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Mechanisms of α IIb β 3 Biogenesis in the Megakaryocyte: A Proteomics Approach

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1. Introduction

Platelets play a central role in hemostasis and thrombosis, initiating clot formation in response to vessel wall damage. Platelet aggregates are formed at sites of injury by the binding and crosslinking of the integrin α IIb β 3 to fibrinogen, von Willebrand factor and other soluble ligands. Platelets also form pathological thrombi, and the resulting arterial occlusion can lead to myocardial infarction or stroke. Inhibition of α IIb β 3 binding can decrease the formation of pathologic thrombi, and α IIb β 3 has become an important pharmacological target. Anti- α IIb β 3 therapies have been highly successful in preventing death following myocardial infarction and percutaneous arterial stent placement.(Topol, Lincoff et al. 2002; De Luca, Ucci et al. 2009) However, attempts to design novel, orally available anti- α IIb β 3 agents have been hampered by what appears to be paradoxical activation of α IIb β 3 by the drug, in some cases leading to an increased risk of mortality in patients who received the drug.(Quinn, Plow et al. 2002)

A novel approach to manipulating α IIb β 3 would be to perturb its post-translational processing and trafficking within the megakaryocyte, prior to platelet formation. Like most membrane proteins, α IIb β 3 is formed by concerted processes of protein sorting and trafficking. Some of the mechanisms underlying α IIb β 3 biogenesis and expression in megakaryocytes have been described, such as the calnexin cycle of protein quality control.(King and Reed 2002; Tiwari, Italiano et al. 2003; El.Golli, Issertial et al. 2005; Lo, Li et al. 2005) Clues to the stringent protein quality control of α IIb β 3 biogenesis come from the study of patients with defective or absent α IIb β 3, who manifest the mucocutaneous bleeding disorder Glanzmann thrombasthenia. A subset of patients with mutations in the α IIb gene produce full-length α IIb that retains the ability to form a complex with β 3 but is retained within the cell and degraded, resulting in disease. These patients demonstrate the existence of stringent quality control mechanisms acting post-translationally to control α IIb β 3 biogenesis and expression. A clearer understanding of these mechanisms may lead to new possibilities of anti-integrin therapy.

Toward the goal of identifying proteins involved in α IIb β 3 biogenesis we performed a proteomic analysis of proteins interacting with α IIb in megakaryocytes cultured from human umbilical cord blood (UCB) and in HEK293 cells expressing α IIb and β 3.

Megakaryocyte proteins were captured by poly-histidine tagged α IIb, or by photoreactive crosslinking followed by immunoprecipitation with anti- α IIb mAbs, and analyzed by mass spectrometry.

The α IIb and β 3 subunits are synthesized as a single-chain precursors in the chaperone-rich folding environment of the endoplasmic reticulum (ER) (Fig 1). The precursor α IIb, pro- α IIb, is glycosylated under control of the calnexin cycle of protein folding. The β 3 precursor is also glycosylated but does not appear to interact with the calnexin cycle. The two precursors heterodimerize to briefly form pro- α IIb β 3, and then pro- α IIb is cleaved by one or more members of the furin family of proteases in the Golgi. Cleavage of pro- α IIb to mature α IIb marks the exit of α IIb from the ER, and this cleavage occurs only when pro- α IIb is in complex with β 3. (Bray, Rosa et al. 1986; Rosa and McEver 1989) Both α IIb and β 3 are synthesized in excess of what will finally be processed to mature α IIb β 3. The calnexin cycle exerts stringent quality control over α IIb production and up to one half of all pro- α IIb is targeted to the proteasome for degradation in megakaryocytes. Excess β 3 is degraded by a non-proteasomal mechanism. Inhibition of the proteasome in megakaryocytes resulted in a build-up of pro- α IIb that was not being degraded, but had no apparent effect on the level of mature α IIb in the α IIb β 3 complex. Thus, the expression level of mature α IIb β 3 is not simply a result of the stoichiometry of production vs. degradation. Rather there appears to be a concerted mechanism that controls how much pro- α IIb β 3 will be converted into mature α IIb β 3, and this mechanism is not grossly responsive to excessive levels of pro- α IIb or β 3.

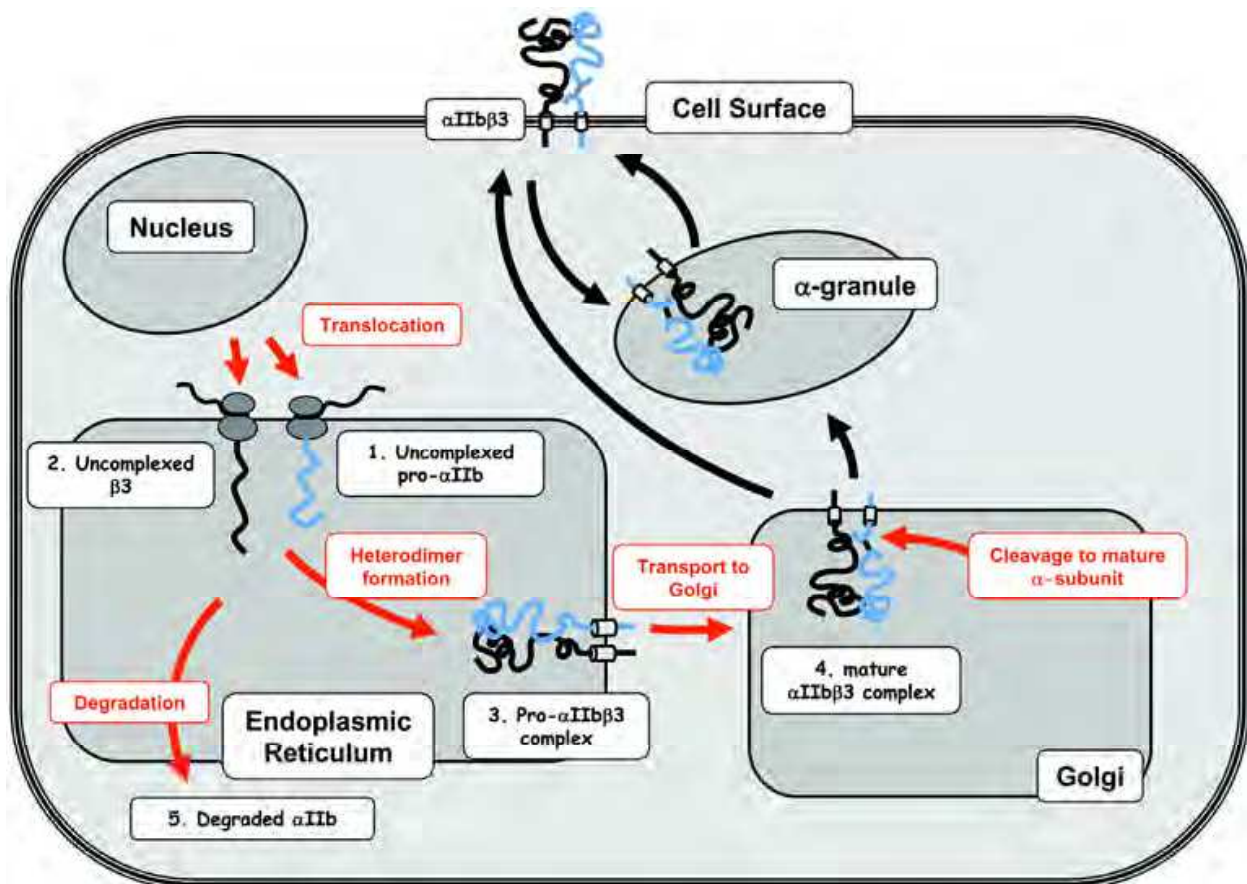


Fig. 1. Biogenesis of α IIb β 3

We hypothesized that whatever proteins underlie this mechanism must be interacting with pro- α IIb and/or pro- α IIb β 3 complex, but not with the mature α IIb β 3 complex. We chose a proteomics approach to identify these interacting proteins. In order to enrich our assay for proteins that preferentially bound pro- α IIb or pro- α IIb β 3, we used an α IIb subunit harboring R858G and R859G mutations that eliminates furin cleavage and traps α IIb in its pro- α IIb form.(Kolodziej, Vilaire et al. 1991) This α IIbR858G/R859G construct can form a complex with β 3, but only in small amounts compared to normal α IIb β 3. In addition, while some of the mutant α IIb β 3 complexes reach the cell surface and can mediate adherence to immobilized fibrinogen, the proportion of α IIbR858G/R859G reaching the surface is very small compared to normal α IIb.(Kolodziej, Vilaire et al. 1991) Thus, α IIbR858G/R859G is a “nearly normal” mutant α IIb subunit that is primarily retained within the cell, making it a useful bait to capture the proteins involved in that process.

The hsp40 type chaperone protein, DNAJC10, was captured by both the normal α IIb and α IIbR858G/R859G subunits, and was evaluated as a putative α IIb interacting protein.. We report that DNAJC10 interacted with pro- α IIb and β 3 in megakaryocytes, and appears to promote the degradation of pro- α IIb. Notably, while α IIb β 3-DNAJC10 interaction was evident in megakaryocytes, specific interaction was not detectable in HEK293 cells transfected with α IIb cDNA, suggesting megakaryocyte specificity. Knockdown of DNAJC10 by siRNA increased α IIb β 3 surface expression in UCB derived megakaryocytes, indicating that DNAJC10 negatively regulates α IIb β 3 surface expression. Thus, DNAJC10- α IIb interaction represents a novel post-translational mechanism regulating α IIb β 3 surface expression.

2. Materials and methods

2.1 Antibodies

The antibodies used in this study were: 10E5 (anti- α IIb β 3 complex); B1B5 (anti- α IIb);7H2 and B36 (anti- β 3) (all 4 generous gifts from Dr Barry Coller); CA3 (anti- α IIb), anti-V5 epitope, and anti-Myc epitope (all three Millipore, Temecula,CA); anti-DNAJC10 (Genetex, Irvine, CA); M148 (anti- α IIb) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse IgG, and rabbit IgG (both Jackson ImmunoResearch Inc, West Grove, PA).

