NO, that compete with the endogenous ligand. In the presence of exogenous ligands, hexacoordinated hemoglobins pass from a reduced form to a state of extramolecular hexacoordination, always with a low spin. In such conditions, even reduced Myoglobin and Hemoglobin pass to hexacoordinated low spin state, for which the spectral differences between penta- and hexacoordinated hemoglobins become less obvious and are attributable to different surroundings in which the chromophores are. Usually the residue which completes the coordination bonds of the iron in the absence of ligands it is the exogenous histidine E7 (called "distal"), which in most cases is also present in the pentacoordinated hemoglobin, but which does not directly bind the heme: as mentioned above, the E7 has a function of stabilize the complex when it binds a ligand, by forming with it a hydrogen bond. Despite the coordination bonds of heme iron are complete even in the absence of exogenous ligands, hexacoordinated hemoglobins are able to bind O_2 and other diatomic molecules with high affinity, compared to that of other hemoglobins and myoglobin (Kundu et al., 2003). To explain this feature has been speculated that the distal histidine easily dissociates from the iron but remains close enough to stabilize the ligand forming a strong hydrogen bond with it (Hargrove et al., 2000; Arredondo-Peter et al., 1997). However, the distal histidine causes a slowdown on the binding process, and its dissociation may require a structural rearrangement that is also extended to the distal pocket (Milani et al., 2005). The deoxy proteins may be presented in the purely hexacoordinated form or can be created an equilibrium with the pentacoordinated state of heme iron particularly in cases in which there are extensive conformational changes between the two species. The constant equilibrium can depend on several factors; apart from the temperature (Uzan et al., 2004). Recently it has been observed that the fraction of the hexacoordinated form in the Ngb and in other proteins depends on the pressure and

viscosity of the medium in which it's located (Hamdane et al., 2005). Regarding the oxidized form of this type of hemoglobins, by measures of spectra in the UV-visible absorption is evidence of maintaining a shape in hexacoordinated low spin, in all similar to that of the oxidized form of the cytochromes of the type His-Fe-His. Contrary to the metHb, metMb and any of the hexacoordinated hemoglobins studied so far, a coordination with a molecule of water was observed in the oxidized form. For water is deprotonable, this kind of link may show pH-dependent spectral properties. It would appear that almost all members of hemoglobins, without exogenous ligands, remain in the hexacoordinated state either in the reduced and in the oxidized form. Today, the note and solved structures indicate that the axial ligands are the same regardless of the state of oxidation.

The group of hemoglobins with intramolecular hexacoordination is a subject of considerable and recent interest in the context of the globin superfamily. Both truncated and mini hemoglobin, and protein with "classic" globin folding belong to this group. Ngb is an example of hexacoorinated globin.

Isoniazid (that represents a first line anti-tuberculosis medication in prevention and treatment) binds reversibly the ferric and ferrous *M. tuberculosis* trHb type N with a simple bimolecular process, which perturbs the heme-based spectroscopic properties. Isoniazid (Inh) binds to the heme-Fe atom, suggesting a direct role of Inh in impairing crucial functions of *Mycobacterium tuberculosis*, such as the scavenging of reactive nitrogen and oxygen species and metabolism (Ascenzi et al., 2013). But to date there is not data about an interaction between Inh and Ngb.

2.FLOTILLIN

Flotillins, also called Reggies, are integral membrane proteins that plays a key role in assembling rafts and signal transduction. There exist two isoforms of Flotillin: Flotillin-1 and -2, with an homology of 47% (Slaughter et al., 2003). Both isoforms are evolutionarily well conserved and ubiquitously expressed from fly to man (Malaga-Trillo E. et al, 2009).
Initially it was thought that Flotillin was an integral membrane protein present in caveolae, but following studies on the myeloid cell line THP1 have revealed that in absence of Caveolins, Flotillins become structural proteins which assist the assembly of the rafts. They are proteins that interact with membrane domains rich in cholesterol and sphingolipids and are used as markers of lipid rafts (Morrow et al., 2005). Thus, if co-expressed in the caveolae, Flotillins can interact with Cav-1 and additionally serve as functional substitutes in Cav-1 deficient cells (such as breast cancer cells MCF-7) (Staubach et al., 2011).
The gene of Flotillin-1 is located on chromosome 6 and encodes a protein of 47 kDa, mainly expressed in neurons and hematopoietic cells lacking in Caveolin.

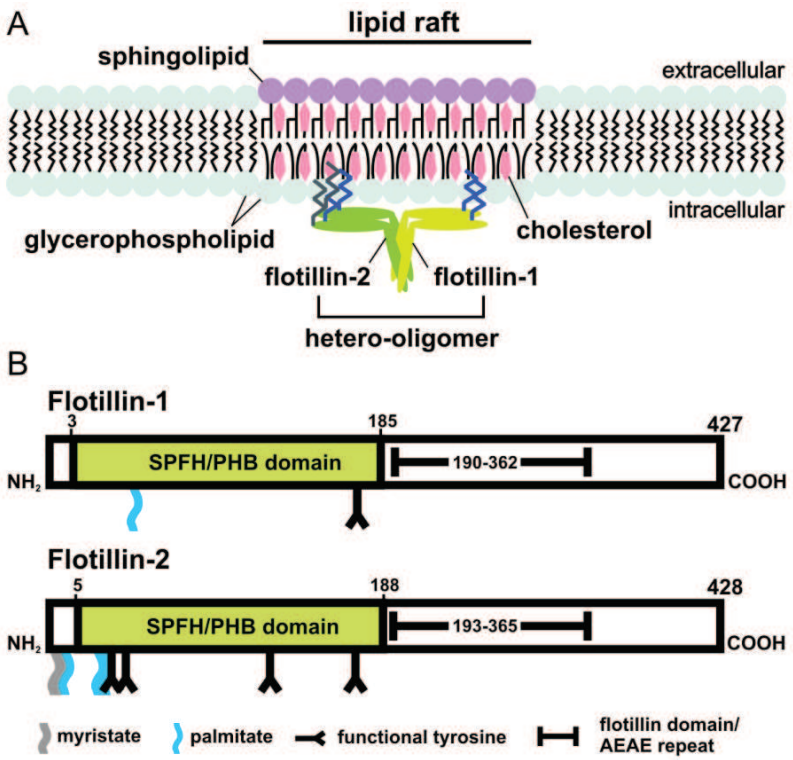


Figure 10: *Flotillins localization (A) and structure (B), (Kurrle et al, Intech, 2012).*

Its location is highly dynamic: it is also expressed in the membrane of phagosomes, endosomes and in the nucleus.

Flotillin-2 instead is a protein of 42 kDa, present in the plasma membrane of epithelial cells and is concentrated mostly in regions of cell-cell contact. It plays an important role in the regulation of signal mediated by raft, including growth factor receptors and adhesion to the extracellular matrix (Slaughter et al., 2003). The Flotillin-2 associates with the membrane after miristoylation in Gly2 and multiple palmitoylation of cysteine residues present at the N-terminus region, which constitute the insertion of the trans-membrane domain in membrane regions with saturated fatty acids modified at N-terminus.

Mutations of Gly2 block miristoylation and palmitoylation of Flotillin-2 so that it becomes soluble and unable to bind to the membrane (Morrow et al., 2005).

Flotillins are proteins that consist of a single polypeptide of 428 amino acids; the N-terminal portion has a region homologous to the Stomatin and Prohibitin (PHB) domain which includes amino acids 83-226. On the contrary the C-terminus region has a small repeating pattern (Morrow et al., 2005). Many studies show that the protein containing the domain PHB have a high affinity for lipid rafts and, since it is present in many proteins, it could represent an important sequence that allows the entry of proteins in rafts.

Flotillin-1 is palmitoylated at Cys34, an essential modification for its localization in the plasma membrane: in fact, mutations of this conserved cysteine residues at the N-terminus reduce palmitoylation and the subsequent incorporation of Flotillin-1 in the plasma membrane. Moreover, the N-terminal region has two hydrophobic regions (aa 10-36 and 134-151): the first region interacts with membrane rafts, the latter is required for the localization in the plasma membrane. The C-terminal region of Flotillin-1 which extends from aa 328 to aa 355 is an α-helix that may form a triple helix wrapped with other monomers of Flotillin.

Flotillin-1 is associated with the membrane compartments of the endocytic pathway (lysosomes and phagolysosome). Traffic Flotillin presents an unusual feature: it reaches the plasma membrane following the N-terminal palmitoylation but not with classical secretory pathway.

Proteins containing the PHB domain have two hydrophobic domains and a trans-membrane domain at the N-terminal that is missing in Flotillin (Morrow et al., 2005). The difference at the N-terminus contributes to differentiate the strategy to target those proteins. In the passage to the plasma membrane Flotillin does not pass trough secretory organelles, unlike the other proteins containing the N-terminus transmembrane domain,

such as Stomatin. This sequence at N-terminus functions as signal for the insertion of the protein that is synthesizing in the endoplasmic reticulum and then will move to the Golgi (Morrow et al., 2005). The absence of this signal sequence induces Flotillin to an independent Golgi traffic (Morrow et al., 2002). The association between Flotillin and the plasma membrane is mediated by two hydrophobic regions in the PHB domain. Thus, the PHB domain plays an important role in the distribution of different groups of membrane proteins having specialized domains with specific lipid composition (Liu et al., 2005). Flotillin function is associated to its localization in rafts and is involved in many cellular processes (Liu et al., 2005):

1. It takes part in the entrance of glucose into the cells stimulated with insulin. It is involved in the activation of the insulin receptor and recruitment of the glucose transporter: Cbl protein is linked to the insulin receptor through one of three SH3 domains of CAP. Following the activation of the insulin receptor and phosphorylation of Cbl, the complex CAP-fosfoCBL moves in rafts forming a ternary complex with Flotillin. This association is required for the signal transduction induced by insulin that regulates the translocation of the glucose transporter (GLUT4) from the cytoplasm to the membrane to require glucose (Chiang et al., 2001).
2. It induces the regeneration of neurons: Pyk2 is a protein associated with the growth factor receptors tyrosine kinase and regulates cytoskeletal remodeling, a key event in the growth of neurites. Pyk2 is associated with Cbl and Argbp2. The expression of Pyk2, Cbl and Argbp2 facilitates the growth of neurites induced by growth factors. Flotillin, binding Argbp2 that binds Cbl and Pyk2 with SH3 domain, drags this complex in lipid rafts (Haglund et al.,2004).
3. It participates in the maturation of phagosomes. Studies show that Flotillin is located in phagosomes after that phagosomes have acquired the lysosome-associated membrane glycoprotein (LAMP 1) following the fusion with endocytic structures. The absence of Flotillin in primary phagosomes indicates that it is not in their plasma membrane; indeed, immunofluorescence studies demonstrate that Flotillin is located in the endocytic structures and is associated with phagosomes only after the fusion of these with late endosomes. The mechanisms are still unclear, but the presence of Flotillin in mature phagosomes and its absence in primary phagosomes suggests a possible role of Flotillin in the maturation of phagosomes (Dermine et al., 2001).

4. It induces cell proliferation of PC3 cells (cell cultures of cancerous prostate). Flotillin, after stimulation with mitogenic agents, translocates to the nucleus and stimulates the proliferation. This process seems to be mediated by a protein PTOV-1, over-expressed in prostatic tumor cells, that translocates to the nucleus of cancer cells. The involvement of Flotillin in cell proliferation is shown by using RNA interference (i.e., gene silencing by small RNAs): silencing both Flotillin-1 and PTOV-1 genes leads to a reduction of cell proliferation. In addition to this, over-expression of Flotillin stimulates proliferation, but the proliferative effect is evident only in the presence of PTOV-1 (Santamaria et a., 2005).
5. It is involved in a Clathrin-independent endocytic pathway. The mechanisms of this process are still not very clear. Some studies show that Flotillin, Caveolin and Clathrin define different regions of the membrane that can be internalized. These three regions can be differentiated according to the mechanism of internalization: Flotillin-dependent endocytosis requires an increase in expression of Flotillin itself.
   Flotillin localization in endocytic structures is demonstrated by immunofluorescence studies with monoclonal antibodies anti Flotillin and by studies of RNA interference (RNAi). Confocal microscopy analysis demonstrates that Flotillin is located on the plasma membrane and on endocytic structures, and Flotillin's RNAi reduces the signal in these regions. The endocytosis of dextran also shows the co-localization of dextran and Flotillin in endosomes and the co-location of dextran and LAMP1 in lysosomes, suggesting that Flotillin is present in the endocytic pathway. Analysis by fluorescence microscopy, also reveal that regions of the membrane containing Flotillin-1 labeled with the fluorescent protein EGFP internalize in a different way compared to clathrin coated vesicles and caveolae. This further endocytic mechanism is demonstrated by the absence of Flotillin in transferrin positive endocytic structures because transferrin internalizes through the classic endocytic clathrin-dependent mechanism (Oleg et al., 2006).

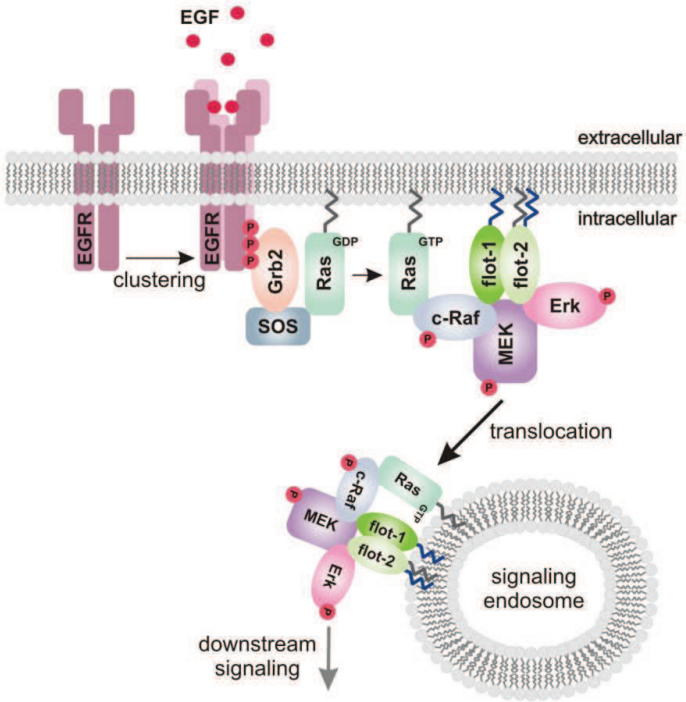


Figure 11: *Flotillin's functions in Receptor TK and endocytosis (Kurrle et al Intech, 2012).*

These data suggest that Flotillin interacts with a small family of adapter proteins that carry a homology domain to Sorbina (SoHO). In mammals three proteins with this domain have been identified: the CAP protein (protein associated with c-Cbl), Vinexina and binding proteins Argbp2. All these proteins have the N-terminus domain SoHO which is a module that links both the Flotillin-1 and the C-terminus domain of the SRC homology (SH3), and allows them to act as adapter proteins. The SH3 domain is rich in Proline and recognizes proteins that have the amminoacidic sequence PxxP (P = proline; x = amino acid). Additionally, proteins with the SoHO domain form a bound between lipid rafts and signaling proteins or cytoskeleton.

The presence of the PHB domain and the ubiquitous expression of Flotillin suggest its importance. One hypothesis could be that Flotillin participates in the generation of the rafts and the stabilization of proteins contained in the rafts themselves through transient ties or with high affinity (Kimura et al., 2001).

In addition Flotillins interact with several proteins.

Flotillin-1 is a crucial molecule in IgE receptor-mediated mast cell activation, and involved in the activation of Lyn (Kato et al. 2006).

The Flotillin-1 interaction with CAP, Vinexin α and ArgBP2 (member of the SoHo family) indicates that Flotillin-1 related to the organization of cytoskeleton (Kimura et al. 2001).
The Flotillin-1 interaction with Ngb suggests that Flotillin-1 might recruit Ngb to rafts to preventing neuronal death (Wakasugi et al., 2004).
Flotillin-2 interacts with F-actin thanks to its SPFh domain regulating its lateral mobility at plasma membrane (Langhorst et al., 2007).
Flotillin-2 interacts with kinesis KIF9 and Flotillins knockdown reduced matrix degradation by macrophage podosomes, so Flotillin and KIF9 can regulate matrix degradation by podosomes (Comfine et al, 2011).
Flotillins are associated also with several cytoskeletal proteins (particularly myosin IIa and spectrin), therefore they play an important roles during neutrophil migration in uropod formation and in the regulation of myosin IIa (Ludwig et al, 2010).
Flotillins are involved also in tumor progression and in neurodegenerative diseases.
Both Flotillin-2 protein and mRNA were increased in tumorogenic and metastatic melanoma cell line *in vitro*. SB2 melanoma cells altered to highly tumorigenic and metastatic in nude mice after transfection of Flotillin-2. These cells proliferated fast in absence of serum and thrombin enhanced their migration. Additionally, the expression of protease activated receptor 1 (PAR-1) mRNA increased in this cells (Hazarika et al., 2004). In this way Flotillin.-2 may play an important role in affecting tumor progression through interacting with PAR-1.
Several studies revealed that Flotillins play a role in the pathogenesis of neurodegenerative diseases (BSE, scrapie and CJD), Parkinson's and Alzheimer's diseases (AD).
Prion diseases are caused by misfolding of cellular prion protein (PrP^c). It was showed that PrP^c was closely associated with Flotillins at plasma membrane in lymphocytes. Furthermore, cross-linking of PrP^c appeared in its clustering in the region of the preformed Flotillin cap (Stuermer et al., 2004). Flotillins were found in lipid-rich vescicles from jurkat T cells together with PrP^c (Reuter et al., 2004). Additionally, scrapie prion protein PrP^{Sc} is localized in Flotillin-1 positive late endosomes in the central nervous system cells (Pimpinelli et al., 2005). So, clustering of PrP may contribute to the spreading of prion diseases.
Flotillin-1 was overexpressed in the substantia nigra of Parkinson's patients (Jacobowitz et al., 2004). Cellular amyloid β-protein (Aβ, a phatological hallmark of AD) is accumulated in Flotillin-1 positive endocytic vescicles. Moreover, Flotillin-1 associated with extracellular Aβ plaques in AD patient brain sections (Rajendran et al., 2007). Sratins, that

reduced the Aβ load by modulating the processing of the amyloid beta precursor protein and reduced the prevalence of AD, also reduced the expression of Flotillin-1 (Kirsch et al., 2003). So, these may indicate an association of Flotillin-1 with AD.

Lipid rafts

Biological membranes are complex two-dimensional structures arising from the assembly of various species of lipid and membrane proteins.

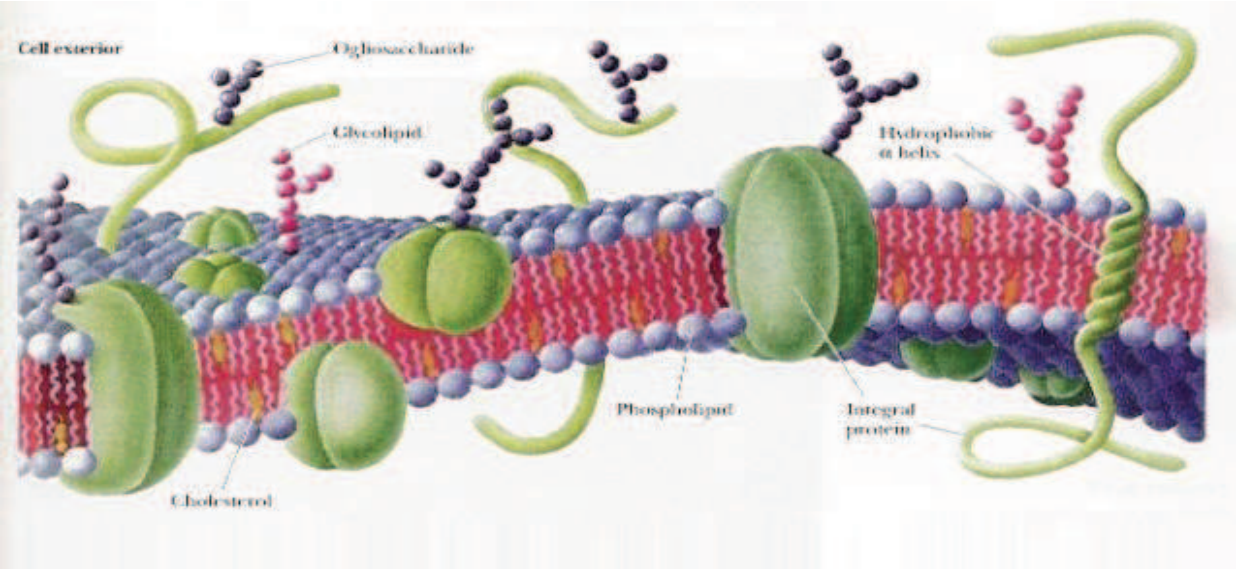


Figure 12: *Plasma membrane structure.*

The fluid mosaic model proposed by Singer and Nicolson (Singer et al., 1972), according to which the cell membranes are fluid and characterized by a random distribution of the molecular components (lipids and proteins) to form a homogeneous structure, has been revisited in years. In fact, several studies suggest that , instead of being fluid and uniform, plasma membranes are characterized by the presence of highly specialized regions, particularly enriched in sphingolipids and cholesterol, named Rafts. These microdomains play an important role in various cellular processes such as membrane trafficking, signal transduction and regulation of the activity of membrane proteins.

Originally called detergent-resistant membranes (DRM), the lipid rafts are defined small (10-200 nm), heterogeneous, highly dynamic, enriched in cholesterol and sphingolipids domains which act as platforms both to concentrate and segregate signaling molecules (Simons et al., 1997) and to compartmentalize cellular processes. In fact, the cell membranes are complex in the composition but highly precise in the purpose.

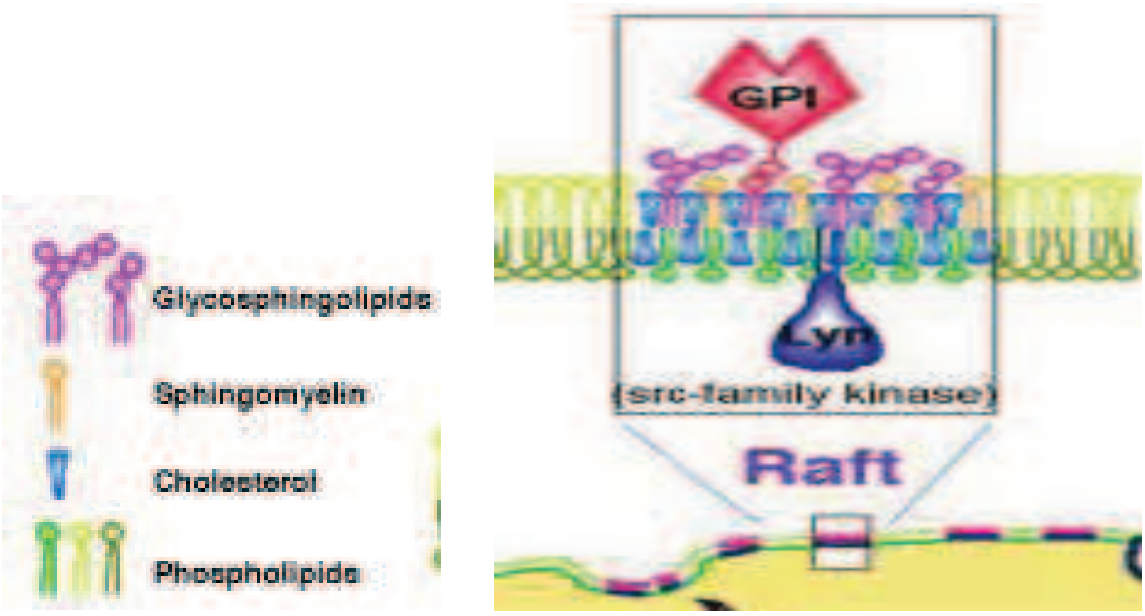


Figure 13: *Lipid rafts structure.*

Literature proposes different models of rafts:

1. they are formed by the spontaneous association of cholesterol and sphingolipids to give a platform for membrane that isolates proteins;
2. they are shells of few lipids that assemble around proteins to give a complex ordered;
3. they are small and unstable complexes of membranes containing at least three molecules present in a region of stabilized structures;
4. they are small lipid molecules dynamically assembled with protein monomers to give stable lipid rafts (Le Roy et al., 2005).

They exist in continuity with no rafts regions of the membrane where the lipid acyl chains are less packed. So, in spite of the fluid mosaic model, lipid in the bilayer exist in different phases with different fluidity: gel, liquid ordered (Lo) and liquid disordered (Ld or Lα).

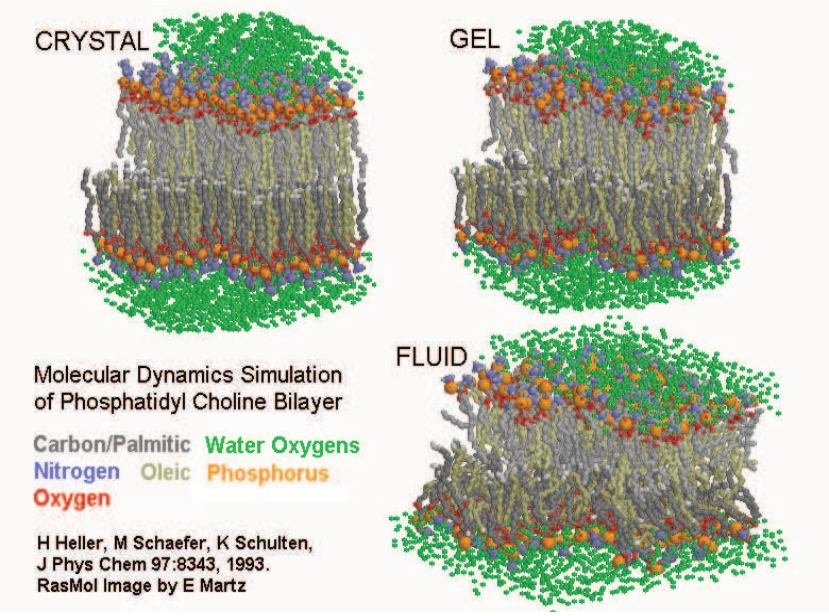


Figure 14: *The three possible phases of the plasma membrane*

Biological membranes of living cells are, in fact, in the fluid state: above the Tm (temperature of Melting), the membrane is located in the liquid-disordered, semi-fluid state, since the hydrocarbon tails of the lipids are free to move and the double bonds of phospholipids, highly unsaturated, introduce folds into hydrocarbon tails that inhibit a close association of the chains. In lipid microdomains, instead, the largely saturated hydrocarbon tails of sphingolipids are closely associated to form a semi-solid gel; however, the high concentration of cholesterol, which intercalates between the chains of saturated fatty acids, inhibits the formation of a perfectly crystalline structure.

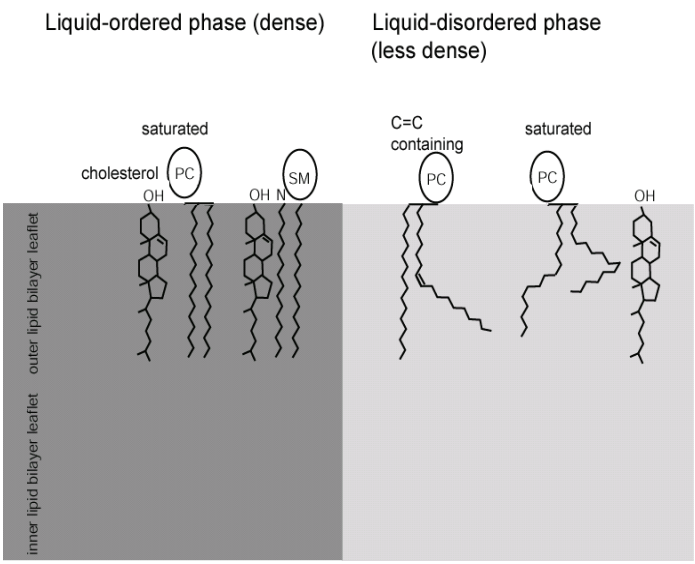


Figure 15: *Liquid-order phase and liquid-disorder phase*

Accordingly, the microdomains exist in a liquid-ordered state, less fluid and thicker (because the acyl chains of sphingolipids are straighter), than the membrane surrounding, with which coexist (Brown 2000).

