# Contaminating reactivity of a monoclonal CCAAT/Enhancer Binding Protein β antibody in differentiating myoblasts

**Hamood AlSudais**
University of Ottawa
**Nadine Wiper-Bergeron** ( Nadine.WiperBergeron@uottawa.ca )
University of Ottawa https://orcid.org/0000-0001-8448-3246

**Research note**

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# Abstract

Objective CCAAT/Enhancer Binding proteins (C/EBPs) are transcription factors involved in the regulation of a variety of cellular processes. We used the Abcam Recombinant Anti-C/EBP beta antibody (E299) to detect C/EBPβ expression during myogenesis. Though the antibody is monoclonal, and the immunogen used is highly specific to C/EBPβ, we identified an intense band at 23 kDa on western blot that did not correspond to any of the known isoforms of C/EBPβ, or family members predicted to cross-react. Absent in myoblast cells overexpressing C/EBPβ, the band was present when C/EBPβ was knocked down, confirming specificity for a protein other than C/EBPβ. The objective of this work was to identify the contaminating reactivity. Results We performed immunoprecipitation followed by mass spectrometry to identified myosin light chain 4 (MYL4) as the unknown band, suggesting that the Abcam monoclonal antibody directed against C/EBPβ is not pure, but contains a contaminating antibody against MYL4. Caution should be used when working in cells lines that express MYL4 to not confound the detection of MYL4 with that of the C/EBPβ LIP isoform.


# Introduction

Antibody specificity is key to rigorous and reproducible research findings. Antibodies can be polyclonal, meaning a mixture of antibodies secreted by several clones of B cells in response to an antigen, or monoclonal, where a single clone of B cells is used to produce an antibody with an affinity to a defined epitope. Good antibodies are characterized by the lack of non-specific bands especially in the molecular weights of interest. Further, monoclonal antibodies are known to have high specificity and less background noise, as well as consistency from batch to batch.

Monoclonal antibodies are produced by inoculating mice with a peptide antigen to elicit an immune response. The recovered splenocytes are fused to myeloma cells and then expanded into individual clones to generate hybridomas[1]. All hybridomas thus have only a single specificity dictated by the epitope and any cross-reactivity is due to similarity between the inoculating sequence and other proteins[2].

Our laboratory is interested in the regulation of myogenesis by the bzip transcription factor CCAAT/Enhancer Binding Protein beta (C/EBP). C/EBPβ is widely expressed and has been shown to play a role in cell differentiation, apoptosis and inflammation[3,4]. *Cebpb* is an intronless gene that produces three protein isoforms from a single mRNA though leaky ribosomal scanning: Liver-enriched Activator protein * (LAP*), LAP, and LIP (Liver-enriched inhibitory protein)[4–6].

To detect the expression of all protein isoforms of C/EBPβ, antibodies specific to the C-terminus are required. Beginning in 2014, we began validation experiments for a monoclonal anti-C/EBP antibody (E299, abcam, ab32358). Our research focuses on muscle stem cells, called satellite cells (SCs), that confer regenerative potential to skeletal muscle[7,8]. In response to muscle injury, satellite cells become activated, proliferate, differentiate and fuse to form myofibers that express contractile proteins[8]. In healthy muscle, satellite cells express C/EBP which inhibits myogenic differentiation[9,10]. Upon induction

of differentiation, C/EBPβ expression dramatically decreases, allowing differentiation to proceed[9–11]. We observed that the anti-C/EBP antibody (E299, ab32358) also detects myosin light chain 4 (MYL4) in differentiating myoblasts and in other cell lines. Because MYL4 protein is detected at approximately 23 kDa, this contaminating band can be confused with the LIP isoform of C/EBPβ and should be used with caution in tissues that express MYL4, including skeletal and cardiac muscle.

# Methods

*Cell Culture.* C2C12 myoblasts (ATCC) were grown in DMEM with 10% FBS (GM, growth media) and differentiation was induced by switching confluent cells to DMEM with 2% HS. Mouse primary myoblasts were isolated and cultured as previously described[9] and maintained on Matrigel-coated plates in DMEM (Wisent) with 20% FBS (Wisent), 10% HS (Sigma), 10 ng/ml basic fibroblast growth factor and 2 ng/ml hepatocyte growth factor (Peprotech). To induce differentiation, confluent cultures were switched to differentiation media (DMEM, 2% FBS, 10% HS). *In vitro* CreER$^{tm}$ activity was induced in primary myoblasts from *Cebpb*$^{fl/fl}$*Pax7*$^{wt/wt}$ (WT) and *Cebpb*$^{fl/fl}$*Pax7*$^{CreER/wt}$ (cKO) with 2μM 4-OH tamoxifen (Sigma) for 48h. Retroviruses were generated in Phoenix Ampho packaging cells (ATCC) and retroviral transductions performed as previously described[9].