2.2 Human Umbilical Cord Blood culture

This study used human UCB from the US National Cord Blood Bank that was deemed not suitable for clinical use due to low white blood cell number. Mothers who donate their UCB to the National Cord Blood Bank sign a consent giving permission to donate the UCB to research if it is inadequate for clinical use and will be discarded. Because the donated units are completely de-identified and are not collected prospectively specifically for research, the New York Blood Center Institutional Review Board (IRB), which oversees research ethics at the National Cord Blood Bank, considers their use exempt from IRB review. Human UCB was prepared as previously described.(Mitchell, Li et al. 2006) Briefly, leukocytes were separated from 1-3 units of human UCB judged to be inadequate for clinical purposes (generously provided by the New York Blood Center) by Dextran 70 sedimentation (Amersham Biosciences, Piscataway, NJ) for 1h, and then enriched for CD34+ progenitor

cells by negative selection using a combination of antibodies against maturation/lineage-specific markers (RosetteSep, StemCell Technologies, Vancouver, BC) concomitant with density sedimentation using Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ). These cells were cultured in serum-free medium (StemCell Technologies, Vancouver, BC) with 50 ng/ml thrombopoietin (TPO) plus 1 ng/ml SCF (both Millipore, Temecula, CA) for 3 days in 10 cm dishes in a 37°C incubator. At this point a portion of the cells will have died. The remaining living population of larger cells was gently washed and replated in fresh media with the same cytokines in 10 cm dishes and left until use (Day 8 or 9). Fresh media was added on day 6. We have previously reported that under these conditions the UCB differentiate into a population of large cells with > 90% expressing α IIb β 3, > 80% expressing GPIb, about 50% expressing α 2 β 1. (Mitchell, Li et al. 2007) For experimental use the cells were gently harvested, pelleted at 300 rpm for 5 min, or let settle by gravity, and gently resuspended in the appropriate buffer.

2.3 HEK293 cell culture

HEK293 cell lines (American Type Culture Collection (ATCC), Manassas, VA) that stably expressed human α IIb β 3 receptors were established as previously described. (Mitchell, Li et al. 2006) Transfections were performed using Lipofectamine 2000 (Gibco-BRL, Carlsbad, CA) according to the manufacturer's instructions, followed by selection in media containing 500 μ g/ml G418 for 2-4 weeks. To obtain a population of cells uniformly expressing high levels of α IIb β 3, cells were labeled with the mAb 10E5 (anti- α IIb β 3) and sorted using a MoFlo cell sorter (Beckman Coulter, Fullerton, CA).

2.4 Immunoprecipitation and biosynthetic labeling

Samples were prepared as previously described and all steps were performed on ice unless otherwise stated. (Mitchell, Li et al. 2006) Briefly, cells (either day 8 megakaryocytes derived from UCB cells or HEK293 cells) were lysed in 1% Brij 98 lysis buffer containing protease inhibitors and 20 μ M N-methylmaleimide (NEM). Lysates were precleared with protein-G Sepharose beads (Amersham Biosciences, Piscataway, NJ), and then equivalent amounts of protein, usually 400 μ g, were incubated one h at 4°C with one or more of the antibodies listed above (4 μ g/reaction). Samples were incubated with protein-G Sepharose beads for one h at 4°C, washed twice, and incubated with SDS sample buffer for 10 min at 100°C. Reduced samples contained 10% beta mercaptoethanol (Sigma, Thermo Scientific, Rockford, IL). Samples were subjected to SDS-PAGE on 7% gels, and the gels were either stained for mass spectrometry identification (described below) or transferred to PVDF membranes for immunoblotting. Non-specific binding was determined by performing parallel immunoprecipitation with mouse or rabbit IgG in each experiment. In preliminary experiments, the production of α IIbR858G/R859G and β 3 was confirmed in the transfected HEK293 cell line by immunoprecipitation with both anti- α IIb and anti- β 3 mAbs followed by immunoblot. For biosynthetic labeling, cells were incubated for 30 min at 37°C in methionine/cysteine-free medium, followed by pulse-labeling for 15 min at 37°C in medium containing ³⁵S-methionine/cysteine (300 μ Ci/10 cm plate). The pulse was terminated by incubation in medium containing unlabeled methionine/cysteine (1 mg/ml each) and the cells were incubated at 37 °C until lysis in 1% Triton-X 100 lysis buffer. Following cell lysis, supernatants were prepared as above. Gels were dried and exposed to film. For inhibition of

the proteasome cells were incubated in the proteasome inhibitor MG132 (10 μ M)(Sigma_Aldrich, St Louis, MO) in normal growth medium at 37C and then immediately lysed.

2.5 Histidine-tag/Nickel bead pulldown assay

HEK293 cells expressing poly-histidine tagged α IIb cDNA (in vector pEF1/V5-His) and β 3 cDNA (in vector pcDNA3.1)(Mitchell, Li et al. 2007) were lysed in 1% Triton, 150 mM NaCl, 10 mM imidazole buffer (lysis buffer) on ice for 30 m, centrifuged for 30m at 4C, and then the supernatant was reacted with 50 μ l of a 6:4 slurry of Ni beads:imidazole buffer (Qiagen, Inc. Valencia, CA) to bind the histidine-tagged subunits to the nickel beads. After incubating for 30 m the beads were washed four times with 10 mM imidazole lysis buffer by using a magnetic chamber to isolate the beads. Next, fresh whole cell lysates (1 mL) of UCB-derived megakaryocytes in 1% Triton, 150 mM NaCl, 10 mM imidazole buffer were incubated with the washed, Ni-bound α IIb for 1 h, and then the beads were washed twice with 1 mL lysis buffer containing 20 mM imidazole. Ni-bound proteins were eluted with 250 mM imidazole and the entire eluate was subjected to SDS-PAGE on a 7% gel followed by staining with Imperial Stain (Pierce, Thermo Scientific, Rockford, IL). Control experiments were run in parallel in which no megakaryocyte lysate was added to the beads, but all other steps remained the same. These controls aimed to identify the remaining HEK293 cell proteins still bound to either the beads or to α IIb after the washing steps, before incubation with megakaryocyte lysate. Experimental and control gels were run simultaneously, and the lanes were excised and analyzed by mass spectrometry.

2.6 Photocrosslinking amino acids

UCB cells were cultured as described above for eight days, then were washed and incubated for 24 hours in leucine- and methionine- free medium containing photoreactive methionine and leucine, dialyzed FBS (both Pierce, Thermo Scientific, Rockford, IL), and 50 ng/ml TPO (Millipore, Temecula,CA). The cells were exposed to 345 nm UV light for 15 minutes to crosslink the photoreactive amino acids, and harvested immediately, according to manufacturer's instructions. Whole cell lysates were immunoprecipitated with antibodies specific for α IIb or β 3, and the proteins were separated by SDS-PAGE using the same protocol as for the histidine-tag affinity capture. Controls were simultaneously immunoprecipitated with non-immune IgG. Experimental and control lanes were excised and analyzed by mass spectrometry.

2.7 RNAi

HEK293 cells stably expressing high levels of α IIb and β 3 were transfected with 100 nM siRNA duplexes (Dharmacon, Thermo Scientific, Rockford, IL and Qiagen, Valencia, CA) using Dharmafect-1 reagent (Dharmacon, Thermo Scientific, Rockford, IL), then analyzed by flow cytometry using a FACSanto (Becton Dickinson,) at 48-96 h after transfection. Cultured UCB cells were transfected twice, on culture days 3 and 5, with 100 nM of siRNA duplexes or controls also using Dharmafect-1 reagent, and then analyzed 48 - 72 h later by flow cytometry. In order to identify individual transfected cells by FACS, cells were co-transfected with 10 nM of fluorescent-labeled non-coding siRNA (Qiagen, Valencia, CA). In

some experiments, Cy3-labeled siRNA duplexes were used (Dharmacon, Thermo Scientific, Rockford, IL). Controls were: no treatment, transfection reagent but no siRNA duplex, Cy3-labeled negative control siRNA duplex only (Qiagen), siControl non targeting siRNA duplex, and siRNA duplex against cyclophilin B (positive control) (both Dharmacon).

2.8 Quantitative RT-PCR

For analysis of RNA content, cells were collected in RNAlater (Applied Biosystems, Foster City, CA) and RNA was purified using the RNEasy mini kit (Qiagen, Valencia, CA). For some analyses, siRNA transfected cells were sorted for expression of the fluorescent marker using a MoFlo sorter, and the sorted cells were subjected to QRT-PCR. Analysis was performed on an ABI 7900 or an ABI 7700 thermocycler/ fluorescence analyzer (Applied Biosystems, Foster City, CA) using the SYBR green probe (Qiagen or Invitrogen) and Quantitect primer assays (Qiagen). Relative mRNA levels were calculated using the $\Delta\Delta C_t$ method that corrects for GAPDH expression in all samples. Fold-reduction was corrected for percent transfection in each experiment.

2.9 Immunofluorescence analysis

Mks were cytopspun onto poly L-lysine-coated glass coverslips and fixed in methanol for 5 minutes at RT. Cells were then washed with saponin buffer (PBS containing 0.02% BSA, 0.005% saponin), then blocked with saponin buffer for 1 hour at RT. Cells were then incubated with the primary antibody diluted in saponin buffer for 1 hour at RT. After washing with PBS, cells were incubated with the appropriate conjugated secondary antibody in PBS for 1 hour at RT. Specimens were mounted with Prolong gold (Invitrogen). Images were acquired using a Zeiss LSM 510 META (Axiovert 200M Inverted Microscope Stand) confocal laser scanning microscope through a 100x objective. Each set of staining conditions was repeated 3 times. Background fluorescence was measured by incubating fixed cells with the secondary antibody only and then acquiring images with the exact settings used to obtain the experimental images.