As tighter packaging of membranes is due to the saturated hydrocarbon chains of sphingolipids and phospholipids present in rafts domains more than to unsaturated fatty acids of the phospholipids in non-raft domains. Sphingolipids have long and saturated acyl chains that increase the interactions between two sides of the membrane and with cholesterol. In fact, the steroidal ring of cholesterol promotes interactions with unsaturated acyl chains of fatty acids more than with saturated chains. Moreover, unlike glycerophospholipids, the binding area between the hydrophilic polar head of fatty acid and sphingosine groups contains both acceptors and donors of hydrogen bonding, thus increasing the possibility of interactions, either with cholesterol and with other sphingolipids (Lingwood et al., 2010).

The presence of cholesterol is particularly important to improve the order of conformational acyl chains, reduce the area o the membrane and increase the thickness of the bilayer, thus contributing to define the density of the lipids packaging. Therefore, cholesterol is effective in reducing the empty space in the region of the acyl chains, allowing to increase the rigidity of binding with increasing concentration of cholesterol. Moreover it has been shown that cholesterol is also able to modify the membrane fluidity. This is important because a change in the fluidity may be related to changes in the structure and activity of membrane proteins (Perttu et al., 2007).

Although a large fraction of the proteins surface has been discovered in liquid-disordered membrane regions, some proteins prefer to be distributed in the rafts domains. Typical examples include glycosylphosphatidylinositol-anchored proteins, the tyrosine kinase Src, palmitoylated and myristoylated proteins like Flotillin, cholesterol binding proteins such as Caveolins, the α subunit of the G protein, phospholipids binding proteins, transmembrane proteins and signal proteins such as SHC and GRB2 (Resh et al., 1999).

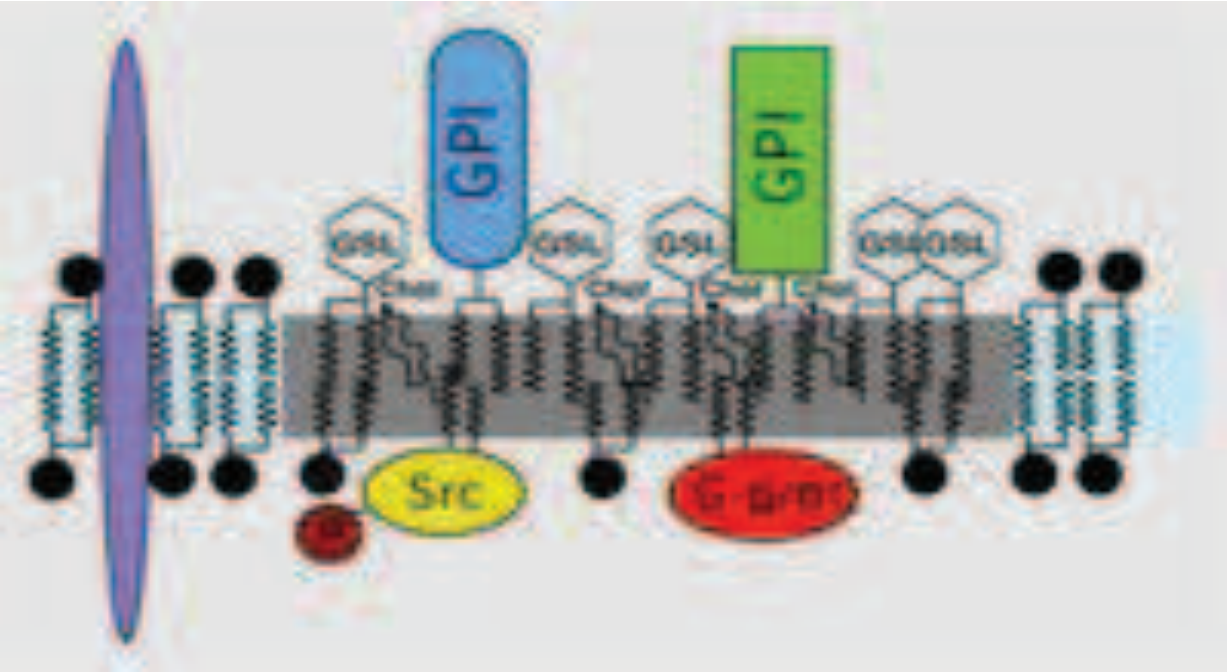
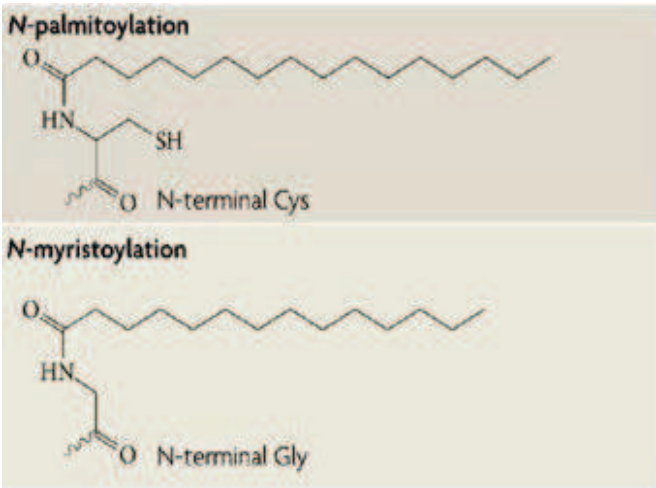


Figure 16: *Lipid-protein interactions in raft.*

Some proteins are typical of specialized rafts, others are distributed in all the rafts. So not only lipids but also proteins are not uniformly distributed in the plasma membrane. The palmitoylation and myristoylation (addition of hydrocarbon chains of sixteen and fourteen carbon atoms respectively) may increase the affinity of the protein for rafts, but is not sufficient for the association.



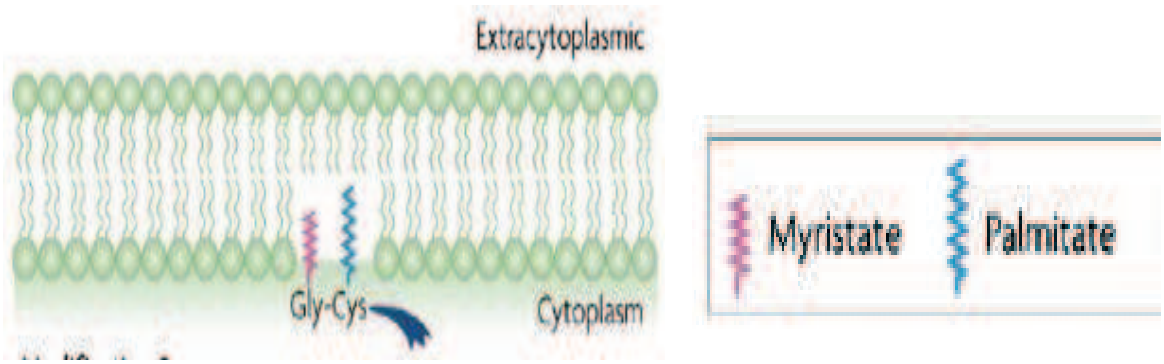


Figure 17: *Palmitoylation and myristoylation at N-terminal portion.*

A necessary condition for entering into rafts is the interaction with structural proteins that are essential for the assembly of rafts, as Caveolin and Flotillin. Flotillin interacts with active GPI-anchored proteins, present in microdomains of the plasma membrane and allows the combined interaction among signaling molecules. The Cav-1 in caveolae interacts through its domain CSD with the G-protein, the endothelial nitric oxide synthase (eNOS), adenylate cyclase and a series of tyrosine kinase (Src family members, MAPK, protein kinase A and C). The binding of signal protein with CSD regulates signal transduction trapping patterns.

The association of proteins with rafts can be both constitutive and transient. In fact, some proteins are associated and dissociated from microdomains with a mechanism regulated by their state of activation. For example, an isoform palmitoylated of H-Ras-GDP resides in lipid microdomains, where it interacts specifically with CAV-1. After linking with the GTP, active H-Ras segregates from markers rafts and this redistribution to non-raft domains is required for an efficient Raf activation (Prior et al., 2001).

Since rafts domains are highly dynamic both lipids and proteins can move with different kinetics.

Being very small rafts, they cannot be identified by techniques such as fluorescence microscopy. Direct evidences of rafts in vivo are mainly based on monitoring movements of membrane proteins or different distributions of fluorescent probes. However, it is difficult to develop experiments that use living cells, which complicates the determination of the effective amount of present rafts, as well as their exact lipid composition, size and half-life (Perttu et al., 2007).

The first method used to biochemically define lipid rafts was based on their resistance to solubilization with Triton X-100 at 4° C. When a non-ionic detergent such as Triton X-100

is added to cells, fluid regions of the membrane are dissolved and the lipid rafts remain intact and can thus be extracted. This is because, in the tightly packed state, lipid-lipid interactions are more stable than lipid-detergent interaction. Therefore, these parts of the membrane are shown in low density fractions after sucrose gradient centrifugation (a technique that, still today, remains crucial to identify and purify different lipids), (Nakahata et al., 2003). These DRM fractions are aggregates of rafts domains and therefore do not represent the native state of lipid rafts in cell membranes. In fact, small rafts can sometimes be stabilized by protein-protein interactions to form larger platforms thanks to cholesterol, the "glue" that binds together these domains.

The distribution of rafts depends on the type of cell: they are abundant in the membrane, in the biosynthetic and endocytic pathway. The rafts present on the plasma membrane are endocytated and form primary endosomes (Mukherjee et al., 2000). Those endosomes can be directly recirculated on the membrane or indirectly via recirculating endosomes that convey primary endosomes first to the Golgi and then to the membrane (Puri et al., 1999).

Although all lipid rafts have a characteristic composition, these microdomains are heterogeneous in their fat and protein content; indeed, there are several subclasses of lipid microdomains characterized by a different distribution of proteins, which determines the morphology and function (Pike et al., 2003). In addition, the lipid microdomains on the cytosolic surface of the plasma membrane do not consist of sphingolipids that are exclusively present on the extracellular side of the membrane.

According to morphological and biochemical criteria, only Caveolae have been well characterized among all microdomains. As shown by the figure below, they are microinvaginations of the plasma membrane, of 50-100 nm in diameter.

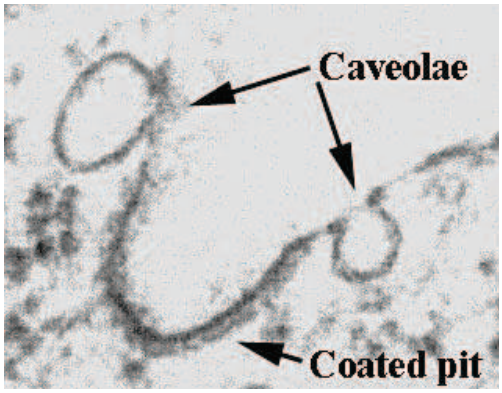


Figure 18: *Electron microscope image of caveolae on the plasma membrane of NIH3T3 cells.*

Markers of caveolae are Caveolins, integral membrane proteins of 21-24 kDa, responsible for the function, biogenesis and stabilization of caveolar invaginations. The family of Caveolins consists of three different proteins: CAV-1, -2, -3.

Caveolins have specific properties that are important not only for the selective localization to the caveolae but also for the formation of these structures. Cav-1 directly binds the two main constituents of lipid microdomains, cholesterol and sphingolipids, both *in vivo* and *in vitro*, conditions, and is modified by palmitoylation at three different parts of its C-terminal domain; these modifications guide the Cav-1 to caveolae and stabilize its localization. In addition, Cav-1 is capable of homo-oligomerization or hetero-oligomerization, such as Cav-2 and Flotillin-1, guiding the formation of caveolae (Razani et al., 2001).

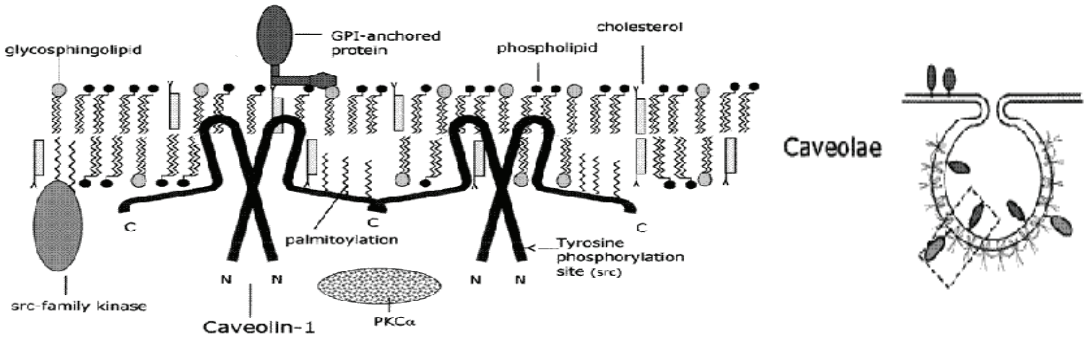


Figure 19: *Caveolin structure in caveolae.*

Llipid rafts, contrary to caveolae, are morphologically distinct from the surrounding membrane. They are lipid platforms of 50-300 nm, derived from the aggregation of sphingolipids and cholesterol, which lead to a separation from the rich side of glycerophospholipids; protein markers of these microdomains are the GPI-anchored proteins and Flotillin-1, but there is not Cav-1 (Simons et al., 1997).

Flotillin was identified for the first time in membrane domains rich in Cav-1 and purified from lung tissue; therefore, Flotillins define a new family of transmembrane proteins associated with caveolae and may contribute, together with Cav-1, to the structural organization of the caveolar membranes.

Caveolins and Flotillins, when co-expressed in the same cells, form a stable hetero-oligomeric complex. However, the expression of Flotillin-1 and -2 is not related to the patterns of expression of the members of the Caveolins family. In fact, cells which do not express Cav-1 and consequently do not possess morphologically distinct caveolae,

continue to express Flotillins. Therefore, the expression of Flotillin-1 and -2 is not sufficient to generate mature invaginate caveolae; but this does not preclude a role in the formation of domains similar to caveolae, but slightly larger. In fact they were lipid microdomains purified from cells and tissues without caveolins. These microdomains or lipid rafts were purified according to their insolubility in Triton X-100.

Flotillin-1 is probably involved in the assembly of lipid rafts and represents a protein marker of these microdomains in cells that do not express Cav-1 and which do not contain caveolae (e.i. hematopoietic cell lines, cells of the nervous system) (Volonte et al., 1999).

In order to make a classification of different membrane lipid microdomains two major classes can be identified:

1. one class that contains a structural protein Caveolin, responsible for the formation of real caveolae and which may also contain Flotillin;
2. another class that lacks Caveolin, but expresses other specific proteins of lipid rafts, including Flotillin-1.

It is not known if the two types of domains can have different functions and lipid rafts themselves are heterogeneous in their protein composition.

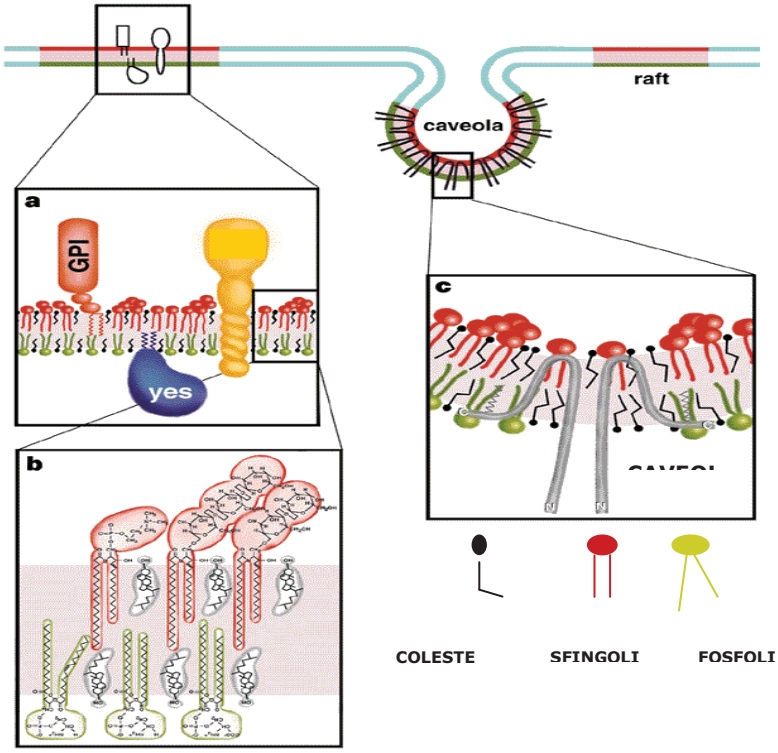


Figure 20: Model of organization of lipid rafts and caveolae in plasma membrane.

3.HEMATOPOIESIS

Hematopoiesis is an highly orchestrated process by which immature precursor cells develop into new mature blood cells, which includes red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (Orkin et al. 1995,1996). It begins early in embryonic development and continues throughout the lifetime of an organism.

In developing embryos, blood formation occurs in aggregates of blood cells in the yolk sac. When bone marrow develops, it, with its intersinusoidal spaces, assumes the task of forming most of the blood cells for the entire organism. However, maturation, activation, and some proliferation of lymphoid cells occurs in secondary lymphoid organs (spleen, thymus and nodes). In children hematopoiesis occurs in the marrow of the long bones, in adults it occurs mainly in the pelvis, cranium, vertebrae and sternum.

The sinusoids (venous channels) feed into the marrow venous drainage system, they are lined with specialized fenestrated endothelial cells. These cells produce growth factors and cytokines, which influence proliferation and differentiation of hematopoietic cells and thus play an important regulatory role. Mature blood cells enter the blood stream by passing through the sinusoidal wall to get into the sinuses. The bone marrow stroma contains many different cell types, including macrophages, fibroblasts, endothelial cells, smooth muscle cells, T-lymphocytes, monocytes. These cells, in combination with components of the extracellular matrix and basement membranes as well as a plethora of soluble and membrane-bound cytokines and growth factor, form the so-called hematopoietic inductive microenvironment (HIM), which maintains the functional integrity of this complex system of resident and circulating cells (Trentin, JJ. 1971).

All different types of blood cells are derived from a small common pool of totipotent cells, called hematopoietic stem cells (HSCs). These cells have the unique properties to give rise to new hematopoietic stem cells (self-renewal) and generate primitive progenitors that are programmed to differentiate. This process is called steay-state hematopoiesis or constitutive hematopoiesis.

During or after cell division the two daughter cells of a HSC have to decide their fate.

They can either choose the same (symmetric division) or different (asymmetric division) fate. Thus, they can either choose to remain as HSCs, commit to differentiation, to die by apoptosis and also to stay in the bone marrow or migrate to periphery (Domen, J and Weissman, IL., 1999). The HSC cell-fate decision involves a complex interplay between

intrinsic genetic processes of blood cells and their environment (Brown G. et all, 1985; Orkin SH., 1995; Singh, H., 1996).

When the totipotent hematopoietic stem cells choose to divide through asymmetric cell division, advance in their differentiative program gradually producing specific receptors and/or molecular markers, which reflect both the degree of differentiation achieved and their ability to recognize certain differentiation factors (cytokines). In this way, gradually are characterized different progenitor cells that are still immature but "committed", directed towards one or other of mature cell lines. By defining their differentiation programs, progenitor cells lost their ability to self-renewal and moving toward the desired phenotype to become, at the same time, more mitotically active.

HSCs can be divided into a long-term subset (LT-HSC), capable of indefinite selfrenewal, and a short-term subset (ST-HSC) that self-renews for a defined interval. LT-HSCs divide into ST-HSCs that give rise to the briefly self-renewing multipotent progenitors (MPPs) which then differentiate into oligolineage-restricted progenitors through functionally irreversibile maturation steps (Fig 21) (Morrison, SJ. Et al., 1997).

Two kinds of oligolineage-restricted progenitors have been identified: the common lymphoid progenitors (CLPs), which at clonal level are restricted to give rise to T lymphocites, B lymphocites and natural killer cells (Kondo, M. et al., 1997), and the common myeloid progenitors (CMPs) which are progenitors for thr myelo-erythroid lineages (Akashi, K. et al., 2000). CMPs give rise to myelomonocytic progenitors (GMPs), which in turn produce monocyte/macrophages and granulocytes, and to megacaryotic/erithroid progenitors, which differentiate into megakaryocytes/platelets and erythrocyte.

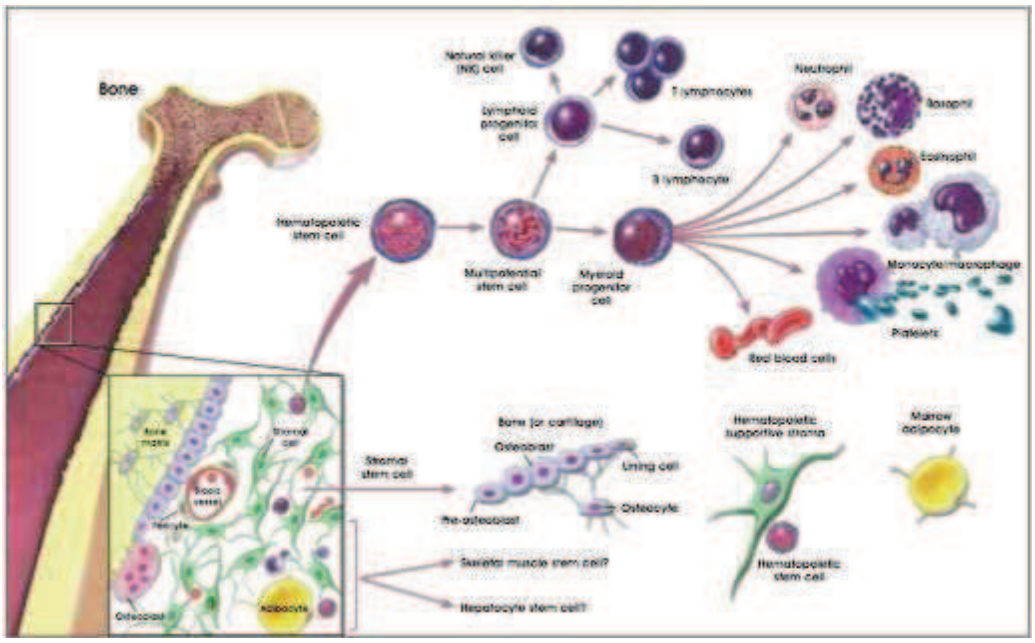


Figure 21: *Hematopoietic stem cell differentiation in the bone marrow microenvironment.*

Lymphopoiesis and myelopoiesis differentiation are stepwise processes cheracterizide by the alternate expression of growth factors, their receptors and transcriptional regulators. Transcription factors present in a particular cell are characteristic of the hematopoietic line to which it belongs and its stage of development and maturation. So the complex interaction between transcription factors, co-regulatory molecules and specific sequences of DNA binding, determine the line-specific gene expression and subsequent cell differentiation. The self-renewal of the stem cell population in the bone marrow, the proliferation and differentiation of hematopoietic progenitor cells, their survival and also all functional activities of the circulating mature forms are subject to regulation by a cascade of proteins that are generally known as growth factors or cytokines. These factors are products of stromal cells and other cells and they are produced through both autocrine and paracrine mechanisms. Cytokines mediate positive and negative effects on multiple cellular functions by engaging a specific receptor and activating a variety of signaling pathways (Ogawa, M., 1993, Zhu J., Emerson SG., 2002).

They are many and of different origin:

- **Stem cell factor** (SCF): it binds to the c-Kit receptor and plays a role in the regulation of HSCs in the bone marrow stem cell niche increasing the adhesion capacity of HSCs to ECM proteins and stromal cells (Broudy VC., 1997).

- **FLT_3 ligand** (FLT_3-L): the ligand of FLT_3 receptor. This is a tyrosine kinase receptor that was at first noted on stem cells and committed lymphoid precursors (Matthews W. et al., 1991). Its ligand (FL) was shown to be an active proliferative stimulus for stem and developing dendritic cells, particularly when acting in synergy with other growth factors (Vigon I. et al., 1992).
- **Multylineage colony stimulating factor** (multi-CSF o IL-3): it stimulates the differentiation of multipotent hematopoietic stem cells into myeloid progenitor cells (Metcalf, D. 2008).
- **Macrophage colony stimulating factor** (M-CSF): it's involved in the proliferation, differentiation and survival of monocytes, macrophages and bone marrow progenitor cells (Stanley ER., 1975).
- **Granulocyte-macrophage colony stimulating factor** (GM-CSF): stimulates stem cells to produce granulocytes (neutrophils, eosinophils and basophils) and monocytes (Metcalf D., 2008).
- **Granulocyte colony stimulating factor** (G-CSF): it initiates proliferation and differentiation into mature granulocytes (Metcalf D. and Nicola NA., 1983).
- **EPO**: it is the humoral regulator of red cell formation.
- **IL-5**: it is the major regulator of red cell formation.
- **TPO**: regulates platelet production (Lok, S. et al., 1994).

The most important feature of cytokines was their polyfunctionality. They were not simply proliferative stimuli but also had actions affecting survival, differentiation, commitment, induction of maturation and functional activation of mature cells, they may also facilitate the interactions between stem cells and elements in the microenvironment including extracellular matrix (ECM) components (Kinashi T. and Springer TA., 1994). Newly discovered cytokines including Wnt and the notch ligand family may also have important effects on stem cell biology (Milner LA and Bigas A. 1999; Van Den Berg DJ. Et al 1998). Chemokines are another class of compounds that in hematopoiesis function as positive and negative regulators of proliferation, cell trafficking and homing (Christopherson K 2nd, Hromas R. 2001). Other important environmental regulators of hematopoiesis include the ECM components, hematopoietic and non-hematopoietic cells, nutrients and vitamins and a variety of physiologic processes. ECM components provide a scaffold for colocalizing progenitors and HSCs with a wide array of positive and negative cytokines and other growth regulators. In addition, ECM and stromal components may directly mediate signaling to HSCs to activate growth, protect cells from apoptosis or modulate responses to

positive and negative regulatory factors. HSCs and progenitors binding to these ECM components is mediated by adhesion molecules, including integrins, selctins and mucins. Hematopoietic and nonhematopoietic cells that regulate hematopoiesis are NK cells, T cells, macrophages, fibroblasts, osteoblasts (Taichman RS. Et al., 2000). These cells may produce important growth factors, facilitate engraftment or induce apoptosis.

In addition to this wide array of environmental factors that regulate hematopoiesis, a number of intrinsic genetic events are critical to determine cell fate. The Rb family, cyclins, Hox and other gene families appear to regulate proliferation and self-renewal of early hematopoietic cells. The Bcl family and Fas receptor with its ligand, caspases, regulate apoptosis in hematopoietic cells. Among progenitors, lineage commitment is accompanied by loss of expression of genes associated with unrelated lineages (i.e. Hox genes), (Park, IK. et al. 2002). This promiscuous gene expression by HSC may provide a framework for stochastic fluctuations in expression of signaling or transcriptional complexes, which ultimately are amplified or repressed to cement lineage choice. These findings support the so called stochastic model according to which HSC randomly commit to either self-renew or differentiate. Citokines present in the bone marrow milieu do not direct this choise per se, but do allow survival and proliferation of the cells that ultimately develop into mature lineages (Wagers, AJ et al., 2002). Earlier, the instructive model suggested, on the contrary, that differentiation of cells into one of several lineages critically depends on the nature of factors acting on these cells at a particular time, at a particular concentration and/or in a particular sequence (Trentin JJ., 1971).

Finally gene-regulatory microRNAs can modulate hematopoietic cell differentiation and proliferation and also the activity of hematopoietic cells, in particular those related to immune function (Garzon R. and Croce, CM.2008).

Alterations in the balance between self-renewal and differentiation can lead to the emergence of cells that survive and growth of normal cells and hence to the establishment of leukemias.

4.CELLULAR STRESS CONDITIONS

Cellular stress is a condition in which physical, chemical, or biological agents can cause abnormalities in the cellular physiology by creating a condition of cytotoxicity that often results in death.