*Western Analysis.* Whole cell extracts prepared from primary myoblasts or C2C12 cells were resolved on a 15% SDS-PAGE gel, transferred to PVDF membrane (Bio-Rad), and probed with the following primary antibodies: C/EBP (E299, ab32358, Abcam), MYL (F5, sc–365243, Santa Cruz), MYL12A/B (A–10, sc–376606, Santa Cruz), and Cyclophilin B (ab16045, Abcam). The ChemiDoc$^{TM}$ MP system (Bio-Rad) was used to detect chemiluminescence.

*Immunoprecipitation.* Differentiated C2C12 myoblasts were collected in lysis buffer (50mM Tris-base pH 7.5, 150mM NaCl, 1% NP–40 and 1X protease inhibitor) and agitated for 20 min at 4ºC. Samples were spun for 10 min at 4000g and supernatants were collected and precleared with magnetic protein-G-dynabeads (Invitrogen). Approximately 1 mg of protein was incubated with 4 μg of anti-C/EBPβ (ab32358) or non-specific IgG (Invitrogen) for 3h at 4ºC, while 3% of protein was kept for loading input. Immunoprecipitates were captured using Protein-G-beads and extensively washed in 50mM Tris-base pH 7.5, 150mM NaCl and 1% NP–40 and pellets were resuspended in lysis buffer with 5X SDS loading buffer and resolved on a 15% SDS gel for proteomics analysis.

*Protein Identification by LC-MS/MS.* Proteomics analysis was performed at the Ottawa Hospital Research Institute Proteomics Core Facility. Proteins were digested in-gel using trypsin (Promega) according to the method of Shevchenko[13]. Peptide extracts were concentrated by Vacufuge (Eppendorf) and LC-MS/MS was performed using a Dionex Ultimate 3000 RLSC nano HPLC (Thermo Scientific) and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). MASCOT software version 2.6 (Matrix Science, UK) was used to infer peptide and protein identities from the mass spectra. The observed spectra were matched against mouse sequences from SwissProt (version 2016–09) and against an in-house database of common contaminants. The results were exported to Scaffold (Proteome Software, USA).

# Results

*Anti-C/EBPβ antibody from abcam (ab32358) detects three C/EBP isoforms in myoblasts.* To validate the abcam anti-C/EBPβ in myogenic cultures, we performed western blot of C2C12 myoblasts, which express low levels of endogenous C/EBPβ, retrovirally transduced to express the LAP isoform (C/EBPβ-LAP), the LIP (C/EBPβ-LIP) isoform, all C/EBPβ isoforms (β) or with empty virus (pLXSN). The antibody successfully detected endogenous LAP, and the LAP (at ~36 kDa) and LIP (at ~17 kDa) isoforms in LAP- and LIP-overexpressing cells, respectively. Both isoforms were detected in cells overexpressing the full length C/EBP sequence (Fig. 1A). Thus ab32358 can detect the three isoforms of C/EBP in mouse myoblast cells with high specificity.