2.10 Mass spectrometry data analysis

The protein gel bands were excised from the SDS-PAGE. The gel bands were reduced with 10 mM of DTT and alkylated with 55 mM iodoacetamide, and then digested with Sequence Grade Modified Trypsin (Promega, Madison, Wisconsin) in ammonium bicarbonate buffer at 37° overnight. (Kumarathasan, Mohottalage et al. 2005) The digestion products were extracted twice with 0.1% trifluoroacetic acid and 50% Acetonitrile and 1.0% trifluoroacetic acid respectively. The extracted mixture was dried by Speed-Vac and redissolved in 10 μ L 0.1% trifluoroacetic acid. Half of the extracts were injected by LC-MS/MS analysis. For LC-MS/MS analysis, each digestion product was separated by a 60 min gradient elution with the Dionex U3000 capillary/nano-HPLC system (Dionex, Sunnyvale, California) at a flow rate of 0.250 μ L/min that is directly interfaced with the Thermo-Fisher LTQ-Obritrapp mass spectrometer (Thermo Fisher, San Jose, California) operated in data-dependent scan mode. The analytical column was a home-made fused silica capillary column (75 μ m ID, 100 mm length; Upchurch, Oak Harbor, Washington) packed with C-18 resin (300 A, 5 μ m, Varian, Palo Alto, California). Mobile phase A consisted of 0.1% formic acid, and mobile phase B

consisted of 100% acetonitrile and 0.1% formic acid. The 60 min gradients with 250 nL/min flow rate for B solvent went from 0 to 55% in 34 minutes and then in 4 min to 80%. The B solvent stayed at 80% for another 8 min and then decreased to 5% in 8 min. Another 6 min was used for equilibration, loading and washing. The mass acquisition method was one FT-MS scan followed by 6 subsequent MS/MS scan in the ion trap. The FT-MS scan was acquired at resolution 30,000 in the Orbitrap. The six most intense peaks from the FT full scan were selected in the ion trap for MS/MS. The selected ions were excluded for further selection for 180 seconds. The following search parameters were used in all MASCOT searches: maximum of 1 missed trypsin cleavages, cysteine carbamidomethylation, methionine oxidation. The maximum error tolerance for MS scans was 10 ppm for MS and 1.0 Da for MS/MS respectively. Proteins were designated as "hits" if they matched at least 2 distinctive peptides with a MASCOT score of at least 40. For proteins matching the same sets of peptides, only the protein with the greater percentage of coverage was selected. In the one case where 2 isoforms could not be distinguished (HSP70A/B), both proteins are reported. Proteins identified in the control gels were considered "negative," and these proteins were removed from further analysis. The interaction data were further analyzed using the Cytoscape (Cline, Smoot et al. 2007) software and publically available protein interaction databases (e.g. INTACT, NCBI, UniProt) to generate a network of first-degree interactions with α IIb. The network was further expanded to include proteins that were previously reported to interact with α IIb and known interactions between any of the proteins identified in our primary assays.

2.11 Statistical analysis

Flow cytometry data was summarized as the geographic mean fluorescence intensity (MFI) of antibody binding, and normalized to the MFI of control siRNA treated cells, so that the experimental MFI is expressed as a percentage of the control MFI. The overall percent change of the replicates was expressed as the average percent change $\pm$ the confidence interval. The two-sided, paired t-test was used to determine whether there were differences in antibody binding between the different experimental groups. In the siRNA experiments, relative mRNA levels were calculated using the $\Delta\Delta$ Ct method that corrects for GAPDH expression.

3. Results

3.1 The α IIb interactome

Proteins putatively interacting with α IIb were isolated from UCB derived megakaryocytes and from HEK293 cells expressing α IIb β 3, and these proteins were analyzed by mass spectrometry. Samples were processed by the two methods depicted in **Figure 2**. In the first method, recombinant α IIb or α IIbR858G/R859G subunits bearing a polyhistidine tag were expressed in HEK293 cells along with normal β 3. The cells were lysed with 1% Triton buffer, and the lysates were reacted with Ni beads that bind polyhistidine. The Ni beads were washed using a magnetic separator to reduce non-specific binding to the Ni-bound α IIb, and then incubated with fresh whole cell lysate of umbilical cord blood derived megakaryocyte. Since we are interested in the early process of α IIb β 3 formation and intracellular trafficking we used megakaryocytes at day 8 or 9 of culture; at that point in our

system $\alpha\text{IIb}\beta 3$ is highly expressed but there is not yet any proplatelet formation. The Ni-bound histidine-tagged αIIb and αIIb -bound proteins were washed twice and then were eluted from the Ni beads with 250 mM imidazole in a 1% Triton buffer. Simultaneous control experiments were performed without megakaryocyte lysate in order to identify proteins non-specifically binding to either the Ni beads or the polyhistidine tag. The proteins thus collected were separated by one dimensional SDS-PAGE on a 7% gel. After staining, the experimental and control lanes were cut out and analyzed by mass spectrometry. This entire process was repeated in three separate experiments with normal αIIb , and in four separate experiments with $\alpha\text{IIbR858G/R859G}$.

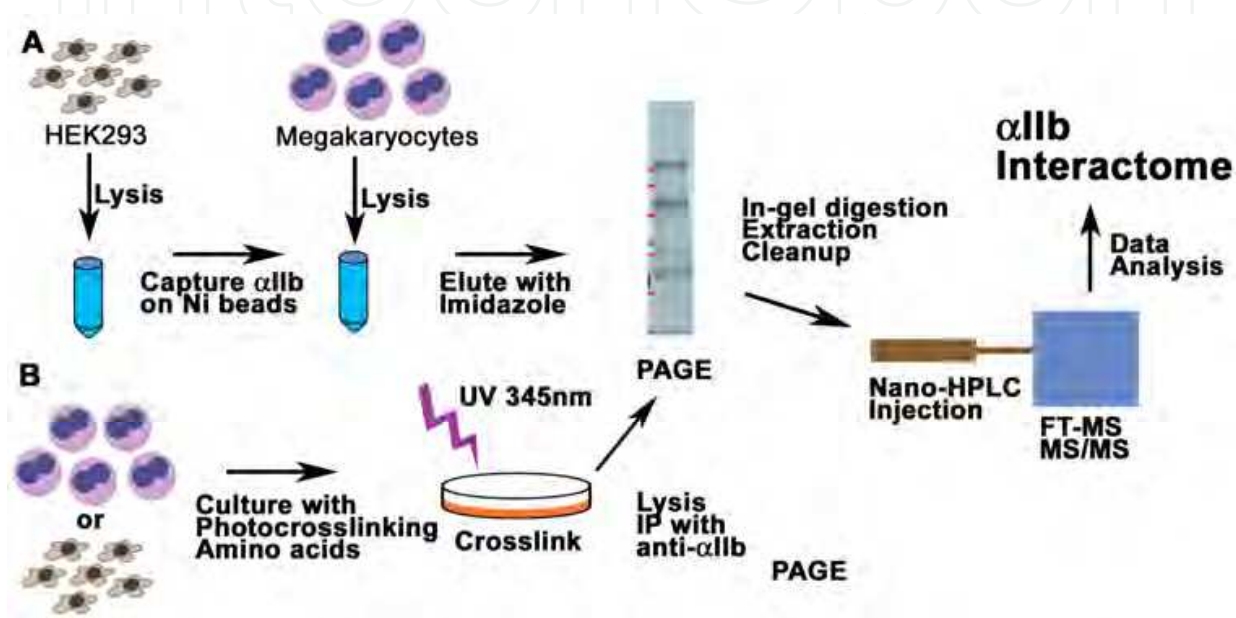


Fig. 2. Methods used to isolate and identify αIIb -interacting proteins. A): Polyhistidine-tagged αIIb subunits were captured on Ni beads and then incubated with whole cell lysate of day 8 UCB-derived megakaryocytes. Protein complexes thus captured were processed as described in the text, and then the proteins were identified by FT-MS and MS/MS. B) Crosslinking experiments were performed on day 8 UCB-derived megakaryocytes and transfected HEK293 cells. Cells were fed photoactivatable methionine and leucine, exposed to UV light and lysed immediately. Samples were processed for mass spectrometry as described.

The second strategy used photo-activated amino acids to crosslink αIIb to its binding partners (**Figure 2B**). UCB-derived megakaryocytes were starved for methionine and leucine, then fed photo-activatable methionine and leucine overnight, and then exposed to UV light to crosslink the amino acids of neighboring proteins. The megakaryocytes were immediately lysed and the αIIb -bound proteins were immunoprecipitated by anti- αIIb mAb. These proteins were separated by one dimensional SDS-PAGE and then the appropriate bands were cut out and analyzed by mass spectrometry. Because we were looking only for proteins crosslinked to αIIb , only the portions of the bands with $M_r > 120$ (M_r of the mature αIIb subunit) were analyzed. Five separate crosslinking experiments were performed with UCB-derived megakaryocytes and two on HEK293 cells expressing $\alpha\text{IIbR858G/R859G}$ and normal $\beta 3$.

Minimum criteria for protein inclusion in data analysis were at least 2 distinctive peptides with a MASCOT score of at least 40, and absence of the protein in the control lanes. Importantly, since we were interested in proteins that are more abundant in the megakaryocyte than in HEK293 cells, even proteins known to interact with α IIb β 3, such as talin, were removed from the final results because they were present in the control lanes. Proteins identified in the Ni bead extraction experiments that are known to harbor natural polyhistidine sequences (such as the DEAH boxes) that could independently bind to the Ni beads were also excluded. Combining the results of both methods, 98 proteins were identified as potentially interacting with the normal α IIb subunit, and 79 proteins were identified as putatively interacting with the α IIbR858G/R859G subunit (Table 1). These 163 proteins putatively constitute a portion of the α IIb interactome, a network of protein-protein interactions relevant to the trafficking and function of α IIb in megakaryocytes (Figure 3).

Proteins Captured with α IIbR858G/R859G								
Gene Symbol	Entrez Gene ID	SwissProt Acc No.	Protein Acc No.	Description	# Pep	% Cov	#. Expt	
		P35579		MYOSIN, HEAVY				
MYH9	4627	Q60FE2	gi 12667788	POLYPEPTIDE 9, NON-MUSCLE	70	45	4	
LMNA	4000	P02545	gi 5031875	LAMIN A/C	37	61	3	
		P14866		HETEROGENEOUS NUCLEAR				
HNRNPL*	3191	A6NIT8	gi 11527777	RIBONUCLEOPROTEIN L	33	53	4	
		Q6NTA2		STROMAL INTERACTION MOLECULE 1				
STIM1	6786	Q13586	gi 21070997	CYTOSKELETON-ASSOCIATED PROTEIN 4	28	46	2	
CKAP4	10970	Q07065	gi 19920317	HEAT SHOCK PROTEIN 90kDa ALPHA, CLASS A	27	55	2	
		P08238	gi 154146191	MEMBER 1	21	36	1	
HSP90AB1	3326			SPLICING FACTOR 3A, SUBUNIT 1, 120kDa	16	32	1	
SF3A1	10291	Q15459	gi 53831995					
		B3KTT5						
		P08107		HEAT SHOCK 70kDa				
HSPA1A*	3303	A8K5I0	gi 5123454	PROTEIN 1A	15	34	3	
		Q8WWM7						
ATXN2L*	11273	A8K1R6	gi 119572372	ATAXIN 2-LIKE	14	20	2	
		Q6PK16		MYOSIN, HEAVY				
		P35580		POLYPEPTIDE 10, NON-MUSCLE				
MYH10	4628	Q9BWG0	gi 41406064		13	9	1	
				DNAJ (HSP40) HOMOLOG, SUBFAMILY C, MEMBER 10				
DNAJC10*	54431	Q8IXB1	gi 24308127	PARASPECKLE	13	21	3	
				COMPONENT 1				
PSPC1	55269	Q8WXF1	gi 109240550	SPLICING FACTOR	13	35	3	
				PROLINE/GLUTAMINE-RICH (POLYPYRIMIDINE TRACT BINDING PROTEIN				
		P23246		ASSOCIATED)				
SFPQ	6421	Q86VG2	gi 119627829		12	35	1	
SF1*	7536	Q14820	gi 42544123	SPLICING FACTOR 1	12	20	2	