4a.OXIDATIVE STRESS

Reactive oxygen species (ROS) have been considered for long time toxic side byproducts of the aerobic metabolism from which the cell is defended by adopting scavenging enzymatic (catalase, glutathione peroxidase/reductase and superoxide dismutase) and non-enzymatic (vit.E, vit.C, glutathione and uric acid) systems. Moreover ROS play also a role as physiological secondary intracellular messengers (Finkel 1998). The production of ROS by phagocytic cells constitutes an essential mechanism of defense against external agents and ROS are produced similarly to the stimulation of growth factors that are involved in cell proliferation (Finkel, 2000). Therefore, pathophysiological conditions related to ROS activity can depend on both an imbalance in the radical amount and on different factors, such as genetic or dietary or environmental, that activate radical scavenging systems: this condition is defined "oxidative stress". Thus oxidative stress is that condition in which the dynamic balance between oxidative and reductive cellular processes is shifted in favor of oxidative processes, with the production of numerous radical species (Sies 1991).

It is induced both by dangerous events, such as oxidative DNA damage and lipid peroxidation, and by physiologic adaptative mechanisms and regulation of intracellular signal transduction.

Free radicals are highly unstable and reactive molecules, characterized by the presence of an unpaired electron in their outer orbital. Among free radicals, the most known species are those in oxygen content (ROS) as the anion superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) and are due to environmental factors (UV rays, pollution, drugs, etc...) and endogenous factors (electron transport system in the mitochondria, activity of phagocytic cells).

Anion superoxide $O_2^{\cdot -}$ The superoxide anion is produced by the one-electron reduction of a molecular diatomic oxygen, reaction that is frequently in nature. With a unpaired electron, the superoxide anion is a free radical and, as well as molecular oxygen, has paramagnetic characteristics.

It can be formed through the following mechanisms:

1. metal-dependent oxidations of neurotransmitters such as epinephrine and norepinephrine and of some thiol compounds;
2. interactions of molecular oxygen with electrons that occasionally escape from the respiratory chain, especially in the transition between the oxidation-reduction of CoQ and cytochromes, at the level of NADH-ubiquinone reductase and ubiquinone-cytochrome c reductase enzymes;
3. a direct production during some specific enzymatic reactions catalyzed by xanthine oxidase, tryptophan and indolamines deoxyigenase.

The superoxide anion, although cytotoxic, may cause limited damages since it is not able to cross the mitochondrial membrane, because negatively charged; it can also be inactivated by the protective enzyme superoxide dismutase (SOD), which converts it to hydrogen peroxide, and other compounds such as vitamin A, able to sequester it (Halliwell et Gutteridge, 1990).

$2\ O_2^{\cdot -} + 2\ H^+ \qquad H_2O_2 + O_2$ [1]

Hydrogen peroxide H_2O_2 By the dismutation reaction [1] hydrogen peroxide, which is not normally toxic since is quickly neutralized by the catalase [2] and glutathione peroxidase [3] is produced.

$H_2O_2 + 2\ e^- + 2\ H^+ \quad 2\ H_2O$ [2]

$H_2O + 2\ GSH \qquad 2\ H_2O + GSSG$ [3]

The hydrogen peroxide can be formed enzimatically, as shown before [1], as well as not enzimatically in mitochondria and peroxisomes. It is present in a range of concentrations between 10^{-9} and 10^{-7} M, and it is able to quickly cross cellular membranes and thus move to other districts where it can activate the peroxidation process.

Hydroxyl radical HO$^{\cdot}$ A molecule of superoxide anion $O_2^{\cdot -}$ and a molecule of hydrogen peroxide, can combine to form a molecule of oxygen, a hydroxyl radical $OH^{\cdot}$ and a ion hydroxyl ion according to the reaction of Haber-Weiss [4], catalyzed by ions Fe^{2+}or Cu^+(Kehrer, 2000) (Cuzzocrea et al.,2001).

$O_2^{\cdot -} + H_2O_2 \qquad O_2 + HO^{\cdot} + OH^-$ [4]

To produce the hydroxyl radical the presence of the ferrous ion Fe^{2+}, is fundamental, even though the metal is normally bound to proteins of transport and storage (transferrin, ferritin), or functional proteins (Hemoglobin, Myoglobin, Neuroglobin) in the form of ferric ion Fe^{3+}. To make the Fe^{2+} ion free is sufficient the presence of high amounts of superoxide anion [6] or a lower pH level due, for example, to the production of lactic acid (ischemia or anoxia). Thus the ferrous ion can oxidize again by reacting with H_2O_2 and forming $HO^{\cdot}$ according to the Fenton reaction:

$Fe^{3+} + O_2^{-\cdot}$ $\quad$ $Fe^{2+} + O_2$ [5]

$Fe^{2+} + H_2O_2$ $\quad$ $Fe^{3+} + OH^- + OH^{\cdot}$ [6]

The net result of these two reactions is that of Haber-Weiss that occurs in our bodies. The hydroxyl radical generated in the reactions [4] is, among the oxygen radicals, the most toxic molecule because is highly reactive and lack of any endogenous mechanism of inactivation. It is responsable for the initial phase of the peroxidation reaction that occurs in body tissues. The hydroxyl radical is, in fact, able to damage all cellular macromolecules: proteins, nucleic acids, glycosaminoglycans and especially polyunsaturated fatty acids of phospholipidic membranes.

Singlet oxygen $^1O_2$ This chemical form of oxygen is not a real free radical but an electronically excited form of O_2, able to quickly attack many molecules, including polyunsaturated fatty acids: it differs from the fundamental state (triplet) of the molecular oxygen for reversing the spin direction of an electron in the outermost valence orbital. In vitro studies it shown that singlet oxygen can oxidize different organic molecules, such as membrane lipids, proteins, nucleic acids , carbohydrates, and thiols. There are four main mechanisms of reaction:

1. reacting with phenolic compounds to form hydroperoxydyenons;
2. energy transfer reaction that transform $^1O_2$ in $^3O_2$ with compounds such as carotenoids, bilirubin, tocopherol, phenols, nickel complexes and ions;
3. addition reactions with systems of conjugated dienes (reaction Diels-Alder) to form cyclic endoperoxides;
4. addition reactions with the double carbon-carbon bond in unsaturated olefins to form hydroperoxide.

Singlet oxygen can be formed after an exposure to ultraviolet radiation (320-380 nm) or during the activation of macrophages in immune response. Myeloperoxidase, a heme-protein, uses hydrogen peroxide to convert the chlorine into hypochlorous acid [7]; the latter reacts again with H_2O_2 to form singlet oxygen [8].

$H_2O_2 + Cl^-$ $HClO + OH^-$ [7]

$H_2O_2 + HClO$ $^1O_2 + H_2O + Cl^- + H^+$ [8]

The singlet oxygen appears to be formed even during the lipid peroxidation, giving development of chemiluminescence.

The mechanism by which ROS damage the lipid membranes has been well characterized and consequently the oxidative damage has often been associated exclusively to the reaction of peroxidation of membrane lipids (Esterbauer et al., 1991). Actually the ROS are also able to degrade proteins and nucleic acids, with consequences also lethal. As for the proteins, ROS can lead to oxidation of their skeleton causing the fragmentation or oxidation of their amino acids, or even to the formation of protein-protein crosslinks. While the oxidative damage to nucleic acids can be repaired with high efficiency through mechanisms of deletion and insertion, repairing the damage of the protein appears to be limited to the enzymatic reduction of oxidized sulfur-containing amino acids: in fact only cysteine and methionine are subjected to reversible oxidation. The repair of other types of oxidize proteins has not been demonstrated: the damage of the protein becomes, therefore, the target of various endogenous proteases such as cathepsin C, calpain, trypsin, and especially proteasome (Grune et al., 1995; Grune et al., 1996).

Nucleic Acid

As part of the cellular damage caused by reactive oxygen species, the oxidized form of DNA is potentially the most dangerous result, since these alterations are often associated with genetic mutations and the development of cancer. It has been emerged also an increasingly clear link between ROS-mediated alterations to DNA and the aging process, the pathogenesis of mellitus diabetes, certain diseases of the liver and other pathology whit an inflammatory etiology. Examples of damage to nucleic acids are, among others, the formation of intermolecular DNA-DNA or DNA-protein bonds and oxidative modifications to caused by nitrogenous bases. The most reactive species are pyrimidine bases, cytosines and thymines, which may run into saturation or ring opening with hydroxylation of the latter. This implies the loss of the aromaticity and planarity,

determining distortions in the geometry of the DNA. In addition the oxidation of thymine can lead to the formation of so-called "thymine dimers". A common oxidative alterations of purine concerns instead the hydroxylation in position 8 of guanosine and detachment of the base nitrogen from sugars. It was estimated that the urinary excretion of 8-hydroxy-deoxyguanosine (8-OHdG) increases after exercises and is considered a measure of the DNA oxidation in response to free radicals (Urso et al.,2003). If damaged bases are removed and repaired before the cellular division, there will be no permanent damage. If, instead, the system repair is prone to errors, the cellular generation will have a defective DNA molecule in which a nitrogenous base is eliminated or replaced by an improper base.

Proteins

Proteins are also a target for free radicals. For proteins the damage can be divided into reversible, such as the oxidation of thiol groups of methionine to sulfoxide, and irreversible, the breakage ring of istidine and tryptophan and the hydrolysis of the peptidic bond in the presence of pralines. The latter particularly harms the collage, rich in proline and hydroxyproline. The SH groups of cysteine residues are the most exposed to collisions radical: radicals tiili (RS•), that are formed, can dimerize or oxidize to RSO_2, causing damages to the structure and function of proteins themselves.

In particular they can be attacked by proteins with an enzymatic function, the phosphofructokinase, that belong to the mitochondrial respiratory chain, and have a fundamental importance for the production of energy in the cell.

The oxidation of protein seems to be also responsible, at least in part, of diseases such as atherosclerosis, ischemia–reperfusion and aging.

Carbohydrates

Free radicals quickly react with carbohydrates by easily extracting hydrogen atoms: deoxyribose, ribose, proteoglycans and heteropolysaccharides (hyaluronic acid) can be degraded by oxidative attacks. This is especially detrimental to proteoglycans, molecules of high molecular weight that are part of the parenchyma tissue, which undergo fragmentation and depolymerization with structural and functional irreversible damages.

Lipids

Lipids are mainly important in membranes that surround each cell. In lipids the oxidative action proceeds with a radical mechanism, in the carbonic chain, called lipid peroxidation. For years, the lipid peroxidation has been associated with the chemistry of oils and the phenomenon of fat rancidity, and the interest in peroxidative processes was confined mainly in industry and food technology.

However, the auto-oxidative reaction of organic compounds has a paramount importance for all living organisms in the oxygen atmosphere whose survival depends on organic molecules.

Lipid peroxidation is developed through three consequential phases:

•initiation: In-In 2 In·
In·+R-H In-H+R·

•propagation: R·+O_2 ROO·
ROO·+R-H ROOH+R·

•termination: 2 ROO· Products

In the first, the initiation, a radical specie, produced by a suitable initiator at the speed Ri, generates the radical R •.

In the propagation step the radical R • reacts with molecular oxygen to give the peroxyl radical, which in turn attacks the substrate, generating a new R • radical that propagates the chain.

In the termination step, the radical specie may combine in different ways giving not radical products, that interrupt (ending) therefore the oxidative chain. Given that, in the stage of propagation, the cyclic production of alkyl and peroxyl radicals, can involve hundreds or even thousands of units, leading to the formation of a radical initiator, which can be a random event, and to the oxidative destruction of numerous molecules of the substrate.

Deleterious effects of the auto-oxidation reaction at the level of biological membranes are now widely known: newly formed conjugated double bonds modify the steric arrangement of the phospholipidic chains, and fatty acid molecules itself undergo oxidative cleavage with formation of cyclic and non-cyclic compounds.

Moreover, products of fatty acids oxidation, mainly consisting of peroxides and hydroperoxides, are usually polar compounds that tend to migrate towards the surface of the lipid bilayer and lead to a reduction in the fluidity of the membrane .

The final result of such modification is a profoundly altered membrane, more permeable and with reduced biological activity of inserted proteins, which implies negative effects on exponing antigens and receptors, and on the enzymatic activity of membrane proteins.

4b.DNA DAMAGE

Cells have developed specific mechanisms to recognize sites of DNA damage and to promote the recruitment of DNA-repair proteins. When DNA damage is present, checkpoints are activated and the cell-cycle progression is slowed down or arrested to promote the DNA damage repair. Once the DNA damage has been removed, cells resume their proliferation.

An inability to repair DNA damage leads to a programmed cell death (apoptosis) or causes cell entrance into permanent cell cycle arrest (senescence).

Central in the DNA-damage response (DDR) are two protein kinases of the PIKK (Phosphatidil Inositol 3-Kinase like-kinase) family, ATM (Ataxia Telangiectasia Mutated protein kinase) and ATR (Ataxia telangiectasia and Rad3 related), with distinct but partially overlapping functions (Shiloh, 2003). These kinases are activated by DNA damage-binding proteins, like RPA or the MRE11-RAD50-NBS1 (MRN) complex (Uziel et al, 2003), which act as "sensor" of damage.

Once activated, ATM and ATR phosphorylate a range of factors, including checkpoint kinases ChK1 and ChK2, that then target various effectors involved in DNA repair and cell-cycle progression (Bartek et al., 2007).

Another target of PIKKs is the histone H_2AX, which is phosphorylated (y- H_2AX) extensively in the chromatin flanking sites of damage.(Fernandez-Capetillo et al.,2004). y-H_2AX induces changes in chromatin structure that facilitate the accumulation of DNA-repair and checkpoint proteins, favouring the DDR.

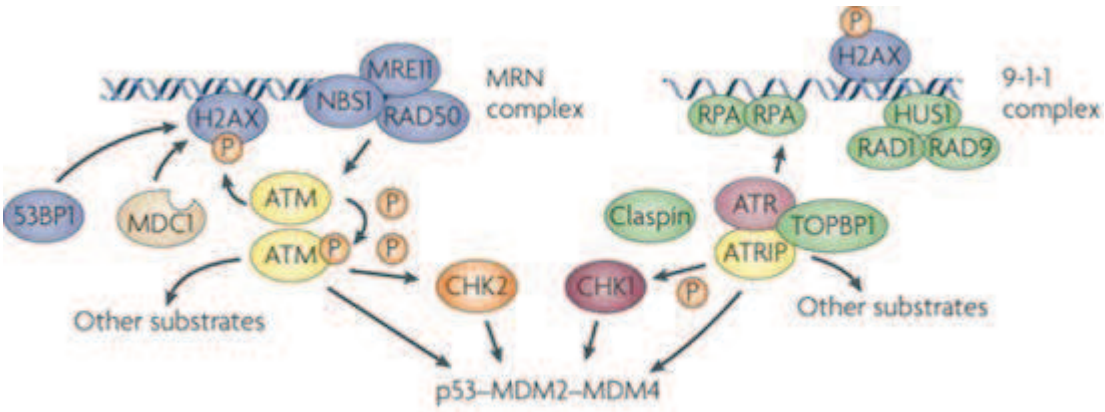


Figure 22: *DNA damage response signaling pathway* (Meek, Nat Rev Cancer, 2009).

The DNA double-strand breaks (DSB) are the most deleterious among DNA modifications: they can lead in fact to cell death or cancer if improperly repaired (Khanna et al.,2001). DSBs can be induced by reactive oxygen species, ionizing radiation (IR), and certain anti-cancer drugs. NHEJ (Non-Homologous End Joining) and HR (Homologous Recombination) are the two different mechanisms by which cells repair DSBs.

NHEJ witch simply pieces together the broken DNA ends, has no such requirement and can, therefore, be operational in all phases of the cell cycle and is the predominant repair pathway in mammalian cells. Main components of NHEJ are the Ligase IV/XRCC4, Ku complex and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Ligase IV/XRCC4 is recruited to DNA ends via interaction with Ku and it is responsible for NHEJ ligase activity (Grawunder et al., 1997). The Ku complex is a heterodimer formed by Ku70 and Ku86 (also known as Ku80) subunits, with high affinity for the ends of dsDNA, implicated in alignment and synapsis of DNA ends and recruitment of additional factors important for processing of DNA breaks (Dyan and Yoo, 1998). DNA-PKcs is a member of the phosphoinositide-3-kinase-related (PIKK) family, which physically associates with the Ku heterodimer forming a catalytically active DNA-PK holoenzyme. The DNA-PK complex has serine/threonine kinase activity. Ku recruits DNA-PKcs to a DSB, leading to a rapid activation of DNA-PK in response to DNA damage (Falck et al., 2005). The activation of this kinase appears to require a direct interaction of DNA-PKcs with free DNA. Little is known about DNA-PKcs function in DSB rejoining or its phosphorylation targets.

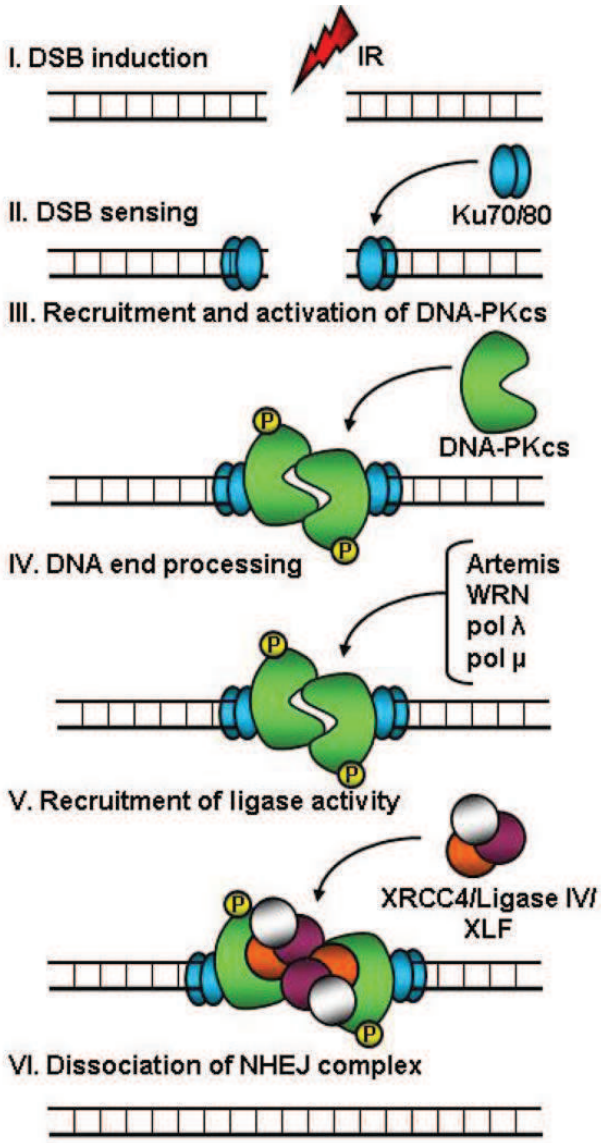


Figure 23: *Simplified overview of non-homologous end joining (Mukherjee et a.l*, Semin Radiat Oncol, *2010).*

In vitro studies indicate that autophosphorylation of DNA-PKcs is critical for a correct NHEJ, implementing the role of DNA-PKcs in DNA ligation, by remodeling of the DNA-end- bound DNA-PK complex (Block et al., 2004). DNA-PK phosphorylates histones H_2AX and H_1, and in this way it facilitates the NHEJ reaction by modifying the local chromatin environment to provide access to other DNA repair complexs at DSBs (Kysela et al., 2005). DNA-PK also recruits Artemis, a specific structural endonuclease that mediates ligation of the ends (Ma et al., 2005).

During S and G_2 phases of the cell cycle on the other hand, when identical sister chromatids are present, HR is the preferred pathway for repair of DSBs, which can be a consequence of stalled replication forks (Haber, 2000).

After recognition of the damage site HR entails recruitment of repair proteins that work in concert with the DNA damage signaling machinery to slow down fork progression before the cell enters G_2. The MRN complex is one of the first complex recruited to the lesion that instantly activate ATM and ATR kinases triggering signals that will halt the cell cycle to permit DNA repair (Carson et al., 2003). In a different way from NHEJ, HR needs an intact 3' single strand overhangs to repair DNA. Indeed DNA blunt end must be processed for HR re-joining otherwise in the case of stalled fork long tracks of single strand DNA (ssDNA) is exposed. In both of case Replication Protein A (RPA) coats ssDNA to protect him and to inhibit formation of secondary structures. RPA coating enhances recruitment of ATR and RAD17 binding to the ssDNA, which leads to recruitment of the 9-1-1 complex (RAD9-RAD1-HUS1) (Zou and Elledge, 2003; Zou et al., 2003). This complex recruits and stimulates Flap endonuclease 1 (FEN1) and DNA polymerase β which collaborate to restart a stalled fork (Wang et al., 2004). In case of break, displacement loop (D loop) formation occurs as a consequence of homologous duplex DNA invasion by the ssDNA, process that is catalyzed by RAD51 and stimulated by RAD52, RAD55/RAD57 and RAD54 (Alexeev et al., 2003); (Sugawara et al., 2003; Sung, 1997). Next, using homologous sequence as template, the invading strand primers DNA synthesis results in the formation of double Holliday junction that will be resolved by RAD51C-XRCC3-dependent resolvase activity. Afterwards the disrupted genetic information at the DSBs will be restored.

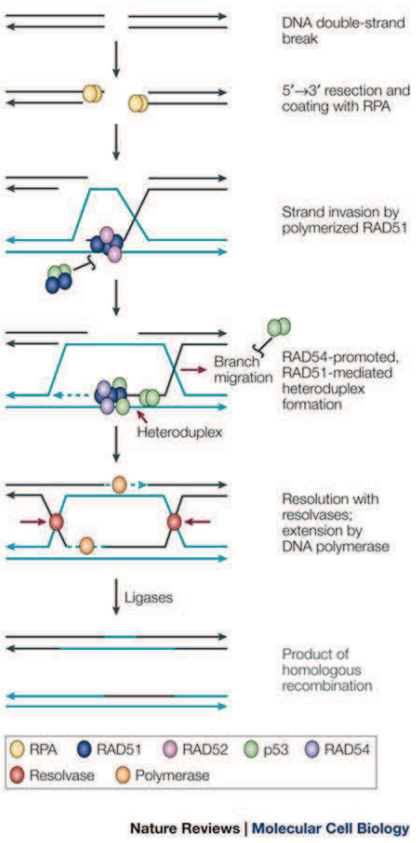


Figure 24: *Simplified overview of Homologous Recombination (Sengupta et al.,Nature Reviews Molecular Cell Biology, 2005).*

Although is not clear any role in the NHEJ by p53 dependent control of HR both *in vitro* and *in vivo*, its inactivation results in a spontaneously enhanced and stress-induced HR (Dudenhoffer et al., 1998). The effect of p53 on HR can be either by itself or in combination with HR proteins, or a combination of both, and can be localized on heteroduplex structure or at Holliday junction level with RECQ elicase interaction. By specific binding with heteroduplex recombination intermediate and specific mismatch recognition, wild-type p53 alone might check the fidelity of HR events (Dudenhoffer et al., 1998), a very important effect to suppress eventual tumorogenic genome rearrangements. On the other hands several specifics HR protein is demonstrated to interact with p53, even if the most studied is its direct association and inhibition of RAD51 (Linke et al., 2003; Sturzbecher et al., 1996).

Eukaryotic genome duplication depends on a complex coordination of progressive events to initiate chromosome replication and distribute fully replicated chromosomes into the daughter cells. Chromosomal duplication is restricted to the synthesis phase (S phase) of

the cell cycle, where replication initiates from multiple specific sequences distributed along each chromosome and called replication origins. Only a subset of all possible origins initiate the synthesis in a given S phase, but these origins are activated in a temporal order that is, in general, conserved from one cell cycle to the next (McCarrol et al., 1988). Checkpoints monitor chromosome duplication and respond to damages or defects by altering cellular metabolism in order to promote fidelity. In the absence of such safeguards, agents that challenge DNA synthesis by altering levels of necessary raw materials or by promoting damage could jeopardize chromosomal duplication and negatively affect the integrity of the genome. One such agent is hydroxyurea (HU). HU affects DNA synthesis by reversibly inhibiting ribonucleotide reductase (RNR), so it prevents the reduction of ribonucleotides to deoxyribonucleotides (deoxynucleoside triphosphates [dNTPs]). HU starves the DNA polymerase at the replication forks for dNTPs.

HU reduces purine dNTP pools in a variety of mammalian cells (Skoog et al., 1971; Gandhi et al., 1998); but conflicting data exist regards its modulation of pyrimidine dNTP pool levels, even for purine dNTPs, HU generates a complete depletion of the dGTP or dATP pools (Skoog et al., 1971; Adams et al., 1971). More commonly, HU results in only partial depletion of the purine dNTP pools (Tyrsted et al., 1982; Gandhi et al., 1998). The complicated, often reciprocal, changes in individual dNTP pools that occur in mammalian cells treated with HU may be due to the compensatory activities of deoxyribonucleotide salvage pathways in higher eukaryotes.

In mammalian cells, the mechanism by which HU inhibits replication has been explored by attempting to reverse HU inhibition by administration of exogenous deoxyribonucleosides (Lagergren et al., 1987; Eriksson et al., 1987).

Instead *Saccharomyces cerevisiae* cells lack deoxyribonucleotide kinase activity, so they depend on RNR activity for dNTP production. The presence of high concentrations of HU (200 mM) averts the accumulation of dNTPs that normally occurs as cells enter S phase (Chabes et al., 2003; Koc et al., 2003), blocks S phase progression, and engages the checkpoint to inhibit passage through the cell cycle into a catastrophic mitosis (Nyberg et al., 2002). Furthermore, wild-type cells activate transcription of genes involved in the replication and repair (Foiani et al., 2000; Gasch et al., 2001; Huang et al., 1997) and stabilize the idling replication complex to allow the resumption of fork progression after the stress has been relieved (Desany et al., 1998; Lopes et al., 2001; Sogo et al., 2002; Tercero et al., 2001).

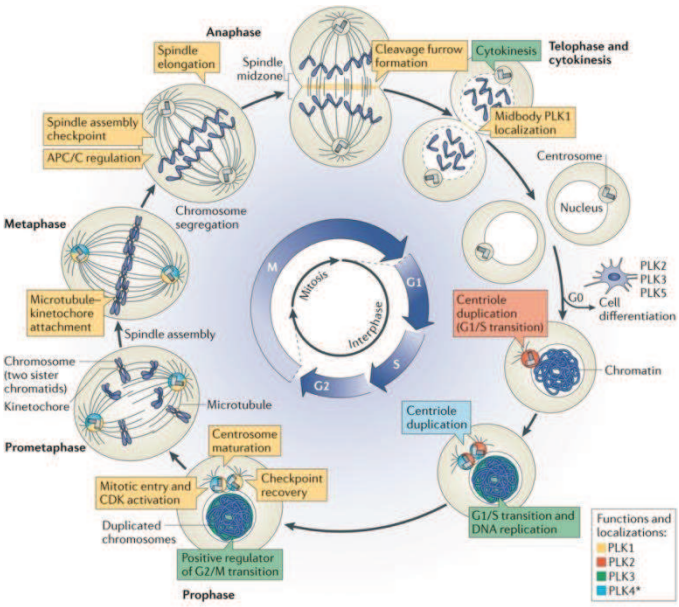


Figure 25: *The cellular cycle.*

The cellular and chromosomal responses to HU-induced replication stress depend on the *MEC1/RAD53* checkpoint pathways (Allen et al., 1994; Desany et al., 1998; Gasch et al., 2001; Lopes et al., 2001; Santocanale et al., 1998; Tercero et al., 2001; Weinert et al.,1994). The checkpoint kinases Mec1 and Rad53 act sequentially in a pathway that, among other things, appears to boost or preserve dNTP levels in cells treated with HU or other DNA-damaging agents. In response to DNA damage, the Mec1/Rad53 pathway positively regulates RNR activity. It induces transcription of the large and small subunit *RNR* genes (Elledge et al., 1993). It is needed for the redistribution of the RNR small subunit (Rnr2) from the nucleus to the cytoplasm, where it can join with the large subunit (Rnr1) to form the holoenzyme (Yao et al., 2003). It inactivates the RNR inhibitor Sml1 (Zhao et al., 1998; 2001; 2002). The Mec1/Rad53 pathway also negatively regulates replication origin activity in HU-treated cells. In wild-type yeast, HU blocks DNA chain initiation at late replication origins (27, 31). In *rad53* mutants, HU does not arrest the firing of late origins (Santocanale et al., 1998; Lengronne et al., 2001). The occurrence of DNA synthesis at late origins in *rad53* cells indicated that HU-treated cells must have a residual pool of dNTPs.