*Abcam32358 detects a non-specific band in differentiating myoblasts.* We next evaluated the ability of ab32358 to detect endogenous C/EBP expression in proliferating and differentiating myoblasts. C/EBPβ expression is highest in proliferating myoblasts, and is rapidly downregulated after induction to differentiate in low serum conditions. C2C12 myoblasts were expanded in high serum medium for 48 hours (growth medium, GM) then differentiated in low serum medium for 96 hours (differentiation medium, DM). Consistent with our previous findings[9–11], C/EBP-LAP is the most predominantly expressed isoform in myoblasts and its expression decreased 24 hours after induction to differentiate (Fig. 1B). However, beginning at 48h of differentiation, a strong band, far more intense that the signal for C/EBPβ, was detected at ~ 23 kDa and this band was not detected in proliferating myoblasts even with longer exposure time (Fig. 1B). According to the data sheet for the antibody and BLAST analysis of the provided epitope, the antibody could detect C/EBP and C/EBP epsilon. However, the molecular weight of the observed band does not overlap with any of the C/EBP isoforms (42 and 30 kDa) or the C/EBP epsilon isoforms (32, 27 and 14 kDa). To determine if the 23 kDa band represented a novel C/EBP isoform, we examined the expression of this band in myoblast overexpressing C/EBP (Fig. 1C). Over the course of differentiation, the band was only detected in empty vector control cells at 72 hours of differentiation, while it remained undetectable in cells overexpressing C/EBPβ (Fig. 1C). We next examined the expression of this band in primary myoblasts isolated from *Cebpb*$^{fl/fl}$*Pax7*$^{wt/wt}$ (WT) or *Cebpb*$^{fl/fl}$*Pax7*$^{wt/Cre}$ (cKO) muscle in which *Cebpb* is excised by Cre expression driven by the *Pax7* promoter, a satellite cell specific protein. Satellite cells were isolated from WT and cKO mice and treated with 4OH-TAM in culture to induce excision of the entire *Cebpb* sequence in cKO cells. Cells were cultured in growth medium for 48h and switched to differentiation medium for 48h (the time course for differentiation of primary myoblasts is much shorter than in C2C12 cells). Knockout efficiency was confirmed by western blot, indicated the absence of C/EBP-LAP protein in cKO myoblasts as compared to WT (Fig. 1D). C/EBP-LAP expression in WT cells was downregulated with differentiation as previously reported[9,10](Fig. 1D). Interestingly, the 23 kDa band was detected in differentiating WT and cKO myoblasts ruling out the possibility that this band is an isoform of C/EBP (Fig. 1D). Since C/EBP is an inhibitor of myogenesis[9,10], the detection of the 23 kDa band correlates with myogenic differentiation (detected only in differentiating myoblasts) and not with C/EBPβ expression (Fig 1C and D).

*ab32358 detects MYL4 in differentiating myoblasts.* To identify the protein causing the 23 kDa band in differentiating myoblasts, we performed an immunoprecipitation (IP) of whole cell extracts from C2C12 myoblasts differentiated for three days using the ab32358 antibody. The 23 kDa band was successfully precipitated using the anti-C/EBP antibody but not by the control IgG as detected by silver staining (Fig. 2A, red box). Western blot analysis of the input and the C/EBP-IP sample confirmed the pull down of the 23 kDa band (Fig. 2B). The excised 23 kDa band was analyzed by mass spectrometry, which identified 16 mouse proteins with molecular weights between 19–23 kDa (Fig. 2C). Based on the spectrum counts, myosin light chain proteins (MYL4, MYL1/3 and MYL12b) were detected at higher levels than others. Similarly, myosin light chain proteins were more highly ranked based on the percentage of amino acids detected by the spectrum. Myosin light chain 4 was detected with 11 exclusive unique peptides, 13 exclusive unique spectra and 72 total spectra with 66% coverage (Fig. 2C). Myosin light chain 1/3 skeletal muscle isoform (MYL1) was detected with 11 unique peptides, 19 unique spectra, 66 total spectra and 82% coverage. MYL12b was also identified with 7 unique peptides and 40% sequence coverage. We used publicly available microarray data from proliferating (GM) and differentiated myoblasts (GSE24811)[12] to determine the expression pattern of the myosin light chain genes. In parallel with myogenin and myosin heavy chain 8 and 3 (markers of terminal differentiation), *Myl4* and *Myl1* were upregulated with myogenic differentiation (Fig. 2D). *Myl12b* gene expression remained stable with differentiation (Fig. 2D).

To confirm the identity of the band detected with the ab32358 antibody, a western blot comparing extracts from undifferentiated myoblasts (GM), differentiated myotubes (D3), extracts from HEK293 cells (human) and recombinant myosin light chain proteins was performed (Fig. 3). The anti-C/EBPβ antibody failed to detect recombinant MYL12B, but did recognize recombinant MYL4 protein, as well as a corresponding band in HEK293 cells (Fig. 3). Human MYL4 is slightly larger and migrates at a higher molecular weight. Loading of recombinant proteins was verified using an antibody that detects MYL4, which also detected the expression of MYL4 in differentiated C2C12 extracts, or MYL12A/B. The anti-C/EBPβ antibody failed to recognize recombinant MYL1 (data not shown). Thus, these findings indicate that the previously unidentified band detected by ab32358 in differentiating myoblasts corresponds to MYL4.