Q15637			HEAT SHOCK 70kDa PROTEIN 5 (GLUCOSE- REGULATED PROTEIN, 78kDa)			
HSPA5*	3309	P11021	gi 119608027	11	37	4
HLF2	4057	P02788	gi 16198357	11	30	1
			P35749			
			Q3MNF1			
			Q3MIV8			
MYH11	4629	Q3MNF0	gi 119574312	11	9	1
			Q14766			
LTBP1	4052	B7ZLY3	gi 46249414	10	8	2
			BINDING PROTEIN 1 PYRUVATE KINASE, MUSCLE			
PKM2*	5315	P14618	gi 127795697	10	28	1
RAVER1*	125950	Q8IY67	gi 123173757	10	29	2
			CARDIOMYOPATHY ASSOCIATED 3			
XIRP2	129446	A4UGR9	gi 61696134	10	16	1
			Q8NE71			
			Q2L6I2			
ABCF1	23	A2BF75	gi 21759807	9	14	1
			POTE ANKYRIN DOMAIN FAMILY, F			
POTEF	728378	A5A3E0	gi 153791352	9	8	1
			P07900			
HSP90AA1	3320	Q86SX1	gi 153792590	8	13	3
			HEAT SHOCK PROTEIN 90kDa ALPHA, CLASS A MEMBER 1			
			INTEGRIN, ALPHA 2B (PLATELET GLYCOPROTEIN IIB OF IIB/IIIA COMPLEX, ANTIGEN CD41)			
ITGA2B*	3674	P08514	gi 119571981	8	17	2
			Q06AH7			
TF*	7018	P02787	gi 110590597	8	18	2
			TRANSFERRIN SPLICING FACTOR 3A, SUBUNIT 2, 66kDa			
SAP 62	8175	A0PJA6	gi 21361376	8	19	1
			Q05DF2			
LYZ	4069	Q15428	gi 4930023	7	70	1
			P61626			
			B2R4C5			
SMAD4	4089	Q13485	gi 4885457	7	12	2
			DPP HOMOLOG 4 (DROSOPHILA) HEAT SHOCK PROTEIN 90kDa BETA (GRP94), MEMBER 1			
HSP90B1	7184	P14625	gi 4507677	7	12	2
CANX	821	P27824	gi 10716563	6	13	1
			Q7Z5C1			
			P98164			
LRP2	4036	Q7Z5C0	gi 32816595	6	1	1
			A6NE14			
			P49368			
CCT3	7203	B3KX11	gi 14124984	6	21	2
			CHAPERONIN CONTAINING TCP1, SUBUNIT 3 (GAMMA) HEAT SHOCK 70kDa			
HSPA1L	3305	P34931	gi 21759781	5	15	2
NCL	4691	P19338	gi 189306	5	6	1
			NUCLEOLIN			

B3KM80							
PIP	5304	P12273	gi 4505821	PROLACTIN-INDUCED PROTEIN	5	31	1
SLPI	6590	P03973 P09493 Q9Y427	gi 4507065	SECRETORY LEUKOCYTE PEPTIDASE INHIBITOR	5	28	1
TPM1	7168	O15513	gi 854189	TROPOMYOSIN 1 (ALPHA)	5	10	1
HECTD1	25831	Q9ULT8	gi 118498337	HECT DOMAIN CONTAINING 1	5	5	3
LACRT	90070	Q9GZZ8	gi 15187164	LACRITIN	5	30	1
ACTG3	71	P63261	gi 178045		4	12	1
DMBT1	1755	Q9UGM3	gi 169218264	DELETED IN MALIGNANT BRAIN TUMORS 1	4	11	1
FLG	2312	P20930	gi 62122917	FILAGGRIN	4	2	1
			HETEROGENEOUS NUCLEAR				
HNRNPK	3190	P61978 Q6IBN1 B3KTV0	gi 55958544	RIBONUCLEOPROTEIN K HEAT SHOCK 70kDa	4	11	2
HSPA8*	3312	P11142	gi 5729877	PROTEIN 8	4	8	1
KPNB1	3837	Q14974	gi 119615215	KARYOPHERIN (IMPORTIN)	4	8	1
LCN1	3933	P31025	gi 4504963	BETA 1	4	30	1
			LIPOCALIN 1				
LMAN1*	3998	P49257 P07478	gi 5031873	LECTIN, MANNOSE- BINDING, 1	4	15	1
PRSS2	5645	Q5NV56	gi 74353564	PROTEASE, SERINE, 2 (TRYPSIN 2)	4	15	1
			ALDEHYDE DEHYDROGENASE 18				
ALDH18A1	5832	P54886	gi 76779856	FAMILY, MEMBER A1	4	5	1
NPM3	10360	O75607	gi 5801867	NUCLEOPHOSMIN/NUCLE OPLASMIN, 3	4	12	1
			WW DOMAIN CONTAINING ADAPTOR WITH COILED- COIL				
WAC	51322	Q9BTA9	gi 55664165	ANKYRIN REPEAT DOMAIN	4	5	1
ANKRD24	170961	Q8TF*21 Q96HY3 P62158	gi 16418357	24	4	5	1
CALM1	801	B4DJ51	gi 61680528	CALMODULIN	3	18	1
			COLLAGEN, TYPE VII, ALPHA 1 (EPIDERMOLYSIS BULLOSA, DYSTROPHIC, DOMINANT AND RECESSIVE)				
COL7A1	1294	Q02388 Q59F16	gi 119585300	HEAT SHOCK 60kDa	3	1	1
HSPD1	3329	P10809 P23368	gi 14326412	PROTEIN 1	3	15	2
ME2	4200	Q9BWL6	gi 5822326	MALIC ENZYME 2, NAD(+)- DEPENDENT	3	5	2
PEX1	5189	O43933	gi 4505725	PEROXISOMAL BIOGENESIS FACTOR 1	3	2	1
PRKAA1	5562	Q13131	gi 29124503	PROTEIN KINASE, AMP- ACTIVATED, ALPHA 1	3	5	1

H6PD	9563	O95479	gi 51859374	CATALYTIC SUBUNIT HEXOSE-6-PHOSPHATE DEHYDROGENASE (GLUCOSE 1- DEHYDROGENASE)	3	3	2
PDIA4	9601	Q549T6 P13667	gi 37182276	PROTEIN DISULFIDE ISOMERASE FAMILY A, MEMBER 4	3	2	1
COLEC10	10584	Q9Y6Z7	gi 5453619	COLLECTIN SUB-FAMILY MEMBER 10 (C-TYPE LECTIN)	3	12	1
FOXJ3	22887		gi 114555879	FORKHEAD BOX J3	3	4	1
ARS2	51593	Q9BXP5	gi 33150698	ARS2 PROTEIN	3	5	1
FOXJ2	55810	Q9P0K8	gi 8923842	FORKHEAD BOX J2	3	4	1
UBAP2	55833	Q5T6F2 Q9P0H6	gi 22325364	UBIQUITIN ASSOCIATED PROTEIN 2	3	3	1
KIAA1529*	57653	Q9P1Z9	gi 7959325	KIAA1529	3	2	3
LRRC8E	80131	B3KR78	gi 801893	LEUCINE RICH REPEAT CONTAINING 8 FAMILY, MEMBER E	3	3	1
QRICH2	84074	Q9H0J4 Q86TI2	gi 14149793	GLUTAMINE RICH 2	3	3	1
DPP9*	91039	Q1ZZB8	gi 119589606	DIPEPTIDYL-PEPTIDASE 9 NASOPHARGYNEAL	3	4	2
LPLUNC1	92747	Q8TDL5	gi 40807482	RELATED HYPOTHETICAL PROTEIN	3	6	1
DOK7	285489	Q18PE1	gi 119602869	FLJ33718	3	4	1
SLFN14	342618	P0C7P3	gi 193788704	ORTHOLOG OF MOUSE SCHLAFEN 10	3	5	1
ENO1	2023	P06733	gi 4503571	ENOLASE 1	2	6	1
				X-RAY REPAIR COMPLEMENTING DEFECTIVE REPAIR IN CHINESE HAMSTER CELLS 5 (DOUBLE-STRAND-BREAK REJOINING; KU			
XRCC5	7520	P13010	gi 119590969	AUTOANTIGEN, 80kDa)	2	4	1
WDR1	9948	O75083	gi 12652891	WD REPEAT DOMAIN 1	2	6	1
NLRP1	22861	Q9C000 Q9H5Z7	gi 37927559	NLR FAMILY, PYRIN DOMAIN CONTAINING 1	2	1	1
PPA2	27068	Q9H2U2		PYROPHOSPHATASE			
		A6NKL9	gi 119612395	(INORGANIC) 2	2	1	1
VPS35	55737	Q96QK1	gi 7022978	VACUOLAR PROTEIN SORTING 35	2	2	1

Proteins Captured with normal αIIb							
Gene Symbol	Entrez Gene ID	SwissProt Acc No.	Protein Acc No.	Description	# Pep	% Cov	# Exp
ITGB3*	3690	P05106	gi 183531	INTEGRIN, BETA 3 (PLATELET GLYCOPROTEIN IIIA, ANTIGEN CD61)	25	42	7