4c.PHAGOCYTOSIS

The immune system has developed success progressive complexity of basic mechanisms that help organisms to survive. The immune system responds to offensives using, first of all, sensors that detect the intruder and interact with soluble or membrane-bound molecules present on the host. Some effectors elaborate a response and consequently attack the intruder. Moreover phagocytosis represents a crucial mechanism used by the immune system to defend organisms.

Phagocytosis is the process by which a cell engulfs diverse particulate targets and represents a mechanism of prime importance for all living organisms.

At the end of the nineties, flow cytometry started to be used to study phagocytosis process. Flow cytometry allows the use of small samples to analyze a considerable number of cells in a very short time and to produce quantitative results. Furthermore, it permits a clear distinction between adherent and ingested particles (because some colorants have the property of quenching the fluorescence of target particles that remain outside the cell after the process of phagocytosis, but cannot penetrate the plasmatic membrane of living cells and thus interact with ingested particles) and allows cell evaluation (Esteban et al. 1997).

Another step forward into the study of the phagocytic process was due to the use of specific cell markers for different cell lines.

Cells involved in the phagocytosis are named phagocytes Among phagocytes can be distinguished:

1. "expert or professional phagocytes": monocytes/macrophages, granulocytes, dendritic cells, and lymphocytes, for which the fagocytic activity is a predominant activity;
2. "optional phagocytes": (fibroblasts, mast cells, endothelial cells and other cells) for which phagocytosis is a marginal function.

The phagocytosis is the most ancient and universal tool of defense against foreign material, because unicellular eukaryotes phagocytize food and defend the organisms. Amoebae already show mechanisms that allow recognition, internalization and destruction of foreign material (Dzik et al., 2010). In fact, amoebae and macrophages show similar phagocytic mechanisms such as recognition of the particle by cell surface receptors (Allen et al., 1990) and killing by oxygen radicals (Davies et al., 1991). The phagocytic machinery characterizing the amoeboid protozoans were inherited during the evolution towards innate immunity (Sillo et al., 2008). Although many mechanisms of immunity are common for

invertebrates and vertebrates (phagocytosis, cytotoxicity, lectins, proteinases), others are only used in invertebrates (hemolymph clotting system, melanization) even though the general plan on which they operate is realized in vertebrates as well (Dzik et al., 2010).

Phagocytosis and macropinocytosis are crucial processes of vertebrates that enable cells to test their environment, to remove pathogens and apoptotic bodies and, constantly, to offer immunoprotection. Phagocytosis is also the front-line mechanism by which the immune system eliminates most pathogenic microorganisms. But phagocytosis is also an essential part of tissue homeostasis and remodeling and regulates the expression of different membrane components (Flannagan et al., 2012; Pedrera et al., 1992).

In vertebrates, phagocytosis appears to have developed from having a crucial role in innate immunity to sharing relevant functions in adaptive immunity (Desjardins et al., 2005; Moretti et al., 2014). Initially phagocytosis was defined as the process by which a cell internalized particles bigger than 0.5 micrometers, but today the term phagocytosis is used to describe the process by which cells engulf particles such as bacteria, other microorganisms, aged red blood cells, foreign matter, *etc.*

The phagocytic process consists on a series of connected steps:

1. detection and recognition of the foreign particle;
2. attachment of the foreign particle to the phagocyte;
3. engulfment or internalization of the foreign particle into a vesicle called phagosome;
4. fusion of phagosome with a lysosome and formation of phagolysosome (degranulation of the phagocyte and maturation of compartment through endosomal fusion);
5. intracellular killing and digestion of the particle;
6. in the case of some phagocytes (such as macrophages and dendritic cells) egestion and antigen presentation.

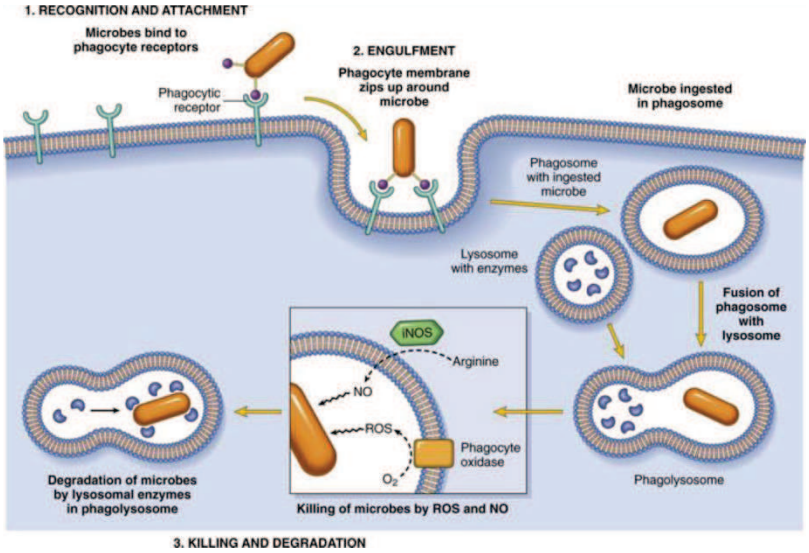


Figure 26: *Phagocytosis process*

The process of phagocytosis is prompted by the interaction of surface molecules from the phagocytic target with receptors present on the phagocytic cells (Desjardins et al., 2005). On the surface of the phagocytes there are many receptors that are able to recognize and decode their cognate ligands expressed on the surface of the phagocytic target and trigger engulfment. These receptors can directly recognize the particle or recognize targets which are coated with opsonic molecules. Initially these ligands were referred to pathogen-associated molecular patterns (PAMPs), but today there is a necessity of including in these patterns the recognition of commensal bacteria and apoptotic and necrotic cells. Therefore, a more inclusive term of "molecular pattern" (MP) was proposed (Moretti et al., 2014). Various kinds of pattern recognition receptors are involved in the identification of foreign factors in vertebrates and invertebrates (Akira et al., 2006). Among such receptors of different evolutionary origins are scavenger receptors and Toll-like receptors.

Phagolysosomes (obtained after the fusion of phagosomes whit lysosomes) are the vesicles where internalized microbes will be killed and degraded by a variety of lysosomal hydrolytic enzymes (proteases, acid phosphatase, ribonucleases, glucosidases,…).

Phagocytosis is known to elicit several antimicrobial mechanisms. Potentially, the most important and well-known mechanism involves the production of reactive oxygen (*i.e.*, superoxide radicals) and nitrogen (*i.e.*, nitric oxide) intermediates, which are known to kill ingested microbes contained in phagolysosomes (Neumann et al., 2001).

Cellular and molecular events that cause the binding of targets to a phagocyte and their engulfment into phagosomes have been largely studied. Recent data suggest that the

process of phagocytosis itself provides important information to myeloid phagocytes about the nature of the targets they are engulfing. Afterwards, this fact helps to tailor the appropriate inflammatory responses.

While unicellular organisms use phagocytosis to obtain food, more complex metazoans have "professional" phagocytes which act as a fundamental mechanism of their immune system. In mammals and other vertebrates, professional phagocytes include polymorphonuclear cells (PMNs), monocytes/macrophages and dendritic cells (DCs) (Neumann et al., 2001; Rabinovitch et al., 1995).

Non-professional phagocytes have more limited phagocytic properties than professional cells. Furthermore, non-professional phagocytes apparently lack the ability of producing microbicide oxygen and nitrogen products upon phagocytosis, and to secrete the cytokines characteristic of professional cells. Other cell types in mammals (*i.e.*, epithelial cells, fibroblasts) are also capable of engulfing particles, albeit with a much restricted capacity (Rabinovitch et al., 1995; Wu et al., 2009).

Mammalian professional phagocytes derive from a common myeloid progenitor cell. Among these professional phagocytes, resident tissue macrophage populations are the first cells that encounter non-self-material, particularly bacteria, and engulf and degrade them by using hydrolytic enzymes and oxidative assault. A diversity of receptors on macrophages are used to detect infection (Zhang et al., 2014). Most of the studies done have revealed that monocytes, macrophages and neutrophils are the main phagocytic cells (Neumann et al., 2001).

During infection, macrophage lineage cells eliminate infiltrating pathogens through a battery of antimicrobial responses, where the efficacy of these innate immune responses is crucial to immunological consequences. Due to the importance of these processes, many intracellular pathogens have developed mechanisms to overcome macrophage defenses, using these immune cells as residences and dissemination strategies.

Granulocytes are leukocytes with a polymorph nucleus and particular granules in the cytoplasm. Precisely, by using the dye affinities of these granules for acid, neutral and basic dyes, they are distinguished in: eosinophils, neutrophils and basophils. It is known that mammalian neutrophils have a crucial role in the host tissue protection by killing and degradating microorganisms and are involved in the inflammatory response.

Neutrophils employ the same two strategies already described for macrophages to trap and kill pathogens: engulfment of microbes (phagocytosis) and secretion of antimicrobials (generation of reactive oxygen and nitrogen species). In 2004, a novel third function was

identified, called neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). NETs are networks of extracellular fibers (primarily composed of DNA from the cells) which bind pathogens. By the formation of NETs neutrophils are able of killing extracellular pathogens while minimizing damage to the host cells. NETs have also been shown to form within blood vessels during sepsis in mammals and intra-vascular NET formation is tightly controlled and is regulated by platelets (Clark et al., 2007).

Dendritic cells (DCs) are specialized antigen presenting cells that bridge innate and adaptive immunity in mammals.

Developmental and functional relationships between B cells and macrophages have long been recognized and several studies probed that mammalian malignant B cell lines can switch into macrophage-like cells, having the capacity to phagocytize large particles (Hanecak et al., 1989). This switch from a lymphoid to a myeloid lineage gave rise to the term of "lineage switching" or "lineage infidelity".

Subsets of B lymphocytes and macrophages shared a closer lineage relationship than what the models of hematopoietic differentiation at that time predicted (Borrello et al., 1996). Thus, it would appear that professional phagocytosis was not restricted to cells of myeloid origin, as it can also be carried out by cells of lymphoid origin.

Until 2006, it was thought that primary B cells were unable to execute phagocytosis.

The processes of phagocytosis in mammalian was influenced by different factors dependent on the environment, first of all the temperature, but also hypoxia, circadian rhythms or ageing,

Therefore phagocytosis is a physiological and fundamental process because: it is the first step in triggering host defense and inflammation, it is necessary for the removal of senescent and apoptotic cells and it is essential for embryonic development and tissue remodeling.

Phagocytosis by phagocytes is therefore the necessary final step of apoptosis. Apoptotic cells are generally rapidly removed, before they lose their membrane integrity, in order to prevent damage caused by an accidental release of their cytotoxic and antigen content which would stimulate the production of auto-antibodies, typical of autoimmune diseases (Wyllie et al., 1980; Savill et al., 1993).

In fact, if the presence of apoptotic cells exceeds the capacity of elimination by macrophages, the cell from apoptotic cell evolves in cell with secondary necrosis resulting in the release of its cellular content and a damage in the surrounding tissues. In addition, the phagocytosis is accompanied by the release of anti-inflammatory signals, such as the

production of transforming growth factor-beta (TGF-beta), the prostaglandin E2 (PGE2) and interleukin 10 (IL10), which are important for the resolution of inflammation and the regulation of immune response (Fadok et al., 1998).

Between the recognition and macrophage cell apoptosis, the latter in the early stages of apoptosis exposes on its surface some changes known as "Eat me signals", such as the exposure of phosphatidylserine (Fadok et al.,1992; Fadok et al., 2001) and the loss of sialic acid of the ends of the oligosaccharide chains of glycoproteins and glycolipids of the membrane (Hall et al., 1994; Dini et al.,1992; Duvall et al., 1985). It is not entirely clear whether the removal of apoptotic cells is a sufficient signal, if their cooperation is necessary or whether there is a specific timeline in their expression on the cell surface. Some studies indicate that exposure of phosphatidylserine on the membrane of apoptotic cells is an early and transient signal. (Spano et al., 2000).

Apoptotic cells larger than 2 microns, require the coordination of activities of different receptors. Two steps are fundamental, on the one hand the "tethering" (recognition and binding), on the other hand the "tickling" (internalization and activation of downstream pathways), (Hoffmann et al., 2001).

The function of recognition and binding is often mediated by integrins, as αvβ5 , αvβ3 (vitronectin receptor) family of β2 integrin (CD11/CD18) and CD36 (integrin-associated protein). All of these receptors contain an RGD motif (Arg-Gly-Glu), such as that used to bond the matrix proteins fibronectin and vitronectin (Giancotti et al., 1999). This ground, however, has different role during phagocytosis, given that this is a recognition site for proteins called "opsonins", soluble proteins secreted by different cell types, including macrophages, which act as a molecular bridge between the receptors and macrophage signals "eat me" of the apoptotic cell. This family of serum proteins includes the annexina1, thrombospondin (TSP), MFG-E8 (Milk fat globule-EGF factor 8), Del-1, β2-glycoprotein-1, protein S and Gas6.

The αvβ3 integrin and CD36 are the main receptors involved in the recognition of apoptotic cells by binding to the thrombospondin opsonin. (Savill et all., 1992; Stern et al., 1996). In particular integrin αvβ3, but not CD36, it is able to bind directly to the thrombospondin domain through its RGD (Savill et al., 1992; Savill et al., 1990).

CD36 instead would play a role in boosting recognition of apoptotic cell. It is possible that this amplification is due to its ability to bind oxidized lipoproteins (Endermann et al., 1993). A leading role has certainly also the reorganization of the cytoskeleton in conveying the two receptors proximally for a perfect synergistic action. This complexity and

redundancy elimination mechanism by phagocytosis of apoptotic cells in mammals denotes its importance and its physiological implications.

Professional phagocytes mediate processes ranging from phagocytosis to tissue homeostasis. This is possible because they effectively engulf and eliminate invading microorganisms. To survive this assault, pathogens have developed an array of countermeasures aimed at avoiding detection (strategies to avoid recognition and uptake by host cell or altered host signaling to promote invasion, manipulation of host cell cytoskeleton) impairing signaling, or paralyzing the machinery that underlies phagocytosis. Some facultative and obligate intracellular bacteria have evolved ways to evade or even exploit autophagy (Tang et al., 2015). Furthermore, certain pathogens benefit from attaching to entering or traversing host cells to establish and spread infection (Sarantis et al., 2012). Other intracellular bacterial pathogens drive the formation of host "pseudo-organelles" that facilitate their replication, survival, or latency. The formation and maintenance of these bacteria-containing vacuoles are dependent on the bacteria's ability to commandeer the host's intracellular membrane system (mainly dynamic compartments involved in exo/endocytic membrane traffic). Additionally, bacterial survival or proliferation inside the vesicles could be augmented by host membrane transport processes subverted by secreted bacterial factors, which facilitate the acquisition of membrane sources and nutrients (Tang et al., 2015). For example, *Leishmania* has evolved ingenious ways to adapt to life in the macrophage. New proteins have recently been found to disrupt processes ranging from antigen cross-presentation to nuclear pore dynamics. Furthermore, *Leishmania* sabotages key metabolic and signaling pathways and induces DNA methylation to turn off genes controlling microbicidal pathways. These novel findings highlight the creative attack employed by *Leishmania* to subvert macrophage function (Arango Duque et al., 2015). Diverse strategies used by different pathogenic bacteria to prevent the bacteria-containing vacuoles from being destroyed via the endolysosomal pathway have been studied in mammals.

AIMS

Ngb is a neuroprotective molecule against hypoxic conditions and oxidative stress related insults that occurs in neurons. In neurons Ngb interacts with Flotillin-1, a protein widely expressed in the hematopoietic system, where enhances myeloid differentiation.
Therefore, in this study we have investigated:

1. Ngb expression in hematopoietic system:
    - Myeloid cells

2. Ngb involvement during myeloid differentiation:
    - Vitamin D_3 induced monocytic differentiation
    - Retinoic Acid induced granulocytic differentiation

3. Ngb role in different stress conditions in the hematopoietic system:
    - Serum deprivation
    - HU induced DNA damage
    - H_2O_2 induced oxidative stress
    - INH induced oxidative stress

4. Ngb and Flotillin-1 relationship in myeloid cells.

MATERIAL AND METHODS

Cell line

Phoenix cells were an adherent cell line derived from epithelial cells of monkey kidney 293T. The genome of these cells has been integrated with plasmids bearing the genes coding for the retrovirus proteins: GAG (membrane glycoprotein), POL (DNA polymerase), ENV (Envelope), necessary for the production of a mature virion. However they were cells defective for the production of the virus as they missing sequence that allows the packaging Ψ. This sequence was present on the retroviral vector Pinco, together with the two LTR sequences that allow replication. Characteristic feature of this cell line was that it was highly transfectable with calcium phosphate. This cell line was maintained in DMEM medium containing 10% fetal bovine serum, 50 units/ml of penicillin/streptomycin antibiotics, 1 mmol/L glutamine at 37°C in 5% CO_2.

THP1 cells were established from patients with acute monocytic leukemia.

U937 cells were established from a patient with generalized diffuse histiocytic lymphoma.

HL60 cells were established from the peripheral blood of a Caucasian patient affect by acute myeloblastic leukemia.

All this tree lines were cell lines in suspension and were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 50 units/ml of penicillin/streptomycin antibiotics, 1 mmol/L glutamine at 37°C in 5% CO_2.

H1299, also known as NCI-H1299 or CRL-5803, was a human non-small cell lung carcinoma cell line derived from the lymph node. These cells had a homozygous partial deletion of the TP53 gene and as a result, didn't express the tumor suppressor p53 protein which in part accounts for their proliferative propensity.

H1975 were non small cell lung cancer from adenocarcinoma.

H1650 were lung cancer cells derived from metastatic site.

All these tree lung cancer cells lines were adherent cells and were maintained in DMEM medium containing 10% fetal bovine serum, 50 units/ml of penicillin/streptomycin antibiotics, 1 mmol/L glutamine at 37°C in 5% CO_2.

Phoenix transfection

For the study of cells overexpressing Flotillin the construct Pinco-*FlotillinEGFP* was introduced for transfection in adherent cells of a packaging line, the Phoenix, in order to synthesize mature retrovirus that was used to infect target cells. It was infected also the VSV-G sequence. The procedure used for transfection was that of Rothenberg and Nolan. They were plated 2 million cells in 10 cm plates in complete DMEM (10% BCS, 1%

penicillin/streptomycin, 1% glutamine) moving the plate at right and left for 4-5 times so as to evenly distribute the cells over the entire surface available. 36-48 hours after plating, when the confluence is around 60-70 %, the cells were transfected with the method of the calcium phosphate [$Ca_3(PO_4)_2$]. It was prepared a first solution containing 20 µg DNA, 124 µl $CaCl_2$ 2 M, 62 µl VSV-G and H_2O up to a final volume of 500 µl. To this solution were added 500 µL of a solution HBS 2X previously prepared and consists of 8.0 g $NaCl_2$, 5 g HEPES, 0.1065 g dibasic Na_2HPO_4, 0.37 g KCl, 1 g dextrose and H_2O up to a final volume of 500 ml. It 'important that all reagents used were at room temperature. The two solutions were mixed using an automatic pipettor for 15 seconds and then let stand at room temperature for 15 minutes so as to produce precipitates of Ca_2PO_4/DNA. The solution HBS/DNA was placed directly on the *Phoenix* in a fresh culture medium containing 25 mM chloroquine, an inhibitor of the release of lysosomal proteins. The plates were moved to cross to facilitate the homogeneous distribution of the solution, and then placed in an incubator at 37° C. Looking at the cells under a light microscope you can see a calcium phosphate precipitate as black particles.

8-12 hours after transfection was changed medium to remove chloroquine that protects DNA from lysed, but it is toxic for the cells. After 24 hours was carried out another change of the medium to prevent acidification of the soil that would make inefficient transfection. Forty-eight hours after transfection, the supernatant was recovered containing retroviral particles complete, filtered through a 0.45 µM and used to infect target cells.

Infection

The infection of the cells was made at a concentration of 10000-200000 cells/ml of viral surnatant. $1x10^6$ cells were pelleted at 1200 rpm for 5 minutes and resuspended in 5 ml of retroviral surnatant. The cells were placed on six-well plates in the presence of *Polybrene* and centrifuged at 1800 rpm for 45 minutes at 33° C. After 2 hours and 15 minutes of incubation at 37° C, it was done a second cycle of infection: the soil was removed and replaced with 5 ml of retroviral surnatant containing *Polybrene* The cells were centrifuged for 45 minutes at 1800 rpm, incubated at 37° C for 2 hours and 15 minutes and resuspended in fresh medium. To increase the percentage of infected cells, they were carried out several cycles of infection. After 48 hours of infection, the infected cells with the construct Pinco-*Flotillin-EGFP* at fluorescence microscopy appear green. The selectable marker, which in this case is represented by the fluorescent protein, it is useful to distinguish infected cells by FACS (Fluorescent Activated Cell Sorter) and separate

them from those not infected. With this method of infection-transfection cells they were obtained THP1 Pinco, THP1 Pinco-*Flotillin*, THP1 Pinco-*Flotillin-EGFP*. By means of Western Blotting experiments it was verified the expression of the fusion proteins studied.

Western Blotting

The samples, obtained from cells lysed or for immunoprecipitation, were sonicated, boiled and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The Ngb, Flotillin, Tubulin and Histone H_4 WB were performed using a Transfer Buffer made with methanol tris and glycine and gels were blotted O/N at 100mA at room temperature. Protein transfer was checked with Ponceau red staining. The membrane was blocked with 3% BSA (for Ngb) or 5% nonfat dried milk (for Tubulin, Flotillin-1 and Histone H_4) 0,1% Tween 20 in TBS for 1 hour at room temperature. Upon removal of the blocking solution, the membrane was briefly washed with TBS-T before incubating with the specific primary antibody over-night at 4°C with agitation. The next day non-binding antibody was removed by four TBS-T washes before exposing the membrane to an horseradish peroxidase (HRP)-coniugated secondary antibody. Following incubation for 1 hour at room temperature, the membrane was washed four times as before. Proteins were detected with Amersham ECL kit (GE Healthcare) or Luminata Crescendo (Millipore).

Primary antibody

The antibodies used for Western Blotting and immunoprecipitation were: mouse monoclonal [13C8] anti-Neuroglobin (Abcam); rabbit polyclonal (H104) anti-Flotillin-1 (Santa Cruz); rabbit polyclonal (TUBA1C) anti-Tubulin (Abgent); rabbit polyclonal anti-Histone H_4 (Millipore).

Secondary antibody

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgG (Amersham Bioscience) were used as secondary antibodies.

Cell differentiation

The study of myeloid differentiation was performed in the following cell lines:

HL60 *Human promyelocytic leukemia cells*, locked to the stage of M_2 myeloblast, when treated with RA or Vitamin D_3.

U937 differentiated to monocyte/macrophage when treated with vitamin D_3.

THP1 differentiated to macrophage when treated with RA or monocyte when treated with vitamin D_3.

Flow cytometry analysis of differentiation markers antigens

Analysis for myeloid maturation with cell-surface markers was performed by Flow cytofluorimetry with Phycoerythrin-labeled antibodies specific for CD11b and CD14. THP1, U937 and HL60 cells were plated at a density of 5x105/ml RPMI plus 10% FBS. Cells were treated with 10^{-6}M RA or 250 nM Vitamin D_3. After incubation at 37°C cells were harvested at day 2, 4 and 6, washed with PBS 1% BSA and stained for 30 minutes with anti-CD11b-PE and IgG2a-PE. Cells were washed with PBS 1% BSA two times and fixed with PBS 1% formaldehyde. Then they were analysed on a FACSCalibur instrument using CELLQUEST software (BD biosciences, Sna Jose, CA, USA). Thirty thousand events were acquired. Live cells were gated based upon forward and side scatter patterns, restricting analysis to gated cells we identified differentiated cells represented on dot plots with CD11b/CD14-PE (FL2) on x-axis and the cell count (event frequency) on the y-axis.

Phagocytosis

Molecular Probes BioParticles product line consist of a series of fluorescently labeled, health or chemically killed bacteria (*E.coli*).

Bacteria were suspended in RPMI 10% FBS, 1% glutamine and 1% penicillin/streptomycin to a final concentration of $1x10^7$ bacteria per ml. In phagocytosis experiments in which a bacterium/THP1 ratio of 10:1 was used, the bacteria were suspended in a final concentration of $5x10^7$ bacteria per ml.

THP1 were added to the incubation mixture containing bacteria in a final concentration of $0,5x10^7$ cells per ml. The bacterium-THP1 mixture was incubated at 37°C for 2 and 4 hours in incubator. The phagocytosis was stopped in PBS with 0,25% Tripan Blue for 1 minutes to quench the surface bound fluorescence. The reaction mixture was washed two times with PBS and then fixed by adding formaldehyde (final concentration 1%) and then analized by Flow cytometry.

Cellular stress induction

a-Oxidative stress

To induce oxidative stress H_2O_2 or Inh diluted in water was added in the cultured medium at various concentrations and for different times. The cells were incubated at 37°C and 5% CO_2. At every time point the cells were harvested, washed with PBS and lysed in Laemmli buffer.

b-Serum deprivation

To induce serum deprivation THP1 and U937 cells were washed two times with PBS and then resuspended in RPMI with 10% or 2,5% or 0,5% or 0,1% FBS; 50 units/ml of penicillin/streptomycin antibiotics; 1 mmol/L glutamine and maintained at 37°C in 5% CO_2 for 48 hours.

c-DNA damage

To induce DNA damage Hydroxyurea diluted in water was added at the cultured medium at the indicated concentrations and for various times. Then the cells were washed with PBS and lysed in Laemmli buffer.

Growth curves

After treatment with Inh THP1, HL60 and U937 cells were put into the flask at 800.000 cells/ml, 500.000 cells/ml and 500.000c/ml respectively, in triplicate. Cells were counted at 24, 48 and 72 hours. Every experiment was repeated at least three times.

Immunoprecipitation

Starting from the Nuclear Extract we recovered the surnatants. The surnatants were pre-cleared with 30µl of Protein-A sepharose (PAS) saturated with BSA 2% in PBS for 2 hour at 4°C with rotation. The surnatant was recovered by pelletting of the PAS, a Bradford assay was performed on the recovered surnatant. Each sample was divided in 3 different aliquots, to have a total nuclear lysate, an unrelated IP sample and a specific IP sample of the same starting sample. The total nuclear lysates was diluted in an equal volume of Laemmli Buller and kept -20°C until use. The unrelated IP and real IP samples were incubated with the antibodies O/N 4°C with rotation. The next day the samples were incubated with 50 µl Protein-A sepharose saturated with BSA 2% in PBS for 2 hours at 4°C. Following incubation, the PAS pellets were washed five times with NET buffer (50

mM Tris-HCl pH 7,5; 150 mM NaCl; 0,1% NP_4O; 1 mM EDTA; 0,25% Gelatin). The immunoprecipitated proteins bound to the PAS were dissolved in SDS Laemmli Buffer.

RESULTS AND DISCUSSION

1- Ngb is expressed in myeloid cells and in lung cancer cell lines

Since the Ngb protective function has been mainly studied in the nervous system and since Flotillin-1 is widely expressed in the hematopoietic system the first purpose was to study Ngb expression in the hematopoietic system.

Western Blotting anti-Ngb conducted on the whole lysates of different hematopoietic cell lines (THP1, U937 and HL60), has revealed that Ngb is widely expressed in this system.

We obtained the same result also in H1299, H1975 and H1650, cell lines derived from lung cancer. Thus Ngb is also expressed in lung cancer cell lines.