# Discussion

The advancement of scientific knowledge and the capacity to build on shared knowledge. At the heart of this is the choice of tools, including antibodies that are validated both by suppliers and researchers. Herein, we describe the validation of an anti-C/EBP antibody that is reported to detect all three C/EBPβ protein isoforms, but also shows specificity for MYL4, which is present in differentiating myoblasts, myotubes and myofibers, and numerous other cell lines, including commonly used cancer cell lines and cardiac muscle. Because the molecular weight of MYL4 is, close to that of the C/EBP-LIP isoform, in tissues and cells that also express MYL4, of which many do, caution must be used when identifying the

LIP isoform, and researchers should use extreme caution when using this antibody to detect C/EBP by immunofluorescence.

Interestingly, the ab32358 antibody also detected a band in HEK293 cells, which was not detected by antibodies that recognize MYL proteins. It is unclear why this is so, but raises the possibility that an additional contaminating specificity exists.

The ab32358 antibody is described as a monoclonal antibody. However, the presence of at least two specificities and the absence of similarity between the epitope and the MYL4 sequence suggests that the hybridoma used to produce this antibody is the most likely source of the contamination, with the presence of at least 2 clones of antibody-producing cells, one for anti-C/EBPβ antibody and one that produces the anti-MYL4 antibody.

# LIMITATIONS

While specificity for C/EBP was verified using protein overexpression and knockdown in myoblasts, in our mass spectrometry experiment, we did not isolate and analyze a gel slice corresponding to the migration of the C/EBP-LAP isoform, which would have further confirmed the dual specificity for the antibody.

## Declarations

## Ethics Approval and Consent to Participate

All animal experimentation was approved by the University of Ottawa Animal Care Committee (protocol #2166) and conformed to the guidelines set out by the Canadian Council on Animal Care.

## Availability of data and material

All data generated or analysed during this study are included in this published article.


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## Consent for publication

All data provided are original and consent for publication from individuals other than the others was not required.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

HA contributed to the conception and design, collection, assembly, analysis and interpretation of data, the manuscript writing and final approval of the manuscript. NWB contributed to the conception and design, analysis and interpretation of data, financial support, the manuscript writing, and the final approval of manuscript.

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# Figures

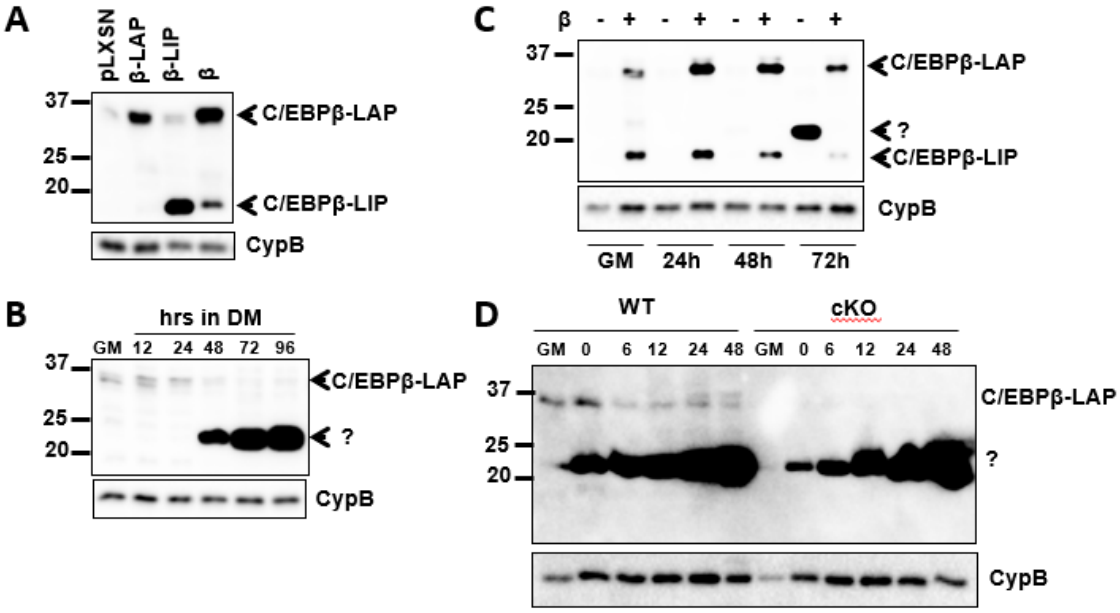
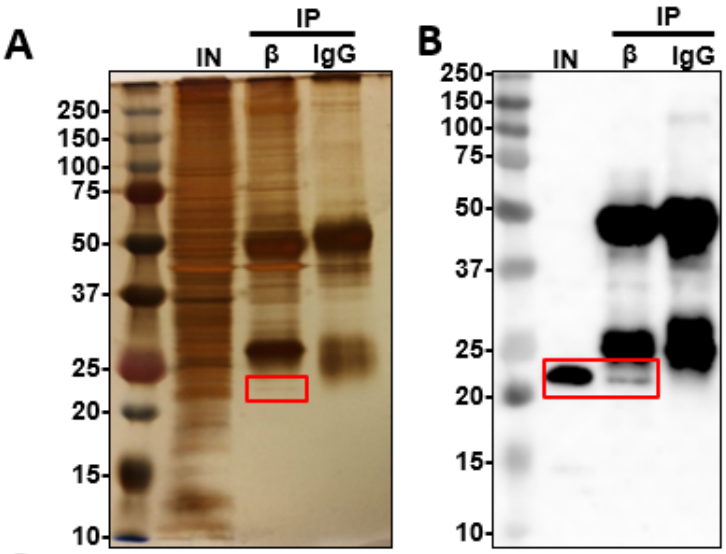