TUBB2C	7284	P49411	gi 20809886	TUBULIN, BETA 2C DEHYDROGENASE E1 AND TRANSKETOLASE	17	35	1
DHTKD1	55526	Q96HY7	gi 119606733	DOMAIN CONTAINING 1 GLUTAMATE	15	36	1
GLUD1	2746	P00367 P14868	gi 183056	DEHYDROGENASE 1 ASPARTYL-TRNA	15	67	3
DARS	1615	P78371	gi 45439306	SYNTHETASE	14	60	3
LMAN1*	3998	P49257	gi 5031873	LECTIN, MANNOSE- BINDING, 1 PROTEIN KINASE, AMP- ACTIVATED, BETA 1 NON- CATALYTIC SUBUNIT	12	36	1
PRKAB1	5564	Q9Y478	gi 4506061	HEAT SHOCK 70kDa PROTEIN 5 (GLUCOSE- REGULATED PROTEIN, 78kDa)	12	38	1
HSPA5*	3309	P11021 P08246 P43626 P43628	gi 16507237	SODIUM CHANNEL, VOLTAGE-GATED, TYPE X, ALPHA	11	30	1
SCN10A	6336	P43632	gi 110835709	TUBULIN, BETA 5	11	5	1
TUBB	10383	P68371	gi 7106439	TUBULIN, BETA 4	11	44	4
TUBB4	23071	Q9BS26	gi 21361322	ALDEHYDE DEHYDROGENASE 18 FAMILY, MEMBER A1 DNAJ (HSP40) HOMOLOG, SUBFAMILY C, MEMBER 10 EXOSOME COMPONENT	10	26	1
ALDH18A1	5832	P54886	gi 76779856	10 UDP-GLUCOSE PYROPHOSPHORYLASE 1 ADP-DEPENDENT	9	18	2
DNAJC10*	54431	Q8IXB1 Q01780	gi 24308127	GLUCOKINASE AMINOLEVULINATE, DELTA-, DEHYDRATASE	9	21	4
EXOSC10	5394	P05156	gi 50301239	CHAPERONIN CONTAINING TCP1, SUBUNIT 2 (BETA)	9	8	1
UGP1	10352	Q9UGM6	gi 48255966	CHAPERONIN CONTAINING TCP1, SUBUNIT 4 (DELTA)	9	33	1
ADPGK	83440	Q9BRR6	gi 31542509	PHENYLALANINE-TRNA SYNTHETASE 2	8	22	1
ALAD	210	P13716	gi 248839	HEAT SHOCK 70kDa PROTEIN 1A	8	22	1
CCT2	10576	P78371	gi 5453603	HEAT SHOCK 70kDa PROTEIN 1B	8	39	1
CCT4	10575	P50991	gi 38455427	HEAT SHOCK 70kDa PROTEIN 9B (MORTALIN-2)	8	28	1
FARS2	10667	O95363	gi 62898407	NUDIX (NUCLEOSIDE DIPHOSPHATE LINKED MOIETY X)-TYPE MOTIF 19	8	24	1
HSPA1A*	3303	B3KTT5	gi 5123454		8	19	1
HSPA1B	3304	P08107	gi 167466173		8	21	1
HSPA9	3313	P38646	gi 12653415		8	17	2
NUDT19	390916	A8MXV4	gi 157739940		8	36	1

AKR7A2	8574	O43488	gi 41327764	ALDO-KETO REDUCTASE FAMILY 7, MEMBER A2 (AFLATOXIN ALDEHYDE REDUCTASE)	7	37	1
CHD9	80205	Q3L8U1	gi 95147342	CHROMODOMAIN HELICASE DNA BINDING PROTEIN 9	7	2	1
FAM175B	23172		gi 148529023	UNKNOWN PROTEIN LOC23172	7	14	1
KIF14	9928	Q15058	gi 7661878	KINESIN FAMILY MEMBER 14	7	4	1
NT5DC2	22978	A8K6K2	gi 12597653	5'-NUCLEOTIDASE DOMAIN CONTAINING 2	7	36	2
P15RS	55197	Q96P16	gi 142385371	REGULATION OF NUCLEAR pre-mRNA DOMAIN CONTAINING 1A	7	20	1
PDLIM1	9260	Q9NR12	gi 13994151	PDZ AND LIM DOMAIN 1 (ENIGMA)	7	37	1
PM20D2	135293	Q8IYS1	gi 58082085	AMINOACYLASE 1-LIKE 2	7	15	1
POLDIP2	26073	Q9Y2S7	gi 7661672	POLYMERASE (DNA- DIRECTED), DELTA	7	26	1
TF*	7018	Q9BVV8	gi 553788	INTERACTING PROTEIN 2	7	12	1
ACTN4	604638	P02787	gi 2804273	TRANSFERRIN	6	9	1
ATXN2L*	11273	Q8WWM7	gi 119572372	ACTININ, ALPHA 4	6	9	1
CCT7	10574	Q99832	gi 62896515	ATAXIN 2-LIKE CHAPERONIN CONTAINING TCP1,	6	23	1
CNDP2	55748	Q96KP4	gi 8922698	SUBUNIT 7 (ETA) CNDP DIPEPTIDASE 2 (METALLOPEPTIDASE M20 FAMILY)	6	11	1
FLJ12529	79869	Q8N684	gi 24432016	PRE-MRNA CLEAVAGE FACTOR I, 59 kDa SUBUNIT	6	12	1
PBEF1	10135	P43490	gi 55960735	PRE-B-CELL COLONY ENHANCING FACTOR 1	6	4	1
PPP1R9A	55607	Q9ULJ8	gi 261244899	PROTEIN PHOSPHATASE 1, REGULATORY (INHIBITOR)	6	5	1
RB1CC1	9821	Q8TDY2	gi 134304845	SUBUNIT 9A, 5 isoforms RB1-INDUCIBLE COILED- COIL 1	6	3	1
SERBP1	26135	Q9H707	gi 66346679	SERPINE1 MRNA BINDING PROTEIN 1	6	16	1
TXNDC4	23352	Q8WXW3	gi 119579327	THIOREDOXIN DOMAIN CONTAINING 4 (ENDOPLASMIC RETICULUM)	6	35	3
ACLY	47	Q5T4S7	gi 38569423	ATP CITRATE LYASE	5	3	1
ACTB	60	Q9NP58	gi 14250401	ACTIN, BETA	5	22	3
CTTN	2017	P53396	gi 2498954	CORTACTIN	5	4	1
CUL-5	8065	Q1KLZ0	gi 67514034	CULLIN 5	5	5	1
FHL1	2273	Q93034	gi 3851650	FOUR AND A HALF LIM	5	3	1

GPHN	10243	Q9NQX3	gi 10880983	DOMAINS 1 GEPHYRIN ISOFORM 1 HETEROGENEOUS NUCLEAR	5	8	1
HNRPH1	3187	P31943	gi 5031753	RIBONUCLEOPROTEIN H1 HEAT SHOCK 70kDa	5	16	1
HSPA8*	3312	P11142	gi 10880983	PROTEIN 8 ISOCITRATE DEHYDROGENASE 3	5	8	3
IDH3A	3419	P50213	gi 5031777	(NAD+) ALPHA PROTEIN KINASE, AMP- ACTIVATED, GAMMA 1	5	13	1
PRKAG1	5571	P54619	gi 2230863	NON-CATALYTIC SUBUNIT RAB INTERACTING LYSOSOMAL PROTEIN- LIKE 1	5	27	1
RILPL1	353116	Q5EBL4	gi 30315660	SIN3A-ASSOCIATED PROTEIN, 130kDa	5	12	1
SAP130	79595	Q9H0E3	gi:25579126	TRYPTOPHANYL TRNA SYNTHETASE 2	5	3	1
WARS2	80139	Q9H7S9	gi 7710154	ADENYLATE CYCLASE 6	5	19	1
ADCY6	112	O43306	gi 168480141	FLJ44216 PROTEIN	4	3	1
C5orf25	375484	Q8NDZ2	gi 196259795	HYPOTHETICAL PROTEIN FLJ39378	4	8	1
CARS2	79587	Q9HA77	gi 119618821	CITRON (RHO- INTERACTING, SERINE/THREONINE KINASE 21)	4	10	1
CIT	11113	O14578	gi 32698687	EUKARYOTIC ELONGATION FACTOR-2 KINASE	4	2	1
EEF2K	29904	O00418	gi 9558749	HYPOTHETICAL PROTEIN FLJ22184	4	4	1
FLJ22184	80164	Q9H6K5	gi 239757129	FILAMIN A, ALPHA (ACTIN BINDING PROTEIN 280)	4	5	1
FLNA	2316	P21333	gi 57284166	FERM AND PDZ DOMAIN CONTAINING 1	4	2	2
FRMPD1	22844	Q5SYB0	gi 239582740	GOLGI ASSOCIATED PDZ AND COILED-COIL MOTIF CONTAINING	4	3	1
GOPC	57120	Q9HD26	gi 9966877	3-HYDROXY-3- METHYLGLUTARYL- COENZYME A SYNTHASE 1 (SOLUBLE)	4	12	1
HMGCS1	3157	Q01581	gi 53734504	HETEROGENEOUS NUCLEAR	4	14	1
HNRNPL*	3191	P14866	gi 133274	RIBONUCLEOPROTEIN L	4	11	2
HTRA2	27429	O43464	gi 21614538	HTRA SERINE PEPTIDASE 2 N-ACETYLGLUCOSAMINE KINASE	4	16	1
NAGK	55577	Q9UJ70	gi 6491737	PYRUVATE KINASE,	4	10	1
PKM2*	5315	P14618	gi 31416989		4	16	1