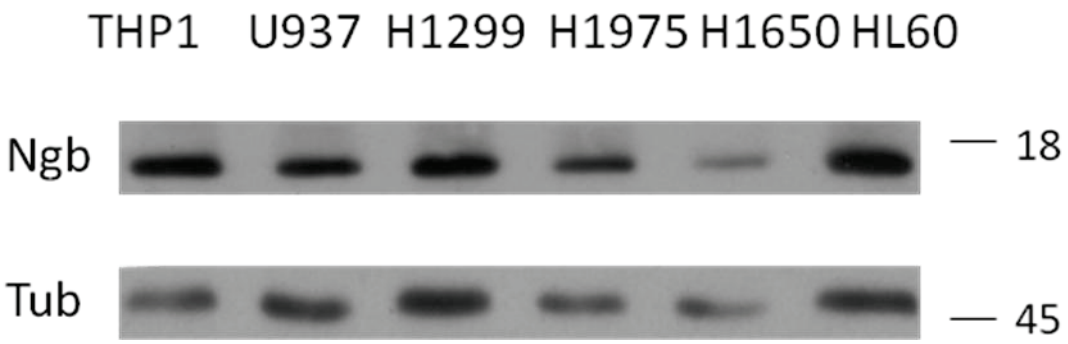


Figure 27: Ngb is expressed in hematopoietic cell lines and in lung cancer cell lines

Western Blotting anti Ngb on samples derived from hematopoietic cell lines (THP1, U937, HL60) and lung cancer cell lines (H1299, H1975, H1650).

Blots were stripped and rehybridized with an anti-Tubulin antibody as loading control.

These results indicate that Ngb expression is not restricted only to the nervous system, but it is expressed also in hematopoietic and lung cancer cells.

2- Ngb expression levels increase during myeloid differentiation

Since Ngb is expressed in hematopoietic cells we evaluated its expression at various stages of the hematopoietic differentiation.

We induced myeloid differentiation in two different ways: with 10^{-6} M Retinoic Acid or with 250 nM Vitamin D_3 at various times.

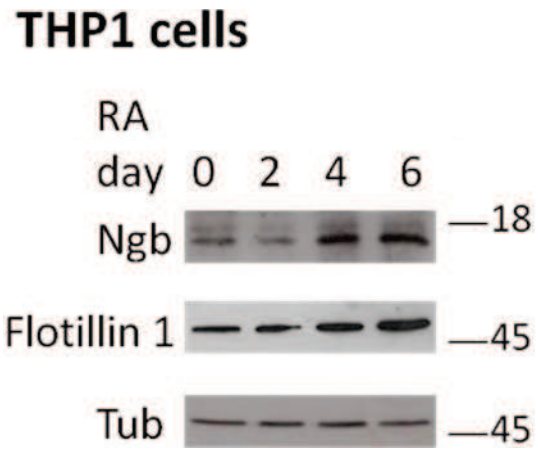
To verify the differentiation state we evaluated the expression of differentiation markers (CD11b and CD14) by flow cytometry at 2, 4 and 6 days of treatment.

Therefore we induced the myeloid differentiation of THP1 cells with 10^{-6} M Retinoic Acid for 6 days.

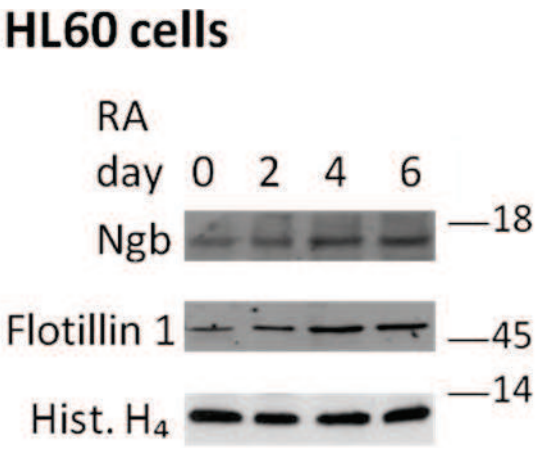
Western Blotting anti-Ngb revealed an increased expression of Ngb starting from day 4 of Retinoic Acid treatment.

The same study of Ngb expression was conducted also in myeloid HL60 cells differentiated by treatment with 10^{-6} M Retinoic Acid for six days. Also in these cells Ngb expression increased already from day 2 of Retinoic Acid treatment.

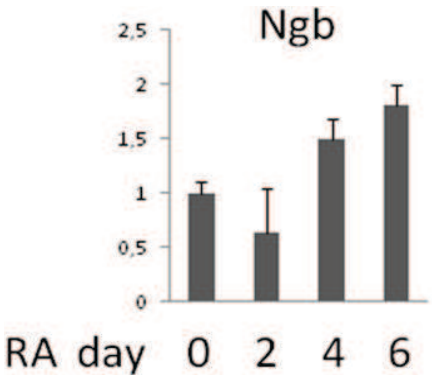
The results obtained show that the increase of Ngb expression and of Flotillin-1 expression follows the same kinetic in both cellular lines analyzed.



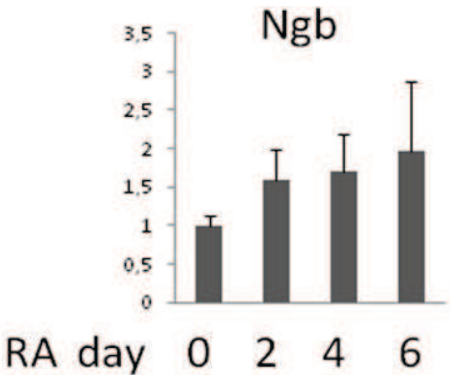
A



B



C



D

Figure 28: Ngb expression levels increase in myeloid differentiated cells.

A, Western Blotting anti-Ngb and anti-Flotillin-1 in Thp1 B, in HL60 cells treated with 10^{-6} M Retinoic Acid for 0, 2, 4, 6 days.

Blots were stripped and rehybridized with an anti-Tubulin antibody as loading control.

C and D, quantification of normalized Western Blotting signals by scanning densitometry, shown as the average of three independent experiments similar to that in A and in B respectively.

Then we tested monocytic/macrophagic differentiation of U937, THP1 and HL60 induced by 250 nM Vitamin D_3 for different days. Also in this experiment, to verify the differentiation state, the expression of differentiation markers (CD11b and CD14) was evaluated by flow cytometry.

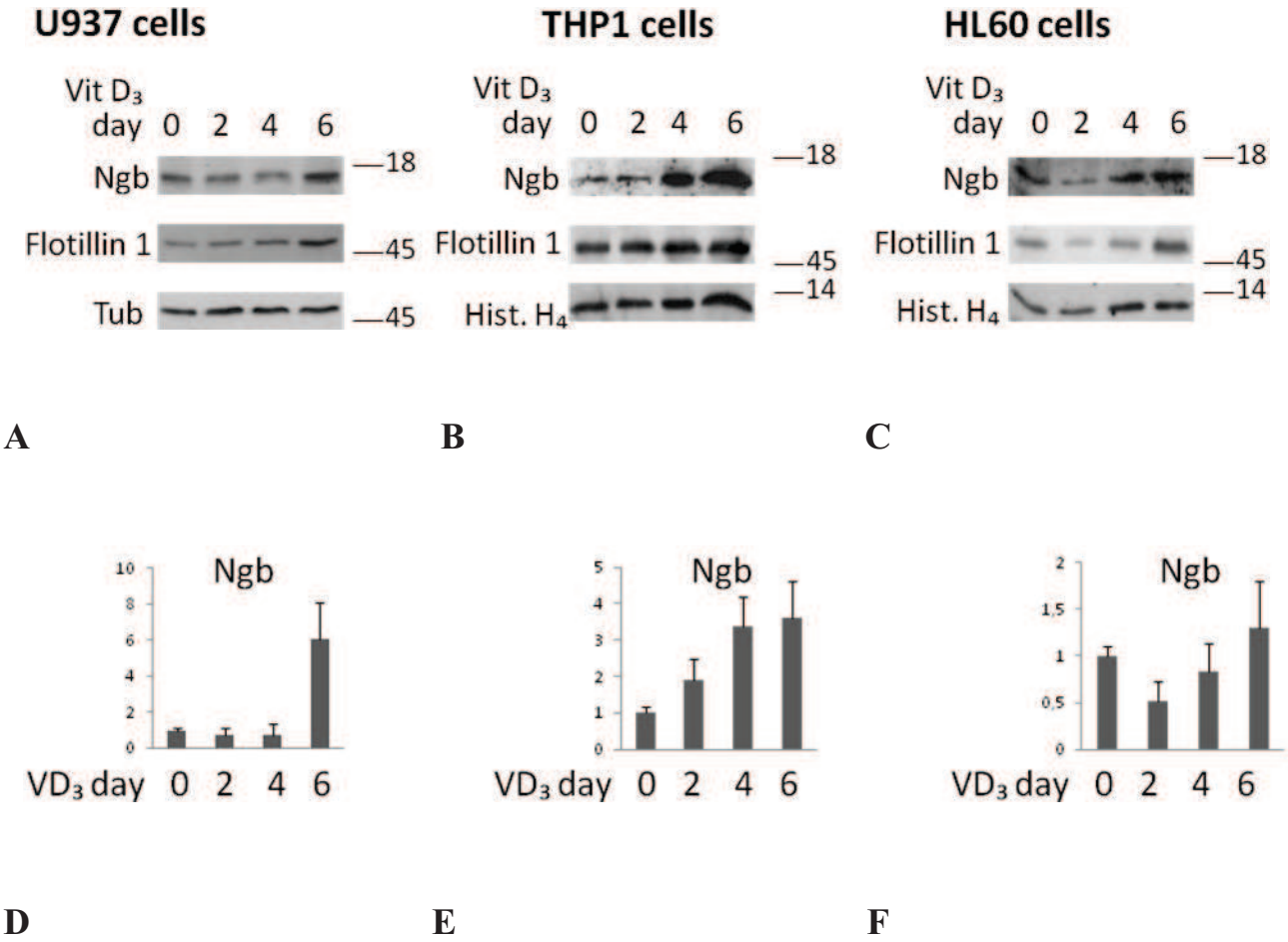


Figure 29: Ngb expression increases in macrophagic differentiated cells.

A, Western Blotting anti-Ngb and anti-Flotillin-1 in U937 B, in THP1 and C, in HL60 cells treated with 250 nM vitamin D_3 for 0, 2, 4, 6 days.

Blots were stripped and rehybridized with an anti-Tubulin or anti-Histone H_4 antibody as loading control.

D, E, F, graphs show Ngb levels obtained by scanning densitometry of the Western blot signals in A, B, and C respectively after normalization to Tubulin or Histone H_4 levels.

The results described above show that Ngb is expressed at higher levels in macrophagic differentiated cells than in immature cells.

3- Neuroglobin expression increases during phagocytosis

The literature suggests that Ngb is an endogenous neuroprotective molecule from oxidative stress related insults in neurons. Several studies have revealed that Ngb protection from oxidative stress is related to its ability in scavenging reactive species. An example of oxidative stress condition in the hematopoietic system is the "oxidative burst" in which macrophages deal with many reactive oxygen species that they produce themselves to combat bacterial and viral infections. Thus we analyzed Ngb expression during phagocytosis in THP1 cells that were induced to macrophagic differentiation.

First of all we induced macrophagic differentiation of THP1 with 10^{-6} M Retinoic Acid for two days. Then to induce phagocytosis in THP1, cells were incubated at 37°C, 5% CO_2 with a red fluorescent *E. Coli* strain diluted in RPMI medium for different hours. Then we evaluated these cells by flow cytometry, because any internalized bacterium makes the THP1 cells fluorescent. In Retinoic Acid differentiated THP1 a high percentage of fluorescent bacteria was phagocytized as showed by significant number of red fluorescent THP1 cells measured by flow cytometry.

Western Blotting anti-Ngb and anti-Flotillin-1 on whole lysates, derived from THP1 that participated in the phagocytosis process, revealed an increased Ngb and Flotillin-1 expression increasing.

These results suggest that these two proteins could have a potential role in this mechanism.

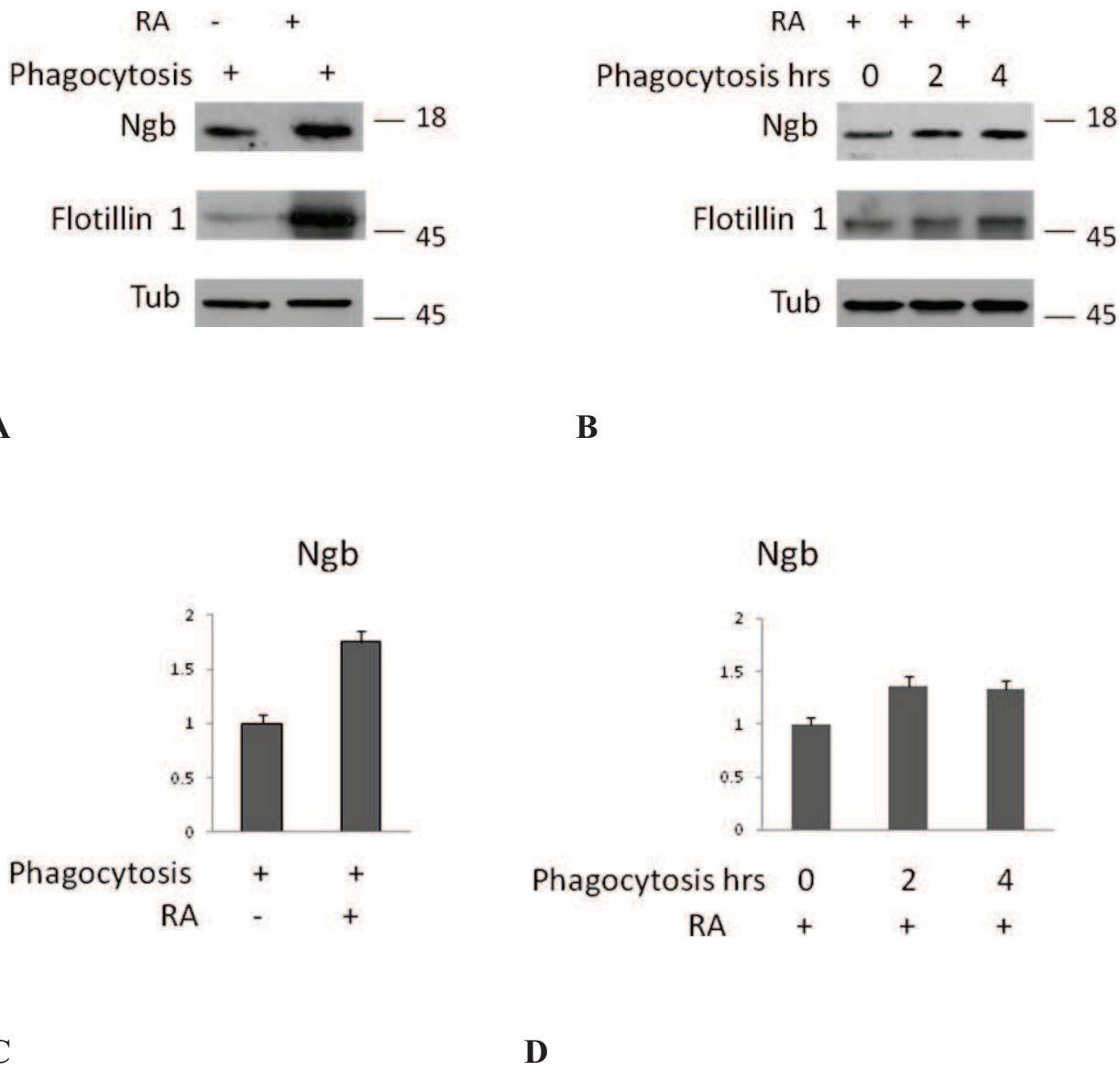


Figure 30: Ngb expression increases during phagocytosis.

A, Western Blotting analysis anti-Ngb and anti-Flotillin-1 from lysates of THP1 untreated (-) or treated (+) with 10^{-6}M Retinoic Acid for 2 days and then four hours incubated with red fluorescent bacteria for phagocytosis.

B, Western Blotting anti-Ngb and anti-Flotillin-1 from lysates of RA treated THP1 during phagocytosis after two and four hours of this mechanism.

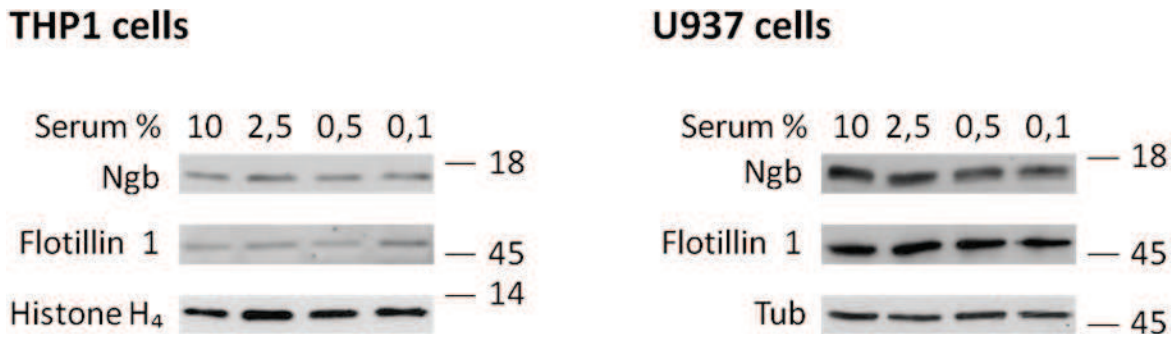
In both experiments Tubulin expression was used as normalize.

C and D, quantification of normalized western blotting signals of Ngb by scanning densitometry.

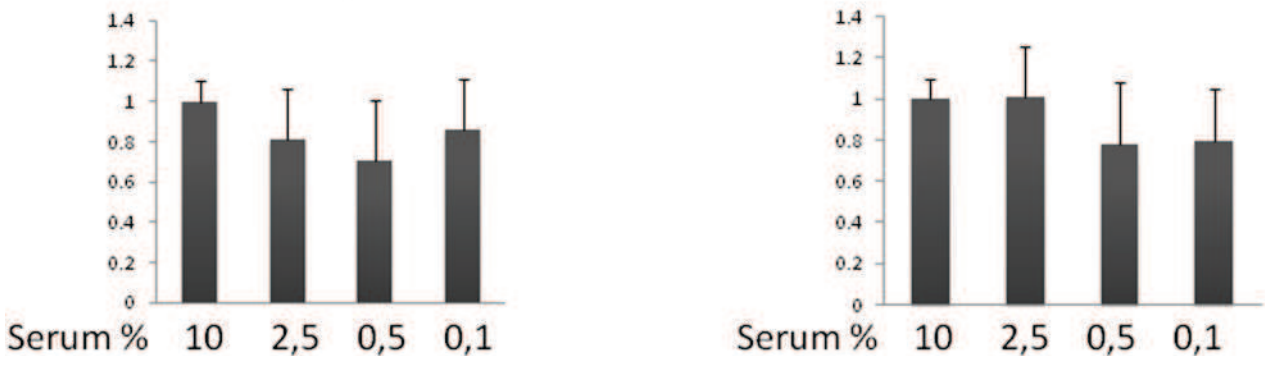
Ngb expression increases during phagocytosis in THP1 and in most of mature macrophagic cells (previously treated with Retinoic Acid).

4- Ngb expression does not change during serum deprivation and DNA damage

Since that Ngb expression is regulated during relevant biological processes in myeloid cells, such as differentiation, we studied Ngb expression under condition that may induce cellular stress or apoptosis. First of all the condition of serum deprivation was studied.
Both THP1 and U937 cells were incubated for 48 hours with percentages of serum (2.5%, 0.5% and 0.1%) lower than that usually used (10%).
Western Blotting anti-Ngb did not reveal any variation in Ngb levels at all percentages of serum tested.



A **B**



C **D**

Figure 31: Ngb expression does not change during serum deprivation.

A, Western Blotting anti-Ngb and anti-Flotillin-1 on whole lysates derived from THP1 and B, U937 cells maintained for 48 hours with different percentages of serum (10; 2.5; 0.5; 0.1 %). A, The expression of Histone H_4 and B, Tubulin proteins were used as loading control.

C and D, quantification of normalized Western Blotting signals by scanning densitometry.

Then we evaluated another stress condition, the DNA damage induced by hydroxyurea. Hydroxyurea (HU) is a DNA replication inhibitor that negatively affects both the elongation and initiation phases and triggers the "intra-S phase checkpoint" (HU blocked DNA synthesis and prevents the dNTP pool expansion).

THP1, U937 and HL60 cells were treated with 2 mM HU for 48 hours. Western Blotting anti-Ngb did not reveal any variation in Ngb levels as a consequence of HU treatment.

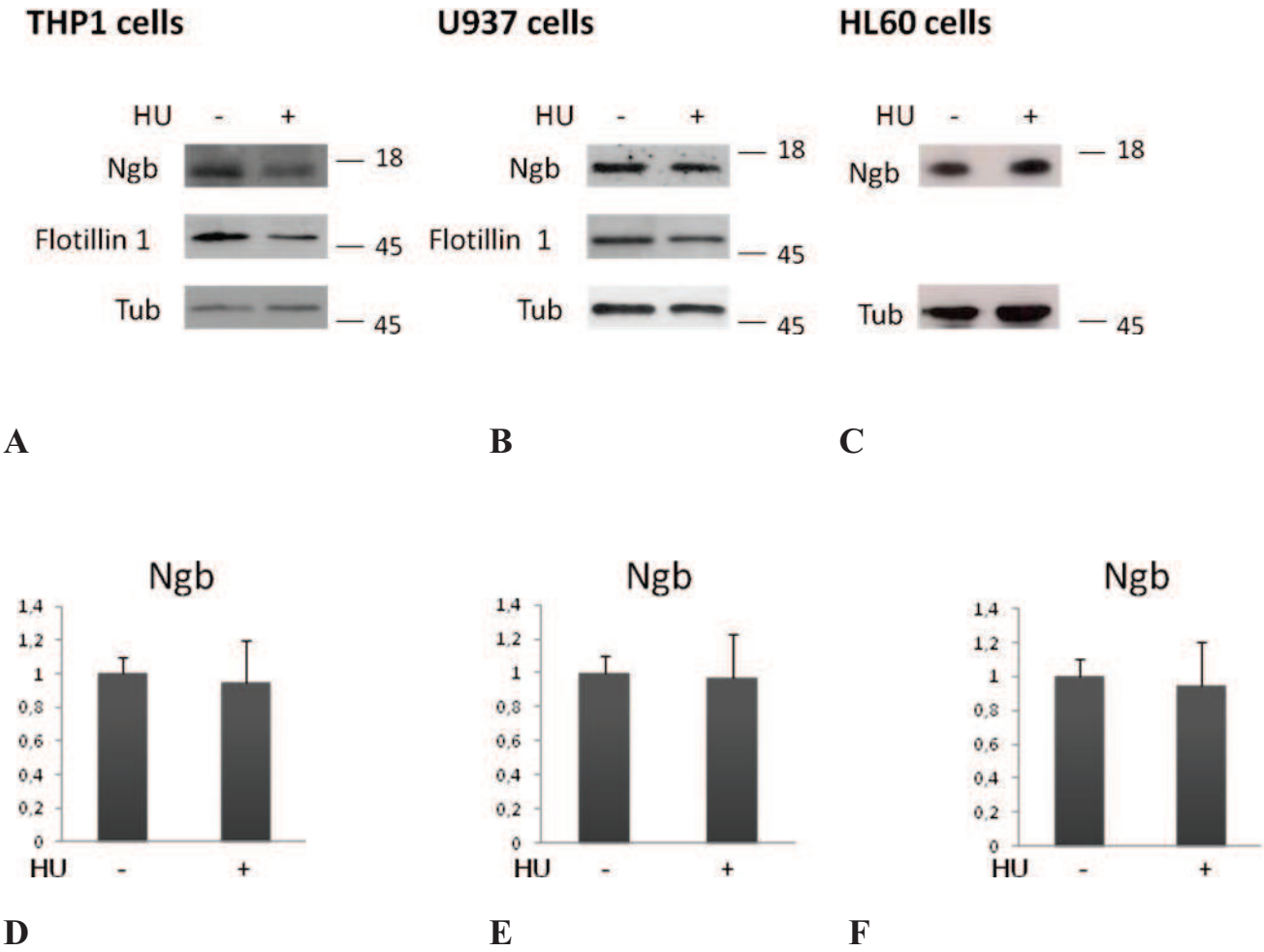


Figure 32: Ngb expression does not change during DNA damage induced by hydroxyurea.

A, Western Blotting anti-Ngb and anti-Flotillin-1 on whole lysates derived from THP1, B, U937 and C, HL60 cells treated for 48 hours with 2 mM HU.

The Tubulin expression was used as a normalization protein for all the experiments.

D, E and F quantification of normalized Ngb levels by scanning densitometry.

These results suggest that there is not any involvement of Ngb during serum deprivation and DNA damage.

5- Ngb expression increases during oxidative stress conditions in hematopoietic cells

To date, Ngb is known to have a protective function from oxidative stress in neurons. Ngb expression in the hematopoietic system suggests that Ngb may have the same protective role from oxidative stress also in hematopoietic cells. Therefore we studied the role of Ngb during H_2O_2-induced oxidative stress in some hematopoietic cell lines (U937, THP1 and HL60).

We treated THP1 and U937 cells with different hydrogen peroxide concentrations at different times.

We started by testing lower concentrations (0,5 and 1 mM) starting from6 and 24 hours of treatment up to 5 days. However none of these conditions showed a significant variation of Ngb expression after Western Blotting analysis.

In contrast, we observed a considerable increased expression of Ngb (in both cell lines) at 6 hours of treatment with 2 mM H_2O_2.