**Figure 1**

Anti-C/EBP antibody E299 (ab32358) detects a ~23 kDa band in differentiating myoblasts. (A) C/EBP protein expression in proliferating C2C12 myoblasts that were retrovirally transduced to express the LAP isoform of C/EBP (-LAP), the LIP isoform (-LIP), the full length C/EBP () or with empty virus (pLXSN).

(B) C/EBP protein expression in proliferating (growth medium, GM) or differentiating (differentiation medium, DM, 12-96h) C2C12 myoblasts. Migration of the unexpected band is indicated by “?”. (C) C/EBP protein expression in proliferating (GM) or differentiating (24-72h) C2C12 myoblasts that were retrovirally transduced to express C/EBP () or with empty virus (pLXSN). (D) C/EBP protein expression in proliferating (GM) or differentiating (0-48h) primary myoblasts isolated from Cebpbfl/flPax7+/+ (WT) and Cebpbfl/flPax7 CreER/+ (cKO) mice.




D

| | LOG2 | | |
|---|---|---|---|
| | GM | D2 | D5 |
| *Myog* | 0.0 | 2.0 | 1.2 |
| *myh8* | 0.0 | 4.8 | 4.0 |
| *Myh3* | 0.0 | 3.5 | 2.8 |
| *Myl4* | 0.0 | 2.9 | 2.8 |
| *Myl1* | 0.0 | 2.0 | 2.0 |
| *Myl12b* | 0.0 | 0.2 | -0.1 |