				MUSCLE			
PRDX3 SF1*	10935 7536	Q05940 P30048 Q15637	gi 14250063 gi 46362557	PEROXIREDOXIN 3 SPLICING FACTOR 1	4 4	20 5	1 1
				TRANSLOCASE OF INNER MITOCHONDRIAL			
TIMM50	92609	Q3ZCQ8	gi 48526509	MEMBRANE 50 HOMOLOG GLUCOSYLTRANSFERASE	4	6	1
AER61	285203		gi 39930530	AER61 C3orf64	3	6	1
CSNK2A2 DPP9*	1459 91039	Q14012 Q86TI2	gi 4503097 gi 114657671	CASEIN KINASE 2, ALPHA PRIME POLYPEPTIDE DIPEPTIDYL-PEPTIDASE 9	3 3	6 2	1 1
				DISHEVELLED, DSH HOMOLOG 2			
DVL2	1856	O14641	gi 55665917	(DROSOPHILA) ENGULFMENT AND CELL	3	2	1
ELMO1	9844	O60895	gi 86788139	MOTILITY 1 GLYCERALDEHYDE-3- PHOSPHATE	3	5	1
GAPDH	2597	P04406 Q16678	gi 31645	DEHYDROGENASE	3	18	1
KIAA0895	23366	Q8NCT3	gi 154426319	KIAA0895 PROTEIN	3	6	1
LUC7L2	51631	Q9Y383	gi 4929587	CGI-59 PROTEIN	3	5	1
MPO	4353	P05164	gi 88180	MYELOPEROXIDASE	3	5	2
PEG10	23089	Q86TG7 P05388	gi 94421473	PATERNALLY EXPRESSED 10	3	11	1
RPLP0	6175	Q9NQX7	gi 12654583	RIBOSOMAL PROTEIN, LARGE, P0	3	12	1
SEC13	6396	A8MV37 Q15436	gi 119584482	SEC13-LIKE 1 (S. CEREVISIAE)	3	11	1
SEC23A	10484	Q92736	gi 22477159	SEC23 HOMOLOG A (S. CEREVISIAE)	3	3	1
SHROOM3	57619	Q8TF72	gi 57284166	SHROOM3 F-ACTIN BINDING PROTEIN	3	2	4
UBR4	23352		gi 9367763	ZINC FINGER, UBR1 TYPE 1	3	3	1
A2M	2	P01023	gi 177872	ALPHA-2- MACROGLOBULIN	2	1	1
ABCA13	154664	Q86UQ4	gi 8928549	ATP-BINDING CASSETTE, SUB-FAMILY A (ABC1), MEMBER 13	2	5	2
CORO1A	11151	P31146	gi 5902134	CORONIN, ACTIN	2	4	1
KIAA1529*	57653	Q9P1Z9	gi 7959325	BINDING PROTEIN, 1A KIAA1529	2 2	2 2	1 4
				MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 7 INTERACTING			
MAP3K7IP2	23118	Q9NYJ8	gi 14149669	PROTEIN 2	2	2	1
RANBP10	57610	Q6VN20	gi 40538736	RAN BINDING PROTEIN 10	2	4	1
RAVER1*	125950	Q8IY67	gi 123173757	RAVER1	2	4	1
TUBA1A	203068	P07437	gi 340021	TUBULIN, ALPHA	2	2	3
ZNF703	80139		gi 13376610	ZINC FINGER PROTEIN 703	2	3	1

Table 1. Proteins captured with αIIb in megakaryocytes and HEK293 cells. Results are separated according to the αIIb subunit they were captured with. Column labels: Gene

Symbol, EntrezGene ID, Protein Accession Number and Description are from the NCBI database. SwissProt Accession Number is from the UniProt database. Number of Unique Peptides is cumulative from all experiments. Coverage percent is cumulative for all experiments. Tally is shown of the total number of experiments in which a protein was identified (No Expts), and whether captured by histidine tag/Ni affinity (Ni-His) or by crosslinking (X-link). Presence or absence in a recently published expression study (HaemAtlas) is indicated. * Proteins captured by both normal and mutant α IIb subunits.

3.2 Gene Ontology analysis

Gene Ontology (GO) analysis using the DAVID Bioinformatics Resources(Dennis, Sherman et al. 2003; Huang, Sherman et al. 2008) revealed enrichment for protein functions related to processing and trafficking, and ER, Golgi or vesicle components (Figure 3). Comparison of the proteins isolated with the normal α IIb subunits *vs* with the α IIbR858G/R859G subunits showed similar distributions of their localization and molecular functions (**Table 2**). Notably, the α IIbR858G/R859G subunits captured a greater percentage of these types of proteins, suggesting that a greater percentage of the α IIbR858G/R859G subunits were associated with the ER, Golgi and vesicles. Comparison of our data set to a recently published analysis of gene expression in megakaryocytes(Watkins, Gusnanto et al. 2009) showed that approximately 70% of the proteins identified herein as potentially interacting with α IIb were identified as expressed in the megakaryocyte transcriptome.

GO function/location	% of total proteins	
	α IIb	α IIbR858G/R859G
Protein transport	31	49
Stress Capone	21	34
Vesicle part	6	14
ER or Golgi part	19	21
Organelle part	27	41
Cytoskeleton	21	29
ATP/GTP binding	31	45

Table 2. Distribution of captured proteins into Gene Ontology categories. Some proteins appear in more than one category.

Despite the similarity in distributions into GO categories pertaining to protein processing and localization, there were only 14 proteins in common between the normal α IIb and α IIbR858G/R859G captured proteins (**Table 1**). This may indicate a difference in the relative amount of time spent by the normal and mutant subunits in the protein processing environment. One protein, DNAJC10, was captured in all experiments by both α IIb and α IIbR858G/R859G. Like other DNAJ proteins, DNAJC10 contains a binding domain for the chaperone BiP, but is unique in that its second domain has four protein-disulfide-isomerase (PDI) consensus sequences. (Cunnea, Miranda-Vizuet et al. 2003; Dong, Bridges et al. 2008) Because disulfide bond rearrangement is important for both biogenesis and function of α IIb β 3, we investigated the putative interaction between α IIb and DNAJC10. To our knowledge, DNAJC10 has not previously been reported to interact with α IIb or β 3.

3.3 DNAJC10 interacts with α IIB β 3 during biogenesis

DNAJC10 coimmunoprecipitated with α IIB and β 3 from UCB derived megakaryocytes using anti- α IIB mAbs 10E5, B1B5, M148 and CA3, and the anti β 3 mAb 7H2. DNAJC10 either interacted with α IIB and β 3 directly or was part of a complex with them (**Figure 3**). Because DNAJC10 was captured by the α IIBR858G/R859G subunit, we explored the possibility that DNAJC10 interacted with α IIB prior to α IIB β 3 complex formation and α IIB cleavage to its mature form. We have previously shown that pro- α IIB is degraded by the proteasome and that proteasome inhibition leads to an increase of pro- α IIB within the cells. (Mitchell, Li et al. 2006) The undegraded pro- α IIB is trapped in a “pre-degradation” state in which normally transient protein interactions, which usually lead to degradation, may become more long-lived and may be captured by co-immunoprecipitation. Incubation of megakaryocytes with MG132 resulted in increased DNAJC10 co-immunoprecipitation with α IIB by all mAbs, but particularly M148 and CA3. Thus, DNAJC10 may associate with α IIB early in biogenesis, before excess pro- α IIB is targeted to the proteasome.

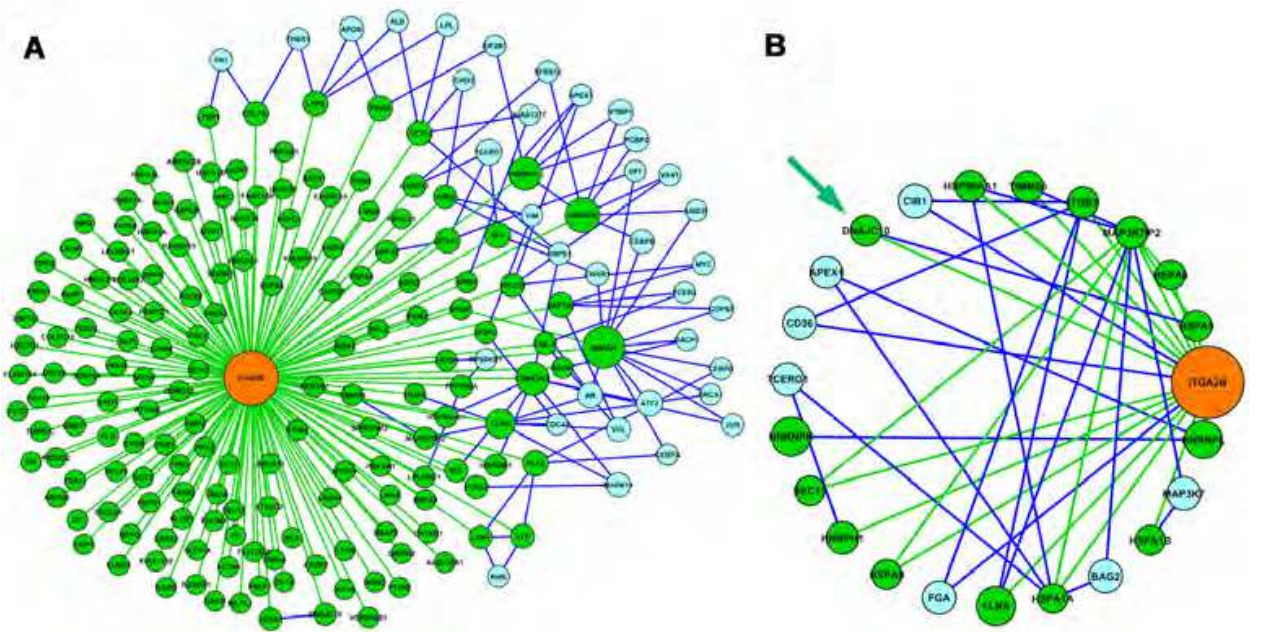


Fig. 3. Network derived from Table 1 data. Proteins are represented as nodes (circles) labeled with their gene symbol, and putative protein interactions with α IIB are indicated by thin green connecting lines. Thick blue lines and blues circles represent interactions or proteins, respectively, retrieved from online databases (Intact and NCBI) using the Cytoscape software. Arrow indicates DNAJC10. Figure prepared using Cytoscape.

To test this possibility we used a panel of conformation-specific mAbs that we have previously used to track the conformational changes of α IIB as it proceeds through biogenesis (**Figure 3**). (Mitchell, Li et al. 2007) Specifically: mAb10E5 binds to the α IIB head region and recognizes both the pro- α IIB β 3 and mature α IIB β 3 complex; mAb 7E3 binds to the β 3 head region and also recognizes both the pro- and mature complex; mAb B1B5 binds to the α IIB tail and preferentially recognizes pro- α IIB and pro- α IIB β 3 complex, and mAb M148 preferentially recognizes mature α IIB β 3 but its epitope is not known. (Mitchell, Li et al.

2007) To determine whether DNAJC10 interacts with α IIb during a specific stage of biogenesis, α IIb was immunoprecipitated from megakaryocytes in the presence of MG132 using this panel of conformation-specific mAbs. DNAJC10 was most strongly coimmunoprecipitated by mAb B1B5, suggesting that DNAJC10 preferentially interacts with pro- α IIb or the pro- α IIb β 3 complex. In contrast, DNAJC10 coimmunoprecipitated less well with mAb M148, suggesting less interaction with mature α IIb. DNAJC10 also interacted with the precursor and/or mature α IIb β 3 complex, since it coimmunoprecipitated with the complex-specific mAb10E5. Since the ratio of pro- α IIb β 3 to mature α IIb β 3 is small in megakaryocytes,(Mitchell, Li et al. 2007) this binding pattern is consistent with DNAJC10 having bound preferentially to the small amount of pro- α IIb β 3 present in the cells. Together these findings suggest that DNAJC10 preferentially interacted with pro- α IIb and the pro- α IIb β 3 complex, rather than the mature complex.