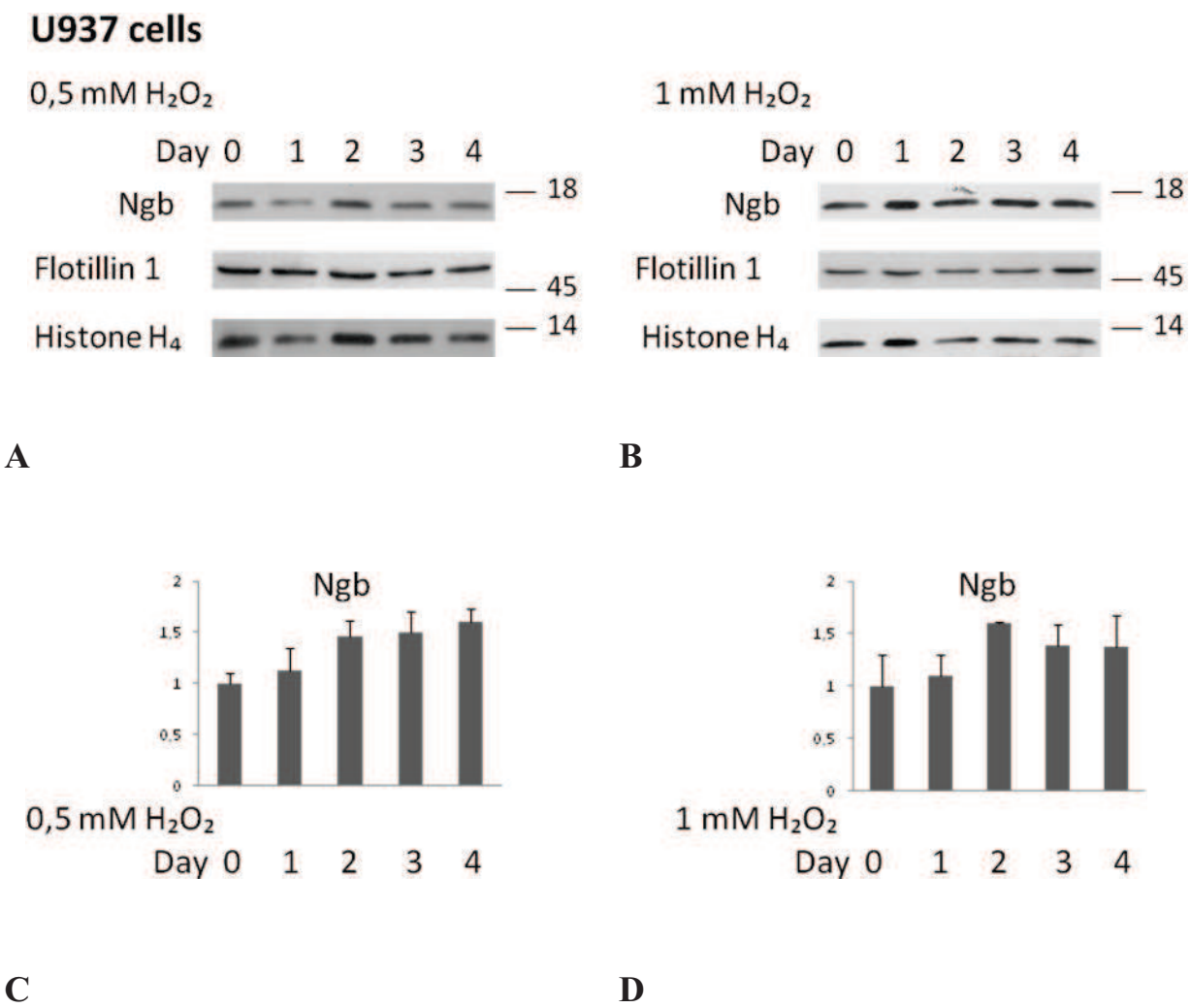
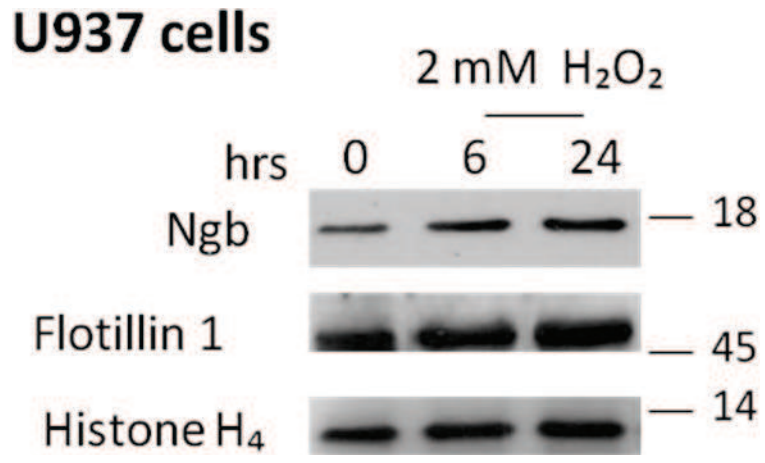


Figure 33: Ngb expression does not change during low doses of H_2O_2in U937

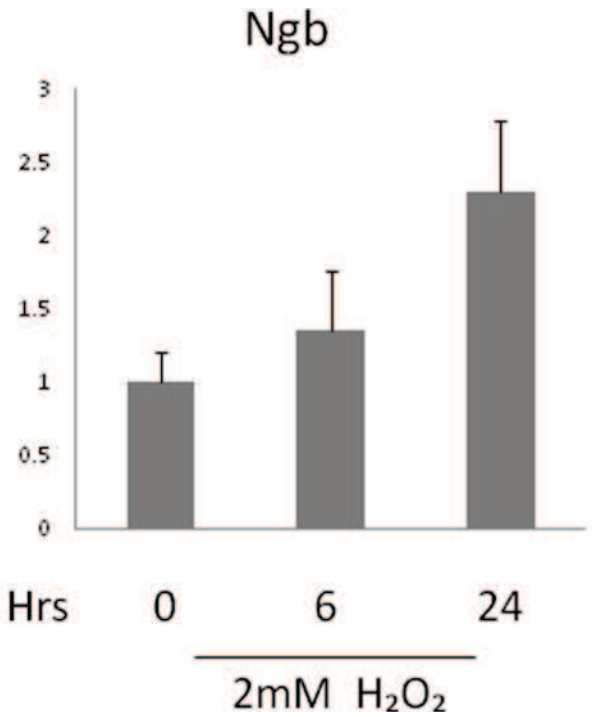
A, Western Blotting anti-Ngb and anti-Flotillin-1 on whole lysates derived from U937 cells incubated with 0,5 mM H_2O_2 and B, 1mM H_2O_2 for different days (0, 1, 2, 3, 4).

Blots were stripped and rehybridized with an anti-Histone H_4 antibody as loading control.

C and D, quantification of normalized Western Blotting signals of Ngb by scanning densitometry.



A



B

Figure 34: Ngb expression increases during high doses of H_2O_2 treatment in U937.

A, Immunoblot anti-Ngb and anti-Flotillin-1 on whole lysates derived from U937 cells incubated with 2 mM H_2O_2 for 0, 6 and 24 hours.

Histone H_4 expression was used to normalize the samples.

B, quantification of normalized Western Blotting signals of Ngb by scanning densitometry.

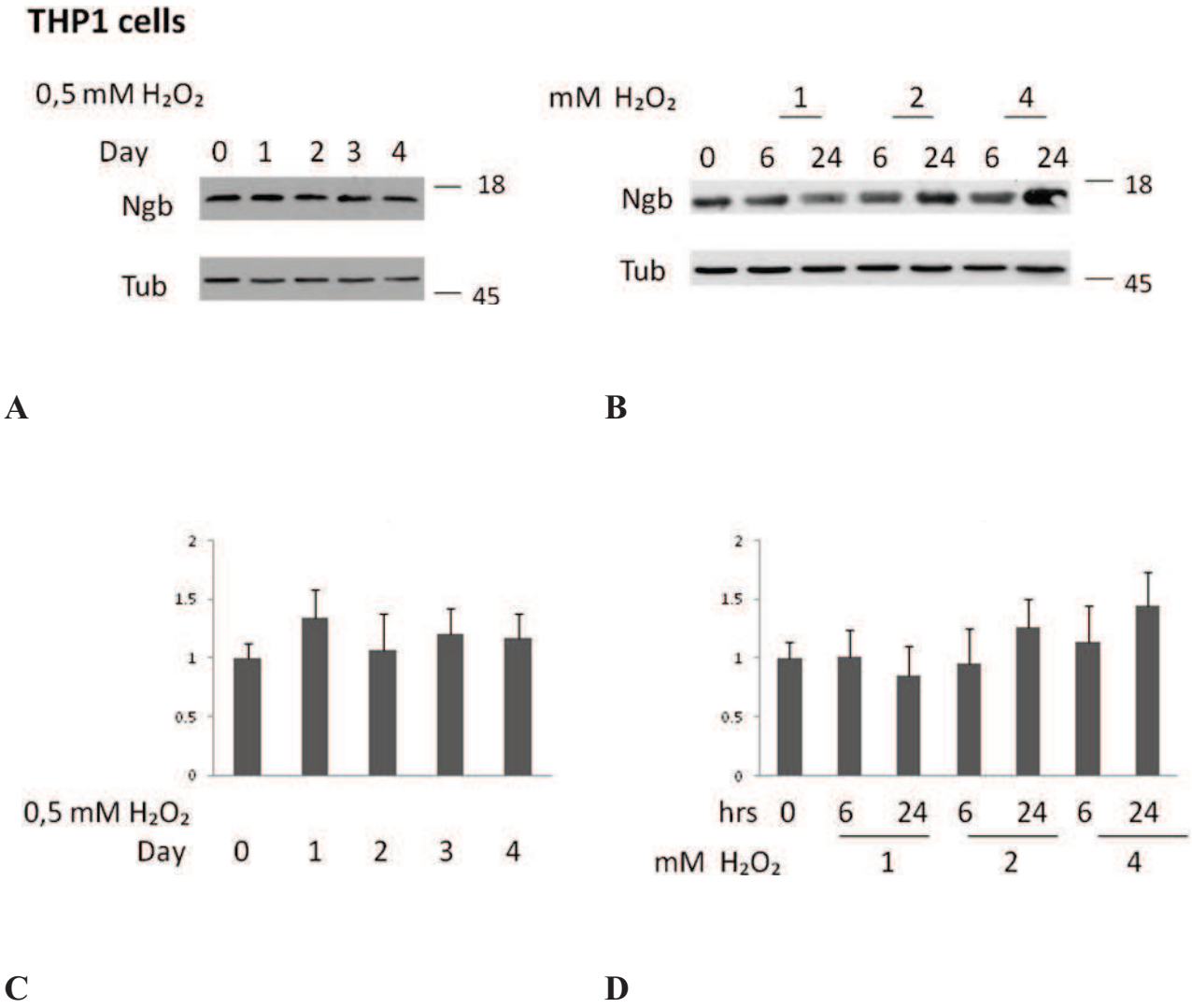


Figure 35: Ngb expression increases during H_2O_2 treatment of THP1.

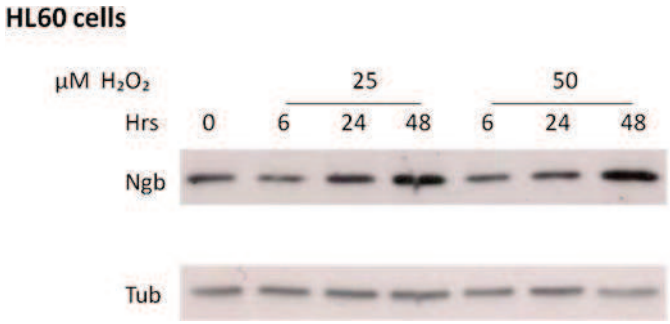
A, Western Blotting anti-Ngb on whole lysates derived from THP1 cells incubated with 0,5 mM H_2O_2 for 0, 1, 2, 3, 4 days .

B, Western Blotting anti-Ngb on whole lysates derived from THP1 cells incubated with 1, 2, 4 mM H_2O_2 for 0, 6, 24 hour .

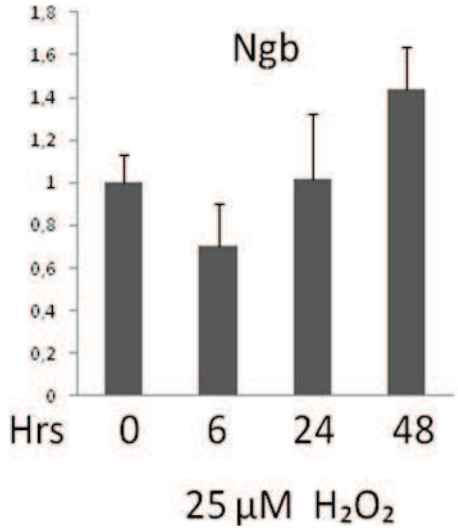
Tubulin protein expression was used to normalize protein content of each sample.

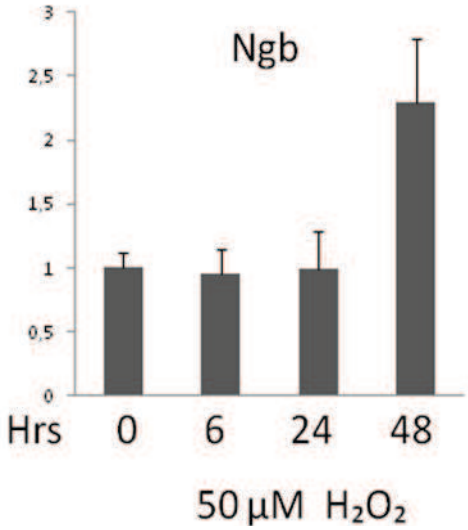
C and D, quantification of normalized Western Blotting signals by scanning densitometry, shown as the average of three independent experiments similar to that in A and in B respectively.

We studied the effect of H_2O_2 induced stress on Ngb expression also in Hl60 cell line. In this cell line we observed the same Ngb expression increase but at lower concentrations of H_2O_2. In fact, the results show the increase of Ngb at 48 hours of treatment with 25 µM H_2O_2 in HL60 cells. The increase is more significant with 50 µM H_2O_2, showing a dose-dependent expression of the protein



A





B **C**

Figure 36: Ngb expression increases during H_2O_2 treatment of HL60.

Western Blotting anti-Ngb on whole lysates derived from HL60 cells incubated with 25 and 50 µM H_2O_2 for 0, 6, 24 and 48 hours.

Tubulin protein expression was used to normalize protein content of each sample.

B and C quantification of Ngb by scanning densitometry normalized with Tubulin expression levels.

Such results suggest that, a part from the protection in neurons, Ngb could protect hematopoietic cells, too, from oxidative stress.

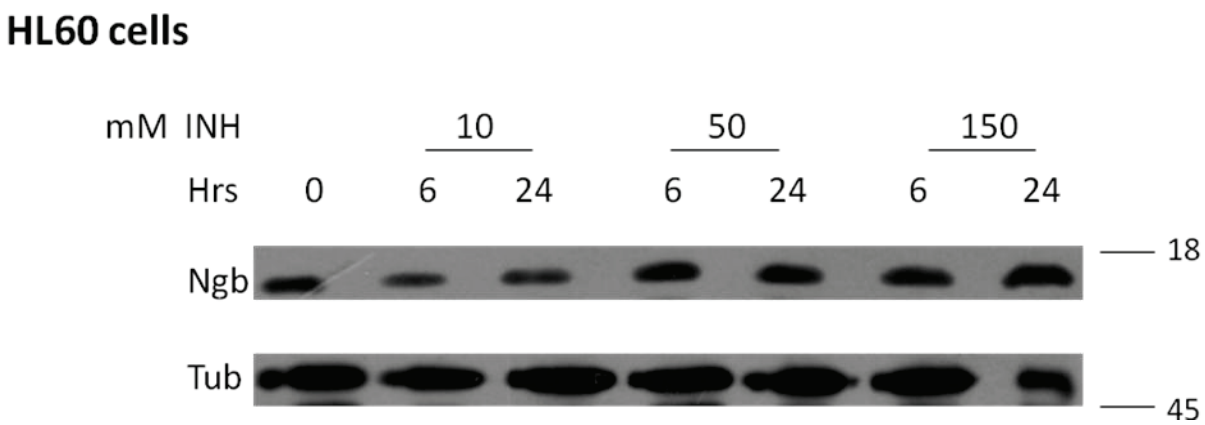
6- Ngb expression increases during Inh treatment

Several drugs induce oxidative stress, such as Inh that binds reversibly ferric and ferrous *Mycobacterium tuberculosis* trHb type N. This binding perturbs the heme-based functional and spectroscopic properties of the protein.

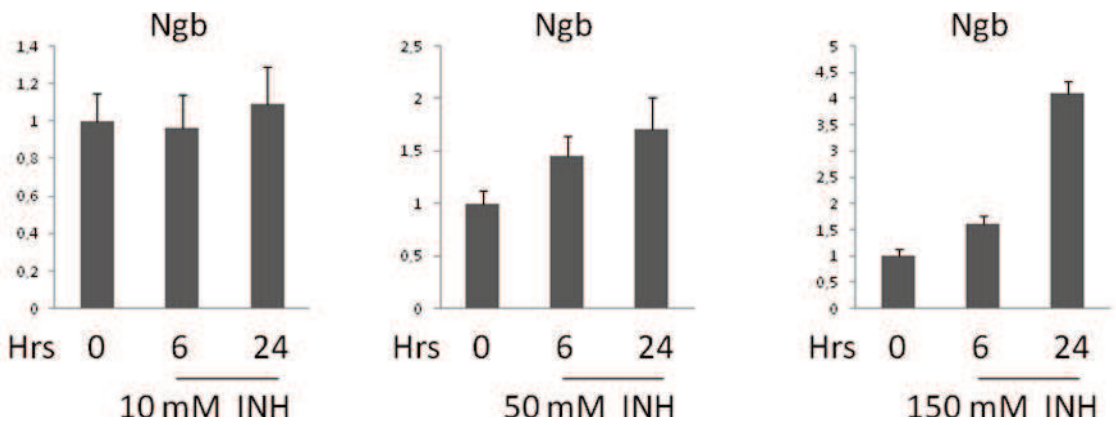
Inh is a heme ligand, displaying a higher affinity for the ferric form. Also Ngb has a heme group and it is converted in ferric state during oxidative stress. These oxidative stress-induced structural changes are essential for Ngb protective activity.

Therefore we analyzed Ngb expression during Inh treatment of hematopoietic cell lines.

We treated HL60 and U937 cells with different concentrations of Inh at different times and then we evaluated the Ngb expression level and cellular growth.



A



B **C** **D**

Figure 37: Ngb expression increases during Inh treatment of HL60 cell lines.

A, Western Blotting anti-Ngb and anti-Tubulin on whole lysates of HL60 treated with 10 - 50 - 150mM Inh for 0 – 6 – 24 hours.

B,C and D, graphs show Ngb expression levels normalized with Tubulin expression.

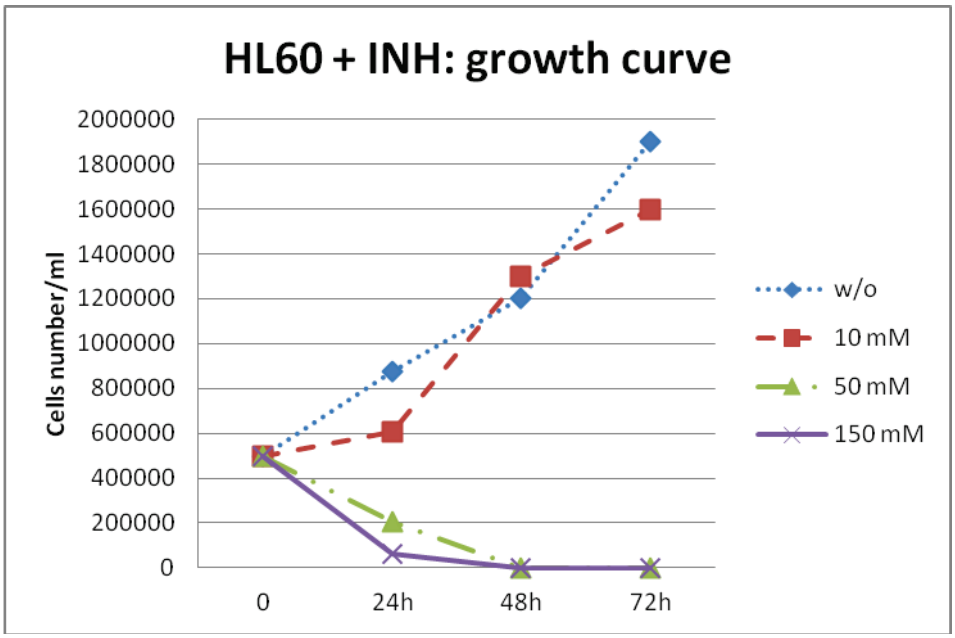
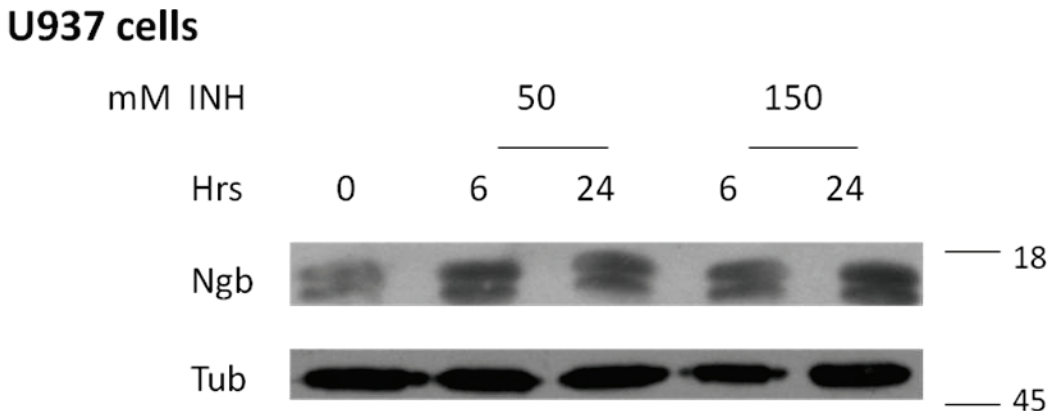


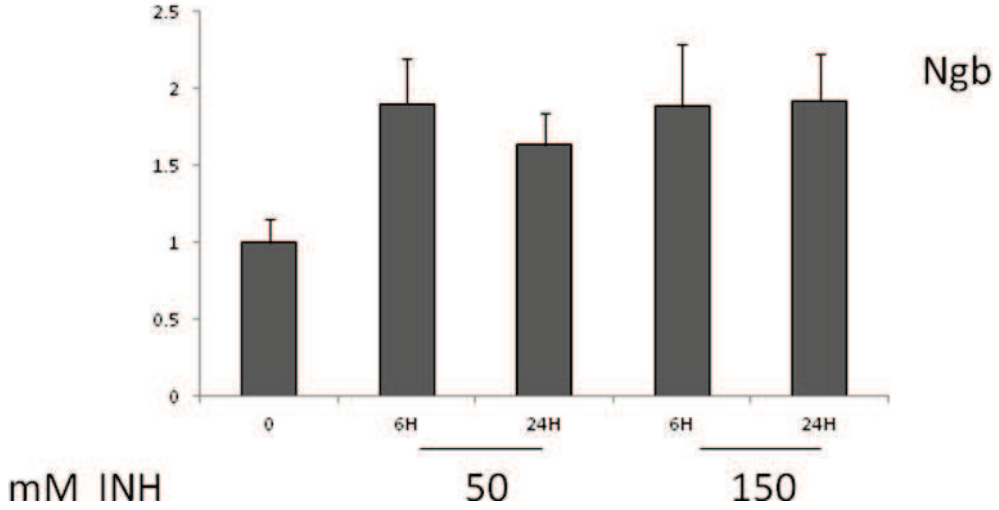
Figure 38: Inh at higher doses causes severe reduction in cellular growth

Growth curve of HL60 cells untreated or treated with Inh at 10mM - 50mM - 150mM for the indicated times.

We conducted the same experiments in U937 cell lines.



A



B

Figure 39: Ngb expression increases during Inh treatment in U937 cell lines.

A, Western Blotting anti-Ngb and anti-Tubulin on whole lysates of HL60 treated with 50 and 150mM Inh for 0 – 6 – 24 hours.

B, Quantification of Ngb by scanning densitometry.

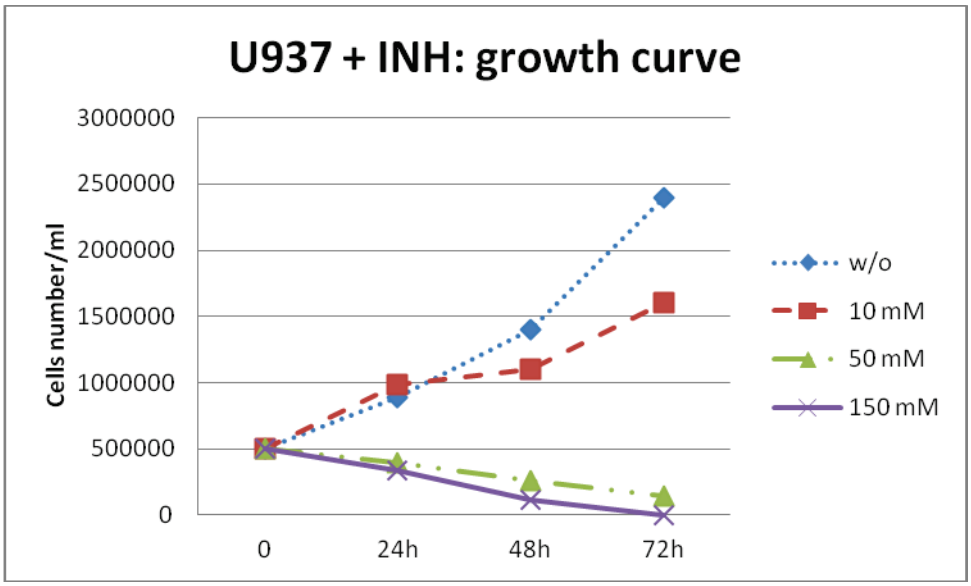


Figure 40: Inh at higher doses causes severe reduction in cellular growth.

Growth curve of U937 cells untreated or treated with Inh at 10mM - 50mM - 150mM for the indicated times.

Ngb expression increases also during Inh induced oxidative stress

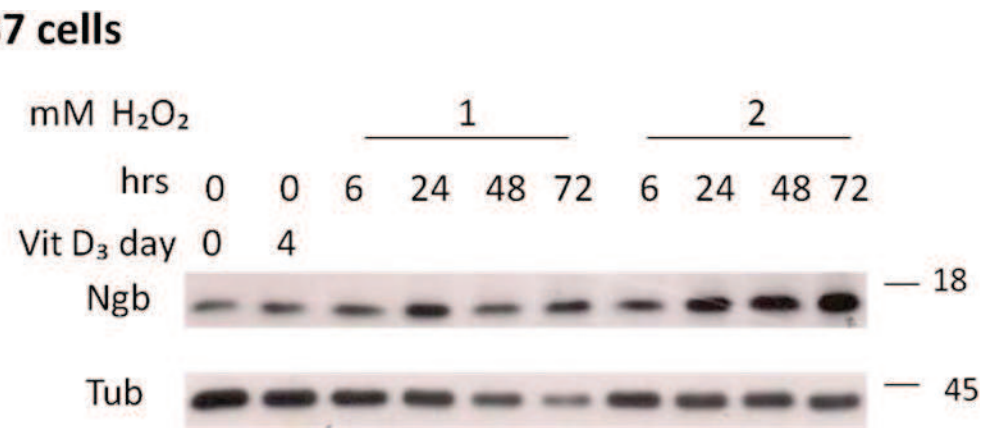
7- Oxidative stress effect on Ngb expression is increased by vitamin D_3-induced differentiation in U937 cells

From previous experiments we have observed that Ngb expression increases during myeloid differentiation and under oxidative stress conditions.

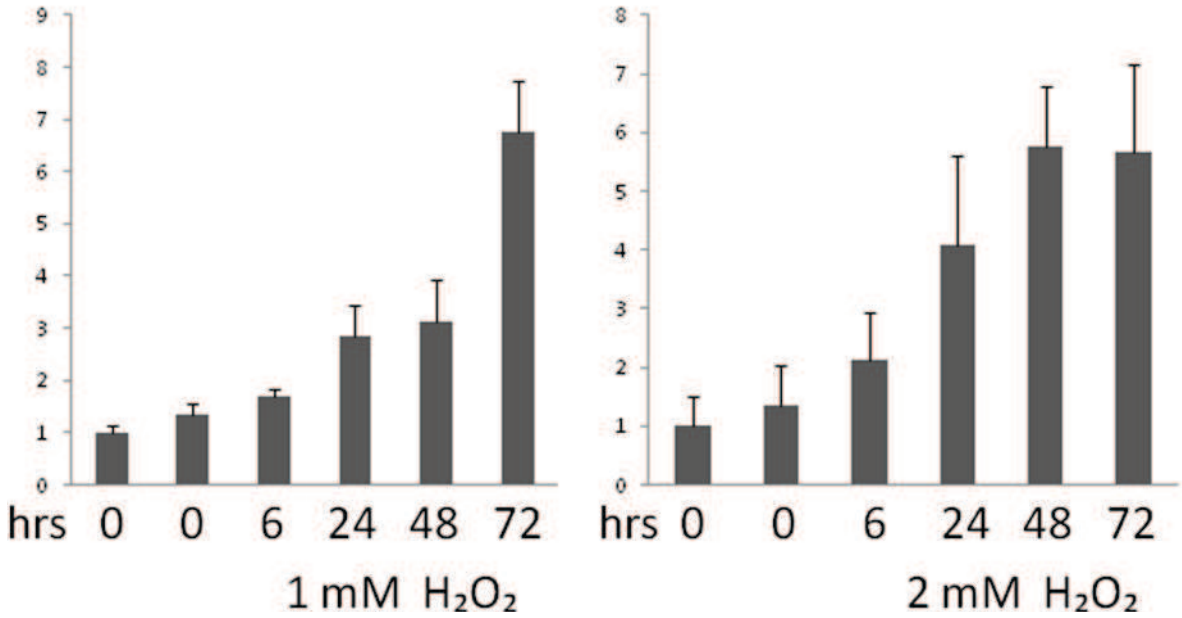
On the basis of these results, we combined the two situations and then estimated Ngb expression during oxidative stress in monocytic cells compared to undifferentiated cells.

We induced monocytic differentiation in U937 cells with 250 nM Vitamin D_3 for four days and then we induced oxidative stress testing two different H_2O_2 concentrations (1 and 2 mM) at different times (6 – 24 – 48 – 72 hours).

Western Blotting analysis revealed a further Ngb expression increase.