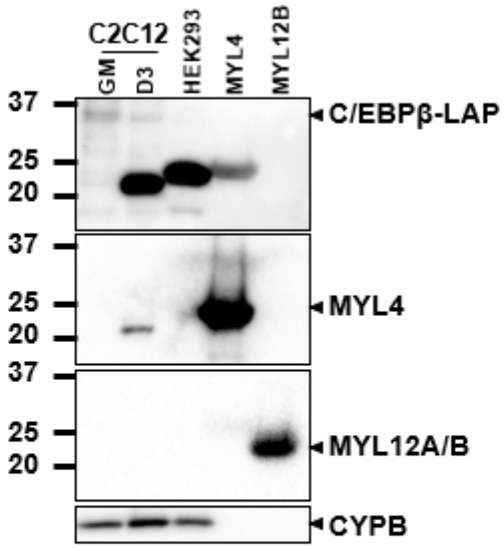
C

| Sequence Coverage | Protein | Accession | Categor | Bio Sar | MS/MS | Prob | %Spec | #Pe | #Unique | #Spec | %Cov | m.w. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MYL4_MOUSE [Myosi | Myosin light chain 4 OS | MYL4_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 0.00833 | 11 | 13 | 72 | 0.66 | 21 kDa |
| MYL1_MOUSE [Myosi | Myosin light chain 1/3, | MYL1_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 0.00763 | 11 | 19 | 66 | 0.82 | 21 kDa |
| ML12B_MOUSE [Myo | Myosin regulatory light | ML12B_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 0.00231 | 7 | 7 | 20 | 0.4 | 20 kDa |
| RS5_MOUSE [40S ribo | 40S ribosomal protein | RS5_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 0.00185 | 7 | 7 | 16 | 0.32 | 23 kDa |
| MYL1_MOUSE [Myosi | Myosin light chain 3 OS | MYL3_MOUSE | Uncateg | DP688 | DP688-1 | 34% | 0.00162 | 0 | 0 | 14 | 0.17 | 22 kDa |
| PRDX1_MOUSE [Pero | Peroxiredoxin-1 OS=M | PRDX1_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 0.0015 | 6 | 7 | 13 | 0.3 | 22 kDa |
| RS7_MOUSE [40S ribo | 40S ribosomal protein | RS7_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 0.0015 | 4 | 4 | 13 | 0.23 | 22 kDa |
| RL17_MOUSE [60S rib | 60S ribosomal protein | RL17_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 0.00104 | 5 | 6 | 9 | 0.24 | 21 kDa |
| TAGL2_MOUSE [Trans | Transgelin-2 OS=Mus m | TAGL2_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 9.25E-04 | 3 | 4 | 8 | 0.17 | 22 kDa |
| PEBP1_MOUSE [Phos | Phosphatidylethanolar | PEBP1_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 8.10E-04 | 4 | 4 | 7 | 0.3 | 21 kDa |
| PRDX2_MOUSE [Pero | Peroxiredoxin-2 OS=M | PRDX2_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 6.94E-04 | 4 | 4 | 6 | 0.23 | 22 kDa |
| RAB7A_MOUSE [Ras-r | Ras-related protein Rab | RAB7A_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 5.78E-04 | 2 | 2 | 5 | 0.12 | 23 kDa |
| EIF3K_MOUSE [Eukary | Eukaryotic translation | EIF3K_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 5.78E-04 | 2 | 3 | 5 | 0.16 | 25 kDa |
| ATPO_MOUSE [ATP sy | ATP synthase subunit O | ATPO_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 4.63E-04 | 2 | 2 | 4 | 0.1 | 23 kDa |
| SRSF3_MOUSE [Serine | Serine/arginine-rich sp | SRSF3_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 4.63E-04 | 2 | 2 | 4 | 0.14 | 19 kDa |
| RL18A_MOUSE [60S r | 60S ribosomal protein | RL18A_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 3.47E-04 | 2 | 2 | 3 | 0.15 | 21 kDa |
| PSB6_MOUSE [Protea | Proteasome subunit be | PSB6_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 2.31E-04 | 1 | 1 | 2 | 0.05 | 25 kDa |
| RAP1A_MOUSE [Ras-r | Ras-related protein Rap | RAP1A_MOUSE | Uncateg | DP688 | DP688-1 | 100% | 2.31E-04 | 2 | 2 | 2 | 0.14 | 21 kDa |

## Figure 2

. LC-MS/MS analysis of the 23 kDa band identifies 16 mouse proteins. (A) Migration of proteins immunoprecipitated by anti-C/EBP antibody (ab32358) or non-specific rabbit IgG from differentiating C2C12 myoblasts, stained with silver stain. Input is 3% of material used for immunoprecipitation. The rectangle highlights the bands that were subject to MS analysis. (B) Western blot analysis of the anti-

C/EBP IP samples performed as in (A) with a rectangle identifying the unknown band. (C) Scaffold Viewer data for mouse proteins detected by MS from (A). (D) Myog,Myh8, Myh3, Myl4, Myl1 and Myl12b gene expression in proliferating (GM) primary myoblasts and differentiated for 2 and 5 Days (D2 and D5) obtained from publicly available microarray data (GSE24811)12. Data are presented as Log2 to the GM samples.




## Figure 3

Anti-C/EBP antibody (ab32358) detects MYL4 in differentiating myoblasts. Western blot of extracts from proliferating myoblasts (GM), 3-day differentiated myoblasts (D3), HEK293 cells with human recombinant MYL4 (ab115722) and human recombinant MYL12B (ab128438) probed with anti-C/EBP (ab32358), anti-MYL (sc-365243) and anti-MYL12A/B (sc-376606). Cyclophilin B is used as loading control.