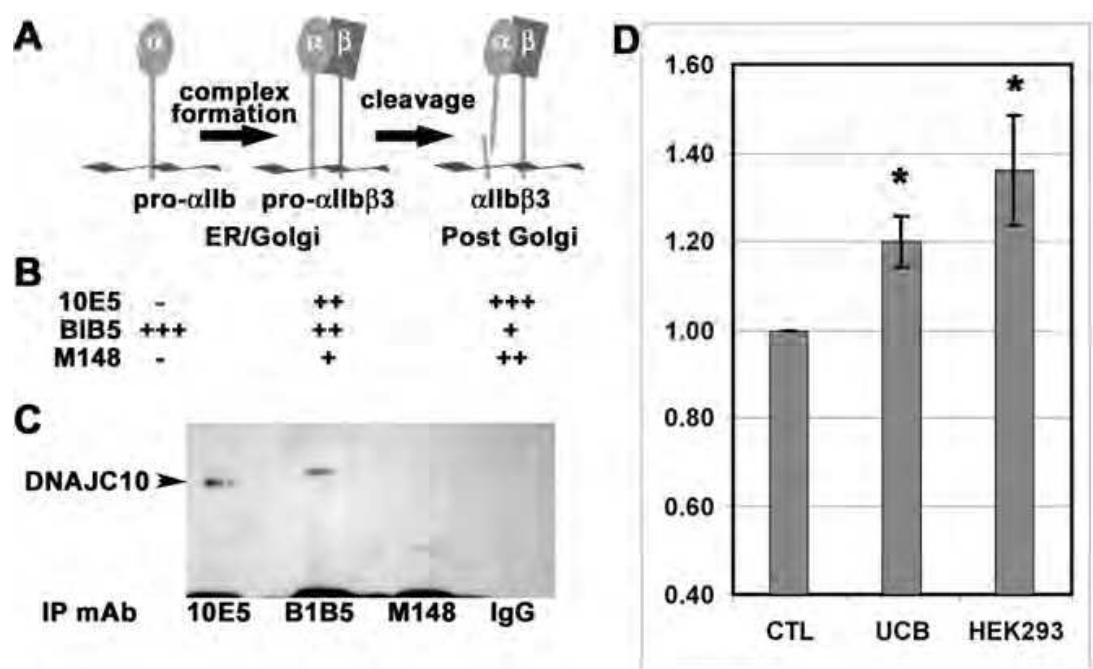


Fig. 4. Interaction of DNAJC10 with α IIb β 3 during biogenesis. A) Schematic of changing conformations of α IIb β 3 as it progresses through biogenesis. B) Changing specificity of 3 mAbs against α IIb as it progresses through distinct conformations during biogenesis. B1B5 preferentially recognizes pro- α IIb, while M148 shows preference for mature α IIb, and 10E5 recognizes the heterodimer complex.C) Immunoprecipitation and Western blot of proteins isolated from megakaryocytes, as described in text. DNAJC10 coimmunoprecipitated preferentially with α IIb that was pulled down by 10E5 and B1B5, but not M148, suggesting that DNAJC10 preferentially interacts with pro- α IIb and pro- α IIb β 3. The last lane is an IgG control. D) siRNA mediated knockdown of DNAJC10 increased surface expression of α IIb β 3 as measured by flow cytometry using an Alexa647-labeled anti- α IIb β 3 mAb (10E5). Expression was increased by 25 +\ - 11 % (p=0.02, n=4) in UCB-derived megakaryocytes (UCB) and by 35 +\ - 12 % (p=0.01, n=3) in HEK293 cells (HEK293) compared to control siRNA transfection (CTL).

3.4 DNAJC10 depletion increases surface expression of α IIb β 3

To determine whether the α IIb-DNAJC10 interaction had physiological relevance, we assessed its impact on the surface expression of α IIb β 3 in megakaryocytes. siRNA mediated knockdown of DNAJC10 was performed on both human megakaryocytes derived from UCB and on HEK293 cells expressing normal α IIb and β 3. At least an 80% decrease in RNA level was achieved (data not shown). Knockdown of DNAJC10 increased α IIb β 3 surface expression on megakaryocytes by 25% +/- 11% (n = 4, p = 0.02), and on HEK293 cells expressing α IIb β 3 by 35% +/- 12% (n=4, p = 0.01) (Figure 3D).

3.5 Intracellular localization of DNAJC10 in megakaryocytes

DNAJC10 was localized within megakaryocytes by immunofluorescence microscopy (Figure 5). The distribution of DNAJC10 was compared to that of α IIb, β 3, and the ER and Golgi compartments. Both α IIb and β 3 were distributed throughout the ER as well as on the cell surface. DNAJC10 had a diffuse punctate distribution in the periphery of the cell, away from the nucleus, and colocalized with only a portion of α IIb and β 3, consistent with their presumably transient interaction. There was partial overlap between the ER marker calnexin and DNAJC10, suggesting that part of the DNAJC10 distribution is outside the ER or at least

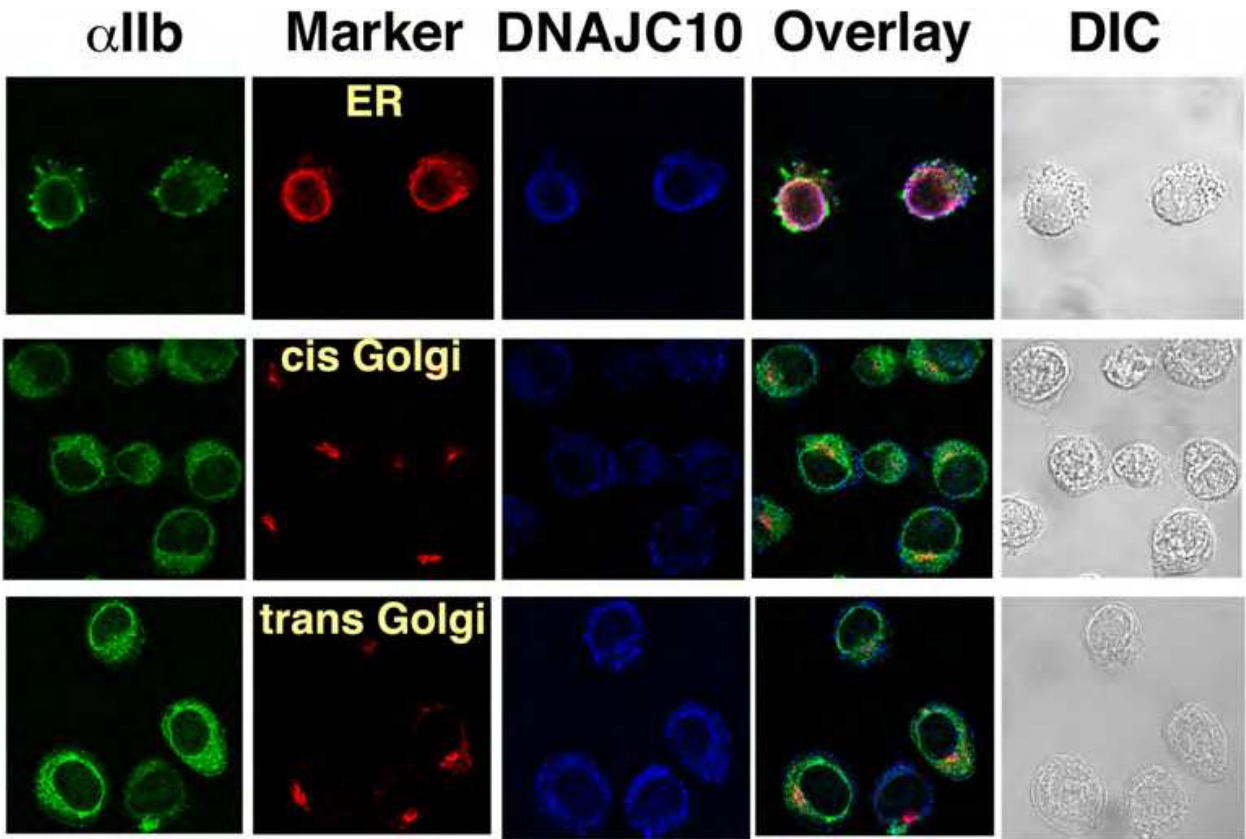


Fig. 4. Intracellular localization of DNAJC10. Cultured megakaryocytes were fixed and immunostained as described in the text. DNAJC10 (Blue) exhibited a punctate staining in the periphery of the cells, away from the nucleus. There was partial overlap of DNAJC10 with calnexin (Red) staining. α IIb (Green) was distributed throughout the ER and cell surface. While α IIb partially colocalized with the cis- and trans-Golgi, DNAJC10 did not.

separate from the distribution of calnexin in the ER. This finding was surprising since DNAJC10 has the ER-retention signal KDEL. DNAJC10 did not localize with markers for the cis- or trans-Golgi compartments.

4. Discussion

We have used a proteomics approach to identify novel proteins interacting with α IIb in megakaryocytes. Two different constructs of α IIb, representing normal α IIb and pro- α IIb subunits, were used to capture proteins interacting with both nascent and mature subunits. Megakaryocytes were derived from human UCB and used on day 8, which in our system yields high expression α IIb β 3 on the surface but no proplatelet formation. (Mitchell, Li et al. 2006) In all, 163 proteins were identified as potentially interacting with α IIb subunits; 98 were captured with normal α IIb and 79 with α IIbR858/G859G, with 14 overlapping (Table 1). Day 8 megakaryocytes express very high amounts of α IIb, most of which is mature α IIb β 3, resulting in a relatively small proportion of nascent α IIb. However, a large proportion of the mutant α IIbR858/G859G subunits are retained within the cell and degraded. Thus the difference between the two protein lists could partly be due to their differences in localization within the cell. This may be reflected in the larger proportion of ER and Golgi related proteins that were captured with the mutant subunit (Table 2). Only a few intracellular proteins have been reported to interact with α IIb and α IIb β 3, most notably talin, calnexin, and calreticulin (Intact and NCBI). Our two-step protein capture method was designed to isolate proteins with low affinity binding to α IIb, such as calnexin, while screening out higher affinity binding proteins, such as talin and β 3. In accord with this expectation, both talin and β 3 were identified in both the control and experimental lanes, and so were excluded from the final interaction list, while calnexin and calreticulin were identified only in the experimental lanes. Surprisingly, while DNAJC10 readily coimmunoprecipitates with α IIb β 3 from megakaryocytes, we have been unable to replicate this finding in HEK293 cells transfected with α IIb and β 3, despite an abundance of DNAJC10 in HEK293 cells (data not shown). While this is in no way conclusive, it is suggestive of cell-specific interaction of α IIb β 3 and DNAJC10 in megakaryocytes. Comparison of our experimental results with previously reported platelet proteomic data and α IIb β 3 interaction data showed good correlation. About 70% of the proteins identified in our screens were reported as “present” in platelets in the Haem Atlas, a proteomic analysis of platelet protein content (Watkins, Gusnanto et al. 2009).