A



B **C**

Figure 41: Ngb expression increases under oxidative stress conditions in differentiated myeloid cells.

A, Western Blotting anti-Ngb in U937 cells treated for four days with 250 nM Vitamin D_3 and then with 1 or 2 mM H_2O_2 for 6, 24, 48, 72 hours.

Tubulin expression was used as normalize .

B and C, graphs show quantification of Ngb levels by scanning densitometry.

Oxidative stress effect on Ngb expression is more evident in differentiated U937 cells

8- Flotillin-1 knockdown decreases Ngb expression

Flotillin-1 interacts with Ngb in the neural tissue. Previous results, obtained in our laboratory, have shown that Flotillin-1 expression increases during myeloid differentiation. To understand Ngb and Flotillin-1 relationship, we evaluated the influence of Flotillin-1 e on Ngb expression. For this purpose we used U937, THP1 and HL60 cells that have been previously infected with a retrovirus harboring the gene FLO-1. So immunoblot analysis anti-Ngb in Flotillin-1 over-expressing U937, THP1 and HL60 cell lines revealed that there was not any variation of Ngb expression compared to wild type cells. We could assume that Flotillin-1 acts by stabilizing the Ngb without increasing its expression.

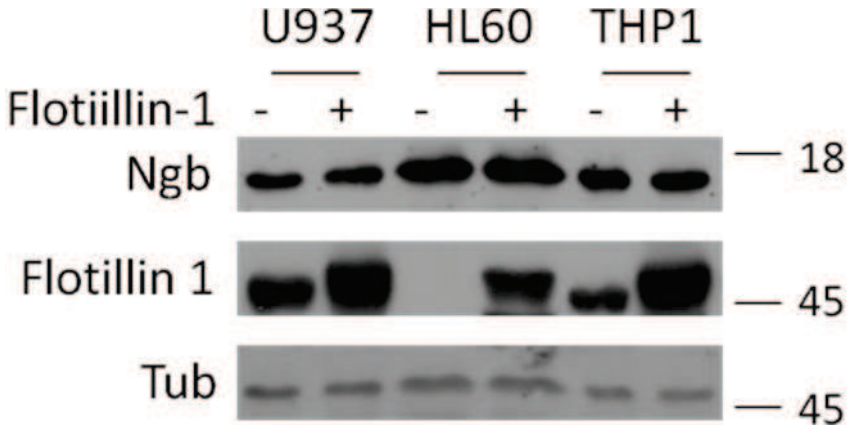


Figure 42: Ngb expression does not change in Flotillin-1 overexpressing cell lines.

Western Blotting anti-Ngb and anti-Flotillin-1 on whole lysates derived from wild type cell lines and from cell lines where Flotillin-1 was overexpressed.

Western Blotting anti-Tubulin was used as normalize.

To investigate the role of Flotillin-1 in regulating Ngb, we studied its expression in cells where Flotillin-1 was downregulated. We have generated cell clones where Flotillin-1 expression was inhibited by specific shRNA with lentiviral infection. Thus we analyzed Ngb expression by Western Blotting and we observed that Ngb expression decreases in THP1 and U937 clones where Flotillin 1 silenced.

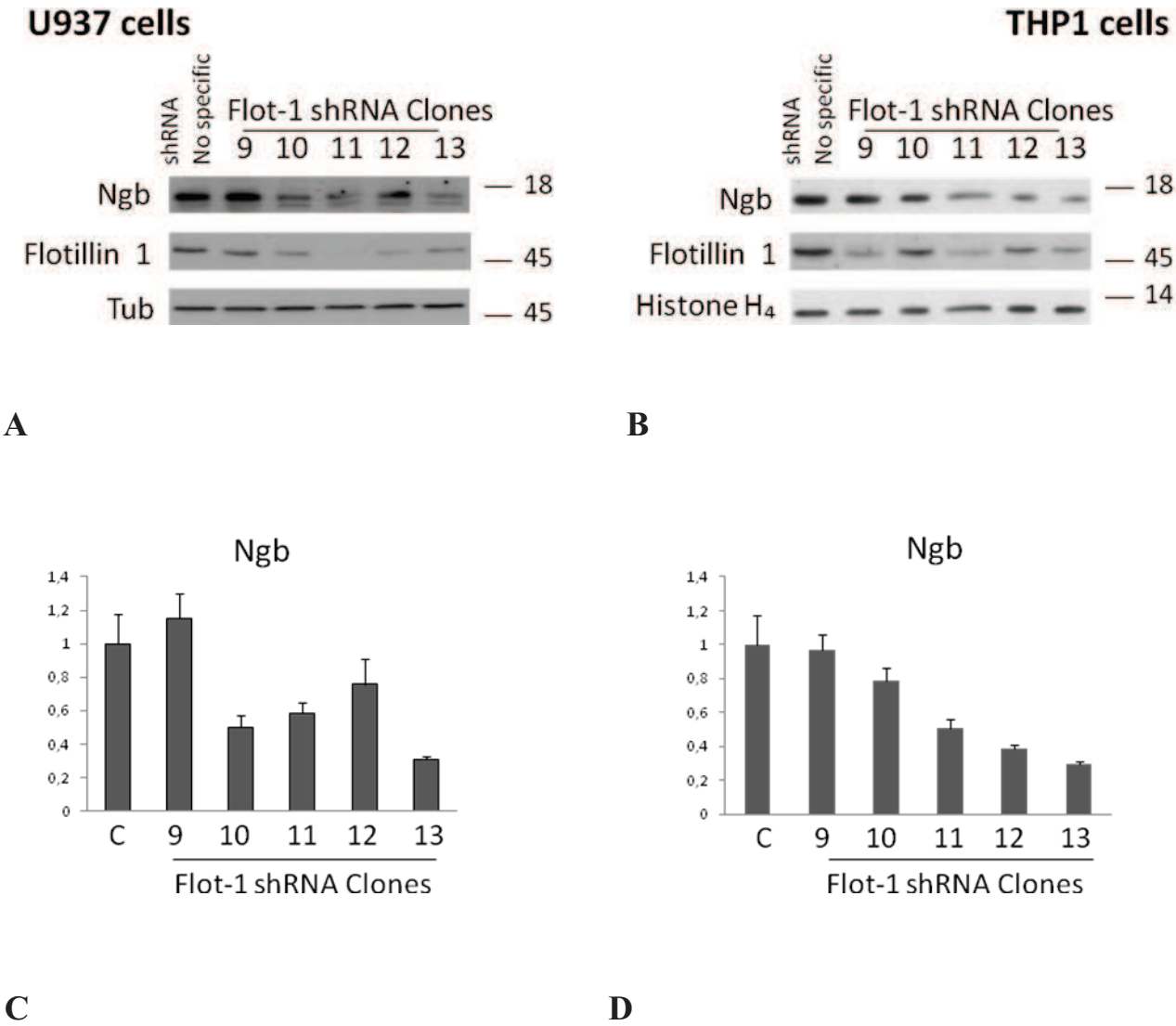


Figure 43: Ngb expression decreases in Flotillin-1 knock-down cell lines.

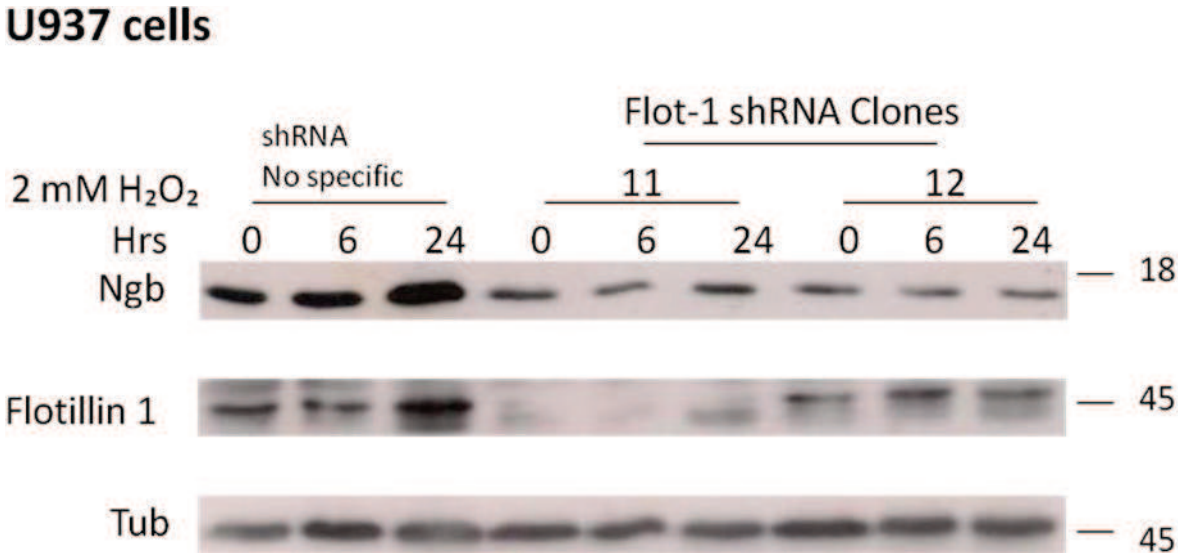
A, Western Blotting anti-Ngb and anti-Flotillin-1 on lysates derived from U937 clones where Flotillin-1 was silenced by shRNA. Tubulin protein expression was used to normalize protein content of each sample.

B, Western Blotting anti-Ngb and anti-Flotillin-1 on lysates derived from THP1 clones where Flotillin-1 was silenced by shRNA. Histone H_4 protein expression was used to normalize protein content of each sample.

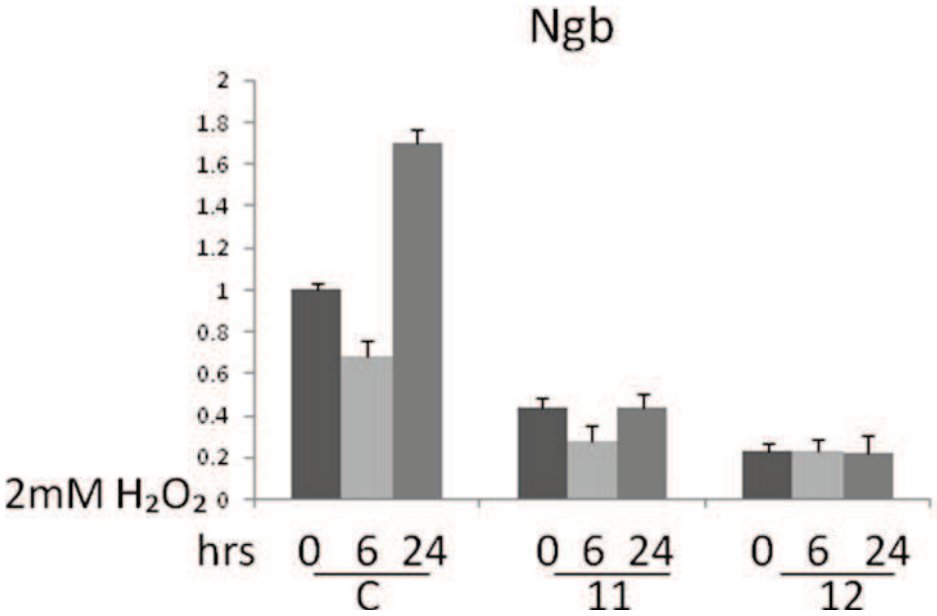
Both in the panel A that in panel B no specific shRNA was used as negative control.

C and D, quantification of Ngb levels by scanning densitometry in immunoblot A and B respectively after normalization with Tubulin or Histone H4 levels.

It was very interesting to see that, during oxidative stress conditions in all the cell lines studied, there was not only a Ngb expression increase but also a Flotillin-1 expression increase. Consequently Flotillin-1 could protect Ngb during oxidative stress. To confirm this hypothesis we evaluated Ngb expression levels during oxidative stress in clones where Flotillin-1 expression was inhibited by specific shRNA with lentiviral infection.



A



B

Figure 44: Ngb expression decreases during H_2O_2 treatment in U937 clones where Flotillin-1 has been silenced.

Western Blotting anti-Ngb and anti-Flotillin-1 1 on whole lysates derived from U937 clones where Flotillin-1 was silenced by shRNA, incubated with 1 mM (Fig. A) and 2 mM (Fig. B) H_2O_2 *for 0, 6 and 24 hours.*

Histone H_4 protein expression was used to normalize protein content of each sample.

Both in the panel A that in panel B no specific shRNA was used as negative control.

B, quantification of Ngb levels by scanning densitometry in immunoblot A after normalization with Tubulin levels.

Flotillin-1 knock-down decreases Ngb expression also during oxidative stress conditions. Thus the absence of Flotillin-1 prevents the increasing of Ngb.

9- Ngb physically interacts with Flotillin-1 in an oxidative stress dependent manner in myeloid cells

The results discussed above suggest that Flotillin-1 has a protective role on Ngb. To confirm this, we have investigated if there was an interaction between these two proteins.

To this end, we performed co-immunoprecipitation experiments using an anti-Flotillin-1 antibody, followed by Western Blotting with an anti-Ngb antibody. This was performed both in basal conditions and after treatment with 2 mM H_2O_2 for 6 hours in THP1 cells.

A positive result was observed only after H_2O_2 treatment, so Ngb interacts with Flotillin-1 in myeloid cells in an oxidative stress dependent manner.

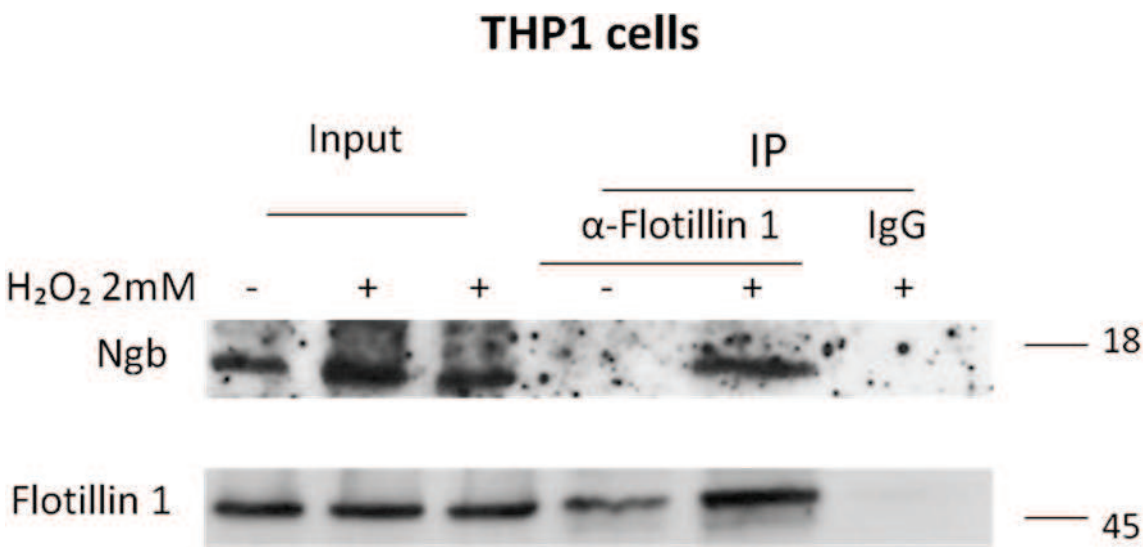


Figure 45: Oxidative stress promotes interaction between Ngb and Flotillin-1.

Co-Immunoprecipitation and Western Blotting experiment from THP1 cells before (-) and after (+) 2 mM H_2O_2 treatment. IP indicates immunoprecipitating antibody. Immunoprecipitates were analyzed by Western Blotting with the indicated antibodies. The firsthree lanes are whole cell-lysates showing the expression of Ngb and Flotillin-1 in THP1 cells. Ig indicates immunoprecipitation with control negative antibody.

Therefore we observed a physically interaction between Ngb and Flotillin-1 only under oxidative stress conditions.

Flotillin-1 is a component of lipid rafts, containing also heterotrimeric G proteins. As Ngb interacts with Gα, Flotillin-1 might recruit Ngb to lipid rafts stabilizing its expression as a mean of preventing cellular death.

CONCLUDING REMARKS

and

FUTURE PERSPECTIVES

On the basis of these results we can conclude that Ngb is expressed also in the analyzed myeloid cell lines and not only in neurons of the central and peripheral nervous system. Ngb expression was investigated also in some lung cancer cell lines where we observed a considerable Ngb expression level.

By analyzing Ngb expression at various stages of the hematopoietic differentiation, we have observed that Ngb expression increases during myeloid differentiation and, moreover, during macrophagic differentiation.

Since Ngb has a protective function against oxidative stress in neurons, related to its ability in scavenging reactive species, we have investigated Ngb expression during oxidative stress conditions that may occur in myeloid cells, such as the "oxidative burst" during phagocytosis. We observed a considerable increase in the Ngb expression during the phagocytosis process in THP1 and during phagocytosis in mature macrophagic cells, too.

Afterwards under oxidative stress conditions, such as H_2O_2- or Inh-induced oxidative stress, Ngb expression increases in a dose and time dependent manner. During H_2O_2-induced oxidative stress, we observed an arised Ngb expression especially in differentiated U937.

Moreover we did not observe any modification in the Ngb expression during different conditions of cellular stress, such as serum deprivation and HU-induced DNA damage. These results suggested a possible protective function of Ngb in myeloid cells (as well as in neurons) only under specific oxidative stress conditions.

To understand the relationship between Ngb and Flotillin-1in myeloid cells, we have evaluated the influence of Flotillin-1 expression on Ngb. So Flotillin-1 knock-down cells showed a decrease in the Ngb expression, while the overexpression ofFlotillin-1 did not change Ngb levels, suggesting a possible protective function of Flotillin-1 on Ngb.

Finally, to confirme this possible role of Flotillin-1 on Ngb we investigated if there was an interaction between these two proteins. We found that Ngb physically interacts with Flotillin-1 in an oxidative stress dependent manner in myeloid cells: in fact, only the H_2O_2 treatment has promoted the interaction between these two proteins.

According to the results discussed above, further investigations could be conducted on Ngb expression and its functions:

-Ngb expression in $CD34^+$ hematopoietic progenitors cells;

-Ngb-Flotillin-1 co-localization by confocal microscopy

-protective role of Ngb during oxidative stress in myeloid cells through its silencing and/or overexpression.

REFERENCES

Adams, R. L., Berryman, S., and Thomson, A. *Biochim. Biophys. Acta.* 1971**;** 240**:** 455-462.

Akira, S. TLR signaling. (2006) *Curr. Top Microbiol. Immunol.* 2006; 31; 1-16.

Alexeev, A., Mazin, A. and Kowalczykowski, S.C. Rad 54 protein processes chromatin-remodeling activity stimulated by the Rad51-ssDNA nucleoprotein filament. *Nat Struct Biol. 2003;* 10: 182-186.

Allen, P.G., Dawidowicz, E.A. Phagocytosis in *Acanthamoeba*: I.A mannose receptor is responsible for the binding and phagocytosis of yeast. *J. Cell Physiol.* 1990; 145: 508-513.

Allen, J.B., Z. Zhou, W. Siede, E.C. Friedberg, S.J. Elledge. The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. *Genes Dev*. 1994; 8**:** 2401-2415.

Arango Duque, G., Descoteaux, A. A *Leishmania* survival in the macrophage: where the ends justify the means. *Curr. Opin. Microbiol.*, 2015; 26: 32-40.

Bartek, J., Bartkova, J. and Lukas, J. DNA damage signaling guards against activated oncongenes and tumor progression. *Oncogene.* 2007; 26: 7773-7779.

Arredondo-Peter, R., M.S.Hargrove, G.Sarath, J.F.Moran, J.Lohrman, J.S.Olson, R.V.Klucas. Rice hemoglobins. Gene cloning, analysis, and O2-binding kinetics of a recombinant synthesized in Escherichia Coli. *Plant Physiol*. 1997; 115: 1259-1266.

Ascenzi P., Coletta A., Cao Y., Trezza V., Leboffe L., Fanali G., Fasano M., Pesce A., Ciaccio C., Marini S., Coletta M. Isoniazid inhibits the heme-based reactivity of *Mycobacterium tuberculosis* truncated hemoglobin N. *Plos one.* 8: 1-17.

Block, W. D., Merkle, D., Meek, K. and Lees-Miller, S. P. Selective inhibition of the DNA-dependent protein kinase (DNA-PK) by the radiosensitizing agent caffeine. *Nucleic Acids Res. 2004;* 32: 1967-1972.

Bolognesi, M., Bordo, D., Rizzi, M., Tarricone, C., Ascenzi, P. Nonvertebrate hemoglobins: structural bases for reactivity. 1997; *Prog Bioohys Mol Biol.* 68: 29-68.

Brown D. A. & London E. Structure and function of sphingolipid- and cholesterol-rich membrane Rafts. *J. Biol. Chem*. 2000; 275: 17221-17224.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y.,

Weiss, D.S., Weinrauch, Y., Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. *Science*. 2004; 303: 1532-1535.

Brunori, M., Nitric oxide, cytochrome-c oxidase and myoglobin. *Trends in Biochemical Sciences.* 2001; 26(1): 21-23.

Brunori M., Giuffre A., Forte E., Mastronicola D., Barone M.C., Sarti P. Control of cytochrome c oxidase activity by nitric oxide. Biochim. Biophys. Acta 2004; 1655: 365–371.

Bumester T., Weich B., Reinhardt S., Hankein T. A vertebrate globin expressed in the brain. *Nature.* 2000; 407: 520-523.

Burmester T., Hankeln T. Neuroglobin: a respiratory protein of the nervous system. *News Physiol. Sci.* 2004; 19; 110–113.

Burmester T., Gerlach F., Hankeln T. Regulation and role of neuroglobin and cytoglobin under hypoxia. *Adv. Exp. Med. Biol.* 2007; 618: 169–180.

Chabes, A., B. Georgieva, V. Domkin, X. Zhao, R. Rothstein, and L. Thelander. Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. *Cell*. 2003; 112**:** 391-401.

Chiang S.H., C. A. Baumann, Mokoto Kanzaki, D. C. Thurmond, T. Watson, C. L. Neudauerk, Ian G. Macarak, J. E. Pessin, A. R. Saltiel. (2001) Insulin-stimulated GLUT4 traslocation requires the CAP-dependent activation of TC10. *Nature*. 2001; 19: 944-948.

Clark, S.R., Ma, A.C., Tavener, A.S., McDonald, B., Goodarzi, Z., Kelly, M.M., Patel, K.D., Chakrabarti, S., McAvoy, E., Sinclair, G.D., *et al*. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in endotoxemic and septic blood. *Nature Med.* 2007; 13: 463-469.

Comfine S., Himmel M., Koop P., Azzouzi KEI, Wiesner C., Kruger M., Rudel T., Linder S. The kinesin KIF9 and reggie/flotillin proteins regulate matrix degradation by macrophage podosomes. *Mol Biol Cell* 2011; 22(2): 202-215.

Davies, B., Chattings, L.S., Edwards, S.W. (1991) Superoxide generation during phagocytosis by *Acanthamoeba castellanii*: similarities to the respiratory burst of immune phagocytes. *J. Gen. Microbiol.* 1991; 137: 705-710.

Dermine J-F, S. Dulcos, J. Garen, F. ST-Louis, S. Rea, R. G. Parton, M. Desjardins. Flotillin-1-enriched Lipid Raft Domains Accumulate on Maturing Phagosomes. *J. Biol. Chem*. 2001; 276: 18507-18512.

Desany, B.A., A.A. Alcasabas, J.B. Bachant, and S.J. Elledge. Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. *Genes Dev*. 1998; 12**:** 2956-2970.

Desjardins, M., Houde, M., Gagnon, E. Phagocytosis: The convoluted way from nutrition to adaptive immunity. *Immunol. Rev.* 2005; 207: 158-165.

Dewilde S., Kiger L., Burmester T., Hankeln T., Baudin-Creuza V., Aerts T., Marden M.C., Caubergs R., Moens L. Biochemical characterization and ligand binding properties of neuroglobin, a novel member of the globin family. *J. Biol. Chem*. 2001; 276: 38949–38955.

Dini L., Autuori F., Lentini A., Oliverio S., Piacentini M. The clearance of apoptotic cells in the liver is mediated by the asialogly coprotein receptor. *FEBS Lett*. 1992; 296: 174-178.

Dudenhoffer, C., Rohally, G., Will., K., Deppert, W. and Wiesmuller, L. Specific mismatch recognition in heteroduplex intermediates by p53 suggests a role in fidelity control of homologous recombination. *Mol Cell Biol. 1998;* 18: 5332-5342.

Duvall E., A.H. Wyllie, R.G. Morris. Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology*. 1985**;** 56: 351-358.

Dynan, W.S. and Yoo, S. Interaction of Ku protein and DNA-dependent protein kinase catalytic subunit with nucleic acids. *Nucleic Acids Res.* 1998; 26: 1551-1559.

Dzik, J.M. The ancestry and cumulative evolution of immune reactions. *Acta Biochim. Pol. 2010;* 57: 443-466.

Elledge, S.J., Zhou, Z., Allen, J.B., Navas, T.A. *BioEssays* 1993; 15**:** 333-339.

Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidised low density lipoprotein. *Biol Chem*. 1993; 268: 11811-6.

Eriksson, S., Skog, S., Tribukait, B., and Wallstrom, B. *Exp. Cell Res.* 1987; 168**:** 79-88.

Esteban, M.A., Meseguer, J. Factors influencing phagocytic response of macrophages from the sea bass (*Dicentrarchus labrax* L.): an ultrastructural and quantitative study. *Anat. Rec*. 1997; 248: 533-541.

Fadok V.A., D.R. Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, P.M.

Henson. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol*. 1992; 148: 2207-2216.

Fadok V.A., D.L. Bratton, A. Konowal, P.W. Freed, J.Y. Westcott, P.M. Henson. Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-BETA, PGE2, and PAF. *J Clin Invest.* 1998; 101: 890-898.

Fadok V.A., A. de Cathelineau, D.L. Daleke, P.M. Henson, D.L. Bratton. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem*. 2001; 276: 1071-1077.

Fernandez-Capetillo, O., Lee, A., Nussenzweig, M. and Nussenzweig, A. H_2AX: the histone guardian of the genome. *DNA Repair (Amst).* 2004; 3: 959-967.

Flannagan, R.S., Jaumouillé, V., Grinstein, S. The cell biology of phagocytosis. *Annu. Rev. Pathol*. 2012; *7*: 61-98.

Foiani, M., A. Pellicioli, M. Lopes, C. Lucca, M. Ferrari, G. Liberi, M. Muzi Falconi, and P. Plevani**.** 2000. DNA damage checkpoints and DNA replication controls in *Saccharomyces cerevisiae*. *Mutat. Res*. 2000; 451**:** 187-196.

Fordel E., Thijs L., Martinet W., Lenjou M., Laufs T., Van Bockstaele D., Moens L., Dewilde S. Neuroglobin and cytoglobin overexpression protects human sh-sy5y neuroblastoma cells against oxidative stress-induced cell death. *Neurosci. Lett.* 2006; 410: 146–151.

Fordel E., Thijs L., Moens L., Dewilde S. Neuroglobin and cytoglobin expression in mice. Evidence for a correlation with reactive oxygen species scavenging. *FEBS J.* 2007; 274: 1312–1317.

Gandhi, V., Plunkett, W., Kantarjian, H., Talpaz, M., Robertson, L. E., and O'Brien, S. *J. Clin. Oncol.*1998; 16**:** 2321-2331.

Gasch, A. P., M. Huang, S. Metzner, D. Botstein, S. J. Elledge, and P. O.Brown. 2001. Genomic expression responses to DNA-damaging agents and the regulatory role of the yeast ATR homolog. Mec1p. *Mol. Biol. Cell.* 2001**;** 12**:** 2987-3003.

Giancotti, F.G. and Ruoslahti, E. Integrin signaling. *Science.*1999; 285: 1028-1032.

Gilman A.G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 1987; 56: 615–649.

Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T.E., Mann, M. and Lieber, M.R. Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. *Nature.* 1997; 388: 492-495.

Geuens E., Brouns I., Flamez D., Dewilde S., Timmermans J.P., Moens L. A globin in the nucleus! *J. Biol. Chem.* 2003; 278: 30417–30420.

Haber, J.E. Partners and pathways repairing a double-strand break. *Trends Genet.* 2000; 16: 259-264.

Haglund K., Kovic-Dikic I.I., Simokaw N., Gary D., K. and I. Dikic. Recruitment of Pyk2 and Cbl to lipid rafts mediates signals important for actin reorganization in growing neuritis. *Journal of Cell Science*. 2004; 1117: 2557-2568.

Hall S.E., J.S. Savill, P.M. Henson, C. Haslett. Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a annose/fucose-specific lectin. *J Immunol*. 1994; 153: 3218-3227.

Hamdane, D., L.Kiger, G.H.B.Hoa, S.Dewilde, J.Uzan, T.Burmester, T.Hankeln, L.Moens, M.C.Marden. High pressure enhances hexacoordination in neuroglobin and other globins. *J Biol Chem*. 2005; 280: 36809-14.

Hanecak, R., Zovich, D.C., Pattengale, P.K., Fan, H. Differentiation *in vitro* of a leukemia virus-induced B-cell lymphoma into macrophages. *Mol. Cell Biol.* 1989; 9: 2264-2268.

Hardison, R.C. A brief history of hemoglobins: plant, animal, protist, and bacteria. *PNAS*, 1996, 93: 5675-5679.

Hardison, R., Hemoglobins from bacteria to man: evolution of different patterns of gene expression. *Journal of Experimental Biology*. 1998; 201(8): 1099-1117.

Hargrove, M., E.Brucker, B.Stec, G.Sarath, R.Arredondo-Peter, R.Klucas, J.Olson, J.G.Phillips. Crystal structure of a nonsymbiotic plant hemoglobin. *Structure.* 2000; 8(9): 1005-1014.

Hazarika P., McCarry M.F., Prieto V.G., George S., Babu D., Koul D., Bar-Eli M., Duvic M. Up-regulation of Flotillin-2 is associated with melanoma progression and modulates expression of the Thrombin receptor protease activated receptor 1. *Cancer Res.* 2004; 64(20): 7361-7369.

Hepler J.R., Gilman A.G. G proteins. *Trends Biol. Sci*. 1992;17: 383–387.

Hoffmann, P.R. et al. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J. Cell Biol.* 2001; 155: 649-659.

Huang, M., Z. Zhou, and S. J. Elledge. The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. *Cell.* 1998; 94**:** 595-605.

Hundahl C., Stoltenberg M., Fago A., Weber R.E., Dewilde S., Fordel E.. Danscher G. Effects of short-term hypoxia on neuroglobin levels and localization in mouse brain tissues. *Neuropathol. Appl. Neurobiol.* 2005; 31: 610–617.

Jacobowitz D.M., Kallarakal A.T. Flotillin-1 in the substantia nigra of the Parkinson brain and a predominant localization in catecholaminergic nerves in the rat brain. *Neurotox Res.* 2004; 6(4): 245-257.

Jin K., Mao X.O., Xie L., Khan A.A., Greenberg D.A. Neuroglobin protects against nitric oxide toxicity. *Neurosci. Lett.* 2008; 430: 135–137.

Jin K., Mao Y., Mao X., Xie L., Greenberg D.A. Neuroglobin expression in ischemic stroke. *Stroke* 2010; 41: 557–559.

Kato N., Nakanishi M., Hirashima N., Flotillin-1 regulates IgE receptor-mediated signaling in rat basophilic leukemia (RBL-2H3) cells. *J Immunol.* 2006; 177(1):147-154.

Khan A.A., Mao X.O., Banwait S., Jin K., Greenberg D.A. Neuroglobin attenuates beta-amyloid neurotoxicity in vitro and transgenic alzheimer phenotype *in vivo*. *Proc. Natl. Acad. Sci. USA.* 2007; 104: 19114–19119.

Khanna, M., Kumar, P., Chugh, L., Prasad, A.K. and Chhabra, S.K. Evaluation of influenza virus detection by direct enzyme immunoassay (EIA) and conventional methods in asthmatic patients. *J Commun Dis.* 2001; 33: 163-169.

Kiger, L., H. Wajcman. L'hémoglobine, des micro-organismes à l'homme : un motif structural unique, des fonctions multiplesHaemoglobin from microorganism to man: a single protein folding, a variety of functions. *Comptes Rendus Biologies*. 2002; 325(12): 1159-1174.

Kimura, Baumann A., Chiang C.A., S. H. & Saltiel, A. R. The Sorbin Homology domain: a motif for the targeting of proteins to lipid rafts. *Proc. Natl. Acad. Sci. USA*. 2001; 98: 9098-9103.

Kirsch C., Eckert G.P., Mueller W.E. Statin effects on cholesterol

microdomains in brain plasma membranes. *Biochem Pharmacol.* 2003; 65 (5): 843-856.

Kitatsuji C., Kurogochi M., Nishimura S., Ishimori K., Wakasugi K. Molecular basis of guanine nucleotide dissociation inhibitor activity of human neuroglobin by chemical cross-linking and mass spectrometry. *J. Mol. Biol.* 2007; 368: 150–160.

Koc, A., L. J. Wheeler, C. K. Mathews, and G. F. Merrill. Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. *J. Biol. Chem*. 2004; 279**:**223-230.

Kundu, S., S.A. Premer, J.A. Hoy, I.James, T.Trent, M.S.Hargrove. Direct Measurement of Equilibrium Constants for High-Affinity Hemoglobins. *Biophys. J.* 2003; 84: 3931-3940.

Kurrle N., John B., Meister M. and Tikkanen R. Function of Flotillins in Receptor Tyrosine Kinase Signaling and Endocytosis: Role of Tyrosine Phosphorylation and Oligomerization. *Intech* 2012; 10: 307-337.

Kysela, B., Chovanec, M. and Jeggo, P.A. Phosphorylation of linker histones by DNA-dependent protein kinase is required for DNA ligase IV-dependent ligation in the presence of histone H_1. *Proc Natl Acad Sci USA.* 2005; 102: 1877-1882.

Lagergren, J., and Reichard, P. *Biochem. Pharmacol.*1987; 36**:** 2985-2991.

Langhorst M.F., Solis G.P., Hannbeck S., Plattner H., Stuermer C.A. Linking membrane microdomeins to the cytoskeleton: regulation of the lateral mobility of reggie-1/flotillin-2 by interaction with actin. *FEBS Lett* 2007; 581(24): 4697-4703.

Larsen G.A., Skjellegrind H.K., Berg-Johnsen J., Moe M.C., Vinje M.L. Depolarization of mitochondria in isolated ca1 neurons during hypoxia, glucose deprivation and glutamate excitotoxicity. *Brain Res.* 2006; *1077*: 153–160.

Le Roy C. and Jeffrey L. Wrana. Clathrin and non-clathrin mediated endocytic regulation of cell signaling. *Molecular and Cellular Biology*. 2005; 6: 112-125.

Lengronne, A., Pasero, P., Bensimon, A., Schwob, E. *Nucleic AcidsRes.* 2001; 29**:** 1433-1442.

Li R.C., Pouranfar F., Lee S.K., Morris M.W., Wang Y., Gozal D.

Neuroglobin protects pc12 cells against β-amyloid-induced cell injury. *Neurobiol. Aging* 2008; 29: 1815–1822.

Lima D.C., Cossa A.C., Perosa S.R., De Oliveira E.M., Da Silva J.A.J., Da Silva F.M.J.,Da Silva I.R., Higa E.M., Da Graca Naffah-Mazzacoratti M., Cavalheiro E.A., *et al.* Neuroglobin is up-regulated in the cerebellum of pups exposed to maternal epileptic seizures. *Int. J. Dev. Neurosci.* 2011; 29: 891–897.

Linke, S.P., Sengupta, S., Khabie, N., Jeffries, B.A., Buchhop, S., Miska, S., Henning, W., Pedeux, R., Wang, X.W., Hofseth, L.J., Yang, Q., Garfield, S. H., Sturzbecher, H.W. and Harris, C.C. p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. *Cancer Res.*2003; 63: 2596-2605.

Lingwood D., Simons K. Lipid rafts as a membrane-organizing principle. *Science.* 2010; 327: 46-50.

Liu J., S.M. De Young, Mei Zhang, Lisa H. Dold and Alan R. Saltiel. The Stomatin/Prohibitin/Flotillin/HflK/C Domain of Flotillin-1 Contains Distinct Sequences That Direct Plasma Membrane Localizzation and Protein Interaction in 3T3-L1 Adipocytes*; J. Biol. Chem*. 2005; 280: 16125-16134.

Liu J., Yu Z., Guo S., Lee S.R., Xing C., Zhang C., Gao Y., Nicholls D.G.; Lo, E.H.;Wang, X. Effects of neuroglobin overexpression on mitochondrial function and oxidative stress following hypoxia/reoxygenation in cultured neurons. *J. Neurosci. Res.* **2009**, *87*, 164–170.

Lopes, M., C. C.R, A. Pellicioli, G. Liberi, P. Plevani, M. Muzi- Falconi, C. S. Newlon, and M. Foiani. The DNA replication checkpoint response stabilizes stalled replication forks. *Nature*. 2001; 412**:** 557-561.

Ludwig A., Otto G.P., Riento K., Hams E., Fallon P.G., Nichols B.J. Flotillin microdomains interact with the cortical cytoskeleton to control uropod formation and neutrophil recruitment. *J Cell Biol.* 2010; 1971 (4): 771-781.

Ma, Y., Schwarz, K. and Lieber, M. R. The Artemis: DNA-PKcs endonuclease cleaves DNA loops, flaps and gaps. *DNA Repair (Amst).*2005; 4: 845-851.

Malaga-Trillo E, Solis GP, Schrock Y, Geiss C, Luncz L, Thomanetz V, et al. Regulation of embryonic cell adhesion by the prion protein. *PLoS Biol*. 2009; 7: 576-590.

McCarroll, R. M., and W. L. Fangman. Time of replication of yeast centromeres and telomeres. *Cell*. 1988; 54**:** 505-513.

Milani, M., A.Pesce, M.Nardini, H.Ouellet, Y.Ouellet, S.Dewilde, A.Bocedi, P.Ascenzi, M.Guertin, L.Moens, J.M.Friedman, J.B.Wittenberg, M.Bolognesi. Structural bases for heme binding and diatomic ligand recognition in truncated hemoglobins. *Journal of Inorganic Biochemistry*. 2005; 99(1): 97-109.

Moffett S., Brown D.A., Linder M.E. Lipid-dependent targeting of G proteins into rafts. *J. Biol. Chem*.2000; 275: 2191–2198.

Moncada S., Erusalimsky J.D. Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat. Rev. Mol. Cell Biol.* 2002; 3: 214–220.

Moretti, J., Blander, J.M. Insights into phagocytosis-coupled activation of pattern recognition receptors and inflammasomes. *Curr. Opin. Immunol.*2014; 26: 100–110.

Morrow I.C., Rea S., Martin S., Prior I.A., Prohska R., Hancock J.F., James D.E., Parton R. G. Flotillin-1/Reggie-2 Traffics to Surface Rafts Domains via a Novel Golgi-independent Pathway. *J. Biol. Chem.* 2002; 277: 48834-48841.

Morrow I.C. and Parton R. G. Flotillins and the PHB Domain Protein Family: Rafts, Worms and Anaesthetics. *Traffic*. 2005; 6: 725-740.

Mukherjee S. e Maxfield. Role of membrane domains in endocytic lipid trafficking. *Traffic.* 2000; 1: 203-211.

Nakahata N. & Ohkubo S. Lipid rafts and their analytical methods. *J. Stage*. 2003; 122: 419-425.

Neumann, N.F., Stafford, J.L., Barreda, D., Ainsworth, A.J., Belosevic, M. Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Dev. Comp. Immunol.* 2001; 25: 807–825.

Nicholls D.G., Budd S.L. Mitochondria and neuronal survival. *Physiol. Rev.* 2000; 80. 315–360.

Nyberg, K. A., R. J. Michelson, C. W. Putnam, and T. A. Weinert**.** Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet*. 2002; 36**:**617-656.

Oleg O. Glebov, Nicholas A. Bright and Benjamin J. Nichols. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cell. *Nature Cell Biology* 2006; 8: 46-54.

Pedrera, I.M., Collazos, M.E., Ortega, E., Barriga, C. *In vitro* study of the phagocytic processes in splenic granulocyte of the tench (*Tinca tinca*, L.). *Dev. Comp. Immunol*. 1992; 16: 431–439.

Perttu S. Niemelä, Samuli Ollila, Marja T. Hyvönen, Mikko Karttunen, Ilpo Vattulainen. Assessing the nature of lipid raft membranes. *PLoS Computational Biology*. 2007; 3: 304-312.

Perutz, M.F. Regulation of oxygen affinity of hemoglobin: influence of structure of the globin on heme iron. 1979; *Annu Rev Biochem.* 48: 327-386.

Pesce, A., M.Couture, S.Dewilde, M.Guertin, K.Yamauchi, P.Ascenzi, L.Moens, M. Bolognesi. A novel two-over-two alpha-helical sandwich fold is characteristic of the truncated hemoglobin family. *The EMBO Journal*. 2000; 19(11): 2424-2434.

Pesce, A., M. Nardini, S.Dewilde, E.Geuens, K.Yamauchi, P.Ascenzi, A.F.Riggs, L.Moens, M.Bolognesi, The 109 Residue Nerve Tissue Minihemoglobin from Cerebratulus lacteus Highlights Striking Structural Plasticity of the a-Helical Globin Fold. *Structure.* 2002; 10: 725-735.

Pesce, A., M.Bolognesi, A.Bocedi, P.Ascenzi, S.Dewilde, L.Moens, T.Hankeln, T.Burmester. Neuroglobin and cytoglobin.Fresh blood for the vertebrate globin family. *EMBO reports*. 2002; 3(12): 1146–1151.

Pesce, A., M. Bolognesi, P. Ascenzi, and M. Milani. Truncated hemoglobins: trimming the classical 'three-over-three' globin fold to a minimal size. *Biochemistry and Molecular Biology Education*. 2001; 29(3): 123-125.

Petit P.X., Goubern M., Diolez P., Susin S.A., Zamzami N., Kroemer G. Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: The impact of irreversible permeability transition. *FEBS Lett.* 1998; 426: 111–116.

Pike L. J. Lipid Rafts. Heterogeneity on the high seas. *Biochem. J.* 2003

Pimpinelli F., Lehmann S., Maridonneau-Parini I. the scrapie prion protein is present in flotillin-1-positivevescicles in central- but not peripheral- derived neuronal cell lines. *Eur J Neurosci.* 2005; 21 (8): 2063-2072.

Prior et al. GTP-dependent segregation of H-Ras from lipid Rafts is required for biological activity. *Nat. Cell. Biol*. 2001; 3: 368-375.

Puri V. et al. Cholesterol modulates membrane traffic along the endocytic pathway in sphingolipid-storage diseases. *Nature Cell Biology*. 1999; 1: 386-388.

Rabinovitch, M. Professional and non-professional phagocytes: an introduction. *Trends Cell Biol.* 1995; 5: 85–87.

Rajendran L., Knobloch M., Geiger K.D., Dienel S., Nitsch R., Simons K.,

Konietzko U. Increased abeta production leads to intracellular accumulation of abeta in flotillin-1-positive endosomes. *Neurodegener Dis* 2007; 4 (2-3): 164-170.

Razani B. & Lisanti M. P. Caveolins and caveolae: molecular and functional relationships. *Exp. Cell. Res*. 2001; 271: 36-44.

Resh M. D. Fatty acylation of proteins: new insights into membrane targeting of myristoilated and palmytoilated proteins. *Biochim. Biophis. Acta*. 1999; 145: 1-16.

Reuss S., Saaler-Reinhardt S., Weich B., Wystub S., Reuss M.H., Burmester T., Hankeln T. Expression analysis of neuroglobin mRNA in rodent tissues. *Neuroscience* 2002; 115: 645–656.

Reuter A., Binkle U., Stuermer C.A., Plattner H. PrPc and reggies/flotillins are contained in and released via lipid-rich vescicles in Jurkat T cells. *Cell Mol Life Sci.* 2004; 61 (16): 2092-2099.

Rumfelt, L.L., McKinney, E.C., Taylor, E., Flajnik, M.F. The development of primary and secondary lymphoid tissues in the nurse shark *Ginglymostoma cirratum*: B-cell zones precede dendritic cell immigration and T-cell zone formation during ontogeny of the spleen. *Scand. J. Immunol.* 2002; 56: 130-148.

Santamaria A., Castellanos E., Gomez V., Benedict P., Piqueras J. R., Morote J., Reventos J., Thomson T.M. and Paciucci A. PTOV-1 enables, the nuclear trans location and Mitogenic Activity of Flotillin-1, a major protein of lipid rafts. *Molec and Cell Biology*. 2005; 1900-1911.

Santocanale, C., J.F. Diffley**.** A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature* 1998; 395**:** 615-618.

Sarantis H., Grinstein, S. Subversion of phagocytosis for pathogen survival. *Cell Host Microb*. 2012; 12: 419–431.

Savill J, Dransfield I, Hogg N, Haslett C. Vitronectin receptor mediated phagocytosis of cell undergoing apoptosis. *Nature*. 1990; 343: 170-3.

Savill J., N. Hogg, Y., Ren, C. Haslett. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition f neutrophils undergoing apoptosis. *J Clin Invest*. 1992; 90: 1513-1522.

Savill J., V. Fadok, P. Henson, C. Haslett. Phagocite recognition of cells undergoing apoptosis. *Immunol Today.* 1993; 14: 131-136.

Schmidt M., Giessl A., Laufs T., Hankeln T., Wolfrum U., Burmester T. How

does the eye breathe? Evidence for neuroglobin-mediated oxygen supply in the mammalian retina. *J. Biol. Chem.* 2003; 278: 1932–1935.

Schmidt-Kastner R., Haberkamp M., Schmitz C., Hankeln T., Burmester T. Neuroglobin mrna expression after transient global brain ischemia and prolonged hypoxia in cell culture. *Brain Res.* 2006; 1103: 173–180.

Shang A., Zhou D., Wang L., Gao Y., Fan M., Wang X., Zhou R., Zhang C. Increased neuroglobin levels in the cerebral cortex and serum after ischemia-reperfusion insults. *Brain Res.* 2006; 1078: 219–226.

Shao G., Gong K.R., Li J., Xu X.J., Gao C.Y., Zeng X.Z., Lu G.W., Huo X. Antihypoxic effects of neuroglobin in hypoxia-preconditioned mice and sh-sy5y cells. *Neurosignals* 2009; 17: 196–202.

Shiloh, Y. ATM and related protein kinases: safeguardian genome integrity. *Nat Rev Cancer.* 2003; 3: 155-168.

Sillo, A., Bloomfield, G., Balest, A., Balbo, A., Pergolizzi, B., Peracino, B., Skelton, J., Ivens, A., Bozzaro, S. Genome-wide transcriptional changes induced by phagocytosis or growth on bacteria in *Dictyostelium*. *BMC Genomics*. 2008; doi:10.1186/1471-2164-9-291.

Simon M.I., Strathmann M.P., Gautam N. Diversity of G proteins in signal transduction. *Science* 1991; 252: 802–808.

Simon Staubach and Franz-Georg Hanisch . Lipid rafts: signaling and sorting platforms of cells and their roles in cancer. *Expert Rev. Proteomics*. 2011; 8 (2): 263-277.

Simons K., Ikonen E. Functional rafts in cell membranes. *Nature*. 1997; 387: 569–572.

Sims N.R., Anderson M.F. Mitochondrial contributions to tissue damage in stroke. *Neurochem. Int.* 2002; 40: 511–526.

Singer, SJ & Nicolson; GL. The fluid mosaic model of the structure of cell membranes. *Science.* 1972; 175: 720-731.

Sogo, J. M., M. Lopes, M. Foiani. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science* 2002; 297**:** 599-602.

Skoog, L., and Nordenskjold, B. *Eur. J. Biochem.* 1971; 19**:** 81-89.

Slaughter N., Laux I., Xiaolin Tu, Whitelegge J., Zhu X., Effros R., Bickel P.

and Nel A. The flotillins are integral membrane proteins in lipid rafts that contain TCR-associated signaling components: implication for the T-cell activation. *Clinical immunology*. 2003; 108: 138-151.

Spano A., Sciola L., Monaco G., Barni S. Relationship between actin microfilamento and plasma membrane changes during apoptosis of neoplastic cell lines in different culture conditions. *Eur. J Histochem*. 2000; 44 (3): 255-67.

Stern M., J. Savill, C. Haslett. Human monocyte derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by alpha V beta 3/CD36/Thrombospondin recognition mechanism and lack of phlogistic response. *Am J Pathol*. 1996; 149: 911-921.

Stuermer CA, Langhorst MF, Wiechers MF, Legler D.F., von Hanwehr S.H., Guse A.H., Plattner H. PrP capping in T cells promotes its association with the lipid raft proteins reggie-1 and reggie-2 and leads to signal transduction. *Faseb J.* 2004; 18(14):1731-1733.

Sturzbecher, H. W., Donzelmann, B., Henning, W., Knippschild, U. and Buchhop, S. p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction. *Embo J.* 1996; **15**: 1992-2002.

Sugawara, N., Wang, X. and Haber, J. E. In vivo roles of Rad52, Rad54 and Rad55 proteins in Rad51-mediated recombination. *Mol Cell*. 2003; 12: 209-219.

Sun Y., Jin K., Mao X.O., Zhu Y., Greenberg D.A. Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. *Proc. Natl. Acad. Sci. USA*. 2001; 98: 15306–15311.

Sun Y., Jin K., Peel A., Mao X.O., Xie L., Greenberg, D.A. Neuroglobin protects the brain from experimental stroke *in vivo*. *Proc. Natl. Acad. Sci. USA*. 2003; 100: 3497–3500.

Sun Y., Jin K., Mao X.O., Xie L., Peel A., Childs J.T., Logvinova A., Wang, X., Greenberg D.A. Effect of aging on neuroglobin expression in rodent brain. *Neurobiol. Aging* 2005; 26: 275–278.

Sung, P. Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. *Genes Dev.*1997; 11: 1111-1121.

Tang, B.L. Bacteria-containing vacuoles: subversion of cellular membrane traffic and autophagy. *Crit. Rev. Eukaryot Gene Expr.* 2015; 25**:** 163–174.

Tercero, J.A., J.F. Diffley**.** Regulation of DNA replication fork progression

through damaged DNA by the Mec1/Rad53 checkpoint. *Nature*. 2001; 412**:** 553-557.

Tyrsted, G. *Biochem. Pharmacol.* 1982; 31**:** 3107–3113.

Uzan, J., S.Dewilde, T.Burmester, T.Hankeln, L.Moens, D.Hamdane, M.C.Marden, L.Kiger. Neuroglobin and other hexacoordinated hemoglobins show a weak temperature dependence of oxygen binding. *Biophys. J.* 2004; 87: 1186-1195.

Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L. and Shiloh, Y. Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J.* 2003; 22: 5612-5621.

Van Doorslaer S., Dewilde S., Kiger L., Nistor S.V., Goovaerts E., Marden M.C., Moens L. Nitric oxide binding properties of neuroglobin. A characterization by epr and flash photolysis. *J. Biol. Chem.* 2003; 278: 4919–4925.

Volonte D., Galbiati F., Li S., Nishiyama K., Okamoto T. & Lisanti M. P. Flotillins/Cavatellins are differentially espresse in cells and tissues and form a hetero-oligomeric complex with Caveolins in vivo. *J. Biol. Chem*. 1999; 274: 12702-12709.

Wakasugi K., Nakano T., Morishima I. Oxidized human neuroglobin as a heterotrimeric Gα protein guanine nucleotide dissociation inhibitor. *J. Biol. Chem*. 2003; 278: 36505–36512.

Wakasugi K., Nakano T., Kitatsuji C., Morishima I. Human neuroglobin interacts with flotillin-1, a lipid raft microdomain-associated protein. *Biochem Biophys Res Commun.* 2004; *318(2): 453-460.*

Wakasugi K., Kitatsuji C., Morishima I. Possible neuroprotective mechanism of human neuroglobin. *Ann. N.Y. Acad. Sci*. 2005; 1053: 220–230.

Wang, R. C., Smogorzewska, A. and De Lange, T. Homologous recombination generates T-loop-sized deletions at human telomeres. *Cell.* 2004; 119: 355-368.

Watanabe S., Takahashi N., Niroyuki U., Wakasugi K. Human neuroglobin functions as an oxidative stress-responsive sensor for neuroprotection. *Journal of Biological Chemistry.* 2012; 287: 30128-30138.

Wei X., Yu Z., Cho K.S., Chen H., Malik M.T., Chen X., Lo E.H., Wang X., Chen D.F. Neuroglobin is an endogenous neuroprotectant for retinal ganglion cells against glaucomatous damage. *Am. J. Pathol.* 2011; 179: 2788–2797.

Weinert, T.A., G.L. Kiser, L.H. Hartwell. Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. *Genes Dev*. 1994; 8**:** 652-665.

Wyllie A.H., J.F, Kerr, A.R. Currie; Cell death: the significance of apoptosis. *Int Rev Cytol*. 1980; 68: 251-306.

Wystub S., Laufs T., Schmidt M., Burmester T., Maas U., Saaler-Reinhardt S., Hankeln T., Reuss S. Localization of neuroglobin protein in the mouse brain. *Neurosci. Lett.* 2003; *346*: 114–116.

Wu, Y., Wu, W. Wong, W.M., Ward, E. Thrasher, A.J., Goldblatt, D., Osman, M., Digard, P., Canaday, D.H., Gustafsson, K. Human gamma delta T cells: a lymphoid lineage cell capable of professional phagocytosis. *J. Immunol.* 2009; 183: 5622–5629.

Yao, R., Zhang, Z., An, X., Bucci, B., Perlstein, D.L., Stubbe, J., Huang, M. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100: 6628-6633.

Yu Z., Liu N., Liu J., Yang K., Wang X. Neuroglobin, a novel target for endogenous neuroprotection against stroke and neurodegenerative disorders. *Int. J. Mol. Sci.* 2012; 13: 6995-7014.

Yu Z., Liu N., Wang Y., Li X., Wang X. Identification of neuroglobin-interacting proteins using yeast two-hybrid screening. *Neuroscience* 2012; 200: 99–105.

Yuyama K., Sekino-Suzuki N., Sanai Y., Kasahara K. Translocation of activated heterotrimeric G protein $G\alpha_o$ to ganglioside-enriched detergent-reristant membrane rafts in developing cerebellum. *J. Biol. Chem.* 2007; 282: 26392–26400.

Zhang C., Wang C., Deng M., Li L., Wang H., Fan M., Xu, W., Meng F., Qian L., He F. Full-length cdna cloning of human neuroglobin and tissue expression of rat neuroglobin. *Biochem. Biophys. Res. Commun.* 2002; 290: 1411–1419.

Zhang W.H., Wang H., Wang X., Narayanan M.V., Stavrovskaya I.G., Kristal B.S., Friedlander R.M. Nortriptyline protects mitochondria and reduces cerebral ischemia/hypoxia injury. *Stroke* 2008; 39: 455–462.

Zhang W., Tian Z., Sha S., Cheng L.Y., Philipsen S., Tan-Un K.C. Functional and sequence analysis of human neuroglobin gene promoter region. *Biochim. Biophys. Acta* 2011; 1809: 236–244

Zhang, L., Wang, C.C. Inflammatory response of macrophages in infection. *Hepatobiliary Pancreat Dis. Int.* 2014; 13: 138–152.

Zhao, X., Muller, E.G., and Rothstein, R. *Mol. Cell.* 1998; 2**:** 329-340.

Zhao, X., Chabes, A., Domkin, V., Thelander, L., Rothstein, R. *EMBO J.* 2001; 20**:** 3544-3553.

Zhao, X., Rothstein, R. *Proc. Natl. Acad. Sci. U. S. A.* 2002; 99**:** 3746-3751.

Zhu S., Stavrovskaya I.G., Drozda M., Kim B.Y., Ona V., Li M., Sarang S., Liu A.S., Hartley D.M., Wu D.C. *et al.* Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature* 2002; 417: 74–78.

Zou, L. and Elledge, S.J. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science*. 2003; 300: 1542-1548.

Zou, L., Liu, D. and Elledge, S. J. Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc Natl Acad Sci USA.* 2003*;* 100: 13827-13832.