Two protein capturing strategies were used, each with strengths and weaknesses. The two-cell pull-down assay using Ni beads to capture poly-histidine-tagged α IIb, allowed the use of mutant cDNA constructs, such as α IIbR858G/R859G, as bait. However, by introducing a protein synthesized in HEK293 cells as bait, there was the potential for false positive identification of proteins that associated with α IIb in the HEK293 cells but not in megakaryocytes. Since the interactions of chaperone proteins are typically of low affinity, these proteins were most likely cleared by the washing steps and did not appear in the control lanes. Another source of false positives was non-specific binding to the Ni beads. Proteins with poly-His sequences (such as DEAH boxes) or naturally occurring Ni binding activity (such as keratin) could have bound to the beads, constituting false positives. However, virtually all of these potential false positives appeared in the control lanes as well and were excluded from analysis. The photoreactive crosslinking assay was intended to

capture proteins in situ with α IIb in megakaryocytes. While crosslinking experiments typically produce high numbers of false positives, in our experiments we identified low numbers of proteins from both the experimental and control lanes. The low yield may be due in part to the short crosslinking time used. We found that more than 15 minutes of UV exposure caused excessive protein degradation, while shorter exposure resulted in low crosslinking activity.

Of the proteins captured using both α IIb and α IIbR858G/R859G the Hsp40-type chaperone protein, DNAJC10 (ERdj5), was notable due to its disulfide isomerase activity, since both α IIb and β 3 require disulfide bond rearrangement for both biogenesis and function. (Shen, Meunier et al. 2002; Cunnea, Miranda-Vizuite et al. 2003; Dong, Bridges et al. 2008) Among its several functions, the ER chaperone protein BiP protects nearly-folded proteins against aggregation by binding to exposed hydrophobic patches. (Hendershot 2004) The Hsp40 chaperones bind to BiP and increase its efficiency of ATP hydrolysis, which allows BiP to release its substrate. DNAJC10 has been shown to be induced during ER stress, and may assist in delivering misfolded ER proteins to the proteasome for degradation. (Shen, Meunier et al. 2002; Cunnea, Miranda-Vizuite et al. 2003; Dong, Bridges et al. 2008)

DNAJC10 coimmunoprecipitated with both α IIb and β 3 subunits in megakaryocytes, suggesting that it may bind the α IIb β 3 complex. The immunoprecipitation pattern obtained using a panel of conformation-specific mAbs (Mitchell, Li et al. 2007) indicated that DNAJC10 preferentially interacted with pro- α IIb or pro- α IIb β 3 rather than mature α IIb β 3. Together these findings suggest that DNAJC10 interacted with pro- α IIb up to the point of complex formation, but not after pro- α IIb cleavage (Figure 2). Thus DNAJC10 appears to be present and interacting with α IIb at a critical decision point during α IIb β 3 biogenesis, i.e. when pro- α IIb will either form the mature α IIb β 3 complex or be targeted to degradation.

Surprisingly, the distribution of DNAJC10, which has a KDEL ER-localization signal, was not confined to the ER, as judged by the distribution of calnexin. To determine if DNAJC10 was cycling to the Golgi and back, as many ER packaging proteins do, we looked for colocalization of DNAJC10 with cis and trans Golgi markers, and found none. The identity of the organelle(s) where DNAJC10 resides remains to be determined.

Depletion of DNAJC10 by siRNA resulted in an increase in surface expression of α IIb β 3 on both human megakaryocytes and transfected HEK293 cells (**Figure 2D**). Since DNAJC10 depletion led to an increase in α IIb β 3 surface expression, it appears to be a negative regulator of α IIb β 3 surface expression. These findings make DNAJC10 an interesting and potentially targetable protein for perturbing α IIb β 3 biogenesis.

5. Conclusion

While the details of DNAJC10- α IIb interaction remain to be investigated, the current findings provide proof of principle that manipulation of early events in α IIb biogenesis can result in altered expression levels of the mature α IIb β 3 receptor, thereby setting a precedent for a novel approach to integrin-related therapy. These studies also support the validity of the data set, although other putative interactions must be explored for greater validation. We hope that the data set created will be a useful tool for studying integrin and megakaryocyte biology.

By deciphering the α IIb β 3 biogenesis pathway we hope to gain an inroad into controlling the level of α IIb β 3 expression on platelets with the long-term goal of developing novel anti-thrombotic therapies. These types of therapy would not just inactivate the circulating platelets, but would modulate the megakaryocytes to make less adhesive platelets. One can imagine a scenario where patients at high risk of heart attack or stroke could be maintained on a drug that decreases their platelet α IIb β 3 expression. Below a certain level of expression, platelet activation and aggregation would be diminished but not completely eliminated, resulting in an overall decrease in platelet thrombus formation but not complete loss of platelet function. This type of therapy could potentially have a greater safety profile than current therapies that summarily inactivate circulating platelets.

This study also assembles some of the wide range of research methods available to hematology research. No single technique could have discovered, validated and explored the function of DNAJC10 in megakaryocytes: rather, a broad range of methods was required. This wide variety is part of what makes research exciting and underscores the benefits of collaboration.

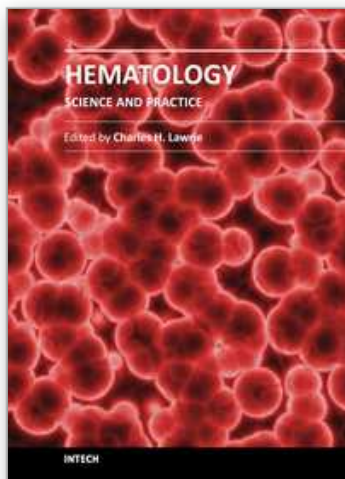
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7. References

- Bray, P. F., J. P. Rosa, et al. (1986). "Biogenesis of the platelet receptor for fibrinogen: Evidence for separate precursors for glycoproteins IIb and IIIa." *Proc Natl Acad Sci USA* 83: 1480-1484.
- Cline, M. S., M. Smoot, et al. (2007). "Integration of biological networks and gene expression data using Cytoscape." *Nat. Protocols* 2(10): 2366-2382.
- Cunnea, P. M., A. Miranda-Vizuite, et al. (2003). "ERdj5, an Endoplasmic Reticulum (ER)-resident Protein Containing DnaJ and Thioredoxin Domains, Is Expressed in Secretory Cells or following ER Stress." *Journal of Biological Chemistry* 278(2): 1059-1066.
- De Luca, G., G. Ucci, et al. (2009). "Benefits from small molecule administration as compared with abciximab among patients with ST-segment elevation myocardial infarction treated with primary angioplasty: a meta-analysis." *J Am Coll Cardiol* 53(18): 1668-73.
- Dennis, G., B. Sherman, et al. (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4(5):P3.
- Dong, M., J. P. Bridges, et al. (2008). "ERdj4 and ERdj5 Are Required for Endoplasmic Reticulum-associated Protein Degradation of Misfolded Surfactant Protein C." *Molecular Biology of the Cell* 19(6): 2620-2630.
- El.Golli, N., O. Issertial, et al. (2005). "Evidence for a Granule Targeting Sequence within Platelet Factor 4." *Journal of Biological Chemistry* 280(34): 30329-30335.

- Hendershot, L. M. (2004). "The ER function BiP is a master regulator of ER function." *Mt Sinai J Med* 71(5): 289-97.
- Huang, D. W., B. T. Sherman, et al. (2008). "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources." *Nat. Protocols* 4(1): 44-57.
- King, S. M. and G. L. Reed (2002). "Development of platelet secretory granules." *Seminars in Cell & Developmental Biology* 13(4): 293-302.
- Kolodziej, M. A., G. Vilaire, et al. (1991). "Study of the endoproteolytic cleavage of platelet glycoprotein IIb using oligonucleotide-mediated mutagenesis." *J Biol Chem* 266: 23499-23504.
- Kumarathasan, P., S. Mohottalage, et al. (2005). "An optimized protein in-gel digest method for reliable proteome characterization by MALDI-TOF-MS analysis." *Anal Biochem* 346(1): 85-9.
- Lo, B., L. Li, et al. (2005). "Requirement of VPS33B, a member of the Sec1/Munc18 protein family, in megakaryocyte and platelet {alpha}-granule biogenesis." *Blood* 106(13): 4159-4166.
- Mitchell, W. B., J. Li, et al. (2006). "alphaIIbbeta3 biogenesis is controlled by engagement of alphaIIb in the calnexin cycle via the N15-linked glycan." *Blood* 107(7): 2713-2719.
- Mitchell, W. B., J. Li, et al. (2007). "Mapping early conformational changes in alphaIIb and beta3 during biogenesis reveals a potential mechanism for alphaIIbbeta3 adopting its bent conformation." *Blood* 109(9): 3725-32.
- Quinn, M. J., E. F. Plow, et al. (2002). "Platelet glycoprotein IIb/IIIa inhibitors: recognition of a two-edged sword?" *Circulation* 106(3): 379-85.
- Rosa, J.-P. and R. P. McEver (1989). "Processing and assembly of the integrin, glycoprotein IIb-IIIa, in HEL cells." *J Biol Chem* 264: 12596-12603.
- Shen, Y., L. Meunier, et al. (2002). "Identification and characterization of a novel endoplasmic reticulum (ER) DnaJ homologue, which stimulates ATPase activity of BiP in vitro and is induced by ER stress." *J Biol Chem* 277(18): 15947-56.
- Tiwari, S., J. E. Italiano, Jr., et al. (2003). "A role for Rab27b in NF-E2-dependent pathways of platelet formation." *Blood* 102(12): 3970-9.
- Topol, E. J., A. M. Lincoff, et al. (2002). "Multi-year follow-up of abciximab therapy in three randomized, placebo-controlled trials of percutaneous coronary revascularization." *Am J Med* 113(1): 1-6.
- Watkins, N. A., A. Gusnanto, et al. (2009). "A HaemAtlas: characterizing gene expression in differentiated human blood cells." *Blood* 113(19): e1-9.



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